A novel *Bacillus sphaericus* binary toxin active against Bin-resistant *Culex* mosquito larvae

by

Gareth Wyn Jones B.Sc.

Being a thesis presented in accordance with the regulations governing the award of the degree of Philisophiae Doctor in the University of Wales.

Date: May, 2006

Cardiff School of Biosciences,

Cardiff University.

UMI Number: U584835

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI U584835
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Candidate ID number:	990491198
Candidate's last name:	Jones
Candidate's first names:	Gareth Wyn

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed ......G.W. Jones............... (candidate) Date ....6/10/06.................

STATEMENT 1

This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD.

Signed ......G.W. Jones............... (candidate) Date ....6/10/06.................

STATEMENT 2

This thesis is the result of my own independent investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended

Signed ......G.W. Jones............... (candidate) Date ....6/10/06.................

Signed .... (supervisor) Date ....

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed ......G.W. Jones............... (candidate) Date ....6/10/06.................

Summary

Some *Bacillus sphaericus* strains (e.g. IAB59, LP1G and 47-6B) can overcome resistance in *Culex* mosquito larvae, raised against the well characterised binary toxin from this bacterium. A common spore protein (P49), of approximately 49 kDa, produced by these strains has previously been proposed to be responsible for this toxicity. Protein fingerprint analysis of sporulated cultures of these strains identified a number of candidate toxins. Their N-terminal sequences were determined and used to design degenerate oligonucleotide probes. Southern blotting, cloning and colony hybridisation allowed the identification of clones containing genes encoding the putative toxins Cry49Aa1 (P49) and Cry48Aa1 (P135) from IAB59. The 1,395 bp *cry49Aa1* gene encodes a protein of 53.3 kDa, showing homology to BinA and BinB from *B. sphaericus* as well as Cry36Aa1 and the Cry35 binary toxins from *Bacillus thuringiensis*. The 3,534 bp *cry48Aa1* gene encodes a 135.6 kDa protein showing homology to the three-domain Cry toxins from *B. thuringiensis*, including the mosquitocidal Cry4Aa and Cry4Ba from *B. thuringiensis* subsp. *israelensis*. Individual expression of these proteins in an acrystalliferous *B. thuringiensis* subsp. *israelensis* strain, followed by bioassays against mosquito larvae revealed no toxicity. However, a Cry48Aa1/Cry49Aa1 combination was toxic to both Bin-susceptible and Bin-resistant *Culex quinquefasciatus* larvae. *Aedes aegypti* and *Anopheles gambiae* mosquito larvae were insensitive to the combination, as were a range of other dipteran, coleopteran and lepidopteran insects.

The components of this novel binary toxin from *B. sphaericus* are highly conserved among strains able to overcome resistance. Differential processing of Cry48Aa1 by *C. quinquefasciatus* and *A. aegypti* larval gut proteinases is not responsible for the non-toxicity towards the latter mosquito. Cry49Aa1 and Cry48Aa1 form bipyramidal and amorphous crystals respectively at sporulation and their expression involves RNA polymerase factor σ^E in *B. subtilis*. Discovery of Cry49Aa1 and Cry48Aa1 may prove central in the development of strategies to avoid resistance development against *B. sphaericus* in *Culex* populations.

CONTENTS

Page

Summary i

Contents ii

Abbreviations ix

Chapter 1 Introduction 1

1.1 Biological control of vector mosquitoes 2

1.2 *B. sphaericus* 3

1.2.1 The Bin toxin of *B. sphaericus* 5

1.2.2 The Binary toxin (*bin*) genes 7

1.2.3 BinA and BinB variants among *B. sphaericus* strains 10

1.2.4 BinA and BinB binary toxins 14

1.2.5 Proteolytic processing of the Bin toxin 16

1.2.6 Binary toxin mode of action 18

1.2.7 Mtx toxins 19

1.2.7.1 Mtx 1 19

1.2.7.2 Mtx2 and Mtx3 24

1.3 *Bacillus thuringiensis* 25

1.3.1 *B. thuringiensis* subsp. *israelensis* toxins 28

1.3.2 Cry toxin production 28

1.3.3 Structure and sequence similarities among the three domain Cry toxins 31

1.3.4 Cry toxin mode of action 37

1.3.5 Cry34/Cry35 binary toxins 38

1.3.6 Cyt toxins 39

1.3.7 Other *B. thuringiensis* toxins 41

1.4 Mosquito resistance to *B. sphaericus* 42

1.5 Other bacterial toxins 46

1.6 Project aims 48

Chapter 2 Materials and methods **49**

2.1 Materials **50**

2.1.1 Cloning and expression vectors 50

2.1.2 *E. coli* host strains 50

2.1.3 *Bacillus sphaericus* strains 51

2.1.4 *Bacillus thuringiensis* subsp. *israelensis* host strain 51

2.1.5 *Bacillus subtilis* strains 52

2.1.6 Media 52

2.1.7 Enzymes 53

2.1.8 Antisera 53

2.1.9 Molecular weight standards 53

2.1.10 Other materials 55

2.2 Molecular biology methods **56**

2.2.1 Preparation of plasmid DNA 56

2.2.2 Submarine agarose gel electrophoresis 56

2.2.3 DNA modifying enzymes and restriction endonucleases 56

2.2.4 Recovery of DNA fragments from agarose gels 57

2.2.5 Precipitation of DNA 58

2.2.6 Polymerase chain reaction (PCR) amplification of DNA 58

2.2.6.1 Basic PCR for cloning and screening 58

2.2.6.2 Inverse PCR 59

2.2.7 Ligation of DNA into plasmid vectors 60

2.2.8 Colour screening of transformant bacterial colonies for inserts 61

2.2.9 Preparation of *E. coli* glycerol stocks 61

2.2.10 Double stranded DNA sequencing 62

2.2.11 Introducing specific mutations into target DNA 62

2.2.11.1 QuikChange site-directed mutagenesis 62

2.2.11.2 Site-specific mutagenesis by Overlap Extension 63

2.2.12 Isolation of *B. sphaericus* total DNA 63

2.2.13 Detection of *B. sphaericus* megaplasmids 64

2.3	**Transformation of bacterial cells by plasmid DNA**	**66**
2.3.1	Preparation of competent *E. coli* cells	66
	2.3.1.1 Calcium chloride method	66
	2.3.1.2 Electrocompetent method	67
2.3.2	Transformation of *E. coli* by plasmid DNA	68
	2.3.2.1 Calcium chloride method	68
	2.3.2.2 Electroporation	68
2.3.3	Packaging of recombinant cosmids into bacteriophage λ particles	69
2.3.4	Electrotransformation of *B. thuringiensis* by plasmid DNA	70
2.3.5	Electrotransformation of *B. subtilis* by plasmid DNA	71
2.4	**DNA-DNA hybridisation methods**	**72**
2.4.1	Preparation of DIG labelled DNA probes	72
	2.4.1.1 3' DIG labelling of oligonucleotides	72
	2.4.1.2 PCR generation of DIG labelled probes	72
2.4.2	Transfer of target DNA to membranes for DNA-DNA hybridisation	72
	2.4.2.1 Capillary transfer by Southern blot	72
	2.4.2.2 Colony hybridisation	73
	2.4.2.3 Dot blotting of DNA onto membranes	74
2.4.3	Hybridisation of probes to DNA immobilised on membranes	74
	2.4.3.1 3' DIG labelled oligonucleotide probes	75
	2.4.3.2 PCR generated DIG labelled probes	76
2.5	**Biochemistry methods**	**76**
2.5.1	SDS-polyacrylamide gel electrophoresis (SDS-PAGE)	76
2.5.2	Immunological detection of recombinant proteins by Western blotting	77
2.5.3	Preparation of protein samples for N-terminal sequencing	78
2.5.4	Analytical scale expression in *E. coli* and sample preparation of recombinant protein	79
2.5.5	Analytical scale expression of soluble and insoluble fractions	80
2.5.6	Purification of crystal proteins from *B. sphaericus* and recombinant *B. thuringiensis* subsp. *israelensis* 4Q7	80
2.5.7	Quantification of crystal protein concentration	81
2.5.8	Electron microscopy of spore-crystal samples	82
2.5.9	Preparation of larval gut extract	82

2.5.10 *In vitro* toxin processing 83

2.6 Insect bioassays 83

2.6.1 Mosquito larvae 84

2.6.2 *Anthonomus grandis* 85

2.6.3 *Anticarsia gemmatalis* 85

2.6.4 *Spodoptera frugiperda* 86

2.6.5 *Plutella xylostella* 86

2.6.6 *Chironomus riparius* 87

Chapter 3 Discovery and cloning of a putative toxin from *B. sphaericus* 88

3.1 Introduction 89

3.2 Construction of a *B. sphaericus* IAB59 cosmid library 91

3.3 Screening of IAB59 cosmid library against *C. quinquefasciatus* larvae 94

3.4 Discussion 94

3.5 Protein fingerprint comparison and light microscope analysis of different *B. sphaericus* spore cultures 95

3.6 Amino terminal sequencing of putative toxins 98

3.7 Design of degenerate oligonucleotides based on the N-terminal P49 sequence 101

3.8 Detection of *p49* by Southern hybridisation 105

3.9 Cloning of DNA fragments containing *p49* sequence 107

3.9.1 Colony hybridisation 107

3.9.2 Inverse PCR 111

3.10 DNA sequencing of the putative toxin gene *p49* 114

3.11 Analysis of the deduced *p49* gene sequence 117

3.12 Analysis of the P49 protein sequence 121

3.13 Sequencing of the *p49* gene from strains LP1G and 47-6B 124

3.14 Discussion 128

Chapter 4 Recombinant expression and bioassay of the putative crystal toxin, P49, from *B. sphaericus* IAB59 **130**

4.1 Introduction 131

4.2 Expression of P49 in *E. coli* 131

4.2.1 Expression of P49 as a GST fusion 132

4.2.1.1 Cloning of *p49* into a pGEX expression vector 132

4.2.1.2 Expression of the P49-GST fusion protein 136

4.2.2 Expression of P49 using the pET vector system 139

4.2.2.1 Cloning of *p49* into a pET22b expression vector 139

4.2.2.2 Expression of P49 using the pET system 144

4.2.3 Bioassay of *E. coli* expressing P49 against *C. quinquefasciatus* larvae 146

4.2.4 Discussion 146

4.3 Expression of P49 in *B. thuringiensis* subsp. *israelensis* 147

4.3.1 Cloning of *p49* into pHT304 148

4.3.2 Expression from pHTP49 in *B. thuringiensis* subsp. *israelensis* 149

4.3.3 Discussion 152

4.4 Bioassay of P49 153

4.5 Discussion 154

Chapter 5 Discovery of an additional putative crystal toxin from *B. sphaericus* IAB59 **156**

5.1 Introduction 157

5.2 Protein based analysis of *B. sphaericus* strains 158

5.2.1 Protein fingerprint comparison of *B. sphaericus* spore cultures 158

5.2.2 Amino terminal sequencing of P43 160

5.2.3 Crystal protein profile of *B. sphaericus* NHA15b 160

5.2.4 Discussion 163

5.3 Sequencing of p49H5 and p49H11 165

5.3.1 Subcloning of the *Hin*dIII fragment in clone p49H5 165

5.3.2 Sequencing of p49H5 and p49H11 by "walking-out" 169

5.4 DNA sequence analysis of the 15.6 kb *Hin*dIII fragment from IAB59 171

5.5 Sequence analysis of the gene encoding P135 from IAB59 179

5.6 Analysis of the P135 protein sequence 184
5.7 Sequencing of the *p135* gene from strains LP1G, 47-6B and NHA15b 186
5.8 Discussion 191

Chapter 6 Recombinant expression and bioassay of the putative crystal toxin, P135, from *B. sphaericus* **193**
6.1 Introduction 194
6.2 Expression of P135 in *B. thuringiensis* subsp. *israelensis* 194
6.2.1 Cloning of *p135* into pHT304 194
6.2.2 Expression from pHTP135 in *B. thuringiensis* subsp. *israelensis* 195
6.2.3 Expression of P135 using the pSTAB vector 197
6.3 Bioassay of P135 202
6.4 Preparation of P49 and P135 crystal stocks for bioassay and characterisation 203
6.5 Discussion 204

Chapter 7 Characterisation of the Cry48Aa1/Cry49Aa1 binary mosquitocidal toxin from *B. sphaericus* IAB59 **206**
7.1 Distribution of the genes encoding Cry48 and Cry49 proteins 207
7.1.1 Detection of *cry48* and *cry49* genes by dot blot and PCR 207
7.1.2 Sequencing of *cry48* and *cry49* genes from strains NHA15b and 2173 214
7.1.2.1 Sequencing of *cry49Aa* from strains NHA15b and 2173 214
7.1.2.2 Sequencing of *cry48Ab2* from strain 2173 215
7.1.3 Discussion 220
7.2 Regulation of Cry48Aa1 and Cry49Aa1 synthesis 223
7.2.1 Cry48Aa1 and Cry49Aa1 expression in *B. subtilis* σ factor mutants 224
7.2.2 Discussion 229
7.3 Cry48Aa1 and Cry49Aa1 toxin specificity 231
7.3.1 Insect bioassays 231
7.3.2 Discussion 234
7.4 Electron microscopic analysis of Cry48Aa and Cry49Aa crystal toxins 235
7.5 Molecular modelling of Cry48Aa1 237
7.5.1 General features of the Cry48Aa1 model 238

7.6 Cry48Aa1 and Cry49Aa1 toxin processing 241

7.6.1 SDS-PAGE analysis of Cry48Aa1 and Cry49Aa1 processing 242

7.6.2 Bioassay of *C. quinquefasciatus* gut activated toxin against *A. aegypti* 246

7.6.3 Discussion 246

7.7 Chapter summary 252

Chapter 8 General discussion 254

Acknowledgements 267

References 268

Appendices 301

Publications 316

Abbreviations and conventions

aa	amino acids
β-mercaptoethanol	2-hydroxyethylmercaptan
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	Bovine Serum Albumin
CIP	Calf intestinal alkaline phosphatase
CSPD	disodium 3-(4-methoxyspiro (1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan)-4-yl) phenyl phosphate
CTAB	Hexadecyltrimethyl ammonium bromide
C-terminal	Carboxy terminal
Da	Dalton
dATP (dCTP, dGTP, dTTP, dUTP)	2'-deoxyadenosine 5'triphosphate (and similarly for cytidine, guanosine, thymidine and uridine)
DBTBS	Database of transcriptional regulation in *Bacillus subtilis*
DIG	Digoxigenin
dNTP	Mixture of dATP, dCTP, dGTP and dTTP
EDTA	Ethylenediamine tetra acetic acid
IPTG	Isopropyl β-D-thiogalactopyranoside
g	gravity
GPI	Glycosyl-phosphatidylinositol
GST	Glutathione S-transferase
kb	Kilobase pairs
LB	Luria Bertani
LC_{50}	Fifty percent lethal concentrations
LMP	Low Melting Point
MCS	Multiple cloning site
MW	Molecular Weight
nt	Nucleotides
N-terminal	Amino terminal
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PVDF	Polyvinylidene difluoride
RBS	Ribosome binding site
RLCq/C3-41	Resistant laboratory-selected *Culex quinquefasciatus* colony/Raised against *B. sphaericus* strain C3-41
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SLCq	Susceptible laboratory *Culex quinquefasciatus* colony
SSC	Standard saline citrate
TE	Tris/EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TCA	Trichloroacetic acid
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
WHO	World Health Organisation

CHAPTER 1

Introduction

1.1 Biological control of vector mosquitoes

Mosquitoes are a constant threat to health and having moved into the 21st century a mosquito-borne disease, malaria, remains one of the major killers in the world. There are almost 500 million cases of malaria each year, resulting in nearly 3 million deaths, caused by parasites such as *Plasmodium falciparum* and *Plasmodium vivax* (WHO, 1998). Mosquito transmission of nematodes such as *Wuchereria bancrofti* and *Brugia malayi* also means that over 120 million people are infected with the causative agents of lymphatic filariasis (WHO, 2000a). In addition, hundreds of thousands of people each year suffer from the diseases; yellow fever, dengue fever, St. Louis encephalitis, Japanese encephalitis and West Nile virus, resulting from infection by mosquito-borne viruses. Anopheliae and Culicidae mosquitoes are the major vectors of such diseases (Porter *et al.*, 1993) with *Anopheles* mosquitoes, of the former subfamily, transmitting malaria and filarial parasites. Members of the *Culex* genus are vectors of filariasis and Japanese encephalitis while *Aedes* mosquitoes transmit yellow fever, dengue viruses, as well as filarial nematodes. Attempts to control mosquito populations using broad range chemical pesticides have been successful, but have resulted in increased environmental pollution as well as increased populations of resistant mosquitoes. Biological control of mosquitoes provides a target-specific, effective and more environmentally friendly approach. Bacteria such as *Bacillus sphaericus* are particularly suited to this role due to their ability to survive in many habitats. While some chemical insecticides such as DDT exert their toxicity at an adult level, *B. sphaericus* and similar agents target the larval stages of the mosquito life cycle and thus have the advantage of controlling potential vectors before they can disperse and transmit diseases (Killeen *et al.*, 2002). The specificity of these larvicides results in no damage to non-target organisms, including

humans, and thus allows for trouble-free application in the field (WHO, 2000b). Integration of biological control with chemical control measures such as insecticide treated bednets (Lengeler, 2000; Roberts *et al.*, 2000) would allow the targeting of multiple stages of the mosquito life cycle. In a recent study (Fillinger *et al.*, 2003), it was shown that the minimum doses of commercially available VectoBac® (*B. thuringiensis israelensis*) and VectoLex® (*B. sphaericus*) required to eliminate *Anopheles gambiae* larvae in a habitat were low (200g/ha). A similar study (Brown *et al.*, 2004) also showed VectoLex® to be effective in control of *Culex quinquefasciatus* and confirmed no unwanted effects on nearby shrimp and fish species. This provides much encouragement for vector control programmes against *A. gambiae*, a malaria vector, and *C. quinquefasciatus.*

1.2 *B. sphaericus*

B. sphaericus is a gram positive, spore-forming bacterium distributed in soil and aquatic environments. Lacking several biochemical pathways, it cannot metabolise sugars and relies on other simple sources of carbon such as acetate, a feature that is useful for isolation of *B. sphaericus* strains from environmental samples (White and Lotay, 1980; Massie *et al.*, 1985). Based on percentage of DNA homology, *B. sphaericus* strains are divided into five groups, with group II being subdivided into group IIa and IIb (Krych *et al.*, 1980). Group IIa contains all the mosquitocidal strains and are related by greater than 79% DNA homology. Flagellar (H) serotyping (de Barjac *et al.*, 1985) and bacteriophage typing (Yousten, 1984) are other methods employed for the classification of *B. sphaericus* strains. Mosquitocidal strains have been divided into the flagellar serotypes: 1a, 2a2b, 3, 5a5b, 6, 9a9c, 25, 26a26b, and 48. However, in contrast to early beliefs, there seems little correlation

between serotype and toxicity, with the exception of serotype 5a5b that harbours only high toxicity strains (Priest *et al.*, 1997).

The first *B. sphaericus* mosquitocidal strain was discovered in 1964 (strain K) but was of low toxicity (Kellen *et al.*, 1965). The search for higher toxicity strains continued, with a strain of significantly greater toxicity (SSII-1) being reported by Singer in 1973 (Singer, 1973). Its discovery prompted the search for further strains, with the isolation of strain 1593, in Indonesia in 1976, representing the discovery of the first high-toxicity strain (Singer, 1977). This was followed by the discovery of more high toxicity strains such as 2297 from Sri Lanka (Wickremesinghe and Mendis, 1980), 2362 from Nigeria (Weiser, 1984), strains IAB59, IAB881 and IAB872 from Ghana (de Barjac *et al.*, 1988; Thiery *et al.*, 1992) and LP1G from Singapore (Liu *et al.*, 1993). All high toxicity *B. sphaericus* strains form parasporal crystals at sporulation.

B. sphaericus strains show toxicity towards the larval stages of many mosquito genra (*Culex*, *Aedes*, *Anopheles*, *Mansonia* and *Psorophora*) (Delecluse *et al.*, 2000). In general *B. sphaericus* strains are highly toxic to *Anopheles* and *Culex* larvae and show low toxicity, or no toxicity, towards *Aedes* larvae. However, mosquitocidal specificity depends on mosquito species and can vary within a genus (Berry *et al.*, 1993). While *B. sphaericus* SSII-1 toxicity was found throughout all stages of growth and was lost when cells were heated above 80°C for 12 min (Myers and Yousten, 1978; Myers *et al.*, 1979), strain 1593 toxicity was more stable and was found to increase at sporulation (Myers *et al.*, 1979). Also, strain 2297 showed about a 1,000-fold increase in toxicity during sporulation (Yousten and Davidson, 1982). Furthermore, mutants blocked at early stages of sporulation failed to make crystals and were poorly mosquitocidal (Charles *et al.*, 1988). The source of the *B. sphaericus*

spore associated toxicity was later found to be the binary toxin (Baumann *et al.*, 1987; Hindley and Berry, 1987; Baumann *et al.*, 1988), which is composed of two polypeptides: BinA (also known as P42, with a molecular weight of 41.9 kDa) and BinB (also known as P51, with a molecular weight of 51.4 kDa). Sporulation independent toxicity was found to be the result of the vegetatively expressed toxins; Mtx1 (100 kDa) (Thanabalu *et al.*, 1991), Mtx2 (31.8 kDa) (Thanabalu and Porter, 1996) and Mtx3 (35.8 kDa) (Liu *et al.*, 1996).

Most high toxicity strains, for example all strains form serotype 5a5b, 2297 from serotype 25 and IAB59 from serotype 6, produce both the binary toxin and Mtx toxins (Liu *et al.*, 1996; Thanabalu and Porter, 1996; Priest *et al.*, 1997). Lower toxicity strains such as SSII-1 from serotype 2a2b produce only Mtx toxins, while others such as 2173 and 2377 produce none of the currently identified toxins. Strains such as 2173 and 2377 of serotype 26a26b provide an opportunity to identify new toxins and the prospect of using multiple *B. sphaericus* strains in the field to reduce the development of resistance currently observed.

1.2.1 The Bin toxin of *B. sphaericus*

Early work to identify the mosquitocidal toxins of *B. sphaericus* were hampered by the inability to isolate sufficient quantities of crystal to permit fractionation or purification of its toxic components. The toxic factor was determined to be associated with the bacterial cell since culture supernatants, after centrifugation, were inactive, with cells retaining toxicity in the pellet (Myers and Yousten, 1980). Breakage of *B. sphaericus* 1593 spores showed the toxicity to be associated with the cell wall fraction and that toxicity was stable to treatment with trypsin, pronase, urea (8M, 30 min), heating (80°C, 12 min), sonication, refrigeration, lyophilisation and

freezing (Myers and Yousten, 1980). An alkaline extraction method, adapted from the successful solubilisation of δ-endotoxin from *B. thuringiensis*, resulted in the successful solubilisation of toxin from *B. sphaericus* 1593 with an apparent molecular weight of 35-54 kDa (Davidson, 1983). Concordantly, crystals purified from *B. sphaericus* 2362 by centrifugation through 48% (w/v) NaBr were found to contain five proteins with apparent molecular weights of 43, 63, 98, 110 and 125 kDa, with all but the 43 kDa and 63 kDa proteins being eliminated by solubilisation under alkaline conditions (Baumann *et al.*, 1985). The 43 kDa and 63 kDa proteins were subsequently purified and their antigenic determinants detected in the non-toxic 110 kDa and 125 kDa proteins observed in the purified crystal preparations (Broadwell and Baumann, 1986). It was thus initially proposed that the 110 kDa and 125 kDa proteins were protoxins activated during sporulation to yield the 43 and 63 kDa proteins, but they were later thought to be surface layer proteins (Bowditch *et al.*, 1989). However, much more recent data points to the 110 kDa and 125 kDa protein bands observed by SDS-PAGE to be oligomers of BinA and BinB (Smith *et al.*, 2005). Purified 43 kDa protein was found to be toxic to *Culex pipiens* mosquito larvae (LC_{50} of 35 ng/ml) whereas the 63 kDa protein was non-toxic (Baumann *et al.*, 1985). The 43 and 63 kDa proteins were also found to have different amino acid compositions, no immunological cross-reactivity, net opposite charges at pH 7.5 and different susceptibility to processing by larval midgut proteinases (Baumann *et al.*, 1985). The 63 kDa protein was rapidly degraded while the 43 kDa protein appeared to be slowly processed to a 40 kDa product. Separate treatment of the 43 kDa protein with mosquito gut proteinases, trypsin or α-chymotrypsin confirmed processing to a 40 kDa product, causing a 54 fold increase in toxicity (to LC_{50} of 1 μg/ml) to culture-grown *Culex quinquefasciatus* cells (Broadwell and Baumann, 1987; Davidson *et al.*,

1987). Proteolytic processing was also shown not to be involved in host specificity, with gut extracts from non-susceptible *Aedes aegypti* larvae being as effective as highly sensitive *C. quinquefasciatus* in toxin activation (Broadwell and Baumann, 1987; Davidson *et al.*, 1987). N-terminal sequencing of the purified 43 kDa protein resulted in determination of the first 40 amino acid residues (Baumann *et al.*, 1985). The mode of action of the toxin was proposed to involve ingestion of the crystal-spore complex before its solubilisation under alkaline conditions in the larval gut and its activation by gut proteinases. Proteins immunologically related to the 43 kDa and 63 kDa protein were detected in strains 1593, 1691 and 2297 (Baumann *et al.*, 1985). Preparation of antibodies against the 1593 larvicidal protein followed by their immobilisation for use in immunoaffinity purification of the toxins gave rise to the isolation of proteins with molecular weights of 42.6, 44.1, 50.7 and 51.3 kDa, with an LC_{50} of 8.3 ng/ml against *Culex* larvae (Narasu and Gopinathan, 1986).

1.2.2 The Binary toxin (*bin*) genes

The gene encoding the 41.9 kDa BinA protein was identified in *B. sphaericus* strain 1593 DNA by Southern blot (Hindley and Berry, 1987) using degenerate probes designed from the 40 amino acid N-terminal sequence of the 43 kDa protein of strain 2362 (Baumann *et al.*, 1985). Sequencing of the gene for BinA from other strains was quick to follow (Berry and Hindley, 1987; Hindley and Berry, 1988). A 3.5 kb *Hin*dIII fragment was later cloned from a genomic library of strain 2362 that contained two open reading frames for 41.9 kDa and 51.4 kDa proteins arranged in an operon (Baumann *et al.*, 1987; Baumann *et al.*, 1988). Sequence analysis (Baumann *et al.*, 1988) confirmed the 41.9 kDa (BinA) and 51.4 kDa (BinB) to correspond to the proteins previously purified from *B. sphaericus* 2362 (Baumann *et al.*, 1985) with

apparent molecular weights of 43 kDa and 63 kDa respectively. The nucleotide sequence of the 3,479 bp operon was found to be highly conserved between 2362, 1593, 2297, IAB59 and 2317.3 (Berry *et al.*, 1989). Strains from H5a5b (1593, 2362 and 2317.3) had identical nucleotide sequences for the operon while the sequences from 2297 (H25) and IAB59 (H6) differed by 25 and 7 nucleotides, resulting in 8 and 6 amino acid substitutions respectively. Interestingly, no genes coding for BinA and BinB were detected by Southern blot analysis in the low toxicity strains SSII-1 and Kellen K (Baumann *et al.*, 1987), suggesting a different source for the toxicity of these strains.

The organisation of the bin operon is shown in figure 1.1 and consists of *binB* lying upstream of *binA* with a 174-176 bp intergenic region and putative Shine-Dalgarno sequences lying upstream of the initiating ATG of both genes (Hindley and Berry, 1987; Baumann *et al.*, 1988). A G+C rich hairpin loop followed by a row of Ts, forming a characteristic transcriptional terminator, is located downstream of *binA*. The lack of a transcriptional terminator in the intergenic region suggests co-transcription of *binA* and *binB* (Baumann *et al.*, 1988). This is further supported by the expression of both BinA and a β-galactosidase-BinB fusion protein when their genes were cloned downstream of the *lac* promoter, regulated by a β-galactosidase inducer in *Escherichia coli* (Baumann *et al.*, 1987).

It has been shown that the binary toxin genes are located on the bacterial chromosome (Porter *et al.*, 1993), at least in some strains, where a binary toxin gene probe failed to hybridise to purified plasmid DNA while hybridisation to binary toxin genes on the chromosome was observed (Liu *et al.*, 1993).

Expression of the binary toxin genes occurs during sporulation in *B. subtilis* (Baumann and Baumann, 1989), while *lacZ* fusion to the *bin* promoter have revealed

that transcription begins immediately before the end of exponential growth in both *B. sphaericus* and *B. subtilis* (Ahmed *et al.*, 1995). More recently, experiments using mutants of *B. sphaericus* strain 2362 have emphasised the importance of the early sporulation genes, *spo0A* and *spoIIAC*, in expression of the binary toxin genes (El-Bendary *et al.*, 2005). Spo0A and RNA polymerase factor σ^F, the protein products of *spo0A* and *spoIIAC* respectively, are known to be essential for the expression of a number of sporulation specific genes and either directly or indirectly are required for the production of multiple sigma factors in *B. subtilis*, such as σ^E, σ^F, σ^K and σ^H (Haldenwang, 1995; El-Bendary *et al.*, 2005). The RNA polymerase sigma factor(s) involved in transcription of the *bin* operon in *B. sphaericus* have not yet been determined.

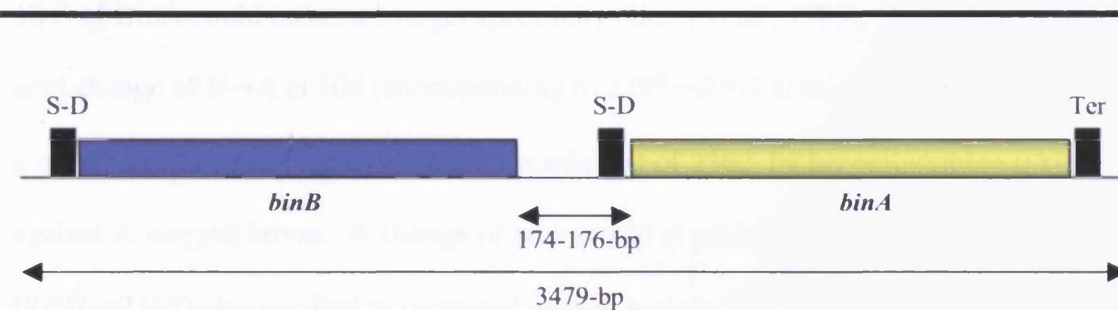


Figure 1.1 Schematic representation of the binary toxin genes, *binA* and *binB*. Putative Shine-Dalgarno (S-D) sequences and transcriptional terminator sites (Ter) are shown.

1.2.3 BinA and BinB variants among *B. sphaericus* strains

All *bin* operons sequenced to date encode two proteins of 41.9 kDa (370 amino acids) and 51.4 kDa (448 amino acids) respectively. The 3,479-bp toxin coding and surrounding regions of strains 1593, 2362 and 2317.3 are identical, while the sequences of the IAB59, 2297 and LP1G variants differ by up to 25 nucleotides (Berry and Hindley, 1987; Hindley and Berry, 1987; Baumann *et al.*, 1988; Hindley and Berry, 1988; Berry *et al.*, 1989; Priest *et al.*, 1997; Humphreys and Berry, 1998). At the protein level this corresponds to five and six potentially variant amino acids in the sequences of BinA and BinB respectively, giving rise to four *bin* variants as summarised in table 1.1 (Humphreys and Berry, 1998). The change of only a few amino acids in BinA or BinB can have a substantial effect on host specificity and toxicity levels. For example, by expressing mutant toxins of strain 2297 in *E. coli*, Berry *et al.* concluded that amino acids within the variable region (position 99 and 104) of BinA could influence target specificity (Berry *et al.*, 1993). A BinA amino acid change of S→A at 104 (corresponding to 2297→2362 at this position) resulted in a switch of activity of the 2297 toxin towards that of 2362, by becoming more toxic against *A. aegypti* larvae. A change of amino acid at position 99 from F→V (2297→2362) also resulted in increased overall toxicity towards *A. aegypti* larvae. It was thus predicted that these variable amino acids, focused around position 100, were involved in toxin-receptor interaction in the larval gut (Berry *et al.*, 1993). Consistent with these results, the BinA4/BinB4 variant seen in strain LP1G bestows on this strain a lower level of toxicity towards *C. quinquefasciatus* compared to all the other Bin variants, in having a serine at position 93 instead of a leucine (table 1.1) (Humphreys and Berry, 1998). Further mutational studies indicated that this L→S change in LP1G was responsible for the lower toxicity of this strain and that introducing a L→S

mutation into BinA2 from strain 1593 also resulted in significant loss of toxicity (Yuan *et al.*, 2001).

BinA and BinB share only a low sequence identity except for in the four conserved blocks described by Baumann *et al.* (Baumann *et al.*, 1988; Baumann *et al.*, 1991), as shown in figure 1.2. It was thus suggested that these two proteins came from a single ancestor by gene duplication followed by sequence divergence and, possibly, functional specialization (channel formation for BinA and receptor binding for BinB) (Baumann *et al.*, 1991; de Maagd *et al.*, 2003). Until recently, the Bin toxin components did not show homology to any other toxins. However the discovery of the Cry34/Cry35 binary toxins from *B. thuringiensis* (Moellenbeck *et al.*, 2001; Ellis *et al.*, 2002; Schnepf *et al.*, 2005) has added a homologue of BinA and BinB, the Cry35 binary toxin component (de Maagd *et al.*, 2003), to the database. Additionally, the Cry36 protein shows homology to BinA and BinB (de Maagd *et al.*, 2003).

Gene	Base position	Amino acid position	Nucleotide and amino acid in strain							
			IAB59 (Bin1)		2362 (Bin2)		2297 (Bin3)		LP1G (Bin4)	
binB	700	69	G	**Ala**	T	Ser	T	Ser	T	Ser
	705	70	A	Lys	C	Asn	C	Asn	C	Asn
	824	110	**T**	**Ile**	C	Thr	C	Thr	T	**Ile**
	1206	239	G	Ala	G	Ala	G	Ala	A	Ala
	1435	314	C	**His**	C	Leu	T	**Tyr**	C	**His**
	1436	314	A	**His**	T	Leu	A	**Tyr**	A	**His**
	1446	317	G	**Leu**	T	Phe	G	**Leu**	G	**Leu**
	1455	320	C	Ser	T	Ser	C	Ser	C	Ser
	1660	389	T	Leu	T	Leu	A	**Met**	A	**Met**
	1677	394	G	Ser	G	Ser	G	Ser	A	Ser
binA	2139	42	C	Ile	C	Ile	T	Ile	T	Ile
	2169	52	T	Asn	T	Asn	C	Asn	C	Asn
	2253	80	C	Ala	C	Ala	T	Ala	T	Ala
	2291	93	T	Leu	T	Leu	T	Leu	C	[illegible]
	2308	**99**	**G**	**Val**	**G**	**Val**	**T**	**Phe**	**G**	**Val**
	2323	**104**	**G**	**Glu**	**G**	**Ala**	**T**	**Ser**	**T**	**Ser**
	2324	**104**	**A**	**Glu**	**C**	**Ala**	**C**	**Ser**	**C**	**Ser**
	2386	125	C	His	C	His	A	Asn	A	Asn
	2412	133	T	Leu	T	Leu	C	Leu	C	Leu
	2417	135	A	Tyr	A	Tyr	T	**Phe**	T	**Phe**
	2490	159	A	Ser	A	Ser	T	Ser	T	Ser
	2643	210	C	Thr	C	Thr	G	Thr	G	Thr
	2745	243	C	Ile	C	Ile	T	Ile	T	Ile
	2813	267	G	Arg	G	Arg	A	Lys	A	Lys

Table 1.1 Bin toxin sequence comparison between the four variants. Amino acid residues differing from the reference type, Bin2, are highlighted yellow with those involved in host determination also shown in bold. The L→S amino acid change in BinA, conferring lower toxicity on the Bin4 variant is highlighted green. Adapted from Humphreys and Berry (1998).

```
BinA2                        MRNLDFIDSFIPTEGKYIR---VMDFYNSEYPFCIHAPSAPNGDI
BinB2       MCDSKDNSGVSEKCGKKFTNYPLNTTPTSLNYNLPEISKKFYNLKNKYSRNGYGLSKTEF

BinA2       MTEICSRENNQYFIFFPT-----------DDGRVIIANRHNGSVFTGEAT-------SVV
BinB2       PSSIENCPSNEYSIMYDNKDPRFLIRFLLDDGRYIIADRDDGEVFDEAPTYLDNNNHPII

BinA2       SDIYTGSPLQFFREVKR------TMATYYLAIQNPESATDVRALEPHSHELPSRLYYTNN
BinB2       SRHYTGEERQKFEQVGSGDYITGEQFFQFYTQNKTRVLSNCRALDSRTILLSTAKIFPIY

BinA2       --IENNSNILISNKEQIYLTLPSLPENEQYPKTPVLSGIDDIG--PNQSEKSIIGSTLIP
BinB2       PPASETQLTAFVNSSFYAAAIPQLPQTSLLENIPEPTSLDDSGVLPKDAVRAVKGSALLP

BinA2       CIMVSD-FISLGERMKTTPYYYVKHTQYWQSMWSALFPPGSKETKTEKSGITDTSQISMT
BinB2       CIIVHDPNLNNSDKMKFNTYYLLEYKEYWHQLWSQIIPAHQTVKIQERTGISEVVQNSMI

BinA2       DGINVSIGADFGLRFGNKTFGIKGGFTYDTKTQITNTSQLLIETTYTREYTNTENFPVRY
BinB2       EDLNMYIGADFGMLFYFRSSGFKEQITRGLNRPLSQTTTQLGERVEEMEYYNSNDLDVRY

BinA2       TGYVLASEFTLHRSDGTQVNTIPWVALNDNYTTIARYP----------------------
BinB2       VKYALAREFTLKRVNGEIVKN--WVAVDYRLAGIQSYPNAPITNPLTLKHTIIRCENSY

BinA2       ---HFASEPLLGNTKIITDDQN
BinB2       DGHIFKTPLIFKNGEVIVKTNEELIPKINQ
```

Figure 1.2 Alignment of BinA and BinB, highlighting the four conserved blocks. The BinA2 and BinB2 variants are shown with identical residues highlighted yellow, except for the identical residues forming the conserved blocks which are highlighted green (block A), blue (block B), magenta (block C) and orange (block D).

1.2.4 BinA and BinB binary toxins

Early studies indicated that BinA alone, purified from *B. sphaericus* 2362, was toxic to mosquito larvae (LC_{50} of 35 ng/ml) and cultured *C. quinquefasciatus* cells (LC_{50} of 1μg/ml) while BinB was not toxic, even at a concentration of 100μg/ml (Baumann *et al.*, 1985; Broadwell and Baumann, 1987). However, the LC_{50} of 35 ng/ml for BinA toxicity against *C. pipiens* larvae in contrast to 6 ng/ml for the crystal toxin, suggested a role for BinB in contributing to toxicity. Further experiments, where both toxins were cloned and expressed in *E. coli* and *B. subtilis*, showed that both components were required for toxicity and that cells expressing either BinA or BinB alone were non-toxic (Broadwell *et al.*, 1990a; Broadwell *et al.*, 1990b; Davidson *et al.*, 1990). It was also shown that an equimolar amount of BinA and BinB yielded greatest toxicity (LC_{50} of 15.4 ng/ml at 24 h) (Davidson *et al.*, 1990; Nicolas *et al.*, 1993). Interestingly, Nicolas *et al.* found that BinA expressed alone in recombinant *B. thuringiensis* subsp. *israelensis*, under the control of the *cytA* promoter, was larvicidal (LC_{50} of 1.9 μg/ml at 24 h, 300 ng/ml at 48 h), however toxicity was greatly enhanced by the addition BinB (Nicolas *et al.*, 1993). Davidson *et al.* proposed that the reason for the toxicity observed for the 43 kDa protein, corresponding to BinA, purified by Baumann *et al.* (Baumann *et al.*, 1985) was due to contamination with degradation products of BinB (Davidson *et al.*, 1990). This was confirmed by absence of toxicity of the 43 kDa protein when pre-treated with antiserum to BinB (Baumann and Baumann, 1991).

Subsequent binding studies confirmed the requirement for both components of the toxin to be present for toxicity. Radiolabelled toxins bound at equimolar amounts to the gastric caecum and posterior midgut of larvae (Davidson, 1988; Davidson *et al.*, 1990; Charles *et al.*, 1997). It was also found that BinA does bind to *A. gambiae* gut

membrane without BinB, although BinB greatly enhances BinA binding (Charles *et al.*, 1997). Further information regarding toxin binding was provided by Oei *et al.* who showed that regional binding of BinA to larval midgut was dependent on BinB, and that BinA alone exhibited only weak binding (Oei *et al.*, 1992). Continuing experiments using N- and C-terminal deletions of BinA and BinB showed the N-terminus of BinB to be involved in an association with the N-terminus and C-terminus of BinA (Clark and Baumann, 1990; Oei *et al.*, 1990; Oei *et al.*, 1992). Mutational analysis further complemented this work by showing that replacement of certain amino acid residues within the N- and C-terminal regions of both toxin components abolished toxicity (Shanmugavelu *et al.*, 1998) and that while mutations in the N- and C-terminal regions of BinA abolished toxicity, toxin binding was not affected (Elangovan *et al.*, 2000). Variants mutated at either the N- or C-termini of BinB and BinA were non-toxic when combined with their wild-type binary partner. Interestingly, a mixture of both the N-terminal and C-terminal mutants of either Bin component, combined with its wild-type partner, was able to complement each other functionally to restore toxicity, suggesting that the Bin components form multimeric structures allowing functional complementation to occur (Shanmugavelu *et al.*, 1998; Delecluse *et al.*, 2000). Mutation of BinA at a C-terminal residue, R^{312}, resulted in complete loss of toxicity (Elangovan *et al.*, 2000). These results may indicate a role for the N-terminus of BinB in larval midgut receptor binding and the C-terminus of BinA, specifically residue R^{312}, in toxicity. It is predicted that amino acid 93 of BinA is positioned in an α-helix and is thus sensitive to modification. Disruption of the predicted helix by such a modification as the L→S modification, seen in the lower toxicity Bin4 variant of LP1G, may disturb a BinA-BinB interaction and thus may be the cause of the lower toxicity of this variant (Yuan *et al.*, 2001).

Recombinant expression of BinA and BinB individually in *B. subtilis*, *B. sphaericus* or *B. thuringiensis* acrystalliferous hosts results in the production of amorphous crystals (Charles *et al.*, 1993; Nicolas *et al.*, 1993). However, when BinA and BinB are expressed together in *B. sphaericus* and *B. thuringiensis* more native crystals are produced (Charles *et al.*, 1993; Nicolas *et al.*, 1993).

1.2.5 Proteolytic processing of the Bin toxin

Clark and Baumann, and Davidson *et al.* confirmed the proteolytic processing of BinA to a 39 kDa protein and BinB to a 43 kDa protein (Clark and Baumann, 1990; Davidson *et al.*, 1990) as previously found by Broadwell and Baumann (Broadwell and Baumann, 1987) and Davidson *et al.* (Davidson *et al.*, 1987). Incubation of BinA with trypsin, α-chymotrypsin and mosquito gut extract also resulted in processing to a 39 kDa product (Davidson *et al.*, 1987). While the processed BinA product of 39 kDa was toxic to cultured *C. quinquefasciatus* cells, the 43 kDa processed form of BinB was non-toxic (Davidson *et al.*, 1987; Baumann and Baumann, 1991). The possibility that the toxic activity of the 39 kDa product was a result of contamination with BinB was ruled out by pre-incubation of the 39 kDa protein with antiserum for BinB (Baumann and Baumann, 1991; Baumann *et al.*, 1991). The apparent lack of contribution of BinB, at the time, raised questions as to its role. The importance of BinB for toxicity in the physiological environment of the larval gut was later determined through the binding studies (Davidson *et al.*, 1990; Oei *et al.*, 1992) described above.

Deletion experiments corresponding to BinB cleavage at potential trypsin and chymotrypsin cleavage sites showed that 32 and 53 aa can be removed from the N- and C-termini (Clark and Baumann, 1990), and that 10 and 17 aa can be removed

from the N- and C-termini of BinA (Broadwell *et al.*, 1990c) without loss of toxicity. Figure 1.3 summarises the findings of these experiments. Experiments performed by Oei *et al.* provided additional information for these deletion mutants, revealing that between 34-39 aa and 52-54 aa can be deleted from the N- and C-termini of BinB and no more than 6 aa and at least 17 aa can be removed from the C- and N-termini of BinA without loss of the essential cores required for toxicity (Oei *et al.*, 1990).

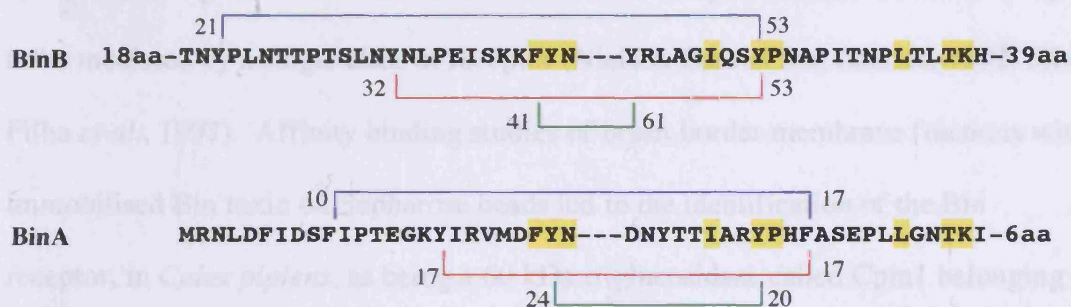


Figure 1.3 Summary of N- and C-terminal deletions of BinA and BinB at possible proteinase cleavage sites. Blue lines indicate proteins equivalent to the predicted products of processing in the larval gut, corresponding to potential chymotrypsin sites. Red lines represent the smallest constructed deletions of BinA and BinB that retained toxicity. Green lines represent the largest proteins constructed that lacked toxicity. Both protein sequences are aligned with identical residues highlighted in yellow. Adapted from Baumann *et al.* (Baumann *et al.*, 1991). Numbers correspond to the number of amino acids deleted from the termini.

1.2.6 Binary toxin mode of action

The localisation of Bin to the gastric caecum and posterior midgut (Davidson *et al.*, 1990), its proteolytic processing (Broadwell and Baumann, 1987; Davidson *et al.*, 1987; Clark and Baumann, 1990; Davidson *et al.*, 1990) and its solubilisation under alkaline pH (Davidson, 1983; Baumann *et al.*, 1985) indicate early events in the mode of action. Larval ingestion of crystal-spore complexes is followed by solubilisation in the alkaline larval gut and processing of the solubilised toxin by gut proteinases. Binding studies using brush border membrane fractions from *Anopheles* and *Culex* mosquito showed the interaction of the binary toxin with the larval midgut to be mediated by a single class of receptor (Nielsen-Leroux and Charles, 1992; Silva-Filha *et al.*, 1997). Affinity binding studies of brush border membrane fractions with immobilised Bin toxin on Sepharose beads led to the identification of the Bin receptor, in *Culex pipiens*, as being a 60 kDa α-glucosidase called Cpm1 belonging to the α-amylase family, that is membrane bound via a glycosyl-phosphatidylinositol (GPI) anchor (Silva-Filha *et al.*, 1999; Darboux *et al.*, 2001). Studies also showed that Bin was able to bind Cpm1 when expressed in the Sf9 insect cell line (Darboux *et al.*, 2002) and in mammalian epithelial cells (Pauchet *et al.*, 2005). It was also recently determined that the different Bin toxin variants from strains IAB59 (Bin1) and 2362 (Bin2) share the same receptor (Silva-Filha *et al.*, 2004). Membrane studies (Cokmus *et al.*, 1997; Schwartz *et al.*, 2001) have confirmed earlier theories of Bin's larvicidal activity by pore formation. Schwartz *et al.* showed that both BinA and BinB form pores, with BinA forming larger channels. BinB facilitated the BinA channel forming effect, with a 1:1 mixture being more effective and forming pores more akin to the large BinA channels than the smaller BinB channels (Schwartz *et al.*, 2001).

1.2.7 Mtx toxins

Mtx toxins are expressed at the vegetative stage of cell growth and are widely distributed among both high toxicity (*bin*$^+$) and low toxicity (*bin*$^-$) *B. sphaericus* strains (Thanabalu *et al.*, 1991; Liu *et al.*, 1996; Thanabalu and Porter, 1996; Priest *et al.*, 1997). These toxins were cloned in attempt to understand the larvicidal nature of the *bin*$^-$ strains, in particular strain SSII-1 (Singer, 1973).

1.2.7.1 Mtx 1

Mtx1 was cloned from *B. sphaericus* strain SSII-1, by screening of cosmid clones of SSII-1 total DNA in *E. coli*, for toxicity towards *C. quinquefasciatus* (Thanabalu *et al.*, 1991). Subcloning of a toxic clone and sequence analysis revealed an open reading frame for a 100 kDa toxin designated Mtx1. The C-terminal region of Mtx1 has internal repeats (Thanabalu *et al.*, 1992) typical of ricin-like beta trefoil repeats thought to be involved in carbohydrate binding (Hazes and Read, 1995). The N-terminal region has regions of homology to toxins that function by ADP-ribosylation of target G proteins, such as pertussis and cholera toxin (Thanabalu *et al.*, 1992; de Maagd *et al.*, 2003). Mtx1 shows no homology to BinA or BinB. The genes encoding Mtx1 from strains 2297 and LP1G were also recently cloned (Shi *et al.*, 2003; Promdonkoy *et al.*, 2004). Analysis of expression of Mtx1, by fusion of the promoter to *lacZ*, confirmed expression during vegetative growth (Ahmed *et al.*, 1995), consistent with the identification of a putative vegetative promoter upstream of *mtx1* (Thanabalu *et al.*, 1991). The contribution of Mtx1 to *B. sphaericus* toxicity is, however, limited by two factors: i) levels of transcription from the *mtx1* promoter appear to be low (Ahmed *et al.*, 1995) possibly due to repression at an upstream inverted repeat (Thanabalu *et al.*, 1991) and ii) the low levels of Mtx1 produced are

degraded by an endogenous proteinase (Wati *et al.*, 1997). This proteinase, called sfericase, is a calcium dependent subtilisin-like serine proteinase and its crystal structure was recently solved (Almog *et al.*, 2003). Indeed, expression of Mtx1 in strains lacking this proteinase results in improved levels of production (Thanabalu and Porter, 1995).

A 97 kDa form of Mtx1, lacking its predicted signal sequence (Thanabalu *et al.*, 1991), was expressed in *E. coli* and purified as a glutathione S-transferase (GST) fusion protein (Thanabalu *et al.*, 1992). Another form of the toxin, lacking both the putative signal sequence (residues 1-29) and membrane-spanning sequence (residues 43-60) was found to be non-toxic (Thanabalu *et al.*, 1992). Mtx1 was found to have an LC_{50} of 15 ng/ml against *C. quinquefasciatus* larvae after cleavage of the GST fusion tag, similar to that of the Bin toxin. The lower toxicity of the SSII-1 strain thus suggested that the protein was either expressed at a low level or that the protein was unstable (Thanabalu *et al.*, 1992). Incubation of Mtx1 with larval gut extracts revealed that the protein was processed to a 27 kDa N-terminal peptide and a 70 kDa C-terminal peptide, with the N-terminal product containing the region showing homology to ADP-ribosyltransferase toxins and the C-terminal peptide containing the region comprising the ricin-like beta trefoil repeat sequences (Thanabalu *et al.*, 1992). ADP-ribosyltransferase toxins are two subunit (AB) toxins with an enzymatically active A-moiety and a receptor-binding B-moiety that transfer an ADP-ribose from NAD^+ onto target proteins such as G-proteins, altering their function. The 27 kDa N-terminal peptide was shown to be responsible for ADP-ribosylation of two proteins of 38 kDa and 42 kDa in *C. quinquefasciatus*, while the C-terminal peptide was able to cause toxicity in cultured *C. quinquefasciatus* cells (Thanabalu *et al.*, 1993). Both regions are required for toxicity to mosquito larvae.

Kinetic studies identified Glu^{197} of the N-terminal 27 kDa product (amino acid residues 30-264) as the catalytic glutamate that is conserved among ADP-ribosyltransferases (Schirmer *et al.*, 2002a). Mutation of this residue to a glutamine resulted in loss of ADP-ribosyltransferase activity. The C-terminal 70 kDa product of processing was also found to remain non-covalently bound to the 27 kDa enzymatic component, inhibiting its activity (Schirmer *et al.*, 2002a; Carpusca *et al.*, 2004). Production of the 27 kDa unit of Mtx1 (residues 30-264) in *E. coli* results in toxicity to the bacterial cell while an enzymatically inactive mutant of the peptide is successfully expressed (Schirmer *et al.*, 2002a). Later studies suggested that this toxicity was due to ADP-ribosylation of *E. coli* elongation factor Tu (EF-Tu), preventing the formation of an EF-Tu:aminoacyl-tRNA:GTP complex (Schirmer *et al.*, 2002b). This may suggest an important role for the 70 kDa region in autoinhibition of the ADP-ribosyltransferase activity and thus preventing inhibition of protein synthesis in bacterial cells producing Mtx1.

The crystal structure of the catalytic domain of Mtx1 was recently solved (figure 1.4) (Reinert *et al.*, 2006). As the catalytic domain (residues 30-264) is itself toxic to *E. coli*, the protein was expressed including its inhibitory C-terminal linker (shown in red in figure1.4) that is processed from the catalytic domain when the 100 kDa toxin is processed to its 27 kDa and 70 kDa moieties. The protein was also expressed in two mutant forms which were catalytically inactive, to improve expression yield. The folding of the Mtx1 domain is typical of the chainfold of all known ADP-ribosyltransferases (Reinert *et al.*, 2006). From this structure it can be said that Mtx1 is cleaved at an exposed activation loop (shown in green in figure 1.4). This results in the 70 kDa moiety, including the linker region shown in red in

figure 1.4, separating from the 27 kDa catalytic unit allowing NAD^+ to enter its binding site.

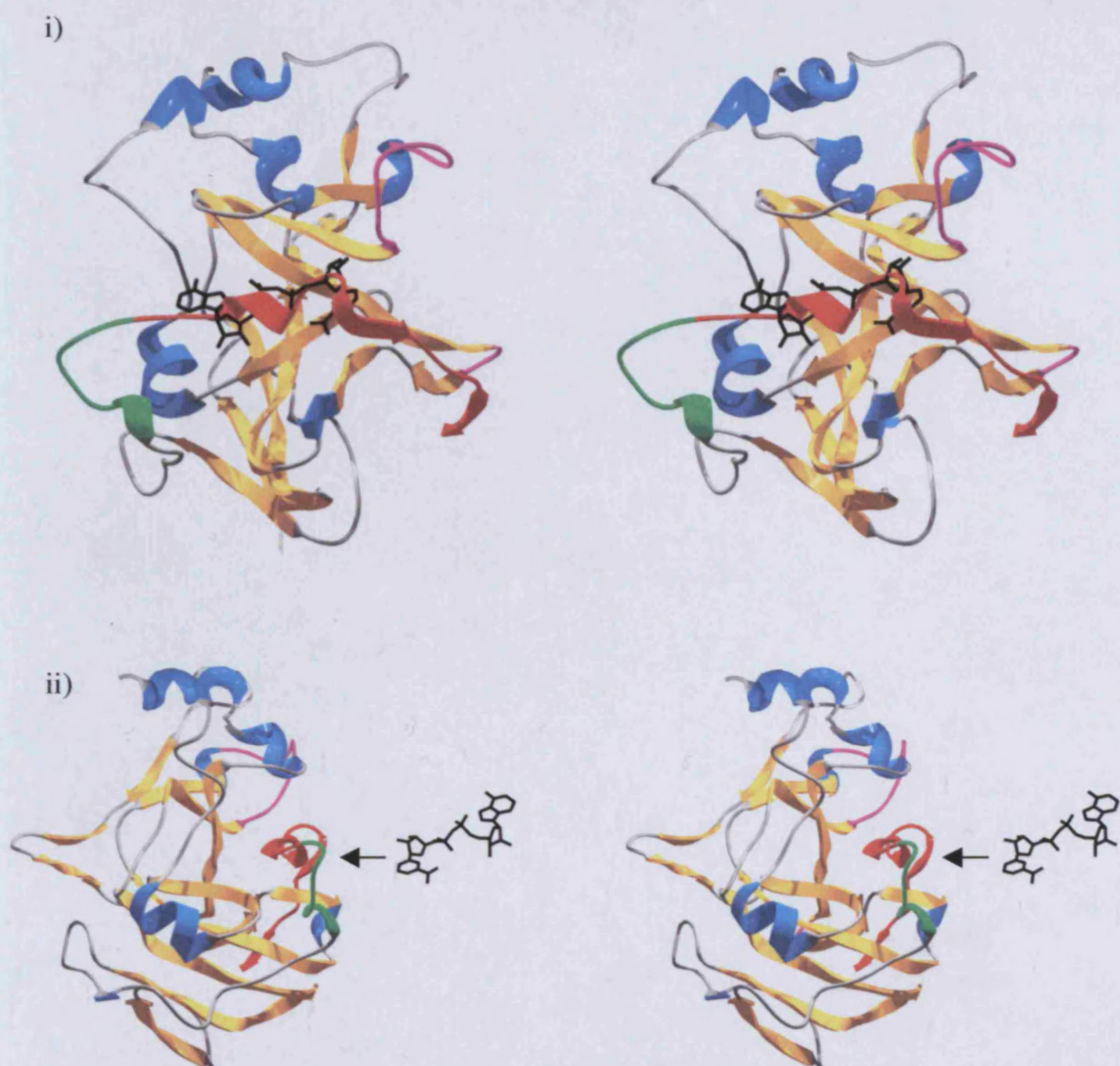


Figure 1.4 Crystal structure of the Mtx1 catalytic domain. The stereo images have α-helices shown in blue and β-sheets shown in gold. The C-terminal linker which occupies the NAD^+ binding site before processing at the activation loop (green) by larval gut enzymes is shown in red. NAD^+ is shown in black. The loop regions coloured in magenta are predicted to contact the target molecule. In (i) the NAD^+ has been placed approximately in its binding location, as described by Reinert *et al.* (Reinert *et al.*, 2006). ii) A view at approximately 90° to (i), rotated about the vertical axis, emphasising the inability of NAD^+ to enter its binding location due to the inhibitory linker (red). Images were created from the Protein data bank accession code 2CB6 for Mtx1 and the NAD^+ molecule was taken from its binding location in cholera toxin (PDB accession 2A5F) using the program DeepView/Swiss-PDB Viewer (Guex and Peitsch, 1996; Guex and Peitsch, 1997).

1.2.7.2 Mtx2 and Mtx3

The genes encoding Mtx2 and Mtx3 were cloned using a similar strategy to that used for the cloning of *mtx1*, by screening size fractionated genomic libraries of *B. sphaericus* SSII-1 against *C. quinquefasciatus* mosquito larvae (Liu *et al.*, 1996; Thanabalu and Porter, 1996). Both genes were shown by Southern hybridisation to be widely distributed amongst the high toxicity and lower toxicity strains of mosquitocidal *B. sphaericus* and their protein products were found to be highly conserved (Liu *et al.*, 1996; Thanabalu and Porter, 1996).

Mtx2 is a protein of 31.8 kDa (Thanabalu and Porter, 1996) while Mtx3 has a molecular weight of 35.8 kDa (Liu *et al.*, 1996). Mtx2, lacking it putative 15 residue N-terminal signal peptide was found to have an LC_{50} of 320 ng/ml at 48 hours against *C. quinquefasciatus* larvae (Thanabalu and Porter, 1996). While these proteins are less well characterised than Bin or Mtx1, studies have shown that the amino acid at position 224 of Mtx2 (a Lys in SSII-1 and Thr in strain 31-2) is a major determinant of toxicity and mosquito host range, while amino acids 279 and possibly 37 and 67 also play an important role (Chan *et al.*, 1996). Mtx2 and Mtx3 are closely related to each other and show homology to the cytotoxin of *Pseudomonas aeruginosa*, the ε-toxin of *Clostridium perfringens*, alpha-toxin of *Clostridium septicum* and aerolysin of *Aeromonas hydrophila* (de Maagd *et al.*, 2003). These toxins function by pore formation and aerolysin and ε-toxin are known to form heptameric complexes leading to pore formation (Lesieur *et al.*, 1999; Miyata *et al.*, 2002). The region of homology between these Mtx toxins and aerolysin includes the region involved in heptamerisation, and may suggest a multimeric pore forming role for Mtx2 and Mtx3 (de Maagd *et al.*, 2003).

1.3 *Bacillus thuringiensis*

B. thuringiensis was first discovered in 1901 and has been commercially available as an insecticide since the late 1950s. Strains have been isolated from many habitats worldwide, including soil, ponds, stored-product dust, insects and plant leaves (Schnepf *et al.*, 1998) and their ability to survive in multiple habitats makes them suitable for their use in the field as biocontrol agents. *B. thuringiensis* spores can survive for several years after application in the field, however, a rapid decline in population and toxicity is observed (Schnepf *et al.*, 1998). *B. thuringiensis*, along with *B. cereus* and *B. anthracis*, is a member of the *B. cereus sensu lato* group of bacteria and is distinguished from the other members by the production of parasporal crystals (Turnbull, 1999; Helgason *et al.*, 2000; Priest *et al.*, 2004).

The first mosquitocidal strain of *B. thuringiensis* was isolated in Israel in 1976 from a stagnant pond that provided a typical breeding site for *Culex pipiens* mosquito larvae (Goldberg and Margalit, 1977). This strain was later serotyped as H14 and designated *B. thuringiensis* serovar *israelensis* (*Bti*). *B. thuringiensis* is a gram positive, spore forming bacterium that forms parasporal crystals at stage II of sporulation. These crystals are responsible for toxicity towards target insects. In the case of *B. thuringiensis* subsp. *israelensis* activity is against Diptera including the Culicidae (mosquito) genera: *Culex*, *Aedes* and *Anopheles*, with higher specificity towards the former two (Delecluse *et al.*, 2000). There are also reports of *B. thuringiensis* subsp. *israelensis* toxicity against lepidopteran and coleopteran insects (Vassal *et al.*, 1993; Mendez-Lopez *et al.*, 2003). Importantly, the parasporal crystals show no toxicity towards vertebrates and other non-target organisms. The *B. thuringiensis* toxins, collectively called δ-endotoxins, consist of the Cry proteins with specific activity, and the Cyt family that have cytolytic and haemolytic activities.

In addition to the toxic crystal proteins produced at sporulation, some *B. thuringiensis* strains produce unrelated insecticidal proteins during the vegetative stage of growth, the VIP toxins (Estruch *et al.*, 1996). Also recently reported was a novel secreted toxin designated Sip1A (Donovan *et al.*, 2006).

Since the discovery of *B. thuringiensis* subsp. *israelensis*, other mosquitocidal strains have been isolated and classed into 3 groups (Charles and Nielsen-LeRoux, 2000; Delecluse *et al.*, 2000). Strains in class 1 show similar larvicidal, haemolytic and crystal polypeptide composition to *B. thuringiensis* subsp. *israelensis*; class 2 strains are nearly as toxic as *B. thuringiensis* subsp. *israelensis* but have different polypeptide compositions; class 3 strains produce polypeptides different to *B. thuringiensis* subsp. *israelensis* and are of low toxicity. A summary of the classification, crystal composition and toxicity of mosquitocidal strains of *B. thuringiensis* is shown in table 1.2.

Class	Strains	Serotype	Mosquitocidal activity			Crystal proteins kDa
			A. aegypti	*A. stephensi*	*C. pipiens*	
1	*israelensis* 1884	H14	+++	+++	+++	135, 125, 68, 28
1	*morrisoni* PG14	H8a, 8b	+++	+++	+++	144, 135, 125, 68, 28
1	*kenyae* LBIT-52	H4a, 4c	+++	ND	+++	135, 125, 68, 28
1	*canadensis* 11S2-1	H5a, 5c	+++	+++	+++	135, 125, 68, 28
1	*entomocidus* LBIT-58	H6	++	ND	+++	135, 125, 68, 28
1	*thompsoni* B175	H12	+++	+++	+++	135, 125, 68, 28
1	*malaysiensis* IMR81.1	H36	+++	+++	+++	135, 125, 68, 28
1	AAT028 K6		+++	+++	+++	135, 125, 68, 28
1	AAT021 B51		+++	+++	+++	135, 125, 68, 28
2	*jegathesan* 367	H28a, 28c	++	+++	++	80, 74-70, 65, 37, 26, 16
2	*medellin* 163-131	H30	++	+++	++	94, 70-68, 30, 28
3	*kurstaki* HD-1	H3a, 3b, 3c	+/-	+	ND	135-130, 66
3	*fukuokaensis* 84-I-1-13	H3a, 3d, 3e	+/-	ND	+/-	90, 86, 82, 72, 50, 48, 37, 27
3	*galleriae* 916	H5a, 5b	+/-	+/-	ND	135-130, 61
3	*canadensis* 89-T-5-9	H5a, 5c	+/-	ND	ND	65, 53, 28
3	*aizawai* ICI	H7	+/-	ND	ND	135-130
3	*darmstadiensis* 73-E10-2	H10a, 10b	+/-	+	+	125, 83, 79, 77, 69, 50, 27
3	*kyushuensis* 74 F6-18	H11a, 11c	+/-	+/-	+/-	140, 85, 80, 70, 66, 50, 27, 15, 14
3	*shandongiensis* 89-ST-1-25	H22	+/-	ND	ND	150, 70-60, 25
3	*higo* 92-KU-137-4	H44	ND	+	+/-	98, 91, 71, 63, 59, 50, 44, 27

Table 1.2 Properties of the mosquitocidal strains of *B. thuringiensis.*
Underlined molecular weights indicate proteins immunologically related to those from *B. thuringiensis* subsp. *israelensis.* +++, LC_{50} values similar to those of *B. thuringiensis* subsp. *israelensis.* ++, + and +/-: LC_{50} values of approx. 2-10, 10-50 and 50-1,500 fold respectively greater than *B. thuringiensis* subsp. *israelensis.* ND: not determined. Taken from Delecluse *et al.* (Delecluse *et al.*, 2000)

1.3.1 *B. thuringiensis* subsp. *israelensis* toxins

Crystals produced at sporulation of *B. thuringiensis* subsp. *israelensis* consist of four major proteins: 135, 125, 68 and 28 kDa in size. Based on the nomenclature for *B. thuringiensis* pesticidal crystal proteins (Crickmore *et al.*, 1998), these toxins have been designated Cry4Ba (135 kDa), Cry4Aa (125 kDa), Cry11Aa (68 kDa) and Cyt1Aa (28 kDa). Further analysis confirmed the presence of two additional toxin genes carried on pBtoxis; *cry10Aa*, encoding a 58 kDa protein (Thorne *et al.*, 1986) and *cyt2Ba*, encoding a 29.8 kDa protein (Guerchicoff *et al.*, 1997). The gene encoding a new putative toxin of 60 kDa, Cyt1Ca, was also recently discovered during the sequencing of pBtoxis (Berry *et al.*, 2002). This protein has an N-terminal half that shows homology to the Cyt toxin but contains an additional C-terminal domain rich in β-trefoil repeats, similar to those found in other bacterial toxins such as Mtx1 from *B. sphaericus* and ricin (Berry *et al.*, 2002; Itsko *et al.*, 2005). However, recent experiments suggest this protein to be non toxic (Manasherob *et al.* manuscript in preparation).

1.3.2 Cry toxin production

The mosquitocidal toxins of *B. thuringiensis* subsp. *israelensis*, unlike the Bin toxin from *B. sphaericus*, are encoded on a large plasmid pBtoxis (127,923 bp) (Berry *et al.*, 2002), and not on chromosomal DNA. The *cry* genes of *B. thuringiensis* are expressed during the stationary phase and their products can account for 20-30% of the dry weight of the spore. One manor in which a bacterium can accumulate a large amount of protein per cell is for expression to be driven by strong promoters in non-dividing cells, a method employed by *B. thuringiensis* during sporulation.

A number of genes encoding Cry toxins have been shown to be regulated by sporulation-specific promoters, including the Cry4Aa (Yoshisue *et al.*, 1993a), Cry4Ba (Yoshisue *et al.*, 1993b) and Cry11Aa (Dervyn *et al.*, 1995) mosquitocidal toxins from *B. thuringiensis* subsp. *israelensis*. The genes encoding the RNA polymerase σ factors that recognise these promoters, σ^{28} and σ^{35}, have been cloned (Adams *et al.*, 1991) and are homologues of the sporulation specific factors σ^{K} and σ^{E} from *B. subtilis* (Haldenwang, 1995). Sporulation independent *cry* gene expression is also observed. For example the *cry3Aa* gene from *B. thuringiensis* subsp. *tenebrionis* was found to be transcribed at a low level during vegetative growth as well as during sporulation and was found to have a promoter similar to those recognised by the vegetative cell factor, σ^{A} (Agaisse and Lereclus, 1994b). Additionally expression of *cry3Aa* in mutant strains was found not to be dependent on sporulation specific sigma factors in *B. subtilis* (Agaisse and Lereclus, 1994a) or *B. thuringiensis* (Salamitou *et al.*, 1996).

Post-transcriptional factors result in the stability of *cry* mRNA, which has an approximate half-life of 10 min, at least five times greater than the average for bacterial mRNA (Agaisse and Lereclus, 1995; Schnepf *et al.*, 1998). It has been demonstrated that the putative stem-loop transcriptional terminator of *cry1Aa* aids in the stability of its mRNA (Wong and Chang, 1986) probably by preventing 3'-5' exoribonuclease degradation of the mRNA (Agaisse and Lereclus, 1995; Schnepf *et al.*, 1998). As well as this 3' stabiliser, STAB-SD sequences have been found upstream of some *cry* genes (Agaisse and Lereclus, 1996). These STAB-SD sequences are perfect Shine Dalgarno sequences and while they may not direct the initiation of translation, they are predicted to bind to the 16S rRNA of the 30S ribosomal subunit, stabilising the transcript. In fact, mutations designed to weaken

the STAB-SD-16S rRNA interaction result in decreased stability of the transcripts (Agaisse and Lereclus, 1996). The STAB-SD sequence has also been shown to improve production levels of other Cry toxins and as a result has been incorporated into the *B. thuringiensis* expression vector pSTAB (Park *et al.*, 1998; Park *et al.*, 1999).

Post-translational factors also contribute to improved protein levels. *B. thuringiensis* produces its Cry toxins, as is the case for the *B. sphaericus* Bin toxin, as proteinase resistant crystals. The C-terminal half of the large 125-140 kDa Cry toxins, such as the Cry1 and Cry4 toxins, are cysteine rich and are thought to be involved in formation of disulphide bonds, contributing to the formation of crystals (Hofte and Whiteley, 1989; Bietlot *et al.*, 1990). Smaller toxins (<70 kDa) that do not posses the conserved C-terminus of the larger toxins are thought to form crystals by using accessory proteins such as Orf2 (e.g. for Cry2Aa) and P20 (e.g. for Cry11Aa) for post-translational stabilisation (Agaisse and Lereclus, 1995). The gene encoding Cry11Aa in *B. thuringiensis* subsp. *israelensis* is found in an operon with P19 and P20 (Dervyn *et al.*, 1995). P20 has been shown to increase levels of Cry11Aa and/or Cyt1Aa by post-translational stabilisation in recombinant *E. coli* (Visick and Whiteley, 1991; Wu and Federici, 1995) and acrystalliferous *B. thuringiensis* (Wu and Federici, 1993). Transgenic *E. coli* are also killed when expressing Cyt1Aa but can survive when coexpressed with P20 (Manasherob *et al.*, 2001). Recombinant expression of Cry2Aa crystals in acrystalliferous strains of *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* requires co-expression of Orf2, with disruption of the *orf2* gene resulting in loss of Cry2Aa crystal production (Crickmore and Ellar, 1992).

1.3.3 Structure and sequence similarities among the three-domain Cry toxins

Protein sequence alignment of Cry toxins has revealed five conserved blocks of amino acids in the mature N-terminal toxic half, as well as three additional blocks in the larger toxins containing the non-toxic C-terminal half removed during processing (Hofte and Whiteley, 1989; Schnepf *et al.*, 1998). Following the elucidation of the crystal structure of a number of Cry toxins, the regions corresponding to these conserved blocks have been mapped. For example, block 1 corresponds to the central helix, α5, of domain 1. Block 2 encompasses helix 7 of domain I and the first β-strand of domain II, block 3 includes the last β-strand of domain II and the beginning of domain III while blocks 4 and 5 lie in the buried strands of domain III.

To date the crystal structures have been determined for the following three-domain Cry toxins; the coleopteran active Cry3Aa (Li *et al.*, 1991) and Cry3Bb1 (Galitsky *et al.*, 2001), lepidopteran active Cry1Aa (Grochulski *et al.*, 1995), the dual-specific Lepidoptera/Diptera Cry2Aa (Morse *et al.*, 2001) and the Diptera specific Cry4Ba (Boonserm *et al.*, 2005) and Cry4Aa (Boonserm *et al.*, 2006). Figure 1.5 shows the crystal structure of the mosquitocidal Cry4Ba toxin from *B. thuringiensis* subsp. *israelensis*. The regions corresponding to the five conserved blocks of amino acids found in Cry toxins are also shown. The solved structures of the Cry toxins reveal that these proteins have three domains. Domain I consists of antiparallel α-helices, of which α5 is the central helix. Domain II consists of antiparallel β-sheets in a "Greek key" topology and domain III comprises antiparallel β-sheets forming a "jelly roll" topology (Schnepf *et al.*, 1998). As the first two helices of Cry4Ba were degraded during crystallisation and are hence missing from the determined structure (Boonserm *et al.*, 2005), domain I of Cry4Aa is shown in figure 1.6(i). A closer look

at domain II and domain III of Cry4Ba are also shown in figure 1.6(ii) and (iii) respectively.

i)

ii)

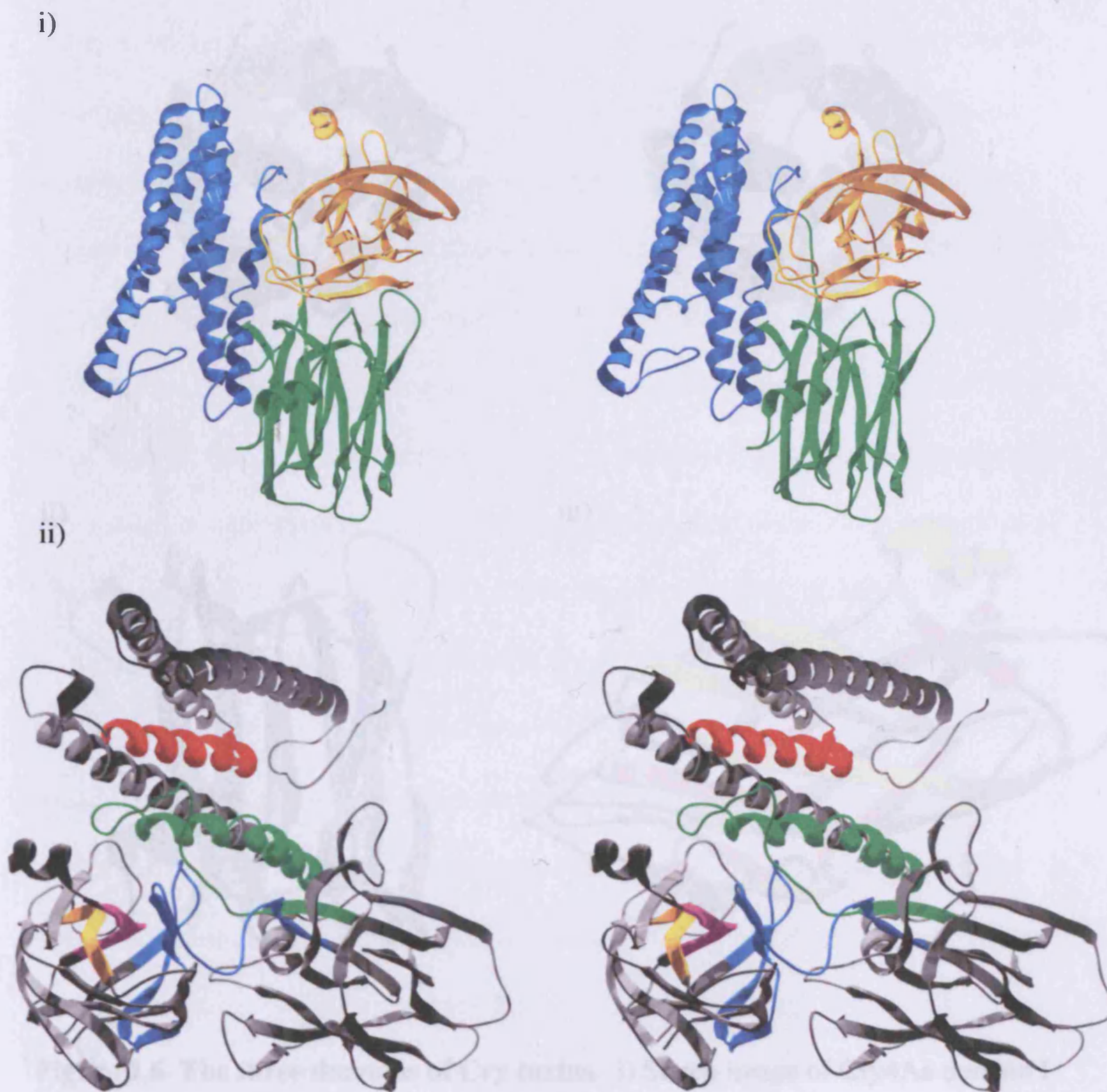
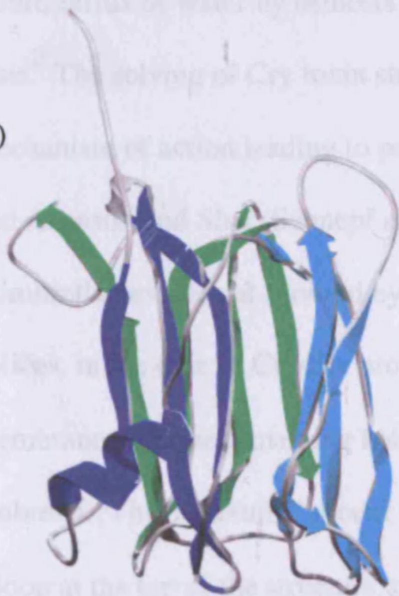


Figure 1.5 Crystal structure of Cry4Ba i) Stereo image of Domain I (blue), domain II (green) and domain III (gold). ii) Stereo image of Cry4Ba with the regions corresponding to the conserved blocks shown: block 1, red; block 2, green; block 3, blue; block 4, orange; block 5, magenta.

i)

ii)



iii)

Figure 1.6 The three domains of Cry toxins. i) Stereo image of Cry4Aa domain I with the individual α-helices coloured: α1, red; α2, green; α3, blue; α4, orange; α5, magenta; α6, light blue; α7, yellow. ii) Domain II of Cry4Ba showing the three antiparallel β-sheets as described by Boonserm *et al.* (Boonserm *et al.*, 2005): sheet 1, cyan; sheet 2, green; sheet 3, dark blue. iii) The domain III β-sandwich of Cry4Ba as described by Boonserm *et al.* (Boonserm *et al.*, 2005). Inner sheets and outer sheets are coloured red and yellow respectively.

Domain I contains hydrophobic and amphipathic helices. The helices of domain I are long enough to traverse a 30Å membrane, with the amphipathic α4-loop-α5 region predicted to undergo a conformational change and insert into the membrane (Boonserm *et al.*, 2005). This hypothesis of membrane pore formation, mediated by domain I, is consistent with early work by Knowles and Ellar where colloid osmotic lysis of cells was shown to be the result of pore formation (Knowles and Ellar, 1987). Pore formation was predicted to result in the influx of ions, and as a result, influx of water by osmosis leading to the swelling of cells and eventual cell lysis. The solving of Cry toxin structures led to the development of predictions of the mechanism of action leading to pore formation (for review of these see Schnepf *et al.* and Aronson and Shai (Schnepf *et al.*, 1998; Aronson and Shai, 2001)). The "Umbrella" model put forward by Li *et al.* (Li *et al.*, 1991) suggests that a pair of helices, in the case of Cry4Ba probably α4-α5 (Boonserm *et al.*, 2005), insert into the membrane with the remaining helices opening on the membrane surface like an umbrella. The "penknife" model suggests that helices α5 and α6, which are joined by a loop at the top of the structure, open like a penknife and insert into the membrane. For either of these models to be correct, a conformational change is required. Li *et al.* (Li *et al.*, 1991) proposed that this may occur following receptor binding and that the change may be transmitted from the receptor binding domain II to domain I via α7 (Knowles, 1994). Another possibility is that an inter-helical loop cleavage by gut proteinases between α5 and α6, as has been observed for Cry4Ba and Cry4Aa (Angsuthanasombat *et al.*, 1993), may facilitate this conformational change and allow membrane insertion to occur. Similar inter-helical loop cleavages have been observed for Cry2Aa (Nicholls *et al.*, 1989) and Cry3Aa (Carroll *et al.*, 1989; Li *et al.*, 1991).

However, removal of the cleavage site in Cry4Aa and Cry4Ba does not have a detrimental effect on toxicity (Angsuthanasombat *et al.*, 1993; Boonserm *et al.*, 2004).

The similarity of the surface exposed loops of domain II to immunoglobulin antigen-binding sites (Schnepf *et al.*, 1998), as well as the variability of this region among the Cry toxins (Boonserm *et al.*, 2005), suggested that they may be involved in receptor binding. Domain II also shows structural similarity to the carbohydrate binding proteins, the lectins, in particular jacalin, which is known to bind carbohydrates via the exposed loops at the apex of its β-prism fold (Schnepf *et al.*, 1998), again suggesting a role in receptor binding for this domain. Indeed, mutations in apical loop 3 of Cry4Ba, by introducing residues from the corresponding region of Cry4Aa, resulted in increased toxicity towards *Culex* larvae while mutations in loops 1 and 2 resulted in loss of toxicity towards *Aedes* and *Anopheles* (Abdullah *et al.*, 2003). Recently, experiments were also performed where the loops of Cry4Aa were altered to mimic those of Cry4Ba (Boonserm *et al.*, 2006). The loop 2 alteration dramatically reduced toxicity while changes in loop 1 and loop 3 yielded Cry4Aa mutants that remained toxic. These results suggests that different regions of Cry4Ba and Cry4Aa may contribute to host receptor binding (Boonserm *et al.*, 2006). Mutations in residues of these loops have also shown changes in binding of the Cry1Ac toxin to insect midgut brush border membrane vesicles (BBMV) (Smedley and Ellar, 1996) further signifying a role for domain II in receptor binding. Exposed loops in domain II of Cry11Aa from *B. thuringiensis* subsp. *israelensis* have been shown to be involved in receptor binding (Fernandez *et al.*, 2005) and a GPI-anchored alkaline phosphatase has been identified as a receptor for Cry11Aa in the *A. aegypti* larval gut (Fernandez *et al.*, 2006). Engineering of the lepidopteran active Cry1Aa, by introducing mutations into the loops of domain II has also resulted in a switch of

specificity towards *C. pipiens* mosquito larvae and loss of toxicity towards its natural target *Manduca sexta* (Liu and Dean, 2006).

Domain III is also thought to be involved in receptor binding. It has been shown that Cry1Aa and Cry1Ab bind tightly to purified aminopeptidase-N (APN) found on *Manduca sexta* epithelial cell membranes (Knight *et al.*, 1994; Masson *et al.*, 1995). Domain III may function as a lectin-like domain that binds to N-acetyl galactosamine (GalNAc) on APN (Burton *et al.*, 1999). Also a 210 kDa cadherin-like receptor has been cloned from *Manduca sexta* (Vadlamudi *et al.*, 1995). Domain III has also been suggested to prevent further degradation of the Cry toxins after proteolytic processing (Li *et al.*, 1991; Schnepf *et al.*, 1998).

1.3.4 Cry toxin mode of action

As is the case for the *B. sphaericus* Bin toxin, Cry toxins are produced as crystals which are ingested and solubilised by target insects before proteolytic processing by midgut proteinases. In the case of *B. thuringiensis* subsp. *israelensis* toxins, Cry4Aa and Cry4Ba are processed initially to 60-68 kDa proteins before further processing to 46-48 kDa mature toxins (Angsuthanasombat *et al.*, 1992) and Cry11Aa is processed to a 30-35 kDa toxin (Dai and Gill, 1993). The Cyt1Aa toxin, which will be discussed shortly, is processed to a 25 kDa protein (Koni and Ellar, 1994). In general, the Cry toxins are processed by chymotrypsin and trypsin like proteinase activity (Rukmini *et al.*, 2000). The large >125 kDa Cry toxins, such as the Cry1 and Cry4 toxins, are processed at the N-terminus as well as at the C-terminus, the latter resulting in removal of the C-terminal half of the pro-toxin which is not required for toxicity (Rukmini *et al.*, 2000). Differential processing of

the same toxin can also result in different target insect specificity (Haider *et al.*, 1986; Haider and Ellar, 1987a; Haider and Ellar, 1987b).

Proteolytic processing of Cry toxins is followed by receptor binding and gut membrane pore formation resulting in colloid-osmotic lysis of cells (Knowles and Ellar, 1987) and larval death, and is predicted to involve the regions of the toxins described in the previous section. Studies involving activated Cry4Ba toxin have shown that it is capable of permeabilising liposomes (Puntheeranurak *et al.*, 2001) and that ionic channels are formed in planar lipid bilayers (Puntheeranurak *et al.*, 2004). Also, the ability of Cry4Ba to insert into different lipid monolayers, varying in features such as lipid composition and packing density, has been explored (Kanintronkul *et al.*, 2005). More recently atomic force microscopy has provided images of the activated Cry4Ba in lipid bilayers and data suggested that pore formation results from a tetrameric interaction of the toxin molecules (Puntheeranurak *et al.*, 2005).

1.3.5 Cry34/Cry35 binary toxins

The Cry34 and Cry35 insecticidal proteins have been isolated from *B. thuringiensis* strains toxic to *Diabrotica virgifera virgifera* (Western Corn Rootworm) (Moellenbeck *et al.*, 2001; Ellis *et al.*, 2002; Baum *et al.*, 2004; Masson *et al.*, 2004; Schnepf *et al.*, 2005). Both the 14 kDa Cry34 and the 44 kDa Cry35 protein components are required for toxicity. Conserved domain searches, using Cry35Ab1 as a query, revealed that the N-terminal 146 residues contains two repeats of a beta-trefoil carbohydrate-binding domain (Schnepf *et al.*, 2005). The Cry35 proteins show homology to both Bin toxin components from *B. sphaericus* and the coleopteran active Cry36Aa1 toxin from *B. thuringiensis* (Ellis *et al.*, 2002; de Maagd

et al., 2003; Schnepf *et al.*, 2005). As a result the Cry34/Cry35 pair have been termed a *B. thuringiensis* binary toxin.

1.3.6 Cyt toxins

The Cyt toxins, together with the Cry toxins, form crystals at sporulation but differ in that they are able to lyse a wide range of cell types *in vitro* (Schnepf *et al.*, 1998). However, *in vivo*, their toxicity is towards the dipteran larvae (Butko, 2003). The Cyt toxins are able to synergise with the Cry toxins, preventing the emergence of Cry-resistance (Wirth *et al.*, 1997). Recent data suggests that Cyt1Aa from *B. thuringiensis* subsp. *israelensis* synergises with Cry11Aa by functioning as a membrane bound receptor (Perez *et al.*, 2005). Key amino acid residues involved in the Cyt1Aa-Cry11Aa interaction were also determined in this study. Analysis of Cyt toxin protein sequences have revealed four conserved blocks of amino acids (Butko, 2003). Figure 1.7 shows the crystal structure Cyt2Aa1 as determined by Li *et al.* (Li *et al.*, 1996). The structure contains a central core of β-sheet surrounded by two α-helical hairpins. The regions of Cyt2Aa1 mapping to the conserved blocks of amino acids are shown in figure 1.7(ii). The two proposed mechanisms of action of Cyt toxins are described in a review by Butko (Butko, 2003). One model proposes that multimers of a Cyt toxin forms pores in the membrane's lipid bilayer while another proposes a detergent-like disruption of the membrane where the Cyt aggregates cause defects in the lipid packaging.

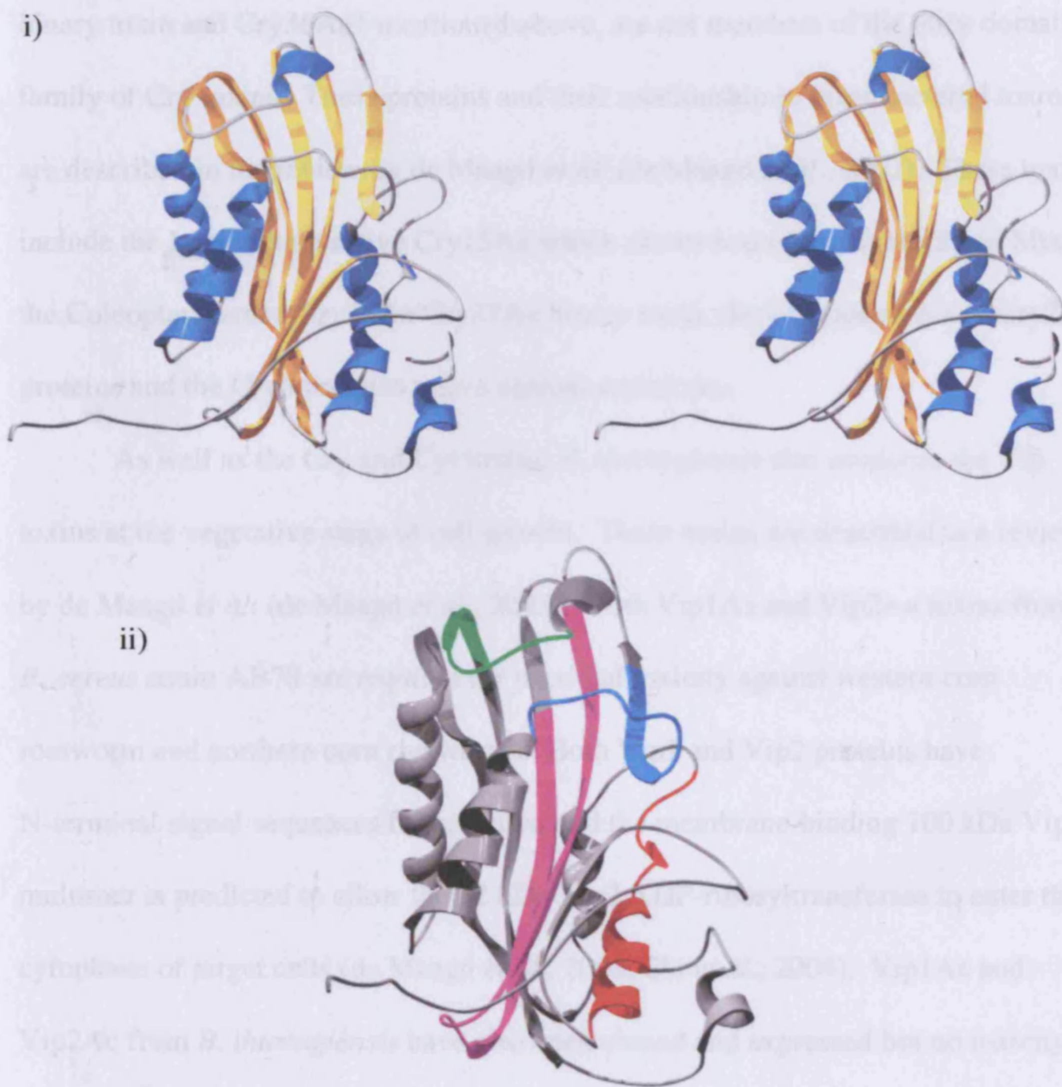


Figure 1.7 X-ray crystallographic structure of Cyt2Aa1. i) Stereo image showing the central β-sheet core in gold and the surrounding α-helices in blue. ii) Cyt2Aa1 showing the regions corresponding to the four conserved blocks of amino acids: block 1, red; block 2, blue; block 3, magenta; block 4, green.

1.3.7 Other *B. thuringiensis* toxins

A number of Cry toxins from *B. thuringiensis*, including the Cry34/Cry35 binary toxin and Cry36Aa1 mentioned above, are not members of the three domain family of Cry toxins. These proteins and their relationship to other bacterial toxins are described in the review by de Maagd *et al.* (de Maagd *et al.*, 2003). These toxins include the Lepidoptera active Cry15Aa which shows homology to Mtx2 and Mtx3, the Coleoptera active Cry23Aa/Cry37Aa binary toxin, the Coleoptera active Cry22 proteins and the Cry6 proteins active against nematodes.

As well as the Cry and Cyt toxins, *B. thuringiensis* also produces the Vip toxins at the vegetative stage of cell growth. These toxins are described in a review by de Maagd *et al.* (de Maagd *et al.*, 2003). Both Vip1Aa and Vip2Aa toxins from *B. cereus* strain AB78 are required for maximal toxicity against western corn rootworm and northern corn rootworms. Both Vip1 and Vip2 proteins have N-terminal signal sequences for secretion and the membrane-binding 100 kDa Vip1 multimer is predicted to allow the 52 kDa Vip2 ADP-ribosyltransferase to enter the cytoplasm of target cells (de Maagd *et al.*, 2003; Shi *et al.*, 2004). Vip1Ac and Vip2Ac from *B. thuringiensis* have also been cloned and expressed but no toxicity was detected, either individually or in combination, against a range of lepidopteran and coleopteran larvae (Shi *et al.*, 2004). The genes encoding Vip3Aa and Vip3Ab were cloned from *B. thuringiensis* and the protein products were found to contain 5 variant amino acids (Estruch *et al.*, 1996). Bioassay of the 88.5 kDa Vip3A proteins revealed toxicity to a number of lepidopteran insect larvae (Estruch *et al.*, 1996). Immunolocalisation experiments showed that Vip3A binds to the midgut epithelium of susceptible larvae (Yu *et al.*, 1997). The gene encoding Vip3Aa16, also known as Vip3LB, was recently cloned and determined to be toxic against the Mediterranean

Flour Moth, *Ephestia kuehniella* (Mesrati *et al.*, 2005). The 91 kDa Vip3Ba1 protein from *B. thuringiensis* has also been expressed in recombinant form and was found to be non-toxic against the range of larvae bioassayed (Rang *et al.*, 2005).

Recently a novel *B. thuringiensis* toxin, active against coleopteran larvae, which is secreted into the culture was identified and named Sip1A (secreted insecticidal protein) (Donovan *et al.*, 2006). The 41 kDa plasmid-encoded protein has some sequence similarity with Mtx3 from *B. sphaericus* and its putative protein secretion signal (residues 1-30) has been identified.

1.4 Mosquito resistance to *B. sphaericus*

While the arsenal of toxins produced by *B. thuringiensis* subsp. *israelensis* delays any emergence of resistance (Georghiou and Wirth, 1997) the dependence of the *B. sphaericus* spore on a single toxin, Bin, has resulted in the selection of Bin-resistant mosquito populations. Field resistance to *B. sphaericus* has been recorded in Brazil (10 fold), India (150 fold), France (10,000 fold), China (25,000 fold) and Tunisia (2,000 fold) (for review see Charles and Nielsen-LeRoux (Charles and Nielsen-LeRoux, 2000)). Mosquito populations with resistance levels of >100,000 fold have also been selected under laboratory conditions (Pei *et al.*, 2002; Yuan *et al.*, 2003).

The development of resistance to *B. sphaericus* is not particularly surprising due to the low number of toxins present in the sporulated cultures used in the field. General rules of resistance management mean that multiple toxins with different modes of action or individual toxins capable of binding multiple receptors are more successful, requiring multiple and simultaneous mutations in the target insect for resistance development. Although the Bin toxin consists of both BinA and BinB, its

mechanism of action, with BinB being the receptor-binding component and BinA the membrane pore former, functions as a single toxin with one toxin-receptor interaction. The lower toxicity Mtx2 and Mtx3 toxins also do not contribute as larvicides in the field due to their expression in the vegetative stage of growth and absence from the spore stage used in biocontrol programmes. The highly potent Mtx1 is also vegetatively expressed (Ahmed *et al.*, 1995) and the low level of protein that accumulates is degraded by the action of an endogenous proteinase (Thanabalu and Porter, 1995; Wati *et al.*, 1997).

In an attempt to understand the mechanism of resistance development to *B. sphaericus*, binding studies were performed (Nielsen-Leroux *et al.*, 1995). In the case of a highly-resistant laboratory selected colony, no toxin binding was observed suggesting a non-functional receptor. For field-resistant colonies from France (high level resistance) and Brazil (low level resistance), toxin-receptor binding was unchanged (Silva-Filha *et al.*, 1997), and furthermore, gut extracts were capable of proteolytically activating the toxin. Therefore, while high selection pressure for resistance in the laboratory results in receptor modification, this does not seem to be the case in field resistant colonies, at least not in receptor binding function, suggesting another mechanism for this resistance. Studies into the inheritance of *B. sphaericus* resistance, by mating resistant and susceptible colonies and backcross mating of the F_1 progeny with the resistant colony, have shown the allele to be recessive (Nielsen-Leroux *et al.*, 1997; Wirth *et al.*, 2000a; Nielsen-Leroux *et al.*, 2002; Oliveira *et al.*, 2004). Following the identification of the larval gut receptor for Bin (Silva-Filha *et al.*, 1999; Darboux *et al.*, 2001) further experiments have revealed that the gene encoding the receptor, Cpm1, in laboratory resistant *C. pipiens* larvae contained a

number of mutations, the critical one being a premature stop codon resulting in loss of the GPI-anchoring site (Darboux *et al.*, 2002).

Studies have shown that the cytolytic Cyt1Aa, although weakly toxic individually, may in particular contribute to the ability of *B. thuringiensis* subsp. *israelensis* to evade the development of resistance thanks to its ability to synergise with the Cry toxins (Wu *et al.*, 1994; Crickmore *et al.*, 1995). For example, sublethal doses of Cyt1Aa were shown to greatly reduce Cry toxin resistance against the *B. thuringiensis* subsp. *israelensis* toxins Cry4Aa, Cry4Ba and Cry11Aa (Wirth *et al.*, 1997). Recent work has also shown that while 1,000 fold resistance developed in *C. quinquefasciatus* larvae selected with Cry11A, a 3:1 mixture of Cry11A:Cyt1A resulted in delay of resistance development, with only 8 fold resistance being induced (Wirth *et al.*, 2005). With such observations, the introduction of *B. thuringiensis* toxins into *B. sphaericus* has been investigated in attempt both to improve toxicity towards low susceptibility targets such as *A. aegypti* (Trisrisook *et al.*, 1990; Poncet *et al.*, 1997; Servant *et al.*, 1999; Wirth *et al.*, 2001; Wirth *et al.*, 2004) and against resistant *Culex* populations (Poncet *et al.*, 1997; Thiery *et al.*, 1998; Servant *et al.*, 1999; Wirth *et al.*, 2001; Wirth *et al.*, 2004). Results were promising with studies showing not only increased toxicity towards resistant *C. quinquefasciatus* larvae but also against *A. aegypti.* Studies have also shown that Cyt1A synergises with the Bin toxin resulting in increased toxicity towards resistant *C. quinquefasciatus* (Wirth *et al.*, 2000b) larvae as well as greatly suppressing the emergence of resistance (Wirth *et al.*, 2005). The disadvantage of introducing *B. thuringiensis* toxins into *B. sphaericus* on plasmids or by homologous recombination into the chromosome is their classification as recombinant, which is problematic for their development for use in the field. As such, a recent study has also introduced the toxin coding plasmid,

pBtoxis, from *B. thuringiensis* subsp. *israelensis* into *B. sphaericus* by a natural mating technique to determine whether a non-recombinant approach may be a viable method for preventing resistance development, as well as improving toxicity towards *A. aegypti* larvae (Gammon *et al.*, 2006). Increased toxicity towards both resistant *Culex* larvae and *A. aegypti* was observed but plasmid maintenance was unstable.

All records of *B. sphaericus* field resistance to date have been against strains 2362, 1593 and C3-41 (all serotype H5a5b) and it has been shown that cross-resistance occurs between different strains (Rodcharoen and Mulla, 1996; Pei *et al.*, 2002), including strains that produce distinct Bin variants such as 2362 (Bin2) and 2297 (Bin3) (Wirth *et al.*, 2000a; Yuan *et al.*, 2003). However, studies have identified strains such as IAB59, LP1G, 47-6B and IAB872 that have the ability to overcome *C. quinquefasciatus* larval resistance developed after exposure to strains 2362 and C3-41 (Wirth *et al.*, 2000a; Shi *et al.*, 2001; Pei *et al.*, 2002; Shi *et al.*, 2003; Yuan *et al.*, 2003). The binary toxin of strain IAB872, cloned for expression in an acrystalliferous *B. thuringiensis* subsp. *israelensis*, shows no toxicity against Bin-resistant mosquito larvae, suggesting that the strain carries unknown toxins (Shi *et al.*, 2001). Similarly it has been shown that the Bin1 variant of strain IAB59 recognises the same receptor as the Bin2 toxin from 2362 (Silva-Filha *et al.*, 2004) and is not toxic to a resistant *C. quinquefasciatus* colony raised against strain 2362 (Pei *et al.*, 2002), again suggesting that unknown toxins are present in strain IAB59. The ability of these strains to overcome Bin toxin resistance holds promise for their development for use in the field. Other potential approaches would be to use recombinant *B. sphaericus* strains expressing toxins from *B. thuringiensis* subsp. *israelensis* (Federici *et al.*, 2003), the use of *B. sphaericus* rather than *B. thuringiensis* being particularly beneficial due to its higher resistance to UV light and ability to

persist longer in polluted waters (Silapanuntakul *et al.*, 1983; Nicolas *et al.*, 1987; Yousten *et al.*, 1992). Other approaches that would avoid the use of recombinant bacteria would be to use a rotation strategy of *B. sphaericus* and *B. thuringiensis* in the field, however, investigations point to a mixture of both bacteria rather than a rotation strategy being more effective in delaying resistance (Zahiri and Mulla, 2003).

1.5 Other bacterial toxins

Bacterial toxins have a number of mechanisms by which they exert their toxicity, for example: damaging cell membranes, inhibiting protein synthesis, activating second messenger pathways, inhibiting neurotransmitter release, or activating the host immune response (Schmitt *et al.*, 1999). Some toxins having such mechanisms are described below.

Anthrax is a disease caused by inhalation of spores of the bacterium *Bacillus anthracis*. Spores replicate in the blood without an evident immune response, leading to death, suggesting that the pathogen impairs the host immune system (Abrami *et al.*, 2005). The pXO1 plasmid-encoded lethal factor (LF), edema factor (EF) and protective antigen (PA) make up the anthrax toxin components. PA (83 kDa) is the component responsible for receptor binding to target cells and after LF (90 kDa) and EF (89 kDa) promoted heptamerisation of PA, internalisation of the PA receptor by endocytocis occurs. PA pore formation of intraluminal vesicles within early endosomes allows LF and EF translocation across the pore into the lumen of the vesicle where they are predicted to refold. The protection of LF and EF in the intraluminal vesicle from proteinases, allows a "Trojan horse" like delivery of LF and EF to late endosomes, and finally, the cytoplasm (Abrami *et al.*, 2005). Release of EF into the cytoplasm results in increased levels of cAMP and LF is responsible for

proteolysis of mitogen-activated protein kinase kinases (MAPKKs). Pathogenesis involves inhibition of the immune system.

The Botulinum and tetanus neurotoxins are produced by the gram positive bacteria *Clostridium botulinum* and *Clostridium tetani* respectively. There are seven botulinum neurotoxin serotypes (A-G) and together with the tetanus neurotoxin they form the clostridial neurotoxin (CNT) family (Turton *et al.*, 2002). The CNTs are produced as a 150 kDa single chain polypeptide and are post-translationally cleaved to yield active toxins composed of a 50 kDa A subunit (light chain) and a 100 kDa BC subunit (heavy chain) linked by a disulphide bond. The CNTs, through their heavy chain, bind to the presynaptic membrane of cholinergic motor neurons and are internalized by endocytosis. The botulinum toxin, light chain, cleaves proteins responsible for release of acetylcholine at the neuromuscular junction causing inhibition of neurotransmission. After internalisation, the tetanus neurotoxin migrates to the central nervous system by retrograde vesicular transport and enters inhibitory interneurons. Proteolysis of proteins responsible for release of glycine and gama-amino-butyric acid results in muscle contraction and hence spastic paralysis (Schmitt *et al.*, 1999; Turton *et al.*, 2002).

Staphylococcus aureus produces the 33 kDa α-toxin which binds to target membranes and forms a non-lytic pre-pore heptamer complex which undergoes a conformational change allowing insertion into the membrane (Schmitt *et al.*, 1999). The α-toxin pore allows small molecules and ions to traverse the membrane, leading to swelling of cells and cell death, and osmotic lysis in the case of erythrocytes. Aerolysin, from *Aeromonas hydrophila*, is another toxin that forms a heptamer prior to pore formation (Parker *et al.*, 1996).

As is the case for Mtx1, many bacterial toxins such as Diphtheria toxin, exotoxin A from *Pseudomonas*, cholera toxin, pertussis toxin, *E. coli* heat-liable toxin, Botulinum C2 and Botulinum C3 toxins function by ADP-ribosylation of target proteins (for review see Krueger and Barbieri (Krueger and Barbieri, 1995)). In general, these toxins consist of a receptor binding subunit(s) (B subunit) and a catalytic subunit (A subunit) which transfers an ADP-ribose from NAD^+ onto a target protein such as a G-protein (guanine binding protein) or a translational elongation factor, inhibiting the GTPase of G-proteins and host-cell protein synthesis respectively.

1.6 Project aims

As described above, instances of Bin-resistance have been recorded in populations of *Culex* mosquito larvae and thus threatens the effectiveness of *B. sphaericus* control of *Culex* larvae. However, more recently strains have been discovered (e.g. IAB59, LP1G and 47-6B) that have the ability to overcome this resistance (Yuan *et al.*, 2003). While a common protein of approximately 49 kDa was suggested as a putative toxin (Nielsen-LeRoux *et al.*, 2001; Pei *et al.*, 2002; Yuan *et al.*, 2003; Silva-Filha *et al.*, 2004), attempts to clone the gene for this candidate toxin proved unsuccessful (Nielsen-LeRoux personal communication). Therefore, the objective of this study was to identify, clone and characterise the toxic factor(s) from the *B. sphaericus* strains able to overcome Bin-resistance in *Culex* mosquito larvae. Such information may prove invaluable in designing strategies to prevent the emergence of resistance to *B. sphaericus* in the field.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Cloning and expression vectors

The cloning vectors pGEM-T (Promega Ltd, Southampton, UK) and pUC18 (Yanisch-Perron *et al.*, 1985) were used for general cloning. The *E. coli* expression vector pGEX-4T-2 was purchased from Amersham Biosciences UK Ltd. (Bucks, UK). A modified pET22b vector, for expression of N-terminal His-tagged proteins, was donated by Mr. Tim Winterburn (Cardiff School of Biosciences, Cardiff University). The *B. thuringiensis-E. coli* shuttle vector, pHT304 (Arantes and Lereclus, 1991), was a kind gift from Dr. Didier Lereclus (Institut Pasteur, Paris, France). The *B. thuringiensis* expression vector pSTAB (Park *et al.*, 1998; Park *et al.*, 1999) was received from Professor Brian Federici (Department of Entomology, University of California Riverside, USA). The cosmid pHC79 (Hohn and Collins, 1980) was used for construction of genomic libraries. Clones pHT680 and pHT684 allowing expression of BinA and BinB respectively in *B. thuringiensis* subsp. *israelensis* 4Q7 were kind gifts from Dr. Christina Nielsen-LeRoux (INRA, Paris, France).

2.1.2 *E. coli* host strains

All genetic manipulations were carried out in the *E. coli* strain DH5α (Invitrogen Ltd., Paisley, UK). Cosmid genomic DNA libraries were prepared in *E. coli* strain LE392 (Promega Ltd, Southampton, UK). Protein expressions were carried out in the *E. coli* strain BL21(DE3)pLysS (Novagen, AMS Biotechnology, Milton Keynes, UK). The genotype of these strains can be seen in table 2.1

E. coli strain	Genotype	Reference
DH5α	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	(Hanahan, 1983)
LE392	F^- *hsd*R574 (r_k^-, m_k^+) *sup*E44 *sup*F58 *lac*Y1 *gal*K2 *gal*T22 *met*B1 *trp*R55	(Promega, 1996)
BL21(DE3)pLysS	F^- *omp*T $hsdS_B$(r_B^-, m_B^-) *gal dcm lon* λ(DE3) *lacIQ/placUV5-T7gene1* pLysS (Cam^R)	(Studier and Moffatt, 1986)

Table 2.1 Genotype of *E. coli* strains used in this study

2.1.3 *Bacillus sphaericus* strains

All *B. sphaericus* strains were obtained from the International Entomopathogenic *Bacillus* Centre (Institut Pasteur, Paris, France), except for strain NHA15b which was a kind gift from Dr. Christina Nielsen-LeRoux (Institut Pasteur, Paris, France).

2.1.4 *Bacillus thuringiensis* subsp. *israelensis* host strain

B. thuringiensis subsp. *israelensis* strain 4Q7 (also known as 4Q2-81) is a plasmid cured host and, therefore, does not carry the genes for insecticidal proteins. This strain was obtained from the International Entomopathogenic *Bacillus* Centre (Institut Pasteur, Paris, France).

2.1.5 *Bacillus subtilis* strains

B. subtilis mutant strains 1S38 (*spoIIIC94 trpC2*), 1S60 (*leuB8 spoIIG41 tal-1*) and 1S86 (*sigF1 trpC2*) were acquired from the *Bacillus* Genetic Stock Centre (Department of Biochemistry, The Ohio State University, Columbus, Ohio, USA).

2.1.6 Media

Luria Bertani (LB) nutrient medium was prepared as described by Sambrook *et al.* (Sambrook *et al.*, 1989) and the reagents were purchased from Sigma Chemical Ltd. (Poole, Dorset, UK). Culture media were sterilised by autoclaving at 121°C (975kPa) for 20 min.

NYSM broth (Myers and Yousten, 1980) consisted of 13g/l nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, UK) and 0.5g/l yeast extract (Difco Laboratories, Oxford, UK). The medium was autoclaved at 121°C (975kPa) for 20 min and after cooling, the following reagents were added to the final concentrations shown: 0.7mM $CaCl_2$, 1mM $MgCl_2$, 50μM $MnCl_2$ from 1,000 fold stock solutions previously filter sterilised through 0.22μm nitrocellulose filters (Millipore Ltd, Watford, Hertz, UK).

Embrapa sporulation medium contained 8g/990ml nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, UK), 1g/990ml yeast extract (Difco Laboratories, Oxford, UK) and 1g/990ml K_2HPO_4 (Fisher Scientific UK Ltd., Loughborough, UK). To this, 10ml of a salt solution (100mM $CaCO_3$, 40mM $MgSO_4$, 3.6mM $FeSO_4$, 3.6mM $MnSO_4$, 3.5mM $ZnSO_4$) was added and the pH adjusted to 7.0. The medium was autoclaved at 121°C (975kPa) for 20 min.

Antibiotics, when required, were added to media at the final concentrations of: ampicillin (100μg/ml), chloramphenicol (34μg/ml), erythromycin (15μg/ml) and streptomycin (150μg/ml). Antibiotics were added to media by dilution of 1,000 fold stock solutions, after the temperature of the media had cooled to below 50°C. Antibiotic stock solutions were stored at -20°C.

2.1.7 Enzymes

Calf intestinal alkaline phosphatase, T4 DNA ligase and *Taq* DNA polymerase were purchased from Promega Ltd (Southampton, UK). Easy–A®, a proofreading DNA polymerase which adds 3'-A overhangs to amplicons, was purchased from Stratagene (California, USA). Lysozyme, proteinase K, trypsin and α-chymotrypsin were purchased from Sigma Chemical Ltd. (Poole, Dorset, UK). All restriction endonucleases were purchased from New England Biolabs (Beverly, MA, USA).

2.1.8 Antisera

The mouse monoclonal Penta-His antibody, specifically recognises a stretch of five consecutive histidine residues on His-tagged proteins and was purchased from Qiagen Ltd. (Crawley, West Sussex, UK). The goat polyclonal Anti-GST antibody specifically recognises glutathione S-transferase (GST) and was used in the detection of recombinant GST-fusion proteins. This antibody was purchased from Amersham Biosciences UK Ltd. (Bucks, UK).

2.1.9 Molecular weight standards

The λ *Hin*dIII and φX174 *Hae*III DNA markers were purchased from Promega Ltd. (Southampton, UK). Prestained broad range molecular weight protein

standards were purchased from New England BioLabs (Beverly, MA, USA). The molecular weights of the protein standards and base pair length of the DNA markers can be seen below.

Prestained protein marker, broad range – New England BioLabs

Protein	Apparent MW (Da)
MBP-β-galactosidase	175,000
MBP-paramyosin	83,000
Glutamic dehydrogenase	62,000
Aldolase	47,500
Triosephosphate isomerase	32,500
β-Lactoglobulin A	25,000
Lysozyme	16,500
Aprotinin	6,500

DNA markers

λ DNA/*Hind*III (bp)	φX174 DNA/*Hae*III (bp)
23,130	1,353
9,416	1,078
6,557	872
4,361	603
2,322	310
2,027	281
564	271
125	234
	194
	118

2.1.10 Other materials

Routine laboratory chemicals were purchased from Sigma Chemicals Ltd. (Poole, Dorset, UK) and Fisher Scientific UK Ltd. (Loughborough, UK). Suppliers of more specialised chemicals and equipment are shown below or in the appropriate sections of the text.

Reagents and Equipment	Suppliers
QIAprep® Spin Miniprep Kits	Qiagen, Crawley, West Sussex, UK
ProtoGel® (30%(w/v) acrylamide, 0.8%(w/v) bisacrylamide solution)	National Diagnostics, Manville, NJ, USA
0.1cm, 0.2cm and 0.4cm electroporation cuvettes	Bio-Rad Laboratories, Hertfordshire, UK
DNeasy® Tissue Kit	Qiagen, Crawley, West Sussex, UK
QIAquick® Gel Extraction Kits	Qiagen, Crawley, West Sussex, UK
QIAEXII® Gel Extraction Kit	Qiagen, Crawley, West Sussex, UK
QIAGEN® Genomic-Tip 500/G	Qiagen, Crawley, West Sussex, UK

2.2 Molecular biology methods

2.2.1 Preparation of plasmid DNA

Plasmid DNA was purified from 3ml of an overnight culture of a plasmid bearing strain of *E. coli* (incubated at 37°C, 200 rpm) using the QIAprep® Spin Miniprep Kit as described in the manufacturer's protocol. This method is a modification of the alkaline lysis method (Birnboim and Doly, 1979) that uses a resin purification step for rapid isolation of high quality DNA.

2.2.2 Submarine agarose gel electrophoresis

Agarose gels were used both analytically and for preparative purification of DNA fragments (section 2.2.4). Agarose (Bioline Ltd., London, UK) was dissolved, by boiling, in TAE buffer (40mM Tris acetate, 2mM EDTA, pH 8.3) to the desired final concentration, ranging from 0.5% to 2%, depending on the size of the DNA fragments to be separated. The solution was allowed to cool to 50°C before the addition of ethidium bromide solution (20mg/ml) to a final concentration of 0.5μg/ml. The gel solution was poured into clean electrophoresis trays and well-forming combs were inserted before the gel was allowed to set. Gels were run submerged in TAE buffer containing 0.5μg/ml ethidium bromide at 5-10V/cm after sample loading, until the desired separation was achieved.

2.2.3 DNA modifying enzymes and restriction endonucleases

DNA modifying enzymes and restriction endonucleases were used in accordance with the manufacturer's instructions. Restriction digests were analysed by submarine electrophoresis (section 2.2.2). Digest fragment sizes were estimated by

comparison of their migration relative to λ *Hin*dIII and/or ϕX174 *Hae*III DNA markers.

2.2.4 Recovery of DNA fragments from agarose gels

DNA fragments to be purified were excised from 1-2% agarose gels, depending on fragment sizes, following submarine electrophoresis (section 2.2.2). DNA extraction was performed using the QIAquick® Gel Purification Kit (Qiagen Ltd., Crawley, West Sussex, UK) in accordance with the manufacturer's protocol.

DNA fragments of greater than 10 kb were purified using QIAEXII® Gel Extraction Kit (Qiagen Ltd., Crawley, West Sussex, UK) according to the manufacturer's protocol.

DNA fragments of 20-40 kb, used for genomic library construction, were purified from LMP-agarose. An equal volume of TE buffer was added to the excised gel in a microfuge tube and the tube incubated at 65°C until the gel was fully melted. The DNA was purified by extracting once with an equal volume of TE saturated phenol, followed by two extractions with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1(v:v:v)). The DNA was precipitated by addition of sodium acetate buffer, pH 5.2 (0.3M final concentration) and 0.7 volumes of isopropanol. The mixture was gently mixed by inversion, the DNA harvested by centrifugation (17,000 x g, 15 min) and the pellet rinsed with 70% ethanol. The DNA pellet was left to air dry and was finally resuspended in nuclease free water or TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) at room temperature, overnight. To avoid shearing of the large DNA fragments all mixing steps were carried out gently, by inversion.

2.2.5 Precipitation of DNA

DNA was precipitated by addition of sodium acetate buffer, pH 5.2 to a final concentration of 0.3M and 0.7 volumes of isopropanol. The sample was mixed by vortexing. DNA was harvested by centrifugation (17,000 x g, 15 min) and the pellet rinsed with 70% ethanol. The DNA pellet was left to air dry and was finally resuspended in the required volume of nuclease-free water or TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0).

2.2.6 Polymerase chain reaction (PCR) amplification of DNA

2.2.6.1 Basic PCR for cloning and screening

In sterile Thermowell™ PCR tubes (Corning Incorporated, New York, USA) a mixture consisting of PCR buffer (10mM Tris-HCl, 50mM KCl, 2mM $MgCl_2$, 0.1% Triton® X–100, pH 9.0), dNTPs (0.2mM of each; dGTP, dTTP, dCTP, dATP), the appropriate forward and reverse oligonucleotide primers (0.5μM each) and template DNA (typically 1–10ng per 50μl reaction) were mixed with nuclease-free water to a final volume of 25μl or 50μl. *Taq* Polymerase (Promega, Southampton, UK) was added to a concentration of 2.5 units per 50μl reaction mix. Template DNA was typically double stranded plasmid DNA but for the screening of large numbers of transformant colonies, bacterial cells were added directly to the PCR mixture. Tubes were spun briefly in a microfuge, before the PCR was carried out using a Biometra® T3 thermocycler (Whatman Biometra®, Goettingen, Gemrnany). Denaturation, annealing, extension temperatures and numbers of cycles are described in the appropriate sections of text. PCR products were analysed by submarine gel electrophoresis and purified if required (sections 2.2.2 and 2.2.4).

To avoid the introduction of mutations into amplified PCR products required for cloning, the proofreading DNA polymerase Easy–A (Stratagene, California, USA) was used according to the manufacturer's recommendations.

2.2.6.2 Inverse PCR

Inverse PCR was used to amplify DNA of unknown sequence flanking one end of a region of known DNA sequence, against which primers were designed. The procedure was carried out as described previously (Triglia *et al.*, 1988; Hartl and Ochman, 1996). Details of primer design and template DNA digestion with restriction endonucleases are described in the appropriate sections of the text.

Template genomic DNA (5μg) was digested with the appropriate restriction enzyme (section 2.2.3). The resulting digested DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1(v:v:v)), followed by precipitation with sodium acetate and isopropanol (section 2.2.5), and resuspension of the DNA to a final concentration of 100μg/ml. Intramolecular ligations of the cleaved genomic DNA to form circularised DNA were prepared using a range of template DNA concentrations. Digested DNA (0.1-1μg/ml), DNA ligase buffer (Promega Ltd., Southampton, UK) and T4 DNA ligase (3 units) (Promega Ltd., Southampton, UK) were added to nuclease-free water in a final reaction volume of 100μl and the ligation mixture was incubated at 16°C for 16 hours. The resulting circularised DNA (1-10ng) was used as template in a 50μl PCR (section 2.2.6.1) using primers designed to anneal at the region of known DNA sequence. The PCR products were analysed by agarose gel electrophoresis (section 2.2.2) and reactions yielding a single DNA fragment were purified (section 2.2.4), cloned (section 2.2.7) and sequenced (section 2.2.10).

2.2.7 Ligation of DNA into plasmid vectors

DNA inserts and plasmid vectors were digested with the appropriate restriction endonucleases. The digest products were separated by gel electrophoresis and the required products purified as described in section 2.2.4 before ligation. Usually a vector:insert molar ratio of 1:4 was added to 1x DNA ligase buffer (Promega Ltd., Southampton, UK) in a 10μl final reaction volume. T4 DNA ligase (3 units) (Promega Ltd., Southampton, UK) was added and the resulting mixture incubated at 16°C for 16 hours.

PCR products were ligated into the pGEM-T vector using the pGEM-T Vector Systems Kit ™ (Promega Ltd., Southampton, UK) according to the manufacturer's instructions. The pGEM-T vector is supplied in linearised form, with overhanging 3' thymidines at the insertion point allowing efficient ligation of PCR products generated by certain thermostable polymerases that add a single 3' deoxyadenosine in a template independent manner to the end of PCR products (Zhou *et al.*, 1995; Zhou and Gomez-Sanchez, 2000).

After incubation of the ligation reactions, generally 1μl of the ligation mixture was transformed into *E. coli* DH5α by electroporation (as described in section 2.3.2.2) and where possible blue-white screening (section 2.2.8) of colonies was performed to identify positive recombinants.

Preparation and ligation of partially digested genomic DNA into cosmid pHC79 was carried out as described by Sambrook *et al.* (Sambrook *et al.*, 1989) and is described in the appropriate section of text.

2.2.8 Colour screening of transformant bacterial colonies for inserts

Recombinant clones, after transformation of ligation reactions, were identified by colour screening using IPTG/X-Gal indicator plates. This method was only used when successful ligation of an insert into a cloning vector resulted in the interruption of the β-galactosidase coding sequence at the MCS (multiple cloning site). IPTG (100μl of 100mM) and X-Gal (20μl of 50mg/ml; Promega Ltd, Southampton, UK) were spread onto the surface of LB agar plates containing the appropriate antibiotics for selection. The plates were incubated at 37°C for 30 min to allow absorption to occur. Cultures containing transformant bacterial clones (section 2.3.2) were spread onto the plates and incubated at 37°C overnight. Blue colonies were ignored while white colonies indicating successful insert-vector ligation were selected for further analysis.

2.2.9 Preparation of *E. coli* glycerol stocks

Glycerol stocks were prepared of *E. coli* cosmid and plasmid libraries for long term storage at -80°C. An overnight culture of *E. coli* (0.7ml), grown in LB medium containing the appropriate antibiotics, was thoroughly mixed with 0.3ml of sterile 50% glycerol. *E. coli* cells stored in 15% glycerol are stable at -80°C for many years after flash freezing in liquid nitrogen. Glycerol stocks were used to streak LB agar plates when regeneration of a working stock was required. A sterile inoculating loop was used to pick fragments of frozen cell-glycerol mixture, avoiding thawing of the glycerol stock, and streaked onto LB agar plates containing the appropriate antibiotics.

2.2.10 Double stranded DNA sequencing

Nucleotide sequence of DNA was determined using an automated adaptation of the dideoxy method of Sanger *et al.* (Sanger *et al.*, 1977). Automated sequencing of plasmid DNA was carried out by Lark™ (Hope End, Takeley, Essex, UK) using high throughput PE Biosystems sequencers. Chromatograms produced by the sequencers were analysed using the computer program EditView 1.0.1 ABI Automated DNA Sequence Viewer (Applied Biosystems, CA, USA).

2.2.11 Introducing specific mutations into target DNA

2.2.11.1 QuikChange Site-directed mutagenesis

Rapid site directed mutagenesis of target DNA was achieved using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, California, USA) according to the manufacturer's recommendations. This procedure is based on that described by Hemsley *et al.* (Hemsley *et al.*, 1989), using complementary primers carrying the desired mutation to amplify template plasmid DNA by PCR. PCR products containing the desired mutation are selected by using the restriction enzyme *Dpn*I, which specifically cleaves at $G^{Me6}ATC$ sequences in methylated and hemimethylated DNA generated by *dam*$^{+}$ bacteria. The undigested amplified DNA containing the desired mutation was transformed into *E. coli* (section 2.3.2.2), the plasmid DNA purified (section 2.2.1) from selected transformant bacterial colonies and sequenced (section 2.2.10) to confirm the successful introduction of a desired mutation. Primer sequences and PCR cycling parameters are described in the appropriate section of text.

2.2.11.2 Site-specific mutagenesis by Overlap Extension

The introduction of specific mutations into genes using Overlap Extension PCR (Ho *et al.*, 1989) was carried out as described in Sambrook and Russell (Sambrook and Russell, 2001). The procedure involves using a pair of primers to amplify a fragment of DNA containing the desired mutation along with the upstream region of DNA. A second PCR is used to amplify a DNA fragment containing the same mutation along with the downstream gene sequence. The two PCR products are used in a final PCR where the overlapping, mutated regions anneal together and the 3'ends of both strands are extended by proofreading DNA polymerase activity. Subsequent cycles of PCR amplify the extended fragments using primers that anneal to the extreme ends of the target gene. The mutated fragment was purified (section 2.2.4), cloned into a suitable vector (section 2.2.7) and sequenced (section 2.2.10) to confirm the introduction of the desired mutation. Primer sequences and PCR conditions are described in the appropriate sections of text.

2.2.12 Isolation of *B. sphaericus* total DNA

Genomic DNA for cosmid library construction was purified based on the method of Ausubel *et al.* (Ausubel *et al.*, 1987). An overnight 100ml culture was harvested by centrifugation (5,000 x g, 10 min) and the pellet resuspended in 9.4ml TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). Lysozyme solution (100μl of 100 mg/ml) was added and incubated at 37°C for 30 min. Complete lysis was achieved by addition of 0.5ml of 10%(w/v) SDS solution and 50μl of Proteinase K solution (20 mg/ml). The mixture was incubated at 37°C for 1 hour before the addition of 1.8ml NaCl solution (5M) and 1.5ml CTAB solution (10%(w/v) CTAB, 0.7M NaCl). This mixture was incubated at 65°C for 20 min. The mixture was

extracted with 1 volume of chloroform:isoamyl alcohol (24:1(v/v)) and centrifuged at 8,000 x g for 10 min. The aqueous layer was retained and the DNA precipitated by addition of 0.6 volumes of isopropanol. The precipitate was transferred to a tube containing 70% ethanol by spooling the DNA with a glass rod. The pellet was harvested by centrifugation at 8,000 x g, the supernatant discarded and the DNA resuspended in 4ml TE buffer overnight. The DNA concentration was adjusted to 100μg/ml before the addition of CsCl (1g CsCl per ml of DNA solution) and 100μl of ethidium bromide solution (10mg/ml). The mixture was transferred to QuickSeal centrifuge tubes (Beckman Coulter Ltd., Buckinghamshire, UK) and after balancing, the tubes were sealed and centrifuged at 390,000 x g for 16 hours at 15°C. The genomic DNA band was visualised using a UV lamp and extracted using a 5ml syringe and 16-G needle, with a 19-G needle inserted to provide an air inlet at the top of the tube. Ethidium bromide was removed from the DNA by extraction with TE saturated butan-1-ol and the CsCl removed by dialysis against two changes of 5 litres of TE buffer. The concentration of DNA was determined and adjusted by addition of TE buffer if required.

Genomic DNA to be used as PCR template or in restriction digests for Southern hybridisation and/or cloning was purified using the DNeasy® Tissue Kit or QIAGEN® Genomic-Tip 500/G (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's protocol.

2.2.13 Detection of *B. sphaericus* megaplasmids

Two methods were used for the detection and isolation of large plasmids in *B. sphaericus*. The first method was a modified version of that described by Jensen *et al.* (Jensen *et al.*, 1995), which is based on the method reported by Kado and Liu

(Kado and Liu, 1981). *B. sphaericus* strains were grown overnight (30°C, 200rpm) in 10ml of LB medium. The cells from 2ml of this culture were pelleted by centrifugation (10,000 x g, 2 min) and resuspended in 100μl E-buffer (40mM Tris-HCl, 2mM EDTA, pH 7.9). Lysozyme was added to a final concentration of 5mg/ml and the mixture incubated at 37°C for 30 min followed by the addition of 200μl lysis solution (50mM Tris-HCl, 15%(w/v) sucrose, 3%(w/v) SDS, freshly adjusted to pH 12.5 with 2M NaOH). The solution was heated to 60°C for 30 min before the addition of 5 units of Proteinase K and incubation at 37°C for 1.5 hours. DNA was extracted by addition of 400μl of phenol:chloroform:isoamyl alcohol (25:24:1(v:v:v)) and a white emulsion was formed by gentle inversion. The emulsion was broken by centrifugation at 17,000 x g for 10 min. The upper aqueous layer was removed to a clean microfuge tube and plasmid profiles were analysed by electrophoresis at 2-3V/cm overnight on a 0.5% agarose gel (section 2.2.2).

The second method involved growing the *B. sphaericus* strains in 50ml Spizizen medium (0.2%(w/v) NH_4SO_4, 1.4%(w/v) K_2HPO_4, 0.6%(w/v) KH_2PO_4, 0.1%(w/v) Na citrate ($2H_2O$), 0.02%(w/v) $MgSO_4$:$7H_2O$, 0.1%(w/v) casamino acids, 0.01%(w/v) yeast extract) to a D_{600} of 0.9-1.1. The culture was centrifuged (8,000 x g, 15 min, 4°C) and washed in 10ml of TES (30mM Tris-HCl, 50mM NaCl, 5mM EDTA, pH 8.0) before resuspension in 2ml TES containing 20%(w/v) sucrose, 2mg/ml lysozyme and 10μg/ml RNase A. The suspension was incubated at 37°C for 1.5 hours, or until spheroplast formation could be confirmed using a light microscope. To this, 3ml of 8%(w/v) SDS in TES buffer was added before incubation at 68°C for 15 min, followed by addition of 1.5ml of 3M Na acetate (pH 4.8) and incubation at -20°C for 30 min. The resulting mixture was centrifuged (20,000 x g, 15 min, 4°C),

the supernatant retained and DNA precipitated by addition of 2 volumes of absolute ethanol and incubation at -20°C overnight. The precipitated DNA was harvested by centrifugation (20,000 x g, 20 min, 4°C) and the pellet left to air dry before resuspension in 100μl TE buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0) by gentle mixing and incubation at room temperature for 2-3 hours. Plasmid profiles were analysed by electrophoresis at 2-3V/cm, overnight in a 0.5% agarose gel (section 2.2.2).

2.3 Transformation of bacterial cells by plasmid DNA

2.3.1 Preparation of competent *E. coli* cells

2.3.1.1 Calcium chloride method

Chemically competent *E. coli* cells were prepared using a simplified version of that described by Sambrook and Russell (Sambrook and Russell, 2001). A glycerol stock of the appropriate *E. coli* strain was used to inoculate 10ml of LB medium, with or without antibiotics as appropriate. The culture was grown for 16 hours with shaking at 37°C, 250 rpm. This culture was diluted 200 fold into fresh LB medium and grown with shaking at 37°C, 250 rpm until the D_{600} reached 0.4. Cells were harvested by centrifugation (3,000 x g, 10 min, 4°C) and were resuspended in half of the original culture volume of ice-cold $CaCl_2$-glycerol solution (100mM $CaCl_2$, 15%(v/v) glycerol) and incubated on ice for 30 min. The cells were harvested as before and resuspended in 0.1 original culture volume of $CaCl_2$-glycerol solution. Cells were incubated on ice for 30 min, divided into 50μl aliquots and flash frozen in liquid nitrogen. Cells were stored at -80°C until use.

2.3.1.2 Electrocompetent method

Electrocompetent *E. coli* DH5α (Dower *et al.*, 1988) were prepared when high efficiency electroporation was required for transformation of ligation reactions, using the method described in Sambrook and Russell (Sambrook and Russell, 2001). An overnight culture of *E. coli*, grown in LB medium (37°C, 250rpm), was diluted 20 fold into two flasks containing 500ml of prewarmed LB medium. The cultures were incubated at 37°C, 300rpm until the D_{600} reached 0.4. The cultures were cooled on ice before harvesting by centrifugation (1,000 x g, 15 min, 4°C) and resuspending in 0.5 original culture volumes of ice-cold sterile water. The cells were again harvested by centrifugation (1,000 x g, 20 min, 4°C) and resuspended in 0.25 original culture volumes of sterile, ice-cold 10%(v/v) glycerol. Cells were harvested again by centrifugation (1,000 x g, 20 min, 4°C) and resuspended in 10ml ice-cold 10%(v/v) glycerol before a final centrifugation (1,000 x g, 20 min, 4°C) and final resuspension in 1ml of ice-cold sterile GYT medium (10%(v/v) glycerol, 0.125%(w/v) yeast extract, 0.25%(w/v) tryptone). The D_{600} of a 1:100 dilution of the cell suspension was measured and the cell suspension was diluted to a concentration of 2×10^{10} to 3×10^{10} cells/ml in ice-cold sterile GYT medium (assuming that a D_{600} of 1 equates to approximately 2.5×10^{8} cells/ml). Samples (40μl) of cells were stored at -80°C in sterile microfuge tubes, after flash freezing in liquid nitrogen.

2.3.2 Transformation of *E. coli* by plasmid DNA

2.3.2.1 Calcium chloride method

Samples (50μl) of competent cells (section 2.3.1.1) were thawed on ice before the addition of plasmid DNA (1-50ng) and mixing by gentle pipetting. The cells were incubated on ice for 30 min, subjected to heat shock at 42°C for 2 min and placed on ice for a further 5 min before the addition of LB medium (1ml) and incubation at 37°C for 1 hour. Following incubation, cells were pelleted by centrifugation (6,000 x g, 1 min), resuspended in 100μl of LB medium and spread onto nutrient agar plates containing the appropriate antibiotic for selection. The plates were allowed to stand for 2 min before overnight incubation at 37°C in an inverted position.

2.3.2.2 Electroporation

Samples (40μl) of electrocompetent cells (section 2.3.1.2) were thawed on ice before addition of plasmid DNA (10pg-25ng in a volume of 1-2μl). The cells were left on ice for 30-60 seconds before transfer to an ice-cold electroporation cuvette (0.2cm-gap). The cuvettes were tapped to ensure that the cells-DNA mix reached the bottom of the cuvette and to remove trapped air bubbles. Condensation and moisture was wiped from the cuvette's electrode contacts and an electric pulse was delivered, giving a time constant of 4-5ms using a Bio-Rad Gene Pulser electroporation unit (Bio-Rad Laboratories Ltd, Herts, UK) set at 2.5kV, 200Ω resistance and 20μF capacitance. SOC medium (1ml of 20g/l tryptone, 5g/l yeast extract, 0.5g/l NaCl, 20mM glucose) was immediately added to the cells in the electroporation cuvette before transfer to a 15ml polypropylene tube (Corning Inc., Acton, MA, USA) and incubation at 37°C, 150rpm for 1 hour. Different volumes (10-200μl) of the

electroporated cells were spread onto LB agar plates containing the appropriate antibiotics for selection. The plates were left at room temperature, until the culture liquid was absorbed, and incubated at 37°C overnight in an inverted position.

2.3.3 Packaging of recombinant cosmids into bacteriophage λ particles.

Packaging of genomic DNA:cosmid hybrids into bacteriophage λ particles was carried out using the Packagene® Lambda DNA Packaging System (Promega Ltd., Southampton, UK). Packagene® extracts were thawed on ice and 10μl of a ligation reaction added as soon as thawing was complete. The mixture was incubated at 22°C for 3 hours. After this time, 440 μl of phage buffer (20mM Tris-HCl, 100mM NaCl, 10mM $MgSO_4$, pH 7.4) and 25μl of chloroform were added and the samples gently mixed by inversion. The packaged cosmids were stored at 4°C for up to a week or at –70°C for longer term storage after the addition of gelatin (0.01%(w/v) final concentration) and DMSO (7%(v/v) final concentration).

E. coli LE392 was grown overnight in 10ml of LB medium supplemented with 0.2%(w/v) maltose and 10mM $MgSO_4$ at 37°C, and was used to inoculate (1/100 dilution) 50ml of LB medium containing 0.2%(w/v) maltose and 10mM $MgSO_4$. The culture was incubated with shaking at 37°C, 250 rpm to a D_{600} of 0.6 and the cells were then placed on ice. A 1/100 dilution of packaged cosmids (100μl) in phage buffer was added to 100μl of LB containing 0.2%(w/v) maltose and 10mM $MgSO_4$ and 100μl of the prepared *E. coli* LE392. The mixture was incubated at 37°C for 30 min. LB containing 0.2%(w/v) maltose and 10mM $MgSO_4$ (1ml) was added and the mixture incubated for a further hour before the cells were plated onto LB agar plates containing 60 μg/ml ampicillin and incubated at 37°C overnight.

2.3.4 Electrotransformation of *B. thuringiensis* by plasmid DNA

Transformation of *B. thuringiensis* subsp. *israelensis* 4Q7 was performed from an adapted version of the method of Bone and Ellar (Bone and Ellar, 1989). A colony of *B. thuringiensis* subsp. *israelensis* from an overnight nutrient agar plate was grown in LB medium (10ml) in a 50ml sterile polypropylene tube (Corning Inc., Acton, MA, USA) at 30°C, 200rpm to a D_{600} of 0.2-0.3. The culture was placed on ice for 10 min before centrifugation (2,000 x g, 5 min, 4°C) and gentle resuspension of pelleted cells in ice-cold, filter sterilised 10%(w/v) sucrose (1ml). Cells were harvested and washed twice more in ice-cold 10% sucrose, as before. Following washing, the cells were harvested again, before the final resuspension in 250µl of ice-cold 10%(w/v) sucrose. Cells were kept on ice until electroporation.

Samples (120µl) of cell suspensions were placed in ice-cold 0.4cm electroporation cuvettes (Bio-Rad Laboratories Ltd, Hertz, UK) and 1µg of plasmid DNA was added and mixed by gentle pipetting. Cells were placed back on ice for 10 min before the volume of the mix was made up to 800µl by the addition of ice-cold 10%(w/v) sucrose. An electrical pulse was applied to the cells, giving a time constant of approximately 9ms using a Bio-Rad Gene Pulser electroporation unit (Bio-Rad Laboratories Ltd, Herts, UK) set at 1.8kV, 400Ω resistance and 25µF capacitance. Immediately after electroporation, prewarmed LB medium (1 ml) was added to the cuvette and the contents were transferred to a 15ml sterile polypropylene tube (Corning Inc., Acton, MA, USA) and incubated at 30°C, 200rpm for 1-2 hours. After this time, the cells were harvested (2,000 x g, 5 min), resuspended in 100µl of LB medium (without antibiotic) and plated onto nutrient agar containing the appropriate antibiotic for selection. The plates were incubated overnight at 30°C in an inverted position.

2.3.5 Electrotransformation of *B. subtilis* by plasmid DNA

Transformation of *B. subtilis* was carried out as described by Xue *et al.* (Xue *et al.*, 1999). An overnight culture of *B. subtilis*, grown in LB medium containing 0.5M sorbitol, was diluted 16-fold into fresh, prewarmed LB medium supplemented with 0.5M sorbitol. The culture was incubated at 37°C, 250rpm to a D_{600} of 0.85-0.95. Cells were incubated on ice for 10 min before harvesting by centrifugation (4,000 x g, 5 min, 4°C) and washing four times in ice-cold electroporation medium (0.5M sorbitol, 0.5M mannitol, 10%(v/v) glycerol). After washing the cells were resuspended in 1/40 of the original culture volume of ice-cold electroporation medium. Cells were kept on ice until electroporation.

Plasmid DNA (50ng-0.5μg) was added to 60μl of electrocompetent cells in an ice-cold electroporation cuvette (0.1cm-gap) and was incubated on ice for 1-1.5 min. An electrical pulse was applied to the cells, giving a time constant of 4.5-5ms using a Bio-Rad Gene Pulser electroporation unit (Bio-Rad Laboratories Ltd, Herts, UK) set at 1.6kV, 200Ω resistance and 25μF capacitance. Recovery medium (1ml of LB medium containing 0.5M sorbitol, 0.38M mannitol) was added to the cells immediately after electroporation, the resulting mixture was transferred to 15ml sterile polypropylene tubes (Corning Inc., Acton, MA, USA) and incubated at 37°C, 200rpm for 3 hours. After this time, cells were harvested by centrifugation (4,000 x g) and resuspended in 100μl of LB medium before spreading onto LB agar plates containing the appropriate antibiotics for selection. The plates were incubated at 37°C overnight in an inverted position.

2.4 DNA-DNA hybridisation methods

2.4.1 Preparation of DIG labelled DNA probes

2.4.1.1 3' DIG labelling of oligonucleotides

Oligonucleotides for use in Southern hybridisation were labelled with digoxigenin–ddUTP at the 3' end by the action of terminal transferase (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

2.4.1.2 PCR generation of DIG labelled probes

DNA probes for use in Southern hybridisation were prepared by incorporation of digoxigenin-dUTP (Roche Diagnostics, Mannheim, Germany) into DNA fragments generated by PCR (section 2.2.6.1) according to the manufacturer's recommendations. Briefly, PCR was performed (section 2.2.6.1) using genomic DNA or plasmid DNA as template and a reaction mixture containing dATP, dCTP, dGTP (200μM each), dTTP (130μM) and DIG-dUTP (70μM). Incorporation of DIG into PCR products was confirmed by analysis using agarose gel electrophoresis (section 2.2.2) before continuing with hybridisation.

2.4.2 Transfer of target DNA to membranes for DNA-DNA hybridisation

2.4.2.1 Capillary transfer by Southern blot

Blotting of DNA fragments generated by restriction enzymes and separated by agarose gel electrophoresis onto Hybond-N membrane (Amersham International plc, Bucks, UK) was based on the method of Southern (Southern, 1975). DNA digested

with restriction enzymes (section 2.2.3) was resolved on a 1% agarose gel (section 2.2.2) and the position of molecular weight markers marked by stabbing the gel with a needle dipped in Indian ink. Following electrophoresis, the gel was placed in depurination solution (250mM HCl) for 10 min, denatured in denaturation solution (0.5M NaOH, 1.5M NaCl) for 2 x 15 min, followed by neutralisation in neutralisation buffer (0.5M Tris-HCl, 1.5M NaCl, pH 7.5) for 2 x 15 min. Before blotting by capillary transfer, the gel was equilibrated in 20X SSC (0.3M sodium citrate, 3M NaCl, pH 7.0) for 10 min. A piece of Whatman 3MM paper, presoaked in 20X SSC was placed on top of an overturned gel tray, the ends resting in a reservoir of 20X SSC. The gel was placed, face down, on top of the Whatman 3MM paper. A gel size piece of Hybond-N was placed on top of the gel and pierced at the molecular weight marker positions, followed by two sheets of Whatman 3MM paper and a 10-15cm stack of paper towels. The construction was compressed by placing a glass plate and a 1 kg weight on top of the paper towels and the transfer allowed to proceed overnight. Following transfer, the membrane was briefly rinsed in 2X SSC and the DNA fixed to the membrane by baking at 80°C for 2 hours.

2.4.2.2 Colony hybridisation

The screening of recombinant bacterial colonies, for plasmids containing probe-hybridisable sequence, by colony hybridisation was based on the method of Grunstein and Hogness (Grunstein and Hogness, 1975) and performed as described by Sambrook & Russell (Sambrook and Russell, 2001).

Hybond-N membranes were placed onto agar plates containing bacterial colonies for 3 min and the membrane marked for future re-alignment by stabbing through the filter into the agar with a syringe needle dipped in Indian ink. Membranes

were carefully peeled from the agar and placed colony side up on sheets of Whatman 3MM paper pre-soaked in denaturing solution (0.5M NaOH, 1.5M NaCl) for 15 min, neutralisation buffer (0.5M Tris-HCl, 1.5M NaCl, 1mM EDTA, pH 7.5) for 15 min and 2X SSC for 10 min with a 1 min drying step between each transfer. Membranes were air dried before fixing the DNA by baking at 80°C for 2 hours.

2.4.2.3 Dot blotting of DNA onto membranes

The dot blotting method was used occasionally on genomic DNA samples to determine the presence of a probe-hybridisable sequence.

DNA solutions were firstly denatured at 95°C for 5 min and were then transferred quickly to ice. DNA solutions containing 100-200ng of DNA were allowed to soak slowly onto a Hybond–N membrane in a grid like fashion, keeping the spots as small as possible. DNA was fixed to the membrane by baking at 80°C for 2 hours.

2.4.3 Hybridisation of probes to DNA immobilised on membranes

Hybridisation of DIG labelled probes (Engler-Blum *et al.*, 1993) was carried out according to manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany).

2.4.3.1 3' DIG labelled oligonucleotide probes

For degenerate oligonucleotide probes, hybridisation incubation temperatures (T_{hyb}) were performed at 10°C below minimum T_m for that oligonucleotide, calculated using the equation:

$$T_m\ (^\circ C) \approx 4(G+C) + 2(A+T)$$

Membranes were placed in pre-hybridisation solution (5X SSC, 0.1%(w/v) N-lauroylsarcosine, 0.2%(w/v) SDS, 1%(w/v) dry skimmed milk) for 1 hour at hybridisation temperature. The pre-hybridisation solution was replaced with hybridisation solution (pre-hybridisation solution containing 10pmol/ml probe) and incubated overnight at hybridisation temperature. Following hybridisation the membrane was washed with low stringency buffer (2X SSC, 0.1%(w/v) SDS) for 2 x 5 min at 15-25°C and then high stringency buffer (0.5X SSC, 0.1%(w/v) SDS) for 2 x 15 min at hybridisation temperature before equilibration for 5 min in MA buffer (0.1M Maleic acid, 0.15M NaCl, pH 7.5) containing 0.3%(v/v) Tween® 20. The membrane was placed in blocking solution (1%(w/v) dry skimmed milk in MA buffer) for 30 min before addition of alkaline phosphatase conjugated Anti-DIG antibody (Roche Diagnostics, Mannheim, Germany) (1:5,000 dilution in blocking solution) for 1 hour. After 1 hour the membrane was washed for 2 x 15 min in MA buffer with 0.3%(v/v) Tween® 20 and equilibrated in detection solution (0.1M Tris-HCl, 0.1M NaCl, pH 9.5). Detection was carried out using CSPD (Roche Diagnostics, Mannheim, Germany) by addition of 1ml CSPD solution (1/100 dilution of stock 25mM CSPD in detection buffer) to the membrane. The membrane was wrapped in Clingfilm, placed in a development folder and incubated for 15 min at

37°C. An X-ray film was exposed to the membrane for 20 min and developed using an AGFA Curix 60 automatic developer.

2.4.3.2 PCR generated DIG labelled probes

Hybridisation of DIG labelled probes generated by PCR, to target DNA was performed by a method identical to that described for oligonucleotide probes, with a few exceptions. DIG labelled PCR products were first made single stranded by heating to 95°C for 5 min and then transferred directly to ice before their addition to prewarmed hybridisation solution (2μl PCR labelled DNA per ml). Hybridisation was performed at 68°C.

2.5 Biochemistry methods

2.5.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli (Laemmli, 1970) and gels were cast and run using the Bio-Rad Mini-PROTEAN II system according to manufacturer's instructions. Running gels comprised 10%(w/v) acrylamide, 0.27%(w/v) N-N' methylene bisacrylamide, 375mM Tris-HCl pH 8.8, 0.1%(w/v) SDS, 0.05%(w/v) ammonium persulphate and 13.2mM TEMED. Stacking gels contained 5%(w/v) acrylamide, 0.14%(w/v) N-N' methylene bisacrylamide, 65mM Tris-HCl pH 6.8, 0.1%(w/v) SDS, 0.1%(w/v) ammonium persulphate and 13.2mM TEMED. Electrolyte running buffer contained 0.1%(w/v) SDS, 192mM glycine and 25mM Tris-HCl pH 8.3.

Protein sample buffer (20mM Tris-HCl pH 6.8, 0.2%(w/v) SDS, 5%(v/v) glycerol, 0.003%(w/v) Bromophenol Blue, 286mM β-mercaptoethanol) was

added to samples before boiling in a water bath for 5 min and sample loading onto gels. Gels were run at 200V and stained using Coomassie Blue R-250 solution (0.05%(w/v) in 50%(v/v) methanol and 10%(v/v) acetic acid) for 1-2 hours and destained in a wash solution containing methanol (25%(v/v)) and acetic acid (7%(v/v)). Pre-stained protein markers were subject to simultaneous electrophoresis with samples, allowing molecular weight determination of sample proteins.

2.5.2 Immunological detection of recombinant proteins by western blotting

After SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Amersham International plc, Bucks, UK) by electroblotting. A gel sized piece of Whatman 3MM paper soaked in solution A (300mM Tris base, 20%(v/v) methanol) was placed on the anode terminal followed by two pieces of Whatman 3MM paper pre-soaked in solution B (20mM Tris, 25%(v/v) methanol). A piece of nitrocellulose membrane, pre-soaked in distilled water was placed on top of the Whatman 3MM paper followed by the SDS-PAGE gel, two sheets of Whatman 3MM paper pre-soaked in solution C (25mM Tris base, 20%(v/v) methanol, 40mM ε-amino-n-caproic acid) and the cathode terminal. Electroblotting was preformed at 145mA for 45 min using a semi-dry blotter (Startorius Ltd., Epsom, Surrey, UK).

For detection of His-tagged proteins, the membrane was washed 2 x 10 min in TBS (10mM Tris-HCl, 150mM NaCl, pH 7.5) before being blocked in TBS containing 3%(w/v) BSA for 1 hour. The membrane was then washed 2 x 10 min in TTBS (20mM Tris-HCl, 500mM NaCl, 0.05% Tween 20, pH 7.5) and for 10 min in TBS. The Penta-His antibody (Qiagen Ltd., Crawley, West Sussex, UK) was applied for 1 hour at a 1/1,000 dilution in TBS containing 3%(w/v) BSA. Following this, the

Penta-His antibody was removed by washing 2 x 10 min in TTBS and for 10 min in TBS. A secondary anti-mouse IgG, conjugated to alkaline phosphatase (Sigma Chemicals Ltd., Poole, Dorset, UK), was applied for 1 hour at a 1/10,000 dilution in TBS containing 3%(w/v) BSA. The membrane was washed 4 x 10 min in TTBS before staining with nitro-blue tetrazolium and 5 Bromo-4-chloro-3-indoyl phosphate (Bio-Rad Laboratories, Watford, Herts, UK) under continuous shaking until a colour reaction was observed. The reaction was stopped by washing in distilled water and the membrane was allowed to air dry.

Detection of GST-fusion proteins was performed as described for detection of His-tagged proteins above, except that blocking was performed in PBS (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.3) containing 5%(w/v) dry skimmed milk and 0.1%(v/v) Tween 20, and washing was performed in PBS containing 0.1%(v/v) Tween 20. The primary antibody was Anti-GST antibody (diluted 1/5,000; from Amersham Biosciences UK Ltd., Bucks, UK) and the secondary antibody was anti-goat IgG (diluted 1/10,000), conjugated to alkaline phosphatase (Sigma Chemicals Ltd., Poole, Dorset UK).

2.5.3 Preparation of protein samples for N-terminal sequencing

Before N-terminal sequencing, all the equipment to be used was washed thoroughly and soaked in Milli-Q overnight to remove all traces of glycine. Protein samples to be sequenced were first separated by SDS-PAGE (section 2.5.1) as usual except that the electrolyte running buffer comprised 0.1%(w/v) SDS, 192mM tricine and 25mM Tris-HCl, pH 8.3. Proteins were then transferred onto PVDF membrane (Bio-Rad Laboratories Ltd, Herts, UK), which had previously been hydrated by soaking in methanol followed by submersion in water for a few seconds, by

electroblotting in an identical fashion to that described for western blotting (section 2.5.2). Following transfer, the membrane was stained with 0.1%(w/v) Coomassie blue R-250, 50%(v/v) methanol for 5 min, destained using 50%(v/v) methanol, 10%(v/v) acetic acid for 2 min and then with three washes of deionised water over a period of 5 min. The membrane was allowed to air dry and the required band(s) excised with a sterile scalpel blade. N-terminal sequencing was performed by Alta Bioscience, University of Birmingham, Birmingham, UK.

2.5.4 Analytical scale expression in *E. coli* and sample preparation of recombinant protein

Cultures (10ml) of *E. coli* BL21(DE3)pLysS harbouring expression plasmids were established by inoculation with a single colony picked from an overnight plate. Cultures were incubated at 37°C, 300rpm overnight in LB containing ampicillin and chloramphenicol. A sample (0.5ml) was removed from each culture and added to fresh medium (100ml) containing ampicillin and chloramphenicol. The cultures were incubated at 37°C, 300rpm until a D_{600} of 0.4 was reached at which point expression was induced by addition of IPTG to 1mM. 1ml samples were removed at appropriate time intervals and the D_{600} recorded. The cells of each sample were pelleted by centrifugation (14,000 x g, 1 min) and resuspended in 100μl of 1x protein sample buffer (with 10%(v/v) β-mercaptoethanol) per unit of attenuance. Samples were stored at -20°C prior to analysis by SDS-PAGE and/or western blotting (sections 2.5.1 and 2.5.2 respectively).

2.5.5 Analytical scale expression of soluble and insoluble fractions

A 5ml *E. coli* BL21(DE3)pLysS protein expression sample (section 2.5.4) to be analysed was pelleted by centrifugation (14,000 x g, 3 min) and the medium was discarded. The cell pellet was resuspended in TE buffer to give a D_{600} of 10. The cells from each sample were lysed by three freeze-thaw cycles and sonicated on ice using a Sonics Vibra-Cell Ultrasonic Processor VCX500 (50% amplitude, 3 x 10 second pulses). After sonication, each sample was harvested by centrifugation (14,000 x g, 3 min) and the supernatant, containing soluble proteins, was retained. The pellet, containing insoluble proteins, was resuspended in the same volume of TE as above. To the supernatant and the insoluble suspension, an appropriate volume of 5x SDS protein sample buffer (with 10% (w/v) β-mercaptoethanol) was added. Samples were then boiled for 5 min prior to analysis by SDS-PAGE and/or western blotting (sections 2.5.1 and 2.5.2 respectively).

2.5.6 Purification of crystal proteins from *B. sphaericus* and recombinant *B. thuringiensis* subsp. *israelensis* 4Q7

Crystal proteins were purified from sporulated cultures of wild type *B. sphaericus* strains and recombinant *B. thuringiensis* subsp. *israelensis* 4Q7, transformed with plasmids carrying crystal protein genes, using the method described by Silva-Filha *et al.* (Silva-Filha *et al.*, 1997). Sporulated cultures, grown in Embrapa medium containing antibiotics when necessary, were harvested by centrifugation (20,000 x g, 4°C) and washed in ice-cold 1M NaCl containing 10mM EDTA. The spore suspension was harvested as before and washed twice in ice-cold 10mM EDTA before final resuspension in 1/30 of the original culture volume of ice-cold sterile water. The final spore suspension was sonicated using a Sonics Vibra-Cell Ultrasonic

Processor VCX500 (50% amplitude, 4 x 30 second pulses) before separation by centrifugation (110,000 x g, 15°C, 16 hours) on a discontinuous sucrose gradient (67/72/79/84%(w/v)) using an SW28 Ultracentrifuge rotor (Beckman Coulter Ltd., Buckinghamshire, UK). Purified crystal bands were extracted from the gradient using a 16-G needle and syringe by piercing the centrifuge tube. Crystals were thoroughly washed in sterile distilled water before analysis by SDS-PAGE (section 2.5.1) and/or determination of toxicity by bioassay (section 2.6). Crystal suspensions were stored at -20°C.

2.5.7 Quantification of crystal protein concentration

Purified crystal protein (section 2.5.6) was solubilised in 50mM NaOH for 1 hour at 30°C and any insoluble matter removed by centrifugation. The pH was adjusted by addition of 150mM Tris-HCl, pH 8.0 and the concentration of protein determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd, Herts, UK) according to the manufacturer's protocol, which is based on the method of Bradford (Bradford, 1976). Five dilutions, in triplicate, of the BSA standard were prepared ranging from 1.5 to 10μg/ml and 200μl of the dye reagent concentrate was added to 800μl of the BSA standard dilutions. These solutions were mixed and left at room temperature for 10 min before measuring the absorbance at 595nm using a spectrophotometer. A standard curve was prepared using the known concentration of the BSA and the absorbance values obtained. Different dilutions of the solubilised crystal protein (800μl) were mixed with 200μl of the dye reagent concentrate and left for 10 min at room temperature before the absorbance was measured as before. The concentration of the protein in samples were determined using the standard curve of the BSA protein. Due to the presence of a small amount of contaminating proteins in

the purified crystal toxin, the % of crystal protein in the sample was determined by densitometry after SDS-PAGE (section 2.5.1) using the program LabWorks™ Image Acquisition and Analysis Software (UVP Ltd., Cambridge, UK) for Windows.

The concentration of crystal protein determined above was confirmed by running known amounts of BSA (2.0, 1.5, 1.25, 1.0, 0.75, 0.5μg) with crystal protein on an SDS-PAGE gel. The amount of crystal protein was determined by densitometry compared with the known BSA standards using the program LabWorks™ Image Acquisition and Analysis Software (UVP Ltd., Cambridge, UK).

2.5.8 Electron microscopy of sporulated cultures

Sporulated cultures of *B sphaericus* and recombinant *B. thuringiensis* subsp. *israelensis* carrying the genes for *B. sphaericus* mosquitocidal toxins were analysed in a scanning electron microscope at Embrapa Recursos Genéticos e Biotechnologia, Brasilia, Brazil. Spore suspensions were deposited onto a metallic support, and allowed to dry at room temperature overnight in a microbiological hood. The samples were covered with gold for 180 seconds, using a sputter EMITECH model K550 and observed in a scanning electron microscope.

2.5.9 Preparation of larval gut extract

A. aegypti and *C. quinquefasciatus* larval gut extracts were prepared as described by Thanabalu *et al.* (Thanabalu *et al.*, 1992). Guts were dissected from 20 fourth-instar mosquito larvae and placed into ice-cold microfuge tubes containing 200μl PBS (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.3) following removal of the peritrophic membranes. The guts were homogenised in 1.5ml microfuge tubes using a pestle which exactly fits the tube.

2.5.10 *In vitro* toxin processing

Crystal toxin (10μg) was solubilised in 50mM NaOH for 1 hour at 30°C and any insoluble material removed by centrifugation (17,000 x g, 5 min). The solution was adjusted to 20mM Tris-HCl, 150mM NaCl, 2.5mM $CaCl_2$, pH 8.4 in a final volume of 100μl. Mosquito larva gut extract (10μl; section 2.5.9) or proteolytic enzyme (1μg) was added to the solubilised toxin and incubated at 30°C for 1 hour. The digest products were precipitated by addition of TCA to a 10%(w/v) final concentration and incubated on ice for 20 min. The precipitated protein was harvested by centrifugation (17,000 x g, 15 min, 4°C) and the pellet washed with acetone, precooled to -20°C. The samples were centrifuged (17,000 x g, 5 min, 4°C), the supernatant discarded and the protein pellets allowed to air-dry before resuspension in SDS-PAGE protein sample buffer and analysis of the digest products by SDS-PAGE (section 2.5.1).

2.6 Insect bioassays

Selective bioassays were performed against a range of insects to establish whether toxins, singly or in combination, were toxic to the test species. Further bioassays were performed on sensitive insects to determine fifty percent lethal concentrations (LC_{50}). For lepidopteran and coleopteran bioassays, larvae were fed artificial diets, based on black beans, brewer's yeast, soy protein, wheat germ and casein, prepared at Embrapa Recursos Genéticos e Biotechnologia (Brasilia, Brazil) as recommended by Shmidt *et al.* (Shmidt *et al.*, 2001).

2.6.1 Mosquito larvae

B. sphaericus strains and recombinant *B. thuringiensis* subsp. *israelensis* 4Q7, carrying the genes encoding putative toxins, were assayed for toxicity against *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles gambiae* mosquito larvae. Initial selective bioassays, to determine whether toxicity was observed, were performed by exposing 10 second- or third-instar larvae to 100μl of a fully sporulated culture of *B. sphaericus* or recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 in 10ml of distilled water at 28°C. Mortality was assessed after 24 hours by counting the number of live larvae. Further bioassays, allowing determination of LC_{50} were performed using the method recommended by the World Health Organisation (WHO) (WHO, 1985). Serial dilutions of sporulated cultures or crystal proteins were added to 100ml of distilled water containing 25 third-instar larvae at 28°C. Mortality was assessed after 24 hours and 48 hours by counting the number of living larvae. Two control bioassays containing only distilled water and non-transformed *B. thuringiensis* subsp. *israelensis* 4Q7 were also used. All bioassays were performed in triplicate. LC_{50} values were calculated using probit analysis (Finney, 1971) with the software SPSS 8.0.

When sporulated cultures were used, serial dilutions of the cultures were spread on LB agar plates, after heat treatment (80°C, 20 min) to kill vegetative cells. The plates were incubated at 30°C overnight and colonies were counted, allowing determination of the number of spores/ml of culture and from this, the calculation of LC_{50} values expressed in spores/ml.

Cosmid genomic DNA libraries, prepared in *E. coli* LE392, were also screened for toxicity against *C. quinquefasciatus*. In this instance cultures were incubated overnight (37°C, 250rpm), each culture containing grouped-pools of ten

colonies. The cultures were used in selective bioassays as described above except that 5 second- or third-instar larvae were assayed in 10ml of distilled water.

2.6.2 *Anthonomus grandis*

The artificial diet was autoclaved and allowed to cool to ~50°C before 200μl of a sporulated culture of *B. sphaericus* or recombinant *B. thuringiensis* subsp. *israelensis* 4Q7, carrying the genes encoding putative toxins, was added and mixed. The diet was poured into Petri dishes and allowed to solidify before 60 holes were made into the surface of the set diet. A second instar larva was placed into each hole and the plates incubated at 27°C. Mortality was assessed after 7 days by counting the number of living larvae. Two control bioassays were performed using an artificial diet containing no spore cultures and with *B. thuringiensis* subsp. *israelensis* 4Q7. Bioassays were performed in triplicate.

2.6.3 *Anticarsia gemmatalis*

One cube (approx. 1cm^3) of artificial diet was placed into each 50ml plastic cup. Bacterial culture (150μl) was added to the artificial diets and 10 second instar larvae were placed into each cup. A total of 10 cups were used for each test bioassay and an additional 10 cups were prepared containing no bacterial culture treatment as control. The cups were covered with lids and the bioassays incubated at 27°C. Mortality was assessed at 48 hours, at which time surviving larvae were transferred to new cups containing untreated diet and mortality assessed again at day 6.

2.6.4 *Spodoptera frugiperda*

Bioassays were performed in 24-well tissue culture plates with wells half filled with artificial diet. One half of the plate contained artificial diet treated with 30μl of a sporulated culture of either *B. sphaericus* or recombinant *B. thuringiensis* subsp. *israelensis* 4Q7, carrying the genes encoding putative toxins. The other half of the plate contained the control untreated diet. A single second instar larva was placed in each well, to prevent cannibalism, and the plates covered with a lid to prevent movement of larvae from one well to another. The bioassays were incubated at 27°C and the mortality was assessed after 48 hours. Each surviving larva was placed into an individual 50ml plastic cup containing untreated diet before the cups were covered with lids and incubated at 27°C. Mortality was assessed again at day 6.

2.6.5 *Plutella xylostella*

Young cabbage leaves, approx. 3cm in diameter, were dipped into a dilution of sporulated cultures of *B. sphaericus* or recombinant *B. thuringiensis* subsp. *israelensis* 4Q7, carrying the genes for putative toxins, and hung to air-dry for 1 hour at 25°C. One treated leaf was then placed into each Petri dish and 10 second-instar larvae were placed on each leaf. Control bioassays were prepared by dipping the leaves into water containing no bacterial spores. Bioassays were incubated at 25°C. A fresh untreated cabbage leaf was placed into each Petri dish when treated leaves were consumed or after 48 hours, whichever occurred first. Mortality was assessed after the 48 hour period and again at day 5.

2.6.6 *Chironomus riparius*

Ten first- or second-instar larvae were placed into 10ml of distilled water containing sediments of homogenised Whatman 3MM paper. A fully sporulated culture (100μl) of *B. sphaericus* or recombinant *B. thuringiensis* subsp. *israelensis* 4Q7, carrying the gene encoding putative toxins, was added and the bioassay incubated at 20°C. Mortality was assessed at 24 and 48 hours by counting survivors.

CHAPTER 3

Discovery and cloning of a putative toxin from *B. sphaericus*

3.1 Introduction

The commercial availability of *B. sphaericus* spore formulations and, therefore, its use for biological control of vector mosquitoes, has led to the selection of mosquito populations with resistance to the strains currently in use. Levels of resistance in *Culex* mosquito populations have been reported in Brazil (10 fold), India (150 fold), France (10,000 fold) and China (25,000 fold) (for review see Charles and Nielsen-LeRoux (Charles and Nielsen-LeRoux, 2000)). Mosquito populations with resistance levels of >100,000 fold have been selected under laboratory conditions (Pei *et al.*, 2002; Yuan *et al.*, 2003).

All records of *B. sphaericus* field resistance to date have been against strains 2362, 1593 and C3-41 (all serotype H5a5b) and it has been shown that cross-resistance occurs between different strains (Rodcharoen and Mulla, 1996; Pei *et al.*, 2002), including strains that produce distinct Bin variants such as 2362 (Bin2) and 2297 (Bin3) (Wirth *et al.*, 2000a; Yuan *et al.*, 2003). However, studies have identified strains (primarily IAB59, LP1G, 47-6B) that have the ability to overcome *Culex quinquefasciatus* larval resistance developed after exposure to strains 2362 and C3-41 (Wirth *et al.*, 2000a; Pei *et al.*, 2002; Yuan *et al.*, 2003). The ability of these strains to overcome Bin toxin resistance holds promise for their development for use in the field. Pei *et al.*, have shown that the toxicity of these strains to resistant *Culex* larvae is independent of amino acid sequence changes between Bin variants, with the Bin1 variant from IAB59 unable to overcome a Bin2-resistant *C. quinquefasciatus* colony raised against 2362 (Pei *et al.*, 2002). This has also been supported by receptor binding studies that have shown that Bin1 and Bin2 recognize the same receptor in the *C. pipiens* larval gut and, that laboratory selected high-level resistance is due to loss of the functional receptor (Silva-Filha *et al.*, 2004). The ability of

strains IAB59, LP1G and 47-6B to overcome binary toxin cross-resistance suggests that these strains carry currently unidentified toxin(s). It has also been shown that development of resistance to strain IAB59 is much more difficult than strains C3-41 and 2362, with a much lower level of resistance (<50 fold compared to >140,000) being selected for under intense laboratory selection (Pei *et al.*, 2002; Yuan *et al.*, 2003). Further supporting the new toxin hypothesis is that the Bin4 toxin (from LP1G), containing a L93S amino acid change in BinA compared to all other Bin variants, shows no toxicity against *C. pipiens* larvae susceptible to strain 2362, suggesting that the low level toxicity observed in this strain may be due to unidentified toxin(s) (Yuan *et al.*, 2001). A common spore protein, with an apparent molecular weight of ~49 kDa in strains IAB59, LP1G and 47-6B, has been suggested as a candidate toxin (Nielsen-LeRoux *et al.*, 2001; Pei *et al.*, 2002; Yuan *et al.*, 2003; Silva-Filha *et al.*, 2004) but previous attempts to clone the gene for this putative toxin have proved unsuccessful (Yuan personal communication; Nielsen-LeRoux personal communication).

The aim of this study was to identify, clone and characterise the toxin gene(s) responsible for the ability of strains IAB59, LP1G and 47-6B to overcome Bin cross-resistance. This involved construction and toxicity screening of a genomic DNA library, a method that has proven to be successful in the past for the cloning of the Mtx toxins (Thanabalu *et al.*, 1991; Liu *et al.*, 1996; Thanabalu and Porter, 1996). A second approach compared SDS-PAGE protein profiles of sporulated *B. sphaericus* cultures, followed by N-terminal sequencing of candidate toxins, allowing the design of degenerate primers for use in cloning strategies.

3.2 Construction of a *B. sphaericus* IAB59 cosmid library

High molecular weight total DNA was purified from *B. sphaericus* strain IAB59 according to the method of Ausubel *et al.*, (Ausubel *et al.*, 1987) (section 2.2.12). Conditions were optimised for partial digestion of genomic DNA to yield 20-40 kb fragments, following recommendations by Sambrook and Russell (Sambrook and Russell, 2001). IAB59 DNA was partially digested using the restriction enzyme *Tsp*509I, yielding products suitable for cloning into *Eco*RI linearised vectors. Pilot reactions were prepared on ice, containing varying amounts of restriction endonuclease and a constant amount of genomic DNA, before incubation at 65°C for 10 min. The reactions were stopped by addition of EDTA and the samples subjected to electrophoresis through a 0.5% agarose gel at 1V/cm, at 4°C overnight. The sizes of digest products were compared relative to the mobility of uncut λ DNA and λ *Hin*dIII DNA marker. Fragments of approximately 20-40 kb, appropriate for cloning into cosmid vectors, were formed using 0.035U of *Tsp*509I per μg DNA. Preparative digests with 60 μg of genomic DNA and 2.1U *Tsp*509I under the same conditions as the pilot reaction were prepared and the samples subjected to electrophoresis through a 0.5% LMP-agarose gel at 1V/cm, at 4°C overnight. DNA fragments of approximately 20-40 kb were excised from the gel and purified (section 2.2.4) before ligation (section 2.2.7) into linearised and CIP treated pHC79 cosmid vector.

The vector pHC79 (figure 3.1) is a 6.4 kb cosmid suitable for the cloning of DNA fragments in the range of 40 kb and has unique restriction sites for *Eco*RI, *Cla*I, *Bam*HI, *Sal*I, *Eca*I and *Pst*I (Hohn and Collins, 1980). The cosmid vector was linearised with *Eco*RI and treated with CIP to prevent self ligation, before extraction (section 2.2.4) from an agarose gel after electrophoresis (section 2.2.2). The size

fractionated IAB59 DNA fragments (1.25 μg) were ligated into the linearised pHC79 (1 μg), using T4 DNA ligase (3U) in a 10μl reaction volume and incubated overnight at 15°C. The ligation mix was then packaged into λ heads and transfected into *E. coli* LE392 (section 2.3.3), before selection by plating onto LB agar plates containing 60μg/ml ampicillin. Three hundred colonies, a coverage of approximately three genome equivalents, were obtained and glycerol stocks prepared for each individual colony (section 2.2.9).

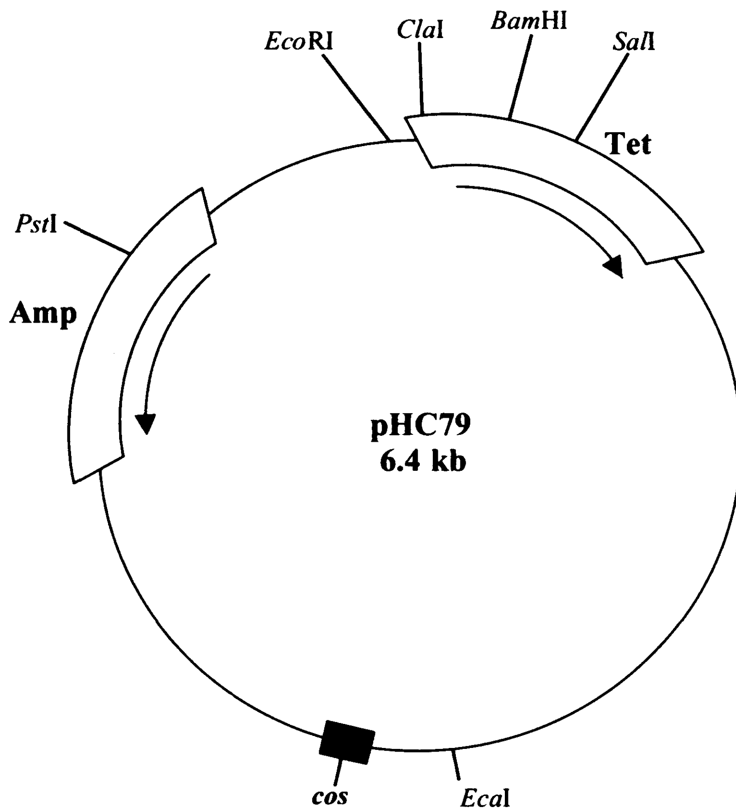


Figure 3.1 Restriction map of cosmid pHC79 (Hohn and Collins, 1980). Amp and Tet indicate the positions of the selectable marker genes for ampicillin and tetracycline resistance respectively. The position of the *cos* sequence from λ is also shown.

3.3 Screening of IAB59 cosmid library against *C. quinquefasciatus* larvae

The IAB59 cosmid library was screened for toxicity against second- or third-instar *C. quinquefasciatus* mosquito larvae to determine whether the gene responsible for the toxicity of this strain towards resistant *Culex* larvae had been cloned (section 2.6.1). Pooled groups of 10 colonies were grown overnight at 37°C in 10ml of LB medium containing 60μg/ml ampicillin. The cells were harvested by centrifugation, washed in 0.7%(w/v) NaCl and resuspended in 1ml of distilled water. The bacterial resuspensions were added to 5 *C. quinquefasciatus* larvae in a 10ml total volume for bioassay, in triplicate. The bioassays were incubated at 28°C and mortality was assessed at 24 hours and again at 48 hours, by counting the number of living larvae. Five pools showed toxicity, however, bioassay of the individual colonies within these pools did not lead to the isolation of any toxic clones. This method relies on a number of criteria being met including: (i) a representative library with at least one copy of each gene, complete with promoter (ii) that the promoter for the toxin is functional in *E. coli* (iii) a sufficient level of production of the toxin by *E. coli* (iv) the stability of the protein product in *E. coli* (v) that the activity of the toxin does not rely on any *B. sphaericus* specific processing.

3.4 Discussion

The failure to isolate any toxic clones from the IAB59 cosmid library suggests that at least one of the criteria mentioned above was not fulfilled. A representative library should not only identify the new gene(s) responsible for toxicity against resistant *Culex* colonies, but also the *bin* genes from IAB59. The *bin* gene is readily transcribed from its own promoter in *E. coli* and the corresponding protein produced at sufficient levels to cause toxicity to *Culex* larvae (Baumann *et al.*, 1987). The lack

of any *bin* derived toxicity in the cosmid library, together with the failure to isolate toxic clone(s) corresponding to previously unidentified toxin(s), suggests that the library is not representative. Restriction enzyme digest analysis of random clones indicated that successful genomic-DNA:cosmid-vector ligations had been accomplished, therefore a larger genome coverage could be attained by increasing the size of the library beyond 300 colonies. This could easily be achieved by transfection of further *E. coli* LE392 cultures as described in section 2.3.3, using the stored stock of packaged cosmid.

Although the vegetatively expressed *mtx1*, *mtx2*, and *mtx3* promoters are recognised by *E. coli* RNA polymerase σ factors (Thanabalu *et al.*, 1991; Liu *et al.*, 1996; Thanabalu and Porter, 1996) and the *bin* operon is readily transcribed in *E. coli* (Baumann *et al.*, 1987), this is not the case for many sporulation genes (Losick *et al.*, 1986). It was therefore possible that the promoter(s) of the new toxin gene(s) from *B. sphaericus* would not be recognised by *E. coli* σ factors, leading to questionable suitability of cosmid library screening as the sole approach towards new toxin cloning. As a result, additional methods were undertaken in conjunction with the screening of the IAB59 cosmid library, namely, N-terminal sequencing of candidate toxins allowing the design of degenerate oligonucleotides for use in cloning strategies.

3.5 Protein fingerprint comparison and light microscope analysis of *B. sphaericus* spore cultures

A spore protein, with an apparent molecular weight of ~49 kDa in strains IAB59, LP1G and 47-6B has been proposed as a toxin (Yuan *et al.*, 2003; Silva-Filha *et al.*, 2004) following protein profile SDS-PAGE analysis of alkali solubilised spore powders. Here, similar analysis was performed by looking at protein profiles of

sporulated cultures of strains IAB59 and LP1G compared with strains 2362 and 1593, that undergo Bin toxin cross-resistance, to determine whether common proteins were present in the former strains that were absent in the latter. *B. sphaericus* strains 2362, 1593, IAB59 and LP1G were grown to sporulation for 72 hours in NYSM medium (30ml; section 2.1.6) at 30°C, 250 rpm, in a 250ml conical flask. Sporulated cultures were then harvested by centrifugation and resuspended in 1/10 original volume of SDS-PAGE sample buffer (section 2.5.1) before boiling for 5 min. The samples (10μl) were analysed by SDS-PAGE (section 2.5.1), through a 10% gel (figure 3.2).

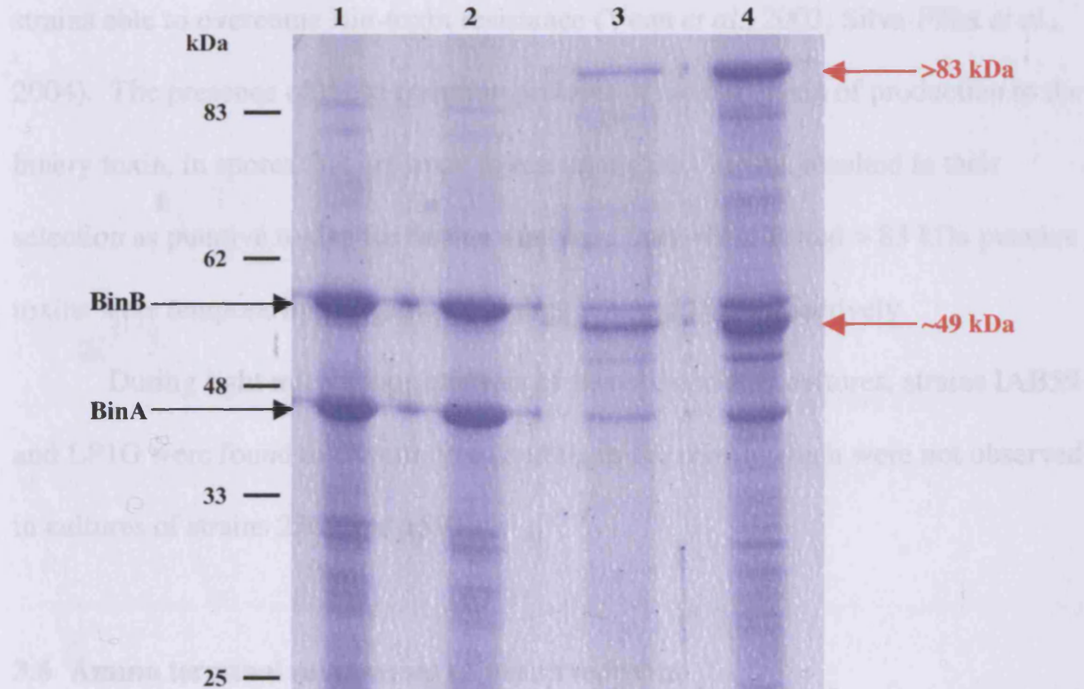


Figure 3.2 Protein profiles of *B. sphaericus* strains. Sporulated cultures of *B sphaericus* strains 2362 (lane 1), 1593 (lane 2), LP1G (lane 3) and IAB59 (lane 4) analysed by SDS-PAGE (10% gel) and stained with Coomassie blue R-250. Two common protein bands present in strains IAB59 and LP1G, but absent in 2362 and 1593, are indicated with red arrows. BinA (42 kDa) and BinB (51 kDa) protein bands are also indicated. The sizes of molecular weight marker bands are shown.

Analysis of IAB59 and LP1G protein profiles revealed multiple bands present in both strains that are absent in 2362 and 1593. The two major bands can be seen in figure 3.2; a high molecular weight band of >83 kDa and a band of slightly lower molecular weight than BinB, consistent with the reporting of a ~49 kDa protein in strains able to overcome Bin-toxin resistance (Yuan *et al.*, 2003; Silva-Filha *et al.*, 2004). The presence of these common proteins at similar levels of production to the binary toxin, in spores that are toxic to resistant *Culex* larvae, resulted in their selection as putative toxins for further analysis. The ~49 kDa and > 83 kDa putative toxins were temporarily assigned the names P49 and P83 respectively.

During light microscope analysis of these sporulated cultures, strains IAB59 and LP1G were found to contain free crystals in the media which were not observed in cultures of strains 2362 and 1593.

3.6 Amino terminal sequencing of putative toxins

Putative toxins were prepared for N-terminal sequencing by their immobilisation onto PVDF membrane. Sporulated cultures were prepared as described in section 3.5 for the preparation of samples for protein profile analysis by SDS-PAGE. Samples (10μl) were separated by SDS-PAGE in tricine buffer and blotted onto PVDF membrane as described in section 2.5.3. Protein bands corresponding to P83 from LP1G and, P49 from both IAB59 and LP1G were excised from the PVDF membrane and subjected to sequencing by Edman degradation, which was performed by Alta Bioscience. The N-terminal sequence deduced for each of the samples can be seen in figure 3.3.

P83 LP1G	A G K S F P D V P A G H W X E D S I
P49 IAB59	M E N Q I K E E F N K N N H G
P49 LP1G	M E N Q I K E E F N K N N H G I P S D C S C I K E

Figure 3.3 N-terminal sequence of putative toxins. The amino terminal sequence of putative toxin proteins P83 (red) from LP1G and P49 (blue) from IAB59 and LP1G. The length of the sequences reflect the positions where further determination of amino acid residues could not be achieved. Failure to assign an identity to an amino acid residue at a particular position is indicated by X.

N-terminal sequence analysis of the putative toxins confirmed that the ~49 kDa protein from IAB59 and LP1G correspond to the same protein, with all 15 N-terminal residues determined before reaction failure being identical. BLAST database searches (Altschul *et al.*, 1990) for sequences showing similarity to the N-terminal sequences of P49 and P83 were performed. No significant matches were found to P49. The N-terminal sequence of P83 shows high sequence identity to the N-terminus of the 95 kDa EA1 S-layer protein from *B. anthracis* (Farchaus *et al.*, 1995). In addition, the 18 aa sequence from P83 is identical, with exception of ambiguous residue X at position 14, to residues 30-47 of the S-layer protein from

B. licheniformis, corresponding to the N-terminus of the mature protein following cleavage of its signal-sequence (residues 1-29) (Zhu *et al.*, 1996). Additional S-layer proteins were also found to show similarity to P84, as shown in figure 3.4.

```
P83 NT               - - - - - - - - - - - - - - - - - - - -  1
EA1 NT B anthracis   - - - - - - - - - - - - - - - - - - - -  1
B licheniformis      M A K T N S Y K K V I A G T M T A A M V 20
B thuringiensis      M A K T N S Y K K V I A G T M T A A M V 20
B cereus E33L        M A K T N S Y K K V I A G T M T A A M V 20
B anthracis          M A K T N S Y K K V I A G T M T A A M V 20

P83 NT               - - - - - - - - - A G K S F P D V P A G 11
EA1 NT B anthracis   - - - - - - - - - A G K T F P D V P A D 11
B licheniformis      A G V V S P V A A A G K S F P D V P A G 40
B thuringiensis      A G V V S P V A A A G K S F P D V P A G 40
B cereus E33L        A G V V S P V A A A G K S F P D V P A G 40
B anthracis          A G I V S P V A A A G K S F P D V P A G 40

P83 NT               H W X E D S I                           18
EA1 NT B anthracis   H W G I D S I                           18
B licheniformis      H W A E D S I                           47
B thuringiensis      H W A E G S I                           47
B cereus E33L        H W A E G S I                           47
B anthracis          H W A E G S I                           47
```

Figure 3.4 Sequence alignment of P83 N-terminal aa residues. The N-terminal sequence of P83 is aligned with: the experimentally derived N-terminal sequence of EA1 from *B. anthracis* Delta Sterne-1 and, gene sequencing derived S-layer protein sequences from *B. licheniformis, B. thuringiensis, B. cereus* and *B. anthracis* strains. Residues identical to P83 are highlighted yellow. The known cleavage site of the signal peptide in *B. licheniformis* is indicated by an arrow (Zhu *et al.*, 1996).

N-terminal sequencing of P83 and protein-homology database searches resulted in its designation as a putative S-layer protein, and no further research was performed on this protein. Therefore, the design of degenerate oligonucleotides for use in cloning of the putative toxin from IAB59 and LP1G focused on the N-terminal aa sequence of P49.

3.7 Design of degenerate oligonucleotides based on the N-terminal P49 sequence

Two degenerate oligonucleotides were designed from the 25 aa N-terminal sequence of P49 (figure 3.5). Use of degenerate primers in cloning techniques or as probes are common and have proved useful in the past with the cloning of the gene for BinA (Hindley and Berry, 1987). Primers were designed from the N-terminal aa sequence by choosing regions that allowed for design of oligonucleotides with as low a degeneracy as possible. The 23-mer DP491 and 25-mer DP492 oligonucleotides were designed with degeneracies of 96 and 256 respectively. The two degenerate oligonucleotides were used as primers in PCR and as probes in Southern hybridisation.

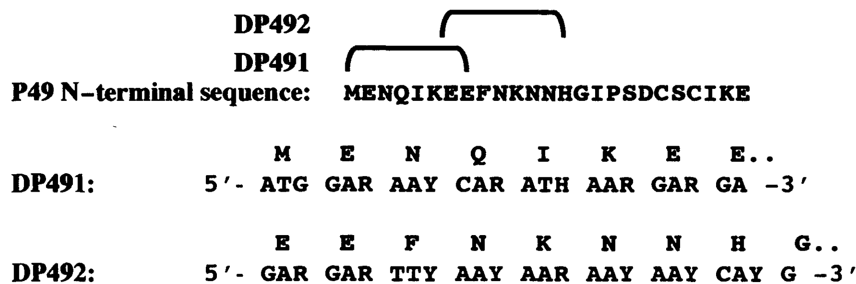


Figure 3.5 Nucleotide sequence of the degenerate oligonucleotides designed against the N-terminal aa sequence of P49. Protein sequences (P49) are shown in blue while DNA oligonucleotides (DP491 and DP492) are shown in red. Degenerate bases are: R = A/G, Y = C/T, H = A/C/T.

Primers DP491 and DP492 were first used in PCR (section 2.2.6.1) to attempt amplification of P49 encoding DNA fragments. Boomerang DNA amplification (Ahern, 1995), where restriction endonuclease digested IAB59 genomic DNA was ligated to synthetic oligonucleotide adapters before PCR, using either DP491 or DP492, was performed but no products were obtained (figure 3.6, data not shown). Additionally, these primers were used in PCR with M13remoteF or M13remoteR (see appendix) vector based primers to attempt amplification of the gene encoding P49 from plasmid libraries in pUC18. Libraries were created by digesting IAB59 genomic DNA separately with the restriction enzymes *Eco*RI, *Bam*HI and *Hin*dIII

(section 2.2.2). Individual ligation reactions were prepared (section 2.2.7) of the digest reaction products (500ng) and pUC18 (500ng) linearised with the same restriction enzymes and treated with CIP. After overnight incubation at 16°C, the ligations were used as template in PCR. Multiple reactions, using either primer DP491 or DP492 in all combinations with either M13remoteF or M13remoteR, were prepared using a range of annealing temperatures from 48°C to 60°C. PCR conditions were an initial denaturation at 95°C for 5 min, 30 cycles of 95°C (1 min), annealing for 1 min, and primer extension at 72°C (2 min), followed by a final extension at 72°C for 10 min. In most reactions, multiple non-specific PCR products were obtained. A few reactions yielded single PCR products which, on cloning and sequencing (sections 2.2.7 and 2.2.10 respectively), were revealed to be non-specific, presumably owing to the degeneracy of the oligonucleotides (data not shown). Attempted amplification of *p49* using the primers DP492 and DP491C (a degenerate primer the reverse complement of DP491) by Inverse PCR (section 2.2.6.2) also yielded no products on analysis by agarose gel electrophoresis (section 2.2.2).

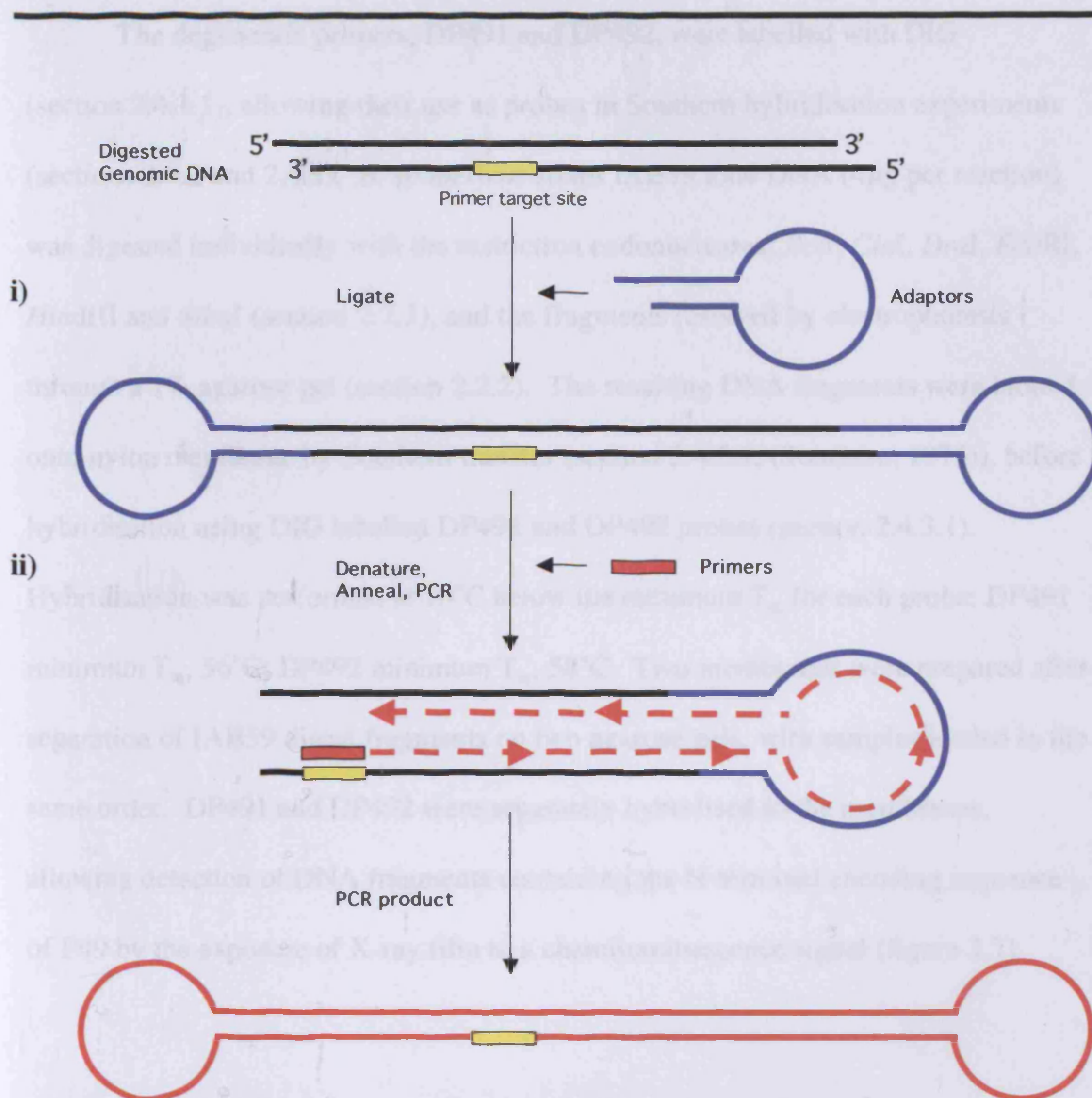


Figure 3.6 Schematic representation of boomerang DNA amplification.

i) Digested IAB59 DNA was ligated to oligonucleotides containing a stem-loop structure and overhanging restriction endonuclease half sites identical to those generated by the restriction enzymes used for IAB59 DNA digestion. ii) Primer extension to amplify target with a single primer.

3.8 Detection of *p49* by Southern hybridisation

The degenerate primers, DP491 and DP492, were labelled with DIG (section 2.4.1.1), allowing their use as probes in Southern hybridisation experiments (sections 2.4.2 and 2.4.3). *B. sphaericus* strain IAB59 total DNA (4μg per reaction) was digested individually with the restriction endonucleases; *Bcl*I, *Cla*I, *Dra*I, *Eco*RI, *Hin*dIII and *Mbo*I (section 2.2.3), and the fragments resolved by electrophoresis through a 1% agarose gel (section 2.2.2). The resulting DNA fragments were blotted onto nylon membrane by Southern transfer (section 2.4.2.1, (Southern, 1975)), before hybridisation using DIG labelled DP491 and DP492 probes (section 2.4.3.1). Hybridisation was performed at 10°C below the minimum T_m for each probe: DP491 minimum T_m, 56°C; DP492 minimum T_m, 58°C. Two membranes were prepared after separation of IAB59 digest fragments on two agarose gels, with samples loaded in the same order. DP491 and DP492 were separately hybridised to the membranes, allowing detection of DNA fragments containing the N-terminal encoding sequence of P49 by the exposure of X-ray film to a chemiluminescence signal (figure 3.7).

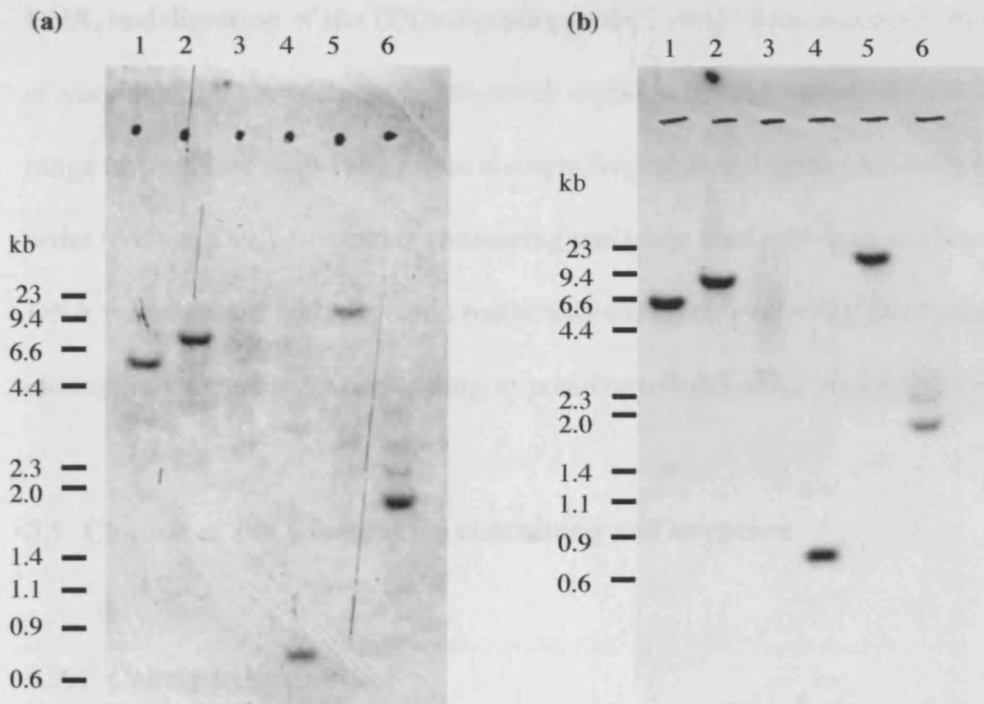


Figure 3.7 Southern blot analysis of *B. sphaericus* IAB59 total DNA digests with DIG labelled DP491 (a) and DP492 (b) oligonucleotide probes. *B. sphaericus* IAB59 total DNA (4μg per lane) digested with *Bcl*I (lane 1), *Cla*I (lane 2), *Dra*I (lane 3), *Eco*RI (lane 4), *Hin*dIII (lane 5) and *Mbo*I (lane 6), and hybridised with probes corresponding to the N-terminus of P49. The sizes of the digest fragments were estimated relative to the migration of λ *Hin*dIII and φX174 *Hae*III DNA markers.

As can be seen from figure 3.7, Southern blot analysis of digested IAB59 total DNA with probes DP491 and DP492 resulted in a common hybridisation banding pattern, with very little background. In both hybridisation experiments, no signal was obtained from IAB59 DNA digested with *Dra*I. After analysis it was concluded that

this was due to the *Dra*I digest not going to completion, resulting in partial digest of DNA, and digestion of the DNA carrying probe hybridisable sequence into fragments of varying sizes. In such cases, the probe signal is diluted due to its spread over a range of fragment sizes rather than a single fragment size giving a strong signal. In order to clone DNA fragments containing partial or total *p49* gene sequence, IAB59 DNA was digested with the same restriction enzymes, excluding *Dra*I, allowing the cloning of fragments corresponding to positive hybridisation signals into pUC18.

3.9 Cloning of DNA fragments containing *p49* sequence

3.9.1 Colony hybridisation

IAB59 total DNA (8μg) was digested in separate reactions with *Bcl*I, *Cla*I, *Eco*RI, *Hin*dIII and *Mbo*I and the reaction products separated by agarose gel electrophoresis (sections 2.2.3 and 2.2.2). DNA digest products, of sizes approximately corresponding to DP491 and DP492 Southern hybridisation signals, were recovered from the agarose gels (section 2.2.4) before ligation into linearised and phosphatase treated pUC18 vector (section 2.2.7). *Eco*RI digest products were cloned into *Eco*RI linearised vector, *Bcl*I and *Mbo*I fragments were cloned into *Bam*HI linearised vector, while *Hin*dIII digested DNA was cloned into *Hin*dIII cut pUC18. *Cla*I digest products were stored at -20°C for later use should they be required, due to there being no *Cla*I compatible sites in pUC18. After overnight incubation of the ligation reactions at 16°C, the products were transformed into *E. coli* DH5α by electroporation (section 2.3.2.2) and colonies containing IAB59 DNA-vector ligations were screened for using IPTG/X-Gal indicator plates (section 2.2.8). From the resulting plates, plasmid libraries were created by selecting

white colonies and spotting onto LB agar plates in a grid like fashion, and incubating at 37°C overnight. Colony hybridisation was performed using probes DP491 and DP492 to identify transformants containing N-terminal P49 encoding gene sequence (section 2.4.2.2), the grid like pattern of colonies facilitating cross-reference between hybridisation signals and positive transformants (figure 3.8). Hybridisation conditions differed to those used in the Southern hybridisation in that DP491 (5pmol/ml) and DP492 (5pmol/ml) were combined in the same hybridisation solution, and incubated at 48°C. The cloning strategy for *p49* is summarised in figure 3.9.

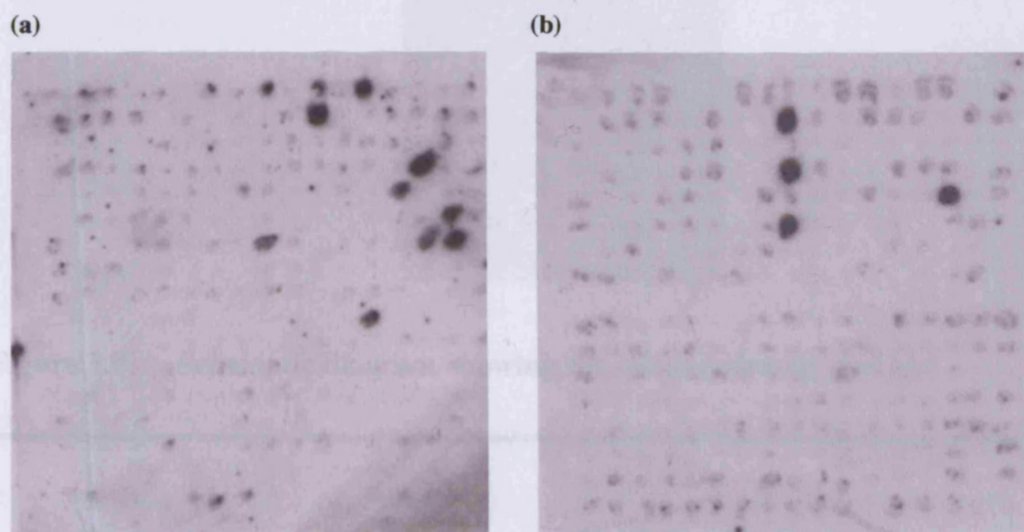


Figure 3.8 Colony hybridisation of *E. coli* plasmid libraries. DNA from *Hind*III (a) and *Eco*RI (b) *E. coli* plasmid libraries were fixed to nylon membranes before hybridisation at 48°C in hybridisation solution containing both DP491 (5pmol/ml) and DP492 (5pmol/ml).

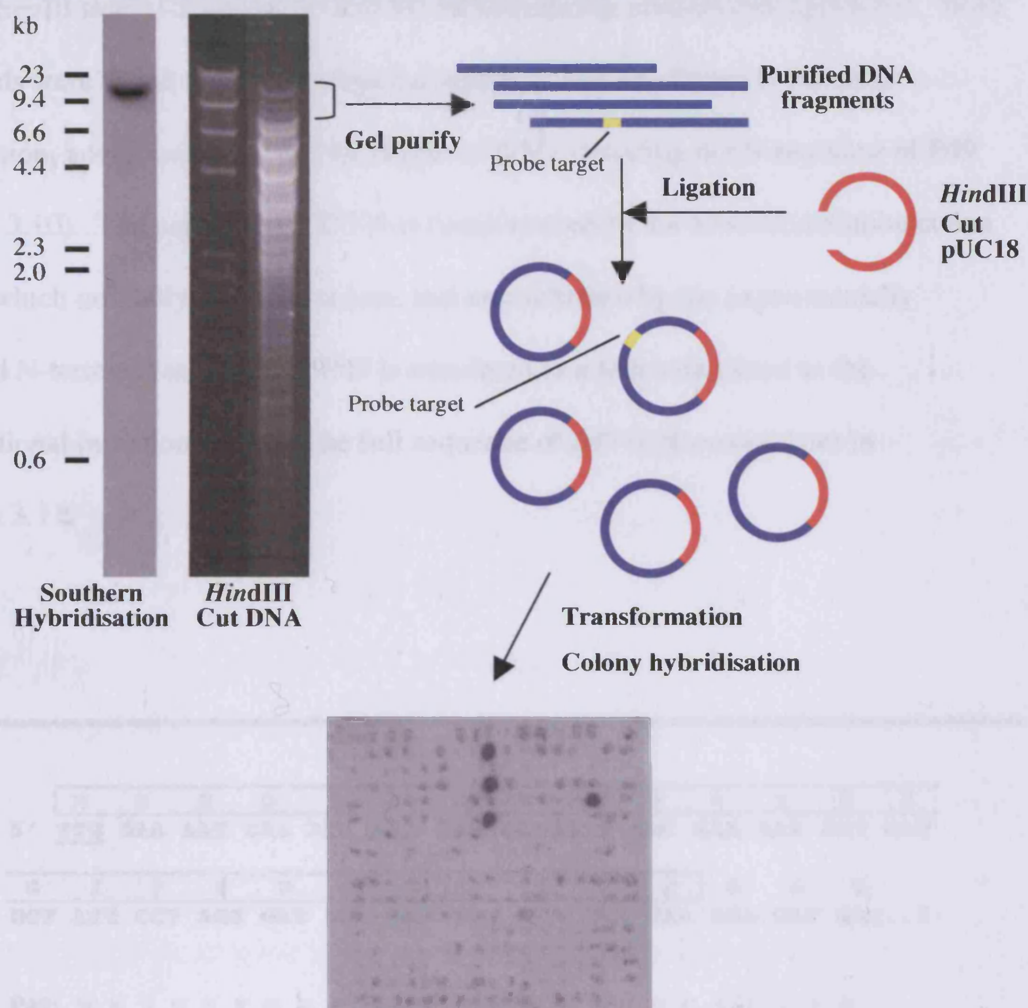


Figure 3.9 Schematic diagram showing the cloning strategy for *p49*

Eleven clones were obtained carrying DP491 and/or DP492 hybridisable DNA sequences within a *Hin*dIII fragment of approximately 15 kb. Four clones were identified containing DP491 and/or DP492 hybridisable DNA within an approximately 800 bp *Eco*RI fragment. Two pUC18 clones containing *Hin*dIII fragments were selected for further analysis and named p49H5 and p49H11. Two clones containing an *Eco*RI fragment were chosen for further analysis and assigned the names p49E1 and p49E2 before double-stranded DNA sequencing (section 2.2.10)

of the *Eco*RI inserts using M13F and M13R sequencing primers (see appendix). Both plasmids were found to contain identical inserts of 763 bp, cloned in the same orientation, and to contain a 477 bp region of DNA encoding the N-terminus of P49 (figure 3.10). The partial *p49* CDS was found to contain the unusual initiation codon TTG, which normally codes for a Leu, and as confirmed by the experimentally derived N-terminal sequence of P49 is translated as a Met when used as the translational initiation codon. The full sequence of *p49* is discussed later in section 3.11.

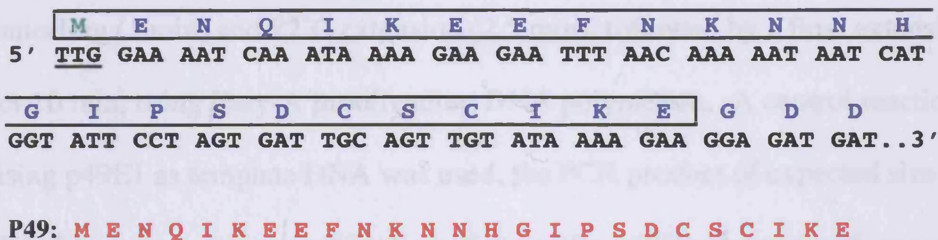


Figure 3.10 5' sequence of *p49*. A stretch of DNA encoding the N-terminus of P49, determined by the sequencing of the *Eco*RI inserts in p49E1 and p49E2 is shown. The DNA sequence is shown in black, with the initiating TTG underlined. The protein sequence deduced from partial *p49* sequence is shown in blue, with residues matching the experimentally derived N-terminal sequence of P49 shown boxed. The Met residue that is incorporated when TTG is used as an initiator codon is shown in green. The experimentally derived N-terminal sequence of P49 is shown in red.

3.9.2 Inverse PCR

Due to the failure to clone the *Mbo*I fragment containing complete or partial *p49* sequence, using the same method as was used for construction p49H5, p49H11, p49E1 and p49E2, inverse PCR amplification (as described in section 2.2.6.2) of this fragment was attempted (figure 3.11). Briefly, IAB59 DNA (5μg) was digested to completion with *Mbo*I before intramolecular ligations of digested products to form circular DNA was achieved using T4 DNA ligase (4U). This circularised DNA was used as template in PCR using primers (Int2F and Int1R, see figure 3.11 for primer sequences and figure 3.14 for full sequence of *p49*) designed based on the known *p49* sequence obtained from sequencing of p49E1 and p49E2. PCR conditions were a 5 min initial denaturation at 95°C, followed by 30 cycles of 95°C (1 min), 60°C annealing (1 min) and 72°C extension (2.5 min), followed by a final extension at 72°C for 10 min, using Easy-A proofreading DNA polymerase. A control reaction comprising p49E1 as template DNA was used, the PCR product of expected size being 3,415 bp representing amplification of the entire p49E1 plasmid with exception of the 28 bp region between the primer binding sites of Int2F and Int1R. Two further control reactions using only Int2F primer, in one case, and Int1R primer in the other were also performed using the circularised DNA as template. A single PCR product of expected size, in comparison with the Southern hybridisation signal from the *Mbo*I digest reaction, was obtained as was a single product of expected size in the control reaction using p49E1 as template DNA (figure 3.12). The inverse PCR product was purified (section 2.2.4) and cloned into the vector pGEM-T (section 2.2.7) before electrotransformation into *E. coli* DH5α (section 2.3.2.2) and plating onto IPTG/X-Gal indicator plates (section 2.2.8). Two positive colonies were selected and plasmids isolated (section 2.2.1). The pGEM-T plasmids, containing an

approximately 2 kb *Mbo*I fragment, were called p49M1 and p49M2 and were sent for DNA sequencing (section 2.2.10) using primers M13F and M13R, and were found to contain identical inserts of 2,010 bp.

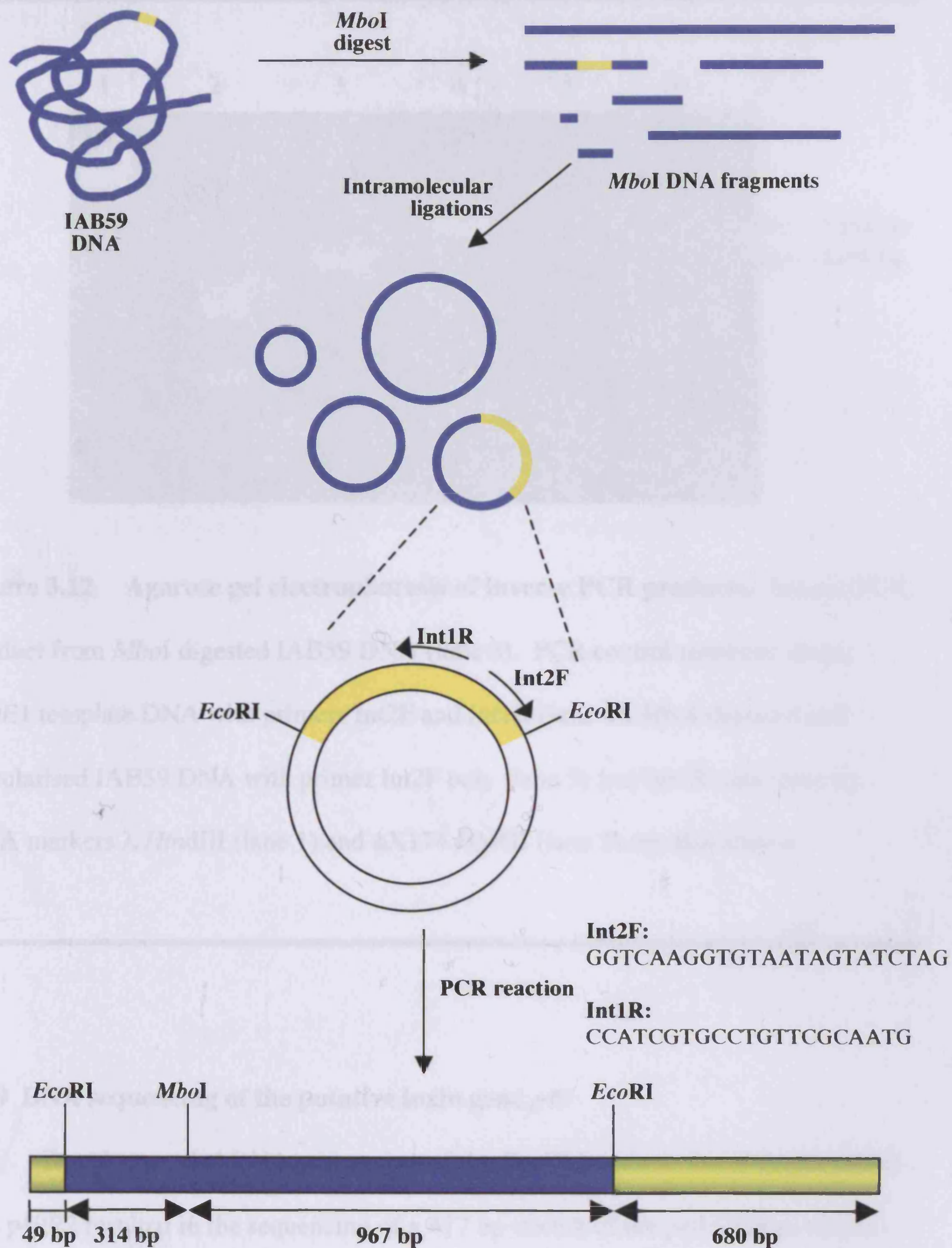


Figure 3.11 Inverse PCR. Schematic representation of inverse PCR amplification of the *Mbo*I DNA fragment containing *p49* DNA. Int2F and Int1R priming sites and primer sequences are shown as are *Mbo*I and *Eco*RI restriction sites. The region of DNA corresponding to the *Eco*RI fragment already sequenced is shown in yellow.

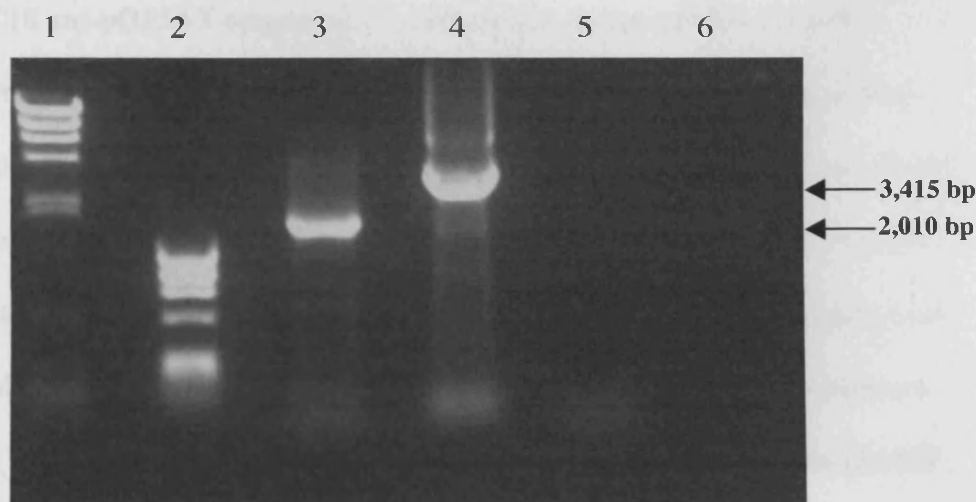


Figure 3.12 Agarose gel electrophoresis of inverse PCR products. Inverse PCR product from *Mbo*I digested IAB59 DNA (lane 3). PCR control reactions using; p49E1 template DNA with primers Int2F and Int1R (lane 4), *Mbo*I digested and circularised IAB59 DNA with primer Int2F only (lane 5) and Int1R only (lane 6). DNA markers λ *Hin*dIII (lane 1) and ϕX174 *Hae*III (lane 2) are also shown.

3.10 DNA sequencing of the putative toxin gene *p49*

Double stranded DNA sequencing of the *Eco*RI inserts in the plasmids p49E1 and p49E2 resulted in the sequencing of a 477 bp stretch of the *p49* CDS as well as 286 bp upstream of *p49*, containing a putative promoter region (see section 3.11). Sequencing of the *Mbo*I inserts in clones p49M1 and p49M2 resulted in the sequencing of 790 bp of *p49*, 477 bp of which were already determined from the sequencing of the *Eco*RI fragments cloned into p49E1 and p49E2, and 1,252 bp of

upstream DNA. Therefore, neither the *Eco*RI or the *Mbo*I DNA fragments cloned into pUC18 and pGEM-T respectively contained the complete CDS for *p49.*

For complete sequencing of the gene encoding the putative toxin, primers were designed to "walk-out" from the derived sequence. Clones p49H5 and p49H11, containing the ~15 kb *Hin*dIII fragment cloned into pUC18 were used as template DNA in the sequencing reactions (section 2.2.10) and the resulting chromatograms were analysed using the program EditView 1.0.1 ABI Automated DNA Sequence Viewer. The complete CDS for P49 was found to be contained within the *Hin*dIII inserts of p49H5 and p49H11. Editing of sequences and assembly of sequencing reaction data into contigs to determine the gene sequence was performed using EditSeq and SeqMan within the Lasergene DNA* software package. The complete sequencing strategy, including the primer sequences designed, can be seen in figure 3.13.

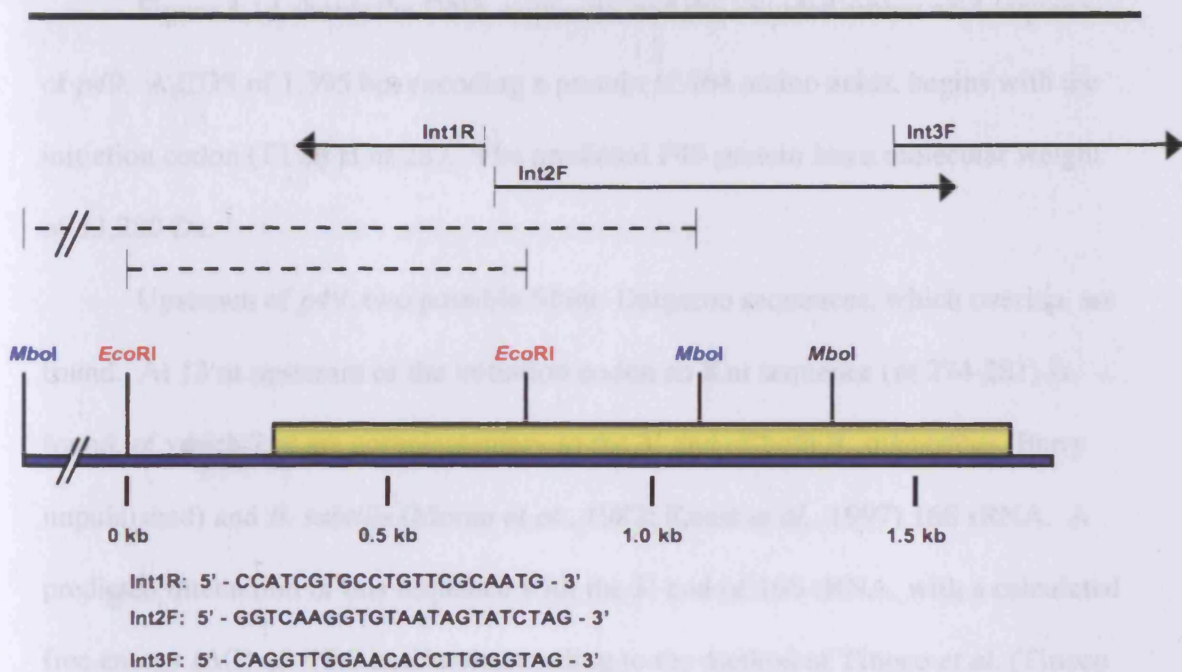


Figure 3.13 Strategy for the sequencing of *p49*. Arrows represent the direction of primer extension during sequencing of the *p49* gene (yellow). Dashed lines show regions previously sequenced from clones p49E1, p49E2, p49M1 and p49M2. Cleavage sites for the restriction enzymes *Eco*RI and *Mbo*I are shown, with the sites shown in colour indicating cleavage sites used in the construction of p49E1, p49E2, p49M1 and p49M2.

3.11 Analysis of the *p49* gene sequence

Figure 3.14 shows the DNA sequence, and the encoded amino acid sequence, of *p49*. A CDS of 1,395 bp, encoding a protein of 464 amino acids, begins with the initiation codon (TTG) at nt 287. The predicted P49 protein has a molecular weight of 53,280 Da.

Upstream of *p49*, two possible Shine-Dalgarno sequences, which overlap, are found. At 13 nt upstream of the initiation codon an 8 nt sequence (nt 274-281) is found, of which 7 nt are complementary to the 3' end of both *B. sphaericus* (Berry unpublished) and *B. subtilis* (Moran *et al.*, 1982; Kunst *et al.*, 1997) 16S rRNA. A predicted interaction of this sequence with the 3' end of 16S rRNA, with a calculated free energy (ΔG) of -15.8 kcal/mol according to the method of Tinoco *et al.* (Tinoco *et al.*, 1973), is shown in figure 3.15(i). In addition, 17nt upstream of the initiation codon, a 14nt sequence is found (nt 270-283), in which 11 out of the 14 nt are complementary to the 3'-16S rRNA sequence of *B. sphaericus* and *B. subtilis*. A proposed interaction of this sequence with 16S rRNA, with a ΔG of -13 kcal/mol can be seen in figure 3.15(ii). The lower ΔG of the former 8 nt sequence, compared to the latter 14 nt sequence, suggests this sequence as a putative Shine-Dalgarno, shown boxed in red in figure 3.14.

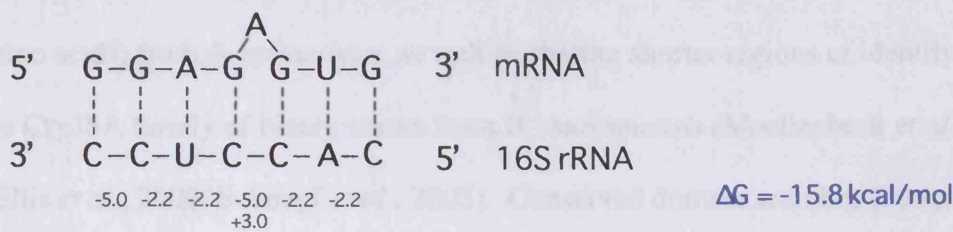
The sequence upstream of *p49* was analysed for possible promoter sequences using the predictive database search of the DBTBS (http://dbtbs.hgc.jp) (Makita *et al.*, 2004) and by carrying out manual analysis of the sequence. Putative promoter sequences similar to those found in *B. subtilis* were identified, most noticeable was the similarity of the sequences GTAATAAT (nt 211-218) and CATATATA (nt 232-239) to the -35 σ^E consensus sequence, G(g/t)(c/a)ATATT, and -10 σ^E consensus sequence, CATACANT, respectively. The sigma factor, σ^E, is active in the

B. subtilis mother cell that has advanced approximately 2 hours into sporulation, after processing of the inactive precursor, pro-σ^E, which is expressed from the *spoIIG* operon at the onset of sporulation (Haldenwang, 1995). The discovery of a σ^E-like promoter upstream of *p49* is, therefore, consistent with the presence of P49 in the *B. sphaericus* spore. Also, other mosquitocidal toxins from *B. thuringiensis* subsp. *israelensis* have been shown to be regulated by RNA polymerase associated with sporulation specific σ^{35} and σ^{28} factors, homologues of σ^E and σ^K from *B. subtilis* respectively (Yoshisue *et al.*, 1993a; Yoshisue *et al.*, 1993b; Dervyn *et al.*, 1995). A recent study on asporogenic *B. sphaericus* 2362, containing mutations in the *spo0A* and *spoIIAC* genes, has also shown the importance of sporulation-specific σ factors and promoters in the expression of the Bin toxin in the *B. sphaericus* spore (El-Bendary *et al.*, 2005). Another sequence showing similarity to a sporulation specific promoter was also identified. The sequences GCATA (nt 126-130) and TACCATATTG (nt 145-154) are similar to the σ^G consensus sequences GHATR (-35) and GGNCATXHTA (-10) respectively. RNA polymerase associated with σ^G is involved in the transcription of genes in the later stages of sporulation (Haldenwang, 1995). In addition to the σ^E and σ^G-like promoter sequences identified upstream of *p49*, a promoter-like sequence overlapping with the predicted σ^E promoter showing similarity to the σ^A consensus recognition sequences was also found. Factor σ^A, a homologue of *E. coli* σ^{70}, is most abundant in vegetatively growing *B. subtilis* cells, its main role being involved in transcription of vegetatively expressed genes. The sequence TTGTAA at nt 209 to 214 is similar to the -35 σ^A consensus, TTGACA, and the sequence TATATT at nt 236 to 241 is identical to the -10 σ^A consensus. In deviation from the consensus, the -35 and -10 σ^A-like sequences upstream of *p49* are separated by 21 nt, rather than the usual

17-18 nt. However, both *spoIIG* and *spoIIE* have -35 and -10 σ^A consensus sequences separated by 22 and 21 bp respectively, that have been shown to be transcribed under the influence of σ^A during the early stages of sporulation (Kenney *et al.*, 1989; York *et al.*, 1992). In addition, the activity of RNA polymerase associated with σ^A has been shown to persist into the early stages of sporulation. The ability of σ^A to recognise the sporulation-specific *spoIIG* and *spoIIE* promoters may suggest a role for σ^A in the transcription of *p49* during sporulation.

```
GAATTCAGGAATCAAGCTGCATAAAGTATTTTTATTATTTCCACTGTCTACTTGACGGGGAGCACTTCGTTATTTTTGTTCCATAGTCGAGTGTAACTTC  100

GGTTGTTTGGTCGTTTTCATCTGATGCATACTCAATTGTATTTGTACCATATTGAATACTTTCTAAATTTTTCTCATTTTTTAATACACGTGCGTTAATA  200

TCTACTCTTTGTAATAATTCAAGTGTATGGGCATATATATTAAAAGTAATACAATTTTTAAATTATTGATTAGGGAGAGTGGAGCTTTGGAAAATCAAAT  300
                                                                                          M  E  N  Q  I
AAAAGAAGAATTTAACAAAAATAATCATGGTATTCCTAGTGATTGCAGTTGTATAAAAGAAGGAGATGATTATAACTCTTTAACAGAGATAAATGCTAAA  400
 K  E  E  F  N  K  N  N  H  G  I  P  S  D  C  S  C  I  K  E  G  D  D  Y  N  S  L  T  E  I  N  A  K
GAGTTTTCTTATTGTAGTCCAAATATGTTTAATTTAAATTTGCCAGAACAAAGTACTAGATTTCAAACAATAGCTTCAATACATTCAAACAATTGTAGTT  500
E  F  S  Y  C  S  P  N  M  F  N  L  N  L  P  E  Q  S  T  R  F  Q  T  I  A  S  I  H  S  N  N  C  S
TTGAAATTCTCAATAACGACCCAGGGTATATATATGGGGATTCTGTAGATGGAGAATGTAGAATTGCAGTAGCCCACAGAGAACTTGGCAATGGTTTAGA  600
F  E  I  L  N  N  D  P  G  Y  I  Y  G  D  S  Y  D  G  E  C  R  I  A  V  A  H  R  E  L  G  N  G  L  E
GCGAACTGGGGACGACAGATTTTTATTTATTTTTTATGCTCTAGATAATAATAATTTTATCATTGCGAACAGGCACGATGGTTTTGTTTTGCAATTTTTG  700
 R  T  G  D  D  R  F  L  F  I  F  Y  A  L  D  N  N  N  F  I  I  A  N  R  H  D  G  F  V  L  Q  F  L
ATAGCAAATGGTCAAGGTGTAATAGTATCTAGAGAATATCAACCAAATATTCATCAAGAATTCACTATACAATCAATTAACTCTGACACTTTTAGGTTGC  800
I  A  N  G  Q  G  V  I  V  S  R  E  Y  Q  P  N  I  H  Q  E  F  T  I  Q  S  I  N  S  D  T  F  R  L
ATTCACGTGATACTAATACTTTCGCTACTGTCTGCTGGGCACAGTTCAACAGTTGGACAAAAATTGTTTCAAGGGTTGATAATCCTGGTGCACCTAATGC  900
H  S  R  D  T  N  T  F  A  T  V  C  W  A  Q  F  N  S  W  T  K  I  V  S  R  V  D  N  P  G  A  P  N  A
GAACTTGAAACATCGTTCACTTCTTACGGATATAAATATGCCACAATTACCAAGTTTAACACCATTACAACCATTACCACGATTAACTGAATTAGAAGAT  1000
 N  L  K  H  R  S  L  L  T  D  I  N  M  P  Q  L  P  S  L  T  P  L  Q  P  L  P  R  L  T  E  L  E  D
GGAGGCCTATCACCTGCTCAAGCACCAAGAGCTATTATAGGAAGAACACTTATTCCATGTTTATTTGTAAATGATCCTGTCTTAAGACTTGAAAATAGGA  1100
G  G  L  S  P  A  Q  A  P  R  A  I  I  G  R  T  L  I  P  C  L  F  V  N  D  P  V  L  R  L  E  N  R
TTAAACAAAGTCCATATTATGTATTAGAACATAGACAATATTGGCACAGAATATGGACAGATATTTTTACTGCTGGGGAGAGAAGAGAATATCGTGAAGT  1200
I  K  Q  S  P  Y  Y  V  L  E  H  R  Q  Y  W  H  R  I  W  T  D  I  F  T  A  G  E  R  R  E  Y  R  E  V
TACAGGAATAAATAATAATGCTCAAAATGATATGAATAAGATGATAAATATAACAATAGGTGCAGATGGACCAAATCGTTTGCGTTTTGGAAATCTTTCT  1300
 T  G  I  N  N  N  A  Q  N  D  M  N  K  M  I  N  I  T  I  G  A  D  G  P  N  R  L  R  F  G  N  L  S
ACACCATTTAGACAACAAATTATAGATAATTCAAATACTTTAGGATCTTTCGCAAATACTAATTATGGAACAAGAACTGATATAGTAAATGTTTTTAATA  1400
T  P  F  R  Q  Q  I  I  D  N  S  N  T  L  G  S  F  A  N  T  N  Y  G  T  R  T  D  I  V  N  V  F  N
GTGAATTTCACCAAGTAAGATATGCAAGATTTGTTAAAGCATATGAATATAGATTAACTCGTGCTGATGGATCACAGGTTGGAACACCTTGGGTAGTTTT  1500
S  E  F  H  Q  V  R  Y  A  R  F  V  K  A  Y  E  Y  R  L  T  R  A  D  G  S  Q  V  G  T  P  W  V  V  L
AGACCGTAAGGAAATGGATTTAAGAACATACCCACATAATATGGCAATAACCTTAGAAAATGTGAAAATTGACAATGCAGATAATAGTTATGATTTATCA  1600
 D  R  K  E  M  D  L  R  T  Y  P  H  N  M  A  I  T  L  E  N  V  K  I  D  N  A  D  N  S  Y  D  L  S
ATATGGAAAACCCCACTAAAATTAAAAGATGGTAAAATTATTATAGAGAATCATGAAAATTCAAAGCCATATTATAATTAATAATTTTCATTTTAAAAAT  1700
I  W  K  T  P  L  K  L  K  D  G  K  I  I  I  E  N  H  E  N  S  K  P  Y  Y  N  .
ATCCTAGGTAGTAATTAAAATTTGGCGCACCTCATATAGCCGTGTATTAATCATTTTTAATCAAATATA  1769
```

Figure 3.14 DNA sequence of the putative toxin gene *p49*. The predicted Shine-Dalgarno sequence is boxed in red and the putative σ^E, σ^G and σ^A promoters are shown with green, orange and red lines above the sequence respectively. The initiation codon (blue line) and the deduced P49 aa sequence is shown. This DNA sequence was later submitted to EMBL GenBank as part of accession no. AJ841948.



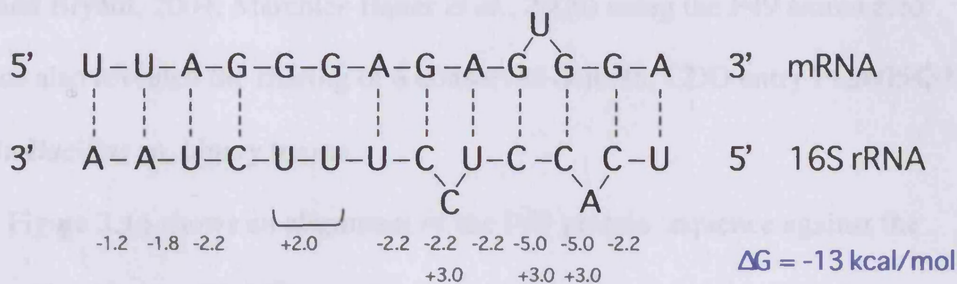


Figure 3.15 Two proposed interactions between the Shine-Dalgarno sequence of the *p49* transcript and the 3' end of 16S rRNA. Free energies (ΔG in kcal ± 10%) for the base paired, the interior loops and the bulge loop regions are shown below the predicted interaction. Values for ΔG were calculated according to Tinoco *et al.* (Tinoco *et al.*, 1973). The lower energy of (i) (ΔG = -15.8 kcal/mol), compared to (ii) (ΔG = -13.0 kcal/mol), favours this interaction between the mRNA and 16S rRNA.

3.12 Analysis of the P49 protein sequence

BLAST searches (Altschul *et al.*, 1990) were performed using the deduced 464 aa sequence of P49. Results indicated that P49 shows homology to a number of insecticidal toxins from *B. sphaericus* and *B. thuringiensis*. P49 shares 34% identity over 441 amino acids with Cry36Aa1 (also known as ET69), a 58.6 kDa coleopteran-active crystal protein from *B. thuringiensis*. In addition, P49 shows

similarity to BinA (31% identity over 321 amino acids) and BinB (27% identity over 469 amino acids) from *B. sphaericus*, as well as sharing shorter regions of identity with the Cry35A family of binary toxins from *B. thuringiensis* (Moellenbeck *et al.*, 2001; Ellis *et al.*, 2002; Schnepf *et al.*, 2005). Conserved domain searches (Marchler-Bauer and Bryant, 2004; Marchler-Bauer *et al.*, 2005) using the P49 amino acid sequence also revealed the sharing of a conserved domain, CDD entry Pfam05431, found in *Bacillus* sp. binary toxins.

Figure 3.16 shows an alignment of the P49 protein sequence against the proteins, identified by BLAST searches, to which it shows greatest homology. Cry35Aa1 is used in the alignment as a representative of the Cry35A family. Protein sequences were aligned using the ClustalW program, left at the default settings. Although BinA and BinB share a low sequence identity, these two proteins have four highly conserved blocks (Baumann *et al.*, 1988). As can be seen from the alignment, P49 shares many of the aa residues conserved in the four blocks, which are shown boxed in figure 3.16. Some of the non identical residues within the blocks also have similar properties to those found in either BinA or BinB. The Cry35 family of binary toxins from *B. thuringiensis* also contain conserved blocks which overlap with those found in the *B. sphaericus* Bin toxin (Ellis *et al.*, 2002). As illustrated in figure 3.17, P49 also shows a closer phylogenetic relationship to Cry36Aa1 and the Bin toxin, than the Cry35 family of binary toxins from *B. thuringiensis*. The phylogenetic tree was drawn by first aligning the protein sequences using the ClustalW program, left at the default settings except that the tree output format was set to PHYLIP distance matrix. The NEIGHBOR program within the PHYLIP software package was then used to construct a phylogenetic tree from this distance matrix. The program TreeViewPPC was used to view the resulting tree in radial format.

```
P49                    MENQIKEEFNKNNHG-------IPSDCSCIKEGDDYNSLTEINAKEFS-
Cry36Aa1    MNVNHGMSCGCGCQQGKEEYNDYHVSNEYRDENPSTTCNSQQGNYEYEQSKETYNNDYQS
BinB2                                           MCDSKDNSGVSEKCGKKFTNYPLN
BinA2
Cry35Aa1

P49         --------------------------------------YCSPNMFN-LNLPEQSTRFQTI
Cry36Aa1    YEYNQQNYNTCGRNQGTMEQESMQKDRNWENANYSGYDGCSPNQLNALNLPDESTRFQKI
BinB2       ---------------------------------------TTPTSLN-YNLPEISKKFYNL
BinA2                                               MRNLDFIDSFIPTEGKYIRV
Cry35Aa1                                                      MLDTNKVYEI

P49         ASIHSNNCS--FEILNNDPGYIYGDSVDGECRIAVAHRELGNGLERTGDDRFLFIFYALD
Cry36Aa1    TNVNTRDSHRVLDMMDVPSGTRLDTRVPPICSQTEFTNTVSNELVSTNHDTQFLIFYQTD
BinB2       KNKYS------------RNGYGLSKTEFPSSIENCPSNEYS--IMYDNKDPRFLIRFLLD
BinA2       MDFYN-------------SEYPFCIHAPSAPNGDIMTEICS------RENNQYFIFFPTD
Cry35Aa1    SNLAN-------------GLYTSTYLSLDDSGVSLMSKKDE----DIDDYNLKWFLFPID
                   A
P49         NNNFIIANRHDGFVLQFLIA--NGQG-VIVSREYQPN-IHQEFTIQSINSDT----FRLH
Cry36Aa1    DSSFIIGNRGNGRVLDVFPS--NRNGYTIVSNVYSGSRNNQRFRMNKASNNQ----FSLQ
BinB2       DGRYIIADRDDGEVFDEAPTYLDNNNHPIISRHYTGEERQKFEQVGSGDYITGEQFFQFY
BinA2       DGRVIIANRHNGSVFTGEAT-------SVVSDIYTGSPLQFFREVKRTMATY------YL
Cry35Aa1    NNQYIITSYGANNCKVWNVK-----NDKINVSTYSSTNSVQKWQIKAKDSSY------II

P49         SRDTNTFATVCWAQFNSWTKIVSRVDNPGAPNANLKHRSLLTD-----INMPQLPSLTPL
Cry36Aa1    TIFKDRVN--ICGHIHNFNAIITATTLGENDSNALFQVQSSTN-----ITLPTLPPRTTE
BinB2       TQNKTRVLSNCRALDSRTILLSTAKIFPIYPPASETQLTAFVNSSFYAAAIPQLPQTSLL
BinA2       AIQNPESATDVRALEPHSHELPSRLYYTNNIENNSNILISNKEQ--IYLTLPSLPENEQY
Cry35Aa1    QSDNGKVLTAGVGQSLGIVRLTDEFPENSNQQWNLTPVQ--------TIQLPQKPKID--
                                       B
P49         QPLPRLTELEDGGLSPAQAPRAIIGRTLIPCLFVNDPVLRLENRIKQSPYYVLEHRQYWH
Cry36Aa1    EPPRALTNINDTGDSPAQAPRAVEGSVLIPAIAVND-VIPVAQRMQESPYYVLTYNTYWH
BinB2       ENIPEPTSLDDSGVLPKDAVRAVKGSALLPCIIVHDPNLNNSDKMKFNTYYLLEYKEYWH
BinA2       PKTPVLSGIDDIG--PNQSEKSIIGSTLIPCIMVSD-FISLGERMKTTPYYYVKHTQYWQ
Cry35Aa1    EKLKDHPEYSETGNINPKTTPQLMGWTLVPCIMVNDSKIDKNTQIKTTPYYIFKKYKYWN
                                                     C
P49         RIWT--DIFTAGERREYREVTGINNNAQNDMNKMINITIGADGPNRLRFGNL---STPFR
Cry36Aa1    RVIS--AILPGSGQTTRFDVN-LPGPNQSTMVDVLDTAITADFRLQFVGSGR---TNVFQ
BinB2       QLWS--QIIPAHQTVKIQERTGISEVVQNSMIEDLNMYIGADFGMLFYFR-----SSGFK
BinA2       SMWS--ALFPPGSKETKTEKSGITDTSQISMTDGINVSIGADFG--LRFGNK---TFGIK
Cry35Aa1    LAKGSNVSLLPHQKRSYDYEWGTEKNQKTTIINTVGLQINIDSGMKFEVPEVGGGTEDIK
                                                   D
P49         QQIIDNSNTLGSFANTNYGTRTDIVNVFNSEFHQVRYARFVKAYEYRLTRADGSQVGTP-
Cry36Aa1    QQIRNGLNILNSTTSHRLGDETRNWDFTN-RGAQGRLAFFVKAHEFVLTRANGTRVSDP-
BinB2       EQITRGLNRPLSQTTTQLGERVEEMEYYNSNDLDVRYVKYALAREFTLKRVNGEIVKN--
BinA2       GGFTYDTKTQITNTSQLLIETTYTREYTNTENFPVRYTGYVLASEFTLHRSDGTQVNTIP
Cry35Aa1    TQLTEELKVEYSTETKIMTKYQEHSEIDNPTNQPMNSIGLLIYTSLELYRYNGTEIKIMD

P49         WVVLDRKEMDLRTYPHN---MAITLENVKIDNADNSYDLSIWKTPLKLKDGKIIIENHEN
Cry36Aa1    WVALDPNVTAAQTFGG----VLLTLEKEKIVCASNSYNLSVWKTPMEIKNGKIYTKNEWN
BinB2       WVAVDYRLAGIQSYPNAPITNPLTLTKHTIIRCENSYDGHIFKTPLIFKNGEVIVKTNEE
BinA2       WVALNDNYTTIARYPH-------------------------FASEPLLGNTKIITDDQN
Cry35Aa1    IETSDHDTYTLTSYPN---------HKEALLLLTNHSYEEVEEITKIPKHTLIKLKKHYF

P49         SKPYYN
Cry36Aa1    TKPNYK
BinB2       LIPKINQ
BinA2
Cry35Aa1    KK
```

Figure 3.16 Protein alignment of P49, Cry36Aa1, BinA2, BinB2 and Cry35Aa1. The number of identical residues (two, pink or grey; three, green; four, blue and all five, yellow) at each position are highlighted. Regions corresponding to the conserved blocks (A, B, C and D) of amino acids found in Bin toxins are shown boxed.

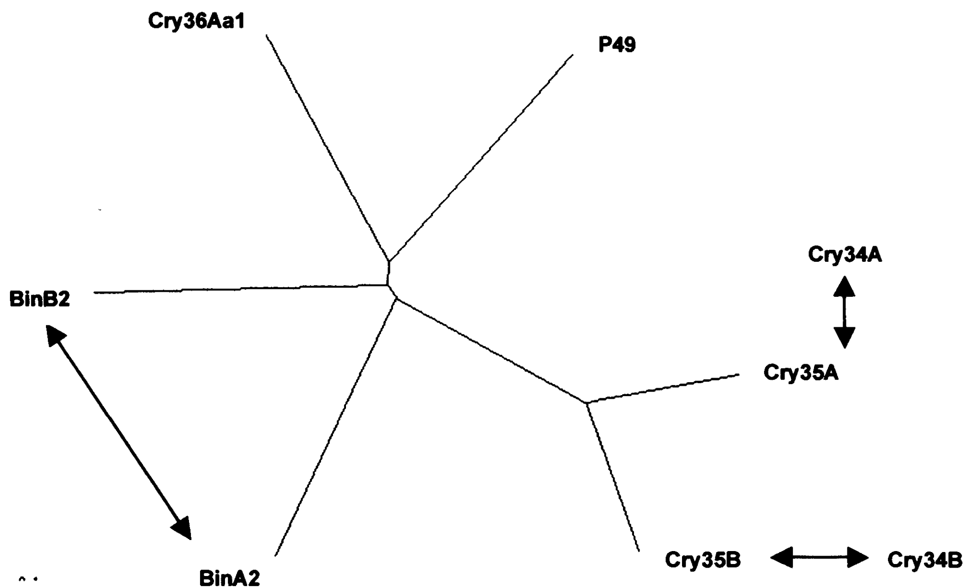


Figure 3.17 Phylogenetic tree showing the relationship between P49 and other insecticidal toxins. Relationship is shown to BinA and BinB from *B. sphaericus* using the Bin2 variant as an example, Cry36Aa1 (also known as ET69) from *B. thuringiensis* and members that make up the Cry35 family of *B. thuringiensis* binary toxins. Double headed arrows indicate a relationship between proteins that function together as binary toxins.

3.13 Sequencing of the *p49* gene from strains LP1G and 47-6B

SDS-PAGE analysis of sporulated cultures of *B. sphaericus* strains IAB59, LP1G and 47-6B, all strains able to overcome Bin toxin resistance, reveals a common spore protein band with an apparent molecular weight of ~49 kDa, as previously stated in section 3.1 and 3.5. The N-terminal sequencing of this protein from strain LP1G (refer back to figure 3.3) has confirmed that it, and P49 from IAB59,

correspond to the same protein, with the first 15 aa being identical. N-terminal sequencing of this protein from 47-6B was not performed at the beginning of this study as the strain was not one of the *B. sphaericus* strains kept at Cardiff University at the time. Primers were designed, based on the IAB59 *p49* gene sequence determined in this study, to attempt amplification of the genes encoding this common protein from LP1G and 47-6B. Total DNA was isolated from strains LP1G and 47-6B (section 2.2.12) and used as template in PCR (section 2.2.6.1) for amplification of *p49*. Primers P49F and P49R (see appendix for sequence) were used to amplify the gene encoding P49 from these two strains using Easy-A proofreading DNA polymerase. Thermocycling conditions were an initial denaturation step at 95°C for 5 min, 15 cycles of 95°C (1 min), 60°C (1 min) and 72°C (1.5 min), followed by a final extension at 72°C for 10 min. The PCR products were run on a 1% agarose gel (section 2.2.2) before purification from the gel (section 2.2.4) and ligation (section 2.2.7) into the vector pGEM-T. The ligation reactions were then transformed into *E. coli* DH5α (section 2.3.2.2.) and colonies were screened, for the successful cloning of *p49*, by colony PCR (section 2.2.6.1), using the same conditions as above except that 30 cycles were performed. Two clones, containing *p49* cloned into pGEM-T, from each strain were sent for sequencing with M13F and M13R primers (section 2.2.10, see appendix for primer sequences). To ensure that any differences that may occur in the *p49* gene sequences between these strains was not due to PCR introduced mutations, the amplification, cloning and sequencing of *p49* from 47-6B and LP1G was repeated.

The sequences of the *p49* gene inserts in the clones from strain 47-6B were found to be both identical to each other and to the *p49* gene from strain IAB59. Thus there is no aa sequence difference between the putative P49 toxin from IAB59 and

47-6B. The sequences of the clones containing the *p49* gene from LP1G were also found to be identical to each other. However, the *p49* gene from LP1G is 1,404 bp in length, compared to 1,395 bp for the *p49* gene from IAB59, encoding a protein of 467 aa and 53.5 kDa. The variation in gene sequence results in amino acid substitutions at 35 positions, one 4 aa insertion and one single aa deletion within the LP1G variant relative to P49 from IAB59, as can be seen in figure 3.18. Of these 35 substitutions, 12 could be considered conservative (K-R, I-L, T-S, D-E, V-I, Y-F) and 7 semi-conservative (A-G, H-R, N-D, L-F, A-S, V-Y). Only two of these amino acid changes are found to occur within the conserved blocks shared with the Bin toxin components; an A→S change at the 3rd aa of block C, and a Y→F at the 3rd aa of block D. The cloning and sequencing of the *p49* genes from strains LP1G and 47-6B confirms that the common spore protein with an apparent MW of 49 kDa identified in strains IAB59, LP1G and 47-6B, as discussed in sections 3.1 and 3.5, correspond to the same protein, P49. The sequence of *p49* from LP1G and its deduced aa sequence can be seen in the appendix and has been submitted to the EMBL GenBank database under accession AM237202.

```
IAB59 P49   MENQIKEEFNKNNHGIPSDCSCIKEGDDYNSLT----EINAKEFSYCSPNMFNLNLPEQS
LP1G  P49   MENQIKEEFNKNNHGIPSDCSCIKEGDDYNSLTEVPSEINAKEFSYCSPNMFNLNLPEQS

IAB59 P49   TRFQTIASIHSNNCSFEILNNDPGYIYGDSVDGECRIAVAHRELGNGLERTGDDRFLFIF
LP1G  P49   TRFQTIGSIHSNNCSFEILNNDPGYIYGDSVAGECRIAVAHRELGNGLERTGDDRFLFIF
                   A
IAB59 P49   YALDNNNFIIANRHDGFVLQFLIANGQGVIVSREYQPNIHQEFTIQSINSDTFRLHSRDT
LP1G  P49   YALDNNNFIIANRHDGFVLQFLIANGQGVIVSREYQPNIRQEFTIQSINSDTFRLHSRDT

IAB59 P49   NTFATVCWAQFNSWTKIVSRVDNPGAPNANLKHRSLLTDINMPQLPSLTPLQPLPRLTEL
LP1G  P49   NTFATVCWAQFNSWTKIVSRVDNPGAPNADLRHRSFL-DINMPQLPSLTPLQPLPRLTGL
                              B
IAB59 P49   EDGGLSPAQAPRAIIGRTLIPCLFVNDPVLRLENRIKQSPYYVLEHRQYWHRIWTDIFTA
LP1G  P49   EDGGLSPAQAPRAIIGRTLIPCLFVNDPVLRLESRIKQSPYYVLEHRQYWHRLWTDIFNA
                                          C
IAB59 P49   GERREYREVTGINNNAQNDMNKMINITIGADGFNRLRFGNLSTPFRQQIIDNSNTLGSFA
LP1G  P49   GERREYREVTGINHNAQNDMNNMINITIGSDGFNRLLFGHLSTPFRQQIISNSNTLGSFA
                                 D
IAB59 P49   NTNYGTRTDIVNVFNSEFHQVRYARFVKAYEYRLTRADGSQVGTPWVVLDRKEMDLRTYP
LP1G  P49   NSNYSSRTESITYLNTEFHQVRFARFVKAYEYRLTRADGTLVGTPWVVLDRKEMDLRTFP

IAB59 P49   HNMAITLENVKIDNADNSYDLSIWKTPLKLKDGKIIIENHENSKPYYN
LP1G  P49   HNMTLNLENVKIVNADNSYDLSVWKTPLKLKDGKIIIENHENSKPYYN
```

Figure 3.18 Alignment of P49 from IAB59 and LP1G. Amino acid residues in the LP1G variant that differ to P49 from IAB59 are highlighted yellow. Gaps inserted into the sequence to maximise the alignment are also highlighted yellow. The regions of P49 which align with the conserved blocks of BinA and BinB, as shown in figure 3.16, are shown boxed.

3.14 Discussion

The *p49* gene, encoding a putative mosquitocidal toxin, was cloned from *B. sphaericus* strain IAB59 by using degenerate probes designed against the N-terminal aa sequence of P49. Sequencing of the gene revealed it to have a size of 1,395 bp, encoding a protein of 464 aa and 53.3 kDa, slightly larger than the apparent 49 kDa, estimated by comparison of mobility relative to molecular weight standards by SDS-PAGE. A predicted RBS and three potential promoters: one showing similarity to the sporulation specific σ^E consensus, another to a σ^G promoter and an atypical σ^A-like promoter, have been identified. The presence of σ^E and σ^G like promoter consensus sequences, as well as an unusual σ^A-like promoter, upstream of *p49* is consistent with the presence of P49 in the *B. sphaericus* spore. Protein database searches have revealed that P49 shows homology to the Cry36Aa1 toxin from *B. thuringiensis* as well as the *B. sphaericus* Bin toxin. Shorter regions of similarity are also shared with the Cry35 family of binary toxins from *B. thuringiensis*. The homology shared between P49 and other insecticidal toxins was encouraging with regard to its predicted role as a mosquitocidal toxin.

The cloning and sequencing of *p49* from strains LP1G and 47-6B has revealed that the amino acid sequence of the encoded protein from LP1G differs from that of IAB59 and 47-6B, which are identical. The effect of these amino acid changes in the LP1G variant is currently unknown. However, the confirmation that P49 is a common protein found in the strains able to overcome Bin toxin resistance, was again encouraging with regard to its putative role as the unidentified toxic factor within these strains.

Light microscope analysis of sporulated cultures revealed the presence of free crystals in the media of strains IAB59 and LP1G, which were not observed in cultures

of strains 2362 and 1593. The small crystals appeared distinct from the spore associated Bin toxin crystal and may suggests that the unidentified toxin(s) from IAB59 and LP1G are produced as crystal(s) at sporulation. The presence of these crystals as well as P49, in both IAB59 and LP1G, may indicate that the putative toxin P49 is a crystal protein.

To determine whether, as hypothesised, P49 is a crystal toxin responsible for the activity of strains IAB59, LP1G and 47-6B towards resistant *Culex* mosquito larvae, a series of bioassays were performed as described in chapter 4. For this, recombinant expression of P49, as described in the following chapter, was performed in preparation for the bioassay. Also, in attempt to gain further knowledge regarding the ~15 kb *Hin*dIII fragment cloned from IAB59 DNA, in p49H5 and p49H11, it was decided to sequence the entire insert, beyond the region encoding P49 determined in this chapter. The sequencing and features of these inserts are described in chapter 5.

CHAPTER 4

Recombinant expression and bioassay of the putative crystal toxin, P49, from *B. sphaericus* IAB59

4.1 Introduction

The *B. sphaericus* BinA and BinB toxin proteins have been produced in recombinant form in both *E. coli* (Davidson *et al.*, 1990; Berry *et al.*, 1993) and *B. thuringiensis* (Nicolas *et al.*, 1993). In addition, the vegetative toxins Mtx1, Mtx2 and Mtx3 have successfully been expressed as GST fusion proteins in *E. coli* (Thanabalu *et al.*, 1992; Liu *et al.*, 1996; Thanabalu and Porter, 1996).

The gene encoding a putative crystal toxin, temporarily named P49, has been successfully cloned from *B. sphaericus* strain IAB59, as described in the previous chapter. To determine whether P49 is the toxic factor responsible for the activity of *B. sphaericus* strains IAB59, LP1G and 47-6B against Bin-resistant *Culex* mosquito larvae, bioassay of P49 against *C. quinquefasciatus* larvae was performed. First, bioassay of P49 in the absence of the potent BinA and BinB combination, against susceptible *C. quinquefasciatus* (SLCq) larvae, would establish whether P49 is toxic. Further assays against Bin-resistant *C. quinquefasciatus* (RLCq/C3-41) larvae would determine whether P49 is the toxic factor accountable for the activity of strains IAB59, LP1G and 47-6B towards resistant *Culex* mosquito populations. To allow for its bioassay in the absence of Bin, recombinant expression of P49 was carried out. As it was unknown which would be the better host for recombinant P49 production, expression was trialled in *E. coli* and in a non mosquitocidal, plasmid cured *B. thuringiensis* subsp. *israelensis* host, 4Q7.

4.2 Expression of P49 in *E. coli*

Over expression of P49 in *E. coli* was attempted by cloning the *p49* gene into the expression plasmids pGEX-4T-2 and a modified pET22b. Cloning the gene for *p49*, in the correct reading frame, into the MCS of pGEX-4T-2 results in the

expression of P49 as a fusion protein with GST. The GST moiety, at the N-terminus of the expressed protein, allows affinity purification of the fusion protein using glutathione, and the GST tag can be removed subsequently by thrombin cleavage. The pET22b vector used here has been modified to encode an N-terminal $(His)_6$ tag. Cloning the *p49* gene, including its stop codon, into the *Nde*I and *Xho*I sites of the modified pET22b results in the production of an N-terminally His tagged P49, the stop codon preventing continued translation into the vector encoded C-terminal His tag.

4.2.1 Expression of P49 as a GST fusion

4.2.1.1 Cloning of *p49* into a pGEX expression vector

Primers were designed for PCR amplification of the gene encoding P49, that would facilitate cloning into the *Bam*HI site in the MCS of pGEX-4T-2. The primer pairs, P49F and P49R, were designed from the 5' and 3' ends of *p49* respectively and the restriction site for *Bam*HI was added to the 5' end of both primers. The nucleotide sequence of P49F and P49R can be seen in figure 4.1, as well as in the appendix. The gene encoding P49 was amplified from p49H5, which contains the ~15 kb *Hin*dIII fragment cloned from IAB59 DNA containing the *p49* gene (section 3.9), by PCR (section 2.2.6.1), using primers P49F and P49R and Easy-A proofreading DNA polymerase. Thermocycling conditions were an initial denaturation step at 95°C for 5 min, 15 cycles of 95°C (1 min), 60°C (1 min) and 72°C (1.5 min), followed by a final extension at 72°C for 10 min. The PCR product was run on a 1% agarose gel (section 2.2.2) before purification from the gel (section 2.2.4) and ligation (section 2.2.7) into the pGEM-T vector. The ligation reaction was then transformed

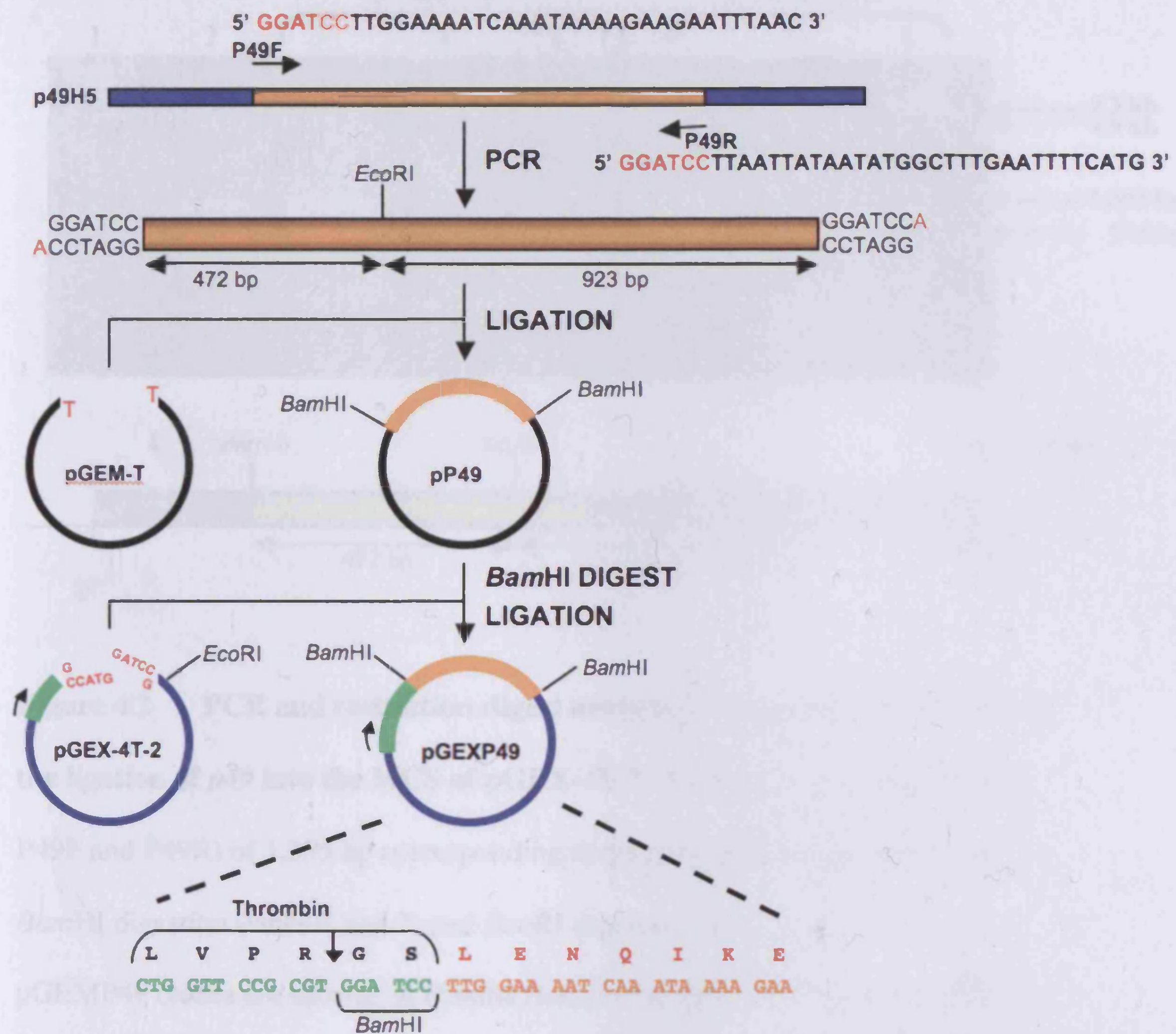


Figure 4.1 Schematic representation of the cloning of *p49* into the pGEX expression vector. P49F and P49R primer sequences, the *p49* gene (orange) and the gene encoding the amino terminal GST (green) are shown. The thrombin recognition sequence and the *Bam*HI recognition sequence are shown in brackets. The cleavage site for removal of the GST tag is indicated by an arrow.

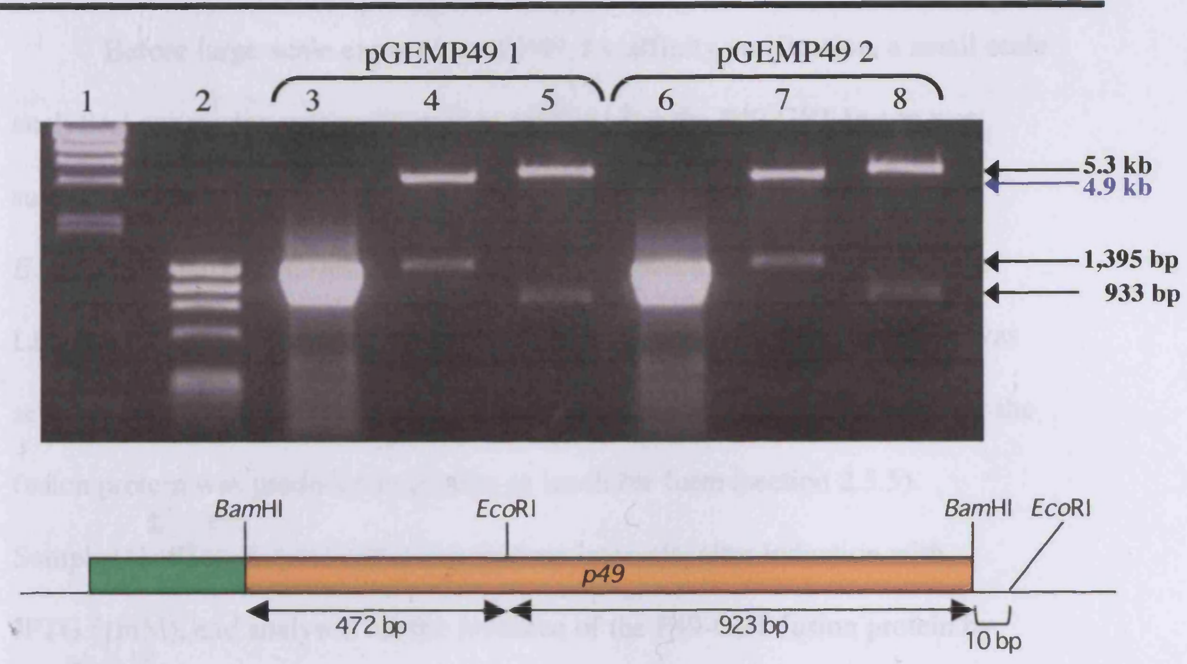


Figure 4.2 PCR and restriction digest analysis of two clones obtained from the ligation of *p49* into the MCS of pGEX-4T-2. PCR products (using primers P49F and P49R) of 1,395 bp corresponding to the *p49* gene (lane 3 and 6) and, *Bam*HI digestion (lanes 4 and 7) and *Eco*RI digestion (lanes 5 and 8) of two pGEMP49 clones are shown. λ *Hin*dIII (lane 1) and φX174 *Hae*III (lane 2) DNA markers were also run. The 1,395 bp fragment in the *Bam*HI digests represents the entire *p49* gene cut from the pGEX-4T-2 vector (4.9 kb). The 933 bp fragment in the *Eco*RI digest consists of the 923 bp fragment from *p49* and 10 bp (including the *Bam*HI site) between the *p49* stop codon and the *Eco*RI cleavage site in the MCS. The 933 bp fragment, and not a 472 bp (+ an additional 10 bp), in the *Eco*RI reaction would indicate that the *p49* gene was cloned into the vector in the correct orientation.

4.2.1.2 Expression of the P49-GST fusion protein

Before large-scale expression of P49, for affinity purification, a small scale analytical expression was performed to confirm that the P49-GST fusion was successfully produced in *E. coli*. The clone pGEXP49 was transformed into *E. coli* strain BL21(DE3)pLysS, as described in section 2.3.2.1, and spread on LB agar plates containing ampicillin and chloramphenicol. A single colony was selected for analytical scale expression (section 2.5.4) and to monitor whether the fusion protein was produced in soluble or insoluble form (section 2.5.5). Samples (1ml) were removed at hourly time intervals, after induction with IPTG (1mM), and analysed for the presence of the P49-GST fusion protein by SDS-PAGE (section 2.5.1), and Western blot (section 2.5.2) using the polyclonal Anti-GST antibody (Amersham Biosciences UK Ltd., Bucks, UK). An immuno-reactive band, with an apparent molecular weight of approx. 79 kDa, was detected by Western blotting (figure 4.3), consistent with the expected size of a fusion protein comprising a 26 kDa GST moiety and the 53 kDa P49. The fusion protein could also be seen by SDS-PAGE analysis, as shown in figure 4.3. Additional immuno-reactive bands, presumably owing to detection of degradation products of the P49-GST fusion protein, could also be seen on the Western blot. Increasing levels of P49-GST could be seen, both by SDS-PAGE analysis and Western blot, from t=0 hours to t=4 hours. Further analysis, performed as described in section 2.5.5, revealed that the majority of the protein was produced in insoluble form. Figure 4.4 shows SDS-PAGE analysis of soluble and insoluble fractions from the expression culture sample taken at t=4 hours post induction with IPTG. Identical analyses were performed for samples taken at t=1, t=2 and t=3 hours, again indicating that the majority of P49-GST was produced in insoluble form (data not shown).

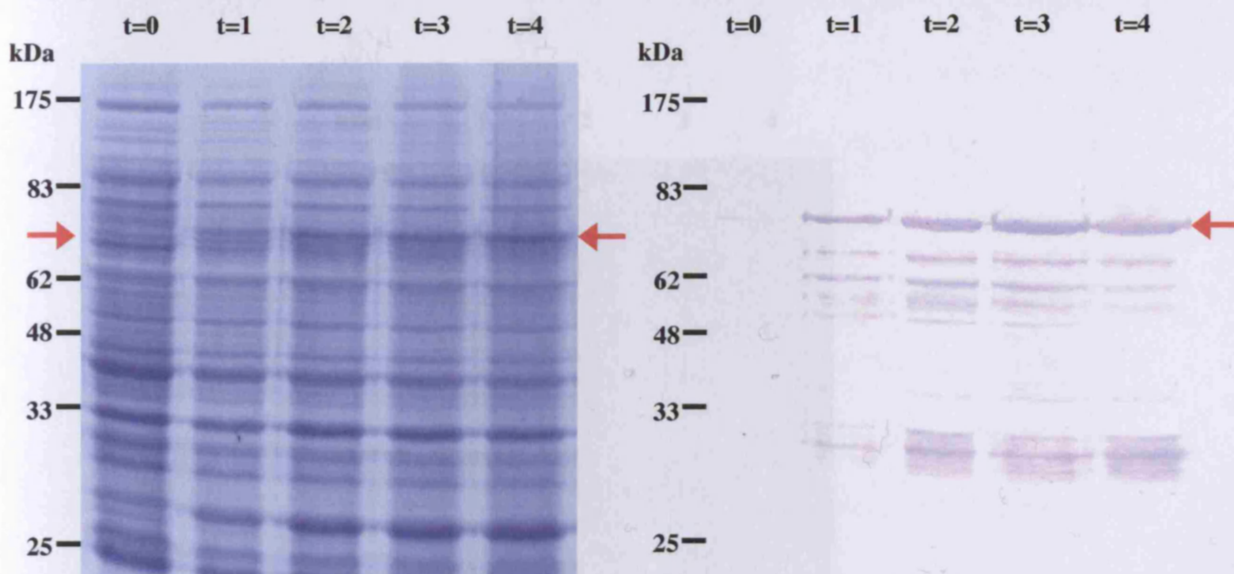


Figure 4.3 SDS-PAGE and Western blot analysis of P49-GST expression.

Analysis of samples following induction of P49-GST in *E. coli* BL21(DE3)pLysS with 1mM IPTG. Protein bands corresponding to P49-GST on the SDS-PAGE gel and blot are indicated by a red arrow. Gel lanes are loaded with samples taken at t=1, t=2, t=3 and t=4 hours post induction. A sample taken immediately before induction is indicated by t=0. The sizes of molecular weight standard proteins are shown.

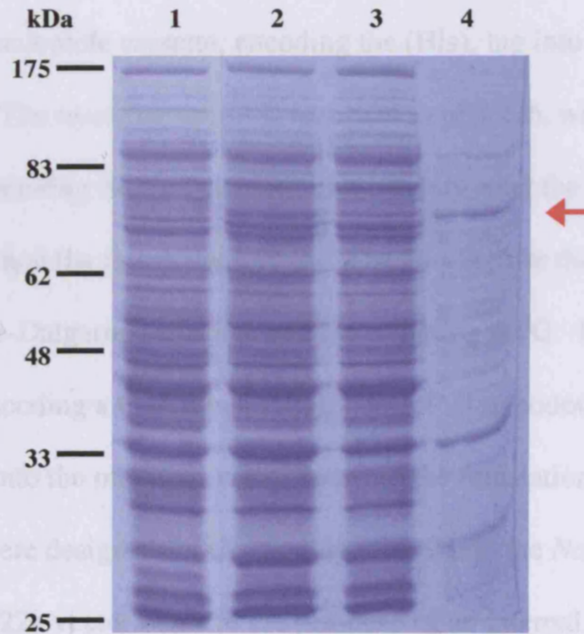


Figure 4.4 SDS-PAGE analysis of soluble and insoluble fractions of the *E. coli* P49-GST expression. Lane 1, culture at t=0 hours; lane 2, culture at t=4 hours; lane 3, soluble fraction of culture at t=4 hours; lane 4, insoluble fraction of culture taken at t=4 hours. The sizes of molecular weight protein standards are shown.

4.2.2 Expression of P49 using the pET vector system

4.2.2.1 Cloning of *p49* into a pET22b expression vector

The pET22b vector used in this study, a kind gift from Mr. T. Winterburn, had been previously modified to encode an N-terminal $(His)_6$ tag. This was achieved by cloning an oligonucleotide cassette, encoding the $(His)_6$ tag into the *Xba*I and *Nde*I sites of pET22b. The resulting vector is identical to pET22b, with exception of i) the DNA sequence encoding the His residues immediately after the ATGAAA coding for the initiating Met and the following Lys and ii) a *Hin*dIII site that was introduced between the Shine-Dalgarno sequence and the initiating ATG. Due to the original pET22b vector encoding a C-terminal $(His)_6$ tag, a 3' stop codon was included in the *p49* gene cloned into the modified vector to avoid the translation of this tag.

Primers were designed for the cloning of *p49* into the *Nde*I and *Xho*I sites of the modified pET22b vector. Due to the presence of an internal *Nde*I recognition sequence at bases 1154-1159 from the initiating TTG, a cloning strategy was designed for removal of the *Nde*I site by overlap extension (section 2.2.11.2), thus allowing digestion of the gene with *Nde*I and *Xho*I for cloning into the modified pET22b vector. A mutation of the CATATG *Nde*I recognition sequence to CCTATG would result in removal of the *Nde*I site, without alteration of the P49 amino acid sequence. Site-specific mutagenesis by overlap extension was performed as described in section 2.2.11.2, and the resulting gene product was cloned into the modified pET22b vector, after digestion of the 5' *Nde*I and 3' *Xho*I sites introduced during the PCR. A Gln residue was also encoded by the 5' primer between the *Nde*I site and the beginning of the sequence coding for P49. This would, if desired, allow removal of the N-terminal His tag using the Qiagen TAGZyme kit (Qiagen, Crawley, West

Sussex) which utilizes an exopeptidase that sequentially removes two aa residues from the N-terminus. The introduced Gln residue, which is refractory to cleavage by this exopeptidase, would prevent continued cleavage into P49. The sequences of the primers used below can be found in the appendix and in the cloning strategy that is summarised in figure 4.5.

Briefly, the region of *p49* containing the undesired *Nde*I site, together with the upstream gene sequence was amplified from p49H5 by PCR (section 2.2.6.1) using the primer PETP49F and the mutagenic primer NDESDMR. A second PCR was performed, using the same template DNA, with primers NDESDMF (a primer the reverse compliment of NDESDMR) and PETP49R to amplify the *p49* region containing the *Nde*I site and the downstream DNA sequence. PCR conditions were an initial denaturation step at 95°C for 5 min, followed by 15 cycles of 95°C (1 min), 55°C (1 min), 72°C (1 min), and a final extension at 72°C for 10 min, using Easy-A proofreading DNA polymerase. Both PCR products were purified by agarose gel electrophoresis (section 2.2.2) followed by extraction from the gel (section 2.2.4). The purified products (see figure 4.6) were then used as template DNA in a final PCR using primers PETP49F and PETP49R, and the same thermocycling conditions as described for the previous two reactions. This final PCR product was digested with *Nde*I and *Xho*I (section 2.2.3), separated by agarose gel electrophoresis, and extracted from the gel before ligation (section 2.2.7) into the modified pET22b which had been pre-cut with *Nde*I and *Xho*I. Following incubation, the ligation mix was transformed into *E. coli* DH5α by electroporation (section 2.3.2.2) and spread onto LB agar plates containing ampicillin for selection of transformants. Colonies were screened for the presence of the *p49* gene by PCR using primers PETP49F and PETP49R and the same thermocycling conditions as described above, except that 30 cycles were

performed rather than 15. A colony yielding a positive PCR product was selected, the plasmid isolated (section 2.2.1) and the insert sequenced (section 2.2.10) using the vector specific primers SF1 and T7, and the Int2F *p49* internal primer (see appendix for primer sequences). The sequencing confirmed that the *p49* gene, lacking the internal *Nde*I site, had been successfully cloned into the modified pET22b vector, and that no PCR introduced mutations had occurred. The resulting clone was named pETP49.

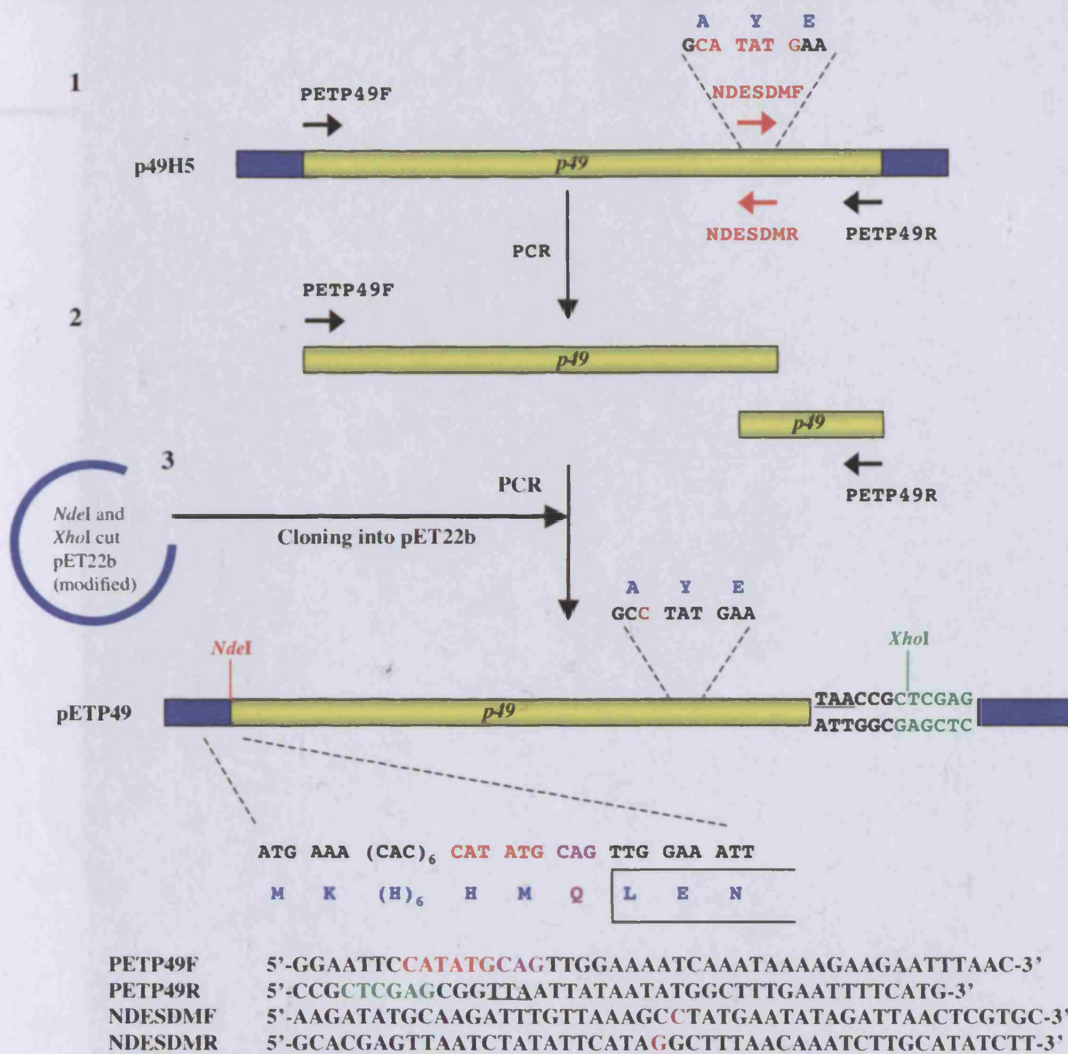


Figure 4.5 Schematic representation of the cloning of *p49* into the modified pET22b for expression using the pET system. 1. Two PCRs were performed for amplification of regions of *p49* from p49H5 using the primer combinations PETP49F/NDESDMR and NDESDMF/PETP49R. 2. A third PCR reaction was performed using the products from the first two PCRs and primers PETP49F and PETP49R. 3. The final product was digested at the primer-introduced *Nde*I and *Xho*I sites and cloned into the modified pET22b, cut with the same restriction enzymes. All *Nde*I sites are shown in red, including the site removed from the wild type *p49* gene. *Xho*I sites are shown in green, the *p49* stop codon is underlined and the protein sequence corresponding to P49 boxed. The primer introduced Gln residue is shown in purple and the altered base introduced by the mutagenic primers is shown in brown.

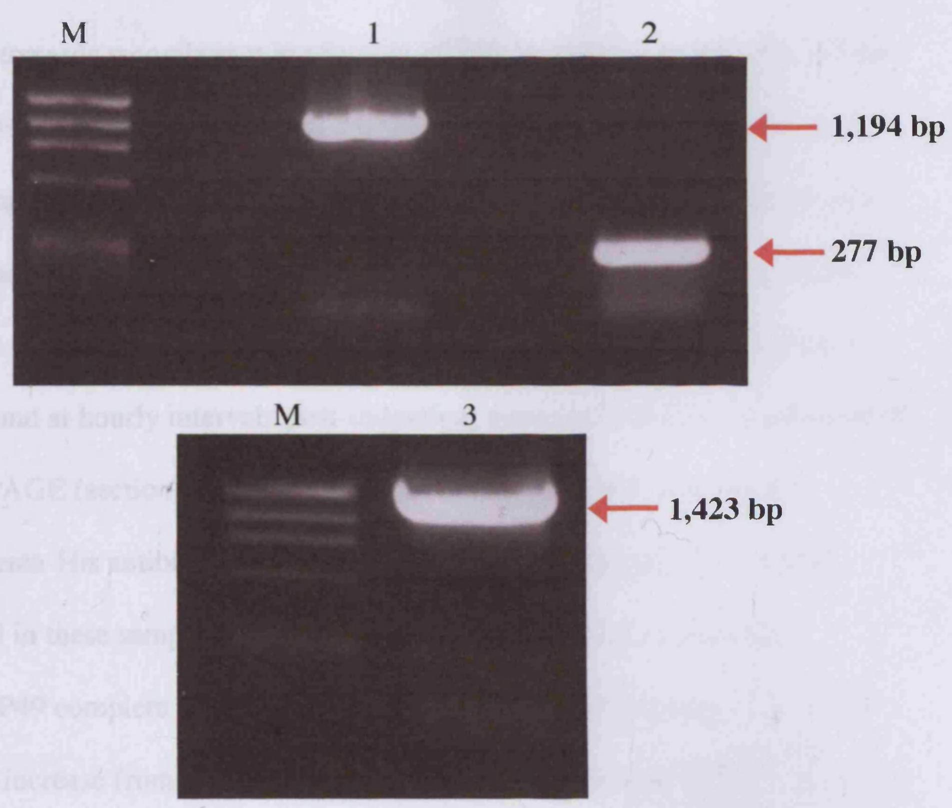


Figure 4.6 Overlap extension PCR removal of the *p49 Nde*I site. Products of the first round PCRs using primers PETP49F and NDESDMR (lane 1) and NDESDMF and PETP49R (lane 2) were used as template in the final PCR with primers PETP49F and PETP49R (lane 3). The visible ϕX174 *Hae*III DNA marker (lanes M) band sizes are 1,353 bp, 1,078 bp, 872 bp, 603 bp and 310 bp.

4.2.2.2 Expression of P49 using the pET system

In an identical manner to that performed for P49 expression as a GST fusion protein, a small scale pET system expression of P49 was also trialled to determine the best approach towards recombinant production of P49 for future experiments. Clone pETP49 was transformed into *E. coli* BL21(DE3)pLysS (section 2.3.2.1) and, from the resulting transformants selected for by growth on LB agar containing ampicillin and chloramphenicol, a single colony was selected for analytical scale expression (section 2.5.4). Samples (1ml), taken immediately before induction by addition of IPTG (1mM) and at hourly intervals post-induction, were analysed for the presence of P49 by SDS-PAGE (section 2.5.1), and Western blot (section 2.5.2) using the monoclonal Penta-His antibody. Figure 4.7a shows the immuno-reactive protein bands detected in these samples. A major band of expected size, considering expression of P49 complete with a His tag, was detected in all the induced samples. Levels of P49 increase from t=0 to t=3 hours, with a slight decrease in P49 seeming to occur between t=3 and t=4. A number of immuno-reactive bands of slightly lower molecular weight were also detected, suggesting slight degradation of P49. Further analysis, performed as described in section 2.5.5, revealed that P49 was produced mainly in insoluble form (figure 4.7b).

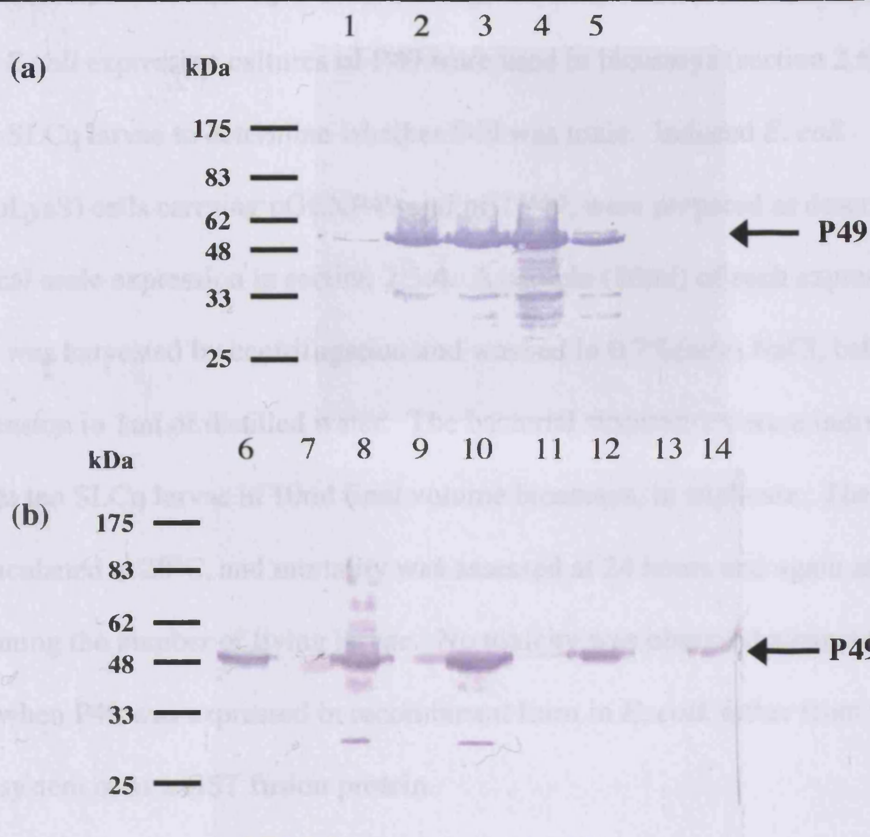


Figure 4.7 Western blot analysis of pETP49 expression. (a) Analysis of whole cell expression samples taken at time points t=0 (lane 1), t=1 (lane 2), t=2 (lane 3), t=3 (lane 4) and t=4 (lane 5) hours post induction with IPTG. (b) Examination of whole cell and soluble *E. coli* BL21(DE3)pLysS pETP49 expression sample extracts. In agreement with the blot shown in (a), immuno-reactive bands, indicating P49 expression, are seen in the whole cell samples at t=1 (lane 6), t=2 (lane 8), t=3 (lane 10) and t=4 (lane 12). No immuno-reactive proteins were detected in the soluble extracts of samples taken at t=1 (lane 7), t=2 (lane 9), t=3 (lane 11) and t=4 (lane 13) hours post induction. Detection of P49, indicating its presence in insoluble form, is seen in the insoluble extract (lane 14) prepared from the sample taken at t=4 hours post induction.

4.2.3 Bioassay of *E. coli* expressing P49 against *C. quinquefasciatus* larvae

E coli expression cultures of P49 were used in bioassays (section 2.6.1) against SLCq larvae to determine whether P49 was toxic. Induced *E. coli* BL21(pLysS) cells carrying pGEXP49 and pETP49, were prepared as described for analytical scale expression in section 2.5.4. A sample (10ml) of each expression culture was harvested by centrifugation and washed in 0.7%(w/v) NaCl, before final resuspension in 1ml of distilled water. The bacterial suspensions were individually added to ten SLCq larvae in 10ml final volume bioassays, in triplicate. The bioassays were incubated at 28°C, and mortality was assessed at 24 hours and again at 48 hours, by counting the number of living larvae. No toxicity was observed against the SLCq larvae when P49 was expressed in recombinant form in *E. coli*, either from the pET vector system or as a GST fusion protein.

4.2.4 Discussion

This section has described the cloning of the gene encoding P49 into pGEX-4T-2 and a modified pET22b expression vector, yielding clones pGEXP49 and pETP49 respectively. Induced *E. coli* BL21(DE3)pLysS cells carrying the pGEXP49 clone were found to express P49 as a fusion with GST. The same *E. coli* strain was also found to express an N-terminal His tagged P49, when transformed with pETP49 and induced with IPTG. Both forms of the recombinant P49 protein were found to be produced predominantly in an insoluble form and were not toxic towards *C. quinquefasciatus* larvae.

To determine whether recombinant production of P49 in *B. thuringiensis* subsp. *israelensis* might yield higher levels of the putative toxin, expression in this host was also explored as described in the following section. Light microscope

analyses of sporulated cultures of *B. sphaericus* strains IAB59 and LP1G have revealed that small crystals are released from the spore following sporulation, and these are distinct from the Bin toxin crystals that are found associated with the spore. These crystals are not found in strains 2362 and 1593, which do not show toxicity against Bin-resistant *Culex* mosquito larvae and do not contain the genes for the putative toxin P49. This has led to the prediction that the putative toxin, P49, is produced as a crystal in the *B. sphaericus* strains showing toxicity to Bin-resistant *Culex* mosquito larvae. If this proved to be the case, a possible advantage of P49 expression in *B. thuringiensis* subsp. *israelensis* could be its production as a more native crystal during sporulation, as is observed for the expression of the *B. sphaericus* Bin toxin crystal in a *B. thuringiensis* host (Nicolas *et al.*, 1993). If P49 is indeed a crystal protein, synthesis of the putative toxin in this form may lead it to adopt a more native fold. It is possible that P49 is unable to assume its native fold in *E. coli*, a factor that may be responsible for its lack of toxicity towards *C. quinquefasciatus* larvae when produced in this host.

4.3 Expression of P49 in *B. thuringiensis* subsp. *israelensis*

Recombinant expression of P49, in an acrystalliferous strain of *B. thuringiensis* subsp. *israelensis,* was attempted under the regulation of its own promoter. The gene encoding P49 and the upstream region predicted to comprise its promoter, as described in the previous chapter, was cloned into the *B. thuringiensis-E. coli* shuttle vector pHT304 (Arantes and Lereclus, 1991). Vector pHT304 contains origins of replication for propagation in both bacteria, and contains genes encoding ampicillin and erythromycin resistance for selection in *E. coli* and *B. thuringiensis* respectively.

4.3.1 Cloning of *p49* into pHT304

The primers P49PROF and P49R were used to amplify the *p49* gene and 201 bp upstream, from clone p49H5, by PCR (section 2.2.6.1) using Easy-A proofreading DNA polymerase. Thermocycling conditions were an initial denaturation at 95°C for 5 min, followed by 15 cycles of 95°C (1 min), 60°C (1 min), 72°C (1 min, 30 sec), and a final extension at 72°C for 10 min. The resulting PCR product was purified, by agarose gel electrophoresis (section 2.2.2) and extraction from the gel (section 2.2.4), before ligation (section 2.2.7) into the cloning vector pGEM-T. The ligation mixture was transformed (section 2.3.2.2) into *E. coli* DH5α and the transformants spread onto LB agar containing IPTG/X-Gal (section 2.2.8) and ampicillin. A white colony, indicating successful vector-insert ligation, was selected and the plasmid isolated. The clone was sequenced (section 2.2.10) using vector primers M13F and M13R and the internal *p49* primer, Int1F, and analysis confirmed that no PCR mutations had occurred. The clone was named pP49PRO.

A *Bam*HI digest (section 2.2.3) of pP49PRO was performed and the products separated by agarose gel electrophoresis (section 2.2.2), followed by purification (section 2.2.4) of the approx. 1.6 kb fragment corresponding to *p49* and its putative promoter. This fragment was cloned (section 2.2.7) into pHT304, pre-cut with *Bam*HI and treated with CIP to prevent self-ligation, and the ligation mix again transformed (section 2.3.2.2) into *E. coli* DH5α. Colonies that formed on LB agar containing ampicillin were screened for the presence of an insert by colony PCR (section 2.2.6.1). A colony yielding a product of correct size was selected and the plasmid isolated, before sequencing (section 2.2.10) with primers M13F, M13R and

Int1F. The insert was found again to contain no mutations and the clone was named pHTP49.

4.3.2 Expression from pHTP49 in *B. thuringiensis* subsp. *israelensis*

The clone pHTP49 was used to transform *B. thuringiensis* subsp. *israelensis* strain 4Q7 by electroporation (section 2.3.4), and the cells spread on LB agar containing erythromycin for selection. Successful transformation was confirmed by colony PCR (section 2.2.6.1) using primers P49PROF and P49R. A single colony, yielding a PCR product of correct size after colony PCR, was selected from the plate and used to inoculate 30ml of Embrapa sporulation medium (section 2.1.6), containing erythromycin, in a 250ml conical flask. A control culture was also prepared by inoculation of 30ml of the same medium, containing no erythromycin, with untransformed *B. thuringiensis* subsp. *israelensis* 4Q7. Both cultures were incubated at 30°C, 250 rpm for 72 hours. Following this time a 1ml sample of each sporulated culture was harvested by centrifugation, the supernatants discarded, and the pellets resuspended in 100μl of SDS-PAGE sample buffer (section 2.5.1). The samples were boiled for 5 min before analysis of 10μl by SDS-PAGE (section 2.5.1), through a 10% gel, to establish whether expression of P49 had occurred. The resulting gel can be seen in figure 4.8, indicating successful expression of P49 in the recombinant *B. thuringiensis* subsp. *israelensis*. Additional protein bands can also be seen on the gel. A number of protein bands of lower molecular weight than P49, which do not appear in the untransformed control, may indicate slight degradation of P49 when expressed in recombinant form in *B. thuringiensis* subsp. *israelensis*. In addition, a protein of much higher molecular weight, appearing above the 83 kDa molecular weight marker and absent from the control culture, was also observed.

Five cycles of N-terminal sequencing of this protein band was performed, as described in section 2.5.3, and a strong signal with no background revealed the sequence (MENQI) to be identical to that of P49, suggesting this band to be a dimer. Analysis of the cultures under a light microscope also revealed the presence of free crystals in the recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) medium, which were not present in the untransformed control culture. A stock of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49), was prepared by dipping a piece of filter paper, sterilised by autoclaving, into the sporulated culture. The filter paper was stored at -20°C. For preparation of new cultures, a small piece was cut from the stock filter paper, with a sterile scalpel blade, and used either to inoculate culture media containing erythromycin, or to streak across the surface of LB agar plates again containing erythromycin.

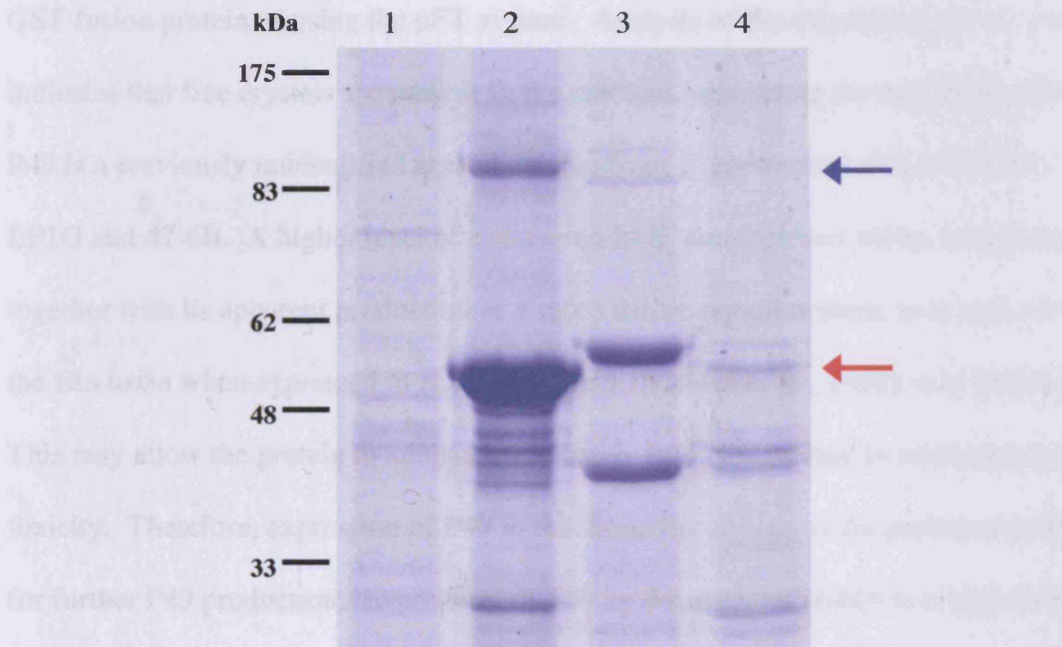


Figure 4.8 Recombinant expression of P49 in *B. thuringiensis* subsp. *israelensis* 4Q7. SDS-PAGE analysis of sporulated cultures of *B. thuringiensis* subsp. *israelensis* 4Q7: untransformed control (lane 1) and transformed with pHTP49 (lane 2). Sporulated cultures of strain 2362 (lane 3) and IAB59 (lane 4), prepared as described for *B. thuringiensis* subsp. *israelensis* 4Q7, are also shown for reference. The position of P49, present in both the recombinant *B. thuringiensis* subsp. *israelensis* and IAB59, is indicated by a red arrow. A dimer of P49, confirmed by N-terminal sequencing, in the recombinant culture is indicated by a blue arrow.

4.3.3 Discussion

Recombinant expression of P49 in *B. thuringiensis* subsp. *israelensis* yields higher levels of P49 than are observed when P49 is expressed in *E. coli*, either as a GST fusion protein or using the pET system. Analysis of the expression culture also indicates that free crystals are present in the medium, supporting the hypothesis that P49 is a previously unidentified crystal protein from *B. sphaericus* strains IAB59, LP1G and 47-6B. A higher level of expression in *B. thuringiensis* subsp. *israelensis*, together with its apparent production as a more native crystal protein, as is seen for the Bin toxin when expressed in *B. thuringiensis* (Nicolas *et al.*, 1993), was observed. This may allow the protein to adopt a more native fold, which may be important for toxicity. Therefore, expression of P49 in this host was chosen as the preferred method for further P49 production. Expression of P49 by this method results in a high level of P49 synthesis, with few contaminating protein bands, as can be seen in figure 4.8. Some of the protein bands of lower MW than P49 may be degradation products of P49, rather than contaminants of unrelated proteins. Also, a higher MW protein band, present in the sporulated culture of *B. thuringiensis* subsp. *israelensis* 4Q7 expressing P49, has been confirmed to be a dimer of P49 and not a contaminant.

4.4 Bioassay of P49

To determine whether P49 shows toxicity against susceptible mosquito larvae, the recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) was used in bioassays against *C. quinquefasciatus* larvae. A small piece of filter paper was cut from the stock filter paper containing spores of *B. thuringiensis* subsp. *israelensis* transformed with pHTP49 and was used to inoculate 30ml of Embrapa sporulation medium (section 2.1.6), containing erythromycin, in a 250ml conical flask. The culture was incubated at 30°C, 250 rpm for 72 hours until sporulation was complete, as judged by light microscopy. These sporulated cultures, containing P49 crystals, were used in a selective bioassay against second or third instar SLCq larvae, as described in section 2.6.1. No toxicity of P49 towards SLCq larvae was observed.

The non-toxicity of P49 could be explained in a number ways: First, that P49 is not responsible for the toxicity of strains IAB59, LP1G and 47-6B towards resistant *Culex* mosquito colonies raised against strains 2362 and C3-41. Second, that P49 functions as a binary toxin with another factor, or finally, that P49 synergises with the Bin toxin in strains IAB59, LP1G and 47-6B. As discussed in chapter 3, P49 shows similarity to other insecticidal toxins, a number of which are binary toxins such as BinA and BinB from *B. sphaericus* and the Cry35 binary toxins from *B. thuringiensis.* The toxicity of strains IAB59, LP1G and 47-6B against Bin-resistant *Culex* larvae could arise from the ability of P49 to complement a non-functional BinA or BinB against Bin-resistant larvae. To determine whether this was the case, the sporulated culture of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) was assayed in combination with spores of *B. thuringiensis* subsp. *israelensis* transformed with either pHT680 or pHT684. Clones pHT680 and pHT684 contain the genes encoding BinA and BinB respectively, under the regulation of the *cyt1Aa* promoter from

B. thuringiensis subsp. *israelensis* (Nicolas *et al.*, 1993). Transformation of clones pHT680 and pHT684 into the host (as described in section 2.3.4) was followed by preparation of sporulated cultures, performed as described above for *B. thuringiensis* subsp. *israelensis* transformed with pHTP49. Selective bioassays (section 2.6.1) of SLCq larvae exposed to a BinA/P49 mix (100μl of each recombinant cultures expressing BinA and P49) and BinB/P49 mix (100μl of each recombinant cultures expressing BinB and P49), in a 10ml assay volume, also showed no toxicity.

Further bioassays were performed to determine whether the ability of strains IAB59, LP1G and 47-6B to overcome Bin-resistance in *Culex* larvae resulted from a synergy between P49 and both components of the Bin toxin. These assays could not be performed using the *C. quinquefasciatus* colony maintained in Cardiff, due to their sensitivity to Bin alone. Therefore, these bioassays were performed by Prof. Yuan Zhiming at the Wuhan Institute of Virology in China, where a resistant *C. quinquefasciatus* colony raised with resistance against *B. sphaericus* strain C3-41 (named RLCq1/C3-41) is maintained, with a resistance ratio of >140,000-fold to Bin (Pei *et al.*, 2002; Yuan *et al.*, 2003). However, bioassay of P49 in combination with Bin (expressed in recombinant form in *B. thuringiensis* subsp. *israelensis*) showed no toxicity against the resistant colony.

4.5 Discussion

This section has described the cloning of *p49* into vectors pGEX-4T-2 and a modified pET22b for expression in *E. coli*, and pHT304 for recombinant expression in *B. thuringiensis* subsp. *israelensis*. While expression studies in *E. coli* BL21(DE3)pLysS proved successful, a higher level of recombinant P49 production was achieved in *B. thuringiensis* subsp. *israelensis* strain 4Q7.

Sporulated cultures of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) were found to contain crystals suggesting, as hypothesised, that P49 is a crystal protein from *B. sphaericus* strains IAB59, LP1G and 47-6B. However, contrary to our previous prediction, P49 shows no toxicity, either alone or in combination with either BinA or BinB against susceptible *C. quinquefasciatus* larvae. In addition, P49 does not synergise with the combined BinA and BinB to cause toxicity against a resistant *C. quinquefasciatus* colony raised against strain C3-41. It seems, therefore, that the putative toxin identified in this study, nominated as the factor responsible for the toxicity of *B. sphaericus* strains IAB59, LP1G and 47-6B against Bin-resistant *Culex* larvae, is not accountable, at least when acting alone, for this toxicity. This suggests that other toxin(s) are present in these strains, allowing them to overcome Bin-resistance. Experiments aimed at the discovery and cloning of further potential toxin(s), from the *B. sphaericus* stains toxic to Bin-resistant mosquito colonies, are described in the following chapter.

CHAPTER 5

Discovery of an additional putative crystal toxin from *B. sphaericus* IAB59

5.1 Introduction

A protein with an approximate molecular weight of 49 kDa has previously been suggested to be the toxic factor from *B. sphaericus* strains IAB59, LP1G and 47-6B towards *Culex* mosquito colonies with resistance raised against strains 2362 and C3-41 (Yuan *et al.*, 2003; Silva-Filha *et al.*, 2004). In this study, the gene encoding this putative toxin, temporarily named P49, has been cloned and expressed in an acrystalliferous *B. thuringiensis* subsp. *israelensis* and determined to be non-toxic towards both susceptible and Bin-resistant *C. quinquefasciatus* larvae. This suggests that strains IAB59, LP1G and 47-6B all carry at least one additional toxin.

During this study, a new *B. sphaericus* strain, named NHA15b (serotype H50), was isolated from Vietnam (Nielsen-LeRoux personal communication). This strain carries the genes coding for P49 but not those encoding the Bin toxin. Bioassay against the RLCq/C3-41 colony as well as SLCq larvae has shown that strain NHA15b is toxic to both. These bioassays have proved that the ability of this strain to overcome Bin-resistance does not involve the Bin toxin acting in concert with another toxic factor, and may suggest a similar situation for strains IAB59, LP1G, 47-6B. Also, the presence of P49 in the newly isolated strain may still point to a role for P49 in overcoming Bin-resistance, and so the toxicity of P49, although non-toxic alone, could not yet be ruled out.

This chapter describes the search for the toxic factor(s) in strains IAB59, LP1G, 47-6B and NHA15b that allows them to overcome Bin-resistance in *Culex* larvae. As described previously in chapter 3, for the cloning of the *p49* gene from IAB59, this involved preparation of SDS-PAGE protein profiles of the strains able to overcome resistance and their comparison to those strains that undergo resistance.

5.2 Protein based analysis of *B. sphaericus* strains

5.2.1 Protein fingerprint comparison of *B. sphaericus* spore cultures

The approach of looking at SDS-PAGE protein fingerprints of sporulated *B. sphaericus* cultures, and comparison of the profiles of those strains able to overcome Bin-resistance with strains 2362 and 1593, two strains against which resistance can develop, proved to be very successful for the cloning of the gene encoding P49. In view of this, the same approach was undertaken for the discovery of further putative toxin(s) from strains IAB59, LP1G, 47-6B and NHA15b. Strains IAB59, LP1G, NHA15b and 2362, as well as *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) for reference, were grown to sporulation for 72 hours in Embrapa sporulation medium (30ml; section 2.1.6) at 30°C, 250 rpm, in a 250ml conical flask. A sample (1ml) of each culture was harvested by centrifugation, the pellet resuspended in 100μl of SDS-PAGE sample buffer (section 2.5.1), and boiled for 5 min. The samples (10μl) were analysed by SDS-PAGE (section 2.5.1), through a 10% gel. As well as P49 being a common protein band in the strains able to overcome resistance, an additional protein, temporarily named P43 according to its approximate molecular weight, was also identified as shown in figure 5.1.

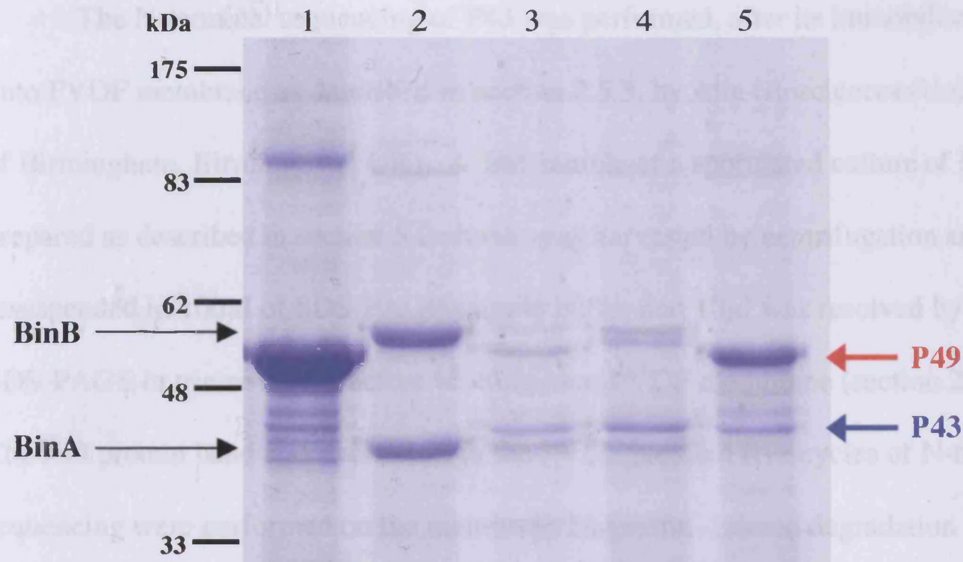


Figure 5.1 SDS-PAGE protein profiles of *B. sphaericus* strains. Sporulated cultures of strains 2362 (lane 2), IAB59 (lane 3), LP1G (lane 4), NHA15b (lane 5) analysed by SDS-PAGE (10% gel). *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) is also shown for reference (lane 1). P49, found in all strains except for 2362, is indicated by a red arrow, with the LP1G variant of LP1G having a slightly higher apparent molecular weight compared to the others. BinA (42 kDa) and BinB (51 kDa) protein bands, present in strains 2362, IAB59 and LP1G, are also indicated. A common protein band in the strains able to overcome resistance, and temporarily assigned the name P43 according to its approximate molecular weight, is indicated by a blue arrow.

5.2.2 Amino terminal sequencing of P43

The N-terminal sequencing of P43 was performed, after its immobilisation onto PVDF membrane as described in section 2.5.3, by Alta Biosciences (University of Birmingham, Birmingham, UK). A 1ml sample of a sporulated culture of IAB59, prepared as described in section 5.2 above, was harvested by centrifugation and resuspended in 100μl of SDS-PAGE sample buffer and 10μl was resolved by SDS-PAGE in tricine buffer before blotting onto PVDF membrane (section 2.5.3). The P43 protein band was excised from the PVDF blot and five cycles of N-terminal sequencing were performed on the membrane fragment. Edman degradation revealed the N-terminal sequence of P43 to be: NNDPG, consistent with P43 being a degradation product of P49 lacking aa residues 1-75 and, providing that no C-terminal processing of P49 or P43 occurs, having a predicted molecular weight of 44.7 kDa. The finding that P43 was a degradation product of P49 and the presence of a protein of the same apparent molecular weight in the *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) sample, suggested that the same degradation was occurring in this recombinant host and resulted in no further analysis being performed on P43.

5.2.3 Crystal protein profile of *B. sphaericus* NHA15b

The recent discovery of *B. sphaericus* strain NHA15b, that carries the genes for P49 but not the *bin* genes, and its ability to cause mortality of Bin-resistant *Culex* mosquitoes prompted a focusing of new toxin discovery on strain NHA15b. Protein profile analysis of the strains able to overcome resistance (figure 5.1) has resulted in analysis of a number of common protein bands, with P49 being selected as a putative toxin, which was later found to be non-toxic. After establishing that P43 is a degradation product of P49, there were no further common protein bands, detectable

by SDS-PAGE protein profile analysis, present for selection as putative toxins. This resulted in the focus on strain NHA15b to determine whether a level of toxin production was present, that was too low for detection by SDS-PAGE, Coomassie stained protein profiles. With the insecticidal toxins being found as crystals at sporulation, as seems to be the case for P49, crystals were purified from strain NHA15b to determine whether further crystal proteins were present. Strain NHA15b was selected, rather than IAB59, to allow bioassay of purified crystals against SLCq larvae, ensuring that any larval toxicity derived from newly identified crystal proteins would not be due to background contamination with Bin.

Sporulated cultures of *B. sphaericus* strain NHA15b were prepared by inoculation of two 2l baffled flasks containing 200ml of Embrapa sporulation medium (section 2.1.6). The flasks were incubated at 30°C, 250 rpm for 72 hours. Complete sporulation of the culture was confirmed by light microscope analysis. Crystals were separated from spores by ultracentrifugation through a discontinuous sucrose gradient as described in section 2.5.6. Briefly, the cultures were washed as described in section 2.5.6 before resuspension in 6.7ml of ice-cold, sterile, distilled water. After sonication the suspension was layered on top of a discontinuous gradient, comprising 7.5ml of each of 67/72/79/84%(w/v) sucrose, and centrifuged using an SW28 Ultracentrifuge rotor (Beckman Coulter Ltd., Buckinghamshire, UK). The three crystal protein bands obtained were separately extracted from the gradients, thoroughly washed and resuspended in 2ml of sterile distilled water, before analysis of 5μl of each crystal protein fraction by SDS-PAGE (section 2.5.1). Figure 5.2 shows a 10% SDS-PAGE gel of the three crystal protein fractions extracted from the gradients. Although a number of low intensity protein bands were observed, that

were presumably spore protein contaminants or degradation products of crystal proteins, the major protein bands were considered to be crystal proteins.

As can be seen from figure 5.2, as well as containing a protein band corresponding to the P49 crystal protein, there is also an additional major protein band, migrating between the 83 kDa and 175 kDa molecular weight standards, suggesting that NHA15b also produces another crystal protein of higher molecular weight than P49. Another protein band, of slightly lower intensity than this high molecular weight putative crystal protein, may correlate to a dimer of P49 that is observed when P49 is produced in recombinant form in *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49), as discussed previously in section 4.3.2 and figure 4.8. Selective bioassays, performed as described in section 2.6.1, of the three crystal protein fractions (50μl of each fraction added to a 10ml bioassay containing 5 *Culex* larvae) revealed that all three fractions caused 100% mortality.

The presence of the high molecular weight crystal protein, in all three toxic fractions from strain NHA15b, led to its selection as a new candidate toxin. N-terminal sequencing of this protein was performed to allow the design of degenerate oligonucleotides for use in cloning strategies. In the first case, five cycles of N-terminal sequencing were performed on the putative crystal toxin band in both fraction 1 and fraction 2. This was carried out to confirm that this protein, present in both fractions, was in fact identical, since it was possible that NHA15b produced two crystal proteins of similar molecular weight. A 5μl sample of fraction 1 and fraction 2 was resolved by SDS-PAGE in tricine buffer before blotting onto PVDF membrane (section 2.5.3). Bands corresponding to the protein, which was predicted although not confirmed to be the same, were excised from the membrane and sent to Alta Bioscience (University of Birmingham, Birmingham, UK) for Edman

degradation. The N-terminal aa sequence for both samples were found to be identical, with an ambiguous residue at position 1 followed by the sequence DINN.

5.2.4 Discussion

A putative, high molecular weight, crystal toxin has been identified by analysis of the crystal protein profile of *B. sphaericus* strain NHA15b. This protein was found to be present in the three fractions of crystal proteins obtained, all of which showed toxicity to SLCq larave. To allow for cloning of the gene encoding this protein, in a similar strategy used for the cloning of the *p49* gene from IAB59 (refer back to chapter 3), the N-terminal sequence of at least 15 aa residues was required for the design of degenerate oligonucleotides with as low a degeneracy as possible. During the preparation of this new putative toxin for N-terminal sequencing, our continued efforts in the sequencing of p49H5 and p49H11, the two clones prepared in chapter 3 (section 3.9) containing an approximately 15 kb *Hin*dIII fragment from strain IAB59, revealed the presence of a 3,534 bp gene downstream of the *p49* gene. Furthermore, the deduced N-terminal sequence of the protein product of this gene is identical to the experimentally derived N-terminus of the new putative crystal toxin from strain NHA15b. The following section describes the sequencing of the identical clones p49H5 and p49H11, followed by analysis of the CDSs found within their approximately 15 kb inserts.

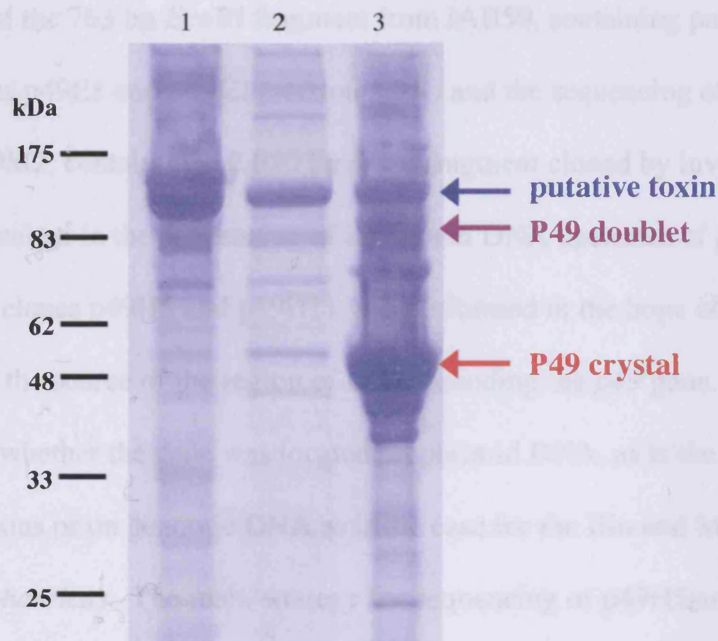


Figure 5.2 SDS-PAGE analysis of NHA15b crystals. Crystals were purified from *B. sphaericus* NHA15b by discontinuous sucrose density gradients and resuspended in water before analysis of 5μl of each band extracted from the gradient by SDS-PAGE. A major protein band corresponding to P49 crystal can be seen in fraction 3 (lane 3), indicated by a red arrow. A major protein band visible in fractions 1, 2 and 3 (lanes 1, 2 and 3 respectively), that may represent a new crystal toxin from strain NHA15b is indicated by a blue arrow. Another protein band that can be seen in fraction 3, present at intermediate levels to P49 and P135, that may be a dimer of P49 is indicate by a purple arrow.

5.3 Sequencing of p49H5 and p49H11

The sequence of a region of p49H5 and p49H11 has been determined during the sequencing of the *p49* gene using primers Int1R, Int2F and Int3F (section 3.10). Also, the sequencing of the 763 bp *Eco*RI fragment from IAB59, containing partial *p49* sequence, in clones p49E1 and p49E2 (section 3.9.1) and the sequencing of clones p49M1 and p49M2, containing a 2,010 bp *Mbo*I fragment cloned by inverse PCR (section 3.9.2) resulted in the sequencing of additional DNA upstream of *p49*. Further sequencing of clones p49H5 and p49H11 was performed in the hope of gaining knowledge on the source of the region of DNA encoding the *p49* gene. For example, determining whether the gene was located on plasmid DNA, as is the case for *B. thuringiensis* toxins or on genomic DNA as is the case for the Bin and Mtx toxin genes from *B. sphaericus*. The main strategy for sequencing of p49H5 and p49H11 was to design primers to "walk-out" from the region of DNA determined during the cloning of the gene encoding P49. However, subcloning of the *Hin*dIII insert in clone p49H5, and sequencing of these clones, was also performed to accelerate the sequencing of the approximately 15 kb insert.

5.3.1 Subcloning of the *Hin*dIII fragment in clone p49H5

A double digest of 1.5μg of p49H5 was performed with restriction enzymes *Eco*RI and *Hin*dIII as well as digestion of the same amount of p49H5 with *Eco*RI alone (section 2.2.3). The resulting digest products were separated by agarose gel electrophoresis (section 2.2.2) for analysis. The resulting gel can be seen in figure 5.3. A product of 2686 bp, found in the double digest, corresponds to the vector pUC18, into which the approx. 15 kb *Hin*dIII fragment was ligated to make clone p49H5. This digest product was, therefore, not selected for subcloning. An

approx. 2 kb fragment present in the double digest but not in the *Eco*RI digest indicated its position at one end of the 15 kb *Hin*dIII insert and, therefore, it was not selected for subcloning as this region could easily be sequenced from p49H5 or p49H11 using vector specific primers. Four products of the double digest reaction, indicted by green arrows in figure 5.3, were selected for subcloning followed by sequencing.

A preparative double digest of p49H5 (5μg), with *Eco*RI and *Hin*dIII, was performed and the four fragments, one of ~5 kb, one ~4 kb, one ~1.5 kb and another ~1 kb, were extracted (section 2.2.4) from an agarose gel after electrophoresis (section 2.2.2). The fragments were cloned (section 2.2.7) into *Eco*RI and CIP (section 2.2.3) treated pUC18. The ligations were transformed into *E. coli* DH5α (section 2.3.2.2), and positive colonies identified after colony PCR using vector specific primers, M13remoteF and M13remoteR (see appendix for primer sequences). PCR conditions were an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C (1 min), 60°C (1 min), 72°C (2.5 min), and a final extension step at 72°C for 10 min. Colony PCR indicated that the cloning of the ~4 kb and ~1 kb fragments were successful, while no products of expected size were obtained by screening of colonies transformed with ligations of the ~5 kb and ~1.5 kb digest products into pUC18. Two positive colonies obtained for each of the ~4 kb and ~1 kb fragments cloned into pUC18 were selected, the plasmids isolated (section 2.2.1) and sent for sequencing with vector specific primers M13F and M13R (see appendix for primer sequences).

Complete sequencing of the approx. 1 kb fragment, named *Eco*RI frag 1K, was achieved with M13F and M13R primers, and both clones were found to contain identical inserts, confirming the sequence of a 1,029 bp region of p49H5. To

complete the sequencing of the approx. 4 kb insert, named *Eco*RI frag 4K, in the other two subclones, the internal primers ECO4KF, ECO4KR and ECO4KInt were designed, the sequences of which can be seen in the appendix. Again, both inserts were found to be identical allowing an additional 4,238 bp region of the approx. 15 kb *Hin*dIII fragment, carrying the gene encoding P49, to be determined. The regions of p49H5 sequenced from the subclones can be seen in the complete sequencing strategy summarised in figure 5.4.

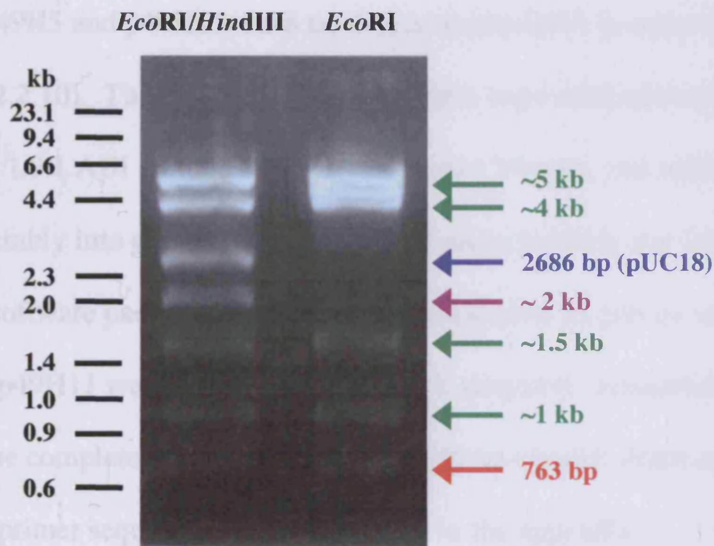


Figure 5.3 Restriction enzyme digest analysis of clone p49H5. The restriction enzymes used in each reaction are indicated above the gel lanes. A fragment of 763 bp, found in both reactions, corresponding to the *Eco*RI DNA fragment in clones p49E1 and p49E2 is indicated by a red arrow. The 2686 bp fragment, in the double digest, corresponding to the vector pUC18, used to construct p49H5, is indicated by a blue arrow. A band of approx. 2 kb, present in the double digest but not in the *Eco*RI digest is indicated by a purple arrow. Four fragments, selected for cloning into pUC18 for sequencing are indicated by green arrows. The size of the molecular weight markers are shown.

5.3.2 Sequencing of p49H5 and p49H11 by "walking-out"

Primers were designed for use in the sequencing of p49H5 and p49H11, based on the regions of the ~15 kb DNA sequenced by subcloning and during the cloning of *p49*. The clones p49H5 and p49H11 were used as template DNA in sequencing reactions (section 2.2.10). The resulting chromatograms were analysed using the program EditView 1.0.1 ABI Automated DNA Sequence Viewer, and editing of sequences and assembly into contigs were performed using EditSeq and SeqMan within the DNA* software package. Of the regions sequenced by primer walking, clones p49H5 and p49H11 were found be identical. A schematic representation of the strategy used for the complete sequencing of the 15,649 bp *Hin*dIII insert can be seen in figure 5.4. The primer sequences can also be seen in the appendix.

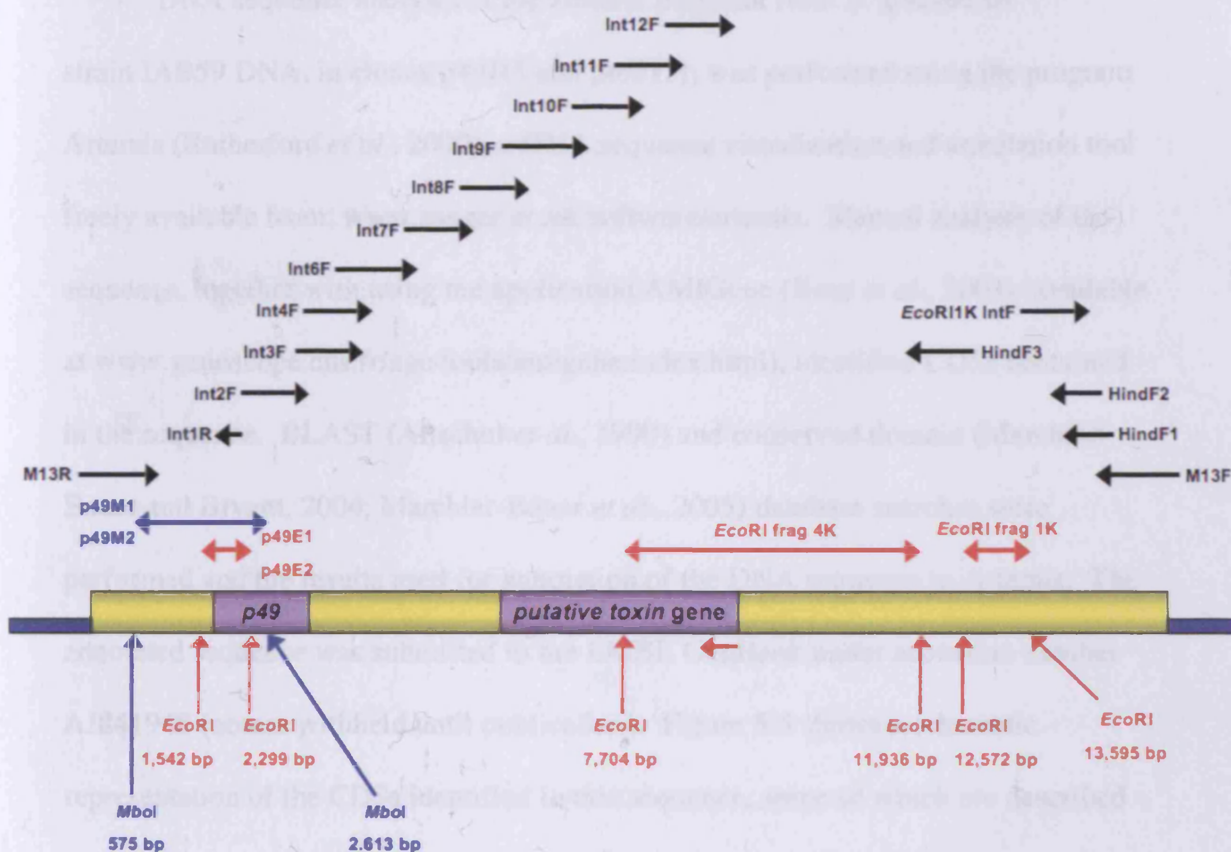


Figure 5.4 Sequencing strategy of the 15,649 bp *Hind*III insert in clones p49H5 and p49H11. The *Hind*III fragment cloned from IAB59 DNA is shown in yellow and the pUC18 vector is shown in blue. Black arrows represent the direction of primer extension during sequencing of the insert. Double headed blue and red arrows represent *Mbo*I and *Eco*RI fragments respectively. Regions corresponding to the inserts cloned in p49M1 and p49M2, and p49E1 and p49E2 are indicated as are the *Eco*RI fragments subcloned for sequencing: *Eco*RI frag 4k and *Eco*RI frag 1K. *Eco*RI (red arrows) and *Mbo*I (blue arrows) cleavage sites within the *Hind*III fragment are indicated. The position of the *p49* gene and the gene coding for a new putative crystal toxin, with an N-terminal aa sequence identical to a predicted crystal toxin from strain NHA15b, is also shown.

5.4 DNA sequence analysis of the 15.6 kb *Hin*dIII fragment from IAB59

DNA sequence analysis of the *Hin*dIII fragment from *B. sphaericus* strain IAB59 DNA, in clones p49H5 and p49H11, was performed using the program Artemis (Rutherford *et al.*, 2000), a DNA sequence visualization and annotation tool freely available from; www.sanger.ac.uk/software/artemis. Manual analysis of the sequence, together with using the application AMIGene (Bocs *et al.*, 2003) (available at www.genoscope.cns.fr/agc/tools/amigene.index.html), identified CDSs contained in the sequence. BLAST (Altschul *et al.*, 1990) and conserved domain (Marchler-Bauer and Bryant, 2004; Marchler-Bauer *et al.*, 2005) database searches were performed and the results used for annotation of the DNA sequence in Artemis. The annotated sequence was submitted to the EMBL GenBank under accession number AJ841948 (access withheld until publication). Figure 5.5 shows a schematic representation of the CDSs identified in this sequence, some of which are described below. The properties of all the CDSs are also summarised in table 5.1.

A number of CDSs with transposase like features were identified. A partial gene, possibly coding for a transposase, CDS 1, is found at the beginning of the sequence, the 3' end of which lies outside the *Hin*dIII fragment. BLAST searches with the encoded protein sequence indicates that it shares 33% identity with the majority of Transposase IS4 from *Alkaliphilus metalliredigenes* QYMF. A conserved domain search also reveals that a partial alignment (35.8% of 232 aa) is shared with the Transposase DDE conserved domain (CDD entry Pfam01619). Three CDSs, annotated as CDS 8-9-10, found from nt 10,590 to nt 9,679 in the reverse direction, are predicted to represent a pseudogene containing a total of four stop codons, which when joined show 53% identity over 216 aa to a transposase from *Bacillus halodurans*. The presence of the stop codons was confirmed by sequencing

of this region of the *Hin*dIII inserts in both clones p49H5 and p49H11 in both directions. The translated products of CDS 11, CDS 12 and CDS 13 also share identity with regions of transposases, and may again represent a pseudogene of an ancestral transposase containing internal stop codons as well as a frameshift. CDS 14, which has a putative upstream RBS but an internal stop codon, may once have encoded a transposase of 158 aa, showing 80% identity with the majority of the IS605/IS200-like transposase from *Staphylococcus epidermidis* ATCC as well as 79% identity over its entire length with a putative IS605/IS200 transposase from *B. sphaericus.* In addition the translated product of this CDS is also predicted, from conserved domain database searches, to comprise the IS200 Transposase conserved domain (CDD entry Pfam01797). However, although the presence of the internal stop codon was confirmed in both clones p49H5 and p49H11, the sequencing of this region in both directions was not performed, therefore, the presence of the stop codon is not certain. Other CDSs with transposase like features are summarised in table 5.1.

Two CDS were identified, that show similarity to genes encoding lipases, and were annotated as a single CDS, CDS 4, containing a frameshift to indicate a predicted pseudogene, the translated product of which shows 61% identity to aa 31-235 and aa 38-242 of lipases from *B. cereus* (a 526 aa lipase) and *B. thuringiensis* subsp. *israelensis* ATCC (a 533 aa lipase) respectively. Sequence analysis suggests that a number of mutations in this region have led to the introduction of stop codons and frameshifts, and that regions of DNA which may previously have encoded the C-terminal region of the lipase are found downstream of CDS 4. The region, where a predicted frameshift has occurred in the *Hin*dIII fragment, was sequenced in both directions confirming the accuracy of sequencing in this region.

A CDS encoding the protein P49, CDS 3, the features of which are described in chapter 3, was found reside within bases 1,828-3,222 of the *Hin*dIII fragment. The putative σ^E-like promoter and the predicted RBS are annotated in the submission. A CDS encoding a protein with an identical N-terminal aa sequence to the putative crystal toxin from *B. sphaericus* NHA15b, the discovery of which is described earlier in this chapter, was found on the reverse strand, from nt 9,432 to nt 5,899 of the insert. The features of this CDS, CDS 7, and its encoded protein of 135.6 kDa, which shows homology to the three domain family of Cry toxins, are described in the following sections, and in table 5.1. As was the case for P49, this putative crystal toxin was assigned the temporary name, P135, according to is approximate molecular weight.

A predicted pseudogene is found at CDS 15, nt 13,292 to nt 12,843, in the reverse direction. The translated product of this CDS shows 50% identity to a putative deletion pseudogene product from the 127,923 bp pBtoxis plasmid from *B. thuringiensis* subsp. *israelensis* (Berry *et al.*, 2002). Like the putative pseudogene located on pBtoxis, the product of this CDS also shows short regions of similarity to crystal toxin proteins and accessory proteins found downstream of crystal toxins, as summarised in table 5.1.

Finally, CDS 16 may encode a possible two-domain protein in which the N-terminal domain (aa 1-349), showing similarity to phosphatidyl inositol-specific phospholipase C, is followed by C-terminal domain (aa 350-487) containing a ricin-like beta-trefoil motif. Ricin-like beta trefoil domains are carbohydrate binding motifs and are found in toxins such as ricin, *Clostridium botulinum* neurotoxin, the mosquitocidal toxin Mtx1 (Thanabalu *et al.*, 1991), the human cancer-cell killing toxins from *B. thuringiensis*, parasporin-3Aa and parasporin-3Ab (also known as

Cry41Aa1 and Cry41Ab1 respectively) (Yamashita *et al.*, 2005), and the putative two domain toxin Cyt1Ca (Itsko *et al.*, 2005) (Manasherob *et al.*, manuscript in preparation). Also, phosphatidyl inositol-specific phospholipase C is predicted to be a virulence factor that contributes to the role of spore toxicity in *B. thuringiensis* and *B. cereus* (Salamitou *et al.*, 2000). Expression of the genes encoding these virulence factors are usually regulated by the protein, PlcR, that recognises the palindromic sequence TATGNAN$_4$TNCATA (Agaisse *et al.*, 1999). However, no binding site for the PlcR regulator was found upstream of the CDS but a potential RBS was identified.

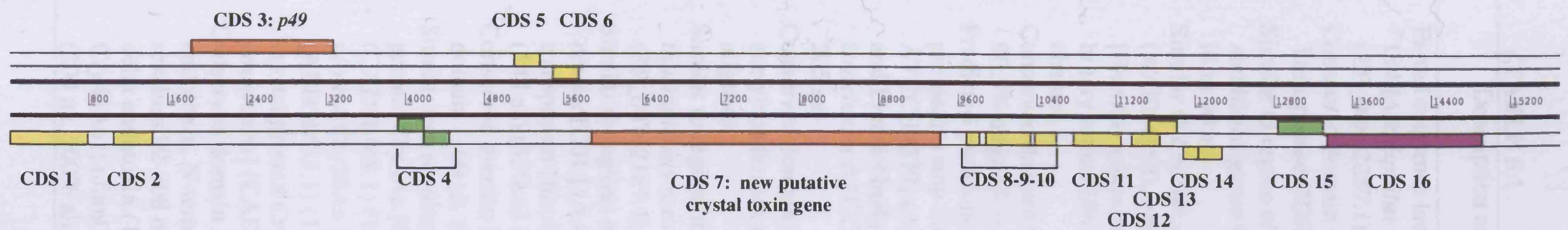


Figure 5.5 Schematic representation of the CDSs identified in the 15,649 bp DNA fragment from IAB59. CDSs having transposase like sequences are shown in yellow. The putative toxins discovered in this work are shown in orange. Predicted pseudogenes are shown in green. An interesting CDS, CDS 16, that may encode a two domain protein is shown in purple.

TABLE 5.1

CDS: Genes	Predicted product	Description and database similarity (accession no.) (% aa identity)
CDS 1	Putative transposase	Partial sequence lacking 3' end of gene, similar to Transposase IS4 [*Alkaliphilus metalliredigenes* QYMF] (ZP_00802287.1) (262 aa) (33% id in 224 aa) Conserved domain hit to pfam01609, Transposase_11, Transposase DDE domain (232 aa, only 35.8% aligned)
CDS 2		Similar to region of Transposase IS4 [*Alkaliphilus metalliredigenes* QYMF] (ZP_00800669.1) (513 aa) (42% id in 94 aa)
CDS 3: *cry49Aa1*	Cry49Aa1 (P49) crystal protein	Similar to Cry36Aa1 (ET69) [*Bacillus thuringiensis*] (AAK64558), both binary toxin components BinA and BinB [*Bacillus sphaericus*] and members of Cry35 family of binary toxins [*Bacillus thuringiensis*], as described in chapter 3. Conserved domain hit to pfam05431, Toxin_10 (199 aa, 90.5% aligned) residues 258-437 of Cry49Aa1
CDS 4	Predicted pseudogene	Predicted pseudogene containing an apparent frameshift and premature stop codon. Similar to Lipase [*Bacillus cereus* ATCC 14579] (AAP09108.1) (526 aa) (61% id in 205 aa) and Lipase family [*Bacillus thuringiensis* serovar *israelensis* ATCC] (ZP_00741507.1) (533 aa) (61% id in 205 aa) Conserved domain hit to COG1075, LipA, predicted acetyltranferases and hydrolases (336 aa, only 57.4% aligned)
CDS 5		Similar to region of N-terminus of Transposase [*Bacillus thuringiensis* serovar *israelensis* ATCC 35646] (ZP_00742187.1) (198 aa) (32% id in 75 aa)
CDS 6		Similar to regions of; transposase, C-terminal region [*Bacillus cereus* E33L] (AAY60214.1) (160 aa) (54% id in 52 aa) and transposase [*Bacillus cereus* G9241] (ZP_00236331.1) (148 aa) (53% id in 62 aa) Conserved domain hit to pfam00665, rve, Integrase core domain (160 aa, only 30% aligned)
CDS 7: *cry48Aa1*	Cry48Aa1 (P135) crystal protein	Similar to a number of Cry toxins including pesticidal crystal protein Cry4Aa [*Bacillus thuringiensis* serovar *israelensis* (CAD30148.1) (1180 aa) (33% id in 1236 aa), crystal protein Cry28Aa [*Bacillus thuringiensis* serovar *finitimus*] (ABB51653.1) (1128 aa) (34% id in 1158 aa) and pesticidal crystal protein Cry4Ba [*Bacillus thuringiensis* serovar *israelensis*] (CAD30095.1) (1136 aa) (33% id in 1193 aa) Conserved domain hit to pfam03945 Endotoxin_N, delta endotoxin, N-terminal domain (225 aa, 87.1% aligned) residues 92-308 of Cry48Aa1; pfam03944 Endotoxin_C, delta endotoxin (138 aa, 100% aligned) residues 521-658 of Cry48Aa1; pfam00555 Endotoxin_M, delta endotoxin (203 aa, 100% aligned) residues 318-511 of Cry48Aa1

TABLE 5.1 - Continued

CDS: Genes	Predicted product	Description and database similarity (accession no.) (% aa identity)
CDS 8-9-10		Three CDSs containing a total four stop codons which when merged show similarity to transposase B of IS655 [*Bacillus haldurans*] (BAD18201.1) (261 aa) (53% id in 216 aa) and transposase [*Enterococcus faecium*] (AAC97536.1) (387 aa) (43% id in 282 aa) Conserved domain hit to pfam00665, rve, Integrase core domain (160 aa, only 68.8% aligned)
CDS 11		Similar to region of Transposase, IS4 [*Syntrophomonas wolfei* str. *Goettingen*] (ZP_00663984.1) (513 aa) (28% id in 124 aa) and transposase [*Methanosarcina acetivorans* C2A] (AAM07572.1) (497 aa) (25% id in 151 aa)
CDS 12		Similar region of to Transposase, IS4 [*Syntrophomonas* wolfei str. *Goettingen*] (ZP_00663984.1) (513 aa) (37% id in 62 aa) and Transposase IS4 [*Alkaliphilus metalliredigenes* QYMF] (ZP_00800669.1) (513 aa) (42% id in 57 aa)
CDS 13		Similar to small region of transposase [*Streptococcus agalactiae*] (AAR12164.1) (465 aa) (61% id in 36 aa) and ISSag8, transposase [*Streptococcus agalactiae* A909] (ABA46182.1) (496 aa) (61% id in 36 aa)
CDS 14	Presence of internal stop uncertain, and therefore, may encode a transposase	Contains an internal stop, shows similarity to IS605/IS200-like transposase [*Staphylococcus epidermidis* ATCC 12228] (AA004610.1) (199 aa) (80% id in 157 aa), transposase, IS200 family [*Staphylococcus aureus* subsp. *aureus* USA300] (YP_494006.1) (161 aa) (79% id in 157 aa) and putative IS605/IS200-like transposase [*Bacillus sphaericus*] (CAI29283.1) (150 aa) (75% id in 150 aa) Conserved domain hit to COG1943, Transposase and inactivated derivatives (136 aa, 97.8% aligned)
CDS 15	Predicted deletion pseudogene	Similar to putative deletion pseudogene product [*Bacillus thuringiensis* serovar *israelensis*] (CAD30105.1) (133 aa) (50% id. in 148 aa), C-terminus of Cry39ORF2 protein [*Bacillus thuringiensis* serovar *aizawai*] (BAB72017.1) (558 aa) and Cry40ORF2 protein [*Bacillus thuringiensis* serovar *aizawai*] (BAB72019.1) (558 aa) (32% id in 95 aa to both), and C-terminus of: Cry20Aa (C20AA_BACUF) (753 aa) (31% id in 91 aa) and Cry11Bb (C11BB_BACTV) (750 aa) (37% in 87 aa)

TABLE 5.1 - Continued

CDS: Genes	Predicted product	Description and database similarity (accession no.) (% aa identity)
CDS 16	Possible two domain protein	Possible two domain protein; N-terminus is similar to 1-phosphatidylinositol phosphodiesterase precursor [*Bacillus thuringiensis* serovar *israelensis* ATCC 35646] (ZP_00741074.1) (433 aa) (44% id in 334 aa); C-terminal half is similar to several Ricin-like beta trefoil domain-containing toxins: Cry protein [*Bacillus thuringiensis*] (BAD35166.1) (810 aa) (39% id in 137 aa), Possible two domain toxin (Cyt1Ca) [*Bacillus thuringiensis*] (CAD30104.1) (525 aa) (37% id in 144 aa), cancer cell killing Cry protein [*Bacillus thuringiensis*] (BAD35163.1) (829 aa) (33% id in 144 aa) and mosquito larvicidal protein (Mtx1) [*Bacillus sphaericus*] (BAD01571.1) (870 aa) (29% id in 163 aa) Conserved domain hit to pfam00388, PI-PLC-X, Phosphatidylinositol-specific phospholipase X, X domain (145 aa, 97.9% aligned) and smart00148 PLCXc, Phospholipase C, catalytic domain (part) (145 aa, 97.9% aligned) and cd00137, PLCc. Pholpholipase C, catalytic domain (298 aa, only 49% aligned)

Table 5.1 Features of the CDSs identified in the 15,649 bp *Hin*dIII fragment from *B. sphaericus* IAB59 DNA, in clones p49H5 and p49H11.

5.5 Sequence analysis of the gene encoding P135 from IAB59

Figure 5.6 shows the DNA sequence, and the deduced amino acid sequence, of *p135*. A CDS of 3,534 bp, encoding a protein of 1177 amino acids begins with the initiation codon ATG at nt 253. The encoded protein has a predicted molecular weight of 135,567 Da.

Two potential Shine-Dalgarno sequences have been identified upstream of the *p135* gene. At 22 nt upstream of the initiation codon an 8 nt sequence (nt 231-238) is found, of which 7 nt are complementary to the 3' end of both *B. sphaericus* (Berry unpublished) and *B. subtilis* (Moran *et al.*, 1982; Kunst *et al.*, 1997) 16S rRNA. A predicted interaction of this sequence with 3'-16S rRNA, with a calculated free energy (ΔG) of -6.8 kcal/mol according to the method of Tinoco *et al.* (Tinoco *et al.*, 1973), is shown in figure 5.7(i). Another potential 8 nt Shine-Dalagarno sequence, found at nt 238-245, is entirely complementary to the 3'-16S rRNA of *B. sphaericus* and *B. subtilis*. A proposed interaction of this sequence with the 3' end of 16S rRNA, with a ΔG of -19.0 kcal/mol, is shown in figure 5.7(ii). It is also possible that an interaction involving both the regions described above occurs with a 3 nt bulge loop occurring between the two sequences, having a ΔG of -20.2 kcal/mol, as shown in figure 5.7(iii) and boxed in red in figure 5.6. The lower ΔG (-19.0 kcal/mol and -20.2 kcal/mol) of the latter two proposed interactions, compared to the first (ΔG of -6.8 kcal/mol) puts forward these sequences as the most likely Shine-Dalgarno sequence.

Analysis of the DNA sequence upstream of *p135* for possible promoter sequences, using the predictive database search of the DBTBS (http://dbtbs.hgc.jp) (Makita *et al.*, 2004) and by carrying out manual analysis, revealed a number of potential promoters. The most striking similarity to *B. subtilis* like promoters were

the sequences AGTATATT (nt 199-206) and CATATATT (nt 220-227), compared to the -35 σ^E consensus G(g/t)(c/a)ATATT and -10 σ^E consensus CATACANT, respectively. The sigma factor, σ^E, is active in the *B. subtilis* mother cell, that has advanced approximately 2 hours into sporulation after processing of the precursor pro-σ^E, which is encoded by the *spoIIG* operon (Haldenwang, 1995). The regulation of *p135* by a σ^E-like homologue in *B. sphaericus* would, therefore, be consistent with the presence of P135 as a spore crystal. Also, two genes encoding mosquitocidal toxins from *B. thuringiensis* subsp. *israelensis*, *cry4Aa* and *cry4Ba*, have been shown to be regulated by sporulation specific σ^{35} and σ^{28} factors, homologues of σ^E and σ^K from *B. subtilis* respectively (Yoshisue *et al.*, 1993a; Yoshisue *et al.*, 1993b). Another two potential promoters, overlapping with the σ^E-like promoter sequence, were also identified using the predictive promoter identification program, DBTBS. Analysis revealed these two additional potential promoter sequences to be low scoring, and manual comparison of the sequences with their suggested σ^F and σ^H consensus sequences confirmed a lower identity than that observed for the σ^E-like sequence. As a result, the σ^E-like sequence was put forward as the most likely promoter identified upstream of the gene encoding P135.

```
CCAGTTTAGTGTTGACATATCAGTTCTTCTTCGAACAATTGAAGGATATGTAATTTTAACCCGATTGGATGTTTACTTCGCCCCAAAAAAATGATTCCCT  100

TTGATAAACAGATTATATTTTTTTAAGCTGTTAGGGAGCATATCACTCTCATGAATATTTAGCTTTTTTATTAATGTAGGAAATTAAAGAAAAAACCTAG  200

TATATTGTTATTTCACCTGCATATATTAATTTAGGAAAAAGGAGGACTGTGTATGGATATCAACAATAATAATGAAAAGGAAATTATTAATTCTCATTTA  300
                                                      M  D  I  N  N  N  N  E  K  E  I  I  N  S  H  L
CTACCATATTCACTTTTAAAAAAATACCCTATTAAGTCTTTACAGAGCACAAATTACAAAGATTGGCTTAATCTGTGTCAAGATTTTAATAAGGATATTG  400
 L  P  Y  S  L  L  K  K  Y  P  I  K  S  L  Q  S  T  N  Y  K  D  W  L  N  L  C  Q  D  F  N  K  D  I
AAAGTTATGATTTGGTAACTGCTGTCTCGAGCGGAACTATTGTCGTAGGGACAATGTTGAGCGCTATTTATGCACCCGCTCTTATAGCTGGTCCTATAGG  500
E  S  Y  D  L  V  T  A  V  S  S  G  T  I  V  V  G  T  M  L  S  A  I  Y  A  P  A  L  I  A  G  P  I  G
AATAATAGGCGCTATCATTATATCTTTTGGAACTCTTCTACCTCTTCTATGGAATGAGAGCGAGAATAACCCTAAAACGACATGGATTGAATTTATTAGA  600
  I  I  G  A  I  I  I  S  F  G  T  L  L  P  L  L  W  N  E  S  E  N  N  P  K  T  T  W  I  E  F  I  R
ATGGGAGAACAGCTTGTTGATAAAACAATATCGCAAACAGTGTTTAATATACTGGAAAGCTATTTAAAAGATTTAAAGGTAAATTTAGTAGATTATGAAA  700
 M  G  E  Q  L  V  D  K  T  I  S  Q  T  V  F  N  I  L  E  S  Y  L  K  D  L  K  V  N  L  V  D  Y  E
AAGCGAAACAAGATTGGATTGAATTGAAAAAACAGCAACTTCCTGGTTCACCTCCCTCAACTAAATTAAGGAATGCTGCAGATATTGCTCATCAAAGACT  800
K  A  K  Q  D  W  I  E  L  K  K  Q  Q  L  P  G  S  P  P  S  T  K  L  R  N  A  A  D  I  A  H  Q  R  L
TGATTCTCTTCATAATAAATTTGCTGAATTGAATAAGTTCAAAGTAGAACCTTATGAAACAATTTTGCTACCGGTTTATGCACAAGCTGCCAACTTACAT  900
  D  S  L  H  N  K  F  A  E  L  N  K  F  K  V  E  P  Y  E  T  I  L  L  P  V  Y  A  Q  A  A  N  L  H
TTAAACTTGTTACAACAAGGGGCTATGTTTGCAGATCAATGGATTGAGGATAAATACTCATCGAGAAATGATACATTTGCTGGAAATTCAAATGATTATC  1000
 L  N  L  L  Q  Q  G  A  M  F  A  D  Q  W  I  E  D  K  Y  S  S  R  N  D  T  F  A  G  N  S  N  D  Y
AGAATTTGTTAAAATCTAGAACAATAACCTATATAAATCATATTGAAAATACTTATCAAAATGGACTTAATTACTTGTGGAATCAACCGGAAATGACGTG  1100
Q  N  L  L  K  S  R  T  I  T  Y  I  N  H  I  E  N  T  Y  Q  N  G  L  N  Y  L  W  N  Q  P  E  M  T  W
GGATATATATAATGAATACCGAACAAAAATGACCATTACTGCATTAGATCTCATGGCATTATTCCCCTTTTATAACAAAGAATTATATGACCCTACAGTG  1200
  D  I  Y  N  E  Y  R  T  K  M  T  I  T  A  L  D  L  M  A  L  F  P  F  Y  N  K  E  L  Y  D  P  T  V
GGTATAAAATCAGAACTTACAAGAGAAATATTTATTAATACACCTGTCGAACCACATTTACACAGGTACTTCAAATTAAGTGAGACAGAAGAAAAACTTA  1300
 G  I  K  S  E  L  T  R  E  I  F  I  N  T  P  V  E  P  H  L  H  R  Y  F  K  L  S  E  T  E  E  K  L
CAAATAATAGTGATTTATTTAAATGGCTAACAAGTCTAAAATTTAGAACGTTATATCAACCTGGCTTCCCTTTTTTAATTGGAAATATGAATAGCTTTAC  1400
T  N  N  S  D  L  F  K  W  L  T  S  L  K  F  R  T  L  Y  Q  P  G  F  P  F  L  I  G  N  M  N  S  F  T
AAATACAAACGGTACACAACTTATCAATAACCAACAGCAGCTTTGGTCGTTTCCTGGAACAACCGAAAATGAAGAAAAATTGTTCCCTTCACCAGCAAAT  1500
  N  T  N  G  T  Q  L  I  N  N  Q  Q  Q  L  W  S  F  P  G  T  T  E  N  E  E  K  L  F  P  S  P  A  N
ATAGATCAAGTTACTATGTATATTTATTATGGTAGCGGATGGGGTATTCCTGAGCCTATTAGTACTACTATAAATAAATTAATTTTTAATCATGATAAAC  1600
 I  D  Q  V  T  M  Y  I  Y  Y  G  S  G  W  G  I  P  E  P  I  S  T  T  I  N  K  L  I  F  N  H  D  K
ATGAGCTAATATCAGAGTATGATGCTGGAAATACCAATGCCCCTACCAGGAGCTTATCTTTAGGTCTCCCAAATCACTATTTATCTTGCTTAAATAGCTA  1700
H  E  L  I  S  E  Y  D  A  G  N  T  N  A  P  T  R  S  L  S  L  G  L  P  N  H  Y  L  S  C  L  N  S  Y
TTACCCTTTAACTGCTACTACGGATGGAATGAATAAAGAAGAACTTAAAATGTATTCGTTTGGATGGACACATAATAGTGTTGATTTTTTAAATGAAATT  1800
  Y  P  L  T  A  T  T  D  G  M  N  K  E  E  L  K  M  Y  S  F  G  W  T  H  N  S  V  D  F  L  N  E  I
AGCAAAGACAAAATTACACAAATTCCTGCAGTAAAAGCCTATCGTTTAACCTCGAACTCTAGGGTAATAAAAGGTCCTAGTCATATTGGTGGTAACTTGG  1900
 S  K  D  K  I  T  Q  I  P  A  V  K  A  Y  R  L  T  S  N  S  R  V  I  K  G  P  S  H  I  G  G  N  L
TTTATCTTAGCGAGAATAGTCAAATGGCTTTAACTTGCAGGTACACAAATTCTTCTCCCCAGGAATATAAAATAAGAATTCGATATGCTTCAAACAGATT  2000
V  Y  L  S  E  N  S  Q  M  A  L  T  C  R  Y  T  N  S  S  P  Q  E  Y  K  I  R  I  R  Y  A  S  N  R  L
GAACATGGGACAATTATTTACAACATTCAGTTCACACCAGTTTGTGCTTCCACCAACTTTCAATCATTTCAATATAGAACAAGCAAAATATGAAGATTAT  2100
  N  M  G  Q  L  F  T  T  F  S  S  H  Q  F  V  L  P  P  T  F  N  H  F  N  I  E  Q  A  K  Y  E  D  Y
GCATATGCTGAATTTCCAGAAAGTATGTCGATTAGAGGTAATTTAAATTCTGATATATTATTAATACTCAATATATTAGCTGGGGGTGAATTACTTCTTG  2200
 A  Y  A  E  F  P  E  S  M  S  I  R  G  N  L  N  S  D  I  L  L  I  L  N  I  L  A  G  G  E  L  L  L
```

```
ATAAAATAGAGTTTATACCATTAACTCAAAAAGTTAAAGATAACCTAGAAAAGGAAAAAATAGATATGTTAAAAAATTTAACAGATTCATTATTTAATAG 2300
 D  K  I  E  F  I  P  L  T  Q  K  V  K  D  N  L  E  K  E  K  I  D  M  L  K  N  L  T  D  S  L  F  N  S
TCCTTCAAAAGATACTTTAAAAATTGATAGTACAGATTATCAAATTGACCAAATCGCTTTTCAAATAGAGTCTATAAATGAAGAAATTAATCCACAAGAA 2400
  P  S  K  D  T  L  K  I  D  S  T  D  Y  Q  I  D  Q  I  A  F  Q  I  E  S  I  N  E  E  I  N  P  Q  E
AAGATGGAATTACTAGATAATATAAAATATGCAAAAAAACTAAATCAATTACGAAATCTTTTATATTCTAGAGAGTCTCAAGCTCAGATAGATTGGTAA 2500
 K  M  E  L  L  D  N  I  K  Y  A  K  K  L  N  Q  L  R  N  L  L  Y  S  R  E  S  Q  A  Q  I  D  W  Y
CAAGTAATGATGTTTCTATTTATCATGGTAAAAAACCATTTAATGACTATACTCTTGTTATGTCAAGAACAAGCTCAAGTTTATCAGAGATTACAGCAAC 2600
 T  S  N  D  V  S  I  Y  H  G  K  K  P  F  N  D  Y  T  L  V  M  S  R  T  S  S  S  L  S  E  I  T  A  T
AAACTATCAAACCTATATTTATAAAAAAATTGAAGAGTCTAAACTAAAACCATATACACGTTACCTGGTAAGAGGTTTCATAAGTAACAGCGAAGATTTA 2700
  N  Y  Q  T  Y  I  Y  K  K  I  E  E  S  K  L  K  P  Y  T  R  Y  L  V  R  G  F  I  S  N  S  E  D  L
GAAATTTTTATTTCTCGATATGAAAATGAAATTCATACTAACATGAATGTTCATGGAGATGACGATACTCTTTTAAATTCAGATATACGACAAAATGAAT 2800
 E  I  F  I  S  R  Y  E  N  E  I  H  T  N  M  N  V  H  G  D  D  D  T  L  L  N  S  D  I  R  Q  N  E
GTGAATCTAAACTTCCAATTATATTTGATGCAACATCACAATATTCGCTGTCTCCAAGTCGTACTTCAGGTATATCTAATCATTCATATTACAATAATGG 2900
 C  E  S  K  L  P  I  I  F  D  A  T  S  Q  Y  S  L  S  P  S  R  T  S  G  I  S  N  H  S  Y  Y  N  N  G
ACATCAGTCATCGTGCAATGACACCCACATATTTTCATTTTCTATTGATACAGGAGAAGTTGATTTTAATAATTATCCTGGTATTGAAATACTATTCAAA 3000
  H  Q  S  S  C  N  D  T  H  I  F  S  F  S  I  D  T  G  E  V  D  F  N  N  Y  P  G  I  E  I  L  F  K
CTTTCAAATACAAATGGGTACGCTTCAATAAGTAATTTAGAAGTAATAGAAGAGCGATTACTAACCGAAGAAGAAAAGCGACAAATTATTCAAATAGAAA 3100
 L  S  N  T  N  G  Y  A  S  I  S  N  L  E  V  I  E  E  R  L  L  T  E  E  E  K  R  Q  I  I  Q  I  E
ATCGATGGAAAGCAAAAAAAGAAAGTCAACGCAATGAAACTGAAAAAATAACTACGCAAGCCCAACAAGCAATAAATAGTCTATTTACGGATACACAATA 3200
 N  R  W  K  A  K  K  E  S  Q  R  N  E  T  E  K  I  T  T  Q  A  Q  Q  A  I  N  S  L  F  T  D  T  Q  Y
TTCAAACTTAAAATTTGAAACAACTAAACAAAATATTACCGAAGCTAATACTATTTTGGAAAACATCCCCTATGTTTACAATGCATTATTACCAACAGAA 3300
  S  N  L  K  F  E  T  T  K  Q  N  I  T  E  A  N  T  I  L  E  N  I  P  Y  V  Y  N  A  L  L  P  T  E
CCAGGTATGAATTTTGTTTTATTTAACAGTTTTAAAGATCAAATAAATAAAGCACACGCATTATATAAAATGAGAAACTTAATTAAGAATGGTGATTTCA 3400
 P  G  M  N  F  V  L  F  N  S  F  K  D  Q  I  N  K  A  H  A  L  Y  K  M  R  N  L  I  K  N  G  D  F
TTAATGATACAAAATATTGGTCTATATCAACAGATGTTAAATTGGAAAAAGTCAACAAGGAAACTATTCTTGTTTTGTCGAGTTGGGAAGCACAAGCATC 3500
 I  N  D  T  K  Y  W  S  I  S  T  D  V  K  L  E  K  V  N  K  E  T  I  L  V  L  S  S  W  E  A  Q  A  S
TCAACAAATACTAGTACAAAAACAAAAACGATACCTACTCCGTGTCATAGCAAAAAAAGAAGATATGGGTAGAGGAAATGTGATAATCAGTGACTGTTTA 3600
  Q  Q  I  L  V  Q  K  Q  K  R  Y  L  L  R  V  I  A  K  K  E  D  M  G  R  G  N  V  I  I  S  D  C  L
AATAATATAGCTAAAATAGATTTTACTCCCCATGATTGTAATATGAACCATATACAAAATTCATCAGAGTTTATTATAAAAACAATACACTTTAGCCCAA 3700
 N  N  I  A  K  I  D  F  T  P  H  D  C  N  M  N  H  I  Q  N  S  S  E  F  I  I  K  T  I  H  F  S  P
ATACTGAGCAAGTACGTATTGATATTGGCCAATCGGATGGTGTATTTAAAGTCGAAAGTATAGAGCTCATTTGCGTTAACTATTAA 3786
 N  T  E  Q  V  R  I  D  I  G  Q  S  D  G  V  F  K  V  E  S  I  E  L  I  C  V  N  Y  .
```

Figure 5.6 DNA sequence of the putative crystal toxin gene *p135*. A stretch of DNA complementary to 3' 16S rRNA, excluding an internal 3 nt non base pairing sequence, representing a possible Shine-Dalgarno sequence is boxed in red. The putative σ^E promoter is shown with a blue line above the sequence, the initiation codon is shown with a black line above the sequence and the deduced P135 amino acid sequence is shown in blue. This DNA sequence was submitted to the EMBL GenBank as part of the 15,649 bp *Hin*dIII fragment DNA sequence, accession number AJ841948.

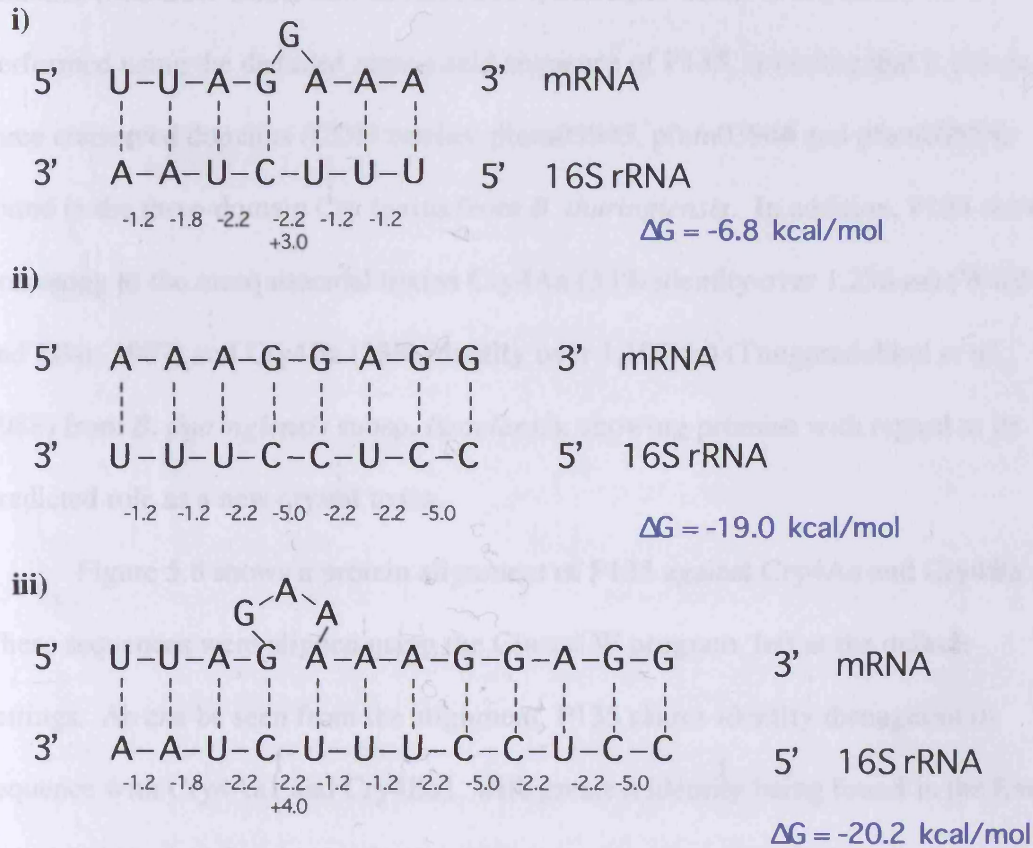


Figure 5.7 Proposed interactions between the Shine-Dalgarno of the *p135* transcript and the 3'end of 16S rRNA. Free energies (ΔG in kcal ± 10%) for the base paired and bulge loop regions are shown below the interactions. Values for ΔG were calculated according to Tinoco *et al.* (Tinoco *et al.*, 1973).

5.6 Analysis of the P135 protein sequence

BLAST database searches (Altschul *et al.*, 1990) and Conserved domain searches (Marchler-Bauer and Bryant, 2004; Marchler-Bauer *et al.*, 2005) were performed using the deduced amino acid sequence of P135, revealing that it shares the three conserved domains (CDD entries: pfam03945, pfam03944 and pfam00555) found in the three-domain Cry toxins from *B. thuringiensis.* In addition, P135 shows homology to the mosquitocidal toxins Cry4Aa (33% identity over 1,236 aa) (Ward and Ellar, 1987) and Cry4Ba (33% identity over 1,193 aa) (Tungpradubkul *et al.*, 1988) from *B. thuringiensis* subsp. *israelensis*, showing promise with regard to its predicted role as a new crystal toxin.

Figure 5.8 shows a protein alignment of P135 against Cry4Aa and Cry4Ba. These sequences were aligned using the Clustal W program, left at the default settings. As can be seen from the alignment, P135 shares identity throughout its sequence with Cry4Aa1 and Cry4Ba1, with greatest identity being found in the five conserved blocks of amino acids shared between the three-domain Cry toxins (Schnepf *et al.*, 1998). A high sequence identity between P135 and both Cry4Aa and Cry4Ba is also found in the three conserved blocks found in the C-terminal halves of the three-domain Cry toxins, a region that is processed from the toxic N-terminal half by insect gut proteinases, and is thought to be important in crystal formation (Hofte and Whiteley, 1989; Bietlot *et al.*, 1990).

```
P135      MDINNNNEKEIINSHLLPYSLLKKYPIKSLQSTNYKDWLNLCQDFNKDIES
Cry4Aa    MNPYQNKNEYETLNASQKKLNISNNYTRYPIENSPKQLLQSTNYKDWLNMCQQN----QQ
Cry4Ba    MN-----------------------SGYPLANDLQGSMKNTNYKDWLAMCENN----QQ

P135      YDLVTAVSSGTIVVGTMLSAIYAPALIAGPIGIIGAIIISFGTLLPLLWNESENNPKTTW
Cry4Aa    YGGDFETFIDSGELSAYTIVVGTVLTGFGFTTPLGLALIGFGTLIPVLFPAQDQS--NTW
Cry4Ba    YG-VNPAAINSSSVSTALKVAGAILK--FVNPPAGTVLTVLSAVLPILWPTNTPTPERVW

P135      IEFIRMGEQLVDKTISQTVFNILESYLKDLKVNLVDYEKAKQDWIELKKQQLPGSPPSTK
Cry4Aa    SDFITQTKNIIKKEIASTYISNANKILNRSFNVISTYHNHLKTWENNPNPQ-NTQDVRTQ
Cry4Ba    NDFMTNTGNLIDQTVTAYVRTDANAKMTVVKDYLDQYTTKFNTWKREPNNQSYRTAVITQ

                                                        1
P135      LRNAADIAHQRLDSLHNKFAELNKFKVEPYETILLPVYAQAANLHLNLLQQGAMFADQWI
Cry4Aa    IQLVHYHFQNVIPELVNSCPPN-PSDCDYYNILVLSSYAQAANLHLTVLNQAVKFEAYLK
Cry4Ba    FNLTSAKLRETAVYFSNLVG---------YELLLLPIYAQVANFNLLLIRDG------LI

P135      EDKYSSRNDTFAGNSNDYQNLLKSRTITYINHIENTYQNGLNYLWNQPEMT------WDI
Cry4Aa    NNRQFDYLEPLPTAIDYYP-VLTKAIEDYTNYCVTTYKKGLNLIKTTPDSNLDGNINWNT
Cry4Ba    NAQEWSLAR--SAGDQLYN-TMVQYTKEYIAHSITWYNKGLDVLRNKSNG------QWIT

                     2
P135      YNEYRTKMTITALDLMALFPFYNKELYDPTVG-----IKSELTREIFINTPVEPHLHRYF
Cry4Aa    YNTYRTKMTTAVLDLVALFPNYDVGKYPIG-------VQSELTREIYQVLNFEESPYKYY
Cry4Ba    FNDYKREMTIQVLDILALFASYDPRRYPADKIDNTKLSKTEFTREIYTAL--VESPSSK-

P135      KLSETEEKLTNNSDLFKWLTSLKFRTLYQPG-FPFLIGNMNSFTNTNGTQLINNQQQLWS
Cry4Aa    DFQYQEDSLTRRPHLFTWLDSLNFYEKAQTTPNNFFTSHYNMFHYTLDNISQ--KSSVFG
Cry4Ba    SIAALEAALTRDVHLFTWLKRVDFWTNTIYQDLRFLSANKIGFSYTNSSAMQ--ESGIYG

P135      FPGTTENEEKLFPSPANIDQVTMYIYYGSGWGIPEPISTTINKLIFNHDKHELISEYDAG
Cry4Aa    NHNVTDKLKS-LGLATNIYIFLLNVISLDNKYLNDYNNISKMDFFITNGTRLLEKELTAG
Cry4Ba    SSGFGSNLTHQIQLNSNVYKTSITDTSSP------SNRVTKMDFYKIDGT---LASYNSN

P135      --NTNAPTRSLSLGLPNHY----------LSCLNSYYPLTATTDGMNK---EELKMYSFG
Cry4Aa    SGQITYDVNKNIFGLPILKRRENQGNPTLFPTYDNYSHILSFIKSLSIPATYKTQVYTFA
Cry4Ba    ITPTPEGLRTTFFGFSTNENTPNQ------PTVNDYTHILSYIKTDVI--DYNSNRVSFA

                              3
P135      WTHNSVDFLNEISKDKITQIPAVKAYRLTSNSRVIKGPSHIGGNLVYLSENS----QMAL
Cry4Aa    WTHSSVDPKNTIYTHLTTQIPAVKANSLGTASKVVQGPGHTGGDLIDFKDH------FKI
Cry4Ba    WTHKIVDPNNQIYTDAITQVPAVKSNFLNATAKVIKGPGHTGGDLVALTSNGTLSGRMEI

                 4
P135      TCRYTNSS--FQEYKIRIRYASN-----RLNMGQLFTTFSSHQ-FVLPPTFNHFN---IE
Cry4Aa    TCQHSNF---QQSYFIRIRYASNGSANTRAVINLSIPGVAELG-MALNPTFSGTD---YT
Cry4Ba    QCKTSIFNDPTRSYGLRIRYAAN--SPIVLNVSYVLQGVSRGTTISTESTFSRPNNIIPT

                                                  5
P135      QAKYEDYAYAEFPES-MSIRGNLNSDILLILN---ILAGGELLLDKIEFIPLTQKVKDNL
Cry4Aa    NLKYKDFQYLEFSN---EVKFAPNQNISLVFNRSDVYTNTTVLIDKIEFLPITRSIREDR
Cry4Ba    DLKYEEFRYKDPFDAIVPMRLSSNQLITIAIQPLNMTSNNQVIIDRIEIIPITQSVLDET

                                           6
P135      EKEKIDMLKNLTDSLFNSPSKDTLKIDSTDYQIDQIAFQIESINEEINPQEKMELLDNIK
Cry4Aa    EKQKLETVQQIINTFYANPIKNTLQSELTDYDIDQAANLVECISEELYPKEKMLLLDEVK
Cry4Ba    ENQNLESEREVVNALFTNDAKDALNIGTTDYDIDQAANLVECISEELYPKEKMLLLDEVK

P135      YAKKLNQLRNLLYSRESQ-AQIDWVTSNDVSIYHGKKPFNDYTLVMSRTSSSLSEITATN
Cry4Aa    NAKQLSQSRNVLQNGDFESATLGWTTSDNITIQEDDPIFKGHYLHMSGAR----DIDGTI
Cry4Ba    NAKQLSQSRNVLQNGDFESATLGWTTSDNITIQEDDPIFKGHYLHMSGAR----DIDGTI

                     7
Cry4Aa    FPTYIFQKIDESKLKPYTRYLVRGFVGSSKDVELVVSRYGEEIDAIMNVPADLNYLYPST
Cry4Ba    FPTYIFQKIDESKLKPYTRYLVRGFVGSSKDVELVVSRYGEEIDAIMNVPADLNYLYPST
P135      YQTYIYKKIEESKLKPYTRYLVRGFISNSEDLEIFISRYENEIHTNMNVHGDDDTLLNSD

                                                               8
P135      IRQNECESKLPIIFDATSQYSLSPSRTSGISNHSYYNNGHQSSCNDTHIFSFSIDTGEVD
Cry4Aa    FDCEGSN-----RCETSAVPANIGNTSDMLYSCQYDTGKKHVVCQDSHQFSFTIDTGALD
Cry4Ba    FDCEGSN-----RCETSAVPANIGNTSDMLYSCQYDTGKKHVVCQDSHQFSFTIDTGALD
```

```
                                8
P135     FNNYPGIEILFKLSNTNGYASISNLEVIEERLLTEEEKRQIIQIENRWKAKKESQRNETE
Cry4Aa   TNENIGVWVMFKISSPDGYASLDNLEVIEEGPIDGEALSRVKHMEKKWNDQMEAKRSETQ
Cry4Ba   TNENIGVWVMFKISSPDGYASLDNLEVIEEGPIDGEALSRVKHMEKKWNDQMEAKRSETQ

P135     KITTQAQQAINSLFTDTQYSNLKFETTKQNITEANTILENIPYVYNALLPTEPGMNFVLF
Cry4Aa   QAYDVAKQAIDALFTNVQDEALQFDTTLAQIQYAEYLVQSIPYVYNDWLSDVPGMNYDIY
Cry4Ba   QAYDVAKQAIDALFTNVQDEALQFDTTLAQIQYAEYLVQSIPYVYNDWLSDVPGMNYDIY

P135     NSFKDQINKAHALYKMRNLIKNGDFINDTKYWSISTDVKLEKVNKETILVLSSWEAQASQ
Cry4Aa   VELDARVAQARYLYDTRNIIKNGDFTQGVMGWHVTGNADVQQIDGVSVLVLSNWSAGVSQ
Cry4Ba   VELDARVAQARYLYDTRNIIKNGDFTQGVMGWHVTGNADVQQIDGVSVLVLSNWSAGVSQ

P135     QILVQKQKRYLLRVIAKKEDMGRGNVIISDCLNNIAKIDFTPHDCNMNHIQNSSEFIIKT
Cry4Aa   NVHLQHNHGYVLRVIAKKEGPGNGYVTLMDCEENQEKLTFT---------SCEEGYITKT
Cry4Ba   NVHLQHNHGYVLRVIAKKEGPGNGYVTLMDCEENQEKLTFT---------SCEEGYITKT

P135     IHFSPNTEQVRIDIGQSDGVFKVESIELICVNY
Cry4Aa   VDVFPDTDRVRIEIGETEGSFYIESIELICMNE
Cry4Ba   VDVFPDTDRVRIEIGETEGSFYIESIELICMNE
```

Figure 5.8 Protein alignment of P135 with Cry4Aa and Cry4Ba, two mosquitocidal toxins from *B. thuringiensis* subsp. *israelensis*. Residues conserved in all three sequences are highlighted yellow, while identical residues present in two of the three sequences are highlighted blue. Regions corresponding to the five conserved blocks found in the processed forms of the three-domain Cry toxins are boxed in black. The three conserved blocks found in the C-terminal part of the pro-toxins are boxed in red.

5.7 Sequencing of the *p135* gene from strains LP1G, 47-6B and NHA15b

Sporulated cultures of *B. sphaericus* strains IAB59, LP1G, 47-6B and NHA15b show toxicity to resistant *C. quinquefasciatus* colonies raised against strains 2362 or C4-31. It has also been confirmed, as described in chapter 3, that P49 is a common crystal protein present in the sporulated cultures of IAB59, LP1G and 47-6B, but is not toxic towards either susceptible or Bin-resistant *C. quinquefasciatus* larvae.

In this chapter, the cloning of a gene encoding a 135.6 kDa putative crystal toxin from IAB59, P135, is described. The N-terminal sequencing of a crystal protein of approximately the same size also confirms the presence of this protein in strain NHA15b, as described in section 5.2.3. Primers were designed to amplify the gene encoding P135 from strain NHA15b based on the sequence of the gene from IAB59. These primers were also used to attempt amplification of the *p135* gene from strains LP1G and 47-6B, to determine whether this new putative toxin is present in all the strains reported to overcome Bin-resistance in *Culex* larvae. The sequences of the forward, P135SeqF, and reverse, P135SeqR, PCR primers can be seen in the appendix. Total DNA was isolated from strains LP1G, 47-6B and NHA15b (section 2.2.12) and used as template in PCR (section 2.2.6.1) to attempt amplification of the gene encoding P135. Thermocycling conditions were an initial denaturation at 95°C for 5 min, followed by 15 cycles of 95°C (1 min), 60°C (1 min), 72°C (3 min), and a final extension at 72°C for 10 min using Easy-A proofreading DNA polymerase. Agarose gel electrophoresis (section 2.2.2) confirmed that single PCR products were obtained with template DNA from NHA15b and 47-6B, but not LP1G. Efforts to optimise PCR conditions for amplification of *p135* from LP1G did not yield any product suggesting that there was no primer annealing site for either or both of P135Seq1F and P135SeqR. As a result, PCR was attempted using the same conditions as described above, using LP1G total DNA and primers P135SeqF and Int9F (see appendix for primer sequences). A single PCR product was obtained suggesting that a variation in the *p135* gene sequence in strain LP1G occurs at the primer annealing site of P135SeqR. Substitution of P135SeqR with Int9F primer in PCR, therefore, resulted in the product from strain LP1G corresponding to a partial gene sequence, lacking a part of the gene encoding the C-terminal end of P135.

Agarose gel electrophoresis (section 2.2.2) and extraction of the PCR products (section 2.2.4) for each strain from the gel was followed by their ligation (section 2.2.7) into the vector pGEM-T. The ligation reactions were then transformed into *E. coli* DH5α (section 2.3.2.2) and a positive clone for each ligation, comprising the *p135* gene from each strain cloned into pGEM-T, was selected after identification by colony PCR using primers M13F and M13R, and the same conditions as above except that 30 cycles were performed using *Taq* DNA polymerase. The plasmid DNA was isolated (section 2.2.1) from the selected colonies and the clones sent for sequencing using the vector primers M13F and M13R, and internal primers Int9F, Int10F, Int11F and Int12F. The sequencing reaction, using primer Int10F, for the clone containing the partial *p135* gene cloned from LP1G failed, due to the LP1G variant not having an annealing site for this primer. An additional primer, named Int10LP1GF, was designed based on the known sequence of this gene from the other sequencing reactions. To ensure that any variation in *p135* gene sequence between these strains was not due to PCR introduced mutations, this cloning was repeated so that two clones, containing *p135* amplified in different PCRs, for each strain was sequenced.

The sequence of the *p135* gene from strains 47-6B and NHA15b were found to be identical to the gene from strain IAB59. As was the case for the gene encoding P49, the LP1G strain carries a gene coding for another variant of the putative crystal toxin P135. Due to the failure to amplify the 3' end of the LP1G variant of *p135*, only a 3,388 bp partial sequence was confirmed. The variation in sequence between the partial *p135* gene from LP1G and the variant found in strains IAB59, 47-6B and NHA15b results in amino acid substitutions at 139 positions, one single aa deletion and one 2 aa deletion in the LP1G variant relative to the other P135 aa sequences. Of

the 139 aa substitutions, 29 could be considered conservative (L-I, I-V, F-Y, E-D, S-T, R-K) and 24 semi conservative (T-V, F-L, N-D, N-E, A-S, E-Q, L-V, G-A). Twenty five of these amino acid substitutions occur within the conserved blocks found in the three-domain Cry toxins. An alignment of the P135 protein from LP1G and the variant found in the other strains can be seen in figure 5.9. The discovery of *p135*, by PCR, followed by its cloning and sequencing from strains LP1G, 47-6B and NHA15b, all strains that are toxic to Bin-resistant *Culex* larvae, was promising with regard to its predicted role in the ability of these strains, and IAB59, to overcome resistance.

```
IAB59/47-6B/NHA15b   MDINNNNEKEIINSHLLPYSLLKKYPIKSLQSTNYKDWLNLCQDFNKDIESYDLVTAVSS
LP1G                 MDINNNNEKEIINSHLLPASLLKKHPIKSLQSTNYKDWLNLCQDFNKDIESYDLVTAVSS

IAB59/47-6B/NHA15b   GTIVVGTMLSAIYAPALIAGPIGIIGAIIISFGTLLPLLWNESENNPKTTWIEFIRMGEQ
LP1G                 GTIVVGTMLSAIYAPAIIAGPIGVIGAIIISFGTLLPLLWSEDENNPKTVWIEFIRMGER

IAB59/47-6B/NHA15b   LVDKTISQTVFNILESYLKDLKVNLVDYEKAKQDWIELKKQQLPGSPPSTKLRNAADIAH
LP1G                 LVDKTISQTVLNILESYLKDLKVNLIDYEKAKQDWIELKKQQLPGSPPSINLRNAADIAH

IAB59/47-6B/NHA15b   QRLDSLHNKFAELNKFKVEPYETILLPVYAQAANLHLNLLQQGAMFADQWIEDKYSSRND
LP1G                 QRLDSLHNKFAELNVFKVASYETILLPVYAQAANLHLNLLQQGAMFADQWIEDKYSPRND

IAB59/47-6B/NHA15b   TFAGNSNDYQNLLKSRTITYINHIENTYQNGLNYLWNQPEMTWDIYNEYRTKMTITALDL
LP1G                 TFAGNSNDYQDLLKSRTITYINHIENTYKDGLNYLWNQPEMTWDIYNEYRTNMTLTALDL

IAB59/47-6B/NHA15b   MALFPFYNKELYDPTVGIKSELTREIFINTPVEPHLHRYFKLSETEEKLTNNSDLFKWLT
LP1G                 LPLFPFYNKELYDPRVGIKSELTREVYINTPVDPHLHRYFKLGETEDKLTNNSELFKWLT

IAB59/47-6B/NHA15b   SLKFRTLYQPGFPFLIGNMNSFTNTNGTQLINNQQQLWSFPGTTENEEKLFPSPANIDQV
LP1G                 SLKFRTFNQPGFPFLIGNMNYFKKTNGTQLINNQQQLWSFPGTTEIE-KLFPSPANIDKV

IAB59/47-6B/NHA15b   TMYIYYGSGWGIPEPISTTINKLIFNHDKHELISEYDAGNTNAPTRSLSLGLPNHYLSCL
LP1G                 TMYIYYGSGWEVPEPISITINKLIFNHHKHGLITEYDAGNTNAPTMGIYVNLPKHYLSCL

IAB59/47-6B/NHA15b   NSYYPLTATTDGMNKEELKMYSFGWTHNSVDFLNEISKDKITQIPAVKAYRLTSNSRVIK
LP1G                 NSYYPLTATTNGMGKEELKMYSFGWTHESVDFLNEISNDKITQIPAVKAYNLNSNSRVIK

IAB59/47-6B/NHA15b   GPSHIGGNLVYLSENSQMALTCRYTNSSPQEYKIRIRYASNRLNMGQLFTTFSSHQFVLP
LP1G                 GPGHIGGNLVYLSDKSQLSLACRYTNSSPQDFLIRIRYASNKRNMVQLFTPFSTHQFVLP

IAB59/47-6B/NHA15b   PTFNHFNIEQAKYEDYAYAEFPESMSIRGNLNSDILLILNILAGGELLLDKIEFIPLTQK
LP1G                 QTFNHLNIEQTKYEDYEYAQLPGSLTINGNVNIDLLFLLNVLDGGELLLDKIEFIPLTQK

IAB59/47-6B/NHA15b   VKDNLEKEKIDMLKNLTDSLFNSPSKDTLKIDSTDYQIDQIAFQIESINEEINPQEKMEL
LP1G                 VKDNLEKEKIDMLKNLTDSLFNSPAKDTLKINSTDYQIDQIAFQIESINEEINTQEKMKL

IAB59/47-6B/NHA15b   LDNIKYAKKLNQLRNLLYSRESQAQIDWVTSNDVSIYHGKKPFNDYTLVMSRTSSSLSEI
LP1G                 LDNIKYAKKLNQLRNLLYSRESQAQIDWVTSNDVSIYHGKKPFNEYTLVMS--GSSLSKI

IAB59/47-6B/NHA15b   TATNYQTYIYKKIEESKLKPYTRYLVRGFISNSEDLEIFISRYENEIHTNMNVHGDDDTL
LP1G                 TSSNYPTYIYKKIEESKLKPYTRYLVRGFISNSDNLEIFISRYENEIHTNMNVHVDDDTL

IAB59/47-6B/NHA15b   LNSDIRQNECESKLPIIFDATSQYSLSPSRTSGISNHSYYNNGHQSSCNDTHIFSFSIDT
LP1G                 LNSYKRQNECESKLPIVFDETSQYPLSPSRTSGISNHSYYNGAQQSSCHDTQIFSFSIDT

IAB59/47-6B/NHA15b   GEVDFNNYPGIEILFKLSNTNGYASISNLEVIEERLLTEEEKRQIIQIENRWKAKKESQR
LP1G                 GDVDFNEYPGIEILFKLSNSNGYASISNLEVIEERLLTEEEKRHIIEIENRWKAKKEIQR

IAB59/47-6B/NHA15b   NETEKITTQAQQAINSLFTDTQYSNLKFETTKQNITEANTILENIPYVYNALLPTEPGMN
LP1G                 NETEKETTQAQQAINNLFTDTQYSKLKFETTKQSISKANAILENIPYVYNSLLPTEPGMN

IAB59/47-6B/NHA15b   FVLFNSFKDQINKAHALYKMRNLIKNGDFINDTKYWSISTDVKLEKVNKETILVLSSWEA
LP1G                 FELFNSFKDQINKAHTLYKMRNSIKNGDFINGTEYWSISTDVKLEKTNIETILVMSSWSA

IAB59/47-6B/NHA15b   QASQQILVQKQKRYLLRVIAKKEDMGRGNVIISDCLNNIAKIDFTPHDCNMNHIQNSSEF
LP1G                 QSSQQILVQKQNRYLLRVIAKKEDMGSGNVTISDCLNNIAKIEFIPHDCNMN

IAB59/47-6B/NHA15b   IIKTIHFSPNTEQVRIDIGQSDGVFKVESIELICVNY
LP1G
```

Figure 5.9 Alignment of P135 from LP1G, and the variant found in strains IAB59, 47-6B and NHA15b. Amino acid residues of P135 from LP1G, that differ from the variant found in other strains are highlighted yellow. The conserved blocks found in the three-domain Cry toxins are shown boxed.

5.8 Discussion

After establishing that P49, a predicted crystal toxin from *B. sphaericus* strain IAB59, was not the toxic factor responsible for the activity of this strain, and strains 47-6B and LP1G, towards Bin-resistant *Culex* larvae, focus turned towards strain NHA15b. Analysis of the crystal protein profile of this *bin*$^{-}$ *B. sphaericus* strain led to the discovery of a high molecular weight protein, present in all three crystal preparation fractions that showed toxicity towards susceptible *Culex* larvae. Subsequently, the experimentally derived N-terminal sequence of this putative crystal toxin was found to be identical to the N-terminus of the translated product of a gene found downstream of *p49*, contained within the 15,649 bp *Hin*dIII fragment cloned from IAB59, in clones p49H5 and p49H11. Sequencing of this 3,534 bp gene revealed that it encodes a 1,177 aa protein of 135.6 kDa, which shows homology to the three domain family of Cry toxins from *B. thuringiensis.* Analysis of the upstream DNA sequence also led to the identification of a putative RBS as well as a predicted σ^{E}-like promoter. The homology shared between this protein, temporarily named P135, and in particular the Cry4Aa and Cry4Ba mosquitocidal toxins from *B. thuringiensis* subsp. *israelensis*, was encouraging with regard to its predicted role as a previously unidentified toxin from the *B. sphaericus* strains that have the ability to overcome Bin-resistance. Also encouraging was the conservation between P135 and the regions of Cry4Aa and Cry4Ba corresponding to the conserved blocks of amino acids shared between the three-domain Cry toxins. This is the first report of a three-domain Cry protein from *B. sphaericus.*

Cloning and sequencing of the gene encoding P135 from strains 47-6B, NHA15b and a partial 3,388 bp sequence of the gene from LP1G, revealed the LP1G

strain to encode a different variant of the putative toxin to the other strains. The finding that the gene encoding P135 was common to the strains known to be able to overcome Bin-resistance in *Culex* larvae was again promising regarding its predicted role as a toxin. The discovery that LP1G encodes a different variant of P135 compared to the other strains, as was the case for P49, may suggest that these proteins in strain LP1G have evolved divergently from the variants found in the other *B. sphaericus* strains.

Sequencing and analysis of the *Hin*dIII insert in clones p49H5 and p49H11 also identified another interesting gene, encoding a protein containing a ricin-like beta-trefoil motif. These carbohydrate binding motifs are found in *B. sphaericus* and *B. thuringiensis* toxins such as Mtx1 (Thanabalu *et al.*, 1991), the human cancer cell specific toxins, parasporin-3Aa (Cry41Aa1) and parasporin-3Ab (Cry41ab1) (Yamashita *et al.*, 2005), and Cyt1Ca (Itsko *et al.*, 2005) (Manasherob *et al.*, manuscript in preparation). The protein product of this gene also has an N-terminal region showing homology to phospholipase C, a predicted virulence factor that contributes to the role of spore toxicity (Salamitou *et al.*, 2000). This gene may prove to be interesting for future research, to determine and characterise its contribution, if any, to *B. sphaericus* toxicity.

To establish whether P135 is the previously unidentified toxin, from strains IAB59, LP1G, 47-6B and NHA15b, responsible for toxicity towards Bin-resistant *Culex* larvae, recombinant expression of P135 in *B. thuringiensis* subsp. *israelensis* 4Q7 followed by bioassays against both susceptible and resistant *Culex* larvae were performed, as described in the following chapter.

CHAPTER 6

Recombinant expression and bioassay of the putative crystal toxin, P135, from *B. sphaericus* IAB59

6.1 Introduction

The crystal protein P49, from IAB59, has been expressed in recombinant form in *B. thuringiensis* subsp. *israelensis* for bioassay against both susceptible and resistant *Culex* mosquito larvae, as described in chapter 4. Also, both BinA and BinB have previously been produced in recombinant form in *B. thuringiensis* (Nicolas *et al.*, 1993). To allow for bioassay of P135, a further putative toxin cloned from *B. sphaericus* IAB59 as described in chapter 5, its expression was again attempted in an acrystalliferous strain of *B. thuringiensis* subsp. *israelensis*, 4Q7. As was the case for P49, bioassay of P135 against susceptible *C. quinquefasciatus* (SLCq) larvae would determine whether this protein is a mosquitocidal toxin. Further bioassays against Bin-resistant *C. quinquefasciatus* (RLCq/C3-41) larvae would establish whether P135, a common spore protein present in the *B. sphaericus* strains able to overcome Bin-resistance, is the toxic component in these strains responsible for their ability to overcome this resistance.

6.2 Expression of P135 in *B. thuringiensis* subsp. *israelensis*

The gene encoding P135, and its upstream putative promoter region, was cloned into the *B. thuringiensis-E. coli* shuttle vector pHT304 (Arantes and Lereclus, 1991), for expression under the regulation of its own promoter. Clone p49H5, containing the 15,649 bp *Hin*dIII fragment from IAB59 DNA, was used as template DNA for amplification of *p135.*

6.2.1 Cloning of *p135* into pHT304

Primers BamP135F and BamP135R were used to amplify the gene encoding P135 and 252 bp upstream, from clone p49H5, by PCR (section 2.2.6.1) using Easy-A

proofreading DNA polymerase. Thermocycling conditions were an initial denaturation at 95°C for 5 min, followed by 15 cycles of 95°C (1 min), 60°C (1 min), 72°C (3 min, 30 s), and a final extension at 72°C for 10 min. The PCR product was purified by extraction (section 2.2.4) from an agarose gel after electrophoresis (section 2.2.2). A *Bam*HI digest (section 2.2.3) of the purified PCR product was performed followed by extraction of the product from an agarose gel after electrophoresis, ready for direct ligation into pHT304 linearised with *Bam*HI and treated with CIP to prevent self-ligation. The resulting ligation reaction was transformed into *E. coli* DH5α by electroporation (section 2.3.2.2) and plated onto LB agar plates containing ampicillin for selection. Colonies were screened for the successful cloning of *p135* into pHT304 by colony PCR (section 2.2.6.1), using the same conditions as above except that 30 cycles were performed using *Taq* polymerase. A positive colony was selected and the plasmid isolated (section 2.2.1) before confirming the integrity of the clone by sequencing (section 2.2.10) using vector specific primers, M13F and M13R, and internal primers Int9F, Int10F, Int11F and Int12F (see appendix for primer sequences). The resulting clone was named pHTP135.

6.2.2 Expression from pHTP135 in *B. thuringiensis* subsp. *israelensis*

The clone pHTP135 was transformed into *B. thuringiensis* subsp. *israelensis* 4Q7 by electroporation (section 2.3.4) before plating onto LB agar plates containing erythromycin for selection. All colonies were confirmed to contain pHTP135 by colony PCR, using primers BamP135F and BamP135R, and the same thermocycling conditions as described for colony PCR in section 6.2.1. A colony was selected for inoculation of 30ml of Embrapa sporulation medium (section 2.1.6)

containing erythromycin, in a 250ml conical flask. A control culture was also prepared by inoculation of untransformed *B. thuringiensis* subsp. *israelensis* 4Q7 into Embrapa sporulation medium without erythromycin. Cultures were incubated at 30°C, 250 rpm for 72 hours, and sporulation confirmed by analysis of a sample by light microscopy. A sample (1ml) of each sporulated culture was harvested by centrifugation, the supernatants discarded and the pellets resuspended in 100 μl of SDS-PAGE sample buffer (section 2.5.1). The resuspended samples were boiled for 5 min before SDS-PAGE analysis (section 2.5.1) of 10μl of the samples, through a 10% acrylamide gel. Expression of P135 could not be detected in these samples after SDS-PAGE analysis, suggesting either that expression of P135 in *B. thuringiensis* subsp. *israelensis* 4Q7 was not possible or that only low level expression was occurring (results not shown).

With detection of P135 in strain NHA15b only possible after purification of crystals from a sporulated culture of this strain, crystal purification from a sporulated culture of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP135) was also performed. For this, *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP135) was used to inoculate two 2l baffled flasks containing 200ml of Embrapa sporulation medium (section 2.1.6), containing erythromycin. The flasks were incubated at 30°C, 250 rpm for 72 hours and complete sporulation confirmed by analysis of a sample by light microscopy. Crystal purification was performed as described in section 2.5.6. A single band was observed in the discontinuous sucrose gradient and was extracted before washing and resuspension in 2ml of sterile distilled water. A 5μl sample of the suspension was analysed by SDS-PAGE (section 2.5.1). A low intensity band of expected size, corresponding to P135, was observed and can be seen in figure 6.1. Although use of this recombinant *B. thuringiensis* subsp. *israelensis* strain was a feasible approach

towards preparation of P135 for bioassay, a higher level of expression was desirable for purification of larger quantities of protein. Light microscope analysis of the sporulated culture of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP135) also revealed the presence of free crystals in the medium, which are not observed in untransformed control cultures of this strain, confirming as predicted the expression of P135 as a crystal protein. A stock of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP135) was prepared by dipping a piece of sterilised filter paper into the sporulated culture. The filter paper was stored at -20°C. For preparation of new cultures of this recombinant strain, a small piece was cut from the stock filter paper with a sterile scalpel blade and used either to inoculate culture media containing erythromycin, or to streak across the surface of LB agar plates containing erythromycin.

6.2.3 Expression of P135 using the pSTAB vector

The vector pSTAB (Park *et al.*, 1998; Park *et al.*, 1999), which was a kind gift from Prof. Brian Federici, is a *B. thuringiensis* expression vector. Expression is driven by the dual *cyt1Aa* promoters and the transcript is stabilised by the vector encoded STAB-SD sequence. The STAB-SD sequence (GAAAGGAGG), sourced from upstream of the *cry3Aa* gene from *B. thuringiensis* subsp. *morrisoni*, is a perfect Shine-Dalgarno sequence that may stabilise the transcript by associating with the 16S rRNA of the 30S ribosomal subunit, and thus prevent degradation by 5'-3' ribonuclease activity (Agaisse and Lereclus, 1996; Schnepf *et al.*, 1998).

Primers PSTABF and PSTABR were used to amplify the gene encoding P135, including a 39 bp region upstream containing the predicted Shine-Dalagarno sequence, from clone p49H5 by PCR. Thermocycling conditions were an initial denaturation at 95°C for 5 min, followed by 15 cycles of 95°C (1 min), 60°C (1 min),

72°C (3 min 30 s), and a final extension at 72°C for 10 min. The amplified product was extracted (section 2.2.4) from an agarose gel after electrophoresis (section 2.2.2). The product was digested at its primer introduced *Sal*I and *Sph*I sites (section 2.2.3) and again purified by recovery of the DNA fragment from an agarose gel following electrophoresis. The *Sal*I and *Sph*I digested PCR product was then ligated into the pSTAB vector, which had been cut with the same restriction enzymes. Electrotransformation of the ligation mixture into *E. coli* DH5α (section 2.3.2.2) was performed and the colonies screened by PCR (section 2.2.6.1), after plating onto LB agar plates containing ampicillin. Colony PCR conditions were identical to those used for amplification of *p135* using PSTABF and PSTABR, except that 30 cycles were performed using *Taq* polymerase. A positive colony was selected and the plasmid DNA purified (section 2.2.1), before confirming the integrity of the clone by sequencing (section 2.2.10) using the vector specific primers M13F and M13R, and internal primers Int9F, Int10F, Int11F and Int12F (see appendix for primer sequences). The clone was named pSTABP135.

The clone pSTABP135 was transformed into *B. thuringiensis* subsp. *israelensis* 4Q7 by electroporation (section 2.3.4) and plated onto LB agar containing erythromycin for selection of recombinants. All colonies obtained were shown to contain pSTABP135, after colony PCR using primers PSTABF and PSTABR and the same thermocycling conditions as described above for colony PCR. Embrapa sporulation medium (30 ml; section 2.1.6) containing erythromycin was inoculated with a single colony of *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135), in a 250 ml conical flask. A control untransformed culture was also prepared in identical fashion, except that no erythromycin was added to the sporulation medium. The cultures were incubated at 30°C, 250 rpm for 72 hours, and sporulation was confirmed

by analysis of a sample by light microscopy. Samples (1ml) were harvested by centrifugation, the supernatant discarded and the pellet resuspended in 100μl of SDS-PAGE sample buffer (section 2.5.1). SDS-PAGE was performed, using 10μl of the samples, after boiling for 5 min. As was the case for expression of P135 under the regulation of its own promoter, no expression of P135 was detected by SDS-PAGE analysis of this sporulated culture (results not shown).

For crystal protein purification of this recombinant strain, two 2l baffled flasks containing 200ml of Embrapa sporulation medium (section 2.1.6), supplemented with erythromycin, were inoculated with recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135). The flasks were incubated at 30°C, 250 rpm for 72 hours until sporulation was complete, as confirmed by light microscopy of a sample of each culture. Crystal purification was performed as described in section 2.5.6. A single band was extracted from the discontinuous sucrose gradient and was washed thoroughly before final resuspension in 2ml of sterile distilled water. A 5μl sample of the suspension was analysed by SDS-PAGE (section 2.5.1) and indicated an improved yield of P135 crystal protein relative to its expression from clone pHTP135. SDS-PAGE analysis of the resulting culture can be seen in figure 6.1, along with analysis of the expression from pHTP135 and fraction 3 obtained by crystal purification from strain NHA15b, as previously shown in figure 5.2. Light microscope analysis of the sporulated culture of *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135), again revealed the presence of free crystals in the medium, confirming its production as a crystal protein. A stock of *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) was prepared by dipping a piece of sterilised filter paper into the sporulated culture. The filter paper was stored at -20°C. For preparation of new cultures of this recombinant strain, a small piece

was cut from the stock filter paper, with a sterile scalpel blade, and used either to inoculate culture media containing erythromycin or to streak across the surface of LB agar plates containing erythromycin.

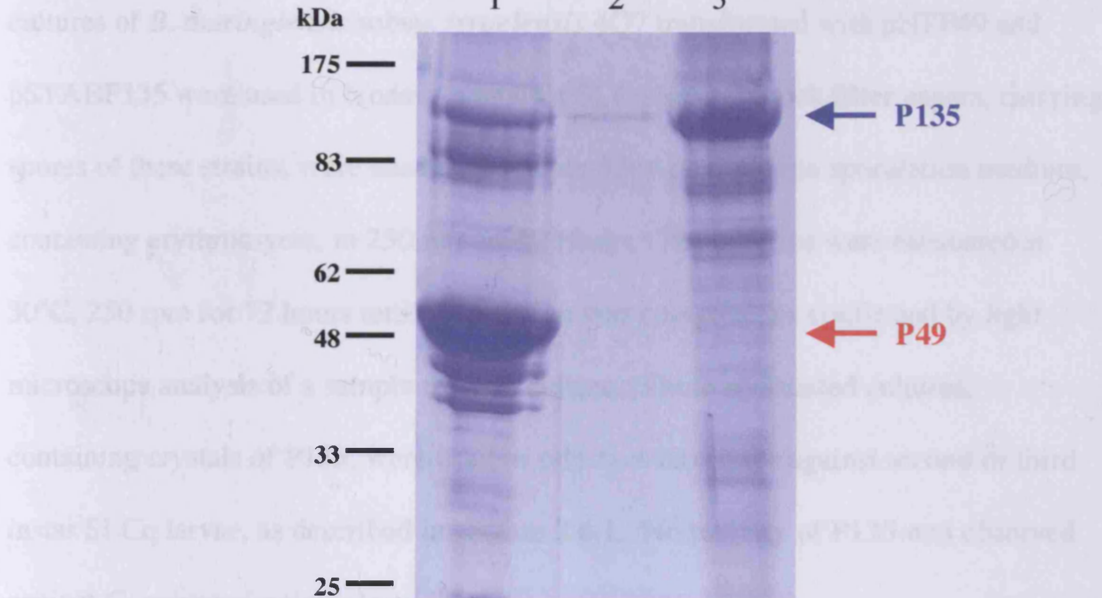


Figure 6.1 Crystal purification of sporulated cultures of *B. sphaericus* strain NHA15b and *B. thuringiensis* P135 expression cultures. The putative toxin, P135, is indicated by a blue arrow, present in *B. sphaericus* strain NHA15b (lane 1), and sporulated cultures of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP135) (lane 2) and *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTAB135) (lane 3). The P49 crystal protein, present in the crystal preparation from strain NHA15b, is indicated by a red arrow. The molecular weight standard marker sizes are shown.

6.3 Bioassay of P135

To determine whether P135 is, as hypothesised, a mosquitocidal crystal toxin present in the *B. sphaericus* strains toxic to Bin-resistant *Culex* larvae, sporulated cultures of *B. thuringiensis* subsp. *israelensis* 4Q7 transformed with pHTP49 and pSTABP135 were used in bioassays against SLCq larvae. Stock filter papers, carrying spores of these strains, were used to inoculate 30ml of Embrapa sporulation medium, containing erythromycin, in 250ml conical flasks. The cultures were incubated at 30°C, 250 rpm for 72 hours until sporulation was complete, as confirmed by light microscope analysis of a sample of each culture. These sporulated cultures, containing crystals of P135, were used in selective bioassays against second or third instar SLCq larvae, as described in section 2.6.1. No toxicity of P135 was observed against *C. quinquefasciatus* larvae.

Bioassays of the *bin*⁻ *B. sphaericus* strain, NHA15b, have shown that this strain is toxic to RLCq/C3-41 larvae (Nielsen-LeRoux personal communication). From this, it can be concluded that the ability of this strain to overcome Bin-resistance is independent of the Bin toxin itself, and may point to a similar situation for strains IAB59, LP1G and 47-6B. However, to ensure that this was the case for strains IAB59, LP1G and 47-6B, bioassays of P135 in combination with BinA and BinB were performed. For this, a sporulated culture of *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) was assayed in combination with spores of *B. thuringiensis* subsp. *israelensis* 4Q7 transformed with either pHT680 and pHT684. Clones pHT680 and pHT684 contain the genes coding for BinA and BinB respectively, under the regulation of the *cyt1Aa* promoter from *B. thuringiensis* subsp. *israelensis* (Nicolas *et al.*, 1993). Selective bioassays (section 2.6.1) of SLCq larvae exposed to a BinA/P135 (100μl of each recombinant culture expressing BinA and

P135) or a BinB/P135 mix (100μl of each recombinant culture expressing BinB and P135) in a 10ml bioassay showed no toxicity.

The lack of toxicity in bioassays of P135 towards SLCq larvae suggest that: i) P135 is not the toxic agent in strains IAB59, LP1G, 47-6B and NHA15b towards Bin-resistant mosquito larvae, or ii) that P135 functions in combination with another toxin component. To determine whether the crystal protein P135, which shows homology to the three-domain Cry toxins, functions in combination with P49, the putative crystal toxin cloned from strain IAB59 as described in chapter 3, bioassays of sporulated cultures of recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 expressing P49 and P135 were performed. Selective bioassays, performed as described in section 2.6.1, of a mixture (100μl of each culture) of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) and *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135), in a 10ml bioassay volume, against SLCq larvae showed 100% mortality. Selective bioassays of the same recombinant strains against RLCq/C3-41 larvae, performed at the Wuhan Institute of Virology (Wuhan, China) by Prof. Yuan Zhiming, also resulted in 100% mortality when P49 and P135 were combined.

6.4 Preparation of P49 and P135 crystal stocks for bioassay and characterisation

Stocks of P49 and P135 crystal toxins were prepared by purification of crystals from multiple litres of sporulated cultures of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) and *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) respectively. The extracted crystal protein bands, from multiple discontinuous sucrose gradients of P49 and P135, were separately pooled and the concentrations of P49 and P135 in the resulting crystal protein suspensions were calculated using the Bio-Rad protein assay kit, as described in section 2.5.7. To confirm the accuracy of

the calculated concentrations, SDS-PAGE analysis of the samples by comparison to known BSA concentrations was performed, as described in section 2.5.7. Briefly, this involved running different amounts of toxin and known BSA concentrations on the same SDS-PAGE gel and comparing protein amounts, after Coomassie staining, by densitometry.

6.5 Discussion

Recombinant production of P135 was achieved in *B. thuringiensis* subsp. *israelensis* 4Q7, with higher yields attained when expressed under the regulation of the dual *cyt1Aa* promoters in the pSTAB vector than under the regulation of its own promoter in pHT304. However, production levels of P135 using the pSTAB vector were still lower than that of P49 when expressed under the regulation of its own promoter in *B. thuringiensis* subsp. *israelensis* 4Q7. The reasons for this are currently unknown but may involve one or more of the following: i) superior stability of the *p49* transcript, ii) higher levels of P49 protein translation, iii) superior stability of the P49 protein compared to P135.

Sporulated cultures of *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) and *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP135) were found to contain free crystals, consistent with the identification of a ~135 kDa crystal protein from strain NHA15b, as described in section 5.2.3. Bioassays of sporulated cultures of these recombinant strains revealed that P135 was not toxic to susceptible *C. quinquefasciatus* larvae but that 100% mortality was observed when P135 was combined with recombinant P49. Toxicity towards Bin-resistant *C. quinquefasciatus* larvae was also observed when P49 and P135 were combined. While three-domain Cry toxins have been shown to synergise with other toxins, P135 is the first report of

a three-domain Cry toxin that functions as a binary toxin with another component, as well as the first report of a three-domain Cry toxin from *B. sphaericus.*

Following the submission of the sequence of the 15,649 bp *Hin*dIII DNA fragment from IAB59, carrying the genes for P49 and P135, to the EMBL GenBank and the confirmation that P49 and P135 are crystal proteins having a toxic effect to a target organism (*C. quinquefasciatus* mosquito larvae), all the criteria required for submission of the protein sequences to the *B. thuringiensis* Pesticidal Crystal Protein Nomenclature Committee were filled. Based on the parameters assigned by the Nomenclature Committee (Crickmore *et al.*, 1998), P49 and P135 from IAB59 have been renamed Cry49Aa1 and Cry48Aa1 respectively. The official names of the Cry48/Cry49 variants from other *B. sphaericus* strains can be found in the appendix. In the following chapters, these newly discovered toxin components are referred to according to their official Cry designations. The full list of Cry and Cyt toxins can be seen at www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/.

Crystal purification from recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) and *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) was performed, allowing use of crystals of Cry49Aa1 and Cry48Aa1 in bioassays against both SLCq and RLCq/C3-41 larvae for determination of LC_{50} values. The characterisation of Cry49Aa1 and Cry48Aa1 are described in the following chapter.

CHAPTER 7

Characterisation of the Cry48Aa1/Cry49Aa1 binary mosquitocidal toxin from *B. sphaericus* IAB59

7.1 Distribution of the genes encoding Cry48 and Cry49 proteins

The genes encoding the Cry48 and Cry49 binary mosquitocidal toxin, previously referred to as P135 and P49 respectively, are found in *B. sphaericus* strains IAB59, LP1G, 47-6B and NHA15b, all strains known to be able to overcome Bin-resistance in *Culex* mosquito larvae, as shown in previous chapters. Another strain, IAB872 (serotype H48), has also been reported to show toxicity against Bin-resistant *Culex* larvae (Shi *et al.*, 2001), with recombinant expression and bioassay of the Bin toxin from this strain in *B. thuringiensis* subsp. *israelensis* showing no toxicity towards resistant larvae, confirming the presence of unidentified toxin(s) in this strain. Strain 2173 (serotype H26a26b), which lacks the Bin toxin and Mtx toxins, is known to be toxic to susceptible and Bin-resistant *Culex* larvae (Wirth *et al.*, 2000a), again pointing to the presence of unidentified toxin(s) in this strain. Experiments were, therefore, performed to determine the distribution of the genes encoding Cry48/Cry49 binary toxins among the *B. sphaericus* strains kept at Cardiff University.

7.1.1 Detection of *cry48* and *cry49* genes by dot blot and PCR

Total DNA was isolated from *B. sphaericus* strains, for use in dot blot hybridisation experiments, using the DNeasy® Tissue Kit (Qiagen, Crawley, West Sussex, UK) as described in section 2.2.12. Probes were prepared, for use in detection of *cry48* and *cry49* genes, by PCR as described in section 2.4.1.2. The 338 nt probe for detection of *cry48* genes was prepared by PCR using primers PSTABF and Int13F, while the 395 nt probe for detection of *cry49* genes was prepared using primers P49F and Int1R (see appendix for primer sequences). The clone p49H5, containing the 15,649 bp *Hin*dIII fragment cloned from IAB59 DNA

and carrying the genes for *cry48Aa1* and *cry49Aa1*, was used as template DNA. Thermocycling conditions were, as recommended by the manufacturer, an initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C (30 s), 60°C (30 s), 72°C (40 s), and a final extension at 72°C for 7 min, using *Taq* DNA polymerase. The agarose gel prepared to confirm successful incorporation of DIG-dUTP into the amplified products can be seen in figure 7.1. The difference in relative mobility of the control PCR products, prepared using a dNTP mixture containing dATP, dCTP, dGTP and dTTP, compared to the products to be used as probes, prepared using a dNTP mix also containing DIG-dUTP, is due to the incorporation of DIG into the products used as probes. Incorporation of DIG into amplified products results in their slower migration through agarose gels, and thus is an effective way of confirming the incorporation of DIG into PCR generated probes. The PCR products, with DIG-dUTP incorporated, in lanes 3 and 6 were selected as probes for detection of *cry49* and *cry48* genes respectively.

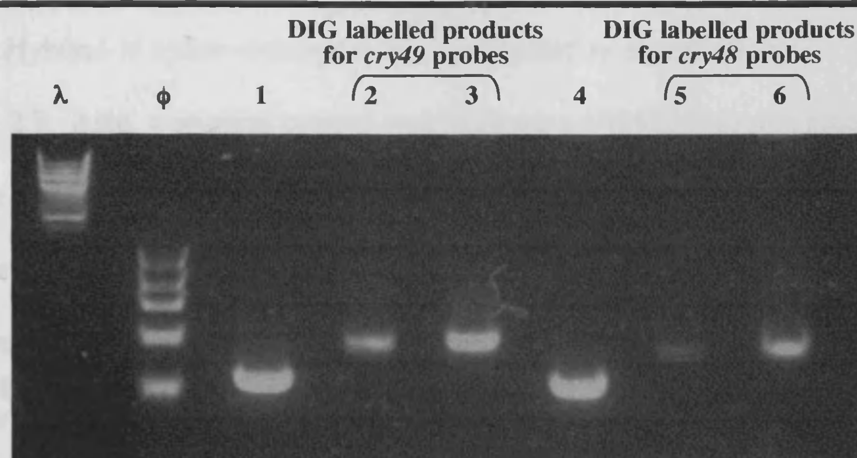


Figure 7.1 PCR generation of DIG labelled probes for hybridisation to *cry48* and *cry49* genes. PCR products obtained using: primers P49F/Int1R and 140 ng of template DNA in the control reaction (lacking DIG-dUTP) (lane 1), primers P49F/Int1R and 14 ng of template DNA in a test reaction containing DIG-dUTP (lane 2), primers P49F/Int1R and 140 ng of template DNA in a test reaction containing DIG-dUTP (lane 3), primers PSTABF/Int13F and 140 ng of template DNA in the control reaction (lacking DIG-dUTP) (lane 4), primers PSTABF/Int13F and 14 ng of template DNA in a test reaction containing DIG-dUTP (lane 5) and primers PSTABF/Int13F and 140 ng of template DNA in a test reaction containing DIG-dUTP (lane 6). The apparent size difference of the products in the test reactions compared to the control reactions is due to the incorporation of DIG into the test products, leading to reduced mobility. The products in lane 3 and lane 6 were used as probes for detection of *cry49* and *cry48* genes respectively. The molecular weight marker lanes are also shown.

Immobilisation of total DNA isolated from *B. sphaericus* (100-200ng per spot), onto Hybond-N nylon membrane, was performed as described in section 2.4.2.3. Also, a positive control spot of clone p49H5 (10ng) was prepared. Briefly, the DNA samples were denatured and spotted onto two Hybond-N membranes in a grid like fashion, before fixing the DNA to the membrane by baking. The DIG labelled PCR products, prepared as described above, were made single stranded by heating to 95°C for 5 min and then used, as described in section 2.4.3.2, individually as probes for detection of *cry48* and *cry49* genes by hybridisation to the dot blot membranes. Hybridisations were performed at 68°C and an X-ray film was exposed to the chemiluminescent signal to identify DNA spots containing probe hybridisable sequence. The resulting X-ray film can be seen in figure 7.2.

Analysis of the dot blots identified the presence of genes for Cry49 proteins in *B. sphaericus* strains IAB59, NHA15b, 47-6B, LP1G, 2173, IAB881, LP14-8, LP18, and LB29. The background signal of the dot blot for detection of *cry48* genes (figure 7.2(ii)) was higher than was obtained for detection of *cry49* genes. This resulted in it being difficult to differentiate absolutely between background and positive signals. However, the most intense signals were observed for strains IAB59, NHA15b, 47-6B, LP1G, 2297, 2173, LP14-8, LP18 and LB29, all except 2297 being found positive for the presence of *cry49* genes also. Interestingly strain 2173, which shows toxicity to *Culex* larvae but does not contain any of the toxins identified to date (Thanabalu *et al.*, 1991; Thanabalu and Porter, 1996; Priest *et al.*, 1997), gave positive hybridisation signals for both *cry48* and *cry49* genes, suggesting that these toxins may be the source of this toxicity. No signal was obtained for strain IAB872, which has the ability to overcome Bin-resistance in *Culex* larvae. However, a signal for the presence of a *cry49* gene was obtained for strain IAB881, of the same serotype

(H3) as LP1G. Also, positive signals were obtained for the presence of both *cry48* and *cry49* genes in strains LP14-8, of the same serotype (H3) as LP1G, LB29, of the same serotype (H26a26b) as 2173 and LP18 (serotype not determined). Due to the high background level obtained in the dot blot for detection of *cry48* genes, a PCR based detection of the *cry48* and *cry49* genes was also performed. Primer combinations P49F/Int1R and PSTABF/Int13F were used for PCR detection of *cry49* and *cry48* genes respectively, using the thermocycling conditions; 95°C for 5 min, followed by 30 cycles of 95°C (1 min), 58°C (1 min), 72°C (30 s), and a final extension at 72°C for 5 min, using *Taq* DNA polymerase and the total DNA purified from *B. sphaericus* strains as template DNA. Clone p49H5 was again used as positive control template DNA. The products of PCR were analysed by agarose gel electrophoresis (section 2.2.2). The resulting agarose gel is shown in figure 7.3.

As can be seen from figure 7.3, PCR confirms the presence of both *cry48* and *cry49* genes in strains IAB59, NHA15b, 47-6B, LP1G, 2173, LP14-8, LP18 and LB29. No PCR product was obtained for the detection of these genes in strain IAB881. However, the strong signal obtained for the presence of a *cry49* gene in this strain by dot blot may suggest that different variants of one, or both, of these genes may be present, resulting in no annealing site for the primers used in PCR. Further research on this strain as well as strain 2297, which showed a signal for detection of a *cry48* gene by dot blot, would determine whether genes related to *cry48Aa* and *cry49Aa* are actually present.

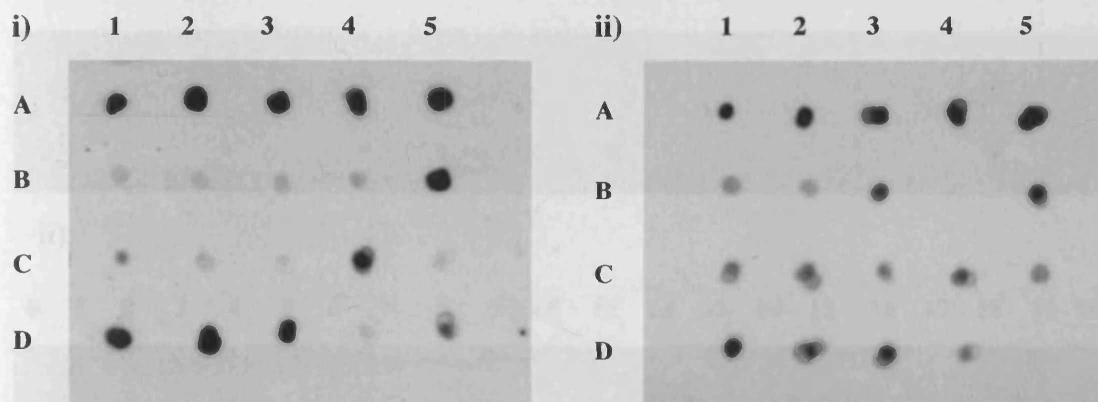


Figure 7.2 Dot blots of total DNA from *B. sphaericus* strains hybridised with probes to detect *cry49* and *cry48* genes. Detection of *B. sphaericus* strains containing the newly identified toxin genes, by hybridisation using *cry49* (i) and *cry48* (ii) specific DIG labelled probes. The DNA spot in position A1 is the positive control, clone p49H5. The DNA spots of total DNA from *B. sphaericus* strains are: IAB59 (A2), NHA15b (A3), 47-6B (A4), LP1G (A5), 2362 (B1), 1593 (B2), 2297 (B3), 2315 (B4), 2173 (B5), SSII-1 (C1), 2627 (C2), Kellen Q (C3), IAB881 (C4), IAB872 (C5), LP14-8 (D1), LP18 (D2), LB29 (D3), PR-1 (D4), 31 (D5).

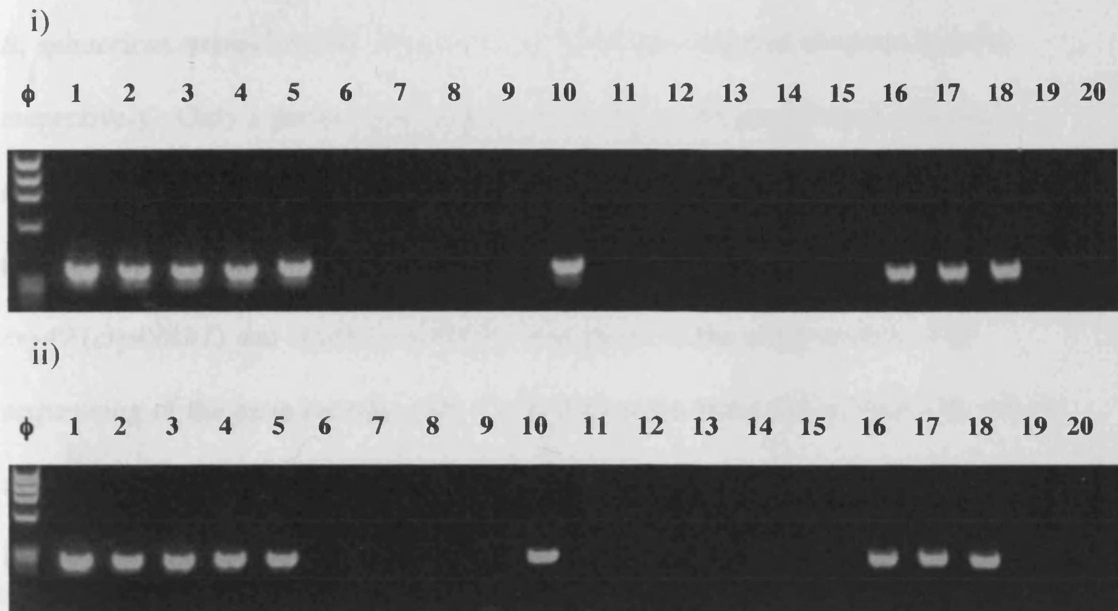


Figure 7.3 PCR detection of *cry48* and *cry49* genes in *B. sphaericus* strains.

Detection of *cry49* (i) and *cry48* (ii) genes by PCR was performed using primers pairs P49F/Int1R and PSTABF/Int13F respectively. Clone p49H5 was used as positive control template DNA for both PCRs (lane 1). Total DNA from *B. sphaericus* strains; IAB59 (lane 2), NHA15b (lane 3), 47-6B (lane 4), LP1G (lane 5), 2362 (lane 6), 1593 (lane 7), 2297 (lane 8), 2315 (lane 9), 2173 (lane 10), SSII-1 (lane 11), 2627 (lane 12), Kellen Q (lane 13), IAB881 (lane 14), IAB872 (lane 15), LP14-8 (lane 16), LP18 (lane 17), LB29 (lane 18), PR-1 (lane 19) and 31 (lane 20) was used as template DNA. The ϕX174 *Hae*III DNA markers can also be seen.

7.1.2 Sequencing of *cry49* and *cry48* genes from strains NHA15b and 2173

The sequencing of the genes encoding Cry49 and Cry48 proteins from *B. sphaericus* strains IAB59, 47-6B and LP1G is described in chapters 3 and 5 respectively. Only a partial gene sequence for the *cry48* gene from LP1G was determined, due to a variation in gene sequence leading to the lack of a 3' binding site for the reverse primer. The LP1G strain was found to carry a different variant of both *cry49* (*cry49Ab1*) and *cry48* (*cry48Ab1*) compared to the other strains. The sequencing of the gene encoding the Cry48Aa3 toxin from strain NHA15b, which again was found to be identical to the variant found in IAB59, is described in chapter 5. However, the sequencing of the gene encoding the Cry49Aa3 toxin from *B. sphaericus* strain NHA15b was not performed at that time, due to this strain coming to our attention after the cloning and sequencing of the *cry49* genes from IAB59, 47-6B and LP1G.

Sequencing of the *cry49Aa3* gene from NHA15b was thus performed to allow comparison of the gene in this strain to the variants sequenced to date. Also, sequencing of both *cry49Aa4* and *cry48Ab2* genes from strain 2173 was performed, as prior to this work the toxic factor(s) from this strain were unknown. As for sequencing of the *cry49* and *cry48* genes from the strains already described in previous chapters, two clones prepared from independent PCRs of each gene was sequenced to confirm that any variation in sequence was authentic and not PCR introduced errors.

7.1.2.1 Sequencing of *cry49Aa* from strains NHA15b and 2173

The sequencing of the *cry49Aa* gene from strains NHA15b and 2173 was performed as described previously, in section 3.13, for the sequencing of the gene

from strains 47-6B and LP1G. The *cry49Aa* genes from strains NHA15b and 2173 were found to be identical to the variant found in IAB59 and 47-6B. The full gene sequence of the *cry49Aa* variants and their protein products can be seen in the appendix. Table 7.1 compares the sequences of the *cry49* gene variants and their protein products in the different *B. sphaericus* strains.

7.1.2.2 Sequencing of *cry48Ab2* from strain 2173

Sequencing of the *cry48Ab2* gene from strain 2173 was performed as described previously, in section 5.7, for the sequencing of the gene from strains 47-6B, NHA15b and LP1G. Attempts to amplify the complete *cry48* gene from strain 2173, using primers P135Seq1F and P135Seq1R, were unsuccessful. Therefore, the sequencing of a partial *cry48* gene from 2173 was performed, as described in section 5.7 for the sequencing of the partial *cry48Aa* gene from LP1G. Analysis of the partial *cry48* gene sequence from 2173 revealed a variation in gene sequence at four bases compared to the partial gene from strain LP1G. This variation results in 3 aa substitutions in the protein produced in strain 2173 compared to the LP1G variant: a N-D change at aa 691 and a E-K at aa 1,051 corresponding to a change in aa sequence to the IAB59 variant at these positions, and a conservative substitution of Y-F at aa 861, a position where the aa sequence of the IAB59 and LP1G variants are identical. The gene sequence of all *cry48* variants, including the partial LP1G and 2173 sequences, and their deduced amino acid products can be seen in the appendix. Figure 7.4 compares the sequences of the Cry48 protein variants in the different *B. sphaericus* strains.

Base	Amino acid	Nucleotide and amino acid in strain			
		IAB59, NHA15b,	47-6B, 2173	LP1G	
12 bp insertion after base 101	4 aa insertion after residue 34	-	---	AGT ACC CAG TGA	Val Pro Ser Glu
188	63	C	Ala	G	Gly
263	88	A	Asp	C	Ala
369	123	T	Asn	C	Asn
467	156	A	His	G	Arg
468	156	T	His	C	Arg
616	206	A	Asn	G	Asp
623	208	A	Lys	G	Arg
633	211	A	Ser	G	Ser
634	212	C	Leu	T	Phe
637	213	C	Leu	T	Leu
3 bp deletion of bases 639-641	1 aa deletion of residue 214	TAC	Thr	-	---
704	235	A	Glu	G	Gly
792	264	T	Pro	A	Pro
809	270	A	Asn	G	Ser
837	279	A	Val	T	Val
865	289	A	Ile	T	Leu
884	295	C	Thr	A	Asn
891	297	G	Gly	A	Gly
903	301	A	Glu	G	Glu
928	310	A	Asn	C	His
930	310	T	Asn	C	His
942	314	T	Asn	C	Asn
954	318	G	Lys	T	Asn
976	326	G	Ala	T	Ser
998	333	G	Arg	T	Leu
999	333	T	Arg	G	Leu
1006	336	A	Asn	C	His
1011	337	T	Leu	G	Leu
1039	347	G	Asp	A	Ser

Table continued on following page

Base	Amino acid	Nucleotide and amino acid in strain			
		IAB59, NHA15b,	47-6B, 2173	LP1G	
1040	347	A	Asp	G	Ser
1065	355	C	Phe	T	Phe
1068	356	A	Ala	G	Ala
1072	358	A	Thr	T	Ser
1081	361	G	Gly	T	Ser
1082	361	G	Gly	C	Ser
1084	362	A	Thr	T	Ser
1092	364	T	Thr	C	Thr
1095	365	T	Asp	A	Glu
1096	366	A	Ile	T	Ser
1097	366	T	Ile	C	Ser
1099	367	G	Val	A	Ile
1103	368	A	Asn	C	Thr
1105	369	G	Val	T	Tyr
1106	369	T	Val	A	Tyr
1110	370	T	Phe	A	Leu
1115	372	G	Ser	C	Thr
1125	375	C	His	T	His
1136	379	A	Tyr	T	Phe
1167	389	A	Arg	G	Arg
1186	396	T	Ser	A	Thr
1190	397	A	Gln	T	Leu
1191	397	G	Gln	T	Leu
1197	399	A	Gly	C	Gly
1244	415	A	Tyr	T	Phe
1245	415	C	Tyr	T	Phe
1258	420	G	Ala	A	Thr
1261	421	A	Ile	C	Leu
1263	421	A	Ile	G	Leu
1265	422	C	Thr	A	Asn
1266	422	C	Thr	T	Asn
1286	429	A	Asp	T	Val
1315	439	A	Ile	G	Val
1332	444	A	Leu	G	Leu
1347	449	T	Gly	G	Gly
1359	453	A	Ile	T	Ile

Table 7.1 Variant nucleotides and encoded amino acids of the *cry49* genes from *B. sphaericus* IAB59, 47-6B, NHA15b, 2173 and LP1G. The numbering is based on the IAB59 reference sequence, starting at the initiating TTG.

```
IAB59/47-6B/NHA15b   MDINNNNEKEIINSHLLPYSLLKKYPIKSLQSTNYKDWLNLCQDFNKDIESYDLVTAVSS
LP1G                 MDINNNNEKEIINSHLLPASLLKKHPIKSLQSTNYKDWLNLCQDFNKDIESYDLVTAVSS
2173                 MDINNNNEKEIINSHLLPASLLKKHPIKSLQSTNYKDWLNLCQDFNKDIESYDLVTAVSS

IAB59/47-6B/NHA15b   GTIVVGTMLSAIYAPALIAGPIGIIGAIIISFGTLLPLLWNESENNPKTTWIEFIRMGEQ
LP1G                 GTIVVGTMLSAIYAPAIIAGPIGVIGAIIISFGTLLPLLWSEDENNPKTVWIEFIRMGER
2173                 GTIVVGTMLSAIYAPAIIAGPIGVIGAIIISFGTLLPLLWSEDENNPKTVWIEFIRMGER

IAB59/47-6B/NHA15b   LVDKTISQTVFNILESYLKDLKVNLVDYEKAKQDWIELKKQQLPGSPPSTKLRNAADIAH
LP1G                 LVDKTISQTVLNILESYLKDLKVNLIDYEKAKQDWIELKKQQLPGSPPSINLRNAADIAH
2173                 LVDKTISQTVLNILESYLKDLKVNLIDYEKAKQDWIELKKQQLPGSPPSINLRNAADIAH

IAB59/47-6B/NHA15b   QRLDSLHNKFAELNKFKVEPYETILLPVYAQAANLHLNLLQQGAMFADQWIEDKYSSRND
LP1G                 QRLDSLHNKFAELNVFKVASYETILLPVYAQAANLHLNLLQQGAMFADQWIEDKYSPRND
2173                 QRLDSLHNKFAELNVFKVASYETILLPVYAQAANLHLNLLQQGAMFADQWIEDKYSPRND

IAB59/47-6B/NHA15b   TFAGNSNDYQNLLKSRTITYINHIENTYQNGLNYLWNQPEMTWDIYNEYRTKMTITALDL
LP1G                 TFAGNSNDYQDLLKSRTITYINHIENTYKDGLNYLWNQPEMTWDIYNEYRTNMTLTALDL
2173                 TFAGNSNDYQDLLKSRTITYINHIENTYKDGLNYLWNQPEMTWDIYNEYRTNMTLTALDL

IAB59/47-6B/NHA15b   MALFPFYNKELYDPTVGIKSELTREIFINTPVEPHLHRYFKLSETEEKLTNNSDLFKWLT
LP1G                 LPLFPFYNKELYDPRVGIKSELTREVYINTPVDPHLHRYFKLGETEDKLTNNSELFKWLT
2173                 LPLFPFYNKELYDPRVGIKSELTREVYINTPVDPHLHRYFKLGETEDKLTNNSELFKWLT

IAB59/47-6B/NHA15b   SLKFRTLYQPGFPFLIGNMNSFTNTNGTQLINNQQQLWSFPGTTENEEKLFPSPANIDQV
LP1G                 SLKFRTFNQPGFPFLIGNMNYFKKTNGTQLINNQQQLWSFPGTTEIE-KLFPSPANIDKV
2173                 SLKFRTFNQPGFPFLIGNMNYFKKTNGTQLINNQQQLWSFPGTTEIE-KLFPSPANIDKV

IAB59/47-6B/NHA15b   TMYIYYGSGWGIPEPISTTINKLIFNHDKHELISEYDAGNTNAPTRSLSLGLPNHYLSCL
LP1G                 TMYIYYGSGWEVPEPISITINKLIFNHHKHGLITEYDAGNTNAPTMGIYVNLPKHYLSCL
2173                 TMYIYYGSGWEVPEPISITINKLIFNHHKHGLITEYDAGNTNAPTMGIYVNLPKHYLSCL

IAB59/47-6B/NHA15b   NSYYPLTATTDGMNKEELKMYSFGWTHNSVDFLNEISKDKITQIPAVKAYRLTSNSRVIK
LP1G                 NSYYPLTATTNGMGKEELKMYSFGWTHESVDFLNEISNDKITQIPAVKAYNLNSNSRVIK
2173                 NSYYPLTATTNGMGKEELKMYSFGWTHESVDFLNEISNDKITQIPAVKAYNLNSNSRVIK

IAB59/47-6B/NHA15b   GPSHIGGNLVYLSENSQMALTCRYTNSSPQEYKIRIRYASNRLNMGQLFTTFSSHQFVLP
LP1G                 GPGHIGGNLVYLSDKSQLSLACRYTNSSPQDFLIRIRYASNKRNMVQLFTPFSTHQFVLP
2173                 GPGHIGGNLVYLSDKSQLSLACRYTNSSPQDFLIRIRYASNKRNMVQLFTPFSTHQFVLP
```

Alignment continued on following page

```
IAB59/47-6B/NHA15b   PTFNHFNIEQAKYEDYAYAEFPESMSIRGNLNSDILLILNILAGGELLLDKIEFIPLTQK
LP1G                 QTFNHLNIEQTKYEDYEYAQLPGSLTINGNVNIDLLFLLNVLDGGELLLDKIEFIPLTQK
2173                 QTFNHLNIEQTKYEDYEYAQLPGSLTINGNVNIDLLFLLNVLDGGELLLDKIEFIPLTQK

IAB59/47-6B/NHA15b   VKDNLEKEKIDMLKNLTDSLFNSPSKDTLKIDSTDYQIDQIAFQIESINEEINPQEKMEL
LP1G                 VKDNLEKEKIDMLKNLTDSLFNSPAKDTLKINSTDYQIDQIAFQIESINEEINTQEKMKL
2173                 VKDNLEKEKIDMLKNLTDSLFNSPAKDTLKIDSTDYQIDQIAFQIESINEEINTQEKMKL

IAB59/47-6B/NHA15b   LDNIKYAKKLNQLRNLLYSRESQAQIDWVTSNDVSIYHGKKPFNDYTLVMSRTSSSLSEI
LP1G                 LDNIKYAKKLNQLRNLLYSRESQAQIDWVTSNDVSIYHGKKPFNEYTLVMS--GSSLSKI
2173                 LDNIKYAKKLNQLRNLLYSRESQAQIDWVTSNDVSIYHGKKPFNEYTLVMS--GSSLSKI

IAB59/47-6B/NHA15b   TATNYQTYIYKKIEESKLKPYTRYLVRGFISNSEDLEIFISRYENEIHTNMNVHGDDDTL
LP1G                 TSSNYPTYIYKKIEESKLKPYTRYLVRGFISNSDNLEIFISRYENEIHTNMNVHVDDDTL
2173                 TSSNYPTYIYKKIEESKLKPYTRYLVRGFISNSDNLEIFISRYENEIHTNMNVHVDDDTL

IAB59/47-6B/NHA15b   LNSDIRQNECESKLPIIFDATSQYSLSPSRTSGISNHSYYNNGHQSSCNDTHIFSFSIDT
LP1G                 LNSYKRQNECESKLPIVFDETSQYPLSPSRTSGISNHSYYNGAQQSSCHDTQIFSFSIDT
2173                 LNSYKRQNECESKLPIVFDETSQFPLSPSRTSGISNHSYYNGAQQSSCHDTQIFSFSIDT

IAB59/47-6B/NHA15b   GEVDFNNYPGIEILFKLSNTNGYASISNLEVIEERLLTEEEKRQIIQIENRWKAKKESQR
LP1G                 GDVDFNEYPGIEILFKLSNSNGYASISNLEVIEERLLTEEEKRHIIEIENRWKAKKEIQR
2173                 GDVDFNEYPGIEILFKLSNSNGYASISNLEVIEERLLTEEEKRHIIEIENRWKAKKEIQR

IAB59/47-6B/NHA15b   NETEKITTQAQQAINSLFTDTQYSNLKFETTKQNITEANTILENIPYVYNALLPTEPGMN
LP1G                 NETEKETTQAQQAINNLFTDTQYSKLKFETTKQSISKANAILENIPYVYNSLLPTEPGMN
2173                 NETEKETTQAQQAINNLFTDTQYSKLKFETTKQSISKANAILENIPYVYNSLLPTEPGMN

IAB59/47-6B/NHA15b   FVLFNSFKDQINKAHALYKMRNLIKNGDFINDTKYWSISTDVKLEKVNKETILVLSSWEA
LP1G                 FELFNSFKDQINKAHTLYKMRNSIKNGDFINGTEYWSISTDVKLEKTNIETILVMSSWSA
2173                 FELFNSFKDQINKAHTLYKMRNSIKNGDFINGTKYWSISTDVKLEKTNIETILVMSSWSA

IAB59/47-6B/NHA15b   QASQQILVQKQKRYLLRVIAKKEDMGRGNVIISDCLNNIAKIDFTPHDCNMNHIQNSSEF
LP1G                 QSSQQILVQKQNRYLLRVIAKKEDMGSGNVTISDCLNNIAKIEFIPHDCNMN
2173                 QSSQQILVQKQNRYLLRVIAKKEDMGSGNVTISDCLNNIAKIEFIPHDCNMN

IAB59/47-6B/NHA15b   IIKTIHFSPNTEQVRIDIGQSDGVFKVESIELICVNY
LP1G
2173
```

Figure 7.4 Alignment of the different Cry48 protein variants. Residues differing from the reference sequence of the variant found in strain IAB59, 47-6B and NHA15b are highlighted yellow.

7.1.3 Discussion

Screening for *B. sphaericus* strains that carry the genes encoding Cry48 and Cry49 proteins, by dot blot and PCR, identified a number of such strains. Of particular interest was the discovery that strain 2173, a strain that shows toxicity towards both susceptible and Bin-resistant *C. quinquefasciatus* larvae (Wirth *et al.*, 2000a) but lacks any of the known toxin genes, carries the genes encoding the newly identified binary toxin. Bioassay of the Cry48Ab2/Cry49Aa4 binary toxin variant produced by 2173 would confirm whether these proteins are the toxic components of this strain. Also interesting was the fact that any strain confirmed by both dot blot and PCR to contain a gene encoding either Cry48 or Cry49, was also found to contain the gene encoding the other component of the binary toxin. This may suggest an evolutionary relationship between the mosquitocidal toxin genes where the two genes were co-acquired by *B. sphaericus* strains.

The failure to detect the genes encoding the new Cry48/Cry49 toxin in *B. sphaericus* strain IAB872, a strain that shows toxicity to Bin-resistant *Culex* larvae (Shi *et al.*, 2001), may point to the presence of unidentified toxin(s) in this strain accountable for its ability overcome resistance. SDS-PAGE analysis of sporulated cultures of this strain, performed as described previously (section 3.5), confirmed the absence of a Cry49 protein (figure 7.5). A protein band for a Cry48 crystal was also not observed on the SDS-PAGE gel, however, detection of this crystal toxin in other *B. sphaericus* stains, as well as recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 expressing this protein, required crystal purification by sucrose density gradient.

Anomalous results were obtained for some of the strains screened for the presence of *cry49* and *cry48* genes. For example, the detection of a *cry49* gene by dot blot in strain IAB881, but not by PCR, suggests that the signal obtained in the dot blot

may be due to non-specific hybridisation. Further analysis, by performing SDS-PAGE of sporulated cultures of this strain as described previously (section 3.5), revealed the absence of a Cry49 protein (figure 7.5), suggesting the dot blot signal to be non-specific. Another strain yielding a hybridisation signal for detection of a *cry48* gene, which was not confirmed by PCR, was strain 2297. The high background signal on this dot blot requires further PCR based detection of the *cry48* and *cry49* genes to be performed to determine whether these genes are present. It would, however, be surprising to find the genes encoding for both Cry48 and Cry49 binary toxin components in strain 2297, since *Culex* larvae resistant to strain 2362 also show cross resistance to 2297 (Wirth *et al.*, 2000a). SDS-PAGE analysis of a sporulated 2297 culture, performed as described previously (section 3.5), revealed that no Cry49 protein was produced (figure 7.5).

The genes encoding the Cry48/Cry49 binary toxins have been identified in a number of *B. sphaericus* strains of different serotypes. Also, although these strains contain both *cry48* and *cry49* genes, their presence seem to be independent of the Bin toxin variant produced by the strain. For example, these new toxin genes are found in strain IAB59 (Bin1, serotype H6), LP1G (Bin4, serotype H3), NHA15b (*bin*$^-$, serotype H50) and 2173 (*bin*$^-$, serotype H26a26b). While the *cry48* and *cry49* genes sequenced to date are highly conserved, being identical in strains IAB59, 47-6B, NHA15b, different variants of the genes are found in strain LP1G. Strain 2173 also has a different variant of *cry48* (*cry48Ab2*), but carries a *cry49Aa* (*cry49Aa4*) gene that is identical to the variant found in strains IAB59, 47-6B and NHA15b.

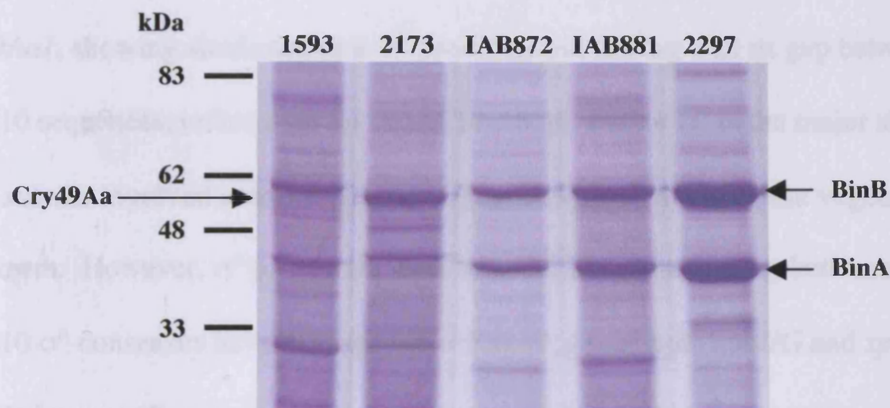


Figure 7.5 SDS-PAGE analysis of sporulated cultures of *B. sphaericus* strains. Protein bands corresponding to BinA and BinB are indicated. A Cry49Aa band can be seen in strain 2173.

7.2 Regulation of Cry48Aa1 and Cry49Aa1 synthesis

Two potential promoters, showing good similarity to the consensus recognition sequences of the sporulation specific sigma factors, σ^E and σ^G from *B. subtilis*, have been identified upstream of the gene encoding Cry49Aa1, as described in section 3.11. Also described is another potential promoter upstream of *cry49Aa1*, showing similarity to a σ^A promoter but having a 21 nt gap between the -35 and -10 sequences, rather than the usual 17-18 nt. Factor σ^A is the major sigma factor in *B. subtilis* involved in transcription of genes expressed during the vegetative stage of growth. However, σ^A promoters with unusual 21-22 nt spacing between the -35 and -10 σ^A consensus have been identified upstream of both *spoIIG* and *spoIIE*, two sporulation specific genes known to be transcribed by RNA polymerase associated with σ^A during the early stages of sporulation (Kenney *et al.*, 1989; York *et al.*, 1992). Thus, regulation of *cry49Aa1* by RNA polymerase associated with *B. sphaericus* homologues of either σ^E, σ^G or σ^A would be consistent with the presence of Cry49Aa1 with the *B. sphaericus* spore. Also, a sequence showing good identity to the σ^E consensus recognition sequence has been identified upstream of the gene encoding Cry48Aa1, as described in section 5.5, again being consistent with the presence of Cry48Aa1 with the *B. sphaericus* spore as a crystal protein. The predictive database search of the DBTBS (http://dbtbs.hgc.jp) (Makita *et al.*, 2004) also identified possible σ^F and σ^H-like promoters upstream of *cry48Aa1*. However, the low score assigned to these promoters due to the lower sequence identity shared with the σ^F and σ^H consensus recognition sequences, compared to the σ^E-like sequence, led to their exclusion as suggested promoters, with a *B. sphaericus* homologue of σ^E appearing to be the most likely sigma factor involved in *cry48Aa1* transcription.

Having identified potential promoters upstream of the two newly identified toxin genes, analysis of their expression in *B. subtilis* mutants, devoid of certain sigma factors, was explored in attempt to identify the σ factor responsible for their regulation. Such an approach has been used in the past for determination of *B. thuringiensis* subsp. *israelensis* sigma factors involved in the transcriptional regulation of the *cry4Ba* and *cry4Aa*, which encode two mosquitocidal toxins that show homology to Cry48Aa (Yoshisue *et al.*, 1993a; Yoshisue *et al.*, 1993b). The *B. subtilis* strains 1S38, 1S60 and 1S86 contain mutations in the genes encoding the sporulation specific σ^K (*sigK* or *spoIIIC*), σ^E (*sigE* or *spoIIG*) and σ^F (*sigF* or *spoIIA*) factors respectively, and were obtained from the *Bacillus* Genetic Stock Centre.

7.2.1 Cry48Aa1 and Cry49Aa1 expression in *B. subtilis* σ factor mutants

Clones pHTP49 and pHTP135, containing the *cry49Aa1* and *cry48Aa1* genes respectively, cloned into the vector pHT304 (Arantes and Lereclus, 1991) as described in sections 4.3.1 and 6.2.1, were used to transform the *B. subtilis* strains 1S38, 1S60 and 1S86. Both clones also contain the putative upstream promoters of the crystal toxin genes. Transformations were performed as described in section 2.3.5. Briefly, this involved growing the *B. subtilis* strains in LB medium supplemented with 0.5M sorbitol to a D_{600} of 0.85-0.95. Cells were washed in ice-cold electroporation medium (0.5M sorbitol, 0.5M mannitol, 10%(v/v) glycerol) before electrotransformation and plating on LB agar plates, containing erythromycin for selection of recombinants. The colonies obtained were also screened by colony PCR (section 2.2.6.1) to confirm successful transformation of the *B. subtilis* strains. Colony PCR was performed using primer pairs P49PROF/P49R and BamP135F/BamP135R, for amplification of regions of pHTP49 and pHTP135

respectively, and thermocycling conditions of 95°C for 5 min, 30 cycles of 95°C (1 min), 60°C (1 min) 72°C (1 min 30 s for pHTP49; 3 min 30 s for pHTP135), and a final extension at 72°C for 10 min, using *Taq* DNA polymerase.

A single colony of each *B. subtilis* mutant transformed with pHTP49 was selected and used to inoculate 30 ml of Embrapa sporulation medium (section 2.1.6) containing erythromycin, in a 250 ml conical flask. The cultures were grown to sporulation at 30°C, 250 rpm for 72 hours, before analysis of Cry49Aa1 expression by SDS-PAGE. For this analysis, a sample (5ml) of each culture was harvested by centrifugation, the supernatant discarded and the pellet resuspended in 100μl of SDS-PAGE sample buffer (section 2.5.1). The samples were boiled for 5 min before analysis of 10μl by SDS-PAGE (section 2.5.1). The resulting SDS-PAGE gels can be seen in figure 7.6(i) and (ii). Cultures of untransformed *B. subtilis* mutant strains were prepared as controls, allowing comparison between the test and control samples to identify the appearance of protein bands corresponding to Cry49Aa1 in the transformants. As can be seen in figure 7.6(i), expression of Cry49Aa1, indicated by a blue arrow, is observed in cultures of *B. subtilis* strains 1S38 and 1S86, corresponding to the σ^K and σ^F mutants respectively. Comparison of the test and control 1S60 cultures reveal two faint protein bands of the same approximate MW as Cry49Aa1 in the test sample that are not present in the control. However, these two protein bands are also present, at similar levels, in the 1S38 and 1S86 transformed strains, in which the level of Cry49Aa1 production is clearly visible. Their presence at similar levels in all three of the *B. subtilis* transformed strains, regardless of the level of Cry49Aa1 production, together with their absence in the untransformed control samples suggest that these proteins may somehow be upregulated due the presence of the introduced plasmids. Another possibility is that these proteins are

encoded on the pHT304 plasmid introduced into the *B. subtilis* mutants, the full sequence of which has not been elucidated. The erythromycin resistance rRNA methylase (*erm*) gene from the conjugative plasmid Tn*1545* of *Streptococcus pneumoniae* (Trieu-Cuot *et al.*, 1990), used in the construction of pHT304 (Arantes and Lereclus, 1991), encodes a protein of 28.8 kDa and, as such, does not correspond to one of these protein bands. Therefore, from Coomassie stained SDS-PAGE analysis, Cry49Aa1 does not seem to be produced in the *B. subtilis* mutant devoid of σ^E.

For the cultures of *B. subtilis* mutant strains transformed with pHTP135, 600ml of Embrapa medium (section 2.1.6) per transformant was prepared and divided into three cultures of 200ml in 2 l baffled flasks, for better aeration. Each culture was inoculated with a single colony and the cultures grown to sporulation at 30°C, 250 rpm for 72 hours. Crystal preparations of the cultures were performed by ultracentrifugation through discontinuous sucrose density gradients, as described in section 2.5.6, followed by SDS-PAGE analysis, to determine whether Cry48Aa1 production had occurred in the *B. subtilis* mutant strains. Untransformed control cultures of the *B. subtilis* strains yielded no crystal protein bands after ultracentrifugation through discontinuous sucrose density gradients. SDS-PAGE analysis of the transformed strains yielding crystal protein bands in sucrose gradients can be seen in figure 7.6(iii). Crystal protein purification followed by SDS-PAGE analysis, confirms Cry48Aa1 production in the *B. subtilis* mutants 1S38 and 1S86, lacking σ^K and σ^F respectively. However, the amount of Cry48Aa1 produced in strain 1S86 was lower than in strain 1S38. *B. subtilis* strain 1S60, corresponding to the mutant devoid of σ^E, did not yield a visible crystal protein band after

ultracentrifugation through sucrose density gradients, suggesting that Cry48Aa1 is not produced, or is produced at a much lower level than usual, in the σ^E mutant.

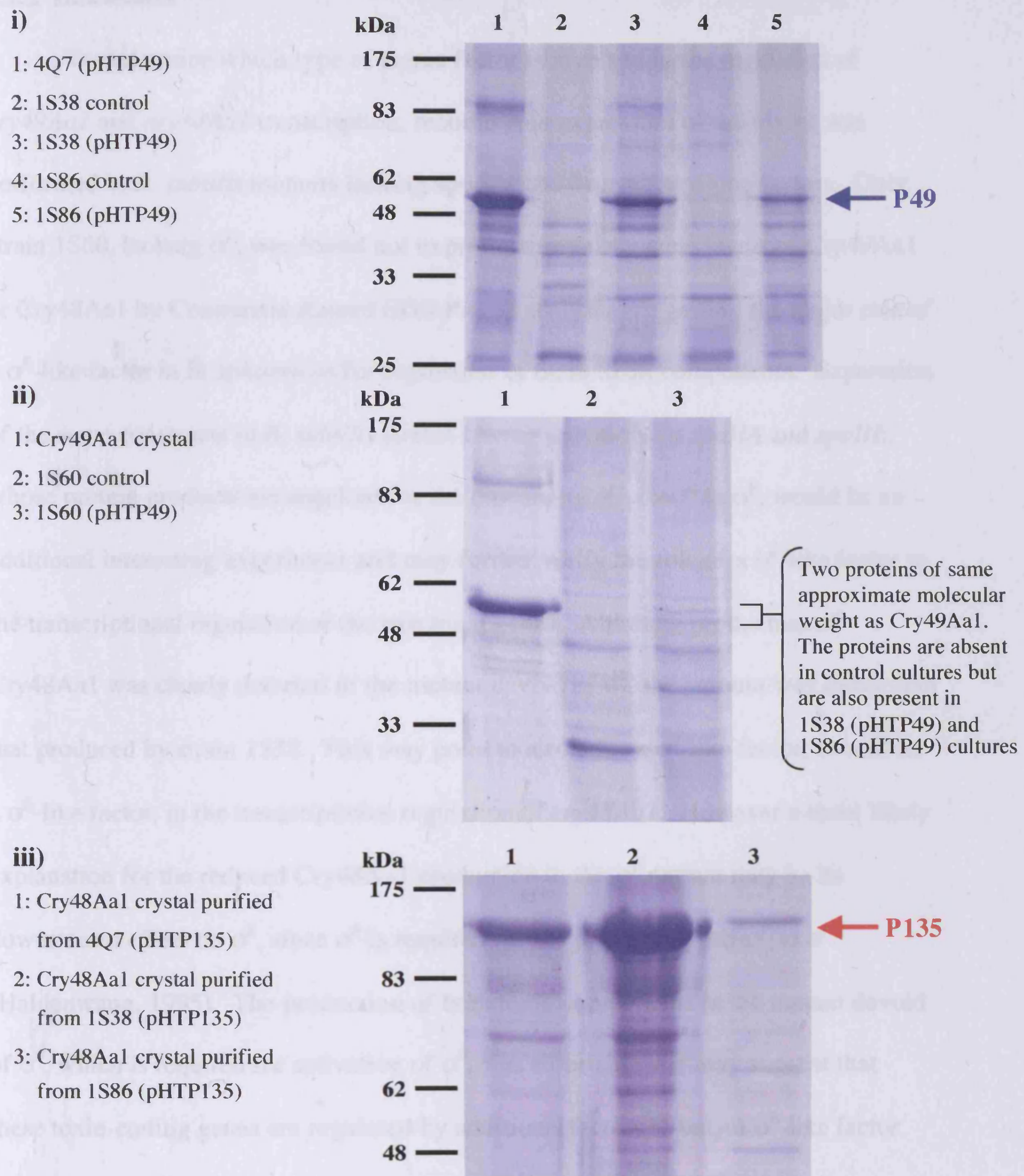


Figure 7.6 SDS-PAGE analysis of Cry49Aa1 and Cry48Aa1 expression in *B. subtilis* σ factor mutants. Untransformed mutant strains were used as controls. No control samples are shown for iii) due to the control cultures not yielding any crystal protein bands after sucrose density gradient ultracentrifugation. Cry49Aa1 and Cry48Aa1, from *B. thuringiensis* subsp. *israelensis* 4Q7 transformed with pHTP49 and pHTP135 respectively, are shown for reference.

7.2.2 Discussion

To determine which type of sigma factor is involved in the regulation of *cry49Aa1* and *cry48Aa1* transcription, recombinant expression of the toxins was performed in *B. subtilis* mutants lacking sporulation-dependent sigma factors. Only strain 1S60, lacking σ^E, was found not to produce any detectable levels of Cry49Aa1 or Cry48Aa1 by Coomassie stained SDS-PAGE analysis, suggesting the major role of a σ^E-like factor in *B. sphaericus* for regulation of these toxin components. Expression of the same constructs in *B. subtilis* strains having mutations in *spoIIA* and *spoIIE,* whose protein products are required for the processing of pro-σ^E to σ^E, would be an additional interesting experiment and may further verify the role of a σ^E-like factor in the transcriptional regulation of the two toxin genes. Although production of Cry48Aa1 was clearly detected in the mutant devoid of σ^F, the amount was lower than that produced by strain 1S38. This may point to a role for a σ^F-like factor, as well as a σ^E-like factor, in the transcriptional regulation of *cry48Aa1*. However a more likely explanation for the reduced Cry48Aa1 production in the σ^F mutant may be its downstream effect on σ^E, since σ^F is required for the processing pro-σ^E to σ^E (Haldenwang, 1995). The production of both toxin components in the mutant devoid of σ^F, which is required for activation of σ^E, was surprising and may suggest that these toxin-coding genes are regulated by additional factors to only a σ^E-like factor.

Factor σ^E has an important role in regulation of the gene encoding σ^K, which is formed by a site-specific recombination event that joins the *spoIVCB* and *spoIIIC* gene into a single cistron (Haldenwang, 1995). Production of both Cry49Aa1 and Cry48Aa1 in the *B. subtilis* mutant 1S38 (lacking σ^K) confirms that the lack of these proteins in the σ^E mutant is not due to a downstream effect of the knockout on σ^K production.

Transcription of *cry4Ba* in *B. thuringiensis* subsp. *israelensis* is regulated by σ^{35}, a homologue of σ^{E} from *B. subtilis* (Yoshisue *et al.*, 1993b). Interestingly a mutation in *spoIIID*, which encodes a mother-cell specific DNA-binding protein, has been shown to prevent transcription of *cry4Ba* in *B. subtilis* (Yoshisue *et al.*, 1993b) and may point to the presence of a homologue of this protein in *B. thuringiensis* subsp. *israelensis*. Given the homology between Cry48Aa1 and Cry4Ba, as well as their apparent regulation by RNA polymerase associated with σ^{E}-like factors, a role for a SpoIIID like protein in *B. sphaericus* for the transcriptional regulation of *cry48Aa1* may also be possible. An interesting experiment that may confirm this possibility would be the expression of *cry48Aa1* under the regulation of its own promoter in a *spoIIID* mutant of *B. subtilis*.

7.3 Cry48Aa1 and Cry49Aa1 toxin specificity

Some Cry toxins have a broader insect specificity than others, such as Cry2Aa from *B. thuringiensis* subsp. *kurstaki* which shows toxicity against two insect orders, Lepidoptera and Diptera (Yamamoto and McLaughlin, 1981; Donovan *et al.*, 1988). BLAST database searches (Altschul *et al.*, 1990), as described in previous chapters, revealed that Cry48Aa1 shows homology to Cry4Ba and Cry4Aa (both mosquito specific toxins) while Cry49Aa1 shows homology to Cry36Aa1 (coleopteran toxin) and BinA and BinB. As a result, cultures of recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 transformed with either pHTP49 or pSTABP135, and thus expressing Cry49Aa1 and Cry48Aa1 respectively, were used in selective bioassays against a range of insects from different orders.

7.3.1 Insect bioassays

Two pieces of stock filter paper, one containing spores of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) and the other *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) spores, were individually used to inoculate two 250ml conical flasks containing 30ml of Embrapa sporulation medium (section 2.1.6) supplemented with erythromycin. The cultures were incubated at 30°C, 250 rpm for 72 hours, until sporulation was complete as confirmed by light microscopy. Also, sporulated cultures of *B. sphaericus* strains IAB59, LP1G, 47-6B and NHA15b were prepared for bioassay in an identical fashion, except that no erythromycin was added to the sporulation media. All the *B. sphaericus* strains and recombinant *B. thuringiensis* subsp. *israelensis*, expressing Cry48Aa1 and Cry49Aa1, were individually bioassayed against all targets. Also, due to Cry48Aa1 and Cry49Aa1 functioning as a binary toxin against *C. quinquefasciatus* larvae, cultures of

B. thuringiensis subsp. *israelensis* 4Q7 (pSTABP135) and *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) were bioassayed in combination against all targets. All bioassays against coleopteran and lepidopteran larvae were performed at Embrapa Recursos Genéticos e Biotechnologia (Brasilia, Brazil), during a visit to Dr. Rose Monnerat's laboratory.

Bioassays of *Anthonomus grandis*, a pest which damages stored grain and is also known as the Mexican cotton boll weevil, involved the addition of 200μl of a sporulated culture of *B. sphaericus* or *B. thuringiensis* subsp. *israelensis* 4Q7 containing either pHTP49 or pSTABP135 to an artificial diet, as described in section 2.6.2. For bioassays of *B. thuringiensis* subsp. *israelensis* 4Q7 transformed with pHTP49 and pSTABP135 in combination, 200μl of each sporulated culture was added to the artificial diet.

Anticarsia gemmatalis, also known as the velvet-bean caterpillar, is an agricultural pest of soybean. Bioassays were performed by adding 150μl of the cultures of each strain to artificial diets, as described in section 2.6.3. Combination bioassays of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) and *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) involved the addition of 150μl of each culture to the artificial diet.

For *Spodoptera frugiperda*, a pest of plants such as field corn and sweet corn, bioassay of individual larvae was required to prevent cannibalism, and was performed as described in section 2.6.4. Either 30μl of a sporulated culture of a *B. sphaericus* strain, *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) or *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135), or in the case of the combination bioassay of Cry48Aa1 and Cry49Aa1, 30μl of each recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 culture, was added to artificial diets.

Bioassays of *Plutella xylostella*, also known as the diamondback moth, and a pest of plants from the family Cruciferae, such as cabbage and cauliflower, were performed as described in section 2.6.5. For this, young cabbage leaves were dipped into dilutions (1/100) of sporulated cultures of the *B. sphaericus* strains, recombinant *B. thuringiensis* subsp. *israelensis* 4Q7, or a combination of both the *B. thuringiensis* subsp. *israelensis* 4Q7 cultures expressing Cry48Aa1 and Cry49Aa1.

Bioassays against the midge *Chironomus riparius* were performed as described in section 2.6.6. Individual bioassays of each sporulated culture (100μl) or a combination of *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) and *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) (100μl of each culture) were carried out.

Bioassays against *Tipula paludosa*, of the order Diptera and also known as Cranefly or Daddy-long-legs, were performed by Dr. Jesko Oestergaard (Institute for Phytopathology, Christian-Albrechts-University Kiel, Germany). The larvae, known as leatherjackets, are pests of grass and agricultural crops.

Bioassays against *Aedes aegypti* and *Anopheles gambiae* mosquito larvae were performed as described in section 2.6.1, as previously performed against *C. quinquefasciatus* larvae. Each sporulated culture (100μl) was added to 10 second or third instar larvae in a 10ml final bioassay volume. For bioassay of Cry48Aa1 and Cry49Aa1 in combination, 100μl of a sporulated culture of each recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 carrying the genes encoding these proteins, were added to the bioassays.

Following incubation of the bioassays, as described for each insect in section 2.6, mortality was assessed by counting the number of surviving larvae. None

of the insects exposed to the cultures tested showed mortality, including the combination bioassays against Cry48Aa1 and Cry49Aa1.

7.3.2 Discussion

Bioassays of Cry48Aa1 and Cry49Aa1 against the range of coleopteran, lepidopteran and dipteran insects described in this chapter, revealed that the binary toxin, which are active against *C. quinquefasciatus* larvae, are non-toxic towards all the other insects tested. The Cry48Aa1/Cry49Aa1 binary combination must, therefore, be designated for the present as specific for only *C. quinquefasciatus* larvae. Most toxins, for example Bin and mosquitocidal Cry toxins such as Cry4Ba, Cry4Aa and Cry11Aa, are active against more than one species of mosquito, which makes the non-toxicity of the Cry48Aa1/Cry49Aa1 combination against *A. aegypti* and *A. gambiae* particularly interesting. Investigations related to this possible specificity are discussed in section 7.6. While the majority of the recently published research on *B. sphaericus* resistance reports the ability of strains such as IAB59, LP1G and 47-6B to overcome resistance against *C. quinquefasciatus* larvae (Wirth *et al.*, 2000a; Pei *et al.*, 2002; Yuan *et al.*, 2003), the same phenomenon is also observed against *C. pipiens* larvae (Silva-Filha *et al.*, 2004) (Silva-Filha personal communication). The ability of the same *B. sphaericus* strains to overcome Bin-resistance in both *C. quinquefasciatus* and *C. pipiens* larvae suggests that the Cry48Aa1/Cry49Aa1 combination is also toxic towards *C. pipiens* larvae. Bioassay of these binary toxin components against *C. pipiens* larvae would confirm whether this is the case.

7.4 Electron microscopic analysis of Cry48Aa and Cry49Aa crystal toxins

As described in previous chapters, light microscope analysis, carried out in this project, has revealed the presence of free crystals in sporulated cultures of the *B. sphaericus* strains that produce Cry48Aa and Cry49Aa. These crystals are distinct from the Bin toxin crystals produced by some of these strains, which are found associated with the spore within the exosporium. However, when the Bin toxin is produced in recombinant form in *B. thuringiensis*, it forms free crystals in the media (Nicolas *et al.*, 1993). Production of Cry48Aa1 and Cry49Aa1 in recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 also leads to the production of free crystals in the medium, as described in chapters 4 and 6.

Electron microscopy was performed on sporulated cultures of *B. thuringiensis* subsp. *israelensis* 4Q7, transformed with pHTP49 and pSTABP135, and *B. sphaericus* strain NHA15b, to observe the Cry49Aa1 and Cry48Aa1 crystals produced by the recombinant strains and to compare their features with the native crystals produced by *B. sphaericus*. Strain NHA15b was chosen as the reference as this strain does not carry the genes for the Bin crystal toxin.

Sporulated cultures of *B. sphaericus* NHA15b, *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49) and *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) were prepared by inoculating 30ml of Embrapa sporulation medium (section 2.1.6) in 250ml conical flasks. For the recombinant cultures, erythromycin was also added to the medium. Cultures were incubated at 30°C, 250 rpm for 72 hours, until sporulation was complete as confirmed by light microscopy. Electron microscopy was performed, as described in section 2.5.8, during a visit to Embrapa Recursos Genéticos e Biotechnologia (Brasilia, Brazil). Figure 7.7 shows the crystal morphologies observed.

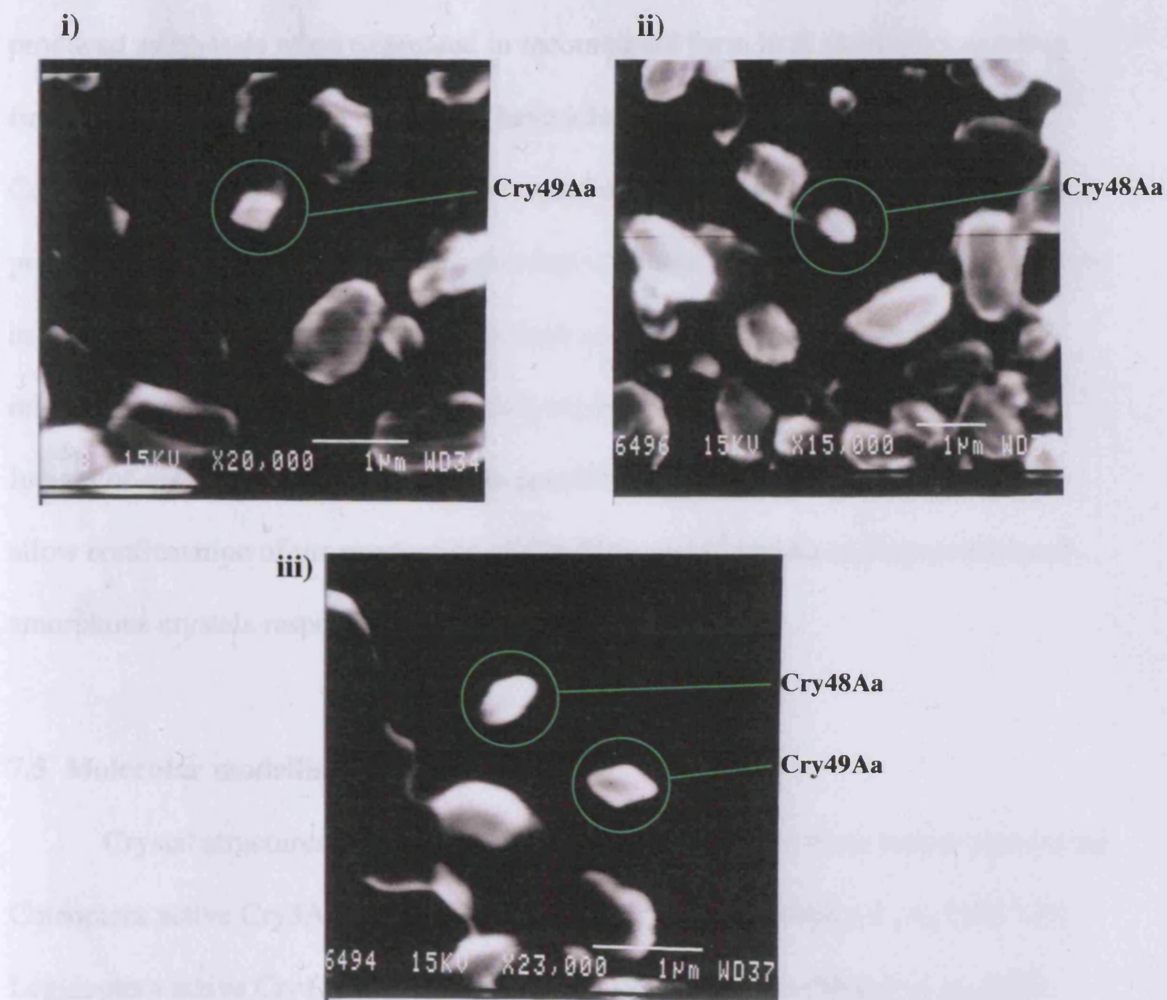


Figure 7.7 Electron micrographs of Cry49Aa and Cry48Aa crystals from recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 and *B. sphaericus* NHA15b. i) *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49), ii) *B. thuringiensis* subsp. *israelensis* 4Q7 (pSTABP135) and iii) *B. sphaericus* strain NHA15b. Crystals of Cry49Aa and Cry48Aa are indicated: the assignment of crystal type in (iii) is based on the morphologies seen in (i) and (ii). The Cry49Aa crystal appears to have a bipyramidal morphology while the Cry48Aa crystal is amorphous.

As can be seen from figure 7.7, both Cry49Aa and Cry48Aa toxins are produced as crystals when expressed in recombinant form in *B. thuringiensis* subsp. *israelensis* 4Q7. Cry49Aa appears to have a bipyramidal morphology, while Cry48Aa seems to be amorphous. The crystals also seem to be of identical size when produced in recombinant form in *B. thuringiensis* compared to their native production in *B. sphaericus*. However, to confirm such an observation, a number of electron micrographs must be taken and crystals lying at 90° to the field of view measured. Images of higher resolution would also provide clearer images of the crystals and allow confirmation of the production of Cry49Aa and Cry48Aa as bipyramidal and amorphous crystals respectively.

7.5 Molecular modelling of Cry48Aa1

Crystal structures of the three-domain Cry toxins have been determined for the Coleoptera active Cry3Aa (Li *et al.*, 1991) and Cry3Bb1 (Galitsky *et al.*, 2001), the Lepidoptera active Cry1Aa (Grochulski *et al.*, 1995), Cry2Aa (Morse *et al.*, 2001) which has dual specificity against Lepidoptera and Diptera, and Cry4Ba (Boonserm *et al.*, 2005) and Cry4Aa (Boonserm *et al.*, 2006) which are Diptera specific. BLAST database searches (Altschul *et al.*, 1990) and amino acid sequence analysis of Cry48Aa1 has revealed that it shows homology to the three-domain Cry toxins, and that it shares sequence identity with the N-terminal half associated with toxicity, characterised in the above crystal structures. With this in mind the homology-modelling server, SWISS-MODEL (Schwede *et al.*, 2003), was used to generate a first approximation 3-dimensional model of Cry48Aa1. This automated procedure involves submission of a primary amino acid sequence to the server, allowing selection of templates based on protein homology. An alignment of the submitted

sequence and the templates is generated, followed by the building of the model backbone, side chain modelling and energy minimization.

7.5.1 General features of the Cry48Aa1 model

Following submission of the Cry48Aa1 aa sequence, modelling was performed using the templates selected by the server. The selected templates, with ExPDB codes 1ji6A, 1w99A, 1dlc and 1ciy, correspond to Cry3Bb1, Cry4Ba, Cry3Aa and Cry1Aa respectively. Analysis of the general features of the Cry48Aa1 model (figure 7.8), by comparison to Cry toxin crystal structures solved to date, was performed in DeepView/Swiss-PDB Viewer (Guex and Peitsch, 1996; Guex and Peitsch, 1997), available at www.expasy.org/spdbv.

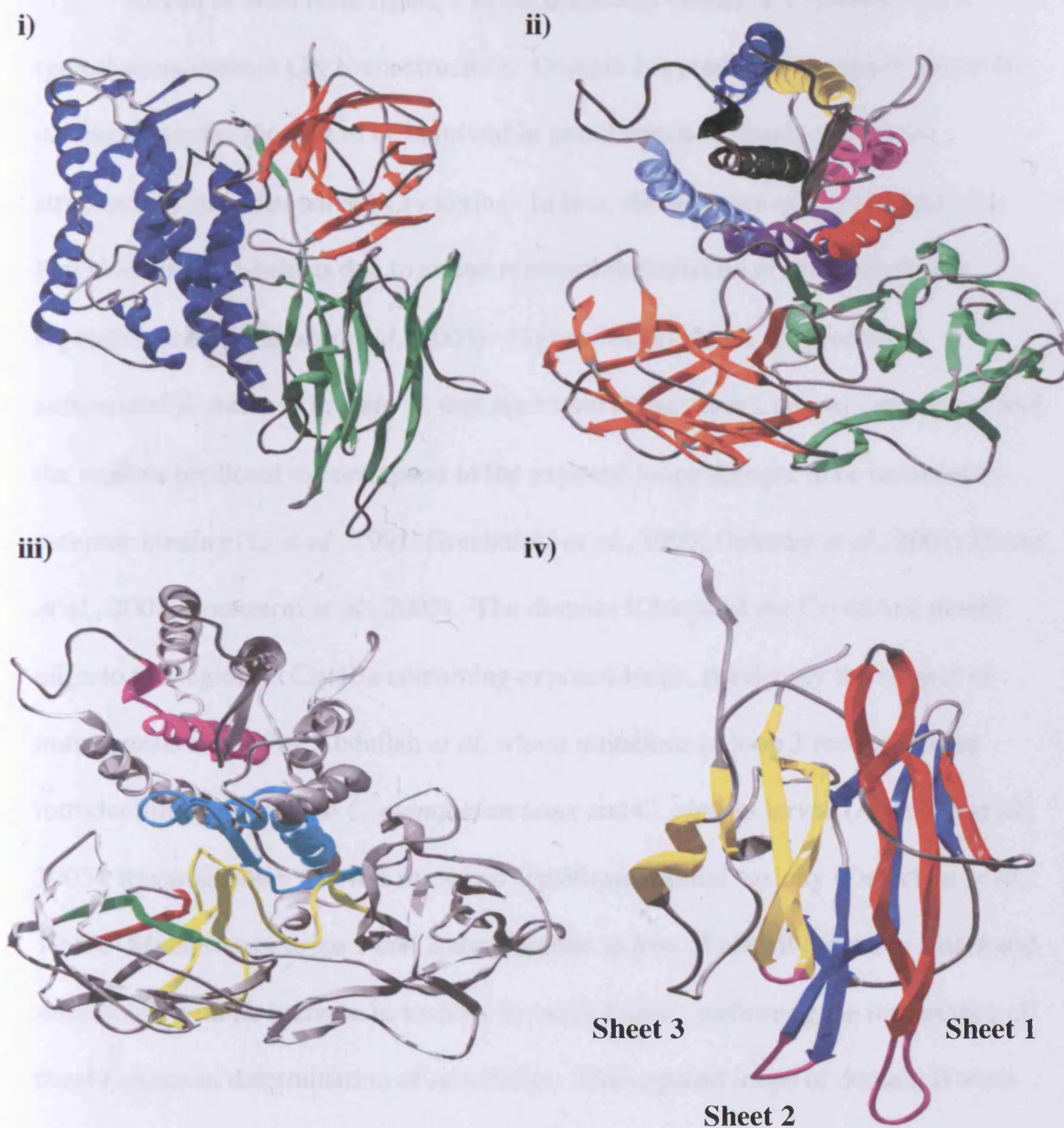


Figure 7.8 Three-dimensional model of Cry48Aa1. i) Domain I (blue), domain II (green) and domain III (orange) are shown. ii) Domains are coloured as for (i) except that the helices of the domain I helix bundle are coloured: $\alpha1$ (red), $\alpha2a$ and $\alpha2b$ (magenta), $\alpha3$ (yellow), $\alpha4$ (dark blue), $\alpha5$ (black), $\alpha6$ (light blue) and $\alpha7$ (violet). iii) model with regions corresponding to the conserved blocks found in the three-domain Cry toxins coloured: block 1 (magenta), block 2 (cyan), block 3 (yellow), block 4 (green) and block 5 (red). iv) Domain II antiparallel β-sheets; sheet 1 (red), 2 (blue) and 3 (yellow) are shown, with the exposed loops coloured in magenta.

As can be seen from figure 7.8, the homology-model of Cry48Aa1 has a typical three-domain Cry toxin structure. Domain I is predicted to contain the seven α-helical bundle, thought to be involved in pore formation, that is seen in the structures of the other solved Cry toxins. In fact, the structure of Cry4Ba lacks the two N-terminal α-helices due to amino terminal degradation of the toxin during crystallisation (Boonserm *et al.*, 2005). Figure 7.8(iv) shows the predicted antiparallel β-sheets in domain II, that are found in the other Cry toxin structures, and the regions predicted to correspond to the exposed loops thought to be involved in receptor binding (Li *et al.*, 1991; Grochulski *et al.*, 1995; Galitsky *et al.*, 2001; Morse *et al.*, 2001; Boonserm *et al.*, 2005). The domain II loops of the Cry48Aa1 model align to the region of Cry4Ba containing exposed loops, previously the subject of mutagenesis studies by Abdullah *et al.* where mutations in loop 3 resulted in the introduction of toxicity to *C. quinquefasciatus* and *C. pipiens* larvae (Abdullah *et al.*, 2003), towards which Cry4Ba shows no significant natural toxicity (Delecluse *et al.*, 1993). Mutations in loops 1 and 2 also resulted in loss of toxicity towards *Aedes* and *Anopheles*, with no increase in toxicity towards *Culex*, confirming the importance of these regions in determination of specificity. The exposed loops of domain II were proposed as a receptor binding epitope after solving the structures of Cry3Aa (Li *et al.*, 1991) and Cry1Aa (Grochulski *et al.*, 1995), the loops of which are larger than those observed in domain II of Cry4Ba (Boonserm *et al.*, 2005), or the model of Cry48Aa1. The smaller loops of domain II in Cry4Ba resulted in Boonserm *et al.* suggesting that these loops may not be the receptor binding epitope of Cry4Ba.

Figure 7.8(iii) shows the regions of the model that correspond to the five conserved blocks of amino acids found in the three-domain Cry toxins. Interestingly these regions map correctly to those described previously; block 1 encompassing

helix 5 of domain I, block 2 consisting of helix 7 of domain I and the first β-strand of domain II, block 3 including the last β-strand of domain II and the beginning of domain III and blocks 4 and 5 lying in the buried strands of domain III (Hofte and Whiteley, 1989; Schnepf *et al.*, 1998). Results from Cry48Aa1 toxin processing experiments, described at the end of the following section, again point to the model having features that are conserved in the solved structures of other Cry toxins.

While obvious care must be taken when considering 3-dimensional models, the general features of the Cry48Aa1 model are very typical of Cry toxin structures. Prior to the confirmation of Cry48Aa1 toxicity, in combination with Cry49Aa1, and in view of the conserved features of the Cry toxin structures solved at the time, the Cry48Aa1 model described here was very encouraging with regard to the predicted toxicity of Cry48Aa1.

7.6 Cry48Aa1 and Cry49Aa1 toxin processing

Cry insecticidal toxins are produced as crystals during sporulation, which are solubilised in the insect gut and undergo proteolytic processing before receptor binding and membrane pore formation occurs. The major gut proteinases involved in Cry toxin processing are trypsin-like and chymotrypsin-like enzymes, while thermolysin-like and elastase-like enzymes have also been reported (Dai and Gill, 1993; Rukmini *et al.*, 2000). Differential processing of the Cry pro-toxins by different larvae has also been shown to determine target insect toxicity (Haider *et al.*, 1986; Haider and Ellar, 1987a; Haider and Ellar, 1987b).

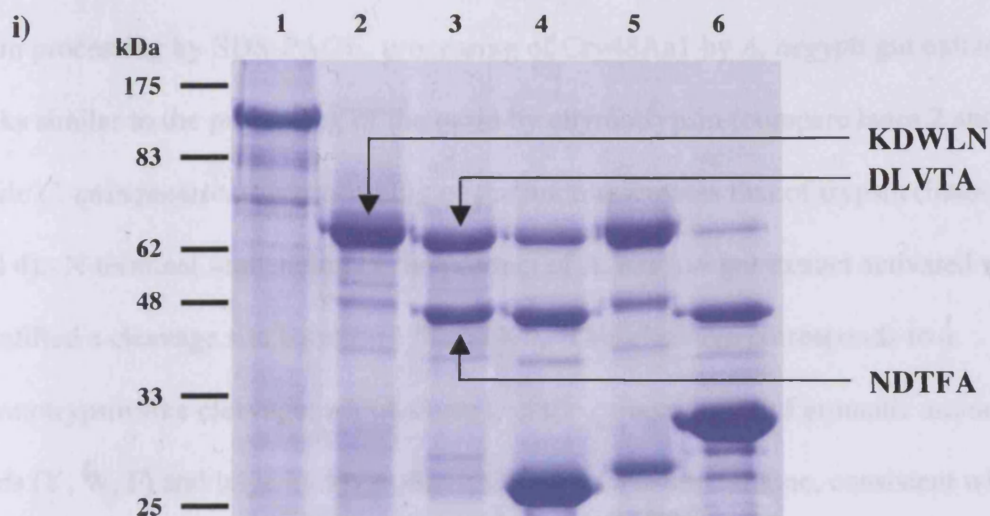
Deletion experiments on Cry4Aa (Pao-intara *et al.*, 1988) and Cry4Ba (Yoshida *et al.*, 1989) from *B. thuringiensis* subsp. *israelensis*, revealed that aa residues 39-677 (72 kDa) and 30-695 (76 kDa) respectively, are required for toxicity

when produced in recombinant form in *E. coli.* However, experiments where Cry4Aa and Cry4Ba were incubated with mosquito gut extracts, as well as trypsin, revealed that the toxins were processed to 46-48 kDa active toxins (Angsuthanasombat *et al.*, 1992). Processing is thought to involve cleavage to a 60-68 kDa protein before further degradation into two fragments of 46-48 kDa and 16-18 kDa (Angsuthanasombat *et al.*, 1991). To determine whether the processing of Cry48Aa1 was similar to that observed for Cry4Aa and Cry4Ba, proteolytic processing reactions were performed. Both Cry48Aa1 and Cry49Aa1 were incubated *in vitro* with *A. aegypti* and *C. quinquefasciatus* larval gut extracts, as well as the enzymes trypsin, chymotrypsin and proteinase K. Activation of the Cry48Aa1/Cry49Aa1 binary toxin, by *A. aegypti* and *C. quinquefasciatus* gut extracts, would determine whether the toxin components are differentially processed in the larval gut of these two mosquitoes, a factor that may be responsible for the lack of toxicity towards *A. aegypti* larvae.

7.6.1 SDS-PAGE analysis of Cry48Aa1 and Cry49Aa1 processing

In vitro toxin processing was performed as described in section 2.5.10. This involved solubilisation of toxin crystals in NaOH, before adjustment of the solution to 20mM Tris-HCl, 150mM NaCl, 2.5,mM $CaCl_2$, pH 8.4 and addition of either *A. aegypti* or *C. quinquefasciatus* gut extract according to the method of Thanabalu *et al.* (Thanabalu *et al.*, 1992). Mosquito gut extracts were prepared from fourth instar larvae as described in section 2.5.9. Analysis of Cry48Aa1 and Cry49Aa1 processed with trypsin, α-chymotrypsin or proteinase K was also performed as described in section 2.5.10. Following the incubation of solubilised toxins with either gut extracts or proteolytic enzymes, the samples were precipitated using TCA and resuspended in SDS-PAGE sample buffer prior to analysis by SDS-PAGE (section 2.5.1). Control

reactions were prepared in an identical manner to the test reactions, except that no gut extract or proteolytic enzymes were added prior to the incubation step. The resulting gels can be seen in figure 7.9. Some of the products of Cry48Aa1 and Cry49Aa1 processing with mosquito larvae gut extract were selected for N-terminal sequencing, allowing the determination of peptide bond cleavage sites during toxin processing. For this, samples were blotted onto PVDF, after SDS-PAGE had been performed in tricine buffer, and the selected protein bands were excised with a sterile scalpel and sent to Alta Bioscience (University of Birmingham, Birmingham, UK) for Edman degradation, as described in section 2.5.3. The N-terminal sequences of the products selected for Edman degradation are shown in figure 7.9.



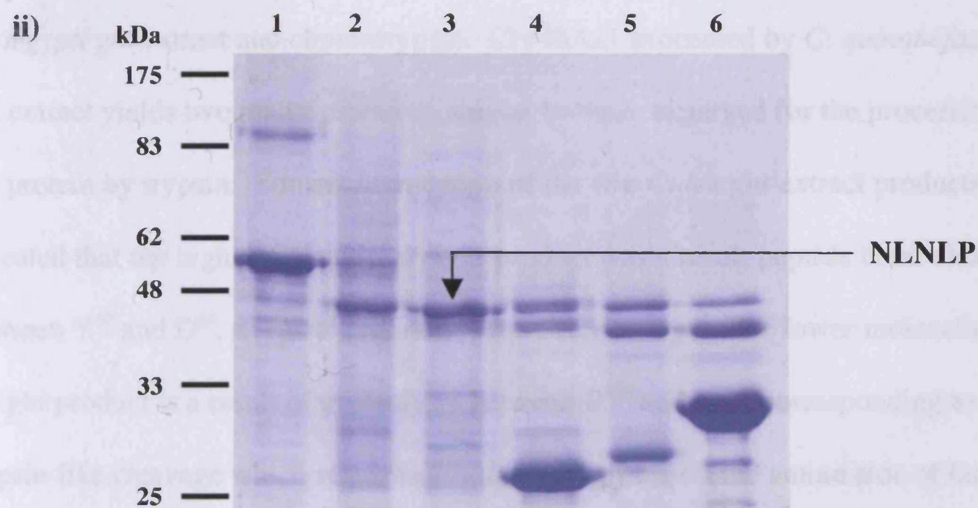


Figure 7.9 Proteolytic processing of Cry48Aa1 and Cry49Aa1. Processing of Cry48Aa1 (i) and Cry49Aa1 (ii) by *A. aegypti* gut extracts (lane 2), *C. quinquefasciatus* gut extracts (lane 3), trypsin (lane 4), chymotrypsin (lane 5) and proteinase K (lane 6). The control, unprocessed Cry48Aa1 and Cry49Aa1 samples are also shown (lane 1). The N-terminal aa residues of the products selected for sequencing are shown.

As can be seen in figure 7.9(i), from comparison of the protein products of toxin processing by SDS-PAGE, processing of Cry48Aa1 by *A. aegypti* gut extract looks similar to the processing of the toxin by chymotrypsin (compare lanes 2 and 5) while *C. quinquefasciatus* processing of the toxin resembles that of trypsin (lanes 3 and 4). N-terminal sequencing of the product of *A. aegypti* gut extract activated toxin identified a cleavage site between Y^{35} and K^{36}. This cleavage corresponds to a chymotrypsin-like cleavage, which occurs on the carboxyl side of aromatic amino acids (Y, W, F) and large hydrophobic residues such as methionine, consistent with the similar protein bands observed by SDS-PAGE analysis of Cry48Aa1 processed by *A. aegypti* gut extract and chymotrypsin. Cry48Aa1 processed by *C. quinquefasciatus* gut extract yields two major products, similar to those observed for the processing of the protein by trypsin. Edman degradation of the two *Culex* gut extract products revealed that the higher molecular weight product was a result peptide bond cleavage between Y^{52} and D^{53}, a typical chymotrypsin-like cleavage. The lower molecular weight product is a result of proteolysis between R^{238} and N^{239}, corresponding to a trypsin-like cleavage which requires a lysine or arginine on the amino side of the scissile bond.

Cry49Aa1 processing (figure 7.9(ii)) appears to show that the toxin component is processed in a similar manner in both *A. aegypti* gut and *C. quinquefasciatus* gut. Similar processing of Cry49Aa1 is also observed when incubated with trypsin, chymotrypsin and proteinase K. N-terminal sequencing of the *C. quinquefasciatus* gut extract activated protein identified the site of processing to be between F^{48} and N^{49}, a chymotrypsin-like cleavage.

7.6.2 Bioassay of *C. quinquefasciatus* gut activated toxin against *A. aegypti*

The processing of Cry48Aa1 by *A. aegypti* and *C. quinquefasciatus* gut extracts was found to be different, with the *Culex* extract producing smaller products than the *Aedes* extracts. To determine whether differential processing of Cry48Aa1 and Cry49Aa1 in *A. aegypti* and *C. quinquefasciatus* larvae is responsible for the toxicity observed against the latter but not the former, bioassays were performed against *A. aegypti* larvae using *C. quinquefasciatus* gut extract activated Cry48Aa1/Cry49Aa1 toxin. Both Cry48Aa1 and Cry49Aa1 (20μg of each), prepared as described in section 2.5.10 and above in section 7.6.1, were incubated with *C. quinquefasciatus* gut extract. Following incubation, both samples were combined in a selective bioassay (section 2.6.1) against ten *A. aegypti* larvae in a 10ml final volume. Control bioassays were also prepared containing no toxin, and a Cry49Aa1/Cry48Aa1 crystal protein mixture added directly to the bioassay. A bioassay using *C. quinquefasciatus* larvae, exposed to the Cry48Aa1/Cry49Aa1 toxin pre-incubated with *C. quinquefasciatus* gut extract was also prepared, as above, to confirm that the processed toxin retained toxicity when fed to susceptible larvae. No toxicity was observed against *A. aegypti* larvae, using either the activated Cry48Aa1/Cry49Aa1 toxin or the crystal protein. Bioassay of the activated toxin against *C. quinquefasciatus* larvae resulted in 100% mortality while no mortality was observed in the control bioassays containing no toxins.

7.6.3 Discussion

Incubation of the toxin components, Cry48Aa1 and Cry49Aa1, with mosquito gut extracts has shown that differential processing of Cry48Aa1 occurs in *A. aegypti* and *C. quinquefasciatus*. However, bioassay of *C. quinquefasciatus* gut extract

activated toxin against *A. aegypti* larvae has revealed that this differential processing does not account for the non-toxicity of the Cry48Aa1/Cry49Aa1 toxin against *A. aegypti*. This may, therefore, suggest that receptor binding may be the factor responsible for the specificity of this toxin towards *C. quinquefasciatus*, with possibly no receptor binding occurring in *A. aegypti* larval gut.

The processing of Cry48Aa1 by *A. aegypti* gut extract results in a chymotrypsin-like cleavage of the toxin between Y^{35} and K^{36}, while processing by *C. quinquefasciatus* results in both chymotrypsin- and trypsin-like processing; between Y^{52} and D^{53}, and R^{238} and N^{239} respectively. According to the homology model of Cry48Aa1, described in section 7.5, the trypsin-like cleavage after R^{238}, that occurs in this toxin after exposure to *C. quinquefasciatus* gut extract, occurs within domain I. This N-terminal domain, comprising an α-helical bundle, is known to be important for toxicity and is predicted to be involved in lysis of midgut epithelial cells by formation of pores. Therefore, a cleavage within this domain might be expected to be detrimental to toxicity. A proteolytic cleavage which occurs at an inter-helical loop in domain I of in Cry4Ba, between helices 5 and 6, has been suggested to assist in allowing the toxin to undergo a conformational change, facilitating the insertion of domain I into the membrane (Angsuthanasombat *et al.*, 1993; Boonserm *et al.*, 2005). Also, a similar inter-helical loop cleavage occurs between helices 3 and 4 of Cry2Aa (Nicholls *et al.*, 1989) and Cry3Aa (Carroll *et al.*, 1989; Li *et al.*, 1991), and helices 5 and 6 of Cry4Aa (Angsuthanasombat *et al.*, 1993). Indeed, based on the model of Cry48Aa1, the cleavage after R^{238} also occurs in a predicted inter-helical loop between α5 and α6, as shown in figure 7.10 and figure 7.11. However, removal of this inter-helical cleavage site in Cry4Ba and Cry4Aa is not detrimental to toxicity, suggesting that processing at this site is not essential for the conformational change

required for pore formation (Angsuthanasombat *et al.*, 1993; Boonserm *et al.*, 2004; Boonserm *et al.*, 2006). The two products of *C. quinquefasciatus* gut extract processing of Cry48Aa1 have estimated molecular weights of 65-70 kDa and 45-48 kDa. However, for exact determination of these molecular weights, mass spectroscopy or C-terminal sequencing of the protein products would be required. These approaches were not pursued due to the expense involved with C-terminal sequencing and the requirement of pure material for mass spectroscopy.

The processing of Cry49Aa1 looks similar, if not identical, when processed with *A. aegypti* and *C. quinquefasciatus* gut extracts. The cleavage site in Cry49Aa1 is between amino acids F^{48} and N^{49}, which leaves a mature toxin of 47.8 kDa if no C-terminal processing occurs. BinA (42 kDa) and BinB (51 kDa) are processed by mosquito gut larvae to 39 kDa and 43 kDa proteins (Broadwell and Baumann, 1987; Broadwell *et al.*, 1990a). Deletion experiments also showed that 32 and 53 aa can be removed from the N- and C-termini of BinB (Clark and Baumann, 1990) and that 10 and 17 aa can be removed from the N- and C-termini of BinA (Broadwell *et al.*, 1990b) without loss of toxicity. Further experiments performed by Oei *et al.* showed that between 34-39 aa and 52-54 aa can be deleted from the N- and C-termini of BinB and that no more than 6 aa and at least 17 aa can be removed from the C- and N-termini of BinA without loss of the essential cores required for toxicity (Oei *et al.*, 1990). The homology shared between Cry49Aa1, BinA and BinB, together with the N-terminal processing of Cry49Aa1 between F^{48} and N^{49}, would be consistent with the *C. quinquefasciatus* gut extract activated toxin component having the minimum requirements for toxicity, as shown in the alignment of the N-terminal sequences of these toxins in figure 7.12. The ability to delete regions from the C-termini of BinA and BinB without loss of toxicity may point to a similar situation for Cry49Aa1.

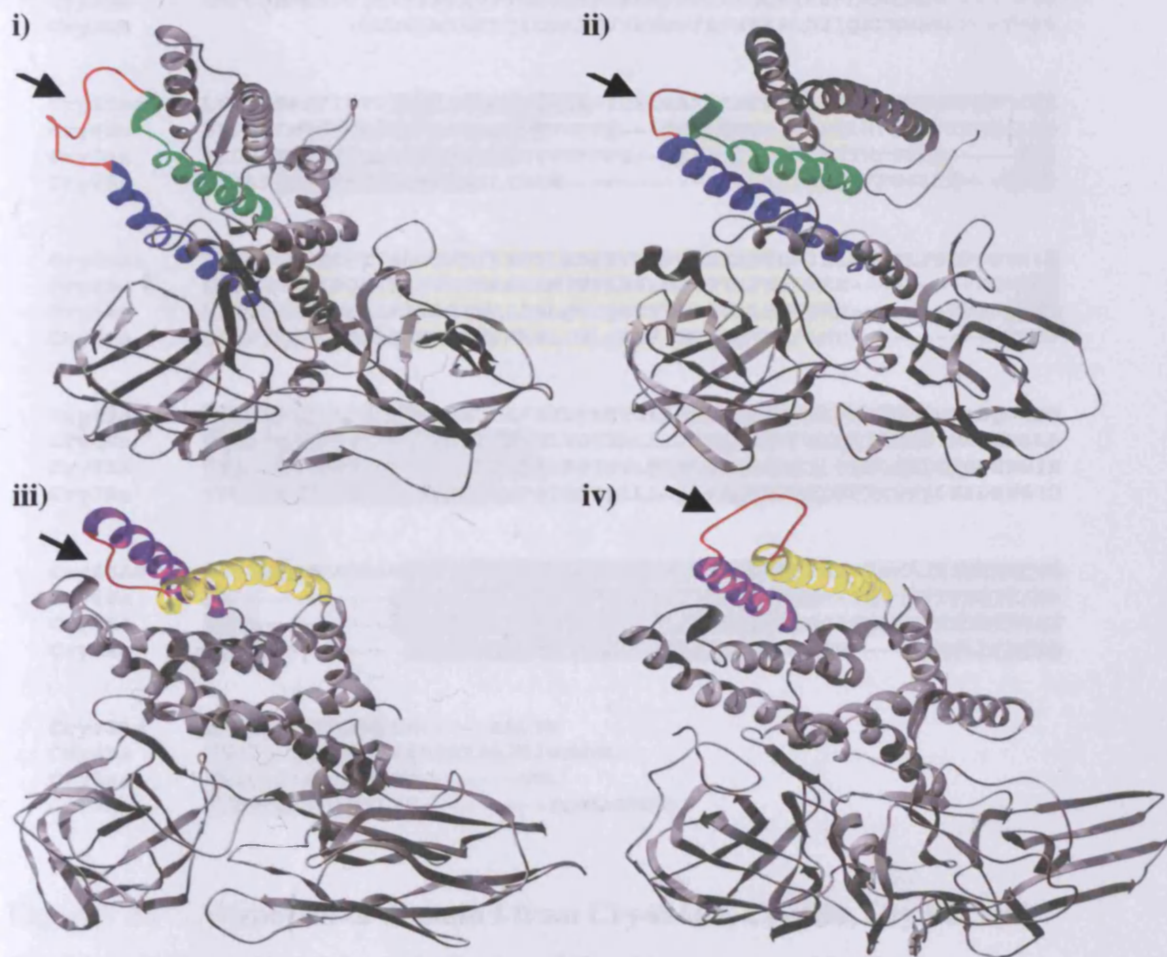


Figure 7.10 Cry toxin inter-helical loops that are cleaved during processing in the larval gut. Cry48Aa1 model (i), Cry4Ba (ii), Cry3Aa (iii) and Cry2Aa (iv) are shown. The inter-helical loops that are cleaved upon processing are indicated by arrows and shown in red. The helices immediately N-terminal and C-terminal to the processed loops are shown in green (α5) and blue (α6) for the Cry48Aa1 model and Cry4Ba, and yellow (α3) and purple (α4) for Cry3Aa and Cry2Aa.

```
Cry48Aa    MDINNNNEKEIINSHLLP-------YSLLKKYPIKSLQSTNYKDWLNLCQDFNKDIESYD
Cry4Ba                       MNS-------GYPLANDLQGSMKNTNYKDWLAMCENN----QQYG
Cry3Aa     MNPNNRSEHDTIKTTENNEVPTNHVQYPLAETPNPTLEDLNYKEFLRMTADN-----NTE
Cry2Aa                  MNNVLNSGRTTICDAYNVVAHDPFSFEHKSLDTIQKEWMEWKR--TDHS

Cry48Aa    LVTAVSSGTIVVGTMLSAIYAPALIAGPIGIIGAIIISFGTLLPLLWNESENNPKTTWIE
Cry4Ba     VNPAAINSSSVSTALKVAGAILKFVNPPA---GTVLTVLSAVLPILWPTNTPTPERVWND
Cry3Aa     ALDSSTTKDVIQKGISVVGDLLGVVGFPFG--GALVSFYTNFLNTIWPSEDP-----WKA
Cry2Aa     LYVAPVVGTVSSFLLKKVGSLIGKR------------ILSELWGIIFPSGSTN---LMQD

Cry48Aa    FIRMGEQLVDKTISQTVFNILESYLKDLKVNLVDYEKAKQDWIELKKQQLPGSPPSTKLR
Cry4Ba     FMTNTGNLIDQTVTAYVRTDANAKMTVVKDYLDQYTTKFNTWKRE---------PNNQSY
Cry3Aa     FMEQVEALMDQKIADYAKNKALAELQGLQNNVEDYVSALSSWQKN-------PVSSRNPH
Cry2Aa     ILRETEQFLNQRLNTDTLARVNAELIGLQANIREFNQQVDNFLNP-----------TQNP

Cry48Aa    NAADIAHQRLDSLHNKFAELNKFKVEPYETILLPVYAQAANLHLNLLQQGAMFADQWIED
Cry4Ba     RTAVITQFNLTSAKLRETAVYFSNLVGYELLLLPIYAQVANFNLLLIRDGLINAQEWSLA
Cry3Aa     SQGRIRELFSQAESHFRNSMPSFAISGYEVLFLTTYAQAANTHLFLLKDAQIYGEEWGYE
Cry2Aa     VPLSITSSVNTMQQLFLNRLPQFQIQGYQLLLLPLFAQAANMHLSFIRDVILNADEWGIS

Cry48Aa    KYSSRNDTFAGNSNDYQNLLKSRTITYINHIENTYQNGLNYLWNQPEMTWDIYNEYRTKM
Cry4Ba     RSAG---------DQLYNTMVQYTKEYIAHSITWYNKGLDVLRNKSNGQWITFNDYKREM
Cry3Aa     KEDI---------AEFYKRQLKLTQEYTDHCVKWYNVGLDKLRGSSYESWVNFNRYRREM
Cry2Aa     AATLR---------TYRDYLRNYTRDYSNYCINTYQTAFRGLNTR----LHDMLEFRTYM

Cry48Aa    TITALDLMALFPFYN-----KELYD
Cry4Ba     TIQVLDILALFASYDPRRYPADKIDNTKL
Cry3Aa     TLTVLDLIALFPLYD-------VRL
Cry2Aa     FLNVFEYVSIWSLFK---------YQSLMVSSG
```

Figure 7.11 Alignment of domain I from Cry48Aa1, Cry4Ba, Cry3Aa and Cry2Aa, showing the seven α-helices and the sites processed in the insect gut. Regions of each protein corresponding to helices α1 (purple), α2a and α2b (orange), α3 (yellow), α4 (green), α5 (magenta), α6 (blue) and α7 (grey) are shown. Regions corresponding to domain I helices for Cry4Ba, Cry3Aa and Cry2Aa were determined from their crystal structures (PDB codes 1w99, 1dlc and 1i5p respectively). Helical regions for Cry48Aa1 were predicted based on the model prepared using SWISS-MODEL (Schwede *et al.*, 2003). Residues shown highlighted in red indicate the sites of processing at the inter-helical loops of domain I, while the residues shown in blue font represent the regions processed from the N-terminus of the pro-toxins.

```
P49        MENQIKEEFNKNNHGIPSDCSCIKEGDDYNSLTEINAKEFSYCSPNMFNLNLPEQSTRFQ
BinB2            MCDSKDNSGVSEKCGKKFTNYPLN------------TTPTSLNYNLPEISKKFY
BinA2                                                  MRNLDFIDS---FI

P49        TIASIHSNNCSFEILNNDPGYIYGDSVDGECRIAVAHRELGNGLERTGDDRFLFIFYALD
BinB2      NLKNKYSRNG-YGLSKTEFPSSIENCPSNEYSIMYDNKDPR-----------FLIRFLLD
BinA2      PTEGKYIRVMDFYNSEYPFCIHAPSAPNGDIMTEICSRENNQ----------YFIFFPTD
```

Figure 7.12 Alignment of the N-termini of Cry49Aa1, BinA and BinB. The amino acid residues shown in red correspond to the region processed from Cry49Aa1 by *C. quinquefasciatus* gut extract. Residues shown in blue can be deleted from BinA and BinB without loss of toxicity, and may correspond to the regions processed from the N-terminus by mosquito larvae (Broadwell *et al.*, 1990b; Clark and Baumann, 1990).

7.7 Chapter summary

This chapter has considered the characterisation of the newly identified toxin in this study, comprising Cry48Aa1 and Cry49Aa1. Screening of *B. sphaericus* strains, by dot blot and PCR, for the presence of *cry48* and *cry49* genes identified a number of strains that carry related sequences. Of particular interest was the discovery of *cry48Ab2* and *cry49Aa4* genes in strain 2173, as although the strain has long been known to be toxic towards *C. quinquefasciatus* larvae (Wirth *et al.*, 2000a), the toxic components of the strain remained unknown. The sequencing of the *cry49Aa* genes from NHA15b and 2173, and partial sequencing of *cry48Ab2* from 2173 was also performed, the nucleotide sequences of which can be seen in the appendix.

Analysis of Cry48Aa1 and Cry49Aa1 production in *B. subtilis* mutant strains devoid of certain sporulation specific σ factors, identified σ^E to be important in the regulation of both toxin genes, with mutants devoid of σ^E showing no, or at least greatly reduced, expression of the toxin components. This suggests that a σ^E-like homologue in *B. sphaericus* might be central in the regulation of these toxin genes. Further studies to determine whether transcripts from *cry48Aa1* and *cry49Aa1* can be detected in the *B. subtilis* mutant lacking σ^E may provide further evidence supporting the importance of a σ^E-like factor in the regulation of the *cry48Aa1/cry49Aa1* toxin genes.

Bioassays of Cry48Aa1 and Cry49Aa1 against a range of lepidopteran, coleopteran and dipteran larvae showed *C. quinquefasciatus* to be the only target insect identified for these toxins. However, the fact that Bin-resistant *C. pipiens* larvae are sensitive to a number of the *B. sphaericus* strains that carry the *cry48Aa* and *cry49Aa* genes (Silva-Filha *et al.*, 2004), may suggest that the Cry48Aa/Cry49Aa

toxin is also active towards this mosquito. Therefore, further bioassays with Cry48Aa1 and Cry49Aa1 could determine whether this toxin pair is active against other species within the *Culex* genus. Bioassays against *A. aegypti* larvae, using *C. quinquefasciatus* gut extract activated Cry48Aa1/Cry49Aa1, also established that differential processing of the toxin between these two mosquito species is not responsible for the specificity towards *C. quinquefasciatus.* This may point to toxin-receptor binding as the determinant of target specificity between these strains. Analysis of toxin activation using mosquito gut extract also revealed a processing site within domain I of Cry48Aa1, predicted to occur between helices α5 and α6, according to a homology-model of Cry48Aa1. Similar processing is observed in other three-domain Cry toxins, as described in section 7.6.3, and has been proposed to facilitate a conformational change that is required for domain I insertion into larval gut cells (Angsuthanasombat *et al.*, 1993; Boonserm *et al.*, 2005). However, it has been determined that processing at this inter-helical loop is not essential for toxicity.

Electron micrographs of the Cry48Aa1 and Cry49Aa1 crystals suggest the latter to have a bipyramidal morphology, while the former is more amorphous. Homology-modelling of the Cry48Aa1 protein sequence, based on the solved structures of Cry3Bb1 (Galitsky *et al.*, 2001), Cry4Ba (Boonserm *et al.*, 2005), Cry3Aa (Li *et al.*, 1991) and Cry1Aa (Grochulski *et al.*, 1995) reveals the predicted structure of the toxin, modelled by SWISS-MODEL, to contain the general features found in the solved crystal structures of other Cry toxins.

CHAPTER 8

General discussion

8.1 General discussion

Some strains of *B. sphaericus*, for example IAB59, LP1G, 47-6B and 2173, have long been known to overcome Bin-resistance in *Culex* mosquito larvae (Wirth *et al.*, 2000a; Pei *et al.*, 2002; Shi *et al.*, 2003; Yuan *et al.*, 2003). Additionally, a *bin*[-] *B. sphaericus* strain called NHA15b, the discovery of which has not been published, also has the ability to overcome Bin-resistance in *Culex* larvae (Nielsen-LeRoux personal communication). While a spore protein with an apparent molecular weight of 49 kDa, present in IAB59, LP1G and 47-6B, had been put forward as a candidate for the toxin responsible for the ability of these strains to overcome resistance (Yuan *et al.*, 2003; Silva-Filha *et al.*, 2004), previous attempts to clone the gene encoding this protein were unsuccessful (Yuan personal communication; Nielsen-LeRoux personal communication). Therefore, prior to this study, little information had been gained regarding the source of this toxicity.

Much of the course of this study involved employing different approaches towards cloning the gene encoding the toxic factor(s) from these strains, particularly strain IAB59. Care was taken to avoid concentrating efforts solely on attempting to clone the gene encoding the putative toxin of ~49 kDa, by also employing a genomic screening approach similar to those used for the cloning of the Mtx toxins from strain SSII-1 (Thanabalu *et al.*, 1991; Liu *et al.*, 1996; Thanabalu and Porter, 1996). However, screening of a cosmid library of strain IAB59 proved unsuccessful in identification of a clone yielding toxicity to *C. quinquefasciatus* larvae. It is possible that this was owing either to a non-representative library or low levels, or lack, of expression of the toxin coding genes in *E. coli.*

The gene encoding the putative ~49 kDa toxin from strain IAB59 was successfully cloned after N-terminal sequencing of the candidate toxin (P49) and use

of degenerate oligonucleotide probes, designed against the determined protein sequence, in Southern blot and colony hybridisation. Partial gene sequence was determined by sequencing of the cloned 763 bp *Eco*RI and 2,010 bp *Mbo*I DNA fragments. Complete gene sequence was determined from a 15,649 bp *Hin*dIII fragment. Recombinant expression of the putative toxin in an acrystalliferous *B. thuringiensis* subsp. *israelensis* resulted in higher yields than were obtained from expression studies in *E. coli* using the pET expression system or when produced as a fusion protein with GST. However, the putative toxin showed no toxicity to *C. quinquefasciatus* larvae following bioassay of sporulated cultures of recombinant *B. thuringiensis* subsp. *israelensis* 4Q7 (pHTP49).

Light microscope analysis of sporulated cultures of strains IAB59, LP1G, 47-6B and NHA15b revealed the presence of free crystals in the media, which were distinct from the Bin toxin crystals that remain associated with the spore within the exosporium. Purification of crystals from the *bin*⁻ strain, NHA15b, followed by SDS-PAGE analysis identified another candidate toxin of approximately 135 kDa. The gene encoding this protein in strain IAB59 was later found also to be contained within the 15,649 bp *Hin*dIII DNA fragment. Recombinant expression of this putative toxin under the regulation of its own promoter in *B. thuringiensis* subsp. *israelensis* 4Q7 was low, therefore, higher expression levels were achieved using the dual *cyt1Aa* promoters and STAB-SD sequence (Agaisse and Lereclus, 1996) in the vector pSTAB (Park *et al.*, 1998; Park *et al.*, 1999). Bioassay of sporulated cultures of this recombinant strain against *C. quinquefasciatus* larvae again revealed that this protein was non-toxic. However, a combination of P49 and P135 was found to be toxic to both Bin-susceptible and Bin-resistant mosquito larvae. Following submission of the protein sequences of P49 and P135 to the Cry toxin nomenclature

committee (Crickmore *et al.*, 1998), the components of this novel *B. sphaericus* binary toxin were assigned the official names Cry49Aa1 and Cry48Aa1 respectively. The requirement of both Cry49Aa1 and Cry48Aa1 for toxicity, as well as the poor production of the latter, may also have been a contributor to the failure to isolate a toxic clone by screening of cosmid libraries of strain IAB59.

As described in chapter 3, the 53.3 kDa Cry49Aa1 protein shows homology to a number of other insecticidal toxins such as BinA and BinB, Cry36Aa1, and members of the Cry35 family of binary toxins from *B. thuringiensis*. The conserved blocks of amino acids shared between BinA and BinB (Baumann *et al.*, 1988) were also well conserved in Cry49Aa1. The homology shared between Cry49Aa1 and components of other binary toxins may have been an early indicator of its role as a binary toxin component. Indeed, although Cry36Aa1 has weak coleopteran toxicity, the homology it shares with the other binary toxins, BinA, BinB and the Cry35 toxins, has led to the suggestion that it too may be part of a yet uncharacterised binary toxin (de Maagd *et al.*, 2003). Light microscope analysis of a sporulated culture of an acrystalliferous strain of *B. thuringiensis* subsp. *israelensis*, expressing Cry49Aa1, confirmed its production as a free crystal released into the culture medium. Electron microscopic analysis revealed a bipyramidal morphology for the Cry49Aa1 crystal.

The Cry48Aa1 toxin, of 135.6 kDa, shows homology to the three-domain Cry toxin from *B. thuringiensis* subsp. *israelensis*. Cry48Aa1 represents the first report of a Cry toxin from *B. sphaericus* as well as the first report of a three-domain Cry toxin that functions as a binary toxin (see below for speculation as to the possible roles of each component of the toxin). The five conserved blocks of amino acids shared among the three-domain Cry toxins are well conserved in Cry48Aa1 as are the three conserved blocks found in the C-terminal half, which is removed upon gut processing.

As for Cry49Aa1, recombinant expression of Cry48Aa1 in *B. thuringiensis* subsp. *israelensis* confirmed its production as free crystals released into the culture medium. Electron microscopic analysis showed the crystal to be amorphous.

Screening of *B. sphaericus* strains for the presence of *cry48* and *cry49* genes, by dot blot and PCR, identified a number of strains yielding positive hybridisation signals and PCR products for both genes: IAB59, NHA15b, 47-6B, LP1G, 2173, LP14-8, LP18 and LB29. Of particular interest was the discovery of genes encoding Cry48Ab2 and Cry49Aa4 proteins in strain 2173, a strain that has long been known to show toxicity towards Bin-resistant *Culex* larvae but produces none of the previously identified toxins (Wirth *et al.*, 2000a). The ability of strain IAB872 to overcome resistance (Shi *et al.*, 2001), and failure to detect *cry48* and *cry49* genes in this strain, may point to the presence of additional, unidentified toxin(s). However, a conflicting study (Nielsen-LeRoux *et al.*, 2001) reports cross-resistance towards strain IAB872. Additional work is required to determine whether this strain produces unidentified toxins. Future studies to identify new toxins may further contribute to knowledge regarding *B. sphaericus* toxins and aid with strategies to avoid resistance development. Both the Cry48 and Cry49 toxins, the genes of which have been sequenced from strains IAB59, LP1G, 47-6B, NHA15b and 2173, are well conserved. The Cry48Aa and Cry49Aa variants found in strains IAB59, 47-6B and NHA15b are identical, while different variants of both binary toxin components are found in strain LP1G (Cry48Ab1 and Cry49Ab1). Strain 2173 produces a Cry49 protein (Cry49Aa4) identical to that found in strains IAB59, 47-6B and NHA15b but produces a different variant of Cry48 (Cry48Ab2) compared to all the *cry48* gene variants sequenced to date. The discovery of *cry48* and *cry49* genes in strains from different serotypes and containing different Bin toxin variants, as well as *bin*$^{-}$ strains, suggests that

B. sphaericus strains have acquired these genes independently of serotype and the chromosomally encoded Bin toxin variant that they produce. Also, all strains were identified as containing both components of the Cry48/Cry49 toxin pair and may suggest that the genes encoding these proteins were co-acquired. The different *cry48* and *cry49* gene variants in LP1G, and *cry48* gene in 2173, may also suggest that the variants found in these strains have evolved divergently from the those found in the other strains.

The discovery of *cry48/cry49* genes in *B. sphaericus* strains, independent of serotype and the Bin toxin variant that they produce, may suggest that the genes have been acquired on mobile genetic elements such as transposons or plasmids. A number of transposase like sequences were discovered within the 15,649 bp *Hin*dIII fragment cloned from strain IAB59, and might support the hypothesis of such an event. The homology shared between the toxin proteins discovered in this study and the Cry toxins of *B. thuringiensis*, which are encoded on large plasmids, may suggest a similar location for the genes encoding the Cry48/Cry49 binary toxin. Preliminary data, obtained after performing megaplasmid detection in *B. sphaericus* strains, as described in section 2.2.13, point to the presence of plasmids in many of the strains carrying the genes for Cry48 and Cry49 (data not shown). While these data are inconclusive, further studies such as megaplasmid curing from these *B. sphaericus* strains, followed by PCR, dot blot or Southern blot based detection of *cry48* and *cry49* genes in the resulting strains, could determine whether these genes are found on the genome or on plasmids.

Cloning of the genes encoding Cry48Aa1 and Cry49Aa1, followed by sequence analysis, identified a number of potential sporulation specific promoters. Expression of these toxins under the regulation of their own promoters in *B. subtilis*

mutants devoid of RNA polymerase factors σ^K, σ^F and σ^E, identified the latter as a key regulator of both *cry48Aa1* and *cry49Aa1* expression. This is consistent with the regulation of other Cry toxin genes, such as *cry4Ba* and *cry4Aa* from *B. thuringiensis* subsp. *israelensis*, by RNA polymerase associated with the sporulation-specific factor σ^{35}, a homologue of σ^E from *B. subtilis* (Yoshisue *et al.*, 1993a; Yoshisue *et al.*, 1993b). Analysis to determine whether any *cry48Aa1* or *cry49Aa1* transcripts can be detected in the *B. subtilis* σ^E mutant would further support the hypothesis that these genes are regulated by a homologue of σ^E in *B. sphaericus*. Also, expression of Cry48Aa1 in a *B. subtilis* mutant of *spoIIID*, which encodes a DNA-binding protein important for transcription of *cry4Ba* in *B. subtilis* (Yoshisue *et al.*, 1993b), would determine whether a homologue of SpoIIID may play a role in expression of this toxin in *B. sphaericus*.

Bioassays of Cry48Aa1 and Cry49Aa1 were performed against a range of coleopteran, lepidopteran and dipteran insect larvae, with toxicity only being observed against *C. quinquefasciatus* larvae. The sensitivity of Bin-resistant *C. pipiens* larvae towards *B. sphaericus* strains that produce the Cry48Aa/Cry49Aa combination (Silva-Filha *et al.*, 2004) also suggest that this novel binary toxin is active against the larvae of this species. The apparent specificity of Cry48Aa/Cry49Aa to *Culex* mosquito larvae suggests that these proteins would be suitable for use in the field, with no detrimental effect to non-target insects. However, to confirm non-toxicity to insects outside the range tested in this study, further bioassays are required. The use of the Cry48/Cry49 binary toxin discovered in this study, in combination with the Bin toxin, should aid with the prevention of resistance development against *B. sphaericus* among *Culex* populations in the filed. Of greater advantage would be the use of recombinant *B. sphaericus* or *B. thuringiensis* subsp. *israelensis* carrying the genes encoding

Cry48Aa, Cry49Aa and Bin as well as the Cry and Cyt toxins encoded by the pBtoxis plasmid. The synergism between this arsenal of toxins and their specificity towards different larval gut receptors would make development of resistance against such a recombinant strain very difficult, requiring multiple and simultaneous mutations within the host. An additional advantage of such a recombinant strain, over the use of only the *B. sphaericus* toxins, would be the toxicity towards *Aedes* mosquito larvae, provided by the *B. thuringiensis* subsp. *israelensis* toxins. Of course, the deliberate release of recombinant bacteria into the environment must be considered carefully as, once released, the bacteria cannot be withdrawn.

While care must be taken when considering structural homology-models, the Cry48Aa1 model, described in chapter 7, exhibits the general features of the Cry toxin structures solved to date and, as such, may point to a similar mode of action. However, although there are numerous reports of synergy among Cry toxins, this is the first report of a three-domain Cry toxin that functions as a binary toxin, which individually shows no toxicity to the target insects bioassayed in this study. The closer phylogenetic relationship between Cry49Aa1 and BinB, which is the Bin component involved in receptor binding (Oei *et al.*, 1992; Charles *et al.*, 1997), compared to BinA may point to Cry49Aa1 having evolved a function of receptor binding. It is possible that Cry49Aa1, having bound to a receptor in the larval gut, may function as a receptor for Cry48Aa1 allowing its domain I helices to insert into the membrane. However, since Cry toxins also have the ability to recognise specific larval midgut receptors, it is possible that only Cry48Aa1, or both Cry48Aa1 and Cry49Aa1, are involved in receptor interaction. To determine the component(s) of the binary toxin that interacts specifically with the *C. quinquefasciatus* larval gut, experiments to determine binding patterns of fluorescently labelled Cry48Aa1 and

Cry49Aa1 could be performed. Additionally, binding assays of labelled Cry48Aa1 and Cry49Aa1 with midgut brush border membrane fractions (BBMF) could identify whether one, or both components of the toxin are involved in receptor interaction and whether one component can enhance the binding of the other protein. Such studies are underway in a collaborating laboratory. Further studies based on these findings could also lead to the identification of the Cry48Aa1/Cry49Aa1 binary toxin receptor. Additionally, since no toxicity is observed towards *A. aegypti* larvae, it would be interesting to determine whether Cry48Aa1 and/or Cry49Aa1 are capable of binding to BBMF from *A. aegypti*. Whatever the case, it is apparent that toxicity requires both proteins, indicating an interaction between Cry49Aa1 and Cry48Aa1. An interesting experiment, which is being pursued in collaboration with another group, is to determine the epitopes of interaction between the two binary components.

Experiments involving incubations of Cry48Aa1 and Cry49Aa1 with mosquito larvae gut extracts from *C. quinquefasciatus* and *A. aegypti* revealed that Cry48Aa1 was differentially processed by the proteinases of these two mosquito species. Processing by *A. aegypti* gut extract appeared to entail a chymotrypsin like activation between Y^{35} and K^{36}, while the *C. quinquefasciatus* gut extract activation between Y^{52} and D^{53} and R^{238} and N^{239} involved both a chymotrypsin- and trypsin-like cleavage. However, feeding *A. aegypti* larvae a Cry48Aa1 and Cry49Aa1 combination activated by *C. quinquefasciatus* gut extract confirmed that this differential processing was not responsible for the lack of toxicity towards the former mosquito species. This may suggest that a toxin-receptor interaction determines target specificity, and this is currently under investigation. According to the model of Cry48Aa1, cleavage between R^{238} and N^{239} within domain I is predicted to occur within an inter-helical loop, similar to that observed for other Cry toxins; Cry4Ba, Cry4Aa, Cry2Aa and

Cry3Aa (Carroll *et al.*, 1989; Nicholls *et al.*, 1989; Li *et al.*, 1991; Angsuthanasombat *et al.*, 1993; Boonserm *et al.*, 2005). It was proposed that this cleavage might aid the conformational changes required for insertion of domain I of these toxins into the gut membrane. However, removal of the inter-helical cleavage site in Cry4Ba and Cry4Aa is not detrimental to toxicity, indicating that this cleavage is not essential for pore formation (Angsuthanasombat *et al.*, 1993; Boonserm *et al.*, 2004; Boonserm *et al.*, 2006). To confirm whether this inter-helical loop processing is required for toxicity of the Cry48Aa1/Cry49Aa1 binary combination, recombinant expression of a Cry48Aa1 mutant with a R238Q substitution could be performed followed by bioassays against *C. quinquefasciatus* larvae.

Preliminary bioassays using purified crystals of Cry48Aa1 and Cry49Aa1 against RLCq/C3-41 larvae, performed by Mr Yang Yankun at the Wuhan Institute of Virology (Wuhan, China) as described in section 2.6.1, indicate an approximate LC_{50} of 150 ng/ml at 48 hours for a 1:1 ratio of Cry48Aa1:Cry49Aa1 (data not shown). This compares to an LC_{50} of 1.8 ng/ml at 48 hours for an equimolar mixture of BinA and BinB against SLCq larvae (Nicolas *et al.*, 1993). Therefore, assuming that the development of resistance to Bin in RLCq does not adversely affect Cry48Aa1/Cry49Aa1 toxicity, these preliminary data indicate that an equimolar mixture of the Cry48Aa1/Cry49Aa1 is approximately 80 fold less toxic than the Bin toxin.

Bioassays performed by Yuan *et al.*, using powders of sporulated cultures of strains IAB59, LP1G and 47-6B, against RLCq/C3-41 larvae (Yuan *et al.*, 2003) provide additional data regarding the toxicity of Cry48/Cry49. The LC_{50} of strain 2362 against SLCq (0.0051mg/l) and the LC_{50} of strains IAB59, LP1G and 47-6B against RLCq/C3-41 (1.584, 1.066 and 2.303mg/l respectively) (Yuan *et al.*, 2003)

suggest that the contribution of Bin to toxicity against SLCq may be >200 fold higher than that of Cry48/Cry49. This does not take into account any possible synergy between Cry48 and/or Cry49 with Bin, as is observed for Cry4Aa and Cry4Ba with Bin (Wirth *et al.*, 2004). To relate this contribution directly to the actual toxicity of the Cry48/Cry49 toxin, we would have to assume an equimolar production of Cry48/Cry49 compared to Bin. While this is not unreasonable with regard to Cry49, the low levels of Cry48 production in *B. sphaericus* results in this assumption being flawed. The Cry48 proteins are not visible by SDS-PAGE analysis of sporulated cultures of strains IAB59, LP1G, 47-6B and NHA15b and purification of crystals from these strains are required for detection of Cry48 protein by SDS-PAGE. Also, for recombinant production of Cry48Aa1 in *B. thuringiensis* subsp. *israelensis*, much better yields were achieved when the toxin was expressed under the regulation of the dual *cyt1Aa* promoters, and STAB-SD sequence, encoded on the pSTAB vector (Park *et al.*, 1998; Park *et al.*, 1999) than were observed for expression of the toxin under the regulation of its own promoter. The non-equimolar production of Cry48/Cry49 in *B. sphaericus* may explain the difference in potency of this toxin in sporulated powders of *B. sphaericus* (>200 fold less toxic than Bin) compared to purified crystal proteins (approximately 80 fold less toxic than Bin). Interestingly, the data of Yuan *et al.* also suggest that the LP1G variant of Cry49/Cry48 is the most toxic. Therefore, while preliminary bioassays of Cry48Aa1/Cry49Aa1 are currently being performed using equimolar amounts, this does not reflect the true ratio of production of these proteins in *B. sphaericus*. In the near future bioassays will be performed, in collaboration with Prof. Yuan Zhiming (Wuhan Institue of Virology, Wuhan, China) and Dr. Rose Monnerat (Embrapa Recursos Genéticos e Biotechnologia, Brasilia,

Brazil), where different ratios of Cry48Aa1:Cry49Aa1 will be tested to determine the ratio yielding maximum toxicity against RLCq/C3-41 and SLCq larave.

A number of experiments, in collaboration with other research groups, are currently under way to provide a better understanding of the how the Cry48 and Cry49 proteins interact and bring about toxicity. Some of these experiments have already briefly been mentioned in this chapter such as: i) determining epitopes of interaction between Cry48Aa1 and Cry49Aa1 ii) investigating which of the novel binary toxin components are responsible for receptor binding and whether toxin-receptor recognition is responsible for target specificity and iii) determining whether Cry48 and Cry49 proteins are plasmid or chromosomally encoded. Additional experiments include determining the pore forming abilities of proteolytically activated Cry48Aa1 and Cry49Aa1 using liposomes as model lipid membranes. Experiments to determine whether Cry48Aa1 and Cry49Aa1 are able to synergise with Cry4Aa and Cry4Ba are also being performed and may provide data supporting the use of recombinant strains of *B. sphaericus*, to prevent the emergence of resistance in *Culex* larvae as well as to improve activity against *A. aegypti*.

While the aims of this study, to identify the toxic factor(s) in strains such as IAB59, LP1G and 47-6B that allow them to overcome Bin-resistance in *Culex* mosquito larvae, were successfully achieved, such discoveries always raise additional questions. For example, what is the mode of action of this new Cry48/Cry49 binary toxin from *B. sphaericus*? Also, the failure to detect *cry48* and *cry49* genes in strain IAB872 suggests that this strain produces further unidentified toxin(s) allowing it to overcome Bin-resistance in *Culex* larvae, and thus provides the opportunity to discover new toxin(s) that may contribute to the fight against resistance towards *B. sphaericus* in *Culex* populations. The work presented in this thesis has contributed

to previous knowledge regarding the strains of *B. sphaericus* able to overcome Bin-resistance in *Culex* mosquito populations, in particular by identifying, cloning and characterising the toxic factors from these strains responsible for this toxicity. These discoveries should aid in the design of strategies to evade development of resistance to *B. sphaericus* in the future.

Acknowledgements

I would like to thank the Cardiff School of Biosciences at Cardiff University for the provision of research facilities and to the BBSRC for financial support during the course of this work.

Over the past three years I have had the pleasure of collaborating with a number of people within the field, most of whom I was lucky enough to meet at the 9th SICONBIOL in Recife, Brazil. I would like to thank Dr. Christina Nielsen-LeRoux, Prof. Yuan Zhiming and Dr. Jesko Oestergaard for their contribution to the work presented in this thesis. A specific mention must be given to Dr. Rose Monnerat for advice with all aspects of the project and for the fantastic welcome I received in Brasilia, muito obrigado.

Thanks to everyone in the CB/JK lab: Dave, Tim, Jon, Yankun and Dimitri. Tim, the perfect timing of your "lazy eyed freak" impression will long be remembered, as will Dave's rendition of "Wild Horses". We had some good times, both in the lab and when sharing those not so few pints of Guinness in the pub.

Gwerthfawrogaf gefnogaeth Gwennol a fy rhieni dros y blynyddoedd diwethaf. Diolch am fy annog I 'mlaen yn ystod yr adegau anodd ac am rhoi lan 'da "Gareth y Grump". Diolch i Gwennol am rhoi lan da'r penwythnosau byr ac i Dad a Mam am yr "arian cwrw"!

Finally, thanks to Dr. Colin Berry for his supervision, inspiration and guidance over the past few years. I thank him for his support with everything concerning this research project and for giving me the experience of working in Brazil. Also, if it were not for Colin I would not have been introduced to musical masterpieces like "Beware Of The Flowers Cause I'm Sure They're Going To Get You Yeah." Thanks Colin.

References

Abdullah, M. A., Alzate, O., Mohammad, M., McNall, R. J., Adang, M. J. and Dean, D. H. (2003) Introduction of *Culex* toxicity into *Bacillus thuringiensis* Cry4Ba by protein engineering. Appl. Environ. Microbiol. 69: 5343-5353

Abrami, L., Reig, N. and van der Goot, F. G. (2005) Anthrax toxin: the long and winding road that leads to the kill. TRENDS Microbiol. 13: 72-78

Adams, L. F., Brown, K. L. and Whiteley, H. R. (1991) Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. J. Bacteriol. 173: 3846-3854

Agaisse, H., Gominet, M., Okstad, O. A., Kolsto, A. B. and Lereclus, D. (1999) PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. Mol. Microbiol. 32: 1043-1053

Agaisse, H. and Lereclus, D. (1994a) Expression in *Bacillus subtilis* of the *Bacillus thuringiensis cryIIIA* toxin gene is not dependent on a sporulation-specific sigma factor and is increased in a *spo0A* mutant. J. Bacteriol. 176: 4734-4741

Agaisse, H. and Lereclus, D. (1994b) Structural and functional analysis of the promoter region involved in full expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis*. Mol. Microbiol. 13: 97-107

Agaisse, H. and Lereclus, D. (1995) How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? J. Bacteriol. 177: 6027-6032

Agaisse, H. and Lereclus, D. (1996) STAB-SD: a Shine-Dalgarno sequence in the 5' untranslated region is a determinant of mRNA stability. Mol. Microbiol. 20: 633-643

Ahern, K. G. (1995) Boomerang DNA amplification. United States Patent No. 5,470,724

Ahmed, H. K., Mitchell, W. J. and Priest, F. G. (1995) Regulation of mosquitocidal toxin synthesis in *Bacillus sphaericus*. Appl. Microbiol. Biotechnol. 43: 310-314

Almog, O., Gonzalez, A., Klein, D., Greenblatt, H. M., Braun, S. and Shoham, G. (2003) The 0.93A crystal structure of sphericase: a calcium-loaded serine protease from *Bacillus sphaericus*. J. Mol. Biol. 332: 1071-1082

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. 215: 403-410

Angsuthanasombat, C., Crickmore, N. and Ellar, D. J. (1991) Cytotoxicity of a cloned *Bacillus thuringiensis* subsp. *israelensis* CryIVB toxin to an *Aedes aegypti* cell line. FEMS Microbiol. Lett. 83: 273-276

Angsuthanasombat, C., Crickmore, N. and Ellar, D. J. (1992) Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism *in vivo*. FEMS Microbiol. Lett. 94: 63-68

Angsuthanasombat, C., Crickmore, N. and Ellar, D. J. (1993) Effects on toxicity of eliminating a cleavage site in a predicted interhelical loop in *Bacillus thuringiensis* CryIVB delta-endotoxin. FEMS Microbiol. Lett. 111: 255-261

Arantes, O. and Lereclus, D. (1991) Construction of cloning vectors for *Bacillus thuringiensis*. Gene 108: 115-119

Aronson, A. I. and Shai, Y. (2001) Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. FEMS Microbiol. Lett. 195: 1-8

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1987) Current protocols in molecular biology, Volume 1. Greene publishing and Wiley interscience

Baker, W., van den Broek, A., Camon, E., Hingamp, P., Sterk, P., Stoesser, G. and Tuli, M. A. (2000) The EMBL nucleotide sequence database. Nucleic Acids Res. 28: 19-23

Baum, J. A., Chu, C. R., Rupar, M., Brown, G. R., Donovan, W. P., Huesing, J. E., Ilagan, O., Malvar, T. M., Pleau, M., Walters, M. and Vaughn, T. (2004) Binary toxins from *Bacillus thuringiensis* active against the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Appl. Environ. Microbiol. 70: 4889-4898

Baumann, L. and Baumann, P. (1989) Expression in *Bacillus subtilis* of the 51- and 42-kilodalton mosquitocidal toxin genes of *Bacillus sphaericus.* Appl. Environ. Microbiol. 55: 252-253

Baumann, L. and Baumann, P. (1991) Effects of components of the *Bacillus sphaericus* toxin on mosquito larvae and mosquito-derived tissue culture-grown cells. Curr. Microbiol. 23: 51-57

Baumann, L., Broadwell, A. H. and Baumann, P. (1988) Sequence analysis of the mosquitocidal toxin genes encoding 51.4- and 41.9-kilodalton proteins from *Bacillus sphaericus* 2362 and 2297. J. Bacteriol. 170: 2045-2050

Baumann, P., Baumann, L., Bowditch, R. D. and Broadwell, A. H. (1987) Cloning of the gene for the larvicidal toxin of *Bacillus sphaericus* 2362: evidence for a family of related sequences. J. Bacteriol. 169: 4061-4067

Baumann, P., Clark, M. A., Baumann, L. and Broadwell, A. H. (1991) *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxins. Microbiol. Rev. 55: 425-436

Baumann, P., Unterman, B. M., Baumann, L., Broadwell, A. H., Abbene, S. J. and Bowditch, R. D. (1985) Purification of the larvicidal toxin of *Bacillus sphaericus* and evidence for high-molecular-weight precursors. J. Bacteriol. 163: 738-747

Berry, C. and Hindley, J. (1987) *Bacillus sphaericus* strain 2362: identification and nucleotide sequence of the 41.9 kDa toxin gene. Nucleic Acids Res. 15: 5891

Berry, C., Hindley, J., Ehrhardt, A. F., Grounds, T., de Souza, I. and Davidson, E. W. (1993) Genetic determinants of host ranges of *Bacillus sphaericus* mosquito larvicidal toxins. J. Bacteriol. 175: 510-518

Berry, C., Jackson-Yap, J., Oei, C. and Hindley, J. (1989) Nucleotide sequence of two toxin genes from *Bacillus sphaericus* IAB59: sequence comparisons between five highly toxinogenic strains. Nucleic Acids Res. 17: 7516

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A. F., Murphy, L., Quail, M. A., Holden, M. T., Harris, D., Zaritsky, A. and Parkhill, J. (2002) Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 68: 5082-5095

Bietlot, H. P., Vishnubhatla, I., Carey, P. R., Pozsgay, M. and Kaplan, H. (1990) Characterization of the cysteine residues and disulphide linkages in the protein crystal of *Bacillus thuringiensis*. Biochem. J. 267: 309-315

Birnboim, H. C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523

Bocs, S., Cruveiller, S., Vallenet, D., Nuel, G. and Medigue, C. (2003) AMIGene: Annotation of Microbial Genes. Nucleic Acids Res. 31: 3723-3726

Bone, E. J. and Ellar, D. J. (1989) Transformation of *Bacillus thuringiensis* by electroporation. FEMS Microbiol. Lett. 58: 171-178

Boonserm, P., Angsuthanasombat, C. and Lescar, J. (2004) Crystallization and preliminary crystallographic study of the functional form of the *Bacillus thuringiensis* mosquito-larvicidal Cry4Aa mutant toxin. Acta Crystallogr. D Biol. Crystallogr. 60: 1315-1318

Boonserm, P., Davis, P., Ellar, D. J. and Li, J. (2005) Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. J. Mol. Biol. 348: 363-382

Boonserm, P., Mo, M., Angsuthanasombat, C. and Lescar, J. (2006) Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-Angstrom resolution. J. Bacteriol. 188: 3391-3401

Bowditch, R. D., Baumann, P. and Yousten, A. A. (1989) Cloning and sequencing of the gene encoding a 125-kilodalton surface-layer protein from *Bacillus sphaericus* 2362 and of a related cryptic gene. J. Bacteriol. 171: 4178-4188

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254

Broadwell, A. H., Baumann, L. and Baumann, P. (1990a) The 42- and 51-kilodalton mosquitocidal proteins of *Bacillus sphaericus* 2362: construction of recombinants with enhanced expression and *in vivo* studies of processing and toxicity. J. Bacteriol. 172: 2217-2223

Broadwell, A. H., Baumann, L. and Baumann, P. (1990b) Larvicidal properties of the 42 and 51 kilodalton *Bacillus sphaericus* proteins expressed in different bacterial hosts: Evidence for a binary toxin. Curr. Microbiol. 21: 361-366

Broadwell, A. H. and Baumann, P. (1986) Sporulation-associated activation of *Bacillus sphaericus* larvicide. Appl. Environ. Microbiol. 52: 758-764

Broadwell, A. H. and Baumann, P. (1987) Proteolysis in the gut of mosquito larvae results in further activation of the *Bacillus sphaericus* toxin. Appl. Environ. Microbiol. 53: 1333-1337

Broadwell, A. H., Clark, M. A., Baumann, L. and Baumann, P. (1990c) Construction by site-directed mutagenesis of a 39-kilodalton mosquitocidal protein similar to the larva-processed toxin of *Bacillus sphaericus* 2362. J. Bacteriol. 172: 4032-4036

Brown, M. D., Watson, T. M., Carter, J., Purdie, D. M. and Kay, B. H. (2004) Toxicity of VectoLex (*Bacillus sphaericus*) products to selected Australian mosquito and nontarget species. J. Econ. Entomol. 97: 51-58

Burton, S. L., Ellar, D. J., Li, J. and Derbyshire, D. J. (1999) N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. J. Mol. Biol. 287: 1011-1022

Butko, P. (2003) Cytolytic toxin Cyt1A and its mechanism of membrane damage: data and hypotheses. Appl. Environ. Microbiol. 69: 2415-2422

Carpusca, I., Schirmer, J. and Aktories, K. (2004) Two-site autoinhibition of the ADP-ribosylating mosquitocidal toxin (MTX) from *Bacillus sphaericus* by its 70-kDa ricin-like binding domain. Biochemistry 43: 12009-12019

Carroll, J., Li, J. and Ellar, D. J. (1989) Proteolytic processing of a coleopteran-specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis.* Biochem. J. 261: 99-105

Chan, S. W., Thanabalu, T., Wee, B. Y. and Porter, A. G. (1996) Unusual amino acid determinants of host range in the Mtx2 family of mosquitocidal toxins. J. Biol. Chem. 271: 14183-14187

Charles, J. F., Hamon, S. and Baumann, P. (1993) Inclusion bodies and crystals of *Bacillus sphaericus* mosquitocidal proteins expressed in various bacterial hosts. Res. Microbiol. 144: 411-416

Charles, J. F., Kalfon, A., Bourgouin, C. and de Barjac, H. (1988) *Bacillus sphaericus* asporogenous mutants: morphology, protein pattern and larvicidal activity. Ann. Inst. Pasteur Microbiol. 139: 243-259

Charles, J. F. and Nielsen-LeRoux, C. (2000) Mosquitocidal bacterial toxins: diversity, mode of action and resistance phenomena. Mem. Inst. Oswaldo Cruz 95: 201-206

Charles, J. F., Silva-Filha, M. H., Nielsen-LeRoux, C., Humphreys, M. J. and Berry, C. (1997) Binding of the 51- and 42-kDa individual components from the *Bacillus sphaericus* crystal toxin to mosquito larval midgut membranes from *Culex* and *Anopheles* sp. (Diptera: Culicidae). FEMS Microbiol. Lett. 156: 153-159

Clark, M. A. and Baumann, P. (1990) Deletion analysis of the 51-kilodalton protein of the *Bacillus sphaericus* 2362 binary mosquitocidal toxin: construction of derivatives equivalent to the larva-processed toxin. J. Bacteriol. 172: 6759-6763

Cokmus, C., Davidson, E. W. and Cooper, K. (1997) Electrophysiological effects of *Bacillus sphaericus* binary toxin on cultured mosquito cells. J. Invertebr. Pathol. 69: 197-204

Crickmore, N., Bone, E. J., Williams, J. A. and Ellar, D. J. (1995) Contribution of the individual components of the delta-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol. Lett. 131: 249-254

Crickmore, N. and Ellar, D. J. (1992) Involvement of a possible chaperonin in the efficient expression of a cloned CryIIA delta-endotoxin gene in *Bacillus thuringiensis*. Mol. Microbiol. 6: 1533-1537

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. and Dean, D. H. (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 807-813

Dai, S. M. and Gill, S. S. (1993) *In vitro* and *in vivo* proteolysis of the *Bacillus thuringiensis* subsp. *israelensis* CryIVD protein by *Culex quinquefasciatus* larval midgut proteases. Insect Biochem. Mol. Biol. 23: 273-283

Darboux, I., Nielsen-LeRoux, C., Charles, J. F. and Pauron, D. (2001) The receptor of *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae) midgut: molecular cloning and expression. Insect Biochem. Mol. Biol. 31: 981-990

Darboux, I., Pauchet, Y., Castella, C., Silva-Filha, M. H., Nielsen-LeRoux, C., Charles, J. F. and Pauron, D. (2002) Loss of the membrane anchor of the target receptor is a mechanism of bioinsecticide resistance. Proc. Natl. Acad. Sci. U. S. A. 99: 5830-5835

Davidson, E. W. (1983) Alkaline extraction of toxin from spores of the mosquito pathogen, *Bacillus sphaericus* strain 1593. Can. J. Microbiol. 29: 271-275

Davidson, E. W. (1988) Binding of the *Bacillus sphaericus* (Eubacteriales: Bacillaceae) toxin to midgut cells of mosquito (Diptera: Culicidae) larvae: relationship to host range. J. Med. Entomol. 25: 151-157

Davidson, E. W., Bieber, A. L., Meyer, M. and Shellabarger, C. (1987) Enzymatic activation of the *Bacillus sphaericus* mosquito larvicidal toxin. J. Invertebr. Pathol. 50: 40-44

Davidson, E. W., Oei, C., Meyer, M., Bieber, A. L., Hindley, J. and Berry, C. (1990) Interaction of the *Bacillus sphaericus* mosquito larvicidal proteins. Can. J. Microbiol. 36: 870-878

de Barjac, H., Larget-Thiery, I., Dumanoir, V. C. and Ripouteau, H. (1985) Serological classification of *Bacillus sphaericus* strains in relation with toxicity to mosquito larvae. Appl. Microbiol. Biotechnol. 21: 85-90

de Barjac, H., Thiery, I., Cosmao-Dumanoir, V., Frachon, E., Laurent, P., Charles, J. F., Hamon, S. and Ofori, J. (1988) Another *Bacillus sphaericus* serotype harbouring strains very toxic to mosquito larvae: serotype H6. Ann. Inst. Pasteur Microbiol. 139: 363-377

de Maagd, R. A., Bravo, A., Berry, C., Crickmore, N. and Schnepf, H. E. (2003) Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu. Rev. Genet. 37: 409-433

Delecluse, A., Juarez-Perez, V. and Berry, C. (2000) Vector active toxins: structure and diversity. Entomopathogenic bacteria: from laboratory to field application 101-125

Delecluse, A., Poncet, S., Klier, A. and Rapoport, G. (1993) Expression of *cryIVA* and *cryIVB* Genes, Independently or in Combination, in a Crystal-Negative Strain of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 59: 3922-3927

Dervyn, E., Poncet, S., Klier, A. and Rapoport, G. (1995) Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. 177: 2283-2291

Donovan, W. P., Dankocsik, C. C., Gilbert, M. P., Gawron-Burke, M. C., Groat, R. G. and Carlton, B. C. (1988) Amino acid sequence and entomocidal activity of the P2 crystal protein. An insect toxin from *Bacillus thuringiensis* var. kurstaki. J. Biol. Chem. 263: 561-567

Donovan, W. P., Engleman, J. T., Donovan, J. C., Baum, J. A., Bunkers, G. J., Chi, D. J., Clinton, W. P., English, L., Heck, G. R., Ilagan, O. M., Krasomil-Osterfeld, K. C., Pitkin, J. W., Roberts, J. K. and Walters, M. R. (2006) Discovery and characterization of Sip1A: a novel secreted protein from *Bacillus thuringiensis* with activity against coleopteran larvae. Appl. Microbiol. Biotechnol. In press.

Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. 16: 6127-6145

El-Bendary, M., Priest, F. G., Charles, J. F. and Mitchell, W. J. (2005) Crystal protein synthesis is dependent on early sporulation gene expression in *Bacillus sphaericus*. FEMS Microbiol. Lett. 252: 51-56

Elangovan, G., Shanmugavelu, M., Rajamohan, F., Dean, D. H. and Jayaraman, K. (2000) Identification of the functional site in the mosquito larvicidal binary toxin of *Bacillus sphaericus* 1593M by site-directed mutagenesis. Biochem. Biophys. Res. Commun. 276: 1048-1055

Ellis, R. T., Stockhoff, B. A., Stamp, L., Schnepf, H. E., Schwab, G. E., Knuth, M., Russell, J., Cardineau, G. A. and Narva, K. E. (2002) Novel *Bacillus thuringiensis* binary insecticidal crystal proteins active on western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Appl. Environ. Microbiol. 68: 1137-1145

Engler-Blum, G., Meier, M., Frank, J. and Muller, G. A. (1993) Reduction of background problems in nonradioactive northern and Southern blot analysis enables higher sensitivity than ^{32}P-based hybridisations. Anal. Biochem. 210: 235-244

Estruch, J. J., Warren, G. W., Mullins, M. A., Nye, G. J., Craig, J. A. and Koziel, M. G. (1996) Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc. Natl. Acad. Sci. U. S. A. 93: 5389-5394

Farchaus, J. W., Ribot, W. J., Downs, M. B. and Ezzell, J. W. (1995) Purification and characterization of the major surface array protein from the avirulent *Bacillus anthracis* Delta Sterne-1. J. Bacteriol. 177: 2481-2489

Federici, B. A., Park, H. W., Bideshi, D. K., Wirth, M. C. and Johnson, J. J. (2003) Recombinant bacteria for mosquito control. J. Exp. Biol. 206: 3877-3885

Fernandez, L. E., Aimanova, K. G., Gill, S. S., Bravo, A. and Soberon, M. (2006) A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. Biochem. J. 394: 77-84

Fernandez, L. E., Perez, C., Segovia, L., Rodriguez, M. H., Gill, S. S., Bravo, A. and Soberon, M. (2005) Cry11Aa toxin from *Bacillus thuringiensis* binds its receptor in *Aedes aegypti* mosquito larvae through loop alpha-8 of domain II. FEBS Lett. 579: 3508-3514

Fillinger, U., Knols, B. G. and Becker, N. (2003) Efficacy and efficiency of new *Bacillus thuringiensis* var *israelensis* and *Bacillus sphaericus* formulations against Afrotropical anophelines in Western Kenya. Trop. Med. Int. Health 8: 37-47

Finney, D. J. (1971) Probit analysis - a statistical treatment of the sigmoid response curve. 3 ed. Cambridge University Press

Galitsky, N., Cody, V., Wojtczak, A., Ghosh, D., Luft, J. R., Pangborn, W. and English, L. (2001) Structure of the insecticidal bacterial delta-endotoxin Cry3Bb1 of *Bacillus thuringiensis.* Acta Crystallogr. D Biol. Crystallogr. 57: 1101-1109

Gammon, K., Jones, G. W., Hope, S. J., de Oliveira, C. M., Regis, L., Silva Filha, M. H., Dancer, B. N. and Berry, C. (2006) Conjugal transfer of a toxin-coding megaplasmid from *Bacillus thuringiensis* subsp. *israelensis* to mosquitocidal strains of *Bacillus sphaericus.* Appl. Environ. Microbiol. 72: 1766-1770

Georghiou, G. P. and Wirth, M. (1997) Influence of Exposure to Single versus Multiple Toxins of *Bacillus thuringiensis* subsp. *israelensis* on Development of Resistance in the Mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. 63: 1095-1101

Goldberg, L. H. and Margalit, J. (1977) A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia ungyiculta, Culex univitatus, Aedes aegypti* and *Culex pipiens.* Mosq. News 37: 355-358

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J. L., Brousseau, R. and Cygler, M. (1995) *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. J. Mol. Biol. 254: 447-464

Grunstein, M. and Hogness, D. S. (1975) Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U. S. A. 72: 3961-3965

Guerchicoff, A., Ugalde, R. A. and Rubinstein, C. P. (1997) Identification and characterization of a previously undescribed cyt gene in *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 63: 2716-2721

Guex, N. and Peitsch, M. C. (1996) Swiss-Pdb Viewer: A fast and easy-to-use PDB viewer for Macintosh and PC. Protein Data Bank Quaterly Newsletter 77: 7

Guex, N. and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modelling. Electrophoresis 18: 2714-2723

Haider, M. Z. and Ellar, D. J. (1987a) Analysis of the molecular basis of insecticidal specificity of *Bacillus thuringiensis* crystal delta-endotoxin. Biochem. J. 248: 197-201

Haider, M. Z. and Ellar, D. J. (1987b) Characterization of the toxicity and cytopathic specificity of a cloned *Bacillus thuringiensis* crystal protein using insect cell culture. Mol. Microbiol. 1: 59-66

Haider, M. Z., Knowles, B. H. and Ellar, D. J. (1986) Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal delta-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. Eur. J. Biochem. 156: 531-540

Haldenwang, W. G. (1995) The sigma factors of *Bacillus subtilis*. Microbiol. Rev. 59: 1-30

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166: 557-580

Hartl, D. L. and Ochman, H. (1996) Inverse polymerase chain reaction. Methods Mol. Biol. 58: 293-301

Hazes, B. and Read, R. J. (1995) A mosquitocidal toxin with a ricin-like cell-binding domain. Nat. Struct. Biol. 2: 358-359

Helgason, E., Okstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I. and Kolsto (2000) *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*--one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66: 2627-2630

Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G. and Galas, D. J. (1989) A simple method for site-directed mutagenesis using the polymerase chain reaction. Nucleic Acids Res. 17: 6545-6551

Hindley, J. and Berry, C. (1987) Identification, cloning and sequence analysis of the *Bacillus sphaericus* 1593 41.9 kD larvicidal toxin gene. Mol. Microbiol. 1: 187-194

Hindley, J. and Berry, C. (1988) *Bacillus sphaericus* strain 2297: nucleotide sequence of 41.9 kDa toxin gene. Nucleic Acids Res. 16: 4168

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77: 51-59

Hofte, H. and Whiteley, H. R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53: 242-255

Hohn, B. and Collins, J. (1980) A small cosmid for efficient cloning of large DNA fragments. Gene 11: 291-298

Humphreys, M. J. and Berry, C. (1998) Variants of the *Bacillus sphaericus* binary toxins: implications for differential toxicity of strains. J. Invertebr. Pathol. 71: 184-185

Itsko, M., Manasherob, R. and Zaritsky, A. (2005) Partial restoration of antibacterial activity of the protein encoded by a cryptic open reading frame (*cyt1Ca*) from *Bacillus thuringiensis* subsp. *israelensis* by site-directed mutagenesis. J. Bacteriol. 187: 6379-6385

Jensen, G. B., Wilcks, A., Petersen, S. S., Damgaard, J., Baum, J. A. and Andrup, L. (1995) The genetic basis of the aggregation system in *Bacillus thuringiensis* subsp. *israelensis* is located on the large conjugative plasmid pXO16. J. Bacteriol. 177: 2914-2917

Kado, C. I. and Liu, S. T. (1981) Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145: 1365-1373

Kanintronkul, Y., Srikhirin, T., Angsuthanasombat, C. and Kerdcharoen, T. (2005) Insertion behavior of the *Bacillus thuringiensis* Cry4Ba insecticidal protein into lipid monolayers. Arch. Biochem. Biophys. 442: 180-186

Kellen, K., Clark, T. B., Lindergren, J. E., Ho, B. C., Rogoff, M. H. and Singer, S. (1965) *Bacillus sphaericus* Neide as a pathogen of mosquitoes. J. Invertebr. Pathol. 7: 442-448

Kenney, T. J., York, K., Youngman, P. and Moran, C. P., Jr. (1989) Genetic evidence that RNA polymerase associated with sigma A factor uses a sporulation-specific promoter in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U. S. A. 86: 9109-9113

Killeen, G. F., Fillinger, U. and Knols, B. G. (2002) Advantages of larval control for African malaria vectors: low mobility and behavioural responsiveness of immature mosquito stages allow high effective coverage. Malar. J. 1: 8

Knight, P. J., Crickmore, N. and Ellar, D. J. (1994) The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. Mol. Microbiol. 11: 429-436

Knowles, B. H. (1994) Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxins. Adv. Insect Physiol. 24: 275-308

Knowles, B. H. and Ellar, D. J. (1987) Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* delta-endotoxins with different insect specificity. Biochim. Biophys. Acta 924: 509-518

Koni, P. A. and Ellar, D. J. (1994) Biochemical characterization of *Bacillus thuringiensis* cytolytic delta-endotoxins. Microbiology 140 (Pt 8): 1869-1880

Krueger, K. M. and Barbieri, J. T. (1995) The family of bacterial ADP-ribosylating exotoxins. Clinical Microbiology Reviews 8: 34-47

Krych, V. K., Johnson, J. L. and Yousten, A. A. (1980) Deoxyribonucleic acid homologies among strains of *Bacillus sphaericus*. Int. J. Syst. Bacteriol. 30:
Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Danchin, A. and et al. (1997) The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature 390: 249-256

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lengeler, C. (2000) Insecticide-treated bednets and curtains for preventing malaria. Cochrane Database Syst. Rev. CD000363

Lesieur, C., Frutiger, S., Hughes, G., Kellner, R., Pattus, F. and van der Goot, F. G. (1999) Increased stability upon heptamerization of the pore-forming toxin aerolysin. J. Biol. Chem. 274: 36722-36728

Li, J., Koni, P. A. and Ellar, D. J. (1996) Structure of the mosquitocidal delta-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J. Mol. Biol. 257: 129-152

Li, J. D., Carroll, J. and Ellar, D. J. (1991) Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. Nature 353: 815-821

Liu, J. W., Hindley, J., Porter, A. G. and Priest, F. G. (1993) New high-toxicity mosquitocidal strains of *Bacillus sphaericus* lacking a 100-kilodalton-toxin gene. Appl. Environ. Microbiol. 59: 3470-3473

Liu, J. W., Porter, A. G., Wee, B. Y. and Thanabalu, T. (1996) New gene from nine *Bacillus sphaericus* strains encoding highly conserved 35.8-kilodalton mosquitocidal toxins. Appl. Environ. Microbiol. 62: 2174-2176

Liu, X. S. and Dean, D. H. (2006) Redesigning *Bacillus thuringiensis* Cry1Aa toxin into a mosquito toxin. Protein Eng. Des. Sel. 19: 107-111

Losick, R., Youngman, P. and Piggot, P. J. (1986) Genetics of endospore formation in *Bacillus subtilis*. Annu. Rev. Genet. 20: 625-669

Makita, Y., Nakao, M., Ogasawara, N. and Nakai, K. (2004) DBTBS: database of transcriptional regulation in *Bacillus subtilis* and its contribution to comparative genomics. Nucleic Acids Res. 32: D75-77

Manasherob, R., Zaritsky, A., Ben-Dov, E., Saxena, D., Barak, Z. and Einav, M. (2001) Effect of accessory proteins P19 and P20 on cytolytic activity of Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* in *Escherichia coli*. Curr. Microbiol. 43: 355-364

Marchler-Bauer, A., Anderson, J. B., Cherukuri, P. F., DeWeese-Scott, C., Geer, L. Y., Gwadz, M., He, S., Hurwitz, D. I., Jackson, J. D., Ke, Z., Lanczycki, C. J., Liebert, C. A., Liu, C., Lu, F., Marchler, G. H., Mullokandov, M., Shoemaker, B. A., Simonyan, V., Song, J. S., Thiessen, P. A., Yamashita, R. A., Yin, J. J., Zhang, D. and Bryant, S. H. (2005) CDD: a Conserved Domain Database for protein classification. Nucleic Acids Res. 33: D192-196

Marchler-Bauer, A. and Bryant, S. H. (2004) CD-Search: protein domain annotations on the fly. Nucleic Acids Res. 32: W327-331

Massie, J., Roberts, G. and White, P. J. (1985) Selective isolation of *Bacillus sphaericus* from soil by use of acetate as the only major source of carbon. Appl. Environ. Microbiol. 49: 1478-1481

Masson, L., Lu, Y. J., Mazza, A., Brousseau, R. and Adang, M. J. (1995) The CryIA(c) receptor purified from *Manduca sexta* displays multiple specificities. J. Biol. Chem. 270: 20309-20315

Masson, L., Schwab, G., Mazza, A., Brousseau, R., Potvin, L. and Schwartz, J. L. (2004) A novel *Bacillus thuringiensis* (PS149B1) containing a Cry34Ab1/Cry35Ab1 binary toxin specific for the western corn rootworm *Diabrotica virgifera virgifera* LeConte forms ion channels in lipid membranes. Biochemistry 43: 12349-12357

Mendez-Lopez, I., Basurto-Rios, R. and Ibarra, J. E. (2003) *Bacillus thuringiensis* serovar *israelensis* is highly toxic to the coffee berry borer, *Hypothenemus hampei* Ferr. (Coleoptera: Scolytidae). FEMS Microbiol. Lett. **226**: 73-77

Mesrati, L. A., Tounsi, S. and Jaoua, S. (2005) Characterization of a novel *vip3*-type gene from *Bacillus thuringiensis* and evidence of its presence on a large plasmid. FEMS Microbiol. Lett. 244: 353-358

Miyata, S., Minami, J., Tamai, E., Matsushita, O., Shimamoto, S. and Okabe, A. (2002) *Clostridium perfringens* epsilon-toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat synaptosomes. J. Biol. Chem. 277: 39463-39468

Moellenbeck, D. J., Peters, M. L., Bing, J. W., Rouse, J. R., Higgins, L. S., Sims, L., Nevshemal, T., Marshall, L., Ellis, R. T., Bystrak, P. G., Lang, B. A., Stewart, J. L., Kouba, K., Sondag, V., Gustafson, V., Nour, K., Xu, D., Swenson, J., Zhang, J., Czapla, T., Schwab, G., Jayne, S., Stockhoff, B. A., Narva, K., Schnepf, H. E., Stelman, S. J., Poutre, C., Koziel, M. and Duck, N. (2001) Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms. Nat. Biotechnol. 19: 668-672

Moran, C. P., Jr., Lang, N., LeGrice, S. F., Lee, G., Stephens, M., Sonenshein, A. L., Pero, J. and Losick, R. (1982) Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. 186: 339-346

Morse, R. J., Yamamoto, T. and Stroud, R. M. (2001) Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure 9: 409-417

Myers, P. and Yousten, A. A. (1978) Toxic activity of *Bacillus sphaericus* SSII-1 for mosquito larvae. Infect. Immun. 19: 1047-1053

Myers, P., Yousten, A. A. and Davidson, E. W. (1979) Comparative studies of the mosquito-larval toxin of *Bacillus sphaericus* SSII-1 and 1593. Can. J. Microbiol. 25: 1227-1231

Myers, P. S. and Yousten, A. A. (1980) Localization of a mosquito-larval toxin of *Bacillus sphaericus* 1593. Appl. Environ. Microbiol. 39: 1205-1211

Narasu, M. L. and Gopinathan, K. P. (1986) Purification of larvicidal protein from *Bacillus sphaericus* 1593. Biochem. Biophys. Res. Commun. 141: 756-761

Nicholls, C. N., Ahmad, W. and Ellar, D. J. (1989) Evidence for two different types of insecticidal P2 toxins with dual specificity in *Bacillus thuringiensis* subspecies. J. Bacteriol. 171: 5141-5147

Nicolas, L., Dossou-Yovo, J. and Hougard, J. (1987) Persistence and recycling of *Bacillus sphaericus* 2362 spores in *Culex quinquefasciatus* breeding sites in West Africa. Appl. Microbiol. Biotechnol. 25: 341-345

Nicolas, L., Nielsen-Leroux, C., Charles, J. F. and Delecluse, A. (1993) Respective role of the 42- and 51-kDa components of the *Bacillus sphaericus* toxin overexpressed in *Bacillus thuringiensis*. FEMS Microbiol. Lett. 106: 275-280

Nielsen-Leroux, C. and Charles, J. F. (1992) Binding of *Bacillus sphaericus* binary toxin to a specific receptor on midgut brush-border membranes from mosquito larvae. Eur. J. Biochem. 210: 585-590

Nielsen-Leroux, C., Charles, J. F., Thiery, I. and Georghiou, G. P. (1995) Resistance in a laboratory population of *Culex quinquefasciatus* (Diptera: Culicidae) to *Bacillus sphaericus* binary toxin is due to a change in the receptor on midgut brush-border membranes. Eur. J. Biochem. 228: 206-210

Nielsen-Leroux, C., Pasquier, F., Charles, J. F., Sinegre, G., Gaven, B. and Pasteur, N. (1997) Resistance to *Bacillus sphaericus* involves different mechanisms in *Culex pipiens* (Diptera:Culicidae) larvae. J. Med. Entomol. 34: 321-327

Nielsen-LeRoux, C., Rao, D. R., Murphy, J. R., Carron, A., Mani, T. R., Hamon, S. and Mulla, M. S. (2001) Various levels of cross-resistance to Bacillus sphaericus strains in Culex pipiens (Diptera: Culicidae) colonies resistant to B. sphaericus strain 2362. Appl. Environ. Microbiol. 67: 5049-5054

Nielsen-Leroux, C., Pasteur, N., Pretre, J., Charles, J. F., Sheikh, H. B. and Chevillon, C. (2002) High resistance to *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae): the complex situation of west Mediterranean countries. J. Med. Entomol. 39: 729-735

Oei, C., Hindley, J. and Berry, C. (1990) An analysis of the genes encoding the 51.4- and 41.9-kDa toxins of *Bacillus sphaericus* 2297 by deletion mutagenesis: the construction of fusion proteins. FEMS Microbiol. Lett. 60: 265-273

Oei, C., Hindley, J. and Berry, C. (1992) Binding of purified *Bacillus sphaericus* binary toxin and its deletion derivatives to *Culex quinquefasciatus* gut: elucidation of functional binding domains. J. Gen. Microbiol. 138 (Pt 7): 1515-1526

Oliveira, C. M., Silva-Filha, M. H., Nielsen-Leroux, C., Pei, G., Yuan, Z. and Regis, L. (2004) Inheritance and mechanism of resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from China and Brazil. J. Med. Entomol. 41: 58-64

Pao-intara, M., Angsuthanasombat, C. and Panyim, S. (1988) The mosquito larvicidal activity of 130 kDa delta-endotoxin of *Bacillus thuringiensis* var. *israelensis* resides in the 72 kDa amino-terminal fragment. Biochem. Biophys. Res. Commun. 153: 294-300

Park, H., Bideshi, D. K., Johnson, J. J. and Federici, B. A. (1999) Differential enhancement of Cry2A versus Cry11A yields in *Bacillus thuringiensis* by use of the *cry3A* STAB mRNA sequence. FEMS Microbiol. Lett. 181: 319-327

Park, H. W., Ge, B., Bauer, L. S. and Federici, B. A. (1998) Optimization of Cry3A yields in *Bacillus thuringiensis* by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. Appl. Environ. Microbiol. 64: 3932-3938

Parker, M. W., van der Goot, F. G. and Buckley, J. T. (1996) Aerolysin - the ins and outs of a model channel-forming toxin. Mol. Microbiol. 19: 205-212

Pauchet, Y., Luton, F., Castella, C., Charles, J. F., Romey, G. and Pauron, D. (2005) Effects of a mosquitocidal toxin on a mammalian epithelial cell line expressing its target receptor. Cell Microbiol. 7: 1335-1344

Pei, G., Oliveira, C. M., Yuan, Z., Nielsen-LeRoux, C., Silva-Filha, M. H., Yan, J. and Regis, L. (2002) A strain of *Bacillus sphaericus* causes slower development of resistance in *Culex quinquefasciatus*. Appl. Environ. Microbiol. 68: 3003-3009

Perez, C., Fernandez, L. E., Sun, J., Folch, J. L., Gill, S. S., Soberon, M. and Bravo, A. (2005) *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. Proc. Natl. Acad. Sci. U. S. A. 102: 18303-18308

Poncet, S., Bernard, C., Dervyn, E., Cayley, J., Klier, A. and Rapoport, G. (1997) Improvement of *Bacillus sphaericus* toxicity against dipteran larvae by integration, via homologous recombination, of the Cry11A toxin gene from *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 63: 4413-4420

Porter, A. G., Davidson, E. W. and Liu, J. W. (1993) Mosquitocidal toxins of bacilli and their genetic manipulation for effective biological control of mosquitoes. Microbiol. Rev. 57: 838-861

Priest, F. G., Barker, M., Baillie, L. W., Holmes, E. C. and Maiden, M. C. (2004) Population structure and evolution of the *Bacillus cereus* group. J. Bacteriol. **186**: 7959-7970

Priest, F. G., Ebdrup, L., Zahner, V. and Carter, P. E. (1997) Distribution and characterization of mosquitocidal toxin genes in some strains of *Bacillus sphaericus*. Appl. Environ. Microbiol. 63: 1195-1198

Promdonkoy, B., Promdonkoy, P., Tanapongpipat, S., Luxananil, P., Chewawiwat, N., Audtho, M. and Panyim, S. (2004) Cloning and characterization of a mosquito larvicidal toxin produced during vegetative stage of *Bacillus sphaericus* 2297. Curr. Microbiol. 49: 84-88

Promega (1996) Promega Protocol and Application guide. Promega Corporation. Available at www.promega.com.

Puntheeranurak, T., Leetacheewa, S., Katzenmeier, G., Krittanai, C., Panyim, S. and Angsuthanasombat, C. (2001) Expression and biochemical characterisation of the *Bacillus thuringiensis* Cry4Ba alpha1-alpha5 pore-forming fragment. J. Biochem. Mol. Biol. 34: 293-298

Puntheeranurak, T., Stroh, C., Zhu, R., Angsuthanasombat, C. and Hinterdorfer, P. (2005) Structure and distribution of the *Bacillus thuringiensis* Cry4Ba toxin in lipid membranes. Ultramicroscopy 105: 115-124

Puntheeranurak, T., Uawithya, P., Potvin, L., Angsuthanasombat, C. and Schwartz, J. L. (2004) Ion channels formed in planar lipid bilayers by the dipteran-specific Cry4B *Bacillus thuringiensis* toxin and its alpha1-alpha5 fragment. Mol. Membr. Biol. 21: 67-74

Rang, C., Gil, P., Neisner, N., Van Rie, J. and Frutos, R. (2005) Novel Vip3-related protein from *Bacillus thuringiensis*. Appl. Environ. Microbiol. 71: 6276-6281

Reinert, D. J., Carpusca, I., Aktories, K. and Schulz, G. E. (2006) Structure of the mosquitocidal toxin from *Bacillus sphaericus*. J. Mol. Biol. 357: 1226-1236

Roberts, D. R., Manguin, S. and Mouchet, J. (2000) DDT house spraying and re-emerging malaria. Lancet 356: 330-332

Rodcharoen, J. and Mulla, M. S. (1996) Cross-resistance to *Bacillus sphaericus* strains in *Culex quinquefasciatus*. J. Am. Mosq. Control Assoc. 12: 247-250

Rukmini, V., Reddy, C. Y. and Venkateswerlu, G. (2000) *Bacillus thuringiensis* crystal delta-endotoxin: role of proteases in the conversion of protoxin to toxin. Biochimie 82: 109-116

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M. A. and Barrell, B. (2000) Artemis: sequence visualization and annotation. Bioinformatics 16: 944-945

Salamitou, S., Agaisse, H., Bravo, A. and Lereclus, D. (1996) Genetic analysis of *cryIIIA* gene expression in *Bacillus thuringiensis*. Microbiology 142 (Pt 8): 2049-2055

Salamitou, S., Ramisse, F., Brehelin, M., Bourguet, D., Gilois, N., Gominet, M., Hernandez, E. and Lereclus, D. (2000) The *plcR* regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus* cereus in mice and insects. Microbiology 146 (Pt 11): 2825-2832

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbour Laboratory Press.

Sambrook, J. and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbour Laboratory Press.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74: 5463-5467

Schirmer, J., Just, I. and Aktories, K. (2002a) The ADP-ribosylating mosquitocidal toxin from *Bacillus sphaericus*: proteolytic activation, enzyme activity, and cytotoxic effects. J. Biol. Chem. 277: 11941-11948

Schirmer, J., Wieden, H. J., Rodnina, M. V. and Aktories, K. (2002b) Inactivation of the elongation factor Tu by mosquitocidal toxin-catalyzed mono-ADP-ribosylation. Appl. Environ. Microbiol. 68: 4894-4899

Schmitt, C. K., Meysick, K. C. and O'Brien, A. D. (1999) Bacterial toxins: Friends or foes? Emerg. Infect. Dis. 5: 224-234

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. and Dean, D. H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 775-806

Schnepf, H. E., Lee, S., Dojillo, J., Burmeister, P., Fencil, K., Morera, L., Nygaard, L., Narva, K. E. and Wolt, J. D. (2005) Characterization of Cry34/Cry35 binary insecticidal proteins from diverse *Bacillus thuringiensis* strain collections. Appl. Environ. Microbiol. 71: 1765-1774

Schwartz, J. L., Potvin, L., Coux, F., Charles, J. F., Berry, C., Humphreys, M. J., Jones, A. F., Bernhart, I., Dalla Serra, M. and Menestrina, G. (2001) Permeabilization of model lipid membranes by *Bacillus sphaericus* mosquitocidal binary toxin and its individual components. J. Membr. Biol. 184: 171-183

Schwede, T., Kopp, J., Guex, N. and Peitsch, M. C. (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res. 31: 3381-3385

Servant, P., Rosso, M. L., Hamon, S., Poncet, S., Del cluse, A. and Rapoport, G. (1999) Production of Cry11A and Cry11Ba toxins in *Bacillus sphaericus* confers toxicity towards *Aedes aegypti* and resistant *Culex* populations. Appl. Environ. Microbiol. 65: 3021-3026

Shanmugavelu, M., Rajamohan, F., Kathirvel, M., Elangovan, G., Dean, D. H. and Jayaraman, K. (1998) Functional complementation of nontoxic mutant binary toxins of *Bacillus sphaericus* 1593M generated by site-directed mutagenesis. Appl. Environ. Microbiol. 64: 756-759

Shi, Y., Xu, W., Yuan, M., Tang, M., Chen, J. and Pang, Y. (2004) Expression of *vip1/vip2* genes in *Escherichia coli* and *Bacillus thuringiensis* and the analysis of their signal peptides. J. Appl. Microbiol. 97: 757-765

Shi, Y., Yuan, Z., Cai, Q., Yu, J., Yan, J. and Pang, Y. (2001) Cloning and expression of the binary toxin gene from *Bacillus sphaericus* IAB872 in a crystal-minus *Bacillus thuringiensis* subsp. *israelensis*. Curr. Microbiol. 43: 21-25

Shi, Y. X., Zheng, D. S. and Yuan, Z. M. (2003) Toxicity of *Bacillus sphaericus* LP1-G against susceptible and resistant *Culex quinquefasciatus* and the cloning of the mosquitocidal toxin gene. Curr. Microbiol. 47: 226-230

Shmidt, F. G. V., Monnerat, R. G., Borges, M. and Carvalho, R. S. (2001) Metodologia de criação de insetos para avaliação de agentes entomopatogênicos. Circular técnica no. 11. Embrapa, Brasília, Brazil.

Silapanuntakul, S., Pantuwatana, S., Bhumiratana, A. and Charoensiri, K. (1983) The comparative persistence of toxicity of *Bacillus sphaericus* strain 1593 and *Bacillus thuringiensis* serotype H-14 against mosquito larvae in different kinds of environments. J. Invertebr. Pathol. 42: 387-392

Silva-Filha, M. H., Nielsen-Leroux, C. and Charles, J. F. (1997) Binding kinetics of *Bacillus sphaericus* binary toxin to midgut brush-border membranes of *Anopheles* and *Culex* sp. mosquito larvae. Eur. J. Biochem. 247: 754-761

Silva-Filha, M. H., Nielsen-LeRoux, C. and Charles, J. F. (1999) Identification of the receptor for *Bacillus sphaericus* crystal toxin in the brush border membrane of the mosquito *Culex pipiens* (Diptera: Culicidae). Insect Biochem. Mol. Biol. 29: 711-721

Silva-Filha, M. H., Oliveira, C. M., Regis, L., Yuan, Z., Rico, C. M. and Nielsen-LeRoux, C. (2004) Two *Bacillus sphaericus* binary toxins share the midgut receptor

binding site: implications for resistance of *Culex pipiens* complex (Diptera: Culicidae) larvae. FEMS Microbiol. Lett. 241: 185-191

Singer, S. (1973) Insecticidal activity of recent bacterial isolates and their toxins against mosquito larvae. Nature 244: 110-111

Singer, S. (1977) Isolation and development of bacterial pathogens of vectors. In *Biological regulation of vectors*, 3-18. DHEW Publication no. (NIH) 77-1180

Smedley, D. P. and Ellar, D. J. (1996) Mutagenesis of three surface-exposed loops of a *Bacillus thuringiensis* insecticidal toxin reveals residues important for toxicity, receptor recognition and possibly membrane insertion. Microbiology 142 (Pt 7): 1617-1624

Smith, A. W., Camara-Artigas, A., Brune, D. C. and Allen, J. P. (2005) Implications of high-molecular-weight oligomers of the binary toxin from *Bacillus sphaericus*. J. Invertebr. Pathol. 88: 27-33

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517

Studier, F. W. and Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189: 113-130

Thanabalu, T., Berry, C. and Hindley, J. (1993) Cytotoxicity and ADP-ribosylating activity of the mosquitocidal toxin from *Bacillus sphaericus* SSII-1: possible roles of the 27- and 70-kilodalton peptides. J. Bacteriol. 175: 2314-2320

Thanabalu, T., Hindley, J. and Berry, C. (1992) Proteolytic processing of the mosquitocidal toxin from *Bacillus sphaericus* SSII-1. J. Bacteriol. 174: 5051-5056

Thanabalu, T., Hindley, J., Jackson-Yap, J. and Berry, C. (1991) Cloning, sequencing, and expression of a gene encoding a 100-kilodalton mosquitocidal toxin from *Bacillus sphaericus* SSII-1. J. Bacteriol. 173: 2776-2785

Thanabalu, T. and Porter, A. G. (1995) Efficient expression of a 100-kilodalton mosquitocidal toxin in protease-deficient recombinant *Bacillus sphaericus*. Appl. Environ. Microbiol. 61: 4031-4036

Thanabalu, T. and Porter, A. G. (1996) A *Bacillus sphaericus* gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. Gene 170: 85-89

Thiery, I., Hamon, S., Delecluse, A. and Orduz, S. (1998) The introduction into *Bacillus sphaericus* of the *Bacillus thuringiensis* subsp. *medellin* Cyt1Ab1 gene results in higher susceptibility of resistant mosquito larva populations to *B. sphaericus*. Appl. Environ. Microbiol. 64: 3910-3916

Thiery, I., Ofori, J., Cosmao-Dumanoir, V., Hamon, S. and de Barjac, H. (1992) New mosquitocidal strains from Ghana belonging to serotypes H3, H6 and H48 of *Bacillus sphaericus*. Appl. Microbiol. Biotechnol. 37: 718-722

Thorne, L., Garduno, F., Thompson, T., Decker, D., Zounes, M., Wild, M., Walfield, A. M. and Pollock, T. J. (1986) Structural similarity between the Lepidoptera- and Diptera-specific insecticidal endotoxin genes of *Bacillus thuringiensis* subsp. "*kurstaki*" and "*israelensis*". J. Bacteriol. 166: 801-811

Tinoco, I., Jr., Borer, P. N., Dengler, B., Levin, M. D., Uhlenbeck, O. C., Crothers, D. M. and Bralla, J. (1973) Improved estimation of secondary structure in ribonucleic acids. Nat. New Biol. 246: 40-41

Trieu-Cuot, P., Poyart-Salmeron, C., Carlier, C. and Courvalin, P. (1990) Nucleotide sequence of the erythromycin resistance gene of the conjugative transposon Tn*1545*. Nucleic Acids Res. 18: 3660

Triglia, T., Peterson, M. G. and Kemp, D. J. (1988) A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res. 16: 8186

Trisrisook, M., Pantuwatana, S., Bhumiratana, A. and Panbangred, W. (1990) Molecular cloning of the 130-kilodalton mosquitocidal delta-endotoxin gene of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus*. Appl. Environ. Microbiol. 56: 1710-1716

Tungpradubkul, S., Settasatien, C. and Panyim, S. (1988) The complete nucleotide sequence of a 130 kDa mosquito-larvicidal delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. Nucleic Acids Res. 16: 1637-1638

Turnbull, P. C. (1999) Definitive identification of *Bacillus anthracis*--a review. J. Appl. Microbiol. **87**: 237-240

Turton, K., Chaddock, J. A. and Acharya, K. R. (2002) Botulinum and tetanus neurotoxins: structure, function and therapeutic utility. TRENDS Biochem. Sci. 27: 552-558

Vadlamudi, R. K., Weber, E., Ji, I., Ji, T. H. and Bulla, L. A., Jr. (1995) Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. 270: 5490-5494

Vassal, J.-M., de Barjac, H., Frutos, R. and Federici, B. A. (1993) Isolation of *Bacillus thuringiensis* subsp. *israelensis* from diseased field-collected larvae of the saturniid moth, *Hylesia metabus*, in French Guiana. FEMS Microbiol. Lett. **107**: 199-203

Visick, J. E. and Whiteley, H. R. (1991) Effect of a 20-kilodalton protein from *Bacillus thuringiensis* subsp. *israelensis* on production of the CytA protein by *Escherichia coli*. J. Bacteriol. 173: 1748-1756

Ward, E. S. and Ellar, D. J. (1987) Nucleotide sequence of a *Bacillus thuringiensis* var. *israelensis* gene encoding a 130 kDa delta-endotoxin. Nucleic Acids Res. 15: 7195

Wati, M. R., Thanabalu, T. and Porter, A. G. (1997) Gene from tropical *Bacillus sphaericus* encoding a protease closely related to subtilisins from Antarctic bacilli. Biochim. Biophys. Acta 1352: 56-62

Weiser, J. (1984) A mosquito-virulent *Bacillus sphaericus* in adult *Simulium damnosum* from northern Nigeria. Zentralbl. Mikrobiol. 139: 57-60

White, P. J. and Lotay, H. K. (1980) Minimal nutritional requirements of *Bacillus sphaericus* NCTC 9602 and 26 other strains of this species: the majority grow and sporulate with acetate as sole major source of carbon. J. Gen. Microbiol. 118: 13-19

WHO (1985) Informal consultation on the development of *Bacillus sphaericus* as microbial larvicide. TDR/BCV/*SPHAERICUS*/ 85.3: 1-24

WHO (1998) Fact Sheet 94. World Health Organization, Geneva, Switzerland.

WHO (2000a) Fact Sheet 102. World Health Organization, Geneva, Switzerland.

WHO (2000b) Microbial pest control. IPCS News: The newsletter of the international programme on chemical safety 12: 2

Wickremesinghe, R. S. B. and Mendis, C. L. (1980) *Bacillus sphaericus* spore from Sri Lanka demonstrating rapid larvicidal activity on *Culex quinquefasciatus*. Mosq. News 40: 387-389

Wirth, M. C., Delecluse, A. and Walton, W. E. (2001) Cyt1Ab1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and B. *thuringiensis* subsp. *israelensis* synergize *Bacillus sphaericus* against *Aedes aegypti* and resistant *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. 67: 3280-3284

Wirth, M. C., Georghiou, G. P. and Federici, B. A. (1997) CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. Proc. Natl. Acad. Sci. U. S. A. 94: 10536-10540

Wirth, M. C., Georghiou, G. P., Malik, J. I. and Abro, G. H. (2000a) Laboratory selection for resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from California, USA. J. Med. Entomol. 37: 534-540

Wirth, M. C., Jiannino, J. A., Federici, B. A. and Walton, W. E. (2004) Synergy between toxins of *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*. J. Med. Entomol. 41: 935-941

Wirth, M. C., Jiannino, J. A., Federici, B. A. and Walton, W. E. (2005) Evolution of resistance toward *Bacillus sphaericus* or a mixture of B. *sphaericus*+Cyt1A from *Bacillus thuringiensis*, in the mosquito, *Culex quinquefasciatus* (Diptera: Culicidae). J. Invertebr. Pathol. 88: 154-162

Wirth, M. C., Walton, W. E. and Federici, B. A. (2000b) Cyt1A from *Bacillus thuringiensis* restores toxicity of *Bacillus sphaericus* against resistant *Culex quinquefasciatus* (Diptera: Culicidae). J. Med. Entomol. 37: 401-407

Wong, H. C. and Chang, S. (1986) Identification of a positive retroregulator that stabilizes mRNAs in bacteria. Proc. Natl. Acad. Sci. U. S. A. 83: 3233-3237

Wu, D. and Federici, B. A. (1993) A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. J. Bacteriol. 175: 5276-5280

Wu, D. and Federici, B. A. (1995) Improved production of the insecticidal CryIVD protein in *Bacillus thuringiensis* using *cryIA(c)* promoters to express the gene for an associated 20-kDa protein. Appl. Microbiol. Biotechnol. 42: 697-702

Wu, D., Johnson, J. J. and Federici, B. A. (1994) Synergism of mosquitocidal toxicity between CytA and CryIVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. Mol. Microbiol. 13: 965-972

Xue, G.-P., Johnson, J. S. and Dalrymple, B. P. (1999) High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus* licheniformis. J. Microbiol. Methods 34: 183-191

Yamamoto, T. and McLaughlin, R. E. (1981) Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taeniorhynchus*. Biochem. Biophys. Res. Commun. 103: 414-421

Yamashita, S., Katayama, H., Saitoh, H., Akao, T., Park, Y. S., Mizuki, E., Ohba, M. and Ito, A. (2005) Typical Three-Domain Cry Proteins of *Bacillus thuringiensis* Strain A1462 Exhibit Cytocidal Activity on Limited Human Cancer Cells. J. Biochem. (Tokyo) 138: 663-672

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119

York, K., Kenney, T. J., Satola, S., Moran, C. P., Jr., Poth, H. and Youngman, P. (1992) Spo0A controls the sigma A-dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. J. Bacteriol. 174: 2648-2658

Yoshida, K., Matsushima, Y., Sen, K., Sakai, H. and Kamano, T. (1989) Insecticidal activity of a pepetide containing the 30th to 695th amino acid residues of the 130-kDa protein of *Bacillus thuringiensis* var *israelensis*. Agric. biol. Chem. 53: 2121-2127

Yoshisue, H., Fukada, T., Yoshida, K., Sen, K., Kurosawa, S., Sakai, H. and Komano, T. (1993a) Transcriptional regulation of *Bacillus thuringiensis* subsp. *israelensis* mosquito larvicidal crystal protein gene *cryIVA*. J. Bacteriol. 175: 2750-2753

Yoshisue, H., Nishimoto, T., Sakai, H. and Komano, T. (1993b) Identification of a promoter for the crystal protein-encoding gene *cryIVB* from *Bacillus thuringiensis* subsp. *israelensis*. Gene 137: 247-251

Yousten, A. A. (1984) Bacteriphage typing of mosquito pathogenic strains of *Bacillus sphaericus*. J. Invertebr. Pathol. 43: 124-125

Yousten, A. A. and Davidson, E. W. (1982) Ultrastructural Analysis of Spores and Parasporal Crystals Formed by *Bacillus sphaericus* 2297. Appl. Environ. Microbiol. 44: 1449-1455

Yousten, A. A., Genthner, F. J. and Benfield, E. F. (1992) Fate of *Bacillus sphaericus* and *Bacillus thuringiensis* serovar *israelensis* in the aquatic environment. J. Am. Mosq. Control Assoc. 8: 143-148

Yu, C. G., Mullins, M. A., Warren, G. W., Koziel, M. G. and Estruch, J. J. (1997) The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. 63: 532-536

Yuan, Z., Rang, C., Maroun, R. C., Juarez-Perez, V., Frutos, R., Pasteur, N., Vendrely, C., Charles, J. F. and Nielsen-Leroux, C. (2001) Identification and molecular structural prediction analysis of a toxicity determinant in the *Bacillus sphaericus* crystal larvicidal toxin. Eur. J. Biochem. 268: 2751-2760

Yuan, Z. M., Pei, G. F., Regis, L., Nielsen-Leroux, C. and Cai, Q. X. (2003) Cross-resistance between strains of *Bacillus sphaericus* but not B. *thuringiensis israelensis* in colonies of the mosquito *Culex quinquefasciatus*. Med. Vet. Entomol. 17: 251-256

Zahiri, N. S. and Mulla, M. S. (2003) Susceptibility profile of *Culex quinquefasciatus* (Diptera: Culicidae) to *Bacillus sphaericus* on selection with rotation and mixture of *B. sphaericus* and *B. thuringiensis israelensis*. J. Med. Entomol. 40: 672-677

Zhou, M. Y., Clark, S. E. and Gomez-Sanchez, C. E. (1995) Universal cloning method by TA strategy. Biotechniques 19: 34-35

Zhou, M. Y. and Gomez-Sanchez, C. E. (2000) Universal TA cloning. Curr. Issues Mol. Biol. 2: 1-7

Zhu, X., McVeigh, R. R., Malathi, P. and Ghosh, B. K. (1996) The complete nucleotide sequence of the *Bacillus licheniformis* NM105 S-layer-encoding gene. Gene 173: 189-194

APPENDICIES

Appendix 1. **Primer sequences (all sequences are written 5'-3')**

Degenerate primers

DP491	5'- ATGGARAAYCARATHAARGARGA -3'
DP492	5'- GARGARTTYAAYAARAAYAAYCAYG -3'
DP491C	5'- TCYTCYTTDATYTGRTTYTCCAT -3

Degenerate bases

R = A/G
Y = C/T
H = A/C/T
D = A/G/T

Vector specific primers (all sequences are written 5' to 3')

M13remoteF	GTTGGGTAACGCCAGGG
M13reomteR	GAAATTGTGAGCGGATAAC
M13F	TCCCAGTCACGACGT
M13R	AACAGCTATGACCATG
SF1	GCTAGTTATTGCTCAGCGG
T7	TAATACGACTCACTATAGGG
pGEXF	GGGCTGGCAAGCCACGTTTGGTG
pGEXR	CCGGGAGCTGCATGTGTCAGAGG

Int1R	CCATCGTGCCTGTTCGCAATG
Int2F	GGTCAAGGTGTAATAGTATCTAG
Int3F	CAGGTTGGAACACCTTGGGTAG
Int4F	TGACAATGCAGATAATAGTTATG
Int6F	GATGACTGTAACTTTCCTCC
Int7F	CTGAACCTTGAACAGTGGAC
Int8F	GTACAAATCCGTATCACCATTTAG
Int9F	GGTTCATATTACAATCATGGGGAG
Int10F	CGTCATCTCCATGAACATTCATG
Int10LP1GF	CCTGAAGTACGACTAGGAGACAG
Int11F	GCATAATCTTCATATTTTGCTTGTTC
Int12F	GATAAGTTGTGTACCGTTTG
Int13F	CATAGAAGAGGTAGAAGAGTTC
HindF1	CATAGCAGGTATTATAATGTTAACG
HindF2	GAAGAACAGCACTAGATCGAGG
HindF3	AGAAACCAGACTGTGGTGCT
EcoRI1KIntF	CATATCCTCTCTGGTGTGTTTCC
ECO4KF	GAGTGTATCAAGTTGATAAATCCATG
ECO4KR	GAAGAGAATCAAGTCTTTGATGAGC
ECO4KInt	CCTCTAATACTTACTAGTGCTTG

P49F	GGATCCTTGGAAAATCAAATAAAAGAAGAATTTAAC
P49R	GGATCCTTAATTATAATATGGCTTTGAATTTTCATG
P49PROF	GGATCCGTCGAGTGTAACTTCGGTTGTTTG
PETP49F	GGAATTCCATATGCAGTTGGAAAATCAAATAAAAGAAGAATTTAAC
PETP49R	CCGCTCGAGCGGTTAATTATAATATGGCTTTGAATTTTCATG
NDESDMF	AAGATATGCAAGATTTGTTAAAGCCTATGAATATAGATTAACTCGTGC
NDESDMR	GCACGAGTTAATCTATATTCATAGGCTTTAACAAATCTTGCATATCTT
P135SeqF	CACCTGCATATATTAATTTAGG
P135SeqR	TTAATAGTTAACGCAAATGAGCTC
BamP135F	GCAAGTGGATCCCCAGTTTAGTGTTGACATATCAG
BamP135R	ATCCGAGGATCCTTAATAGTTAACGCAAATGAGCTC
PSTABF	AGTGCAGTCGACCACCTGCATATATTAATTTAGG
PSTABR	ATGTCAGCATGCTTAATAGTTAACGCAAATGAGCTC

Appendix 2. Gene sequences, protein sequences and GenBank accession numbers

cry49Aa gene sequence and translated amino acid sequence of the variant from strains IAB59 (AJ841948), 47-6B (AM237201), NHA15b (AM237203) and 2173 (AM237204)

```
   1   L  E  N  Q  I  K  E  E  F  N  K  N  N  H  G  I  P  S  D  C
   1  TTGGAAAATCAAATAAAAGAAGAATTTAACAAAAATAATCATGGTATTCCTAGTGATTGC

  21   S  C  I  K  E  G  D  D  Y  N  S  L  T  E  I  N  A  K  E  F
  61  AGTTGTATAAAAGAAGGAGATGATTATAACTCTTTAACAGAGATAAATGCTAAAGAGTTT

  41   S  Y  C  S  P  N  M  F  N  L  N  L  P  E  Q  S  T  R  F  Q
 121  TCTTATTGTAGTCCAAATATGTTTAATTTAAATTTGCCAGAACAAAGTACTAGATTTCAA

  61   T  I  A  S  I  H  S  N  N  C  S  F  E  I  L  N  N  D  P  G
 181  ACAATAGCTTCAATACATTCAAACAATTGTAGTTTTGAAATTCTCAATAACGACCCAGGG

  81   Y  I  Y  G  D  S  V  D  G  E  C  R  I  A  V  A  H  R  E  L
 241  TATATATATGGGGATTCTGTAGATGGAGAATGTAGAATTGCAGTAGCCCACAGAGAACTT

 101   G  N  G  L  E  R  T  G  D  D  R  F  L  F  I  F  Y  A  L  D
 301  GGCAATGGTTTAGAGCGAACTGGGGACGACAGATTTTTATTTATTTTTTATGCTCTAGAT

 121   N  N  N  F  I  I  A  N  R  H  D  G  F  V  L  Q  F  L  I  A
 361  AATAATAATTTTATCATTGCGAACAGGCACGATGGTTTTGTTTTGCAATTTTTGATAGCA

 141   N  G  Q  G  V  I  V  S  R  E  Y  Q  P  N  I  H  Q  E  F  T
 421  AATGGTCAAGGTGTAATAGTATCTAGAGAATATCAACCAAATATTCATCAAGAATTCACT

 161   I  Q  S  I  N  S  D  T  F  R  L  H  S  R  D  T  N  T  F  A
 481  ATACAATCAATTAACTCTGACACTTTTAGGTTGCATTCACGTGATACTAATACTTTCGCT

 181   T  V  C  W  A  Q  F  N  S  W  T  K  I  V  S  R  V  D  N  P
 541  ACTGTCTGCTGGGCACAGTTCAACAGTTGGACAAAAATTGTTTCAAGGGTTGATAATCCT

 201   G  A  P  N  A  N  L  K  H  R  S  L  L  T  D  I  N  M  P  Q
 601  GGTGCACCTAATGCGAACTTGAAACATCGTTCACTTCTTACGGATATAAATATGCCACAA

 221   L  P  S  L  T  P  L  Q  P  L  P  R  L  T  E  L  E  D  G  G
 661  TTACCAAGTTTAACACCATTACAACCATTACCACGATTAACTGAATTAGAAGATGGAGGC

 241   L  S  P  A  Q  A  P  R  A  I  I  G  R  T  L  I  P  C  L  F
 721  CTATCACCTGCTCAAGCACCAAGAGCTATTATAGGAAGAACACTTATTCCATGTTTATTT

 261   V  N  D  P  V  L  R  L  E  N  R  I  K  Q  S  P  Y  Y  V  L
 781  GTAAATGATCCTGTCTTAAGACTTGAAAATAGGATTAAACAAAGTCCATATTATGTATTA

 281   E  H  R  Q  Y  W  H  R  I  W  T  D  I  F  T  A  G  E  R  R
 841  GAACATAGACAATATTGGCACAGAATATGGACAGATATTTTTACTGCTGGGGAGAGAAGA

 301   E  Y  R  E  V  T  G  I  N  N  N  A  Q  N  D  M  N  K  M  I
 901  GAATATCGTGAAGTTACAGGAATAAATAATAATGCTCAAAATGATATGAATAAGATGATA

 321   N  I  T  I  G  A  D  G  P  N  R  L  R  F  G  N  L  S  T  P
 961  AATATAACAATAGGTGCAGATGGACCAAATCGTTTGCGTTTTGGAAATCTTTCTACACCA

 341   F  R  Q  Q  I  I  D  N  S  N  T  L  G  S  F  A  N  T  N  Y
1021  TTTAGACAACAAATTATAGATAATTCAAATACTTTAGGATCTTTCGCAAATACTAATTAT

 361   G  T  R  T  D  I  V  N  V  F  N  S  E  F  H  Q  V  R  Y  A
1081  GGAACAAGAACTGATATAGTAAATGTTTTTAATAGTGAATTTCACCAAGTAAGATATGCA

 381   R  F  V  K  A  Y  E  Y  R  L  T  R  A  D  G  S  Q  V  G  T
1141  AGATTTGTTAAAGCATATGAATATAGATTAACTCGTGCTGATGGATCACAGGTTGGAACA
```

```
 401  P  W  V  V  L  D  R  K  E  M  D  L  R  T  Y  P  H  N  M  A
1201  CCTTGGGTAGTTTTAGACCGTAAGGAAATGGATTTAAGAACATACCCACATAATATGGCA

 421  I  T  L  E  N  V  K  I  D  N  A  D  N  S  Y  D  L  S  I  W
1261  ATAACCTTAGAAAATGTGAAAATTGACAATGCAGATAATAGTTATGATTTATCAATATGG

 441  K  T  P  L  K  L  K  D  G  K  I  I  I  E  N  H  E  N  S  K
1321  AAAACCCCACTAAAATTAAAAGATGGTAAAATTATTATAGAGAATCATGAAAATTCAAAG

 461  P  Y  Y  N  -
1381  CCATATTATAATTAA
```

cry49Ab1 **gene sequence and translated amino acid sequence of the variant from strain LP1G (AM237202)**

```
   1  L  E  N  Q  I  K  E  E  F  N  K  N  N  H  G  I  P  S  D  C
   1  TTGGAAAATCAAATAAAAGAAGAATTTAACAAAAATAATCATGGTATTCCTAGTGATTGC

  21  S  C  I  K  E  G  D  D  Y  N  S  L  T  E  V  P  S  E  I  N
  61  AGTTGTATAAAAGAAGGAGATGATTATAACTCTTTAACAGAAGTACCCAGTGAGATAAAT

  41  A  K  E  F  S  Y  C  S  P  N  M  F  N  L  N  L  P  E  Q  S
 121  GCTAAAGAGTTTTCTTATTGTAGTCCAAATATGTTTAATTTAAATTTGCCAGAACAAAGT

  61  T  R  F  Q  T  I  G  S  I  H  S  N  N  C  S  F  E  I  L  N
 181  ACTAGATTTCAAACAATAGGTTCAATACATTCAAACAATTGTAGTTTTGAAATTCTCAAT

  81  N  D  P  G  Y  I  Y  G  D  S  V  A  G  E  C  R  I  A  V  A
 241  AACGACCCAGGGTATATATATGGGGATTCTGTAGCTGGAGAATGTAGAATTGCAGTAGCC

 101  H  R  E  L  G  N  G  L  E  R  T  G  D  D  R  F  L  F  I  F
 301  CACAGAGAACTTGGCAATGGTTTAGAGCGAACTGGGGACGACAGATTTTTATTTATTTTT

 121  Y  A  L  D  N  N  N  F  I  I  A  N  R  H  D  G  F  V  L  Q
 361  TATGCTCTAGATAATAATAACTTTATCATTGCGAACAGGCACGATGGTTTTGTTTTGCAA

 141  F  L  I  A  N  G  Q  G  V  I  V  S  R  E  Y  Q  P  N  I  R
 421  TTTTTGATAGCAAATGGTCAAGGTGTAATAGTATCTAGAGAATATCAACCAAATATTCGC

 161  Q  E  F  T  I  Q  S  I  N  S  D  T  F  R  L  H  S  R  D  T
 481  CAAGAATTCACTATACAATCAATTAACTCTGACACTTTTAGGTTGCATTCACGTGATACT

 181  N  T  F  A  T  V  C  W  A  Q  F  N  S  W  T  K  I  V  S  R
 541  AATACTTTCGCTACTGTCTGCTGGGCACAGTTCAACAGTTGGACAAAAATTGTTTCAAGG

 201  V  D  N  P  G  A  P  N  A  D  L  R  H  R  S  F  L  D  I  N
 601  GTTGATAATCCTGGTGCACCTAATGCGGACTTGAGACATCGTTCGTTTTTGGATATAAAT

 221  M  P  Q  L  P  S  L  T  P  L  Q  P  L  P  R  L  T  G  L  E
 661  ATGCCACAATTACCAAGTTTAACACCATTACAACCATTACCACGATTAACTGGATTAGAA

 241  D  G  G  L  S  P  A  Q  A  P  R  A  I  I  G  R  T  L  I  P
 721  GATGGAGGCCTATCACCTGCTCAAGCACCAAGAGCTATTATAGGAAGAACACTTATTCCA

 261  C  L  F  V  N  D  P  V  L  R  L  E  S  R  I  K  Q  S  P  Y
 781  TGTTTATTTGTAAATGATCCAGTCTTAAGACTTGAAAGTAGGATTAAACAAAGTCCATAT

 281  Y  V  L  E  H  R  Q  Y  W  H  R  L  W  T  D  I  F  N  A  G
 841  TATGTTTTAGAACATAGACAATATTGGCACAGATTATGGACAGATATTTTTAATGCTGGA

 301  E  R  R  E  Y  R  E  V  T  G  I  N  H  N  A  Q  N  D  M  N
 901  GAGAGAAGAGAGTATCGTGAAGTTACAGGAATAAATCACAATGCTCAAAACGATATGAAT

 321  N  M  I  N  I  T  I  G  S  D  G  P  N  R  L  L  F  G  H  L
 961  AATATGATAAATATAACAATAGGTTCAGATGGACCAAATCGTTTGCTGTTTGGACATCTG
```

```
 341   S  T  P  F  R  Q  Q  I  I  S  N  S  N  T  L  G  S  F  A  N
1021   TCTACACCATTTAGACAACAAATTATAAGTAATTCAAATACTTTAGGATCTTTTGCGAAT

 361   S  N  Y  S  S  R  T  E  S  I  T  Y  L  N  T  E  F  H  Q  V
1081   TCTAATTATTCATCAAGAACCGAATCAATAACTTATTTAAATACTGAATTTCATCAAGTA

 381   R  F  A  R  F  V  K  A  Y  E  Y  R  L  T  R  A  D  G  T  L
1141   AGATTTGCAAGATTTGTTAAAGCATATGAATATAGGTTAACTCGTGCTGATGGAACACTT

 401   V  G  T  P  W  V  V  L  D  R  K  E  M  D  L  R  T  F  P  H
1201   GTTGGCACACCTTGGGTAGTTTTAGACCGTAAGGAAATGGATTTAAGAACATTTCCACAT

 421   N  M  T  L  N  L  E  N  V  K  I  V  N  A  D  N  S  Y  D  L
1261   AATATGACACTGAATTTAGAAAATGTGAAAATTGTCAATGCAGATAATAGTTATGATTTA

 441   S  V  W  K  T  P  L  K  L  K  D  G  K  I  I  I  E  N  H  E
1321   TCAGTATGGAAAACCCCACTGAAATTAAAAGATGGGAAAATTATTATTGAGAATCATGAA

 461   N  S  K  P  Y  Y  N  -
1381   AATTCAAAGCCATATTATAATTAA
```

***cry48Aa* gene sequence and translated amino acid sequence of the variant found in strains IAB59 (AJ841948), 47-6B (AM237205) and NHA15b (AM237206)**

```
   1   M  D  I  N  N  N  N  E  K  E  I  I  N  S  H  L  L  P  Y  S
   1   ATGGATATCAACAATAATAATGAAAAGGAAATTATTAATTCTCATTTACTACCATATTCA

  21   L  L  K  K  Y  P  I  K  S  L  Q  S  T  N  Y  K  D  W  L  N
  61   CTTTTAAAAAAATACCCTATTAAGTCTTTACAGAGCACAAATTACAAAGATTGGCTTAAT

  41   L  C  Q  D  F  N  K  D  I  E  S  Y  D  L  V  T  A  V  S  S
 121   CTGTGTCAAGATTTTAATAAGGATATTGAAAGTTATGATTTGGTAACTGCTGTCTCGAGC

  61   G  T  I  V  V  G  T  M  L  S  A  I  Y  A  P  A  L  I  A  G
 181   GGAACTATTGTCGTAGGGACAATGTTGAGCGCTATTTATGCACCCGCTCTTATAGCTGGT

  81   P  I  G  I  I  G  A  I  I  I  S  F  G  T  L  L  P  L  L  W
 241   CCTATAGGAATAATAGGCGCTATCATTATATCTTTTGGAACTCTTCTACCTCTTCTATGG

 101   N  E  S  E  N  N  P  K  T  T  W  I  E  F  I  R  M  G  E  Q
 301   AATGAGAGCGAGAATAACCCTAAAACGACATGGATTGAATTTATTAGAATGGGAGAACAG

 121   L  V  D  K  T  I  S  Q  T  V  F  N  I  L  E  S  Y  L  K  D
 361   CTTGTTGATAAAACAATATCGCAAACAGTGTTTAATATACTGGAAAGCTATTTAAAAGAT

 141   L  K  V  N  L  V  D  Y  E  K  A  K  Q  D  W  I  E  L  K  K
 421   TTAAAGGTAAATTTAGTAGATTATGAAAAAGCGAAACAAGATTGGATTGAATTGAAAAAA

 161   Q  Q  L  P  G  S  P  P  S  T  K  L  R  N  A  A  D  I  A  H
 481   CAGCAACTTCCTGGTTCACCTCCCTCAACTAAATTAAGGAATGCTGCAGATATTGCTCAT

 181   Q  R  L  D  S  L  H  N  K  F  A  E  L  N  K  F  K  V  E  P
 541   CAAAGACTTGATTCTCTTCATAATAAATTTGCTGAATTGAATAAGTTCAAAGTAGAACCT

 201   Y  E  T  I  L  L  P  V  Y  A  Q  A  A  N  L  H  L  N  L  L
 601   TATGAAACAATTTTGCTACCGGTTTATGCACAAGCTGCCAACTTACATTTAAACTTGTTA

 221   Q  Q  G  A  M  F  A  D  Q  W  I  E  D  K  Y  S  S  R  N  D
 661   CAACAAGGGGCTATGTTTGCAGATCAATGGATTGAGGATAAATACTCATCGAGAAATGAT

 241   T  F  A  G  N  S  N  D  Y  Q  N  L  L  K  S  R  T  I  T  Y
 721   ACATTTGCTGGAAATTCAAATGATTATCAGAATTTGTTAAAATCTAGAACAATAACCTAT

 261   I  N  H  I  E  N  T  Y  Q  N  G  L  N  Y  L  W  N  Q  P  E
 781   ATAAATCATATTGAAAATACTTATCAAAATGGACTTAATTACTTGTGGAATCAACCGGAA
```

```
 281   M  T  W  D  I  Y  N  E  Y  R  T  K  M  T  I  T  A  L  D  L
 841   ATGACGTGGGATATATATAATGAATACCGAACAAAAATGACCATTACTGCATTAGATCTC

 301   M  A  L  F  P  F  Y  N  K  E  L  Y  D  P  T  V  G  I  K  S
 901   ATGGCATTATTCCCCTTTTATAACAAAGAATTATATGACCCTACAGTGGGTATAAAATCA

 321   E  L  T  R  E  I  F  I  N  T  P  V  E  P  H  L  H  R  Y  F
 961   GAACTTACAAGAGAAATATTTATTAATACACCTGTCGAACCACATTTACACAGGTACTTC

 341   K  L  S  E  T  E  E  K  L  T  N  N  S  D  L  F  K  W  L  T
1021   AAATTAAGTGAGACAGAAGAAAAACTTACAAATAATAGTGATTTATTTAAATGGCTAACA

 361   S  L  K  F  R  T  L  Y  Q  P  G  F  P  F  L  I  G  N  M  N
1081   AGTCTAAAATTTAGAACGTTATATCAACCTGGCTTCCCTTTTTTAATTGGAAATATGAAT

 381   S  F  T  N  T  N  G  T  Q  L  I  N  N  Q  Q  Q  L  W  S  F
1141   AGCTTTACAAATACAAACGGTACACAACTTATCAATAACCAACAGCAGCTTTGGTCGTTT

 401   P  G  T  T  E  N  E  E  K  L  F  P  S  P  A  N  I  D  Q  V
1201   CCTGGAACAACCGAAAATGAAGAAAAATTGTTCCCTTCACCAGCAAATATAGATCAAGTT

 421   T  M  Y  I  Y  Y  G  S  G  W  G  I  P  E  P  I  S  T  T  I
1261   ACTATGTATATTTATTATGGTAGCGGATGGGGTATTCCTGAGCCTATTAGTACTACTATA

 441   N  K  L  I  F  N  H  D  K  H  E  L  I  S  E  Y  D  A  G  N
1321   AATAAATTAATTTTTAATCATGATAAACATGAGCTAATATCAGAGTATGATGCTGGAAAT

 461   T  N  A  P  T  R  S  L  S  L  G  L  P  N  H  Y  L  S  C  L
1381   ACCAATGCCCCTACCAGGAGCTTATCTTTAGGTCTCCCAAATCACTATTTATCTTGCTTA

 481   N  S  Y  Y  P  L  T  A  T  T  D  G  M  N  K  E  E  L  K  M
1441   AATAGCTATTACCCTTTAACTGCTACTACGGATGGAATGAATAAAGAAGAACTTAAAATG

 501   Y  S  F  G  W  T  H  N  S  V  D  F  L  N  E  I  S  K  D  K
1501   TATTCGTTTGGATGGACACATAATAGTGTTGATTTTTTAAATGAAATTAGCAAAGACAAA

 521   I  T  Q  I  P  A  V  K  A  Y  R  L  T  S  N  S  R  V  I  K
1561   ATTACACAAATTCCTGCAGTAAAAGCCTATCGTTTAACCTCGAACTCTAGGGTAATAAAA

 541   G  P  S  H  I  G  G  N  L  V  Y  L  S  E  N  S  Q  M  A  L
1621   GGTCCTAGTCATATTGGTGGTAACTTGGTTTATCTTAGCGAGAATAGTCAAATGGCTTTA

 561   T  C  R  Y  T  N  S  S  P  Q  E  Y  K  I  R  I  R  Y  A  S
1681   ACTTGCAGGTACACAAATTCTTCTCCCCAGGAATATAAAATAAGAATTCGATATGCTTCA

 581   N  R  L  N  M  G  Q  L  F  T  T  F  S  S  H  Q  F  V  L  P
1741   AACAGATTGAACATGGGACAATTATTTACAACATTCAGTTCACACCAGTTTGTGCTTCCA

 601   P  T  F  N  H  F  N  I  E  Q  A  K  Y  E  D  Y  A  Y  A  E
1801   CCAACTTTCAATCATTTCAATATAGAACAAGCAAAATATGAAGATTATGCATATGCTGAA

 621   F  P  E  S  M  S  I  R  G  N  L  N  S  D  I  L  L  I  L  N
1861   TTTCCAGAAAGTATGTCGATTAGAGGTAATTTAAATTCTGATATATTATTAATACTCAAT

 641   I  L  A  G  G  E  L  L  L  D  K  I  E  F  I  P  L  T  Q  K
1921   ATATTAGCTGGGGGTGAATTACTTCTTGATAAAATAGAGTTTATACCATTAACTCAAAAA

 661   V  K  D  N  L  E  K  E  K  I  D  M  L  K  N  L  T  D  S  L
1981   GTTAAAGATAACCTAGAAAAGGAAAAAATAGATATGTTAAAAAATTTAACAGATTCATTA

 681   F  N  S  P  S  K  D  T  L  K  I  D  S  T  D  Y  Q  I  D  Q
2041   TTTAATAGTCCTTCAAAAGATACTTTAAAAATTGATAGTACAGATTATCAAATTGACCAA

 701   I  A  F  Q  I  E  S  I  N  E  E  I  N  P  Q  E  K  M  E  L
2101   ATCGCTTTTCAAATAGAGTCTATAAATGAAGAAATTAATCCACAAGAAAAGATGGAATTA

 721   L  D  N  I  K  Y  A  K  K  L  N  Q  L  R  N  L  L  Y  S  R
2161   CTAGATAATATAAAATATGCAAAAAAACTAAATCAATTACGAAATCTTTTATATTCTAGA
```

```
741  E S Q A Q I D W V T S N D V S I Y H G K
2221 GAGTCTCAAGCTCAGATAGATTGGGTAACAAGTAATGATGTTTCTATTTATCATGGTAAA

761  K P F N D Y T L V M S R T S S S L S E I
2281 AAACCATTTAATGACTATACTCTTGTTATGTCAAGAACAAGCTCAAGTTTATCAGAGATT

781  T A T N Y Q T Y I Y K K I E E S K L K P
2341 ACAGCAACAAACTATCAAACCTATATTTATAAAAAAATTGAAGAGTCTAAACTAAAACCA

801  Y T R Y L V R G F I S N S E D L E I F I
2401 TATACACGTTACCTGGTAAGAGGTTTCATAAGTAACAGCGAAGATTTAGAAATTTTTATT

821  S R Y E N E I H T N M N V H G D D D T L
2461 TCTCGATATGAAAATGAAATTCATACTAACATGAATGTTCATGGAGATGACGATACTCTT

841  L N S D I R Q N E C E S K L P I I F D A
2521 TTAAATTCAGATATACGACAAAATGAATGTGAATCTAAACTTCCAATTATATTTGATGCA

861  T S Q Y S L S P S R T S G I S N H S Y Y
2581 ACATCACAATATTCGCTGTCTCCAAGTCGTACTTCAGGTATATCTAATCATTCATATTAC

881  N N G H Q S S C N D T H I F S F S I D T
2641 AATAATGGACATCAGTCATCGTGCAATGACACCCACATATTTTCATTTTCTATTGATACA

901  G E V D F N N Y P G I E I L F K L S N T
2701 GGAGAAGTTGATTTTAATAATTATCCTGGTATTGAAATACTATTCAAACTTTCAAATACA

921  N G Y A S I S N L E V I E E R L L T E E
2761 AATGGGTACGCTTCAATAAGTAATTTAGAAGTAATAGAAGAGCGATTACTAACCGAAGAA

941  E K R Q I I Q I E N R W K A K K E S Q R
2821 GAAAAGCGACAAATTATTCAAATAGAAAATCGATGGAAAGCAAAAAAAGAAAGTCAACGC

961  N E T E K I T T Q A Q Q A I N S L F T D
2881 AATGAAACTGAAAAAATAACTACGCAAGCCCAACAAGCAATAAATAGTCTATTTACGGAT

981  T Q Y S N L K F E T T K Q N I T E A N T
2941 ACACAATATTCAAACTTAAAATTTGAAACAACTAAACAAAATATTACCGAAGCTAATACT

1001 I L E N I P Y V Y N A L L P T E P G M N
3001 ATTTTGGAAAACATCCCCTATGTTTACAATGCATTATTACCAACAGAACCAGGTATGAAT

1021 F V L F N S F K D Q I N K A H A L Y K M
3061 TTTGTTTTATTTAACAGTTTTAAAGATCAAATAAATAAAGCACACGCATTATATAAAATG

1041 R N L I K N G D F I N D T K Y W S I S T
3121 AGAAACTTAATTAAGAATGGTGATTTCATTAATGATACAAAATATTGGTCTATATCAACA

1061 D V K L E K V N K E T I L V L S S W E A
3181 GATGTTAAATTGGAAAAAGTCAACAAGGAAACTATTCTTGTTTTGTCGAGTTGGGAAGCA

1081 Q A S Q Q I L V Q K Q K R Y L L R V I A
3241 CAAGCATCTCAACAAATACTAGTACAAAAACAAAAACGATACCTACTCCGTGTCATAGCA

1101 K K E D M G R G N V I I S D C L N N I A
3301 AAAAAAGAAGATATGGGTAGAGGAAATGTGATAATCAGTGACTGTTTAAATAATATAGCT

1121 K I D F T P H D C N M N H I Q N S S E F
3361 AAAATAGATTTTACTCCCCATGATTGTAATATGAACCATATACAAAATTCATCAGAGTTT

1141 I I K T I H F S P N T E Q V R I D I G Q
3421 ATTATAAAAACAATACACTTTAGCCCAAATACTGAGCAAGTACGTATTGATATTGGCCAA

1161 S D G V F K V E S I E L I C V N Y -
3481 TCGGATGGTGTATTTAAAGTCGAAAGTATAGAGCTCATTTGCGTTAACTATTAA
```

***cry48Ab1* partial gene sequence and translated amino acid sequence of variant found in strain LP1G (AM237207)**

```
   1  M  D  I  N  N  N  N  E  K  E  I  I  N  S  H  L  L  P  A  S
   1  ATGGATATCAACAATAATAATGAAAAGGAAATTATTAATTCTCATTTACTACCGGCTTCA

  21  L  L  K  K  H  P  I  K  S  L  Q  S  T  N  Y  K  D  W  L  N
  61  CTTTTAAAAAAACATCCTATTAAGTCTTTACAGAGCACAAATTACAAAGATTGGCTTAAT

  41  L  C  Q  D  F  N  K  D  I  E  S  Y  D  L  V  T  A  V  S  S
 121  CTGTGTCAAGATTTTAATAAGGATATTGAAAGTTATGATTTGGTAACAGCTGTCTCGAGT

  61  G  T  I  V  V  G  T  M  L  S  A  I  Y  A  P  A  I  I  A  G
 181  GGGACTATTGTCGTAGGTACCATGTTGAGTGCTATTTATGCACCTGCAATTATAGCTGGC

  81  P  I  G  V  I  G  A  I  I  I  S  F  G  T  L  L  P  L  L  W
 241  CCTATAGGAGTAATAGGCGCTATCATTATATCTTTTGGCACTCTTCTACCTCTTCTATGG

 101  S  E  D  E  N  N  P  K  T  V  W  I  E  F  I  R  M  G  E  R
 301  TCTGAGGACGAGAATAACCCTAAAACGGTATGGATTGAATTTATTAGAATGGGAGAGCGC

 121  L  V  D  K  T  I  S  Q  T  V  L  N  I  L  E  S  Y  L  K  D
 361  CTTGTTGATAAAACAATATCGCAAACAGTGTTAAATATACTGGAAAGCTACTTAAAAGAT

 141  L  K  V  N  L  I  D  Y  E  K  A  K  Q  D  W  I  E  L  K  K
 421  TTAAAGGTAAATTTAATAGATTATGAAAAAGCGAAACAAGATTGGATTGAATTAAAAAAA

 161  Q  Q  L  P  G  S  P  P  S  I  N  L  R  N  A  A  D  I  A  H
 481  CAGCAACTTCCTGGTTCACCTCCTTCTATTAACTTAAGGAATGCTGCAGATATTGCTCAT

 181  Q  R  L  D  S  L  H  N  K  F  A  E  L  N  V  F  K  V  A  S
 541  CAAAGACTTGATTCACTTCATAATAAGTTTGCTGAATTGAATGTGTTCAAAGTGGCATCT

 201  Y  E  T  I  L  L  P  V  Y  A  Q  A  A  N  L  H  L  N  L  L
 601  TATGAAACAATTTTGCTACCAGTTTATGCACAAGCTGCCAACTTACATTTAAACTTGTTA

 221  Q  Q  G  A  M  F  A  D  Q  W  I  E  D  K  Y  S  P  R  N  D
 661  CAACAAGGGGCTATGTTTGCAGATCAATGGATTGAGGATAAATATTCACCGAGAAATGAT

 241  T  F  A  G  N  S  N  D  Y  Q  D  L  L  K  S  R  T  I  T  Y
 721  ACATTTGCAGGAAATTCAAATGATTATCAGGATTTGTTAAAATCTAGAACAATAACCTAT

 261  I  N  H  I  E  N  T  Y  K  D  G  L  N  Y  L  W  N  Q  P  E
 781  ATAAATCACATTGAAAATACTTACAAAGATGGACTTAATTACTTATGGAATCAACCGGAA

 281  M  T  W  D  I  Y  N  E  Y  R  T  N  M  T  L  T  A  L  D  L
 841  ATGACGTGGGATATATATAATGAATACCGAACAAATATGACCCTTACTGCATTAGATCTC

 301  L  P  L  F  P  F  Y  N  K  E  L  Y  D  P  R  V  G  I  K  S
 901  TTGCCATTATTTCCTTTTTATAACAAAGAATTATATGATCCTAGAGTTGGTATAAAATCA

 321  E  L  T  R  E  V  Y  I  N  T  P  V  D  P  H  L  H  R  Y  F
 961  GAACTTACAAGAGAAGTATATATTAATACACCTGTAGATCCACATTTACACAGGTACTTC

 341  K  L  G  E  T  E  D  K  L  T  N  N  S  E  L  F  K  W  L  T
1021  AAATTAGGTGAGACAGAAGATAAACTTACAAATAATAGTGAGTTATTTAAATGGCTAACA

 361  S  L  K  F  R  T  F  N  Q  P  G  F  P  F  L  I  G  N  M  N
1081  AGTCTAAAATTTAGAACGTTTAATCAACCTGGATTCCCTTTTTTAATTGGAAATATGAAT

 381  Y  F  K  K  T  N  G  T  Q  L  I  N  N  Q  Q  Q  L  W  S  F
1141  TACTTTAAAAAAACAAACGGTACACAACTTATCAATAACCAACAGCAGCTTTGGTCGTTT

 401  P  G  T  T  E  I  E  K  L  F  P  S  P  A  N  I  D  K  V  T
1201  CCTGGAACAACCGAAATTGAAAAATTGTTCCCTTCACCGGCAAATATAGATAAAGTTACT

 421  M  Y  I  Y  Y  G  S  G  W  E  V  P  E  P  I  S  I  T  I  N
1261  ATGTATATTTATTATGGTAGCGGATGGGAAGTTCCTGAGCCTATTAGTATTACTATAAAT
```

```
 441  K  L  I  F  N  H  H  K  H  G  L  I  T  E  Y  D  A  G  N  T
1321  AAATTAATTTTTAATCATCATAAACATGGGCTAATAACAGAGTACGATGCTGGAAATACC

 461  N  A  P  T  M  G  I  Y  V  N  L  P  K  H  Y  L  S  C  L  N
1381  AATGCCCCTACCATGGGAATATATGTAAACCTCCCAAAACACTATTTATCTTGCTTAAAT

 481  S  Y  Y  P  L  T  A  T  T  N  G  M  G  K  E  E  L  K  M  Y
1441  AGCTATTATCCTTTAACTGCTACTACGAATGGAATGGGTAAAGAAGAACTTAAAATGTAT

 501  S  F  G  W  T  H  E  S  V  D  F  L  N  E  I  S  N  D  K  I
1501  TCGTTTGGATGGACACATGAAAGTGTTGATTTTTTAAATGAAATTAGCAATGACAAAATT

 521  T  Q  I  P  A  V  K  A  Y  N  L  N  S  N  S  R  V  I  K  G
1561  ACACAAATCCCTGCAGTAAAAGCCTATAATTTAAATTCGAACTCTAGGGTAATTAAAGGC

 541  P  G  H  I  G  G  N  L  V  Y  L  S  D  K  S  Q  L  S  L  A
1621  CCTGGTCATATCGGTGGAAACTTGGTTTATCTTAGTGATAAGAGTCAACTATCTTTAGCT

 561  C  R  Y  T  N  S  S  P  Q  D  F  L  I  R  I  R  Y  A  S  N
1681  TGCAGGTACACAAATTCTTCTCCTCAGGATTTTTTAATAAGAATTCGATATGCTTCAAAT

 581  K  R  N  M  V  Q  L  F  T  P  F  S  T  H  Q  F  V  L  P  Q
1741  AAGCGGAACATGGTACAACTATTTACGCCCTTCAGTACACACCAATTTGTGCTTCCACAA

 601  T  F  N  H  L  N  I  E  Q  T  K  Y  E  D  Y  E  Y  A  Q  L
1801  ACTTTCAATCATTTGAATATAGAACAAACAAAATATGAAGATTACGAATATGCTCAACTT

 621  P  G  S  L  T  I  N  G  N  V  N  I  D  L  L  F  L  L  N  V
1861  CCAGGAAGTTTAACGATTAACGGTAATGTAAATATAGATTTATTATTTCTGCTCAATGTA

 641  L  D  G  G  E  L  L  L  D  K  I  E  F  I  P  L  T  Q  K  V
1921  TTAGATGGTGGTGAATTACTTCTTGATAAAATAGAGTTTATACCATTAACTCAAAAAGTT

 661  K  D  N  L  E  K  E  K  I  D  M  L  K  N  L  T  D  S  L  F
1981  AAAGATAACCTAGAAAAGGAAAAAATAGATATGTTAAAAAATTTAACAGATTCATTATTT

 681  N  S  P  A  K  D  T  L  K  I  N  S  T  D  Y  Q  I  D  Q  I
2041  AATAGTCCTGCAAAAGATACTTTAAAAATTAATAGTACAGATTATCAAATTGACCAAATC

 701  A  F  Q  I  E  S  I  N  E  E  I  N  T  Q  E  K  M  K  L  L
2101  GCTTTTCAAATAGAGTCTATAAATGAAGAAATTAATACACAAGAAAAGATGAAATTACTA

 721  D  N  I  K  Y  A  K  K  L  N  Q  L  R  N  L  L  Y  S  R  E
2161  GATAATATAAAATATGCAAAAAAACTAAATCAATTACGAAATCTTTTATATTCTAGAGAG

 741  S  Q  A  Q  I  D  W  V  T  S  N  D  V  S  I  Y  H  G  K  K
2221  TCTCAAGCTCAAATAGATTGGGTAACAAGTAATGATGTTTCTATTTATCATGGTAAAAAA

 761  P  F  N  E  Y  T  L  V  M  S  G  S  S  L  S  K  I  T  S  S
2281  CCATTTAATGAATATACTCTTGTTATGTCAGGATCAAGTTTATCGAAGATTACATCTTCA

 781  N  Y  P  T  Y  I  Y  K  K  I  E  E  S  K  L  K  P  Y  T  R
2341  AACTATCCAACCTATATTTATAAAAAAATTGAAGAGTCTAAACTAAAACCATATACACGT

 801  Y  L  V  R  G  F  I  S  N  S  D  N  L  E  I  F  I  S  R  Y
2401  TACCTGGTGAGGGGTTTTATAAGTAACAGTGACAATTTAGAAATTTTTATATCTCGATAC

 821  E  N  E  I  H  T  N  M  N  V  H  V  D  D  D  T  L  L  N  S
2461  GAAAATGAAATTCATACCAATATGAATGTTCATGTAGACGACGATACTCTATTAAATTCA

 841  Y  K  R  Q  N  E  C  E  S  K  L  P  I  V  F  D  E  T  S  Q
2521  TATAAACGACAAAACGAATGTGAATCTAAACTCCCAATTGTTTTTGATGAAACATCACAA

 861  Y  P  L  S  P  S  R  T  S  G  I  S  N  H  S  Y  Y  N  G  A
2581  TATCCACTGTCTCCTAGTCGTACTTCAGGCATATCTAATCATTCATATTATAATGGTGCA

 881  Q  Q  S  S  C  H  D  T  Q  I  F  S  F  S  I  D  T  G  D  V
2641  CAGCAGTCATCGTGCCATGACACCCAGATATTTTCATTTTCTATTGATACAGGAGACGTT
```

```
 901  D  F  N  E  Y  P  G  I  E  I  L  F  K  L  S  N  S  N  G  Y
2701  GATTTTAATGAATATCCCGGTATTGAAATTCTATTCAAACTTTCTAATTCAAATGGGTAC

 921  A  S  I  S  N  L  E  V  I  E  E  R  L  L  T  E  E  E  K  R
2761  GCTTCAATAAGTAATTTAGAAGTAATAGAAGAGCGATTACTAACCGAAGAAGAAAAACGA

 941  H  I  I  E  I  E  N  R  W  K  A  K  K  E  I  Q  R  N  E  T
2821  CATATTATTGAAATAGAAAATCGATGGAAAGCAAAAAAAGAAATTCAACGCAATGAAACT

 961  E  K  E  T  T  Q  A  Q  Q  A  I  N  N  L  F  T  D  T  Q  Y
2881  GAAAAAGAAACTACGCAAGCACAACAAGCAATAAATAATTTATTTACGGATACACAATAT

 981  S  K  L  K  F  E  T  T  K  Q  S  I  S  K  A  N  A  I  L  E
2941  TCAAAATTAAAATTTGAAACAACTAAACAAAGTATTAGCAAAGCTAATGCTATTTTGGAA

1001  N  I  P  Y  V  Y  N  S  L  L  P  T  E  P  G  M  N  F  E  L
3001  AACATCCCCTATGTGTACAATTCATTATTACCAACAGAACCAGGTATGAATTTTGAATTA

1021  F  N  S  F  K  D  Q  I  N  K  A  H  T  L  Y  K  M  R  N  S
3061  TTTAATAGTTTTAAAGATCAAATAAATAAAGCACACACATTATATAAAATGAGAAATTCA

1041  I  K  N  G  D  F  I  N  G  T  E  Y  W  S  I  S  T  D  V  K
3121  ATTAAGAATGGTGATTTCATCAATGGTACAGAATATTGGTCTATTTCAACAGATGTTAAA

1061  L  E  K  T  N  I  E  T  I  L  V  M  S  S  W  S  A  Q  S  S
3181  CTGGAAAAAACGAACATTGAAACTATTCTTGTTATGTCGAGTTGGAGCGCCCAATCATCT

1081  Q  Q  I  L  V  Q  K  Q  N  R  Y  L  L  R  V  I  A  K  K  E
3241  CAACAAATACTAGTACAAAAACAAAATCGATACCTACTCCGTGTCATAGCAAAAAAAGAA

1101  D  M  G  S  G  N  V  T  I  S  D  C  L  N  N  I  A  K  I  E
3301  GATATGGGTAGTGGAAATGTGACAATCAGTGACTGTTTAAATAATATAGCTAAAATAGAG

1121  F  I  P  H  D  C  N  M  N
3361  TTTATTCCCCATGATTGTAATATGAACC
```

cry48Ab2 partial gene sequence and translated amino acid sequence of variant found in strain 2173 (AM237208)

```
   1  M  D  I  N  N  N  N  E  K  E  I  I  N  S  H  L  L  P  A  S
   1  ATGGATATCAACAATAATAATGAAAAGGAAATTATTAATTCTCATTTACTACCGGCTTCA

  21  L  L  K  K  H  P  I  K  S  L  Q  S  T  N  Y  K  D  W  L  N
  61  CTTTTAAAAAAACATCCTATTAAGTCTTTACAGAGCACAAATTACAAAGATTGGCTTAAT

  41  L  C  Q  D  F  N  K  D  I  E  S  Y  D  L  V  T  A  V  S  S
 121  CTGTGTCAAGATTTTAATAAGGATATTGAAAGTTATGATTTGGTAACAGCTGTCTCGAGT

  61  G  T  I  V  V  G  T  M  L  S  A  I  Y  A  P  A  I  I  A  G
 181  GGGACTATTGTCGTAGGTACCATGTTGAGTGCTATTTATGCACCTGCAATTATAGCTGGC

  81  P  I  G  V  I  G  A  I  I  I  S  F  G  T  L  L  P  L  L  W
 241  CCTATAGGAGTAATAGGCGCTATCATTATATCTTTTGGCACTCTTCTACCTCTTCTATGG

 101  S  E  D  E  N  N  P  K  T  V  W  I  E  F  I  R  M  G  E  R
 301  TCTGAGGACGAGAATAACCCTAAAACGGTATGGATTGAATTTATTAGAATGGGAGAGCGC

 121  L  V  D  K  T  I  S  Q  T  V  L  N  I  L  E  S  Y  L  K  D
 361  CTTGTTGATAAAACAATATCGCAAACAGTGTTAAATATACTGGAAAGCTACTTAAAAGAT

 141  L  K  V  N  L  I  D  Y  E  K  A  K  Q  D  W  I  E  L  K  K
 421  TTAAAGGTAAATTTAATAGATTATGAAAAAGCGAAACAAGATTGGATTGAATTAAAAAAA

 161  Q  Q  L  P  G  S  P  P  S  I  N  L  R  N  A  A  D  I  A  H
 481  CAGCAACTTCCTGGTTCACCTCCTTCTATTAACTTAAGGAATGCTGCAGATATTGCTCAT
```

181 Q R L D S L H N K F A E L N V F K V A S
541 CAAAGACTTGATTCACTTCATAATAAGTTTGCTGAATTGAATGTGTTCAAAGTGGCATCT

201 Y E T I L L P V Y A Q A A N L H L N L L
601 TATGAAACAATTTTGCTACCAGTTTATGCACAAGCTGCCAACTTACATTTAAACTTGTTA

221 Q Q G A M F A D Q W I E D K Y S P R N D
661 CAACAAGGGGCTATGTTTGCAGATCAATGGATTGAGGATAAATATTCACCGAGAAATGAT

241 T F A G N S N D Y Q D L L K S R T I T Y
721 ACATTTGCAGGAAATTCAAATGATTATCAGGATTTGTTAAAATCTAGAACAATAACCTAT

261 I N H I E N T Y K D G L N Y L W N Q P E
781 ATAAATCACATTGAAAATACTTACAAAGATGGACTTAATTACTTATGGAATCAACCGGAA

281 M T W D I Y N E Y R T N M T L T A L D L
841 ATGACGTGGGATATATATAATGAATACCGAACAAATATGACCCTTACTGCATTAGATCTC

301 L P L F P F Y N K E L Y D P R V G I K S
901 TTGCCATTATTTCCTTTTTATAACAAAGAATTATATGATCCTAGAGTTGGTATAAAATCA

321 E L T R E V Y I N T P V D P H L H R Y F
961 GAACTTACAAGAGAAGTATATATTAATACACCTGTAGATCCACATTTACACAGGTACTTC

341 K L G E T E D K L T N N S E L F K W L T
1021 AAATTAGGTGAGACAGAAGATAAACTTACAAATAATAGTGAGTTATTTAAATGGCTAACA

361 S L K F R T F N Q P G F P F L I G N M N
1081 AGTCTAAAATTTAGAACGTTTAATCAACCTGGATTCCCTTTTTTAATTGGAAATATGAAT

381 Y F K K T N G T Q L I N N Q Q Q L W S F
1141 TACTTTAAAAAAACAAACGGTACACAACTTATCAATAACCAACAGCAGCTTTGGTCGTTT

401 P G T T E I E K L F P S P A N I D K V T
1201 CCTGGAACAACCGAAATTGAAAAATTGTTCCCTTCACCGGCAAATATAGATAAAGTTACT

421 M Y I Y Y G S G W E V P E P I S I T I N
1261 ATGTATATTTATTATGGTAGCGGATGGGAAGTTCCTGAGCCTATTAGTATTACTATAAAT

441 K L I F N H H K H G L I T E Y D A G N T
1321 AAATTAATTTTTAATCATCATAAACATGGGCTAATAACAGAGTACGATGCTGGAAATACC

461 N A P T M G I Y V N L P K H Y L S C L N
1381 AATGCCCCTACCATGGGAATATATGTAAACCTCCCAAAACACTATTTATCTTGCTTAAAT

481 S Y Y P L T A T T N G M G K E E L K M Y
1441 AGCTATTATCCTTTAACTGCTACTACGAATGGAATGGGTAAAGAAGAACTTAAAATGTAT

501 S F G W T H E S V D F L N E I S N D K I
1501 TCGTTTGGATGGACACATGAAAGTGTTGATTTTTTAAATGAAATTAGCAATGACAAAATT

521 T Q I P A V K A Y N L N S N S R V I K G
1561 ACACAAATCCCTGCAGTAAAAGCCTATAATTTAAATTCGAACTCTAGGGTAATTAAAGGC

541 P G H I G G N L V Y L S D K S Q L S L A
1621 CCTGGTCATATCGGTGGAAACTTGGTTTATCTTAGTGATAAGAGTCAACTATCTTTAGCT

561 C R Y T N S S P Q D F L I R I R Y A S N
1681 TGCAGGTACACAAATTCTTCTCCTCAGGATTTTTTAATAAGAATTCGATATGCTTCAAAT

581 K R N M V Q L F T P F S T H Q F V L P Q
1741 AAGCGGAACATGGTACAACTATTTACGCCCTTCAGTACACACCAATTTGTGCTTCCACAA

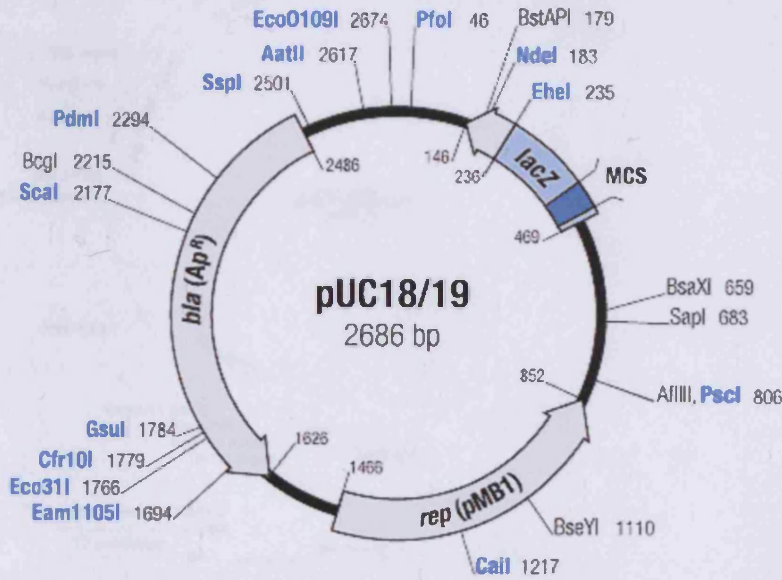
601 T F N H L N I E Q T K Y E D Y E Y A Q L
1801 ACTTTCAATCATTTGAATATAGAACAAACAAAATATGAAGATTACGAATATGCTCAACTT

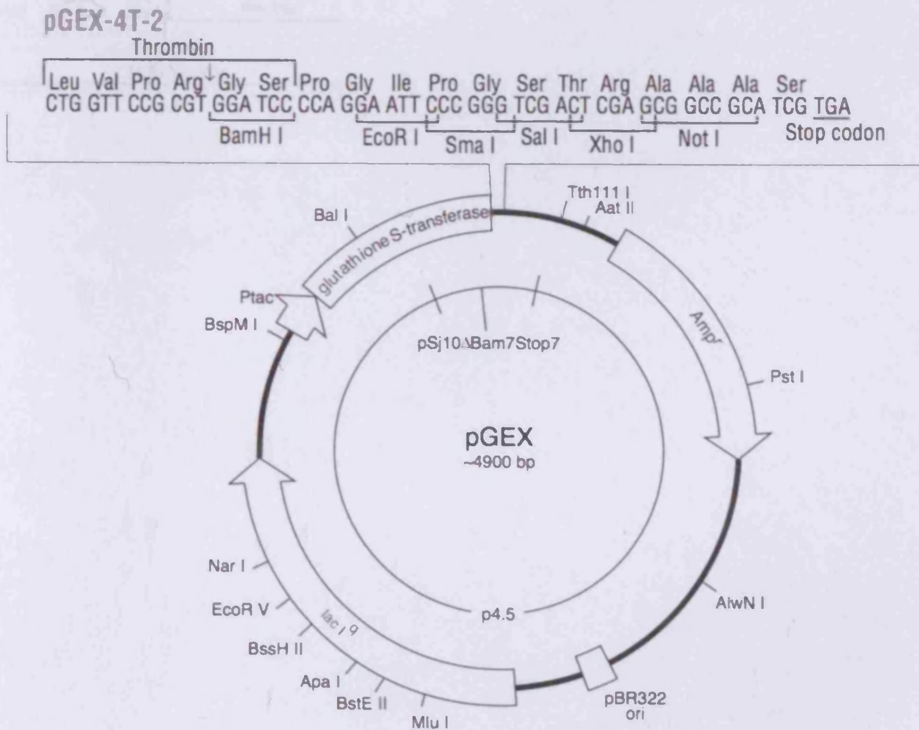
621 P G S L T I N G N V N I D L L F L L N V
1861 CCAGGAAGTTTAACGATTAACGGTAATGTAAATATAGATTTATTATTTCTGCTCAATGTA

```
641   L  D  G  G  E  L  L  L  D  K  I  E  F  I  P  L  T  Q  K  V
1921  TTAGATGGTGGTGAATTACTTCTTGATAAAATAGAGTTTATACCATTAACTCAAAAAGTT

661   K  D  N  L  E  K  E  K  I  D  M  L  K  N  L  T  D  S  L  F
1981  AAAGATAACCTAGAAAAGGAAAAAATAGATATGTTAAAAAATTTAACAGATTCATTATTT

681   N  S  P  A  K  D  T  L  K  I  D  S  T  D  Y  Q  I  D  Q  I
2041  AATAGTCCTGCAAAAGATACTTTAAAAATTGATAGTACAGATTATCAAATTGACCAAATC

701   A  F  Q  I  E  S  I  N  E  E  I  N  T  Q  E  K  M  K  L  L
2101  GCTTTTCAAATAGAGTCTATAAATGAAGAAATTAATACACAAGAAAAGATGAAATTACTA

721   D  N  I  K  Y  A  K  K  L  N  Q  L  R  N  L  L  Y  S  R  E
2161  GATAATATAAAATATGCAAAAAAACTAAATCAATTACGAAATCTTTTATATTCTAGAGAG

741   S  Q  A  Q  I  D  W  V  T  S  N  D  V  S  I  Y  H  G  K  K
2221  TCTCAAGCTCAAATAGATTGGGTAACAAGTAATGATGTTTCTATTTATCATGGTAAAAAA

761   P  F  N  E  Y  T  L  V  M  S  G  S  S  L  S  K  I  T  S  S
2281  CCATTTAATGAATATACTCTTGTTATGTCAGGATCAAGTTTATCGAAGATTACATCTTCA

781   N  Y  P  T  Y  I  Y  K  K  I  E  E  S  K  L  K  P  Y  T  R
2341  AACTATCCAACCTATATTTATAAAAAAATTGAAGAGTCTAAACTAAAACCATATACACGT

801   Y  L  V  R  G  F  I  S  N  S  D  N  L  E  I  F  I  S  R  Y
2401  TACCTGGTGAGGGGTTTTATAAGTAACAGTGACAATTTAGAAATTTTTATATCTCGATAC

821   E  N  E  I  H  T  N  M  N  V  H  V  D  D  D  T  L  L  N  S
2461  GAAAATGAAATTCATACCAATATGAATGTTCATGTAGACGACGATACTCTATTAAATTCA

841   Y  K  R  Q  N  E  C  E  S  K  L  P  I  V  F  D  E  T  S  Q
2521  TATAAACGACAAAACGAATGTGAATCTAAACTCCCAATTGTTTTTGATGAAACATCACAA

861   F  P  L  S  P  S  R  T  S  G  I  S  N  H  S  Y  Y  N  G  A
2581  TTTCCACTGTCTCCTAGTCGTACTTCAGGCATATCTAATCATTCATATTATAATGGTGCA

881   Q  Q  S  S  C  H  D  T  Q  I  F  S  F  S  I  D  T  G  D  V
2641  CAGCAGTCATCGTGCCATGACACCCAGATATTTTCATTTTCTATTGATACAGGAGACGTT

901   D  F  N  E  Y  P  G  I  E  I  L  F  K  L  S  N  S  N  G  Y
2701  GATTTTAATGAATATCCCGGTATTGAAATTCTATTCAAACTTTCTAATTCAAATGGGTAC

921   A  S  I  S  N  L  E  V  I  E  E  R  L  L  T  E  E  E  K  R
2761  GCTTCAATAAGTAATTTAGAAGTAATAGAAGAGCGATTACTAACCGAAGAAGAAAAACGA

941   H  I  I  E  I  E  N  R  W  K  A  K  K  E  I  Q  R  N  E  T
2821  CATATTATTGAAATAGAAAATCGATGGAAAGCAAAAAAAGAAATTCAACGCAATGAAACT

961   E  K  E  T  T  Q  A  Q  Q  A  I  N  N  L  F  T  D  T  Q  Y
2881  GAAAAAGAAACTACGCAAGCCCAACAAGCAATAAATAATTTATTTACGGATACACAATAT

981   S  K  L  K  F  E  T  T  K  Q  S  I  S  K  A  N  A  I  L  E
2941  TCAAAATTAAAATTTGAAACAACTAAACAAAGTATTAGCAAAGCTAATGCTATTTTGGAA

1001  N  I  P  Y  V  Y  N  S  L  L  P  T  E  P  G  M  N  F  E  L
3001  AACATCCCCTATGTGTACAATTCATTATTACCAACAGAACCAGGTATGAATTTTGAATTA

1021  F  N  S  F  K  D  Q  I  N  K  A  H  T  L  Y  K  M  R  N  S
3061  TTTAATAGTTTTAAAGATCAAATAAATAAAGCACACACATTATATAAAATGAGAAATTCA

1041  I  K  N  G  D  F  I  N  G  T  K  Y  W  S  I  S  T  D  V  K
3121  ATTAAGAATGGTGATTTCATCAATGGTACAAAATATTGGTCTATTTCAACAGATGTTAAA

1061  L  E  K  T  N  I  E  T  I  L  V  M  S  S  W  S  A  Q  S  S
3181  CTGGAAAAAAACGAACATTGAAACTATTCTTGTTATGTCGAGTTGGAGCGCCCAATCATCT

1081  Q  Q  I  L  V  Q  K  Q  N  R  Y  L  L  R  V  I  A  K  K  E
3241  CAACAAATACTAGTACAAAAACAAAATCGATACCTACTCCGTGTCATAGCAAAAAAAGAA
```

```
1101   D  M  G  S  G  N  V  T  I  S  D  C  L  N  N  I  A  K  I  E
3301   GATATGGGTAGTGGAAATGTGACAATCAGTGACTGTTTAAATAATATAGCTAAAATAGAG

1121   F  I  P  H  D  C  N  M  N
3361   TTTATTCCCCATGATTGTAATATGAACC
```

Appendix 3. **Maps of commercially available vectors used in this study**





pET-22b(+)
(5493bp)

Ava I(158)
Xho I(158)
Not I(166)
Eag I(166)
Hind III(173)
Sal I(179)
Sac I(190)
EcoR I(192)
BamH I(198)
Nco I(220)
Msc I(225)
BseR I(260)
BspM I(268)
Nde I(288)
Xba I(326)
Bgl II(392)
SgrA I(433)
Sph I(589)
PflM I(696)
ApaB I(798)
Mlu I(1114)
Bcl I(1128)
BstE II(1295)
Bmg I(1323)
Apa I(1325)
BssH II(1525)
Hpa I(1620)
PshA I(1959)
Psp5 II(2221)
Bpu10 I(2321)
BspG I(2741)
Tth111 I(2960)
Bst1107 I(2986)
Sap I(3099)
BspLU11 I(3215)
AlwN I(3631)
Eam1105 I(4106)
Bsa I(4169)
Pst I(4353)
Pvu I(4478)
Sca I(4588)
Dra III(5251)
Bpu1102 I(80)

f1 origin (5027-5482)
Ap (4038-4895)
lacI (764-1843)
ori (3277)

T7 promoter primer #69348-3

T7 promoter

Bgl II — lac operator — *Xba* I — rbs

AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGA

Nde I — *BspM* I — pelB leader — *Msc* I *Nco* I — *BamH* I *EcoR* I *Sac* I

TATACATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGAATTAATTCGGATCCGAATTCGAGCTCC

MetLysTyrLeuLeuProThrAlaAlaAlaGlyLeuLeuLeuLeuAlaAlaGlnProAlaMetAlaMetAspIleGlyIleAsnSerAspProAsnSerSerSer

signal peptidase

Sal I — *Hind* III — *Eag* I *Not* I — *Ava* I* *Xho* I — His·Tag — *Bpu*1102 I

GTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAAC

ValAspLysLeuAlaAlaAlaLeuGluHisHisHisHisHisHisEnd

T7 terminator primer #69337-3

T7 terminator

TAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG

Publications

Jones, G.W., Monnerat, R., Yuan, Z. and Berry, C. Cloning, expression and characterisation of a novel *Bacillus sphaericus* binary toxin, that can overcome resistance to *Culex quinquefasciatus*. **Manuscript in preparation.**

Stein, C., Jones, G.W., Chalmers, T. and Berry, C. **(2006)** Transcriptional analysis of the toxin-coding plasmid pBtoxis from *Bacillus thuringiensis* subsp. *israelensis*. **Appl. Environ. Microbiol. 72 (3): 1771-1776**

Gammon, K., Jones, G.W., Hope, S.J., de Oliveira, C.M.F., Regis, L., Silva-Filha, M.H., Dancer, B.N. and Berry, C. **(2006)** Conjugal transfer of a toxin-coding megaplasmid from *Bacillus thuringiensis* subsp. *israelensis* to mosquitocidal strains of *Bacillus sphaericus*. **Appl. Environ. Microbiol. 72 (3): 1766-1770**

Vlachakis, D.P., Berry, C., Jones, G.W. and Brancale, A. **(2005)** An assay for the biological testing of potential inhibitors for the HCV helicase, Dengue virus helicase and Dengue virus helicase/protease complex (NS3 domain). **Antiviral Research 65: A60**

Monnerat, R., da Silva, S.F., Dias, D.S., Martins, E.S., Praça, L.B., Jones, G.W., Soares, C.M., de Souza Dias, J.M.C. and Berry, C. **(2004)** Screening of Brazilian *Bacillus sphaericus* strains for high toxicity against *Culex quinquefasciatus* and *Aedes aegypti*. **J. Appl. Entomol. 128: 469-473**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2006, p. 1771–1776 Vol. 72, No. 3
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.3.1771–1776.2006
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Transcriptional Analysis of the Toxin-Coding Plasmid pBtoxis from *Bacillus thuringiensis* subsp. *israelensis*

Claudia Stein, Gareth W. Jones, Tanya Chalmers, and Colin Berry*

Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, United Kingdom

Received 13 December 2005/Accepted 14 December 2005

In *Bacillus thuringiensis* subsp. *israelensis* all of the insecticidal toxins are encoded on a single, large plasmid, pBtoxis. Sequencing of this plasmid revealed 125 potential coding sequences, many of which have predicted functions in gene regulation and physiological processes, such as germination. As a first step in understanding the possible role of pBtoxis in its host bacterium, a survey of the transcription of genes with predicted functions was carried out. Whereas many coding sequences, including those previously identified as probable pseudogenes, were not transcribed, mRNA was detected for 29 of the 40 sequences surveyed. Several of these sequences, including eight with similarities to the sequences of known transcriptional regulators, may influence wider gene regulation and thus may alter the phenotype of the host bacterium.

Bacillus thuringiensis subsp. *israelensis* is widely used for control of dipteran pest insects and vectors of human disease, such as mosquitoes and blackflies. This bacterium has an excellent safety record, and there have been no reports of insect resistance in the field. An important factor in this lack of resistance is the complex arsenal of toxins produced by this organism (5), all of which are encoded by a single, large plasmid, pBtoxis. Sequencing of this plasmid (3) revealed 125 coding sequences (CDSs), including the sequences of the previously identified toxin genes (*cry4Aa*, *cry4Ba*, *cry11Aa*, *cry10Aa*, *cyt1Aa*, and *cyt2Ba*); a new, putative toxin gene, *cyt1Ca*, encoding a previously undescribed fusion toxin; several CDSs corresponding to fragments of toxin-encoding sequences; and numerous transposition-associated CDSs. Whereas such sequences accounted for 30% of the CDSs, it is likely that other plasmid CDSs may also have an effect on the host cell phenotype. Several of the CDSs appeared to be related to genes encoding transcriptional regulators in other organisms. Production of such regulators might alter the transcription of genes located both on the plasmid and on the chromosome and thereby have significant effects on the phenotype and, perhaps, on virulence.

As a first step in analyzing the possible roles of such plasmid genes, we performed a transcriptional survey of the pBtoxis genes using previously identified orthologs in other organisms. Clearly, effects on the phenotype can be exerted only through expression of the plasmid CDSs. Thus, we identified potentially important genes that could be investigated further to increase our understanding of the molecular biology of *B. thuringiensis* subsp. *israelensis*.

MATERIALS AND METHODS

Bacterial strains. For this survey, we utilized *B. thuringiensis* subsp. *israelensis* strain 4Q7 (also known as 4Q2-81), a plasmidless strain, in order to prescreen CDS-specific primers to ensure that there were no genomic copies of the genes that might produce false-positive results for pBtoxis-derived gene expression. *B. thuringiensis* subsp. *israelensis* strain 4Q5 (also known as 4Q2-72) has been cured of all plasmids except pBtoxis and was used to determine whether transcripts were produced from CDSs on this plasmid (since full sequences have not been determined for all *B. thuringiensis* subsp. *israelensis* plasmids, the use of strain 4Q5 eliminated false-positive results if related CDSs were present on other plasmids).

CDSs used. Table 1 shows the subset of pBtoxis CDSs surveyed in this study and their relatedness to previously reported genes, as identified during sequencing of the plasmid (3). As a positive control, the gene encoding the Cry11Aa toxin (pBt023) was chosen since production of this protein, like production of Cry4Aa, Cry4Ba, and Cyt1Aa, is known to be significant (13).

Detection strategy. For each CDS surveyed, a pair of oligonucleotide primers was designed so that they had annealing temperatures in the approximate range from 58 to 66°C and produced 128- to 703-bp amplicons from the target sequences (Table 2). The abilities of the primer pairs to produce the desired amplicons were confirmed by colony PCR using vegetative cells of strain 4Q5 as the template; the PCR conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then a final extension at 72°C for 10 min. Each primer pair was then used in a similar colony PCR using strain 4Q7 to test for the presence of related CDSs in the host genomic DNA. As a further control, PCRs were carried out with primer pairs in the absence of any template.

To detect transcription, RNA was isolated from *B. thuringiensis* subsp. *israelensis* 4Q5 cultures grown at 30°C with shaking in NYSM medium (11), using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Samples (1 ml) were removed for RNA isolation at approximately 12, 24, and 36 h after inoculation of 30 ml of medium with one colony from a plate that was incubated overnight. These times were chosen since they corresponded to times when there were rapidly dividing vegetative cells at the early times and when there were cells in different phases of the sporulation process later. RNAs from these different times were then pooled for reverse transcription since it was concluded that the reverse transcription-PCR technique is so sensitive that information concerning the stage specificity of transcription could not be derived reliably. For reverse transcription, Superscript RNase H^- reverse transcriptase (Invitrogen) was used to obtain cDNA from approximately 200 ng of isolated RNA with 12.5 μg/μl random hexamer primers in a 20-μl (final volume) mixture according to the manufacturer's instructions. In each case, a duplicate sample to which no reverse transcriptase was added was prepared as a negative control. This sample was used in subsequent PCRs in parallel with the reverse-transcribed RNA to ensure that there was no bacterial DNA contamination of the RNA samples that would lead to false-positive results. PCR was carried out under the conditions described above for the colony PCR except that the initial step consisting of 95°C for 5 min was omitted. Amplified products were visualized in 1.5% agarose gels (>200-bp amplicons) or 2% agarose gels (<200-bp amplicons) that were stained with ethidium bromide.

RESULTS AND DISCUSSION

Genomic homologs. No CDS-specific primer pairs produced amplicons in template-free controls, indicating that artifactual

* Corresponding author. Mailing address: Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, United Kingdom. Phone: 44-29-20874508. Fax: 44-29-20874116. E-mail: Berry@cf.ac.uk.

TABLE 1. CDSs included in the transcriptional survey

CDS[a]	Predicted product	Transcriptional activity
pBt011	Putative DNA-binding protein	Yes
pBt014	Probable transcriptional regulator	Yes
pBt020	Hypothetical protein	No
pBt021	Cyt1Aa toxin	Yes
pBt023	Cry11Aa crystal toxin	Yes
pBt029	Putative DNA-binding protein	No
pBt031	Putative *N*-acetylmuramoyl-L-alanine amidase (peptidoglycan hydrolase)	Yes
pBt036	Cyt2Ba cytolytic delta-endotoxin	Yes
pBt054	Cyt1Ca possible two-domain toxin	Yes
pBt056	Hypothetical protein (potential pseudogene; matches pBt152 with two frameshifts and an in-frame stop)	No
pBt060	Putative spore germination protein (pseudogene; contains two potential frameshifts)	No
pBt063	Putative spore germination protein (pseudogene; truncated by *IS240* insertion)	No
pBt075	Hypothetical protein; weakly similar to a *Yersinia pestis* plasmid hypothetical protein	Yes
pBt084	Putative spore germination protein; similar to *B. subtilis* GerAC	Yes
pBt085	Putative spore germination protein; similar to *B. subtilis* GerBB	Yes
pBt086	Putative spore germination protein; similar to *B. subtilis* GerKA	Yes
pBt087	Putative 1-phosphatidylinositol phosphodiesterase precursor; contains an in-frame TGA stop after amino acid 86	No
pBt091	Putative transcriptional regulator, ArsR family	Yes
pBt092	Small DNA-binding protein (bacterial histone-like family)	Yes
pBt093	HfQ protein (RNA-binding protein)	No
pBt094	Putative transcriptional regulator; similar to *B. subtilis* transition state regulators	Yes
pBt97	Putative class II aminotransferase	Yes
pBt98	Pyridoxal phosphate-dependent enzyme	Yes
pBt100	tRNA synthetase-related protein	Yes
pBt101	Possible kinase	Yes
pBt102	GntR family transcriptional regulator containing aminotransferase domain	N/D[b]
pBt108	Putative sigma factor, ECF family	Yes
pBt120	Putative DNA-binding protein	Yes
pBt131	Putative ABC transporter permease protein	No
pBt132	Putative ABC transporter ATP-binding protein	N/D
pBt133	Putative ABC transporter exported solute-binding protein	N/D
pBt136	Possible peptide antibiotic precursor	Yes
pBt137	Integral membrane protein (possible peptide antibiotic maturation and biosynthesis protein)	Yes
pBt138	Integral membrane protein (possible accessory factor in peptide antibiotic secretion)	Yes
pBt139	Putative ABC transporter ATP-binding protein	No
pBt145	Putative spore coat-associated protein; similar to *B. subtilis* spore coat-associated protein N CotN	Yes
pBt147	HfQ protein (RNA-binding protein)	No
pBt148	Putative transcriptional regulator; similar to *B. subtilis* transition state regulators	Yes
pBt149	Putative transcriptional regulator, ArsR family	Yes
pBt152	Hemagglutinin-related protein	Yes
pBt156	FtsZ/tubulin-related protein	Yes
pBt157	Putative DNA-binding protein; contains predicted helix-turn-helix motif	Yes
pBt158	Putative transcriptional regulator, MerR family	No

[a] The CDS designations are those described by Berry et al. (3), who describe each CDS and its database homologs in more detail. The transcriptional activity determined in this study is indicated.

[b] N/D, not determined (activity of the gene was not assessed due to the presence of closely related genomic copies of the gene).

products from primers alone were not generated. Prescreening of primer pairs with plasmidless *B. thuringiensis* subsp. *israelensis* strain 4Q7 resulted in production of amplicons with the pBt102, pBt132, and pBt133 oligonucleotide pairs. The resulting fragments were cloned into the vector pGEM-T (Promega) and subjected to DNA sequencing. Analysis of these sequences revealed very closely related genomic homologs of the plasmid target genes (and also homologs in the complete *B. thuringiensis* subsp. *konkukian* genome [accession number AE017355]). As a result, analysis of the expression of the corresponding plasmid CDSs could not be performed reliably. No other primer pairs produced amplicons from strain 4Q7 cells, whereas all other primer pairs produced fragments of the expected sizes when strain 4Q5 cells (containing the pBtoxis plasmid) provided the template. Products of the expected sizes were also generated by reverse transcription-PCR from some but not all CDSs (data not shown), and the amplicons were taken to indicate the transcriptional activities of the genes. In the case of four genes (pBT036, pBt084, pBt108, and pBt138), the amplicon was also cloned and sequenced to verify that the products were derived from the intended genes. In each case, the sequence corresponded to the expected amplicon from pBtoxis. Table 1 shows the results of the transcriptional survey. It should be noted that in our experiments several CDSs were transcriptionally inactive. However, we cannot completely rule out the possibility that these genes are transcribed (for instance, under other growth conditions or under the influence of unknown inducers). Nonetheless, our survey did indicate that there was mRNA production from many of the plasmid genes assessed, as discussed below.

Toxin-related CDSs. As expected, transcripts for the *cry11Aa* (pBt023) and *cyt1Aa* (pBt021) genes were detected, which is consistent with the fact that the corresponding proteins are produced abundantly by *B. thuringiensis* subsp. *israelensis* during sporulation. In addition, transcripts were also detected for the *cyt1Ca* (pBt054) and *cyt2Ba* (pBt036) genes, for which spore-associated proteins have not been found in strain 4Q5 (although possible detection of Cyt2Ba was observed by Western blotting in *B. thuringiensis* subsp. *israelensis* strain 1884 [6]). The reason for the apparently low levels of these Cyt proteins may include instability of the transcript and/or the resultant protein, failure in translation of the message, and, in the case of Cyt2Ba, possible masking of the product on protein gels by Cyt1Aa, which is more abundant but a similar size.

CDS pBt020 encodes a hypothetical protein with no known homologues in the NCBI nonredundant database. We decided to analyze this CDS because of its location in the pBtoxis plasmid, in which it is immediately downstream of the *cyt1Aa* gene, and because it and *cyt1Aa* are convergently transcribed and there are only 2 nucleotides (nt) between their stop codons. As a result, transcriptional read-through from both genes to produce antisense RNA with respect to the other gene is a theoretical possibility. In our experiments, an amplicon was produced using the pBt020 primers; however, our method of cDNA production using random hexamers would have produced an amplicon from this CDS whether RNA was produced as sense RNA (from a putative pBt020 promoter) or as antisense RNA (from the *cyt1Aa* promoter). As a result, further experiments were performed with this pair of CDSs to determine the nature of the transcripts detected in the initial experiment

TABLE 2. Oligonucleotide primer sequences based on the CDS of pBtoxis

CDS	Orientation	Oligonucleotide primer (5′–3′)	T_m (°C)[a]	Amplicon size (bp)
pBt011	Forward	GACAATTATCGAAGTTGAAAAGG	62	430
	Reverse	ACTATTGAATCCTTCCGTTTCC	62	
pBt014	Forward	ACAGTAATCTATCTAAAATTGAGC	62	278
	Reverse	TCTAAAGTTTCACGTAGCTTCC	62	
pBt020	Forward	GAAGAACAAATTTTAGAGAAAGG	60	128
	Reverse	TAGTAGCTATATTTATTAAATATGG	60	
pBt021	Forward	CTGGGGCAAGGAAACTGCTA	62	270
	Reverse	TTAGAGGGTTCCATTAATAGCG	62	
pBt023	Forward	AGATAGTTCTTTAGATACTTTAAG	60	314
	Reverse	CATCACTAACCTCAATAATCC	60	
pBt029	Forward	TATTAGGTTCAGAAAAGAATCCG	62	223
	Reverse	CAACAGAACTTATATTTCTTTCC	60	
pBt031	Forward	GGTGCACATGATTCAGGTGC	62	679
	Reverse	CGATGTGCATTACCTTCAAGG	62	
pBt036	Forward	AGGCATATTGCATTAACAGTTCC	64	411
	Reverse	TGTTTGAGTAGCTGATAAATTACG	64	
pBt054	Forward	GCACTAGTACCTACTACATCC	62	703
	Reverse	GGTTTTGAATACTGTAAGCACG	62	
pBt056	Forward	GATGATTTGTTTAATCCTGAATGG	64	323
	Reverse	TTCATTGTGGGGAAACTTTGC	60	
pBt060	Forward	GAATGAAAATATTGAAACCATCC	60	391
	Reverse	AGAAATAGGTTCCTGAATCG	60	
pBt063	Forward	TTATTCATTCGCTACAACTTGC	60	192
	Reverse	CAAAATAACTCGTTAGAATCTGG	62	
pBt075	Forward	AGGAGATAGTCTAAGATATGTACG	66	324
	Reverse	GTATTACCATTTAGATCTGTGACG	66	
pBt084	Forward	TAATTGCCTTAGATAGAACACC	60	541
	Reverse	GAGATTTTGGTTAAGTACTTTCC	62	
pBt085	Forward	TATTCACTCACTGCAACTGGC	62	623
	Reverse	CTGTATTCGCTATCAGTATTCC	62	
pBt086	Forward	GAATGAGATTGTTGAAACTATCC	62	641
	Reverse	AGTTAGATAATTCTCCTATGCC	60	
pBt087	Forward	GACTTGTGTAACATTAGTAGGC	62	598
	Reverse	TATACCGTATTCTTGGGATGC	62	
pBt091	Forward	CGATCTTAATTATGAACTCGAGC	64	244
	Reverse	CACTTGTTTATCGTCCACTCG	62	
pBt092	Forward	TCAGAATTAATCAAACAAGTCGC	62	257
	Reverse	ACAGCTTCTTTTAATGCTTTTCC	62	
pBt093	Forward	TCTTTGCAGGAACAATTGTTGC	62	180
	Reverse	GAATCGAATCGTCGAGATTGC	62	
pBt094	Forward	GAAAGCAACCGGTATTGTACG	62	270
	Reverse	ACTTTGTATTGTTCTAATTCTTCC	62	
pBt097	Forward	GGCTGAAGAGAAGGCATGGC	64	343
	Reverse	CACAGTGCTCCTAGTCTCTCC	66	
pBt098	Forward	TGCCTATGAGTCATTGGCGG	62	356
	Reverse	CTGCATATGATGCCGCTGAGC	66	

Continued on following page

TABLE 2—*Continued*

CDS	Orientation	Oligonucleotide primer (5′-3′)	T_m (°C)[a]	Amplicon size (bp)
pBt100	Forward	CGAAAATTATATCTCCAAGATCC	62	582
	Reverse	GCATGGAATATGCATATCACC	60	
pBt101	Forward	CTATATGCTAGTGCTGAAGCC	62	403
	Reverse	CATTATCAACGATAACCAATCGC	64	
pBt102	Forward	AGAGGTACACGTGTTAGTACAC	64	358
	Reverse	GTTGTGCGCCTGATGTAATCATA	66	
pBt108	Forward	ACGTTGTGAAACAAATTCTATGG	62	303
	Reverse	GTTCATTCATATTACTAAGTACG	60	
pBt120	Forward	CTATTAGGTTCAGAAAATAATCC	60	246
	Reverse	TAACTCTATTTCTTTGCTCATGC	62	
pBt131	Forward	CCATGCTTGGGATTATTATCG	60	683
	Reverse	AGTTGCATCACTTGGCTTGCG	64	
pBt132	Forward	CGTATTATCAAGGAAAATTGGC	60	455
	Reverse	CGTTGGATTATTCGCAATCGC	62	
pBt133	Forward	CACTAATTGTTATTGTGGCAGC	62	686
	Reverse	TTGGATTTGTGACTTCTGAAGC	62	
pBt136	Forward	TGTTCGAGATTATGGGTTATTTTG	64	218
	Reverse	CCATGCAACAGCTTGTGCTTTTA	66	
pBt137	Forward	CCTTATTATTTGAGGGTATTACG	62	652
	Reverse	GCTGAAATTATGTATAAAATTAGG	60	
pBt138	Forward	CACATAAGTTTGAAATATATATGG	60	425
	Reverse	GTATATTGAATTTTACGAAGCCG	62	
pBt139	Forward	CTACATAATATCTCATTTTCTGC	60	377
	Reverse	GACCAATACAATTTGTGACGC	60	
pBt145	Forward	GAAATTAGGTGCTGGAGTGG	60	446
	Reverse	ATTTTAATCCACCTGTTTCTGC	60	
pBt147	Forward	CGAAATTACAATCATTTCAAAAGG	62	225
	Reverse	AGAATCGAATCGTAGAGATTGC	62	
pBt148	Forward	TGAAAGCAACAGGTATTGTACG	62	252
	Reverse	CAGCTAACAGTTGTTTTAGACC	62	
pBt149	Forward	TCTATATGGATATTGAGTATTACG	62	273
	Reverse	CCTCTATTCTCTTCATGCTCTC	64	
pBt152	Forward	GGAGATTTGACTGCCTTTGG	60	675
	Reverse	CCAATCATTACAATTAACAGCG	60	
pBt156	Forward	AAGAGGCTGATTTATTCGCAGG	64	425
	Reverse	GTTAATTTCATCAGGATCACCG	62	
pBt157	Forward	TGAATAGGGATCACTTTTATACG	62	279
	Reverse	AACACTCTACTATTTCTACACC	60	
pBt158	Forward	TCTGAACTTACTGGGCTTTCG	62	397
	Reverse	TAAGATCCTGATTTCTACGTGC	62	

[a] Approximate denaturation temperatures (T_m) (based on approximations for short sequences) were determined as follows: T_m = 4(G + C) + 2(A + T).

described above. Using the same RNA sample, separate reverse transcriptase reactions in which the random hexamer primers were replaced by the following primers were carried out: pBt020 forward or pBt021 reverse (for subsequent detection of a transcript from *cyt1Aa*) or pBt020 reverse or pBt021 forward (for subsequent detection of a transcript from pBt020). Reactions primed with the latter primers produced no product during PCR with the pBt020 forward and pBt020 reverse primers, in-

dicating that there was no transcription from the pBt020 CDS. In contrast, PCR performed with the pBt021 forward and pBt021 reverse primers produced amplicons of the expected size following reverse transcription with either pBt020 forward or pBt021 reverse. This indicated not only that a transcript was produced from *cyt1Aa* (as expected) but that there was significant read-through into the pBt020 region since pBt020 forward must have primed reverse transcription in this region. Thus, it appears that termination of the *cyt1Aa* transcript does not occur at the stem-loop structure that begins 27 nt downstream of the *cyt1Aa* gene and within the pBt020 CDS (GGTAATATCACAAGT ATAAATACTTGTGGTATTACC; $\Delta G = -20.8$ kcal/mol [17]). This sequence lacks the T tract of a classical factor-independent transcriptional termination sequence, although not all such terminators require the T tract (18). In this case, however, it appears that the feature described above is not sufficient to cause transcript termination. The lack of pBt020 transcription means that this CDS is unable to interfere with production of Cyt1Aa from its gene. Analysis of the region upstream of the pBt020 CDS revealed a region with some similarity to a σ^G-like promoter sequence (−35 GTATA-14 nt-CATATTA, 200 nt upstream of the pBt020 ATG initiation codon; compared to the σ^G consensus G[A/C]AT[A/G]-18 nt-CAT[A/T][A/C]TA [8]) that might permit this gene to be transcribed during sporulation, the period during which the *cyt1Aa* gene would be convergently transcribed. The suboptimal spacing of the features identified may explain the apparent lack of pBt020 transcription in our experiments.

Like pBt020, pBt075 encodes a hypothetical protein. However, protein derived from this gene has been identified in association with spores of a transconjugant *Bacillus sphaericus* strain to which an erythromycin-resistant variant of pBtoxis was transferred (4a). Our experiments also indicated that there was production of transcript from this gene in *B. thuringiensis*, although the function of the protein product remains unknown. The CDS apparently encoding a sigma factor (pBt108, sigma E-like) is transcriptionally active, which supports the suggestion (3) that the pBtoxis plasmid may contribute a supplementary sigma factor of a type involved in toxin gene transcription (4, 19–21) that may aid in the production of its own toxins.

Sporulation-, germination-, and cell division-related CDSs. Several CDSs that might have direct effects on the host phenotype were identified during the sequencing of pBtoxis (3). The present study showed that several such CDSs are transcriptionally active. Two CDSs that may be associated with sporulation, pBt145 (related to *cotN*, which produces a secreted protein incorporated into the spore of *Bacillus subtilis* and may be involved in its production [15, 16]) and pBt031 (which produces a protein that has similarities to cell wall hydrolases), are transcribed from pBtoxis. The plasmid also appears to encode proteins with possible germination functions (pBt084, pBt085, and pBt086). Transcript was detected for each CDS in this group, implying that the CDSs are organized as an operon. To analyze this further, PCRs between CDSs were performed using primers pBt084 reverse and pBt085 forward and, separately, primers pBt085 reverse and pBt086 forward. Amplicons of the expected sizes were produced with each of these primer pairs, indicating that there was cotranscription of the genes and confirming that the CDSs are organized in a single operon. No transcript was produced from the pBt060 and pBt063 CDSs, which are related to *ger* and were postulated to be pseudogenes (3). The effect of the plasmid *ger* genes on the host bacterium will be the subject of a separate report (K. Gammon, C. Berry, and B. N. Dancer, unpublished data). Plasmid-directed production of peptide antibiotic factors may be encoded by pBt136, pBt137, and pBt138. In this study we detected mRNA for each of these CDSs, implying that they may be active in directing antibiotic peptide production. To determine the possible operon organization of these genes, PCRs were performed using primers pBt136 forward and pBt137 reverse and, separately, primers pBt137 forward and pBt138 reverse. A product of the predicted size was obtained with the pBt136 forward and pBt137 reverse primers, indicating that pBt136 and pBt137 are cotranscribed. No product was detected in the PCR performed with primers pBt137 forward and pBt138 reverse, suggesting that pBt138 does not form part of the operon with pBt136 and pBt137. It is possible that this two-gene operon may be regulated by PlcR, the pleiotropic regulator of transcription of several extracellular virulence factors in *B. thuringiensis* (1), since it is preceded by a possible −10 sequence (TATAAT; nt 111884 to 111889) and the conserved palindromic sequence TATGNAN$_4$TNCATA (pBtoxis nt 111835 to 111850) associated with PlcR regulation. PlcR-regulated genes are usually turned on at the end of the vegetative phase in cells grown in rich media, such as NYSM (10). The putative ABC transporter genes (pBt131 to pBt133) are orientated divergently with respect to the peptide antibiotic genes described above and encode proteins with low levels of similarity to Bac components involved in bacteriocin production and secretion. We surveyed the transcription of only pBt131 (since genomic homologs of pBt132 and pBt133 were identified [see above]) and found that this CDS appeared to remain untranscribed (similarly, pBt139, the gene encoding the other predicted ABC-type protein on pBtoxis, was transcriptionally silent). Another CDS, pBt152, encodes a protein related to hemagglutinin, and our results indicated that this gene is transcribed, although the significance of this in *B. thuringiensis* remains to be determined. The putative deletion pseudogene pBt056, which matches pBt152 with two frameshifts and an in-frame stop codon, is not transcribed. The plasmid also contains a gene encoding a protein with similarity to the cell division protein FtsZ (pBt156). This gene appears to be transcriptionally active, and this suggests a possible influence of the plasmid on cell division.

Enzyme-encoding CDSs. CDSs encoding putative enzymatic products were also identified in pBtoxis. Phosphatidylinositol-specific phospholipase C is known to have roles in virulence in *Bacillus cereus* (14). The pBtoxis homolog pBt087, however, contains an in-frame stop codon and appeared in this study to be transcriptionally inactive. The genome of *B. thuringiensis* subsp. *israelensis*, however, contains a distinct but related phosphatidylinositol-specific phospholipase C gene (7) that may be transcribed. CDSs encoding other putative enzymes, including pBt097, pBt098, pBt100, and pBt101, all appear to be transcribed.

Regulator CDSs. The pBtoxis plasmid contains 14 CDSs that may have functions in the regulation of other genes based on their similarity to genes encoding DNA-binding proteins, RNA-binding proteins, or known transcriptional regulators. Many of these 14 CDSs (pBt029, pBt093, pBt120, pBt147, and pBt158) were not

transcribed in our experiments (pBt102 was not examined due to the presence of a genomic homolog). However, pBt011, pBt014, pBt091, pBt092, pBt094, pBt148, pBt149, and pBt157 did produce transcripts and therefore may produce regulatory proteins. Some of these active CDSs have homologues with known roles in bacilli; for instance, pBt149 encodes a protein similar to the PagR protein of the anthrax plasmid pXO1, which is known to regulate other genes in bacilli (9), and pBt094 and pBt148 are related to the gene encoding the *Bacillus subtilis* transition state regulator AbrB, a regulator of other regulator proteins (12). Transcription of the genes encoding the putative regulators described above may have great significance for the phenotype and behavior of *B. thuringiensis* strains. Such effects could parallel the recent report (2) of a plasmid-encoded regulator that alters extracellular proteinase production in *Bacillus anthracis*, which, like *B. thuringiensis*, is a member of the *B. cereus* sensu lato group. Of course, genomic regulators would be expected to influence transcription of plasmid genes, but our results may indicate that there is production of plasmid-encoded regulators that may be able to participate in plasmid-genome "cross talk" to influence expression of both plasmid and genomic loci. *B. thuringiensis* subsp. *israelensis* is by no means unique in *B. thuringiensis*, in which the toxins are encoded on large extrachromosomal elements in most strains. We expect that these plasmids, like pBtoxis, encode not only the toxins that are directly responsible for insect pathogenicity but also a variety of other proteins that may affect the phenotype and behavior of the host organism. In this study, we established that many of the pBtoxis genes with putative functions other than toxicity are transcribed. The production of the corresponding proteins and their roles in host processes and their possible contributions to virulence remain to be established, but in this study we took the first step in the investigation of the molecular role of the toxin-coding plasmid in the host cell.

ACKNOWLEDGMENTS

This work received funding from the BBSRC (G.W.J.) and the European Community through the UK Socrates-Erasmus Council (C.S.).

REFERENCES

1. **Agaisse, H., M. Gominet, O. Økstad, A.-B. Kolstø, and D. Lereclus.** 1999. PlcR is a pleiotropic regulator of extracellular virulence factor expression in *Bacillus thuringiensis*. Mol. Microbiol. **32:**1043–1053.
2. **Aronson, A. I., C. Bell, and B. Fulroth.** 2005. Plasmid-encoded regulator of extracellular proteases in *Bacillus anthracis*. J. Bacteriol. **187:**3133–3138.
3. **Berry, C., S. O'Neil, E. Ben-Dov, A. F. Jones, L. Murphy, M. A. Quail, T. G. Holden, D. Harris, A. Zaritsky, and J. Parkhill.** 2002. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **68:**5082–5095.
4. **Dervyn, E., S. Poncet, A. Klier, and G. Rapoport.** 1995. Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **177:**2283–2291.

4a. **Gammon, K., G. W. Jones, S. J. Hope, C. M. F. de Oliveira, L. Regis, M. H. N. L. Silva Filha, B. N. Dancer, and C. Berry.** 2006. Conjugal transfer of a toxin-coding megaplasmid from *Bacillus thuringiensis* subsp. *israelensis* to mosquitocidal strains of *Bacillus sphaericus*. Appl. Environ. Microbiol. **72:**1766–1770.

5. **Georghiou, G. P., and M. C. Wirth.** 1997. Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. **63:**1095–1101.
6. **Guerchicoff, A., R. A. Ugalde, and C. P. Rubinstein.** 1997. Identification and characterization of a previously undescribed *cyt* gene in *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **63:**2716–2721.
7. **Guttmann, D. M., and D. J. Ellar.** 2000. Phenotypic and genotypic comparisons of 23 strains from the *Bacillus cereus* complex for a selection of known and putative *B. thuringiensis* virulence factors. FEMS Microbiol. Lett. **188:**7–13.
8. **Haldenwang, W. G.** 1995. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. **59:**1–30.
9. **Hoffmaster, A. R., and T. M. Koehler.** 1999. Control of virulence gene expression in *Bacillus anthracis*. J. Appl. Microbiol. **87:**279–281.
10. **Lereclus, D., H. Agaisse, C. Grandvalet, S. Salamitou, and M. Gominet.** 2000. Regulation of toxin and virulence gene transcription in *Bacillus thuringiensis*. Int. J. Med. Microbiol. **290:**295–299.
11. **Myers, P., and A. Yousten.** 1978. Toxic activity of *Bacillus sphaericus* SSII-1 for mosquito larvae. Infect. Immun. **19:**1047–1053.
12. **Phillips, Z. E. V., and M. A. Strauch.** 2002. *Bacillus subtilis* sporulation and stationary phase gene expression. Cell. Mol. Life Sci. **59:**392–402.
13. **Poncet, S., G. Anello, A. Delécluse, A. Klier, and G. Rapoport.** 1993. Role of the CryIVD polypeptide in the overall toxicity of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **59:**3928–3930.
14. **Salamitou, S., F. Ramisse, M. Brehélin, D. Bourguet, N. Gilois, M. Gominet, E. Hernandez, and D. Lereclus.** 2000. The *plcR* regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects. Microbiology **146:**2825–2832.
15. **Serrano, M., R. Zilhao, E. Ricca, A. J. Ozin, C. P. Moran, Jr., and A. O. Henriques.** 1999. A *Bacillus subtilis* secreted protein with a role in endospore coat assembly and function. J. Bacteriol. **181:**3632–3643.
16. **Stover, A. G., and A. Driks.** 1999. Secretion, localization, and antibacterial activity of TasA, a *Bacillus subtilis* spore-associated protein. J. Bacteriol. **181:**1664–1672.
17. **Tinoco, I., Jr., P. N. Borer, B. Dengler, M. D. Levin, O. C. Uhlenbeck, D. M. Crothers, and J. Bralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nat. New Biol. **246:**40–41.
18. **Unniraman, S., R. Prakash, and V. Nagaraja.** 2002. Conserved economics of transcription termination in eubacteria. Nucleic Acids Res. **30:**675–684.
19. **Yoshisue, H., T. Fukada, K.-I. Yoshida, K. Sen, S.-I. Kurosawa, H. Sakai, and T. Komano.** 1993. Transcriptional regulation of *Bacillus thuringiensis* subsp. *israelensis* mosquito larvicidal crystal protein gene *cryIVA*. J. Bacteriol. **175:**2750–2753.
20. **Yoshisue, H., K. Ihara, T. Nishimoto, H. Sakai, and T. Komano.** 1995. Expression of genes for insecticidal crystal proteins in *Bacillus thuringiensis*: *cryIVA*, not *cryIVB*, is transcribed by RNA polymerase containing σ^H and not that containing σ^E. FEMS Microbiol. Lett. **127:**65–72.
21. **Yoshisue, H., T. Nishimoto, H. Sakai, and T. Komano.** 1993. Identification of a promoter for the crystal protein-encoding gene *cryIVB* from *Bacillus thuringiensis* subsp. *israelensis*. Gene **137:**247–251.

Applied and Environmental Microbiology, Mar. 2006, p. 1766–1770 Vol. 72, No. 3
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.3.1766–1770.2006
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Conjugal Transfer of a Toxin-Coding Megaplasmid from *Bacillus thuringiensis* subsp. *israelensis* to Mosquitocidal Strains of *Bacillus sphaericus*

Katherine Gammon,[1] Gareth W. Jones,[1] Steven J. Hope,[1] Cláudia M. F. de Oliveira,[2] Lêda Regis,[2] Maria Helena N. L. Silva Filha,[2] Brian N. Dancer,[1] and Colin Berry[1]*

Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, United Kingdom,[1] and Departamento de Entomologia, Centro de Pesquisas Aggeu Magalhães-FIOCRUZ, Av. Moraes Rêgo s/n, Recife PE 50670-420, Brazil[2]

Received 13 July 2005/Accepted 12 December 2005

Both *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* produce mosquitocidal toxins during sporulation and are extensively used in the field for control of mosquito populations. All the known toxins of the latter organism are known to be encoded on a large plasmid, pBtoxis. In an attempt to combine the best properties of the two bacteria, an erythromycin resistance-marked pBtoxis plasmid was transferred to *B. sphaericus* by a mating technique. The resulting transconjugant bacteria were significantly more toxic to *Aedes aegypti* mosquitoes and were able to overcome resistance to *B. sphaericus* in a resistant colony of *Culex quinquefasciatus*, apparently due to the production of Cry11A but not Cry4A or Cry4B. The stability of the plasmid in the *B. sphaericus* host was moderate during vegetative growth, but segregational instability was observed, which led to substantial rates of plasmid loss during sporulation.

Mosquitoes are the vectors of many deadly and debilitating human diseases, such as malaria, filariasis, and arbovirus infections, including yellow fever and dengue fever. Control of vector mosquito populations often involves the use of insect-pathogenic bacteria which produce highly potent toxins that act specifically against mosquitoes and simulids and which have no effect on nontarget species. At present, only two types of mosquito-pathogenic bacteria are used in control programs in the field, *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis*. Both of these bacteria produce parasporal crystalline inclusion bodies composed of potent mosquito-toxic proteins. The two *Bacillus* species have different properties. *B. thuringiensis* subsp. *israelensis* is toxic as a result of the presence of a 128-kb plasmid, pBtoxis, that carries four *cry* (*cry4Aa*, *cry4Ba*, *cry10Aa*, and *cry11Aa*) and three *cyt* (*cyt1Aa*, *cyt2Ba*, and *cyt1Ca*) toxin genes. The resulting production of an arsenal of toxins appears to be sufficient to prevent the emergence of insect resistance (13). However, *B. thuringiensis* subsp. *israelensis* is more sensitive to UV radiation than *B. sphaericus*, and the duration of effective control is shorter, especially in polluted water (30). In contrast, *B. sphaericus* is less UV sensitive, survives well in polluted water (30, 42), recycles in the environment (11), and is toxic largely by virtue of a chromosomally encoded binary gut toxin (Bin) (4). However, *B. sphaericus* performs poorly against one major vector mosquito, *Aedes aegypti*, and although it is highly active against other important vectors, resistance has been reported in some mosquito species (31). It has been shown that the Cyt1Aa toxin from *B. thuringiensis* subsp. *israelensis* is highly synergistic with the Bin toxin of *B. sphaericus* (37, 38), and introduction of a *cyt1A* gene from another mosquitocidal *B. thuringiensis* strain has been shown to help overcome resistance to *B. sphaericus* (32). Thus, there is significant potential for strain improvement by combination of the additional toxins of *B. thuringiensis* subsp. *israelensis* with the better environmental performance (and extra crystal toxin) of *B. sphaericus*. Several groups have reported success in moving *B. thuringiensis* subsp. *israelensis* toxin genes, one or two at a time, into *B. sphaericus* and thereby enhancing its toxicity (3, 26, 27, 34). However, the use of single genes, while resulting in improved activity, may result in only a slight delay in the development of insect resistance, since Georghiou and Wirth (13) have shown that to prevent resistance to *B. thuringiensis* subsp. *israelensis* toxins, both Cry and Cyt toxins must be expressed. In addition, the strains produced in the previous studies are considered to be both genetically modified and recombinant by virtue of the methods by which the toxin genes were transferred to the *B. sphaericus* host. Optimum toxicity in *B. thuringiensis* subsp. *israelensis* arises from the presence of all of its toxins and is enhanced by the presence of at least two "helper proteins," P19 and P20, which may stabilize the toxins or aid in crystal formation (40). Since all the *B. thuringiensis* subsp. *israelensis* toxins and the two helper proteins are encoded on a single 128-kb plasmid (5), major enhancement of the mosquito-toxic properties of *B. sphaericus* might be attainable by transfer of this plasmid, which would combine the best properties of the two species in a single organism. Transfer of pBtoxis by the classical microbiological mating method could yield transconjugants that would not be considered genetically modified or recombinant. Conjugal transfer of pAMβ1 both within *B. sphaericus* species (7) and between species (23) has been accomplished using filter mating methods. Similarly, conjugation methods for both *B. thuringiensis* subsp. *israelensis* (15, 33) and *B. sphaericus* (7) in liquid media have also been reported, and transfer of a plasmid from *B. thuringiensis* to *B. sphaericus* using such a

* Corresponding author. Mailing address: Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, United Kingdom. Phone: 44-29-2087-4508. Fax: 44-29-2087-4116. E-mail: Berry@cf.ac.uk.

protocol has been achieved (36). In the case of *B. thuringiensis*, it has been demonstrated that in some subspecies the toxin-encoding plasmids themselves are able to conjugate between *B. thuringiensis* strains (14, 35). In this study we obtained proof of the mating transfer strategy concept using a "marked" derivative of pBtoxis, and the results indicated that plasmid transfer to *B. sphaericus* is possible and enhances the toxicity of the resulting transconjugants.

MATERIALS AND METHODS

Bacterial strains. *B. thuringiensis* subsp. *israelensis* was obtained from the commercial product VectoBac 12AS (Valent Biosciences Corporation), *B. thuringiensis* subsp. *israelensis* 4Q7 (also known as 4Q2-81) is a strain cured of all plasmids, and *B. thuringiensis* subsp. *israelensis* 4Q5 (also known as 4Q2-72) is a strain cured of all plasmids except pBtoxis. *B. thuringiensis* subsp. *israelensis* 4Q5::erm is a derivative of 4Q5 in which the *cyt1Aa* gene on pBtoxis was knocked out by in vivo recombination in order to insert the gene encoding erythromycin resistance (8), and it was kindly supplied by A. Delécluse of the Institut Pasteur, Paris, France. *B. sphaericus* strains 1593R$^-$ and 2362R$^-$ were kindly supplied by W. Burke, Arizona State University, and are restriction-negative variants of *B. sphaericus* 1593 and 2362, respectively, which were chosen for use so that transferred plasmid DNA would not be digested in the new *B. sphaericus* hosts. These strains are naturally resistant to chloramphenicol (1).

Transfer of pBtoxis::erm. The transfer strategy which we used relies on microbiological methods of mating rather than genetic engineering techniques and exploits the ability of the natural *B. thuringiensis* subsp. *israelensis* conjugative plasmid pXO16 to assist in the mobilization of other plasmids (17). Triparental mating was carried out using the wild-type VectoBac strain of *B. thuringiensis* subsp. *israelensis* that contains pXO16 to mobilize the pBtoxis::erm plasmid from strain 4Q5::erm. Fresh broth cultures of all strains were incubated at 30°C for 4 to 6 h. For mating, 100 μl of *B. thuringiensis* subsp. *israelensis* was spread onto LB agar plates, followed by 100 μl of the plasmid host *B. thuringiensis* subsp. *israelensis* 4Q5::erm and then 100 μl of the recipient *B. sphaericus* 1593R$^-$ or *B. sphaericus* 2362R$^-$. The plates were incubated overnight at 30°C, and the growth was harvested into 2 ml of quarter-strength Ringer's solution. The suspension was serially diluted in quarter- strength Ringer's solution, and 100 μl was spread onto LB agar containing chloramphenicol (5 μg/ml) and erythromycin (5 μg/ml) to select for *B. sphaericus* that had acquired pBtoxis::erm and then incubated at 30°C for 24 to 48 h. Controls consisting of donors and recipients were plated separately.

PCR with pBtoxis and 16S rRNA genes. To screen for the presence of pBtoxis in potentially transconjugant *B. sphaericus* strains, PCR screening for a number of plasmid genes was carried out. DNA for use in PCRs was isolated using a PUREGENE DNA purification kit for yeast and gram-positive bacteria (GENTRA Systems, Minneapolis, Minn.). Primers for pBtoxis coding sequences, distributed around the plasmid, were used. Primers Dip1A and Dip1B (CAAGCCGCAAATCTTGTGG and ATGGCTTGTTTCGCT ACATC, respectively) and primers Dip2A and Dip2B (GGTGCTTCCTAT TCTTTGGC and TGACCAGGTCCCTTGATTAC, respectively), designed by Carozzi et al. (6), were used to detect the presence of the Cry4A and Cry4B genes, respectively. In addition, the following primers were used: for pBt020, primers GAAGAACAAATTTTAGAGAAAGG and TAGTAGCTA TATTTATTAAATATGG; for pBt054, GCACTAGTACCTACTACATCC and GGTTTTGAATACTGTAAGCACG; for pBt084, TAATTGCCTTAG ATAGAACACC and GAGATTTTGGTTAAGTACTTTCC; for pBt136, TGT TCGAGATTATGGGTTATTTTG and CCATGCAACAGCTTGTGCTTTTA; and for pBt156,AAGAGGCTGATTTATTCGCAGG and GTTAATTTCATC AGGATCACCG. The PCRs were performed with an initial step consisting of 94°C for 5 min, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of primer annealing at 55°C, and 1 min of extension at 72°C and then a final 10-min step at 72°C. Products were analyzed by gel electrophoresis on a 1% agarose gel and visualized under UV light. Fragments of 16S rRNA genes were amplified using primers 63f (CAGGCCTAACACATGCAAGTC) and 1387r (GGGCGG AGTGTACAAGGC), which were designed by Marchesi et al. (20). The PCR was performed using the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then a final 10-min extension at 72°C. The resulting amplicon was subjected to direct sequencing using the same primers in order to elucidate the amplified sequence. The origin of the sequence was verified by BLAST analysis (2) and by direct comparison to the equivalent sequences from *B. sphaericus* and *B. thuringiensis*.

Protein fingerprinting and Western blotting. Transconjugants and parental strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. *B. thuringiensis* subsp. *israelensis* strains were grown in a sporulation medium (nutrient broth, 8 g/liter; yeast extract, 1 g/liter; K_2HPO_4, 1 g/liter; $CaCO_3$, 0.1 g/liter; $MgSO_4 \cdot 7H_2O$, 0.1 g/liter; $FeSO_4 \cdot 7H_2O$, 0.01 g/liter; $MnSO_4 \cdot 7H_2O$, 0.01 g/liter; $ZnSO_4 \cdot 7H_2O$, 0.01 g/liter; pH 7.0).

Cultures were grown for 72 h (with >98% sporulation) and harvested, and samples were resuspended in SDS-PAGE loading buffer. Proteins were separated using the method of Laemmli (18) and a 12% acrylamide resolving gel at 200 V for 40 min.

Proteins from gels were transferred electrophoretically onto nitrocellulose membranes using a mini Protean II electrophoresis cell and a mini transblot module (Bio-Rad). Western blotting was carried out as described by Maniatis et al. (19). Cry proteins were detected with rabbit antisera against Cry4A and Cry4B crystal toxins (kindly provided by A. Delécluse, Institut Pasteur, Paris, France) together with an alkaline phosphatase-linked, anti-rabbit secondary antibody, followed by color development using a Bio-Rad AP conjugate substrate kit. Protein bands of interest that were identified in SDS-PAGE gels were blotted onto a polyvinylidene difluoride membrane from equivalent SDS-PAGE gels prepared with Tricine in place of glycine. Following rapid staining and destaining of the membrane, bands of interest were excised for N-terminal protein sequencing (Alta Bioscience, Birmingham, United Kingdom).

Mosquito colonies. The following *Culex quinquefasciatus* and *A. aegypti* colonies used in this work were maintained in the insectarium of the Centro de Pesquisas Aggeu Magalhães-FIOCRUZ, Brazil: (i) CqSF, a susceptible *C. quinquefasciatus* colony; (ii) CqRL1/2362, a colony derived from CqSF and selected with *B. sphaericus* strain 2362 under laboratory conditions, which had a stable and high level (resistance ratio, >162,000-fold) of resistance (22, 24); and (iii) AeLab, a susceptible *A. aegypti* colony. The insects from all colonies were reared at 26 ± 2°C with 70% relative humidity and with a photoperiod consisting of 12 h of light and 12 h of darkness. Larvae were reared in tap water and fed ground cat biscuits. The adults were maintained with a 10% sugar solution, and females were allowed to feed on chickens.

Bioassays. Transconjugant strains were tested for toxicity to *A. aegypti* larvae and larvae from the binary toxin-sensitive and -resistant colonies of *C. quinquefasciatus* described above. Bioassays were performed with early fourth-instar larvae by using a standard method recommended by the World Health Organization (39). For all bioassays, larvae were exposed to serial dilutions of lyophilized spore-crystal powders of strains by placing groups of 20 larvae in 100 ml of distilled water in 125-ml plastic cups at 26 ± 2°C with the desired concentration of *B. sphaericus* spore powder (from cultures grown in NYSM medium [21]). At least five concentrations that resulted in levels of mortality between 2 and 98% were tested, and mortality was recorded after 48 h of exposure. A control group tested with only water was included in each experiment, and the bioassay was repeated two or three times on different days. One drop of larval food was added to each cup. The 50% lethal concentration, expressed in mg per liter, was determined using probit analysis (12) with the software SPSS 8.0.

Plasmid stability. To determine the stability of pBtoxis::erm in *B. sphaericus* vegetative transconjugants, cultures were grown in NYSM broth without antibiotics for 18 h at 30°C. A sample was taken and serially diluted up to a dilution of 10^{-5} in quarter-strength Ringer's solution, and 20-μl drops were plated onto both NYSM agar supplemented with chloramphenicol (10 μg/ml) to enumerate all *B. sphaericus* cells and NYSM agar supplemented with chloramphenicol (10 μg/ml) and erythromycin (1 μg/ml) to enumerate transconjugants. The plates were incubated at 30°C overnight, and then colonies were counted. Prior to plating cultures were checked by phase-contrast microscopy for the absence of spores. Fresh NYSM broth was inoculated using the previous culture, and the process was repeated.

To determine the stability of pBtoxis::erm in *B. sphaericus* transconjugants through sporulation, transconjugant strains were grown until the level of sporulation was at least 90% (4 days) in LB broth and heated at 70°C for 30 min to inactivate any vegetative cells. The cells and spores (1 ml) were centrifuged at 14,000 × *g* for 4 min and resuspended in 1 ml of quarter-strength Ringer's solution. The suspensions were then serially diluted up to a dilution of 10^{-5} in quarter-strength Ringer's solution, and 20-μl drops were plated as described above for analysis of total and transconjugant *B. sphaericus*. Successive subcultures were derived from colonies grown in the absence of antibiotics. Counts were obtained after 24 h of incubation at 30°C. Fresh LB broth was inoculated with 50 μl of an undiluted culture and incubated at 30°C, and the process was repeated serially.

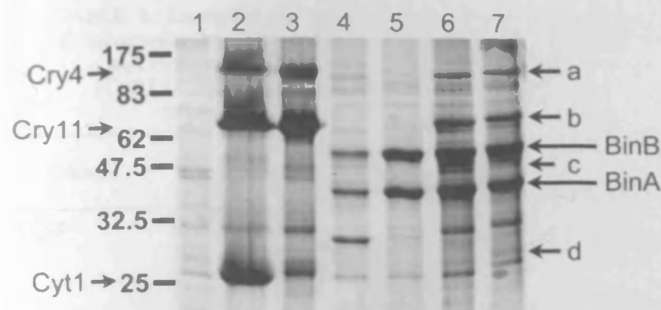


FIG. 1. Protein profiles of lysed sporulated cultures of transconjugant strains. Proteins from *B. thuringiensis* subsp. *israelensis* strains 4Q7 (lane 1), 4Q5 (lane 2), and 4Q5::erm (lane 3), *B. sphaericus* strains 1593R$^-$ (lane 4) and 2362R$^-$ (lane 5), and transconjugants 1593.1 (lane 6) and 2362.5 (lane 7) were examined. The positions of bands for Cry4A and Cry4B (Cry4), Cry11A, Cyt1A, Bin A, and Bin B are indicated, as are the positions of bands that were chosen for N-terminal sequencing, bands a to d; the positions of the molecular weight standards (in kDa) are indicated on the left.

RESULTS AND DISCUSSION

Transconjugant production. Following the on-plate mating described above, a number of colonies were selected on plates containing chloramphenicol (to select for *B. sphaericus* since the parental *B. sphaericus* 1593R$^-$ and 2362R$^-$ strains were chloramphenicol resistant) and erythromycin (to select for the presence of pBtoxis::erm). Two such colonies were selected for further study, and they were designated transconjugant strains 1593.1 and 2362.5. The presence of plasmids in these transconjugants was confirmed by PCR. All pBtoxis primer pairs described above produced amplicons of the expected sizes (results not shown), confirming not only the identity of the plasmid but also, due to the distribution of the primers, that it was likely to be substantially intact. The host bacteria were confirmed to be *B. sphaericus* by their ability to germinate on LB agar plates containing 0.25 M sodium acetate, by their binary toxin production, as judged by SDS-PAGE (see below), and by amplification and sequencing of a portion of the 16S rRNA gene.

Toxin production. Production of the *B. sphaericus* binary toxin and production of the *B. thuringiensis* subsp. *israelensis* Cry toxins were analyzed by SDS-PAGE. Figure 1 shows the protein profiles of lysed sporulated cultures of *B. thuringiensis* subsp. *israelensis* 4Q7, 4Q5, and 4Q::erm, the parental *B. sphaericus* strains, and transconjugants. There are several protein bands in the profiles of the transconjugant strains that are absent from the profiles of the parental *B. sphaericus* strains and appear to match bands in the profile of the *B. thuringiensis* subsp. *israelensis* 4Q5::erm donor. Bands a, b, c, and d in the strain 1593.1 profile (Fig. 1) were chosen for N-terminal sequencing. Band a was present in the profiles of the parental *B. sphaericus* strains but, in Tricine gels used for sequencing, resolved as a doublet with sizes consistent with the sizes of the Cry4Aa and Cry4B toxins (125 kDa and 135 kDa, respectively). However, both bands yielded the same N-terminal sequence, AQLND. No such sequence is encoded by pBtoxis, and the presence of equivalent bands in the parental *B. sphaericus* strains confirmed that this molecule was a host protein. Thus, it appeared that no Cry4 toxins were produced by the transconjugant strains. Band b produced the sequence MEDSS, corresponding to the N terminus of the Cry11Aa toxin, while the faint band c had an ambiguous N-terminal residue that may have been Ala, Met, or Lys, followed by the sequence QEID. The sequence IQEID occurs in the Cry10A sequence encoded by pBtoxis, and Q is residue 128 in this protein. Given the ambiguous first residue of our N-terminal sequence, band c may correspond to a proteolytic product of Cry10A or may be a *B. sphaericus* protein produced under the influence of pBtoxis. This plasmid encodes putative transcriptional regulators (5), some of which have been shown to be transcribed in *B. thuringiensis* subsp. *israelensis* (31a). Band d was another faint band and produced the sequence ALNAQ, corresponding to the sequence encoded by coding sequence Bt075 in the pBtoxis sequence (5) but lacking its initiator methionine residue. This sequence was annotated as a hypothetical protein (with a low level of similarity to a hypothetical protein from *Yersinia pestis*), and this is the first demonstration that this or any other pBtoxis protein not linked to toxicity (i.e., toxin proteins or the P19 and P20 accessory proteins) is actually produced. As indicated above, protein bands (e.g., band a in Fig. 1) in transconjugant strains whose sizes were consistent with the sizes of Cry4A and Cry4B were actually unrelated to these proteins. Western blotting using antisera raised against these proteins also failed to detect Cry4 toxins in transconjugant strains (results not shown). This apparent failure of the transconjugants to express the Cry4A toxin reflects the poor synthesis of this protein when it is encoded on a low-copy-number plasmid in a crystal-minus strain of *B. thuringiensis* subsp. *israelensis* (9). In their study, Delécluse et al. demonstrated that this was not due to low levels of transcription from the Cry4A promoter. In the same system, however, Cry4B did accumulate in *B. thuringiensis* subsp. *israelensis* to high levels (9), in contrast to the results presented here for the *B. sphaericus* transconjugants. Previous studies in which recombinant approaches were used to transfer the *cry4B* or *cry11A* gene to *B. sphaericus* showed that there was expression of both proteins in the recombinant strains (27, 34). However, Cry4B was reported to account for less than 2% of the total protein, while the production of Cry11A could not be assessed since it was reported only from Western blotting experiments (27). Cry11A production was significant, however, when the gene was integrated into the *B. sphaericus* genome by homologous recombination (26). The toxicity of Cry11A is intermediate between the toxicity of Cry4A and the toxicity of Cry4B against both *A. aegypti* and *Anopheles stephensi* (28), but a *cry11A* knockout strain seemed to indicate that it has a particular role in increasing the toxicity of *B. thuringiensis* subsp. *israelensis* against *Culex pipiens* and *A. stephensi* (25). The *cry4A*, *cry4B*, and *cry11A* genes are all transcriptionally controlled by σ^E, while *cry4A* and *cry11A* are additionally controlled by σ^H and σ^K, respectively (10, 41). Perhaps the availability of related transcription factors in *B. sphaericus* influences the different levels of transcription of these genes in *B. sphaericus* compared to *B. thuringiensis* subsp. *israelensis*. Plasmid pBtoxis itself carries a coding sequence (Bt108) that appears to be related to σ^E (5), but its role, if any, in toxin production is still unclear. While the level of production of the Cry toxins appears to be substantially lower in the transconjugants than in *B. thuringiensis* subsp. *israelensis*, the presence of pBtoxis apparently does not decrease the levels of Bin toxin that accumulate in the transconjugants.

TABLE 1. Larvicidal activity of *B. sphaericus* strains against *C. quinquefasciatus* larvae from a susceptible colony (CqSF) and from a colony resistant to *B. sphaericus* 2362 (CqRL1/2362) and against larvae from an *A. aegypti* colony (AeLab) after 48 h of exposure

Colony	Expt	50% lethal concn (mg/liter)[a]	
		B. sphaericus 2362R⁻	*B. sphaericus* 2362.5
CqSF	1	0.013 (0.0045–0.019)[b]	0.012 (0.006–0.022)
	2	0.011 (0.0041–0.031)	0.014 (0.011–0.019)
	3	0.009 (0.0039–0.016)	
	Mean	0.011 (0.0042–0.022)	0.013 (0.008–0.021)
CqRL1/2362	1	>270	0.49 (0.36–0.75)
	2		0.41 (0.27–0.65)
	Mean		0.45 (0.32–0.70)
AeLab	1	>10	1.3 (1.1–1.6)
	2		0.70 (0.51–0.90)
	3		0.47 (0.32–0.61)
	Mean		0.83 (0.63–1.1)

[a] The 50% lethal concentrations were calculated using the software SPSS 8.0 for Windows.
[b] The values in parentheses are limits.

Toxicity. Transconjugant strain 2362.5 and its parent strain, 2362R⁻, were bioassayed with susceptible (CqSF) and resistant (CqRL1/2362) laboratory colonies of *C. quinquefasciatus* and with *A. aegypti* (Table 1). There was not a significant difference in toxicity for the susceptible *C. quinquefasciatus* strain, but this could have been expected since *B. sphaericus* 2362R⁻ is highly toxic to this strain, through the activity of the Bin toxin. Previous studies in which the *cry11A* gene was introduced into the chromosome of *B. sphaericus* strain 2297 showed that there were no (29) or slight (26) increases in toxicity. In both studies, the 2297(::*cry11A*) strains exhibited greatly enhanced toxicity (>100-fold) for *A. aegypti* (which naturally has a low level of susceptibility to *B. sphaericus*). For transconjugant 2362.5 there was also a clear increase in toxicity for this insect. This effect was probably due to the production of Cry11A by the transconjugant since in *B. thuringiensis* subsp. *israelensis* this protein is known to contribute significant mosquitocidal activity against both *A. aegypti* and *C. quinquefasciatus* (25). This effect against *C. quinquefasciatus* was seen most clearly with the Bin-resistant colony, which was much more sensitive to transconjugant 2362.5 than to the parental strain 2362R⁻, confirming previous studies (29) and indicating that expression of Cry11A in *B. sphaericus* is a viable strategy for combating mosquito resistance.

Plasmid maintenance. Transconjugants of strains 1593R⁻ and 2362R⁻ containing pBtoxis::erm were tested to determine the stability of the introduced plasmid by growing them in the presence and absence of selective antibiotics. When strain 2362.5 was used, the pBtoxis plasmid seemed to be relatively stably maintained in vegetative culture without selection (Table 2). However, in spores the plasmid was apparently substantially lost during the initial subculture but appeared to be retained in later rounds (Table 2); thus, with some strain development it might be possible to select strains with more stable maintenance of the plasmid.

Further investigation of plasmid stability in the other transconjugant (1593.1) during successive rounds of subculture overnight with no antibiotic selection showed that there was a general trend toward rapid loss of erythromycin resistance, presumably indicating that there was a loss of pBtoxis in vegetative cells. Overnight cultures grown in the presence of antibiotics to select for pBtoxis::erm generally behaved as expected; i.e., they retained a high level of antibiotic resistance, although for strain 1593.1 less than 1% of the cells seemed to retain erythromycin resistance compared to the total counts.

The variability of the retention of antibiotic resistance and, by implication, plasmid stability suggests that pBtoxis is not just segregationally unstable but also genetically unstable. A further study of genetic changes that may have occurred in pBtoxis in various transconjugants may be required. At a practical level, if such strains are to be used for mosquito control in jurisdictions where genetically manipulated organisms are accepted, it would be best if relatively stable strains could be used and if antibiotic selection could be retained for all stages prior to inoculation of the production culture. With wild-type, unmarked plasmids, monitoring for the presence of plasmids at all stages may be necessary.

B. thuringiensis subsp. *israelensis* plasmid pXO16 has been shown previously to be capable of mobilizing small plasmids that lack both the *mob* gene and the *oriT* site (16). In this work, we showed for the first time that very large plasmids, such as pBtoxis::erm (128 kb), can be mobilized for transfer to distantly related bacteria by using this system. Despite plasmid stability issues, this work provides the first demonstration that toxin-coding plasmids are able to replicate and can be sustained for any period outside the *Bacillus cereus* group of

TABLE 2. Vegetative and spore counts obtained as described in Materials and Methods using *B. sphaericus* transconjugant strain 2362.5

Cells	Subculture	Total cell count (CFU ml^{-1})[a]	Transconjugant cell count (CFU ml^{-1})[a]	% Cells retaining pBtoxis
Vegetative	First	$2.9 \times 10^8 \pm 3.1 \times 10^7$	$3.2 \times 10^8 \pm 2.0 \times 10^7$	111.4
	Second	$5.9 \times 10^9 \pm 5.0 \times 10^8$	$5.9 \times 10^9 \pm 4.1 \times 10^8$	100
	Third	$2.3 \times 10^8 \pm 3.1 \times 10^7$	$2.5 \times 10^8 \pm 2.7 \times 10^7$	106.9
	Fourth	$3.0 \times 10^8 \pm 2.1 \times 10^7$	$3.1 \times 10^8 \pm 2.9 \times 10^7$	103.0
Spores	First	$1.7 \times 10^8 \pm 2.5 \times 10^7$	$8.3 \times 10^7 \pm 2.3 \times 10^6$	48.6
	Second	$1.4 \times 10^8 \pm 1.3 \times 10^7$	$9.7 \times 10^7 \pm 1.3 \times 10^7$	71.1
	Third	$2.1 \times 10^8 \pm 2.2 \times 10^7$	$1.2 \times 10^8 \pm 1.0 \times 10^7$	50.1
	Fourth	$8.5 \times 10^7 \pm 1.9 \times 10^7$	$1.4 \times 10^8 \pm 2.8 \times 10^7$	16.2
	Fifth	$1.4 \times 10^8 \pm 1.8 \times 10^7$	$2.8 \times 10^6 \pm 7.6 \times 10^5$	2.0

[a] The values are means ± standard deviations (n = 6).

bacteria (which includes *B. thuringiensis*). This offers the potential to transfer the toxin-coding plasmids to other species that may have useful environmental properties for enhanced insect control. The existence of Cry toxins in non-*Bacillus* hosts, such as *Clostridium bifermentans* subsp. *malaysia* (25), could perhaps be linked to the natural transfer of such a plasmid during the evolution of the strain. The production of the *B. sphaericus* transconjugants without in vitro intervention using only microbiological mating techniques means that resulting strains that were not marked with antibiotic resistance genes would not be classified as "genetically modified" or "recombinant." This is likely to be very important in regulatory terms in many countries, if a commercializable strain is developed.

ACKNOWLEDGMENT

We acknowledge financial support from the Leverhulme Trust for this study.

REFERENCES

1. **Alexander, B., and F. G. Priest.** 1990. Numerical classification and identification of *Bacillus sphaericus* including some strains pathogenic for mosquito larvae. J. Gen. Microbiol. **136:**367–376.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Bar, E., J. Lieman-Hurwitz, E. Rahamim, A. Keynen, and N. Sandler.** 1991. Cloning and expression of *Bacillus thuringiensis israelensis* δ-endotoxin DNA in *B. sphaericus*. J. Invertebr. Pathol. **57:**149–158.
4. **Baumann, P., M. A. Clark, L. Baumann, and A. H. Broadwell.** 1991. *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxin. Microbiol. Rev. **55:**425–436.
5. **Berry, C., S. O'Neil, E. Ben-Dov, A. F. Jones, L. Murphy, M. A. Quail, T. G. Holden, D. Harris, A. Zaritsky, and J. Parkhill.** 2002. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **68:**5082–5095.
6. **Carrozi, N. B., V. C. Kramer, G. W. Warren, S. Evola, and M. G. Koziel.** 1991. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl. Environ. Microbiol. **57:**3057–3061.
7. **Correa, M., and A. A. Yousten.** 1997. Conjugation by mosquito pathogenic strains of *Bacillus sphaericus*. Mem. Inst. Oswaldo Cruz **92:**415–419.
8. **Delécluse, A., J.-F. Charles, A. Klier, and G. Rapoport.** 1991. Deletion by in vivo recombination shows that the 28-kilodalton polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. J. Bacteriol. **173:**3374–3381.
9. **Delécluse, A., S. Poncet, A. Klier, and G. Rapoport.** 1993. Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal-negative strain of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **59:**3922–3937.
10. **Dervyn, E., S. Poncet, A. Klier, and G. Rapoport.** 1995. Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **177:**2283–2291.
11. **Des Rochers, B., and R. Garcia.** 1984. Evidence for persistence and recycling of *Bacillus sphaericus*. Mosq. News **44:**160–165.
12. **Finney, D. J.** 1971. Probit analysis—a statistical treatment of the sigmoid response curve, 3rd ed. Cambridge University Press, Cambridge, United Kingdom.
13. **Georghiou, G. P., and M. C. Wirth.** 1997. Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. **63:**1095–1101.
14. **González, J. M., B. J. Brown, and B. C. Carlton.** 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc. Natl. Acad. Sci. USA **79:**6951–6955.
15. **Hu, X., B. M. Hansen, Z. Yuan, J. E. Johansen, J. Eilenberg, N. B. Hendriksen, L. Smidt, and G. B. Jensen.** 2005. Transfer and expression of the mosquitocidal plasmid pBtoxis in *Bacillus cereus* group strains. FEMS Microbiol. Lett. **245:** 239–247.
16. **Jensen, G. B., L. Andrup, A. Wilcks, L. Smidt, and O. M. Poulsen.** 1996. The aggregation-mediated conjugation system of *Bacillus thuringiensis* subsp. *israelensis*: host range and kinetics of transfer. Curr. Microbiol. **33:**228–236.
17. **Jensen, G. B., A. Wilcks, S. S. Petersen, J. Damgaard, J. A. Baum, and L. Andrup.** 1995. The genetic basis of the aggregation system in *Bacillus thuringiensis* subsp. *israelensis* is located on the large conjugative plasmid pXO16. J. Bacteriol. **177:**2914–2917.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
19. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
20. **Marchesi, J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, D. Dymock, and W. G. Wade.** 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. **64:**795–799.
21. **Myers, P., and A. Yousten.** 1978. Toxic activity of *Bacillus sphaericus* SSII-1 for mosquito larvae. Infect. Immun. **19:**1047–1053.
22. **Oliveira, C. M., M. H. Silva-Filha, C. Nielsen-Leroux, G. Pei, Z. Yuan, and L. Regis.** 2004. Inheritance and mechanism of resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from China and Brazil. J. Med. Entomol. **41:**58–64.
23. **Orzech, K., and W. Burke.** 1984. Conjugal transfer of pAMβ1 in *Bacillus sphaericus* 1593. FEMS Microbiol. Lett. **25:**91–95.
24. **Pei, G., C. M. F. Oliveira, Z. Yuan, C. Nielsen-Leroux, M. H. Silva-Filha, J. Yan, and L. Regis.** 2002. A strain of *Bacillus sphaericus* causes slower development of resistance in *Culex quinquefasciatus*. Appl. Environ. Microbiol. **68:**3003–3009.
25. **Poncet, S., G. Anello, A. Delécluse, A. Klier, and G. Rapoport.** 1993. Role of the CryIVD polypeptide in the overall toxicity of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **59:**3928–3930.
26. **Poncet, S., C. Bernard, E. Dervyn, J. Cayley, A. Klier, and G. Rapoport.** 1997. Improvement of *Bacillus sphaericus* toxicity against dipteran larvae by integration, via homologous recombination, of the Cry11A toxin gene from *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **63:**4413–4420.
27. **Poncet, S., A. Delécluse, G. Anello, A. Klier, and G. Rapoport.** 1994. Transfer and expression of the *cryIVB* and *cryIVD* genes of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus* 2297. FEMS Microbiol. Lett. **117:**91–96.
28. **Poncet, S., A. Delécluse, A. Klier, and G. Rapoport.** 1995. Evaluation of synergistic interactions among the CryIVA, CryIVB, and CryIVD toxic components of *B. thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. **66:**131–135.
29. **Servant, P., M.-L. Rosso, S. Hamon, S. Poncet, A. Delécluse, and G. Rapoport.** 1999. Production of Cry11A and Cry11Ba toxins in *Bacillus spherius* confers toxicity towards *Aedes aegypti* and resistant *Culex* populations. Appl. Environ. Microbiol. **65:**3021–3026.
30. **Silapanuntakul, S., S. Pantuwatana, A. Bhumiratana, and K. Charoensiri.** 1983. The comparative persistence of toxicity of *Bacillus sphaericus* strain 1593 and *Bacillus thuringiensis* serotype H-14 against mosquito larvae in different kinds of environments. J. Invertebr. Pathol. **42:**387–392.
31. **Silva-Filha, M.-H., L. Regis, C. Nielsen-Leroux, and J.-F. Charles.** 1995. Low-level resistance to *Bacillus sphaericus* in a field-treated population of *Culex quinquefasciatus* (Diptera: Culicidae). J. Econ. Entomol. **88:**525–530.

31a. **Stein, C., G. W. Jones, T. Chalmers, and C. Berry.** 2006. Transcriptional analysis of the toxin-coding plasmid pBtoxis from *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **72:**1771–1776.

32. **Thiéry, I., S. Hamon, A. Delécluse, and S. Orduz.** 1998. The introduction into *Bacillus sphaericus* of the *Bacillus thuringiensis* subsp. *medellin cyt1Ab1* gene results in higher susceptibility of resistant mosquito larva populations to *B. sphaericus*. Appl. Environ. Microbiol. **64:**3910–3916.
33. **Thomas, D. J. I., J. A. W. Morgan, J. M. Whipps, and J. R. Saunders.** 2000. Plasmid transfer between the *Bacillus thuringiensis* subspecies *kurstaki* and *tenebrionis* in laboratory culture and soil and in lepidopteran and coleopteran larvae. Appl. Environ. Microbiol. **66:**118–124.
34. **Trisrisook, M., S. Pantuwatana, A. Bhumiratana, and W. Panbangred.** 1990. Molecular cloning of the 130-kilodalton mosquitocidal δ-endotoxin gene of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus*. Appl. Environ. Microbiol. **56:**1710–1716.
35. **Vilas-Bôas, G. F. L. T., L. A. Vilas-Bôas, D. Lereclus, and O. M. N. Arantes.** 1998. *Bacillus thuringiensis* conjugation under environmental conditions. FEMS Microbiol. Ecol. **25:**369–374.
36. **Wilcks, A., N. Jayaswal, D. Lereclus, and L. Andrup.** 1998. Characterisation of plasmid pAW63, a second self-transmissible plasmid in *Bacillus thuringiensis* subsp. *kurstaki* HD73. Microbiology **144:**1263–1270.
37. **Wirth, M. C., A. Delécluse, and W. E. Walton.** 2001. Cyt1Ab1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and *B. thuringiensis* subsp. *israelensis* synergize *Bacillus sphaericus* against *Aedes aegypti* and resistant *Culex quinquefasciatus* (Dipetera: Culicidae). Appl. Environ. Microbiol. **67:**3280–3284.
38. **Wirth, M. C., B. A. Federici, and W. E. Walton.** 2000. Cyt1A from *Bacillus thuringiensis* synergizes activity of *Bacillus sphaericus* against *Aedes aegypti* (Diptera: Culicidae). Appl. Environ. Microbiol. **66:**1093–1097.
39. **World Health Organization.** 1985. Informal consultation on the development of *Bacillus sphaericus* as microbial larvicide. Publication TDR/BCV/SPHAERICUS/85.3.1–24. World Health Organization, Geneva, Switzerland.
40. **Wu, D., and B. A. Federici.** 1993. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. J. Bacteriol. **175:**5276–5280.
41. **Yoshisue, H., K. Ihara, T. Nishimoto, H. Sakai, and T. Komano.** 1995. Expression of genes for insecticidal crystal proteins in *Bacillus thuringiensis*: *cryIVA*, not *cryIVB*, is transcribed by RNA polymerase containing sigma^{H} and that containing sigma^{E}. FEMS Microbiol. Lett. **127:**65–72.
42. **Yousten, A. A., F. J. Genthner, and E. F. Benfield.** 1992. Fate of *Bacillus sphaericus* and *Bacillus thuringiensis* serovar *israelensis* in the aquatic environment. J. Am. Mosq. Control Assoc. **8:**143–148.

diarrhoea-induced rats were reduced by 54% and 44%, respectively, compared with normal rats. The difference in the Cmax was determined to be statistically significant. In conclusion, the concomitant administration of loperamide had no effect on the PK of celgosivir in normal rats and could be considered a viable treatment option for reducing gastrointestinal effects that may be associated with celgosivir treatment. Since induced-induced rats showed a reduction in castanospermine Cmax and AUC, treatment with loperamide might prevent lowered systemic drug exposure in patients experiencing diarrhea.

105

In Vitro Characterization of Celgosivir, a Clinical Stage Compound for the Treatment of Hepatitis C Viral Infections

Dominique Dugourd, Jeremy Fenn, Raymond Siu, Jacob J Clement, Richard Coulson[1]

MIGENIX Inc., Vancouver, BC, Canada

Celgosivir (MX-3253), a compound in phase II clinical trials for the treatment of chronic Hepatitis C viral (HCV) infections, targets intracellular α-glucosidase I, an endoplasmic reticulum (ER) enzyme that plays a critical role in viral maturation by initiating the processing of N-linked oligosaccharides of viral envelope glycoproteins. The inhibitory activities of celgosivir and its metabolite, castanospermine, were tested against the HCV-surrogate virus, bovine viral diarrhea virus (BVDV), maintained in Madin-Darby bovine kidney (MDBK) cells at various multiplicities of infection (MOI). Celgosivir and castanospermine had EC_{50} values of 2.1 and 13.0 μM, respectively, for blocking virus release in a single cycle assay at an MOI of ~1. In a multiple cycle assay, celgosivir blocked BVDV's cytopathic effect with EC_{50} values of 7.2 and 17.2 μM at MOIs of 0.01 and 0.1, respectively. Similarly, castanospermine blocked BVDV's cytopathic effect, with EC_{50} values of 75 μM and 185 μM at MOIs of 0.01 and 0.1, respectively. When BVDV-infected cells were pretreated for 24 h, viral re-growth times (time for $1 \times \log_{10}$ viral growth) were 4, 4, and 8 h post-treatment for 11, 33 and 100 μM of celgosivir, respectively. With 11, 33 and 100 μM of ribavirin, re-growth times were 4, 8, and 16 h, respectively, compared with 2 h for untreated BVDV-infected cells. This suggests that celgosivir targets the late viral replication stage whereas ribavirin targets early replication. Celgosivir and castanospermine showed minimal cytotoxicity (CC50 >1000 μM) when tested against non-infected human hepatocytes. Celgosivir and its metabolite castanospermine exhibit potent anti-BVDV efficacy and low host cell toxicity. These findings confirm the potential of celgosivir as a Hepatitis C viral therapy in humans.

107

An Assay for the Biological Testing of Potential Inhibitors for the HCV Helicase, Dengue Virus Helicase and Dengue Virus Helicase/Protease Complex (NS3 Domain)

Dimitrios P. Vlachakis[1], Colin Berry[2], Gareth Jones[2], Andrea Brancale[1]

[1]Medicinal Chemistry, Welsh School of Pharmacy, Cardiff University, Wales, UK; [2]Cardiff School of Biosciences, Cardiff University, Wales, UK

Hepatitis C and dengue are enveloped ñ positive-sense RNA viruses. Hepatitis C virus is the major etiological agent of post-transfusion hepatitis worldwide. An estimated 3% of the world's population is infected with HCV according to the World Health Organization. Infection with HCV will most regularly result in chronic hepatitis, which leads to liver cirrhosis, hepatocellular carcinoma and liver failure. Dengue is currently the most important viral disease, transmitted by mosquitoes and afflicting humans worldwide. Clinical symptoms range from mild fevers to a severe hemorrhagic disease. To date, no specific antiviral treatments exist nor are there any vaccines available for either infections. Thus there is an urgent need for new therapies.

The aim of this project is to design and establish an enzymatic assay that will be used to screen for potential inhibitors of the Helicases of the HCV and dengue viruses as well as the Helicase/protease complex of the dengue virus. Helicases are interesting targets for drug design, firstly for their vital function in the viral cell cycle and secondly for the fact that human cells lack helicases capable of unwinding positive sense double stranded RNA. The genes of the HCV Helicase, the dengue virus Helicase and the dengue virus NS3 domain (Helicase and Protease) were incorporated into a pET system expression vector. The vectors carrying the genes were then transformed into *E. coli* cells and the genes were expressed (BL21-pLysS strain). It was determined that both Helicases are produced in the cell without the need for induction. This was confirmed by an expression test with variable concentrations of inducer (0–1 mM IPTG). It was found that the protein was present under all expression systems. However the one induced at 1 mM showed max yield. After induction, the cell suspensions were harvested. SDS–PAGE and His-Tag Western blotting confirmed the existence of the various proteins. Protein isolation was based on the 6(His)-Tags of the three proteins. The proteins were tested for their functionality using specific enzymatic assays.

JEN 128(7) doi: 10.1111/j.1439-0418.2004.00874.469–473

© 2004 Blackwell Verlag, Berlin

Screening of Brazilian *Bacillus sphaericus* strains for high toxicity against *Culex quinquefasciatus* and *Aedes aegypti*

R. Monnerat[1], S. F. da Silva[1], D. S. Dias[2], É. S. Martins[1], L. B. Praça[1], G. W. Jones[3], C. M. Soares[2], J. M. C. de Souza Dias[1] and C. Berry[3]

[1]Embrapa Recursos Genéticos e Biotecnologia, SAIN-Parque Rural, Caixa, postal 02372, 70770-900-Brasilia-DF, Brazil; [2]Bthek Biotecnologia Ltda., SAAN trecho 3 lote 240, Brasília, Brazil; [3]Cardiff School of Biosciences, Cardiff University, Cardiff, Wales, UK

Ms. received: December 30, 2003; accepted June 07, 2004

Abstract: In this work, 246 *Bacillus sphaericus* strains were evaluated against *Aedes aegypti* and *Culex quinquefasciatus* larvae to select the most effective ones to be used as the basis of a national product. All strains were isolated from different regions of Brazil and they are stored in a *Bacillus* spp. collection at Embrapa Genetic Resources and Biotechnology. The selected strains were characterized by biochemical and molecular methods. Based on selective bioassays, 87 strains were identified as toxic to one or both target species. All of these strains contain genes that encode the 42, 51 kDa proteins that constitute the binary toxin and the 100 kDa Mtx1 toxin. All toxic strains presented a very high LC_{50} against *A. aegypti*, so, a product based on any of these *B. sphaericus* strains would not be recommended for use in programmes to control *A. aegypti*. S201 had highest activity against *C. quinquefasciatus*, presenting the lowest LC_{50} and LC_{90} in bioassays.

Key words: *Aedes aegypti, Bacillus sphaericus, Culex quinquefasciatus*, bioassay, biological control, mosquito, vector disease

1 Introduction

Bacillus sphaericus Neide is a bacterial species found commonly in soil and aquatic habitats (Davidson, 1985) and characterized by the production of spherical terminal or subterminal spores in the sporangium. Although most strains of *B. sphaericus* are not pathogenic for insects, the mosquitocidal strains are important tools in mosquito control programs. The first pathogenic strains were isolated from *Culiseta incidens* (Dipt.: Culicidae) larvae and were called strains K and Q (Kellen et al., 1965). Currently many toxic strains are known and many studies have been performed on strains 1593 and 2362 isolated respectively in Indonesia (Singer, 1973) and Nigeria (Weiser, 1984). This organism does not infect non-target invertebrates (including bees) or cold-blooded vertebrates and it is also innocuous to mammals in laboratory tests (Davidson, 1985). The World Health Organization (WHO) recommends the utilization of this bacterium in public health programs (World Health Organisation, 1985).

Almost all mosquito species from the genus *Culex* (Dipt.: Culicidae), some of them vectors of filariasis, are susceptible to *B. sphaericus*, as are members of the genera *Anopheles, Psorophora* and *Mansonia*. The activity of this bacterium against mosquitoes from the genera *Aedes* and *Ochlerotatus* (representing species that used to be defined as *Aedes*), is variable. Some mosquito species are very susceptible and others, particularly *Aedes aegypti*, a major vector of dengue and yellow fever, show low sensitivity. *Bacillus sphaericus* is very effective when used in polluted water, making it a good option to control *Culex quinquefasciatus* and other *Culex*, breeding in polluted water in cities located in tropical and sub-tropical areas. The *B. sphaericus* activity is because of a presence of different kinds of protein toxins that differ both in their composition and time of synthesis. The parasporal crystal of *B. sphaericus*, which is produced during the sporulation phase is a binary toxin composed of two components designated as P51 and P42 on the basis of their molecular masses (Baumann et al., 1987), and now termed BinB and BinA, respectively. The different Mtx toxins, which have molecular masses of 100 kDa (Mtx1) and 32 and 36 kDa (Mtx2 and Mtx3) are expressed during the vegetative growth phase, but low levels of production and instability mean that their toxicity is of minor significance, particularly in the spores that are applied in mosquito control programmes (Thanabalu et al., 1991; Chan et al., 1996; Liu et al., 1996).

Bacillus sphaericus is a very promising microorganism and several laboratories around the world are looking for new strains that may be able to produce novel toxins or which may be more adapted to local environmental conditions, in order to have better effect in the field and which could be used in resistance management (Monnerat and Bravo, 2000).

Embrapa Genetic Resources and Biotechnology has a culture collection of entomopathogenic *Bacillus* spp. in which around 300 *B. sphaericus* strains are stored (Monnerat et al., 2001a). The aim of this work was the

characterization of the most toxic *B. sphaericus* strains for the control of *A. aegypti* and *C. quinquefasciatus* among the Embrapa culture collection to identify strains that could be used as a basis for a Brazilian product.

2 Materials and Methods

2.1 *Bacillus sphaericus* strains

A total of 246 *B. sphaericus* strains were used in this work. They are stored at Embrapa's Culture Collection of Entomopathogenic *Bacillus* spp. and were isolated from soil and water samples collected in different regions of Brazil (Monnerat et al., 2001a).

2.2 Preliminary bioassay

All strains were grown in NYSM (a medium composed of nutrient broth, yeast extract, Mncl2, MgCl2 and Ca Cl2) medium (Yousten, 1984) for 48 h at 28°C and 200 r.p.m. and tested against third-instar larvae of *C. quinquefasciatus* and *A. aegypti*. One millilitre of total culture of each strain was added to 200 ml cups in triplicate with 100 ml of distilled water and 25 larvae of *C. quinquefasciatus* or *A. aegypti*. One cup without bacteria was used as the control. Forty-eight hours later, the numbers of dead larvae were evaluated. The strains that killed more than 50% of the larvae were considered pathogenic (Monnerat et al., 2001b).

2.3 Quantified bioassay

2.3.1 Final whole culture

In order to determine the LC_{50}, the quantified bioassay was performed according to the method recommended by WHO (WHO, 1985), several dilutions of the final culture prepared as described above were used. One millilitre of these dilutions was added into 200 ml cups in triplicate, as for the procedure used in selective bioassays. Forty-eight hours later the numbers of dead larvae were recorded and the LC_{50} was calculated by Probit analysis (Finney, 1971). *Bacillus sphaericus* 2362 (SPH-88, from the Pasteur Institute) was used as standard.

2.3.2 Lyophilized culture

The most toxic strains against *C. quinquefasciatus* were also tested as above, except that lyophilized culture, prepared as described previously (WHO, 1985), was added in place of diluted whole cell cultures. These bioassays were repeated three times.

2.4 Analysis of protein profile

The spore-crystal mixtures of the *B. sphaericus* strains were prepared according to Schenkel et al. (1992). The protein composition of the spore-crystal mixtures was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels.

2.5 Analysis of the presence of *B. sphaericus* toxin genes by polymerase chain reaction (PCR)

The method used was described by Otsuki et al. (1997). *Bacillus sphaericus* strains were grown on NYSM agar for 16 h, at 25°C. Cells were resuspended in MilliQ water and frozen at −80°C for 1 h and then transferred to boiling water for 10 min to lyse the cells.

Primers designed for detection of the binary toxin operon, and the individual *bin* genes, BSN1/BSN2 and BS1/BS2 (*binB*), BSN3/BSN4 and BS3/BS4 (*binA*) and 100.1/100.2 (*mtx1*) toxin were used (Otsuki et al., 1997). Fifteen microlitre of supernatant obtained from cell lysates of the *B. sphaericus* strains were transferred to a 200-μl reaction tube (Bio-Products) containing 0.5 μM of each primer, 0.2 mM of each dNTP, 1x *Taq* polymerase buffer, 1.5 mM $MgCl_2$ and 2.5 U of *Taq* DNA polymerase (Gibco BRL, Life Technologies, Grand Island, NY, USA) in a final volume of 50 μl. PCR amplification was performed with a Programmable Thermal Controller (MJ Research model PTC-100, MJ Research, Inc., Waltham, MA, USA). The conditions used for the PCRs were those described by Otsuki et al. (1997). After amplification, a 15-μl sample of the product from each PCR reaction was subjected to electrophoresis in a 2% (w/v) agarose gel in Tris-borate buffer at 100 V for 1 h and stained with ethidium bromide.

2.6 Sequencing of a bin operon

To determine the sequence of the bin genes from a sample strain, the operon was amplified by PCR using the primers BinF (CAATGATAAGGAGATGAAGA) and BinR (CAT-CTATTAGTTCAAGAATATTG) at an annealing temperature of 60°C. The amplified fragment was cloned into the *Escherichia coli* vector pGEM-T (Promega, Madison, WI USA) for subsequent sequencing using an ABI Prism 3100 capillary sequencer.

3 Results and Discussion

3.1 Toxicities of *B. sphaericus* strains against *C. quinquefasciatus* and *Aedes aegypti* larvae

Single point bioassays were performed on all 246 strains of *B. sphaericus* in the Embrapa collection. Of these strains, 87 strains were considered toxic, killing more than 50% of insect larvae: 69 were toxic only to *C. quinquefasciatus* and 18 to both *C. quinquefasciatus* and *A. aegypti*.

LC_{50} and LC_{90} values, using a final whole culture against the two target species, were subsequently determined only for the 18 strains that presented dual activity (tables 1 and 2).

Against *C. quinquefasciatus*, the LC_{50} values showed a range between 0.38 and 14.5×10^{-6} fcd (final culture dilution), the LC_{90} values showed a range between 1.52 and 72.9×10^{-6} (table 1). The most effective strains were S201 and S162, having respectively LC_{50} of 0.38 and 0.55×10^{-6} fcd and LC_{90} of 1.52 and 2.20×10^{-6} fcd. This result shows that both strains are at least twice as active against this insect than the standard strain *B. sphaericus* 2362 that had an LC_{50} of 2.1×10^{-6} fcd and LC_{90} of 5.4×10^{-6} fcd in our assays (table 1).

Against *A. aegypti*, the LC_{50} values ranged between 0.003 and 0.35 fcd and the LC_{90} between 0.012 and 2.67 fcd (table 2). The most effective strains were S242, S233 and S260, having LC_{50} of 0.003, 0.004 and 0.004 fcd and LC_{90} of 0.012, 0.018 0.019 fcd respectively. Strains 2362 and S201 were the least toxic

***Table 1.** Toxicities of* B. sphaericus *strains against C. quinquefasciatus larvae*

Strains	LC_{50} (fiducial limits 95%)	LC_{90} (fiducial limits 95%)
S201	0.38 (0.28–0.52)	1.52 (1.02–2.74)
S162	0.55 (0.41–0.76)	2.20 (1.46–4.18)
2362	2.12 (1.65–2.87)	5.40 (3.80–9.30)
S242	2.20 (1.70–2.98)	5.47 (3.87–9.40)
S200	2.28 (1.77–3.09)	5.56 (3.94–9.43)
S295	2.28 (1.77–3.09)	5.56 (3.94–9.43)
S260	2.47 (1.91–3.35)	5.74 (4.10–9.46)
S516	2.69 (2.06–3.57)	6.73 (4.89–10.6)
S524	2.69 (2.06–3.57)	6.73 (4.89–10.6)
S15	2.86 (2.19–3.77)	7.58 (5.53–11.7)
S16	3.16 (2.39–4.16)	9.19 (6.67–14.3)
S233	3.16 (2.39–4.18)	7.07 (5.25–10.5)
S444	3.16 (2.39–4.18)	7.07 (5.25–10.5)
S662	3.16 (2.39–4.18)	7.07 (5.25–10.5)
S438	3.54 (2.67–4.62)	8.59 (6.46–12.5)
S64	3.85 (2.84–5.01)	8.70 (6.64–12.2)
S558	4.84 (3.62–6.35)	14.6 (10.6–23.3)
S1	13.7 (9.30–27.7)	66.0 (31.1–428)
S131	14.5 (9.73–31.7)	72.9 (32.9–556)

The unit used was final culture dilution (fcd) × 10^{-6}.

***Table 2.** Toxicities of* B. sphaericus *strains against A. aegypti larvae*

Strains	LC_{50} (fiducial limits 95%)	LC_{90} (fiducial limits 95%)
S242	0.003 (0.002–0.005)	0.012 (0.008–0.022)
S233	0.004 (0.002–0.006)	0.018 (0.011–0.033)
S260	0.004 (0.002–0.006)	0.019 (0.012–0.035)
S295	0.005 (0.003–0.007)	0.025 (0.015–0.049)
S516	0.005 (0.003–0.007)	0.025 (0.015–0.049)
S558	0.005 (0.003–0.008)	0.029 (0.017–0.059)
S200	0.006 (0.004–0.010)	0.037 (0.021–0.082)
S444	0.006 (0.004–0.010)	0.037 (0.021–0.082)
S662	0.006 (0.004–0.010)	0.037 (0.021–0.082)
S438	0.007 (0.004–0.010)	0.040 (0.023–0.090)
S524	0.007 (0.004–0.010)	0.040 (0.023–0.090)
S131	0.009 (0.006–0.015)	0.061 (0.031–0.168)
S15	0.020 (0.013–0.029)	0.140 (0.080–0.310)
S64	0.024 (0.017–0.035)	0.160 (0.096–0.0351)
S16	0.047 (0.033–0.069)	0.230 (0.141–0.450)
S1	0.044 (0.032–0.065)	0.220 (0.130–0.411)
S162	0.062 (0.043–0.095)	0.034 (0.205–0.740)
S201	0.233 (0.165–0.330)	1.30 (0.811–2.66)
2362	0.355 (0.243–0.541)	2.67 (1.47–7.07)

The unit used was final culture dilution (fcd).

presenting LC_{50} values of 0.35 and 0.24 fcd and LC_{90} of 2.67 and 1.30 fcd (table 2).

To check whether higher apparent toxicities were because of a greater toxicity per cell or a higher cell density in the final cultures, LC_{50} and LC_{90}values were also determined for the four most toxic strains against *C. quinquefasciatus* using a lyophilized final whole culture (table 3). In this case, S201 and S242 were the most toxic strains, showing LC_{50} of 1.24 and 1.35 ng/ml and LC_{90} of 4.52 and 7.16 ng/ml of lyophilized material, respectively whilst strain S162 showed similar toxicities to that of 2362 with values of 2.97 and 4.15 ng/ml (LC_{50}) and 26.8 and 35.1 ng/ml (LC_{90}).

***Table 3.** Toxicities of* B. sphaericus *strains against C. quinquefasciatus larvae. Results are expressed in nanogram of lyophilized bacteria/ml*

Strains	LC_{50} (fiducial limits 95%)	LC_{90} (fiducial limits 95%)
S201	1.24 (0.92–1.67)	4.52 (3.12–7.87)
S242	1.35 (0.95–1.90)	7.16 (4.53–14.9)
S162	2.97 (1.92–4.36)	26.8 (16.6–52.2)
2362	4.15 (2.74–6.04)	35.1 (21.7–69.7)

These results confirm *B. sphaericus* S201 and S242 as promising strains and indicates that for strain S201, better growth characteristics as well as higher toxicity per spore may contribute to the higher activity observed.

3.2 Analysis of protein profile by SDS-PAGE

Spore crystal complexes from 87 mosquitocidal *B. sphaericus* strains and the standard strain 2362 were used. All of them presented the same protein profile, showing two major proteins of 51 and 42 kDa (fig. 1). This protein profile is typical of the binary toxin produced by *B. sphaericus* (Baumann et al., 1987).

3.3 Analysis of the presence of *B. sphaericus* toxin genes by polymerase chain reaction

All 87 *B. sphaericus* pathogenic strains produced the expected PCR amplicons of 0.523, 0.720 and 0.700 kb, indicating the presence of genes encoding BinA, BinB and Mtx1, respectively (fig. 2). These results are consistent with the protein profiles for these strains where the presence of 51 and 42 kDa bands, corresponding to the sizes of BinB and BinA respectively, were observed. The presence of 100 kDa

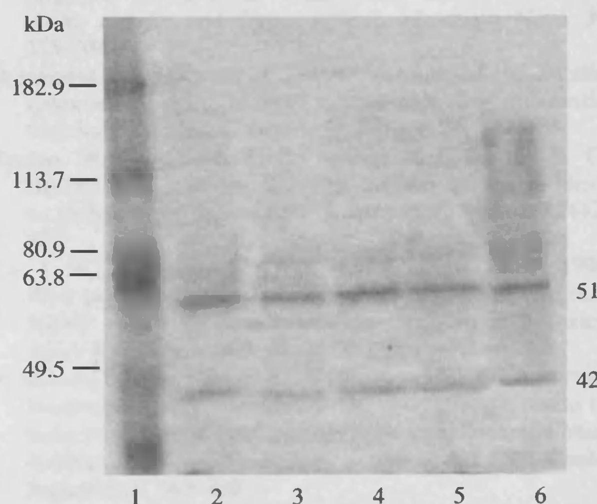


***Fig. 1.** Representative SDS-PAGE of spore-crystal from B. sphaericus strains. 1, molecular marker Gibco BRL; 2, 2362; 3, S242; 4, S233; 5, S260 and 6, S295. All other toxic strains exhibited the same profile (not shown)*

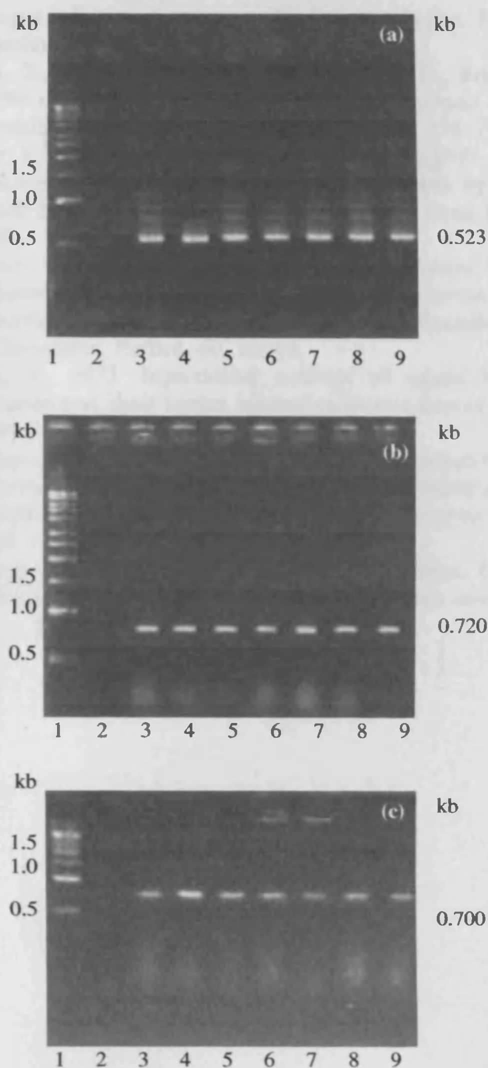


Fig. 2. Representative agarose gel of PCR products obtained with primers BS1/BS2 (a), BSN3/BSN4 (b) and 100.1/100.2 (c). 1, molecular marker 1 kb Pharmacia; 2, negative control; 3, 2362; 4, S200; 5, S233; 6, S242; 7, S260; 8, S295 and 9, S516. All other toxic strains exhibited the same profile (not shown)

bands corresponding to the Mtx1 toxin were not observed on SDS-PAGE and would not be expected in spore crystal mixes due the fact that this is a vegetative protein produced in low amounts with low stability that has not been observed previously in spores (Thanabalu et al., 1992; Thanabalu and Porter, 1995).

3.4 Sequencing of a bin operon

To assess whether this toxicity was because of a new variant of the bin operon, the bin genes from S242 were amplified and sequenced. The results showed that the bin operon in this strain was identical to bin type 2 as found in *B. sphaericus* strains such as 2362 (Humphreys and Berry, 1998). This indicates that the greater toxicity to *A. aegypti* may be because of higher levels of toxin production or to the presence of an extra, unidentified toxin active against this species.

The assays performed in this work demonstrated that the toxic *B. sphaericus* strains isolated from different regions of Brazil all appear to be typical of highly toxic strains of this bacterium as they encode Bin toxins, along with the Mtx1 protein although they present a range virulence levels towards the two mosquito species studied. It is also important to emphasize that the LC_{50} obtained against *C. quinquefasciatus* is not indicative of the relative toxicity against *A. aegypti.* Although many strains are more toxic than 2362 against *A. aegypti*, the LC_{50} are still very high when compared with *Bacillus thuringiensis israelensis* (Goldberg and Margalit, 1997) and *B. thuringiensis medellin* (Orduz et al., 1994) so, a product based on any of these *B. sphaericus* strains would not be recommended for use in programmes to control *A. aegypti.* The compilation of the results shows that S201 is the best strain to be used as a basis of a product against *C. quinquefasciatus*, as this strain presented the lowest LC_{50} and LC_{90} in both kinds of bioassays with a significantly better activity against this mosquito.

References

Baumann, P.; Baumann, L.; Bowditch, L.; Broadwell, A. H., 1987: Cloning of the gene for the larvicidal toxin of *B. sphaericus* 2362: evidence for a family of related sequences. J. Bacteriol. **163,** 738–747.

Chan, S. W.; Thanabalu, T.; Wee, B. Y.; Porter, A. G., 1996: Unusual amino acid determinants of host range in the Mtx2 family of mosquitocidal toxins. J. Biol. Chem. **271,** 14183–14187.

Davidson, E. W., 1985: *Bacillus sphaericus* as a microbial control agent for mosquito larvae. In: Integrated Mosquito Control Methodologies, vol. 2. Ed. by Laird, M.; Miles, J. USA: Academic Press, 213–226.

Finney, D., 1971: Probit Analysis. Cambridge: Cambridge University Press.

Goldberg, L. J.; Margalit, J. A., 1997: Bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegypti* and *Culex pipiens.* Mosquito News **37,** 355–358.

Humphreys, M. J.; Berry, C., 1998: Variants of the *Bacillus sphaericus* binary toxins: implications for differential toxicity of strains. J. Invertebr. Pathol. **71,** 184–185.

Kellen, W. R.; Clark, T. B.; Lindegren, J. E.; Ho, B. C.; Rogoff, M. H.; Singer, S., 1965: *Bacillus sphaericus* Neide as pathogen of mosquitoes. J. Invertebr. Pathol. **7,** 442–448.

Liu, J. W.; Porter, A. G.; Wee, B. Y.; Thanabalu, T., 1996: New gene from nine *Bacillus sphaericus* strains encoding highly conserved 35.8-kilodalton mosquitocidal toxins. Appl. Env. Microbiol. **62,** 2174–2176.

Monnerat, R. G.; Bravo, A., 2000: Proteínas bioinseticidas produzidas pela bactéria *Bacillus thuringiensis*: modo de ação e resistência. In: Controle Biológico, Embrapa Meio Ambiente. Ed. by Melo, I. S.; Azevedo, J. L. São Paulo: Jaguariúna, 163–200.

Monnerat, R.; Silva, S. F.; Silva-Werneck, J. O.; Dias, J. M. C. S., 2001a: Métodos de coleta, isolamento, caracterização e armazenamento de estirpes de *Bacillus sphaericus.* Circular Técnica n° 9, Embrapa, Brasília.

Monnerat, R. G.; Silva, S. F.; Silva-Werneck, J. O., 2001b: Catálogo do banco de germoplasma de bactérias ento-

mopatogênicas do gênero *Bacillus*. Embrapa Recursos Genéticos e Biotecnologia, Brasília.

Orduz, S.; Diaz, T.; Thierry, I.; Charles, J. F.; Rojas, W., 1994: Crystal Proteins from *Bacillus thuringiensis* serovar. *medellin*. Appl. Microbiol. Biotechnol. **40,** 794–799.

Otsuki, K.; Guaycurus, T. V.; Vicente, A. C. P., 1997: *Bacillus sphaericus* entomocidal potential determined by polymerase chain reaction. Mem. Inst. Oswaldo Cruz **92,** 107–108.

Schenkel, R. G. M.; Nicolas, L.; Frachon, E.; Hamon, S., 1992: Characterization and toxicity to mosquito larvae of four *Bacillus sphaericus* strains isolated from Brazilian soils. J. Invertebr. Pathol. **60,** 10–14.

Singer, S., 1973: Insecticidal activity of recent bacterial isolates and their toxins against mosquito larvae. Nature **244,** 110.

Thanabalu, T.; Porter, A., 1995: Efficient expression of a 100-kilodalton mosquitocidal toxin in protease-deficient recombinant *Bacillus sphaericus*. Appl. Environ. Microbiol. **61,** 4031–4036.

Thanabalu, T.; Hindley, J.; Jackson-Yap, J.; Berry, C., 1991: Cloning sequencing and expression of gene encoding a 100-kilodalton mosquitocidal toxin from *Bacillus sphaericus* SSII-1. J. Bacteriol. **173,** 2776–2785.

Thanabalu, T.; Hindley, J.; Berry, C., 1992: Proteolytic processing of he mosquitocidal toxin from *Bacillus sphaericus* SSII-1. J. Bacteriol. **174,** 5051–5056.

Weiser, J. A., 1984: A mosquito-virulent *Bacillus sphaericus* in adult *Simulium damnosum* from northern Nigeria. Zentralbatt Mikrobiologie **139,** 57–60.

World Health Organization, 1985: Informal Consultation on the Development of Bacillus sphaericus as a Microbial Larvicide. Special Programme for Research and Training in tropical Diseases, TDR/BCV/SPHAERICUS/85.3. Geneva

Yousten, A. A., 1984: *Bacillus sphaericus*: Microbiological factors related to its potencial as a mosquito larvicide. Adv. Biotechnol. Process. **3,** 315–343.

Author's address: Rose Monnerat, Embrapa Recursos Genéticos e Biotecnologia, SAIN-Parque Rural, Caixa postal 02372, 70770-900-Brasilia-DF, Brazil. E-mail: rose@cenargen.embrapa.br