Factors influencing the activity of mosquito control agent (*Bacillus thuringiensis* subsp. *israelensis*)

by

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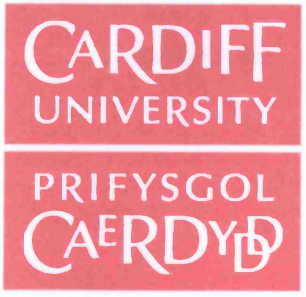
Being a thesis presented in accordance with the regulations governing the award of the degree of Doctor of Philosophy in Cardiff University



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Dedication

To My Lord ALLAH and to my Country LIBYA

Summary

Bacillus thuringiensis subsp. *israelensis* is the most important biological insecticide for the control of mosquito vectors of disease. This bacterium produces toxins during sporulation that cause larval death by lysis of cells in their midgut.
For toxicity, *B. thuringiensis* must be taken into the larval midgut, where a community of other bacteria is already present. The culturable flora from the *Aedes aegypti* mosquito midgut was analysed and its role in larval growth and insect mortality was determined. In contrast to published reports concerning *B. thuringiensis* subsp. *kurstaki*, subsp. *israelensis* caused toxicity and larval death even in the absence of other bacteria.

The pBtoxis plasmid of *B. thuringiensis* subsp. *israelensis* encodes all the mosquitocidal toxins and a number of other coding sequences. The potential effects of selected genes on host phenotype was assessed. No evidence was found for antibiotic production from putative antibiotic synthesis genes. The plasmid also carries potential germination genes organised in a single *ger* operon. Comparison of the germination responses of spores from strains with and without pBtoxis revealed that this plasmid could promote activation of the spores under alkaline conditions but not following heat treatment. Introduction of the *ger* operon on a recombinant plasmid to the plasmidless strain established this operon as the first with an identified role in alkaline activation.

Mosquito midguts provide an alkaline environment and in which enhanced germination may occur. Co-feeding experiments showed that in competition to colonise intoxicated *A. aegypti* larvae, *B. thuringiensis* carrying pBtoxis, are able to outgrow the plasmid-cured strain. This indicates a selective advantage for the presence of pBtoxis. The strain carrying the recombinant *ger* genes also outgrows its plasmidless parent, indicating that the *ger* genes may be responsible for this effect, perhaps by allowing strains a "head-start" by germinating more rapidly in the insect gut.

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Abbreviations

aa	amino acids
ALP	alkaline phospatase
APN	aminopeptidase N
Acridine orange	3,6-bosdimethylaminoacridine
BLAST	Basic Local Alignment Search Tool
BBMV	Brush border membrane vesicles
bp	base pair
BSA	Bovine Serum Albumin
Bti	*Bacillus thuringiensis*. subsp *israelensis*
°C	Degrees Celsius
CADR	Cadherin-like protein
CIP	Calf intestinal alkaline phosphatase
C-terminal	Carboxy terminal
D	Attenuance
Da	Dalton
dATP(dCTP, dGTP, dTTP, dUTP)	2'-deoxyadenosine 5'triphosphate (and similarly for cytidine, guanosine, thymidine uridine)
dNTP	Mixture of dATP, dCTP, dGTP and dTTP
EDTA	Ethylenediamine tetra acetic acid
GalNAc	N-acetyl galactosamine
GPI	Glycosyl-phosphatidylinositol
h	Hour (s)
kb	Kilobase pairs
l	Litre
LB	Luria Bertani
m, μ, g	milli, micro, gram
min	Minute(s)
MW	Molecular Weight
nt	Nucleotides
N-terminal	Amino terminal
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RE	Restriction endonuclease
rep-PCR	Repetitive element PCR
RNase	Ribonuclease
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
sec	second(s)
Sips	Secreted insecticidal proteins
STAB-SD	Shine-Dalgarno Stabilising Sequences
TAE	Tris Acetate buffer
TE	10 mM Tris/1mM EDTA
Tris	2-amino-2- (hydroxymethyl)-1,3-propandiol
UV	Ultraviolet
v/v	volume/volume
Vips	Vegetative insecticidal proteins
w/v	weight/volume
WHO	World Health Organisation
xg	Units of gravitational force

CHAPTER 1

General introduction

1.1 Introduction

Mosquito-borne diseases are found worldwide infecting humans, causing medical concerns and also infecting livestock, causing economic problems. Mosquitoes such as *Anopheles*, *Aedes* and *Culex* are vectors of malaria, dengue fever, yellow fever, lymphatic filariasis and viral encephalitis. Current methods of control of vector mosquitoes used in control programmes such as chemical insecticides e.g. (DDT dichlorodiphenyltrichloro ethane, gammatane and melathion) are losing their effectiveness due to mosquito resistance (Luxananil *et al*, 2001), and harm the environment (Tabashnik, 1994). So to deal with these problems new methods are required to achieve better control of mosquitoes worldwide. Biological insecticides represent an alternative method using insect pathogenic organisms such as the bacterium *Bacillus thuringiensis*, which produce proteins that have specific toxic activity against mosquitoes and blackfly (Schnepf *et al.*, 1998).

Bacterial endospores were first described in 1876 by Fredinand Cohn and also Robert Koch independently (Gould, 2006). Among the most studied spore-forming genera is the genus *Bacillus*. In early investigations, the most studied were *B. cereus* and *B. megaterium,* but in the 1970s and 1980s, *B. subtilis* 168 became the first *Bacillus* species studied genetically, leading to the sequence of its genome (Gould, 2006). The capacity to form endospores is a powerful strategy for survival in adverse environmental conditions. To return to the vegetative state in favourable conditions, spores need to have efficient mechanisms to germinate (Moir *et al.*, 1994). Germination happens when the spore responds to certain molecules in the environment, which indicate the right time for re-establishing the vegetative form (Moir *et al*, 2002).

1.2 *Bacillus cereus* group

Bacillus cereus sensu lato is a complex group of aerobic or facultative aerobic gram-positive spore-forming bacteria that includes six closely related species; *Bacillus thuringiensis, Bacillus cereus, Bacillus anthracis, Bacillus weihenstephanensis, Bacillus pseudomycoides* and *Bacillus mycoides* (Jensen *et al.*, 2003) all belonging to the *Bacillaceae* family (Rasko *et al.*, 2005). *B. cereus* is an opportunistic human pathogen and produces enterotoxins, which cause food poisoning (Sorokin *et al.*, 2006), whereas *B. anthracis* is the causative agent of anthrax (Jensen *et al.*, 2003). *B. thuringiensis* is an insect pathogen, which produces insecticidal crystal inclusions (δ-endotoxins) inside the cell during sporulation (Whiteley *et al.*, 1986). *B. weihenstephanensis* is able to grow at temperatures lower than 8°C, whereas *B. mycoides,* has a rhizoidal growth and makes a specific form of colonies that have a rhizoidal appearance (Sorokin *et al.*, 2006). The most studied species of the group are *B. thuringiensis, B. cereus* and *B. anthracis.*

Analysis of members of the group on a genetic basis by examining DNA sequences, demonstrated that *B. thuringiensis, B. cereus* and *B. anthracis* are similar and can be regarded as variants of one species (Helgason *et al.*, 2000). These species are very similar and cannot be distinguished by standard methods (Carlson *et al.*, 1994; Jensen *et al.*, 2005) including pulsed-field gel electrophoresis (PFGE) and a combination of PCR and restriction endonuclease digestion. Zhong *et al.*, (2007) have also stated that using PFGE to differentiate between *B. thuringiensis, B. cereus* and *B. anthracis* is difficult. The complete genomes of *B. anthracis* and two strains of *B. cereus* confirmed the shared similarity (Rasko *et al.*, 2004; Read *et al.*, 2003).

In spite of this, they have been maintained in the taxonomy as separate species due to different pathogenicity (Priest *et al.*, 2004).

This difficulty distinguishing between the individual species is also due to their ability to exchange their genetic material (Sorokin *et al.*, 2006). The *B. cereus* group shares a large "core-set" of genes between its members (Rasko *et al.*, 2005), and has a large number of plasmids on which the genes responsible for distinguishing phenotypes are located (Helgason *et al.*, 2000). These plasmids are exchangeable between *B. cereus* group members (Jensen *et al.*, 2003). *B. thuringiensis* is phenotypically similar to *B. cereus*, but with the ability to form parasporal crystal protein inclusions during sporulation, and these inclusions display toxic activity against many orders of insects and are used in insect control programmes worldwide (Whiteley and Schnepf, 1986; Delécluse *et al.*, 1991; Sorokin *et al.*, 2006).

The genetic variability in the populations of *B. thuringiensis* is lower than that of *B. cereus*, and this difference in population genetic variability indicates difference in behaviour of strains in reaction to their surrounding environment (Vilas-Bôas *et al.* 2002) and the study of sympatric populations of these strains using Repetitive element polymorphism -PCR fingerprints supports the classification as two separate species of one group (Peruca *et al.*, 2008). Other new genotypic methods will be required to clarify strain similarities and differences among the *B. cereus lato* group (Dworzanski *et al.*, 2010).

1.3 *Bacillus thuringiensis*

B. thuringiensis (Bt) is a member of *Bacillaceae* family. It is an insect pathogen that is found in soil worldwide and has also been isolated from various locations such as water, stored grain and from dead insects including mosquitoes (Garcia-Robles *et al.*, 2001; Rosas-Garcia, 2009). *B. thuringiensis* was introduced as a silkworm larva disease agent by Ishiwata 1901, and named as *B. sotto*.
B. thuringiensis was named after the region of Thuringia in Germany, by Berliner 1911, when he isolated it from diseased Mediterranean flour moth larvae (*Anagasta kuehniella*) in Thuringia (Federici, 2005; Whitely and Schnepf, 1986).

B. thuringiensis did not receive significant attention until its crystal proteins were discovered and Angus (1954) described their capability of killing insects. The protein inclusions are produced in large amounts only during sporulation and constitute up to 25% of the weight of sporulated cells (Agaisse and Lereclus, 1995). Thousands of different strains of *B. thuringiensis* have been identified in screening programmes. These strains may have insecticidal activity against a wide range of insect orders and other organisms such as nematodes and protozoa (de Maagd *et al.*, 2003). *B. thuringiensis* produces several types of toxins; designated α-exotoxin, ß-exotoxin and δ-endotoxins (crystal proteins) (Whiteley and Schnepf, 1986).
The α and ß-exotoxins are non-specific and active against many orders of insects or many cell types, whereas the δ-endotoxins are active against limited host ranges, for instance a limited number of species in one order of insects (Whiteley and Schnepf, 1986).

The definition of Crystal delta-endotoxin (Cry) proteins as stated by Crickmore *et al.*, (1998) is: "parasporal inclusion proteins from Bt that exhibit toxicity toward target organisms, or any protein with sequence similarity to any known Cry protein".

The toxins are deposited in crystalline inclusions during sporulation. These parasporal crystalline inclusions are synthesised as inactive protoxins inside the cell and form a crystal with a size of approximately 1 μm (Grochulski *et al.*, 1995; Rajamohan *et al.*, 1995). These crystal proteins are toxic predominantly to insect larvae and they are called δ-endotoxins or Cry proteins. The insecticidal crystal proteins dissolve in the alkaline environment of the insect midgut and the inactive protoxins are activated by gut proteinases (Rajamohan *et al.*, 1995; Otieno-Ayayo *et al.*, 2008).

1.4 *Bacillus thuringiensis* in control programmes

The limitations of chemical insecticides include: insect resistance; their effects on the environment, non-target and beneficial insects; their contamination of air, food and water supplies (Federici et al., 2003; Park *et al.*, 2005). This illustrates the urgent need to develop new insect control methods, using biological agents such as *B. thuringiensis* (Federici *et al.*, 2003). The insecticide production must take into account the necessity for high quality and efficiency to achieve a product that must be safe and easy to use with a long shelf life (Rosas, 2009).

B. thuringiensis produces insecticidal proteins that are toxic to target insects but do not cause any environmental concerns (Siqueira *et al.*, 2004). *B. thuringiensis* toxins are active against insect larvae belonging to the orders Lepidoptera, Coleoptera

and Diptera (including mosquitoes, which transmit Malaria, dengue and yellow fever; and black flies) (Schnepf *et al.*, 1998, Aronson, 2002). *B. thuringiensis* is a widely applied biological insecticide for control programmes of insects that are crop pests and transmit human and animal diseases (Schnepf and Whiteley, 1981).
B. thuringiensis was applied in the field for the first time in the late 1920s and early 1930s in southeastern Europe (Hungary and Yugoslavia) to control the European corn borer (*Ostrinia nubilalis*) (Schnepf *et al.*, 1998).

The potential for application in the field led to the production of Sporeine in 1938 in France by Laboratoire Libec, but its production was stopped due to the World War II (Lord, 2005). In 1957, *B. thuringiensis* products became commercially available and the first *B. thuringiensis* product that entered the market was Thuricide, which was produced by Valent BioSciences and is still available today (Schnepf *et al.*, 1998; Lord, 2005). Many *B. thuringiensis* strains with the ability to produce insecticidal proteins have been used as environmentally friendly bio-insecticides in control programmes to manage insect pests of plants for more than 50 years and *B. thuringiensis* insecticides are often still the method of choice for such programmes (Betz *et al.*, 2000; Garcia-Robles *et al.*, 2001; Hernandez-Rodriguez *et al.*, 2009).

The genes encoding Cry proteins have been cloned (Schnepf and Whiteley, 1981), and their expression in plants to produce genetically modified crops was started in tobacco and tomato (Barton *et al.*, 1987; Vaeck *et al.*, 1987; Fischhoff *et al.*, 1987). Since then other *cry* gene transformed plants including rice, cabbage and soybean have been produced (Schnepf *et al.*, 1998). In 1996 the first crop plants (cotton, corn and potatoes) that were genetically engineered to express Cry toxins were available in

the United States for sale to growers (Hilbeck *et al.*, 1998; Schnepf *et al.*, 1998). The usefulness of transgenic plants is that they can lead to the reduction of chemical pesticide use in the field and their effects on human health and the environment as well as beneficial insects (Betz *et al.*, 2000). The range of targeted insects can also be expanded to include larvae that are not exposed to sprayed insecticides such as sucking and root-dwelling insects (Schnepf *et al.*, 1998).

Currently there are two types of bacteria, *Bacillus sphaericus* and *B. thuringiensis* subsp. *israelensis* that are used in the field as biopesticides to control the larvae of *Culex, Aedes* and *Anopheles* mosquitoes (Luxananil *et al.*, 2001; Broderick, *et al.*, 2006). *B. sphaericus* produces parasporal crystal proteins known as binary toxins (Bin) with toxic activity against *Culex* larvae. The binary toxin of *B. sphaericus* consists of two proteins, the binding component BinB (51.4-kDa) and the toxin domain BinA (41.9-kDa) (Federici *et al.*, 2003), however, resistance of *Culex* mosquitoes to *B. sphaericus* in the field has been reported (Wirth *et al.*, 2000).

B. thuringiensis subsp. *israelensis* and *Bacillus sphaericus* insecticidal properties are due to the production of insecticidal parasporal crystal proteins during sporulation. The main toxins of *B. thuringiensis* subsp. *israelensis* are Cyt1A (27 kDa), Cry4A (134 kDa), Cry4B (128 kDa) and Cry11A (128 kDa) (Federici *et al.*, 2003: Bravo and Soberón, 2008). *B. thuringiensis* subsp. *israelensis,* beside its specific toxicity towards the larvae of mosquitoes and blackfly, is safe for people, animals, birds and non-target insects (Schnepf *et al.*, 1998). Moreover *B. thuringiensis* subsp. *israelensis* has been used in many countries as a control method of choice for mosquito and blackfly. The problem of insect resistance, which

reduces the effectiveness of chemical control methods and *B. sphaericus*, has not arisen (Becker and Ludwig, 1993; Bravo and Soberón, 2008).

B. thuringiensis subsp. *israelensis* was used intensively in some regions around the world and the problem of insect resistance did not develop (Becker, 2000). The presence of the Cyt1A protein, which synergises with Cry toxins (Pérez *et al.*, 2005a; Bravo *et al.*, 2007: Bravo and Soberón, 2008) in *B. thuringiensis* subsp. *israelensis* parasporal crystals is able to prevent the development of resistance (Georghiou and Wirth, 1997; Wirth *et al.*, 1997). The toxins and their activities will be described in later sections.

There are different commercial products of *B. thuringiensis* subsp. *israelensis* for mosquito and fly control, such as VectoBac® and Teknar®. A *B. sphaericus* product, VectoLex® is also commercially available (Federici *et al.*, 2003). *B. thuringiensis* subsp. *israelensis* was used successfully against the larvae of blackfly *Simulium damnosum*, a vector of the nematode parasite that causes river blindness (Onchocerciasis) in the WHO sponsored Onchocerciasis Control Programme (OCP) from the 1980s to 1990s in Western Africa (WHO, 1999).

B. thuringiensis subsp. *israelensis* products have been used in rotation with chemical insecticides in control programmes of the WHO (Federici, 2005). Although the cost of producing large quantities of the insecticide from *B. thuringiensis* subsp. *israelensis* is still moderately high (Barbosa *et al.*, 2009). Many of the *B. thuringiensis* insecticidal products that are commercially available worldwide are shown in table 1.4.1.

Table 1.4.1 List of some available commercial *B. thuringiensis* insecticidal products

Adapted from Rosas-Garcia (2009) and producer companies' websites.

***Bacillus thuringiensis* strain**	**Insecticidal**	**Target insect**	**Producer company**
Bt subsp. *israelenisis*	VectoBac®	Mosquito/fly larvae	Valent Biosciences Corporation
Bt subsp. *israelenisis*	Teknar®	Mosquito/ black fly larvae	Valent Biosciences Corporation
Bt subsp. *israelenisis*	GnatroI®DG	Sciarid mushroom fly larvae	Valent Biosciences Corporation
Bt subsp. *israelenisis*	Bt-horus	Mosquitoes	Bthek Biotecnologia
Bt subsp. *kurstaki*	CoStar®	Lepidoptera	Certis
Bt subsp. *kurstaki*	Deliver®	Lepidoptera	Certis
Bt subsp. *kurstaki*	Javelin WG	Lepidoptera	Certis
Bt subsp. *kurstaki*	Lepinox WDG	Lepidoptera	Certis
Bt subsp. *kurstaki*	Condor®	Lepidoptera	Certis
Bt subsp. *kurstaki*	Jackpot®TP	Lepidoptera	Certis
Bt subsp. *kurstaki*	Turix WP/ Agree WP	Lepidoptera	Certis
Bt subsp. *kurstaki*	Aquabac™	Mosquitoes and black flies	AFA Environment
Bt subsp. *kurstaki*	DiPel®	Lepidoptera	Valent Biosciences Corporation
Bt subsp. *kurstaki*	XenTari®	Armyworm and diamondback moth larv	Valent Biosciences Corporation
Bt subsp. *kurstaki*	Biobit®	Lepidoptera	Valent Biosciences Corporation
Bt subsp. *kurstaki*	Gnatrol®	Fungus gnat larvae	Valent Biosciences Corporation
Bt subsp. *kurstaki*	Foray®	Lepidoptera	Valent Biosciences Corporation
Bt subsp. *kurstaki*	Thuricide®	Moth larvae and certain leaf-eating worms and gypsy moths	Certis/ Valent Biosciences Corporation
Bt subsp. *kurstaki* strain EG7841	Crymax WDG	Lepidoptera	Certis
Bt subsp. *tenebrionis*	Novodor®	Coleoptera	Valent Biosciences Corporation
Bt subsp. *aizawai*	Agree® WG	Lepidoptera	Certis

1.5 *Bacillus thuringiensis* strains and toxins

In the 1970s, Dulmage discovered *B. thuringiensis* subsp. *kurstaki* strain HD-1, which showed toxic activity against agricultural pests to a greater degree than other *B. thuringiensis* strains known at that time. The strain of *B. thuringiensis* subsp. *israelensis* (Bti) was discovered in the mid 1970s, and showed excellent toxic activity against mosquitoes and blackfly larvae (Goldberg and Margalit, 1977). The control of mosquito and blackfly using *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* led to screening programmes to discover more potent strains (Federici *et al.*, 2003).

As a result of more research, many *B. thuringiensis* strains were discovered such as *B. thuringiensis* subsp. *morrisoni* with toxins that affect larvae of some species in the order Coleoptera (Krieg *et al.*, 1983; Federici *et al.*, 1998), and another *B. thuringiensis* strain, which displayed toxic activity against nematodes, was reported in the late 1980s (Edwards *et al.*, 1988). Other mosquitocidal strains including *B. thuringiensis* subsp. *jegathesan,* which produces seven Cry and Cyt proteins, (Delécluse *et al.*, 2000) have been discovered. Further strains of *B. thuringiensis* active against mites and protozoa were reported (Payne *et al.*, 1994; Feitelson *et al.*, 1992). Moreover some other bacterial species with mosquitocidal activities were reported including *Clostridium bifermentans* (Delécluse *et al.*, 2000).

B. thuringiensis can be grouped according to flagellar antigen types. Serotypes refer to interaction with individual typing-antisera. Strains may react with one or more of these antisera and the patterns of interaction define serovars (eg serotype 3a, 3b and 3c defines serovar *kurstaki* whereas serotype 14 defines strain *israelensis*) (Norris,

1964; de Barjac and Frachon, 1990; Lecadet *et al.*, 1999). In 1990, De Barjac and Frachon included 27 serovars and 34 serotypes in their classification but by 1998, 82 serotypes and 69 serovars of *B. thuringiensis* had been discovered (Lecadet *et al.*, 1999). The current classification of *B. thuringiensis* strains based on H serotype and serological varieties is shown in table 1.11.1.

Different isolates of *B. thuringiensis* exhibit different target insect specificities determined by the profile of toxins they produce different host range. The *B. thuringiensis kurstaki* HD-1 isolate has a broad spectrum of activity due to four endotoxin proteins (Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa) that it encodes. In contrast, the *B. thuringiensis kurstaki* HD73 isolate has a very narrow spectrum of insecticidal activity because it produces only one protein, Cry1Ac (Federici, 2005).

Table 1.5.1 H serotype classification of *B. thuringiensis* strains

Adapted from Lecadet *et al.* (1999).

H antigen	Serovar	H antigen	Serovar	H antigen	Serovar
1	*thuringiensis*	19	*tochigiensis*	44	*higo*
2	*finitimus*	20a, 20b	*yunnanensis*	45	*roskildiensis*
3a, 3c	*alesti*	20a, 20c	*pondicheriensis*	46	*chanpaisis*
3a, 3b, 3c	*kurstaki*	21	*colmeri*	47	*wratislaviensis*
3a, 3d	*sumiyoshiensis*	22	*shandongiensis*	48	*balearica*
3a, 3d, 3e	*fukuokaensis*	23	*japonensis*	49	*muju*
4a, 4b	*sotto*	24a, 24b	*neoleonensis*	50	*navarrensis*
4a, 4c	*kenyae*	24a, 24c	*novosibirsk*	51	*xiaguangiensis*
5a, 5b	*galleriae*	25	*coreanensis*	52	*kim*
5a, 5c	*canadensis*	26	*silo*	53	*asturiensis*
6	*entomocidus*	27	*mexicanensis*	54	*poloniensis*
7	*aizawai*	28a, 28b	*monterrey*	55	*palmanyolensis*
8a, 8b	*morrisoni*	28a, 28c	*jegathesan*	56	*rongseni*
8a, 8c	*ostriniae*	29	*amagiensis*	57	*pirenaica*
8b, 8d	*nigeriensis*	30	*medellin*	58	*argentinensis*
9	*tolworthi*	31	*toguchini*	59	*iberica*
10a, 10b	*darmstadiensis*	32	*cameroun*	60	*pingluonsis*
10a, 10c	*londrina*	33	*leesis*	61	*sylvestriensis*
11a, 11b	*toumanoffi*	34	*konkukian*	62	*zhaodongensis*
11a, 11c	*kyushuensis*	35	*seoulensis*	63	*bolivia*
12	*thompsoni*	36	*malaysiensis*	64	*azorensis*
13	*pakistani*	37	*andaluciensis*	65	*pulsiensis*
14	*israelensis*	38	*oswaldocruzi*	66	*graciosensis*
15	*dakota*	39	*brasiliensis*	67	*vazensis*
16	*indiana*	40	*huazhongensis*	68	*thailandensis*
17	*tohokuensis*	41	*sooncheon*	69	*pahangi*
18a, 18b	*kumamotoensis*	42	*jinghongiensis*		

1.6 Classification of *Bacillus thuringiensis* toxins

B. thuringiensis toxins were classified according to Hofte and Whiteley (1989) in two main families; Cry (crystal) and Cyt (cytolytic) proteins, and the genes encoding them were put in two classes accordingly. Their system was used to organise the nomenclature on the basis of insecticidal activity of the insecticidal proteins, and distinguished four delta-endotoxins and named them using Roman numerals as, CryI, CryII, CryIII and CryIV according to their toxicity against different orders of insects (Hofte and Whiteley, 1989). However, the usefulness of the system declined since it could not accommodate the increasing number of toxin genes discovered (for instance, the CryIIA protein was classified as lepidopteran and dipteran active, and the CryIIB which is not toxic to dipterans was placed in the same class (Crickmore *et al.*, 1998). Therefore, a new nomenclature system was developed based on the amino acid sequence identity instead of toxic activity, and furthermore, the Roman numerals were exchanged for Arabic numerals, and thus CryIAa became Cry1Aa (Crickmore *et al.*, 1998).

In this new classification system (nomenclature scheme), four ranks are defined in the toxin names and are assigned based on sequence identities. Sequences sharing > 45 % identity are assigned to the same primary rank, showing by an Arabic numeral (eg Cry1). Proteins sharing > 78 % identity are given the same secondary rank, shown by a capital letter (eg Cry1A) and proteins with > 95 % identity are given the same tertiary rank, shown with a lower case letter (eg Cry1Aa). The final level in the nomenclature is entry, regardless of sequence identity to other entries (eg Cry1Aa1), this quaternary level is normally omitted. A similar system of nomenclature has been applied to the Cyt and Vip toxins described later.

The Cry group of proteins contains more than 400 endotoxins including holotype toxins that have been discovered and identified through screening programmes (Bravo *et al.*, 2007; Fang *et al.*, 2007; Shu and Zhang, 2009). As of 26th of July 2010, there are 67 families of Cry toxins, classified from Cry1 to Cry67 and the up-to-date full list of *B. thuringiensis* delta-endotoxins can be found at (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html).

Most of the Cry toxins belong to a related group of so-called three domain proteins (de Maagd *et al.*, 2001) that show toxic activity against many insect orders (Coleoptera, Lepidoptera, Hymenoptera and Diptera) and may also be toxic to parasitic nematodes (de Maagd *et al.*, 2001: de Maagd *et al.*, 2003). Although other, un-related lineages also exist within the Cry nomenclature.

1.7 The three- domain Cry toxins

The major family within Cry proteins is the so-called three- domain toxins, which represent nearly 90% of the Cry proteins toxins (Akiba *et al.*, 2009). The 3-domain Cry toxins are produced as protoxins that may be relatively large (approx 130 kDa) or small (approx 70 kDa) (Schnepf *et al.*, 1998). The large protoxins consist of a hydrophilic C-terminal half and a hydrophobic N-terminal half containing the mature toxin sequence (Vazquez-Padron *et al.*, 2004). The C-terminal domain of the larger protoxins is absent from the shorter protoxins so that shorter protoxins are, essentially, homologues of the N-terminal domain of the ~ 130 kDa protoxin.

The amino acid sequences of these homologous regions of the 3-domain Cry proteins revealed the five highly conserved blocks of amino acids in the toxin region (Hofte and Whiteley, 1989). The C-terminal part of the long 3 domain protoxins has additionally three blocks of amino acids outside the toxic core (Schnepf *et al.*, 1998). The C-terminal part of large protoxins is not essential for toxic activity and may be involved in crystal production (de Maagd *et al.*, 2003). So that the two domains may have complementary roles contributing to insecticidal properties of the Cry proteins and crystal formation (Vazquez-Padron *et al.*, 2004). Crystallization of shorter protoxins may require the action of accessory proteins (Agaisse and Lereclus., 1995; Baum and Malvar., 1995).

The structures of six members of the 3-domain family have been determined by X-ray crystallography (Cry1Aa active against Lepidopterans, Cry2Aa toxic to Lepidopterans/ Dipterans, Cry3Aa and Cry3Bb1 active against Coleopterans and Cry4Aa and Cry4Ba active against Dipterans (Li *et al.*, 1991; Boonserm *et al.*, 2005; Grochulski *et al.*, 1995; Morse *et al.*, 2001; Galitsky *et al.*, 2001). Despite the fact that the amino acid sequence homology is relatively low between these proteins (36% between Cry1Aa and Cry3Aa), they share structural similarity (Schnepf *et al.*, 1998; Aronson, 2002). Each structure shows 3 distinct domains in the activated toxin. Illustration of the three dimensional structure of Cry3A toxin (active against Coleopteran larvae) is shown in Figure 1.7.1.

Domain I consists of seven α-helices with the helix α 5 in the centre, encircled by six other α- helices (Li *et al.*, 1991; Schnepf *et al.*, 1998). Domain I is believed to be responsible for membrane insertion and the pore formation process (Walters *et al.*,

1993; Aronson and Shai, 2001) and it has been suggested that a pair of helices (α-4 and α-5) of this domain insert into the membrane and other helices open on the membrane surface like an umbrella (Li *et al.*, 1991). In the case of Cry4Ba, the two helices that insert into the membrane are α-4 and α-5 (Boonserm *et al.*, 2005) this is called the umbrella mode. There is another model called the 'penknife', which suggests that α-5 and α-6 (joined at the top of the structure by a loop) open like a penknife and insert into the membrane. What has been suggested in both models may occur after receptor binding (Li *et al.*, 1991).

Domain II consists of three anti-parallel β-sheets producing a "Greek key" topology, forming a β-prism. Domain II is involved in receptor binding and, therefore, plays a role in the insect specificity (Wu and Dean, 1996; Bravo, 1997). Domain II has exposed loops (at the apices of β-sheets) that vary among Cry proteins and appear similar to the binding sites of immunoglobulin antigen, suggesting that they may have a role in receptor binding (Schnepf *et al.*, 1998; Boonserm *et al.*, 2005).

Figure 1.7.1 The three dimensional structure of Cry3Aa

The three domains, I (green/yellow), II (blue) and III (red). Domain I, helix α 5 is in the centre and surrounded by other α- helices. The two helices *α*-4 and *α*-5 of domain I that insert into the membrane (yellow) in the umbrella model (Boonserm *et al.*, 2005). Domain II (blue) has anti-parallel β-sheets. Domain III (red) is a *β*-sandwich of two anti-parallel sheets forming a jellyroll (Li *et al.*, 1991).

In *B. thuringiensis* subsp. *israelensis* it has been found that exposed loops in the Cry11Aa domain II are involved in receptor binding and the Cry11Aa receptor in *Aedes aegypti* has been identified as a GPI-anchored alkaline phosphatase (Fernandez *et al.*, 2005; 2006). Mutations in domain II affected the binding of the toxin to membrane vesicles, which indicates that this domain is very important in receptor binding and the overall toxicity (Jenkins *et al.*, 2000).

Wu and Dean (1996) studied the role of domain II of Cry3Aa and, using loop residue mutations, they showed that loops 1 and 3 are involved in receptor binding. Rajamohan *et al* studied the role of the Cry1Ab domain II in the toxicity against *M. sexta* and found that loop 2 residues, 368RRP370, are involved in initial binding, whereas residues Phe371 and Gly374 play a role in irreversible binding of the toxin to *M. sexta* (Rajamohan *et al.*, 1995). When residues 365-371 were deleted from Cry1Aa loop2, it abolished the toxicity and initial binding of the toxin to *Bombyx mori* (Lu *et al.*, 1994). Pacheco *et al.* (2009) have shown that loop 3 of Cry1Aa may initiate the binding to APN receptor. The deletion of loop 3 amino acids of Cry1Aa and Cry1Ab toxins reduces their toxicity and affects their ability to achieve initial binding to membrane vesicles in *M. sexta, B. mori and Heliothis virescens* (Rajamohan *et al.*, 1996). Domain swapping work indicates that domain II and also domain III are involved in receptor binding and target specificity (Rajamohan *et al.*, 1996).

Domain III is β-sandwich of two anti-parallel sheets forming a jellyroll and the C-terminal part of domain III may be involved in receptor binding and thus involved in determining insect specificity (Schnepf *et al.*, 1998; Li *et al.*, 1991). Domain III is expected to be involved in receptor binding and may have a role as a lectin-like domain, which binds to N-acetyl galactosamine (GalNAc) on the aminopeptidase N (APN) (Burton *et al.*, 1999). Domain III may prevent degradation of Cry toxins after they have been processed by proteolysis in the midgut (Schnepf *et al.*, 1998). Domain III displays some similarities with other carbohydrate-binding proteins such as the cellulose binding domain of 1,4-β-glucanase C, galactose oxidase, sialidase, β-glucoronidase, or the carbohydrate-binding domain of xylanase U and β-galactosidase (de Maagd *et al.*, 2003). This suggests that there may be an important role of carbohydrate moieties in the three-domain Cry toxins' mode of action (Bravo *et al.*, 2007).

Furthermore the C-terminal domain III might have a role in pore formation (de Maagd *et al.*, 2001), as well as in specificity of target insect and protein stability (Li *et al.*, 1991). Domain III swapping may lead to the production of new toxins with new specificities (Bravo *et al.*, 2007).

1.8 Mode of action of 3 domain Cry toxins

B. thuringiensis crystal proteins (Cry) and cytotoxic proteins (Cyt) are considered as pore forming toxins (PFT) due to their action on target cells. There are two main groups of PFT. The first group is the α-helical toxins, which form trans-membrane pores, among this group are the colicins, exotoxin A, diphtheria toxin and also the three-domain Cry toxins (Parker and Feil, 2005). The second group is the β-barrel toxins that form a β-barrel composed of β-sheet hairpins from each monomer to be inserted into the membrane, and includes aerolysin, α-hemolysin, anthrax protective antigen, cholesterol-dependent toxins such as the perfringolysin O and the Cyt toxins (Parker and Feil, 2005).

Generally these toxins interact with specific cell membrane receptors and are activated by proteinases, inducing the oligomeric structure formation that is insertion-competent and causing insertion into the cell membrane (Parker and Feil, 2005).

The mode of action of Cry toxins has been studied widely using Cry1A toxin, which shows high insecticidal activity against larvae of Lepidoptera (Pérez *et al.*, 2007). The Cry toxin proteins are different in their insecticidal activity and spectra, and also in their primary structure but they appear to have a similar mode of action (Schnepf *et al.*, 1998). Two models have been proposed for the mode of action of Cry toxins, the first is the pore formation model and the second is a signal transduction model (Bravo and Soberón 2008). The pore formation model is believed to happen in larvae of Lepidoptera and Coleoptera and mosquitoes as all have similar receptors (Bravo and Soberón 2008). Some preliminary steps are involved in Cry toxin mode of

action in both models, such as solubilisation, proteolysis, and receptor binding and finally formation of lytic pores (Garcia-Robles *et al.*, 2001).

Cry toxins are produced during sporulation as inactive protoxin forms and become active in a series of stages. First the protein crystal is solubilised in the alkaline conditions of the midgut. Toxic activity is affected by the ability of protoxins to be solubilised (Aronson, *et al.*, 1991). Protoxins are then processed by the proteolytic enzymes of the midgut, which remove some peptides from the N-terminal and C-terminal ends and thus activate the Cry toxins (Gill *et al.*, 1992; Schnepf *et al.*, 1998; Rukmini *et al.*, 2000; Bravo *et al.*, 2007). For example: The 65 kDa Cry11Ba undergoes cleavage inside the mosquito larval midgut to yield products of 30 kDa and 35 kDa; the former is the active core of this Cry toxin (Likitvivatanavong, *et al.*, 2009). The serine proteinase family is the predominant class in the midgut of lepidopteran and dipteran larvae (Johnston *et al*; 1995), and this class is also found in Coleoptera, other proteinases such as cysteine and aspartic proteinases may be more important in some species (Terra and Ferreira, 1994).

After activation, Cry toxins bind to specific receptors on the brush border membranes of midgut epithelial cells in susceptible larvae. Some such receptors are aminopeptidase N (APN), cadherin-like glycoproteins and alkaline phosphatase (ALP) (Schnepf *et al.*, 1998; Burton *et al.*, 1999; de Maagd *et al.*, 2001: Zhang *et al.*, 2008). Some Cry toxins bind to one receptor and others may bind to multiple receptors to trigger their toxic activities (Bravo *et al.*, 2004). In lepidoptera, Cry1A binds to multiple receptors such as cadherins, ALP, APN and glycolipids, whereas Cry11Ba binds to a specific receptor (106 kDa GPI-anchored APN) in the BBMV of *Anopheles*

gambiae larvae (Zhang *et al.*, 2008). In the larval midgut of *Aedes aegypti*, the functional receptor for Cry11Aa is also a GPI-anchored alkaline phosphatase (Fernandez *et al.*, 2006), while in *A. aegypti* and *An. Gambiae*, Cadherin fragments have been shown to synergize the activity of Cry4Ba (Park *et al.*, 2009). It has been suggested that insect resistance is linked to the quality of binding of toxins to BBMV (Schnepf *et al.*, 1998) and change in receptor binding can lead to resistance to Cry toxins (Ferre and Van Rie, 2002).

Domain II has been shown to be involved in receptor binding and Domain III is believed to play a role in receptor binding as well as insect specificity (Schnepf *et al.*, 1998; de Maagd *et al.*, 1996; Bravo *et al.*, 2007) and may also have a role in the insertion of the toxin into the membrane (Dean *et al.*, 1996). Toxin interaction with membrane vesicles involves two steps showing first reversible binding and then irreversible binding (Aronson and Shai, 2001). The irreversible binding step includes strong binding of the toxin with the receptor and also the insertion of the toxin into the membrane (Aronson and Shai, 2001).

Bravo *et al.* (2007) have shown that the two binding steps may happen in lepidoperan larvae after the solubilisation and activation of Cry toxins. The first binding occurs between the Cry toxin and a receptor such as CADR (a cadherin-like protein). This may facilitate further cleavage and formation of an oligomeric form, which will bind to a second receptor such as GPI-APN. After this, the toxin oligomer inserts in the membrane lipid raft and leads to pore formation in the membrane causing lysis of the apical microvillar membrane of midgut epithelial cells in the

target insect (Schnepf *et al.*, 1998; de Maagd *et al.*, 2003; Bravo *et al.*, 2004; Bravo *et al*; 2005; Bravo *et al.*, 2007).

For the pore formation and membrane insertion, several models have been suggested following receptor binding. The first is called the umbrella model, in which the two helices that insert into the membrane are α-4 and α-5 in the case of Cry4Ba and the rest of helices open on the surface of the membrane with their hydrophobic sides toward the membrane forming an umbrella shape (Boonserm *et al.*, 2005; Alzate *et al.*, 2006). This indicates the importance of α-4 and α-5 helices in the toxicity as any mutation in their structure leads to reduction of activity as has been shown by introducing a proline residue in middle of α-4 in Cry4Ba, whereas introduction of proline in the middle of helix α3 did not have any effect on the activity (Uawithya *et al.*, 1998). Cry4Ba, isolated from *B. thuringiensis* subsp. *israelensis*, has high toxic activity against the larvae of *Aedes* and *Anopheles* mosquitoes (Boonserm *et al.*, 2005).

The second model is the 'penknife', which proposed that the α-5 and α-6 helices (joined at the top of the structure by a loop) open like a penknife and insert into the membrane as helical hairpin (Li *et al.*, 1991). The other helices of domain I are not required in the 'penknife'model (Alzate *et al.*, 2006). The mode of action is shown in Figure 1.8.1.

In both proposed models it has been suggested that the conformational changes required for protein translocation are triggered by the binding of domain II to the receptors and interaction of toxins with membrane surface (Li *et al.*, 1991; Alzate

et al., 2006). The umbrella model and penknife model, both were proposed according to the basis of analogous properties with other membrane-translocating proteins that have similar structure (Alzate *et al.*, 2006).

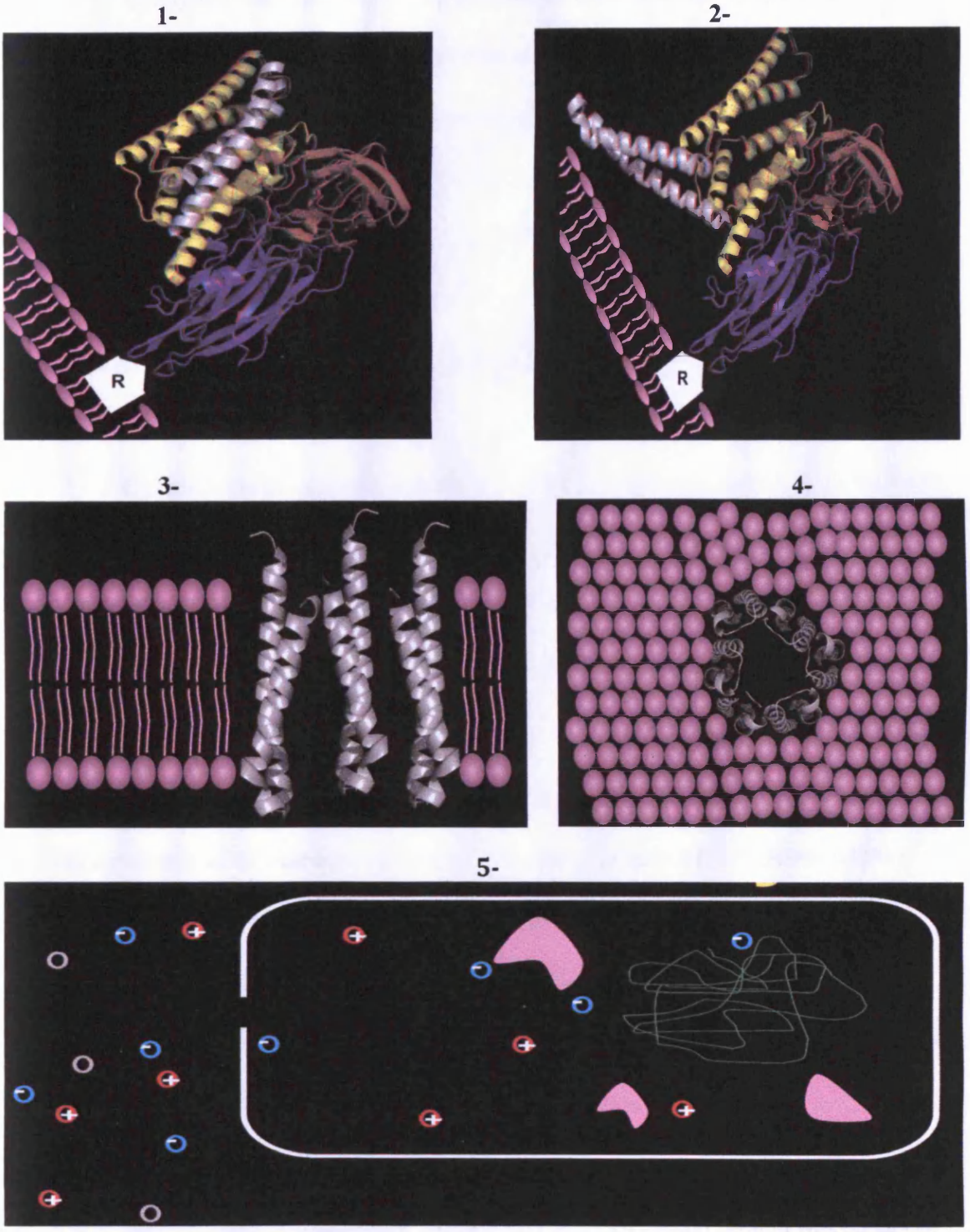
Further Models have been suggested in addition to the umbrella and penknife models, which propose the whole toxin is inserted into the midgut membrane. Alcantara *et al* suggested in addition to helices 4 and 5, that α helix 7 may also be inserted into the membrane allowing passage of ions through the membrane (Alcantara *et al.*, 2001). A recent model proposed by Tomimoto *et al.* for Cry1Aa is called the "Buried Dragon" model. In this model, the mechanism of pore formation is caused by single Cry1Aa molecule, where the binding of Cry1Aa to BBMV involves both C-terminal and N-terminal half of the activated toxin. The parts that strongly embed and integrate with BBMV are α-2 to α-7 helices (Tomimoto *et al.*, 2006).

The pore formation in the cell membrane disrupts the osmotic equilibrium of the cells, leading to colloid - osmotic lysis. The pores allow free equilibration of ions and small molecules but are too small to allow passage of macromolecules. These will result in net influx of water into the cell leading to cell swelling. Subsequent to cell swelling the membrane will stretch and this may cause other pores to enlarge and lead to cell lysis (Knowles and Ellar, 1987).

In the signal transduction model, it has been proposed that the binding of toxin to cadherin triggers a cascade pathway including the activation of G proteins, which in turn will activate the adenylyl cyclase (AC) to increase the level of the cyclic adenosine monophosphate that activates protein kinase A (PKA) then cell death

occurs (Zhang *et al.*, 2006; Bravo and Soberón, 2008). Activation of the AC/PKA signalling pathway will lead to number of cytological events including membrane blebbing and appearance of nuclear ghosts, as well as cell swelling, which will lead to cell lysis (Zhang *et al.*, 2006).

Figure 1.8.1 The mode of action of *B. thuringiensis* Cry toxins



The figure 1.8.1 shows the mode of action in 5 steps that occur after the ingestion of toxins and solubilisation in midgut alkaline conditions and activated by proteinases;
1- The binding of domain II to the specific receptor (R) on the midgut membrane,
2- The two helices α-4 and α-5 of domain I that insert into the membrane (grey),
3- Insertion of the oligomeric toxin into lipid rafts, 4- Pore formation, 5- Efflux of cytoplasmic ions/small molecules while macromolecules remain within.

1.9 The Cyt Toxins

Cytolytic delta-endotoxin (Cyt) proteins are produced during sporulation as parasporal inclusion proteins that exhibit haemolytic and cytolytic activity (Schnepf *et al.*, 1998). Cyt toxins of *B. thuringiensis* are active *in vivo* against larvae of members of the order Diptera (Schnepf *et al.*, 1998), which includes mosquitoes and black flies. Cyt toxins are mainly produced by strains of *B. thuringiensis* that active against mosquitoes (Bravo *et al.*, 2007). The amino acid sequences of Cyt toxins show no homology to Cry family toxins (Butko, 2003).

Cyt toxin proteins act by a different mechanism than Cry toxins, and they have the ability to make a direct interaction with the lipid bi-layer of the cell membrane, which may cause the insertion of Cyt toxins and lead to pore formation causing formation of channels of 1-2 nm in diameter (Knowles *et al.*, 1989; Li *et al.*, 1996). This will induce the leakage of the cytoplasm from the cells, and may cause lysis to many cell types *in vitro* (Schnepf *et al.*, 1998; Butko, 2003). The Cyt1A is the most highly expressed *B. thuringiensis* subsp. *israelensis* toxin but is less specific than other Cry toxins Cry4Aa, Cry4Ba and Cry11Aa (Hofte and Whiteley, 1989).
The Cyt1Aa protein alone has a low toxic activity against mosquito larvae, but when combined with Cry toxins shows a high toxic activity, greater than the combination of Cry4Aa, Cry4Ba and Cry11Aa together (Crickmore *et al.*, 1995).

In *A. aegypti,* the binding to BBMV by Cry11Aa crystal protein was enhanced by membrane-bound Cyt1Aa protein (Bravo *et al.*, 2007) and it has been suggested that the Cyt1Aa toxin protein may enhance the insecticidal activity of Cry11Aa crystal protein, by acting as membrane bound receptor and providing alternative binding sites

for Cry11Aa (Pérez *et al.*, 2005a). This binding would lead to the production of Cry11Aa oligomer and facilitate the pore formation in the gut cell membranes of the susceptible larvae (Pérez *et al.*, 2007). The interaction between Cry11Aa and Cyt1Aa takes place through the same regions of the Cry11Aa domain II loops that are involved in receptor interaction (Fernández *et al.*, 2005; Pérez *et al.*, 2005b).

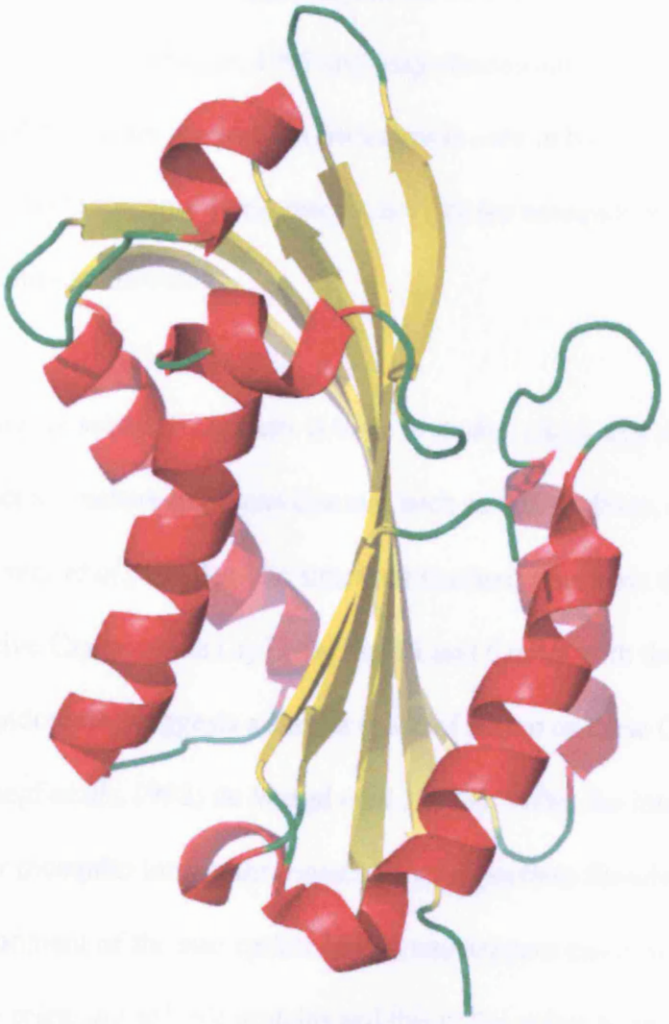
The Cyt1A toxin protein is able to synergize with Cry toxin proteins, and this may be an important factor to avoid the development of insect resistance to *B. thuringiensis* subsp. *israelensis* in field conditions (Georghiou and Wirth, 1997; Wirth *et al.*, 1997; Wirth *et al.*, 2005). Wirth, *et al* (1997) reported that using Cyt1Aa, the resistance of *Culex quinquefasciatus* to Cry toxins was suppressed. Some laboratory studies have reported that larval resistance can be developed against *B. thuringiensis* strains that only produce Cry toxins (Wirth *et al.*, 2005).

Cyt toxins are also synthesized as protoxins and, in order to be activated, small portions of the N-terminus and C-terminus are removed (Li *et al.*, 1996; Gill *et al.*, 1987). For instance, to produce the monomeric protein that is haemolytically active from Cyt2Aa, 32 amino acid residues are removed from the N-terminus and 15 amino acid residues from the C-terminus (Koni and Ellar, 1993). Also it has been shown that the Cyt1Aa acts synergistically with Cry10Aa and enhances its insecticidal activity against 4th instar larvae of *Aedes aegypti* (Hernández-Soto *et al.*, 2009). Thus *B. thuringiensis* subsp. *israelensis* may produce toxins and also their receptors too to suppress resistance to its crystal toxins such as Cry11Aa (Pérez *et al.*, 2005a).

Four similar blocks of amino acids have been found in six Cyt toxins from different subspecies of *B. thuringiensis* by analysing of Cyt protein sequences. These regions correspond to helix a, the loop after helix d and strands 4, 5 and 6 (Butko, 2003). This conservation may be important for the mode of action of Cyt toxins. The structure of the Cyt2Aa protein was determined and it consists of one domain with two outer layers of β-sheet encircled by 2 α-helix hairpins (Li *et al.*, 1996). The Cyt1Aa protein also seems to have a similar structure to Cyt2Aa with 70% identity in their amino acid sequences (Schnepf *et al.*, 1998; Li *et al.*, 1996). The three dimensional structure of Cyt2A toxin is shown in figure 1.9.1.

There are two proposed models of the mode of action for Cyt toxin proteins. The first suggests that Cyt toxins aggregate to cause defects in lipid packaging and the second model suggests that Cyt toxins form pores in the lipid bilayer of the membrane (Butko, 2003). Cyt1Aa has been shown to bind to phospholipids of the membrane (Butko, 2003). Manasherob *et al.* (2003) have shown that the induction of Cyt1Aa, the cytotoxic 27 kDa protein from *Bacillus thuringiensis* subsp. *israelensis* may lead to compaction of the *Escherichia coli* nucleoid in the cell's centre which is associated with the loss of the ability to form a colony.

Figure 1.9.1 The three-dimensional structure of the *B. thuringiensis* cytolytic toxin Cyt2A



The figure shows that Cyt2A consists of only one domain.
There are two α helices (red) surrounding the β- sheet (yellow).
The green colour represents the toxin loops.

1.10 Mode of action of Cry and Cyt toxins in mosquitoes

Bacillus thuringiensis subsp. *israelensis* synthesises a number of Cry and Cyt protoxins that are deposited as parasporal crystal proteins during sporulation. These can act synergistically (Poncet *et at*, 1995 and Angsuthanasombat *et al*, 1992) to cause a potent effect on dipteran larvae and this bacterium is used in biological control programmes worldwide to control pest insects, notably the mosquito vectors of several important human diseases.

B. thuringiensis subsp. *israelensis* is toxic to *Aedes*, *Culex* and *Anopheles* mosquitoes that act as vectors of human diseases such as yellow fever, encephalitis and malaria (Rukmini *et al.*, 2000). The structural similarity between the mosquitocidal active Cry proteins Cry11Aa, Cry4A and Cry4B with the Cry1Aa (active against lepidoptera) suggests a similar mode of action of these Cry proteins in mosquitoes (Schnepf *et al.*, 1998; de Maagd *et al.*, 2003). After the intake of the crystal proteins by mosquito larvae, the ingested crystal proteins dissolve in the alkaline gut environment of the susceptible larvae, and are processed by trypsin-like proteinase activity releasing soluble proteins and this is the active form of the toxins (Rukmini *et al.*, 2000).

In the case of the *B. thuringiensis* subsp. *israelensis* toxins, the 70 kDa Cry11Aa protoxin is processed resulting in 34 and 32 kDa fragments (Dai and Gill, 1993). The Cry4Ba protein (130 kDa) is processed at the N-terminus and at the C-terminus to 60 - 68 kDa proteins then further to 46 - 48 kDa toxin proteins (Angsuthanasombat *et al.*, 1992; 1993). The processing at the C-terminus removes the C-terminal half, which has no role in the toxic activity (Rukmini *et al.*, 2000).

1.11 Other *Bacillus thuringiensis* toxins

B. thuringiensis strains may produce other toxins during their life cycle. These toxins include α-exotoxin, β-exotoxins, secreted vegetative insecticidal proteins (Vip toxins), secreted insecticidal protein (Sip toxins) and Cry43/Cry35 binary toxin (Schnepf *et al.*, 1998; de Maagd *et al.*, 2001; de Maagd *et al.*, 2003; Donovan *et al.*, 2006). There are also other crystal proteins, which are referred to as parasporins (Mizuki *et al.*, 2000). Some of these proteins are described below, and more details can be found in de Maagd *et al.* (2003). These are not 3-domain Cry toxins

1.11.1 The Cry34/ Cry35 binary insecticidal proteins

Another insecticidal crystal protein family was discovered from *B. thuringiensis*. These toxin proteins are called binary toxins because, in order for the toxin to be lethal, both the 14 kDa Cry34 and 44 kDa Cry35 components are necessary (Ellis *et al.*, 2002; Schnepf *et al.*, 2005). Cry34 and Cry35 toxins have been isolated from *B. thuringiensis* strains with toxic activity against western corn rootworm (*Diabrotica virgifera virgifera*) (Ellis *et al.*, 2002). Cry34 and Cry35 proteins are not homologous to the 3-domain crystal proteins, Cyt or Vip of *B. thuringiensis*. However Cry35 proteins are related to BinA/BinB binary toxins and Cry49 from *B. sphaericus* and to Cry36Aa1 (active against Coleoptera) from *B. thuringiensis* (Ellis *et al.*, 2002; de Maagd *et al.*, 2003: Jones *et al.*, 2007).

The Cry34/35 binary toxin proteins are produced during the sporulation phase and are encoded by a single operon (Moellenbeck *et al.*, 2001). Sequence analysis of the *cry34B* and *cry35B* genes has found that these genes have 50 to 60% sequence

identity to each other (Schnepf *et al.*, 2005). These binary toxins have been successfully engineered for expression in corn roots (Moellenbeck *et al.*, 2001) by insertion of the *cry*34Ab1 and *cry*35Ab1 genes (Stein *et al.*, 2009).

1.11.2 α-exotoxin

This toxin is a small protein that is secreted by *B. thuringiensis* during the later stages of development and growth. It is heat-labile and soluble in water, and found to be active against lepidopteran insects if injected and some vertebrates such as mice (Krieg, 1971; Prieto-Samsónov *et al.*, 1997). The α-exotoxin is considered to be a phospholipase C, which affects the phospholipid layer of cell membranes (Heimpel, 1954; Bonnefoi and Béguin, 1959). The detailed mode of action of these toxins is still unclear.

1.11.3 β-exotoxin

The β-exotoxin is a 701 kDa heat stable toxin produced by cells during vegetative growth just before sporulation and secreted into the medium. It referred to as thuringiensin or fly toxin (fly factor) since it has specificity as a general insecticidal agent (Bond, 1969; Liu and Tzeng, 1998). It is toxic to a wide range of insect species of orders including; Lepidoptera, Diptera, Coleoptera and Hymenoptera especially during the molting stage (Bond *et al.*, 1969; Inoue *et al.*, 1977; Gohar and Perchat, 2001). The toxicity is caused because β-exotoxin inhibits synthesis by DNA-dependent RNA polymerase (Hernandez-Rodriguez *et al.*, 2009) by competing for binding sites with ATP and thus leads to the inhibition of the biosynthesis of RNA (Prieto-Samsónov *et al.*, 1997), thereby affecting insect molting and pupation.

There are two forms of β-exotoxin, alternatively named thuringiensin A and thuringiensin B (Kim and Huang, 1970) or β-exotoxin I and β-exotoxin II (Levinson *et al.*, 1990), but the chemical differences between them and the relationship of thuringiensin A to either β-exotoxin I or β-exotoxin II is unclear. Each contains adenine, ribose, phosphate, glucose, and polyhydroxycarboxylic acid moities (Kim and Huang, 1970; Prieto-Samsónov *et al.*, 1997) The structure of β-exotoxin is showing in figure 1.11.3.1.

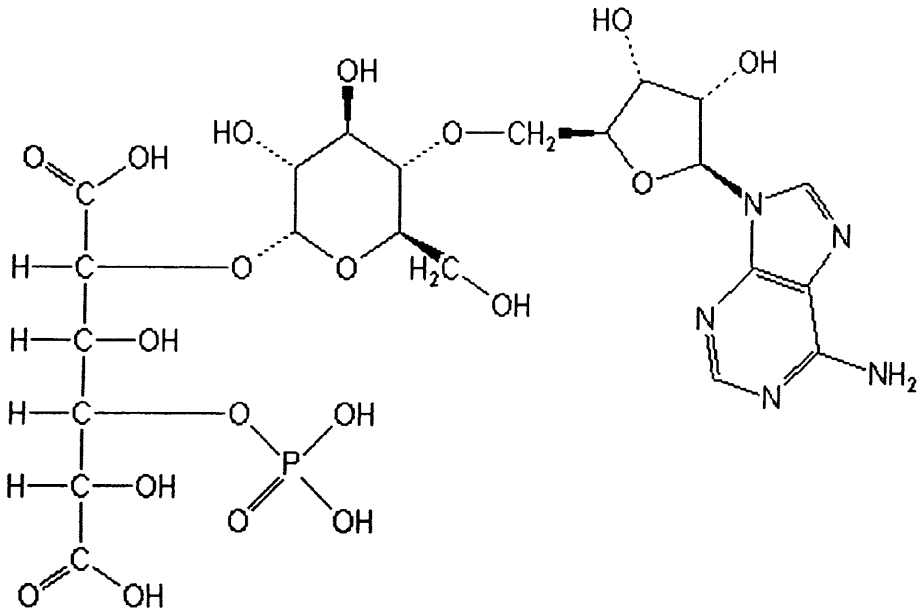


Figure 1.11.3.1 The structure of β-exotoxin of *Bacillus thuringiensis*

Adapted from (Liu and Tzeng, 1998).

Espinasse *et al.*, (2004) have suggested that plasmids may regulate the expression of β-exotoxin. *B. thuringiensis* strains that do not produce Cry toxin proteins have been found to be not capable of β-exotoxin secretion (Espinasse *et al.*, 2002a). This indicates that the production of β-exotoxin is linked to the production of Cry toxins, and suggests that some genetic factors for β-exotoxin are found on the plasmid alongside *cry* genes (Espinasse *et al.*, 2002a). Different size plasmids that encode various Cry toxin proteins have been found to be essential for β-exotoxin I production in many *B. thuringiensis* strains (Levinson *et al.*, 1990; Espinasse *et al.*, 2002b).

It has been suggested that that the β-exotoxin may have the potential to be used in fruit fly pest control programmes (Toledo *et al.*, 1999). But due to its toxicity to vertebrates, β-exotoxin use was stopped in many countries by WHO in 1999 and commercial *B. thuringiensis* products should be produced from strains free of *β*-exotoxin (WHO, 1999; Hernandez-Rodriguez *et al.*, 2009).

However the products of β-exotoxin such as (Muscabac®; Farmos Group, Ovlunsalo Finland; Bitoxibacillin®; Mikrobioprom, Moscow, Russia) were effective in the control of fly larvae when were used at insecticidal doses that do not affect vertebrates (Carlberg *et al.*, 1985; Carlberg, 1986). Thus *β*-exotoxin may be important for the production and the development of bio-pesticides from *B. thuringiensis* (Mac Innes and Bouwer, 2009).

1.11.4 Vegetative insecticidal proteins (Vip toxins)

Many *B. thuringiensis* strains (15% of those tested in one study Estruch *et al.*, 1996) produce vegetative insecticidal proteins (Vips), which are secreted from bacterial cells in the mid-log phase of vegetative growth and during sporulation (Estruch *et al.*, 1996; Jensen *et al.*, 2003; Arora *et al.*, 2003). Many new *vip* genes and Vip proteins have been discovered and have been classified and named in a similar nomenclature system to the *cry* genes and Cry toxins (Crickmore *et al.*, 2010). The full list of Vips and their classification can be found on (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html).

The vegetative insecticidal proteins do not share any amino acid sequence homology with delta-endotoxins and do not form crystals (Estruch *et al.*, 1996; Rice, 1999; Hernandez-Rodriguez *et al.*, 2009) and the Vips are a structurally different insecticidal group of *B. thuringiensis* toxins (Selvapandiyan *et al.*, 2001) with insecticidal activity against wide range of lepidopteran and coleopteran insect larvae (Estruch *et al.*, 1996). The Vip toxins are ingested by the susceptible larvae, activated by midgut proteinases and bind to specific receptors on the cell membrane of the midgut in the insect leading to pore formation and lysis of gut cells (Yu *et al.*, 1997; Lee *et al.*, 2006).

The Vips have a different mode of action from *B. thuringiensis* endotoxins and they are classified into two main groups. Group one consists of two proteins: the 100 kDa Vip1 and 52 kDaVip2 (Warren *et al.*, 1997 cited in Fang *et al.*, 2007; Espinasse *et al.*, 2003), which, in combination, have very high toxicity to the coleopteran western corn rootworm (*Diabrotica virgifera*), but have no activity

against lepidopterans (Han *et al.*, 1999). It has been found that Vip1Aa and Vip2Aa contain N-terminal signal sequences for secretion, and the presence of both toxins is required for insecticidal activity against Western and Northern corn rootworm (de Maagd *et al.*, 2003). Vip1 is believed to bind to specific receptors on cells of the midgut and act to form a pore, whereas the Vip2 ADP-ribosylase modifies actin and prevents polymerisation. Vip2 would pass through this pore and enter the cytoplasm of midgut cells (Estruch *et al.*, 1996; Warren *et al.*, 1996; Carlier, 1990).

The second group contains Vip3 toxins, which are active against a range of lepidopteran larvae (Espinasse *et al.*, 2003). Vip3 toxins have no sequence similarity to Vip1 and Vip2 toxins and can be detected in different stages of cell growth before sporulation (Estruch *et al.*, 1996). The *vip3A* gene encodes an 88 kDa protein, which is secreted into the culture medium (Yu *et al.*, 1997).

The most commonly identified *vip* gene in *B. thuringiensis* is *vip3A*. Vip3Aa1 is toxic to many lepidopteran insect larvae such as *Agrotis ipsilon, Spodoptera frugiperda, Spodoptera exigua, Heliothis virescens,* and *Helicoverpa zea* but it does not have toxic activity against the European corn borer *Ostrinia nubilalis* (Estruch *et al.*, 1996). The initiation of the expression of the *vip3Aa* gene is during mid-log phase and also continues during sporulation (Estruch *et al.*, 1996). Vip3A causes symptoms to susceptible insect after 48-72 h, whereas delta-endotoxins cause similar symptoms and death in 16 -24 h (Yu *et al.*, 1997).

The insecticidal target specificity of *B. thuringiensis* was reduced by deleting *vip3Aa1* gene, indicating that Vip3 can be important for the *B. thuringiensis* strain toxicity (Donovan *et al.*, 2001). Neither Vip3A, Cry1Ac nor Cry2Ab2 inhibit the ability of the others to bind to BBMV (Lee *et al.*, 2006). The receptors of Vip3Aa1 in the insect midgut are not endotoxin receptors such as aminopeptidase N-like and cadherin-like molecules, thus they have different binding sites (Lee *et al.*, 2003). Vip3A is able to cause pore formation after binding to BBMV using a different insecticidal mode of action from Cry toxins (Lee *et al.*, 2003). Having these properties, Vip3A potentially can be used as biological agent to overcome or reduce resistance problems to *B. thuringiensis* insecticidal toxins (Lee *et al.*, 2006; Liu *et al.*, 2007; Fang *et al.*, 2007).

Despite the fact that vegetative insecticidal proteins such as Vip3A have high toxic activity and relatively narrow specificity (Llewellyn *et al.*, 2007), the commercial development of Vip toxins has been slow. This may be because of the small quantities produced in cell cultures and their non-crystal structure makes them unstable (Qin *et al.*, 2010). The fact that vegetative insecticidal proteins are secreted from the cell into the culture medium during growth may also result in limitations to their field applications (Arora *et al.*, 2003). However, the potential is that transgenic plants that express Vip3A may exhibit high efficacy against the cotton bollworm and also Vip3A toxin is safe to use in transgenic plants, as it causes no harm to vertebrates (Brake *et al.*, 2005).

1.11.5 Sip toxins

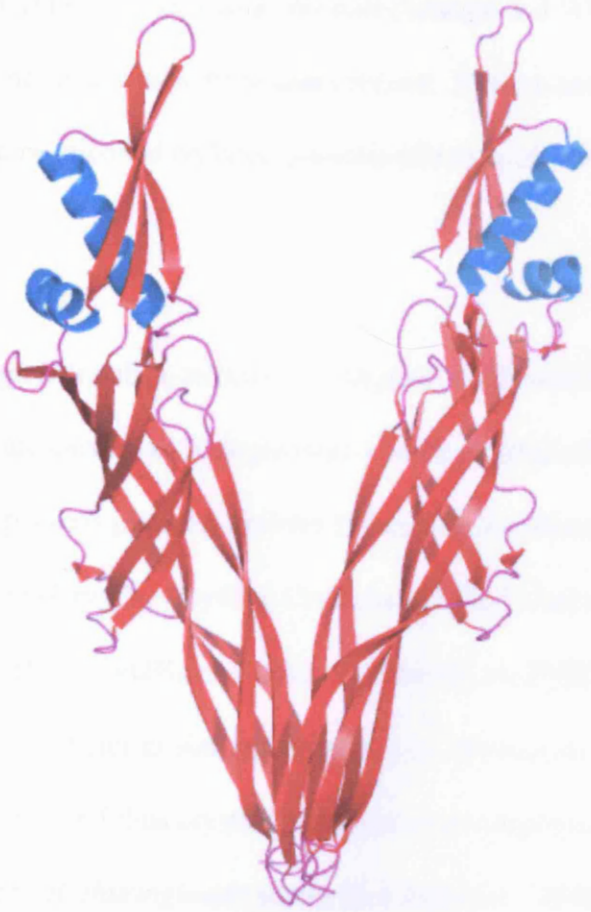
Some *B. thuringiensis* strains have been found to produce another type of toxin called Sip1A (secreted insecticidal protein), which is secreted into the growth medium and this toxin has shown toxic activity against larvae of Coleoptera (Donovan *et al.*, 2006). Sip toxin is a 41 kDa protein that is plasmid encoded and is not related to the Cry, Cyt or Vip toxin proteins and its mechanism of action is still unclear.

1.11.6 Parasporin

This is a novel protein with a unique non-haemolytic cytocidal activity (Mizuki *et al.*, 2000). The parasporin protein has been defined as "*Bacillus thuringiensis* and related bacterial parasporal proteins that are non-hemolytic but capable of preferentially killing cancer cells" (Katayama *et al.*, 2005; Akiba *et al.*, 2009). These toxins have been classified using the system of numbers and letters for Cry, Cyt and Vip toxins (Ohba *et al.*, 2006). More information and full list of PS toxins can be found on the link http://parasporin.fitc.pref.fukuoka.jp/intro.html. One parasporin crystal structure has been solved and the protein resembles the β- pore forming toxin aerolysin (Akiba *et al.*, 2006) figure 1.11.6.1.

Figure 1.11.6.1 The three-dimensional structure of parasporin 26-kDa protein

Adapted from (Akiba *et al.*, 2006)



1.12 *Bacillus thuringiensis cry* toxin genes

The use of *B. thuringiensis* in biological control of vectors and the discovery of new subspecies of *B. thuringiensis* with insecticidal proteins led to the cloning of the genes responsible for the production of these proteins. The first delta-endotoxin gene cloned was from *B. thuringiensis* subsp. *kurstaki* (Schnepf and Whiteley, 1981) and since then many other genes have been characterised. The *cry* and *cyt* genes appear, in most of cases, to be encoded on large plasmids (Berry *et al.*, 2002 and references herein).

In *B. thuringiensis* subsp. *israelensis*, the genes responsible for the expression of delta-endotoxin are carried on a single large 128-kb plasmid pBtoxis (Schnepf *et al.*, 1998). This pBtoxis plasmid contains 125 coding sequences including four *cry* toxin genes encoding (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and three *cyt* toxin genes encoding (Cyt1Aa, Cyt2Ba and Cyt1Ca) (Berry *et al.*, 2002). The plasmids of *B. thuringiensis* strains differ in size and number from strain to strain and many of them are transmissible, and thus crystal protein genes are transmissible and might easily spread between *B. thuringiensis* strains (Schnepf *et al.*, 1998).

The *cry* gene expression for most Cry proteins is controlled at different levels - transcriptional, post-transcriptional and post-translational during sporulation (Agaisse and Lereclus, 1994; Schnepf *et al.*, 1998). Studies on the expression of crystal genes have shown that sporulation specific sigma factors in *B. thuringiensis* control the crystal gene promoters. These sigma factors are σ^E, σ^F, σ^H and σ^K (Dervyn *et al.*, 1995). The promoters of *cry4A* and *cry4B* are activated in the mid phase of sporulation in *B. thuringiensis*. The transcription of crystal genes (*cry4A*, *cry4B* and

cry11A genes) has been found to be controlled by σ^E. furthermore *cry4A* is also controlled by σ^H and *cry11A* is controlled by σ^K (Yoshisue *et al*, 1995; Dervyn *et al*., 1995). In *spo*IIG mutants the transcription of the *cry4B* but not *cry4A* is blocked.

The transcription of *cry4A* is shown to start earlier than *cry4B* and *cyt1A* genes (Yoshisue *et al*, 1995). The synthesis of Cry3A was enhanced when *cry3A* and its upstream STAB-SD mRNA (Shine-Dalgarno Stabilising Sequences) were expressed under the control of the sporulation dependent *cyt1A* promoters. It has also been found that the synthesis of different Cry proteins can be enhanced by such a combination of *cry3A* promoter and its upstream STAB-SD mRNA (Park *et al*., 1998; 1999). The STAB-SD mRNA structure at the 5' end protects the mRNA from degradation and, therefore, increases the protein synthesis as it increases the life of mRNA (Agaisse and Lereclus, 1996). Another study has shown that the expression of *cry3A* toxin gene of *B. thuringiensis* in *B. subtilis* does not rely on a sporulation - specific sigma factor (Agaisse and Lereclus, 1994).

Members of the *cry* gene family have also been discovered in bacterial species other than *B. thuringiensis* such as, *Clostridium bifermentans* subsp. *malaysia*, which was found to harbour *cry16Aa* and *cry17Aa* genes (Barloy *et al*., 1996) and *Paenibacillus lentimorrbus* strain *semadara,* which has the *cry43Ba1* gene (Yokoyama *et al*., 2004).

1. 13 Plasmids

The plasmids in *B. thuringiensis* may vary in number and size from one strain to another (Andrup *et al.*, 2003). In the *B. cereus sensu lato* group the plasmids play a very important role in its pathogenesis and ecology (Rasko *et al.*, 2007). *B. anthracis* has two plasmids pXO1 that encodes toxin production and pXO2 that encodes encapsulation, whereas *B. thuringiensis* contains toxin encoding plasmids, which defines its strains (Rasko *et al.*, 2007).

The *B. cereus* group has a large number of plasmids that are able to exchange between group members (Jensen *et al.*, 2003). The role played by plasmids exchanging genetic materials is important for bacterial evolution (Andrup *et al.*, 2003). Plasmid encoded genes including virulence genes have been shown to switch location between plasmids and chromosomes (Rasko *et al.*, 2005). In *B. cereus* ATCC 10987, there are examples of possible genetic exchange between the chromosome and the plasmid so that identical copies of a transposable element similar to *S. aureus* Tn554 have been found that in the chromosome and plasmid (Bastos and Murphy, 1988). The *bclA* gene in *B. anthracis* is limited to the chromosome and found on some plasmids of *B. thuringiensis* (Andrup *et al.*, 2003), but similar copies of *bclA* in *B. cereus* ATCC 10987 are located on the chromosome and the plasmid (Rasko *et al.*, 2005). The similarity of genes found on other plasmids indicates that genetic exchange between chromosomes and plasmids takes place in *B. cereus* group (Rasko *et al.*, 2005).

The large plasmids are important due to their properties such as delta-endotoxin production, conjugative functions, transposition, and heat-stable exotoxin

production (Jensen *et al.*, 1996; Baum *et al.*, 1990; Ozawa and Iwahana, 1986; Lereclus *et al.*, 1986). The plasmids containing Cry protein genes may in some cases be conjugative and transfer from strain to strain by a conjugation like processes (Gonzalez *et al.*, 1982). Plasmid transfer between *B. thuringiensis* strains has been observed in laboratory, soil and larval insects (Thomas *et al.*, 2000). The transfer of plasmids containing Cry protein genes between *B. thuringiensis* and *B. cereus* strains may happen in the environment and this may lead to new strains with higher insecticidal activity and different ranges of host insects (Helgason *et al.*, 1998).

1.13.1 Plasmid pBtoxis

B. thuringiensis strains can contain up to 17 plasmids (Lereclus *et al.*, 1982) that vary in size from about 2 to 200 kb and usually the insecticidal toxin genes (*cry*) are located on large self-transmissible plasmids (Gonzalez *et al.*, 1982). *B. thuringiensis* subsp. *israelensis* has two large plasmids: the approximately 180 kb pXO16, which is able to mobilise other plasmids (Reddy *et al.*, 1987) and the 128 kb toxin-coding plasmid pBtoxis. This plasmid can be transferred between *B. cereus* group strains (Hu *et al.*, 2005) and into more distantly related *B. sphaericus* (Gammon *et al.*, 2006).

The pBtoxis plasmid was sequenced and revealed 125 possible genes. These include seven known toxins genes (four *cry* genes; *cry*4Aa, *cry*4Ba, *cry*10Aa, and *cry*11Aa and three *cyt* genes; *cyt*1Aa, *cyt*2Ba and *cyt*1Ca) (Berry *et al.*, 2002). The plasmid does not only encode toxin proteins responsible for virulence traits but was also found to encode other proteins with putative roles that may affect the phenotype of the host organism.

The pBtoxis carries a number of genes encoding possible transposons (21 genes), proteins with potential roles in sporulation and germination (5 genes), possible involvement in antibiotic production (3 genes), possible transcription regulators (14 genes), enzymes (5 genes) as well as 13 conserved hypothetical proteins (Berry *et al.*, 2002). Of 40 genes with putative functions, Stein *et al*, (2006) showed 29 genes to be transcriptionally active. These include genes encoding possible peptide antibiotics (pBt136, pBt137 and pBt138), and germination genes (pBt084, pBt085 and pBt086) (Stein *et al.*, 2006). Eight of the 14 CDSs that have similarity with genes encoding transcriptional regulators were also found to be transcribed and these may have roles in modulating transcription in *B. thuringiensis* subsp *israelensis* (Stein *et al.*, 2006).

The origin of replication of pBtoxis is located close to pBt001 (Tang *et al.*, 2006), as proposed by Berry *et al.*, (2002). The pBt001 protein shows 76% amino acid identity to a protein encoded by pXO1-49, which is found near to the putative replication origin of pXO1, the virulence plasmid of *B. anthracis* and probably has a role in plasmid replication (Tang *et al.*, 2006). The replication origin was found to be located on plasmid pBtoxis within a 2.2kb fragment spanning nucleotide positions 124407 to 126636. This region also contains pBt156 and pBt157, which were found to be transcriptionally active (Stein *et al.*, 2006) and may, therefore, also be important players in replication of pBtoxis (Tang *et al.*, 2006).

1.13.2 Regulatory genes

The pBtoxis plasmid harbours 125 genes, among them transcribed genes with potential roles as transcriptional regulators, such as pBt108, which shows homology to sigma E, which is a factor in transcription of toxin genes during the early phase of sporulation (Berry *et al.*, 2002). There are many pBtoxis genes that appear to be similar to genes encoding transcriptional regulators in other organisms, and may have significant effects on virulence and phenotype by altering gene transcription from the plasmid or host DNA (Stein *et al.*, 2006).

1.13.3 Antibiotic production

Many *B. thuringiensis* strains produce antibiotics during their life cycle including a range of bacteriocins (Cherif, *et al.*, 2003). These have an antibacterial activity against other *Bacillus* strains such as *B. cereus* and *B. weihenstephanensis* (Riley and Wertz., 2002; Kamoun, *et al.*, 2005). Also some stains of *B. thuringiensis* and *B. cereus* produce the antibiotic zwittermicin A (Stabb, *et al.*, 1994).

In *B. thuringiensis* subsp. *israelensis,* the plasmid appears to encode proteins related to peptide antibiotics (Berry, *et al.*, 2002). These genes are pBt136, pBt137 and pBt138 and mRNA was detected for these pBtoxis genes (Stein *et al.*, 2006). The CDSs pBt136 and pBt137 appear to form an operon, which may be regulated by PlcR (Stein *et al.*, 2006). When rich medium such as NYSM is used for cell growth, the PlcR -regulated genes tend to turn on at the end of vegetative phase (Lereclus *et al.*, 2000). The role of these putative antibiotic production genes will be investigated in this work (chapter 3).

1.14 The aim of this study

The aim of this study was to investigate different factors other than toxins that are encoded by pBtoxis and may enhance the ability of *B. thuringiensis* subsp. *israelensis* to control mosquitoes and black flies. Factors investigated include the antibiotic production genes and germination genes. In addition the role of the mosquito gut flora in pathogenicity was also explored.

CHAPTER 2

General materials and methods

2.1 Strains used in this study

Several bacterial and fungal strains were used in this study and are shown in Table 2.1.1.

Table 2.1.1 Bacterial and fungal strains

No	Strains	Notes
1	*B. thuringiensis* subsp.*israelensis* strain 4Q5 (also known as 4Q2-72)	Cured of all plasmids except pBtoxis plasmid
2	*B. thuringiensis* subsp. *israelensis* strain 4Q7 (also known as 4Q2-81)	Cured of all plasmids including pBtoxis: Crystal minus strain
3	*B. subtilis* strain IA2	
4	*B. thuringiensis* subsp. *israelensis* strain IPS 78/11	Cured of all plasmids including pBtoxis: Crystal minus strain
5	*B.thuringiensis* subsp.*israelensis* vectobac®	Contains all plasmids of wild-type *B. thuringiensis* subsp. *israelensis* including pBtoxis
6	*B. sphaericus* strain 1593	
7	*B. thuringiensis* subsp. *israelensis* 4Q7 *ger*	Strain 4Q7 transformed with pHT304-*ger* (Section 6.3.3)
8	*E. coli K12*	
9	*E. coli DH5α*	Genotype: DH5alpha =*end*A1, *rec*A1, *gyr*A96, *thi, hsd*R17 (rk–, mk+), *rel*A1, *sup*E44, Δ (*lac-pro*AB), [F′ *tra*D36, *pro*AB, *laq*IqZΔM15].
10	*Enterobacter sp.*	Gut bacteria of our lab reared *Aedes aegypti*
11	*Microbacterium sp. (oxydans)*	Gut bacteria of our lab reared *A. aegypti*
12	*Brevundimonas diminuta*	Gut bacteria of our lab reared *A. aegypti*
13	*Bacillus sp.*	Gut bacteria of our lab reared *A. aegypti*
14	*B. circulans*	Gut bacteria of our lab reared *A. aegypti*
15	*Nitrosococcus sp.*	Gut bacteria of our lab reared *A. aegypti*
16	*Pseudomonas aeruginosa PAO1*	Provided by Prof. Richard Dickinson Cardiff University
17	*Burkholderia multivorans ATCC 17616*	Provided by Dr. Mahenthiralingam, Cardiff University
18	*Saccharomyces cerevisiae* strain *BY4741*	Provided by Dr. Mahenthiralingam, Cardiff University

2.2 Growth media and conditions

All *Bacillus* strains were cultured at 30°C in Embrapa medium to enhance germination. The composition of 1 litre is 8 g Nutrient broth, 1 g yeast extract, 1 g sodium hydrogen phosphate and 10 ml of mineral salts (10 g/l $CaCO_3$: $7H_2O$, 10 g/l $MgSO_4$: $7H_2O$, 1 g/l $FeSO_4$: $7H_2O$, 1 g/l $MnSO_4$: $7H_2O$, $ZnSO_4$: $7H_2O$) adjusted to pH 7, prior to autoclaving (20 minutes at 120°C).

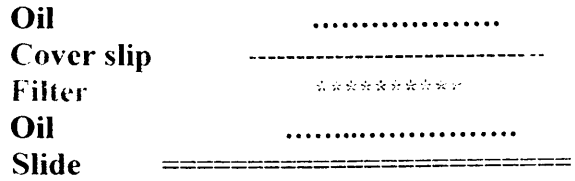
2.3 Bacterial counting

2.3.1 Acridine orange method

Bacterial strains were counted using an acridine orange method to stain bacterial cells in order to facilitate direct counting of cells on a slide using a fluorescence microscope. The fluorochrome acridine orange stains DNA green and RNA red. Dilution series of *B. thuringiensis* subsp. *israelensis* 4Q5kan and *B. thuringiensis* subsp. *israelensis* 4Q7rif cultures were prepared (neat, 10^{-2}, 10^{-4} and 10^{-6}) and 100 µl was added to 10 ml of filter-sterilised 2.5% (v/v) glutaraldehyde, then 100 µl of acridine orange (2 mg/ml) was added to make the final concentration of 20 µg/ml acridine orange. The sample was kept in a dark room for 3 minutes and filtered onto a black, 25 mm nucleopore polycarbonate membrane with 0.2 µm pore size. The filter was washed with 5 ml glutaraldehyde followed by 5 ml of 25 % (v/v) propan-2-ol.

The membrane was then mounted on a microscope slide with a thin layer of spectosol liquid paraffin oil under the filter and on the cover slip as shown in figure 2.1.

Figure 2.1 Slide preparation



The slide was placed under 100x-magnification lens and bacterial cells were counted in the grid for 20 squares and this was repeated in 3 fields of view. The number of cells per ml in the original culture was calculated as follows.

The grid has 100 squares of 10 μM ($10*10^{-4}$ cm) and thus, the area of the small square is 10^{-6} cm^2. The number of bacteria (n) on the whole filter is calculated with the following equation when,

N = average number of bacteria on 20 small squares and r = radius of filter in cm.

$$n = \frac{N * \prod r^2}{10^{-6}}$$

Since 100 μl of bacteria were used, this number is multiplied by 10 to obtain the number of cells per ml and by the dilution ratio to calculate the original cell density.

2.3.2 Drop plate method

Serial dilutions of cultures were prepared and three 20 μl drops were pipetted onto a quarter of one plate. Four such dilutions were drop plated onto each plate. Thus, the first plate would have dilutions from 10^{-1} to 10^{-4} and second plate would have dilutions from 10^{-5} to 10^{-8}. Plates were incubated at room temperature for 24 h. Counting was done as follows: Drops were chosen at dilutions yielding countable numbers (between 30 and 300) of colonies per drop. The average number of colonies

on three drops from 20 µl was multiplied by 50 to obtain the number of cells per ml and by the dilution ratio to calculate the original cell density.

2.4 Production of antibiotic resistant bacterial

The antibiotic resistant strains of *B. thuringiensis* subsp. *israelensis* were produced by selection to facilitate identification after co-feeding. *B. thuringiensis* subsp. *israelensis* 4Q5 was selected using kanamycin while *B. thuringiensis* subsp. *israelensis* 4Q7 was selected with rifampicin, as follows.

From an overnight culture of *B. thuringiensis* subsp. *israelensis*, 100 µl was inoculated into 10 ml of fresh LB containing 0.1 µg/ml antibiotic and incubated over night in a shaking incubator at 30°C. Then 100 µl of this culture was inoculated into 10 ml of fresh LB medium containing 1 µg/ml antibiotic and incubated over night in a shaking incubator at 30°C. Finally 100 µl of this culture was inoculated in fresh LB medium containing 10 µg/ml antibiotic and incubated over night in a shaking incubator at 30°C. These cultures were plated onto LB agar containing 10 µg/ml antibiotic for the isolation of individual, antibiotic- resistant colonies.

2.5 Purification of crystal proteins from *B. thuringiensis* subsp. *israelensis* 4Q5

Crystal proteins were purified from sporulated *B. thuringiensis* subsp. *israelensis* 4Q5 carrying crystal protein genes using the method described by Silva Filha *et al* (Silva Filha *et al*, 1997). Cultures were grown in Embrapa medium with shaking at 30°C for 3-4 days and until more than 90% sporulation, which was judged by phase contrast microscopy. Sporulated cultures were harvested using centrifugation (20,000 x g, 4°C) and then washed in ice-cold 1 M NaCl containing 10

mM EDTA. The spore suspension was harvested as before and washed twice in ice cold 10 mM EDTA and finally re-suspended in 1/30 of the original culture volume of ice-cold sterile water.

The final spore suspension was sonicated (eg using a Sonics Vibra Cell Ultrasonic Processor VCX500 (50% amplitude, 4x30 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 then analysed by SDS Page. Crystal suspensions were stored at - 20°C.

2.6 PCR amplification of 16S rRNA genes

To allow identification of bacterial isolates, fragments of 16S rRNA genes were amplified using two PCR primers; forward primer 63f (5`-CAG GCC TAA CAC ATG CAA GTC- 3`) and reverse primer 1387r (5`- GGG CGG WGT GTA CAA GGC-3`) (Operon) (Marchesi *et al.*, 1998).

PCR reactions (100 µl) were set up as follows: 1µl of template DNA; 20µl of 5x buffer; 2µl of dNTPs; 1 µl each of 10 µM 63f and 1387r primers; 1 µl of *Taq* polymerase (Promega); 8 µl of 25 mM $MgCL_2$; and 67 µl of dH_2O. The reaction was placed in a T3 thermocycler (Biometra®, Germany) and after a 95°C 5 min hot start,

30 cycles of PCR were carried out as follows.

Denaturation	95°C for 1 min
Annealing	55°C for 1 min
Extension	72°C for 1.5 min

Following these cycles, a final extension stage (72°C for 5 min) was programmed.

The products were separated by agarose gel electrophoresis (Section 2.8) to determine the size of the DNA fragments, estimate DNA concentration and to isolate DNA fragments needed for further cloning. Amplified bands of approximately 1,300 bp were seen for most colonies. Products were submitted to Lark Technologies for sequencing using the 63f primer and the DNA sequences were analysed as described below.

2.7 16S rRNA sequences analysis

To analyse 16S rRNA taxonomy of the samples, the sequences of 16S rRNA genes were compared to the non-redundant database at the NCBI homepage by Blast (Altschul *et al*, 1990) analysis (http://www.ncbi.nih.gov/BLAST/Blast.cgi) and also submitted to expasy homepage (www.expasy.org) to determine the most similar database sequence.

2.8 Agarose Gel Electrophoresis

Agarose (Bio-line) (0.6 g) was added to 60 ml of TAE and dissolved by heating using a microwave for one minute. Once cooled, 3 µl of 10 mg/ml Ethidium bromide (Sigma) was added and the gel cast in a standard gel tray before a well- forming comb was inserted. Once the gel had set, the comb was removed

and the gel placed in an electrophoresis gel tank (Biorad) filled with 1x TAE buffer. Loading Buffer was added to the DNA samples, which were loaded along with two markers (Lambda DNA cut with *Hin*dIII and ΦX174 DNA/*Hae*III) (Promega). The gel was run at a constant voltage of 120 V, visualised using a UVP transluminator (Ultra-Violet Products) Gel Doc-It imaging system and an image captured using Labworks software (Jencons-Plc).

2.9 Maintenance of *Aedes aegypti*

Aedes aegypti eggs were obtained from Keele University and hatched in water and were reared at Cardiff School of Biosciences, Cardiff University, Cardiff, UK. Adult mosquitoes were maintained in cages kept in a controlled temperature room at 30°C and 12 h light: 12 h dark photoperiod. Mosquitoes were fed every two days on defibrinated horse blood (TSC Biosciences Ltd, Buckingham, UK) using an artificial blood feeder (Hemotek membrane feeding systems, Lancs. England), and lamb gut was used as the feeding membrane to cover the blood reservoir. Mosquitoes laid eggs on Whatman 3 MM papers in water-filled cups.

Eggs were stored at 4°C until required or hatched in tap water in small plastic containers (10 X 15 X 15 cm). Eggs hatched within 24 hours. Larvae were fed on crushed rabbit pellets and maintained under same conditions as adults. Each larval stage lasted for 2-3 days, thereby; pupil stages were observed 10-14 days after the emergence of larvae and became adults few days later.

CHAPTER 3

Re-analysis of pBtoxis plasmid

3.1 Introduction

Bacillus thuringiensis strains harbour a number of different plasmids that may vary in size from about 2 to 200 kb (Gonzalez *et al.*, 1982). *Bacillus thuringiensis* subsp. *israelensis* has two large plasmids: the approximately 180 kb pXO16, which is capable of mobilising other plasmids (Reddy *et al.*, 1987) and the 128 kb toxin-coding plasmid pBtoxis. This plasmid can be transferred between *B. cereus* group strains (Hu *et al.*, 2005) and into the more distantly related *Bacillus sphaericus* (Gammon *et al.*, 2006).

The original sequence of the pBtoxis 128 kb plasmid was determined in 2002 by Berry *et al.*, and revealed 125 possible genes that include seven known toxin genes (four *cry* genes; *cry4Aa*, *cry4Ba*, *cry10Aa*, and *cry11Aa* and three *cyt* genes; *cyt1Aa*, *cyt2Ba* and *cyt1Ca*) (Berry *et al.*, 2002). The pBtoxis plasmid encodes toxin proteins responsible for virulence traits and was also found to encode other proteins with putative roles that may affect the phenotype of the host organism (Stein *et al*,. 2006). The pBtoxis plasmid also carries a number of other genes encoding possible transposons (21 genes), proteins with potential roles in sporulation and germination (5 genes), possible involvement in antibiotic production (3 genes), possible transcription regulators (14 genes), enzymes (5 genes) as well as 13 conserved hypothetical proteins (Berry *et al.*, 2002).

Due to the fact that database changes and updates are ongoing as new genes and functions are discovered, the pBtoxis plasmid sequence was analysed again to look for new possible functions of its genes.

3.2 Methods

The analysis of the pBtoxis plasmid was carried out at the Sanger Institute (Wellcome Trust) in Cambridge, UK, using Artemis release 10 systems software (for more details on Artemis see http://www.sanger.ac.uk/Software/Artemis/), to search the database for new functions and compare pBtoxis plasmid genes with other sequences in the databases to find new information about pBtoxis genes. This work was done from 24-07-2008 to 30-07-2008.

3.3 Results

The analysis of pBtoxis genes for new functions revealed new information on several coding sequences (CDS). For instance CDS pBt001, which was originally annotated as "unknown function" since no similar CDSs had been found (Berry, *et al;* 2002), was on re-analysis, found to be related to a putative transcriptional regulator as indicated by its HHMPfam domain. Similarly, the pBtoxis coding sequences pBt010, pBt013, pBt020, pBt030, pBt032, pBt034, pBt065, pBt066, pBt113, pBt114, pBt115, pBt116, pBt127, pBt146 and pBt150 were considered as hypothetical proteins and in this analysis they have been found to be conserved hypothetical proteins as they are similar to other genes now present in the database.

The pBtoxis plasmid genes pBt136, pBt137 and pBt138 previously shown to be related to genes involved in the production of an antibiotic peptide AS-48 were, in the re-analysis of the pBtoxis plasmid found to be related to production of bacteriocin (pBt136), bacteriocin maturation (pBt137) and bacteriocin secretion (pBt138). Also pBt139 may be related to bacteriocin transportation, whereas it was annotated as an ATP-binding protein in the previous analysis (Berry, *et al* 2002).

The CDS pBt145 may be a Camelysin precursor and not putative spore coat associated protein as previously suggested. For CDS pBt152, which was originally recorded as a hemagglutinin related protein, the potential as a putative cell killing protein is now noted with the identification of a lectin-like domain in the protein. A full list of CDSs with altered annotations is given in table 3.3.1.

Table 3.3.1: Predicted genes in pBtoxis

Name of Gene	Predicted product	Database similarity (EMBL no.) (%aa identity)	pOX1 homologue (% aa identity)
pBt001	Putative transcriptional regulator	Similar to *Bacillus cereus*; Putative uncharacterized protein; length=378; id 84.8%; E()=2.3e-106; 309 aa overlap; query 1-308; subject 1-309" Similarity with UniProt: Q9X319 (EMBL:AF065404); *Bacillus anthracis*; PXO1-49.; length=227; id 78.5%; E()=1.9e-47; 158 aa overlap; query 152-308; subject 1-158"	pXO1-49 (158in 227 aa)
pBt010	Conserved hypothetical protein	Similarity to *Bacillus thuringiensis* subsp. *galleriae*.; pBt10-like protein.; length=83; id 51.3%; E()=5.7e-12; 76 aa overlap; query 1-76; subject 3-78"	
pBt013	Conserved hypothetical protein	Similarity *Bacillus cereus* G9241; Putative uncharacterized protein.; length=202; id 43.1%; E()=1.5e-35; 202 aa overlap; query 3-201; subject 1-200"	
pBt014	Probable transcriptional regulator	Similarity to *Algoriphagus sp.* PR1.; Transcriptional regulator, PbsX family protein.; length=66; id 43.1%; E()=0.0049; 65 aa overlap; query 1-65; subject 1-65"	
pBt020	Conserved hypothetical protein	Similarity to uncultured archaeon GZfos18H11.; Putative uncharacterized protein.; length=63; id 51.7%; E()=2.7; 29 aa overlap; query 3-31; subject 1-27"	

pBt030	Conserved hypothetical protein	Similarity to *Bacillus thuringiensis* subsp. *israelensis*.; pBt032; Putative uncharacterized protein pBt032.; length=103; id 79.2%; E()=2.2e-25; 96 aa overlap; query 1-96; subject 1-96"	
pBt032	Conserved hypothetical protein	Similarity to *Bacillus thuringiensis* subsp. *israelensis*.; pBt030; Putative uncharacterized protein pBt030.; length=101; id 79.2%; E()=2.6e-24; 96 aa overlap; query 1-96; subject 1-96"	
pBt034	Conserved hypothetical protein	Similar to C-terminal half of *Bacillus anthracis* pXO1-106 TR:Q9X375 (EMBL:AF065404) (126 aa) fasta scores: E(): 2.5e-11, 62.68% id in 67 aa. Similarity with UniProt:Q6I280 (EMBL:AE017225); *Bacillus anthracis*; Putative uncharacterized protein.; length=91; id 87.1%; E()=6.2e-19; 62 aa overlap; query 1-62; subject 24-85"	pXO1-106
pBt063	Putative spore germination protein (pseudogene) Spore germination GerIB-like protein (pseudogene)	similarity with UniProt:A9VG14 (EMBL:CP000903); *Bacillus weihenstephanensis* (strain KBAB4).; Spore germination protein.; length=367; id 68.5%; E()=3.7e-23; 92 aa overlap; query 1-92; subject 1-92" Truncated by IS240 insertion	
pBt065	Conserved hypothetical protein	Similarity with UniProt:A9VFV6 (EMBL:CP000903); *Bacillus weihenstephanensis* (strain KBAB4).; Putative uncharacterized protein.; length=70; id 87.1%; E()=1.2e-24; 70 aa overlap; query 1-70; subject 1-70"	
pBt066	Conserved hypothetical protein	Similarity with UniProt:Q8KNR3 (EMBL:AL731825); *Bacillus thuringiensis* subsp. *israelensis*.; pBt126; Putative uncharacterized protein pBt126.; length=118; id 50.9%; E()=2.8e-18; 110 aa overlap; query 3-112; subject 1-110"	
pBt113	Conserved hypothetical protein	Similarity with UniProt:Q4V1Y8 (EMBL:CP000040); *Bacillus cereus* (strain ZK/E33L).; Uncharacterized protein.; length=129; id 37.8%; E()=1.1e-12; 111 aa overlap; query 25-132; subject 8-117"	

pBt114	Conserved hypothetical protein	Similarity with UniProt:Q4YYW6 (EMBL:CAAI01001627); *Plasmodium berghei*.; Putative uncharacterized protein (Fragment).; length=96; id 44.4%; E()=2.8e+02; 45 aa overlap: query 18-59; subject 19-63"	
pBt115	Conserved hypothetical protein	Similarity with UniProt:Q4AA63 (EMBL:AE017243); *Mycoplasma hyopneumoniae* (strain J/ATCC 25934/NCTC 10110).; rpmF; 50S ribosomal protein L32.; length=65; id 57.9%; E()=15; 19 aa overlap; query 17-35; subject 30-48" Similarity with UniProt:Q5FUN5 (EMBL:CP000009); *Gluconobacter oxydans* (*Gluconobacter suboxydans*).; rpmF; 50S ribosomal protein L32.; length=70; id 45.5%; E()=52; 22 aa overlap; query 18-39; subject 30-51"	
pBt116	Conserved hypothetical exported protein	Similarity with UniProt:Q63B60 (EMBL:CP000001); *Bacillus cereus* (strain ZK/E33L).; Group-specific protein.; length=134; id 71.0%; E()=1.2e-37; 131 aa overlap; query 1-129; subject 1-131" Similarity with UniProt:Q81QA5 (EMBL:AE016879); *Bacillus anthracis*.; Putative uncharacterized protein.; length=134; id 70.2%; E()=2.8e-37; 131 aa overlap; query 1-129; subject 1-131"	
pBt127	Conserved hypothetical protein	Similar in parts to *Bacillus anthracis* gene fragments pxo1-106 TR:Q9X375 (EMBL:AF065404) (126 aa) fasta scores: E(): 4.9e-17, 80.76% id in 78 aa, and to *Bacillus anthracis* pxo1-71 TR:Q9X341 (EMBL:AF065404) (85 aa) fasta scores: E(): 2e-08, 36.98% id in 73 aa, and to *Bacillus anthracis* pxo1-72 TR:Q9X342 (EMBL:AF065404) (101 aa) fasta scores: E(): 3.6e-08, 38.77% id in 98 aa, and to *Rickettsia conorii* hypothetical 12.1 kDa protein rc1156 TR:AAL03694 (EMBL:AE008664) (102 aa) fasta scores: E(): 2e-07, 40.96% id in 83 aa, and to *Rickettsia conorii* hypothetical 8.9 kDa protein rc1157 TR:AAL03695 (EMBL:AE008664) (78 aa) fasta scores: E(): 0.021, 32.81% id in 64 aa"	pXO1-106 pXO1-71 pXO1-72

pBt136	Bacteriocin	Similarity with UniProt:A5H1G9 (EMBL:DQ650653); *Streptococcus uberis.*; ublA; *Bacteriocin uberolysin* precursor; length=76; id 43.8%; E()=5.3e-07; 73 aa overlap; query 1-73; subject 4-76" Similarity with UniProt:Q8GB47 (EMBL:AY164463); *Clostridium beijerinckii* (Clostridium MP).; cirA; Circularin A.; length=72; id 36.0%; E()=19; 75 aa overlap; query 1-73; subject 1-72"	
pBt137	Putative bacteriocin maturation/ biosynthesis protein	Similarity with UniProt:A5H1H0 (EMBL:DQ650653); *Streptococcus uberis.*; ublB; UblB.; length=535; id 22.5%; E()=2.4e-10; 546 aa overlap; query 12-555; subject 14-526" Similarity with UniProt:O52964 (EMBL:D85752); *Enterococcus faecalis* (*Streptococcus faecalis*).; bacB; BacB protein.; length=563; id 21.6%; E()=2.6e-06; 537 aa overlap; query 39-555; subject 45-552" Similar to *Enterococcus faecalis* BacB protein bacB TR:O52964 (EMBL:D85752) (563 aa) fasta scores: E(): 1.3e-06, 22.16% id in 537 aa (also called as48-B TR:O53024 (EMBL:Y12234) AS-48 maturation and biosynthesis protein)"	
pBt138	Putative bacteriocin secretion protein	Similarity with UniProt:Q7WYU0 (EMBL:AJ566621); *Clostridium beijerinckii* (Clostridium MP).; cirC; CirC protein.; length=185; id 30.3%; E()=6.6e-07; 142 aa overlap; query 36-176; subject 44-183"	
pBt139	Putative bacteriocin transporter	Similarity with UniProt:Q7WYT9 (EMBL:AJ566621); *Clostridium beijerinckii* (Clostridium MP).; cirD; ATP-binding protein.; length=221; id 38.5%; E()=6.4e-22; 208 aa overlap; query 10-216; subject 11-217" Similarity with UniProt:A5H1H2 (EMBL:DQ650653); *Streptococcus uberis.*; ublD; UblD.; length=218; id 38.6%; E()=6e-17; 202 aa overlap; query 9-203; subject 5-200	

pBt145	Camelysin precursor calY	Similarity with UniProt:Q8GJ76 (EMBL:AJ514407); *Bacillus cereus*., calY; Camelysin precursor.; length=197; id 56.3%; E()=3.4e-46; 197 aa overlap; query 1-197; subject 1-197" (camelysin is a novel surface metalloproteinase from *Bacillus cereus*)	
pBt146	Conserved hypothetical protein	Similarity with UniProt:A7GUZ2 (EMBL:CP000764); *Bacillus cereus* subsp. *cytotoxis* (strain NVH 391-98).; Group-specific protein.; length=46; id 60.0%; E()=1.8e-06; 45 aa overlap; query 1-45; subject 1-45"	
pBt150	Conserved hypothetical protein	Similarity with UniProt:Q1PS77 (EMBL:DQ242517); *Bacillus thuringiensis* subsp. *tenebrionis*.; Putative uncharacterized protein.; length=194; id 95.9%; E()=9.9e-51; 146 aa overlap; query 1-145; subject 1-146"	
pBt152	Putative cell killing protein (Has a lectin domain)	Similarity with UniProt:Q3ES64 (EMBL:AAJM01000175); *Bacillus thuringiensis* serovar *israelensis* ATCC 35646.; Mosquitocidal toxin protein.; length=501; id 41.0%; E()=1.5e-12; 144 aa overlap; query 328-471; subject 256-392" Similarity with UniProt:Q45868 (EMBL:X79103); *Clostridium botulinum*.; HA-33; HA-33 protein.; length=292; id 32.4%; E()=1.8e-06; 136 aa overlap; query 321-455; subject 146-280"	
pBt156	FtsZ/ tubulin-related protein Plasmid replication protein	Weakly similar to *Pyrococcus kodakaraensis* TubA protein tubA TR:Q9HHD0 (EMBL:AB031743) blast scores: E(): 4e-06, score: 54 21% id, and to *Pyrococcus horikoshii* cell division protein ftsz homolog 3 ftsz3 or ph1335 SW:FTZ3_PYRHO (O59060) blast scores: E(): 5e-05, score: 50 23% id, and to *Bacillus anthracis* pxo1-45 TR:Q9X315 (EMBL:AF065404) blast scores: E(): 3e-04, score: 48 21% id" repX showed limited homology to bacterial FtsZ proteins that are involved in cell division (Tinsley and Khan, 2006)	pXO1-45 (21 in 44 aa)

3.4 Discussion

The analysis of pBtoxis genes for new functions revealed new information on several coding sequences (CDS) that are shown in table 3.3.1. Due to the fact that database changes and updates are ongoing as new genes and functions are discovered, the analysis of the pBtoxis plasmid sequence should be carried out periodically to look for new possible functions of its genes. This analysis was an important start point for studies described in later chapters to select and investigate the function of genes from pBtoxis that may have important functions for the host bacterium.

CHAPTER 4

Antibiotic production by

Bacillus thuringiensis subsp. *israelensis*

4.1 Introduction

Many studies have shown that *Bacillus thuringiensis* strains produce antibiotics including a range of bacteriocins. The bacteriocins are ribosomally synthesized proteins, which cause no harm to the producer bacteria but have an antibacterial activity that acts against closely related bacteria. In this way they function mainly against other *B.* strains such as *B. cereus* and *B. weihenstephanensis* (Riley and Wertz., 2002; Cherif, *et al.*, 2003; Kamoun, *et al.*, 2005; Barboza-Corona, *et al.*, 2007). The bacteriocins can also affect un- related bacterial species (De la Fuente-Salcido, *et al.*, 2008; Birri *et al.*, 2010). There are four classes of bacteriocins (Cherif, *et al.*, 2008).

- Class I: Lantibiotics, are small peptides, which contain unusual amino acids such as lanthionines, and the most widely studied among class I Lantibiotics is Nisin, which is produced by *Lactococcus lactis*. Nisin is active against a wide range of pathogenic bacteria and it is used widely for food preservation.
- Class II: Non-lantibiotics, small heat-stable bacteriocins (Yi *et al.*, 2010), contain unmodified peptides; eg. cystibiotics and thiolbiotics (Cherif *et al.*, 2008). This class recently was subdivided into four subclasses designated as IIa, IIb, IIc and IId (Birri *et al.*, 2010).
- Class III: Bacteriocins in this class are large and thermolabile proteins.
- Class VI: includes complex bacteriocins that contain an undefined mixture of proteins, carbohydrates and lipids (Savadogo *et al.*, 2006).

The bacteriocins are potentially useful in food preservation, as they are capable of suppressing unwanted microbes (Kamoun, *et al.*, 2005). Many *Bacillus* species produce bacteriocins (table 4.1) including *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus megaterium* (Jack *et al.*, 1995).

Table 4.1.1 Bacteriocins produced by *Bacillus* strains

	***Bacillus* strain**	**Bacteriocin**	**Reference**	**Note**
1	*Bacillus subtilis*	Subtilin	Jansen and Hirschmann (1944)	Active against gram-positive bacteria and various pathogenic fungi. (Class I)
2	*Bacillus subtilis*	Subtilosin	Zheng and Slavik, (1999) Shelburne *et al.* (2007)	Active against a wide range of gram-positive and gram-negative bacteria as well as aerobes and anaerobes. (Class I)
3	*Bacillus cereus* ATCC4579	Blis	Risoen, *et al.* (2004)	(Class IIa)
4	*Bacillus coagulans*	Coagulin	Von Tersch and Carlton (1983); Hyronimus *et al.* (1998)	*Active against L. monocytogenes* (the food-borne pathogen). (Class IIa)
5	*Bacillus thuringiensis B439*	Thuricin CD	Rea, *et al.* (2010)	Active against gram-positive spore forming bacteria. Also against types of *Clostridium*, such as *C. difficile*, and against *Listeria monocytogenes*, a gram-positive, food-borne pathogen. (Class IId)
6	*Bacillus thuringiensis* subsp.*thuringiensis* HD2	Thuricin	Favret and yousten (1989)	(Class IId)
7	*Bacillus thuringiensis* B439	Thuricin B439	Ahern *et al.* (2003)	(Class IId)
8	*Bacillus thuringiensis* subsp. *tochigiensis* HD868	Tochicin	Paik *et al.* (1997)	Active against most of 20 typical *B. thuringiensis* strains and a strain of *B. cereus.* (Class IId)
9	*Bacillus thuringiensis* subsp. BMG1.7	Thuricin 7	Chief, *et al.* (2001)	Active against the species of the *B. cereus* group and other gram-positive bacteria. (Class IId)
10	*Bacillus thuringiensis* subsp. *entomocidus* HD9	Entomocin 9	Chief, *et al.* (2003)	*Active against Pseudomonas aeruginosa* and gram-positive bacteria including *Listeria monocytogenes.* (Class IIa)
11	*Bacillus thuringiensis* subsp. *entomocidus*	Entomocin 110	Cherif, *et al.* (2008)	Active against gram-positive bacteria such as *Listeria monocytogenes* and other *Bacillus* species. (Class IIa)

New bacteriocins that are produced by *B. thuringiensis* strains are being discovered and recently identified bacteriocins include morricin 269, kurstacin 287, kenyacin 404, entomocin 420 and tolworthcin 524 (Barboza-Corona, *et al.*, 2007; De la Fuente-Salcido, *et al.*, 2008). Barboza-Corona *et al.* (2009) have tested these new *B. thuringiensis* bacteriocins against *Staphyloccus aureus* isolates and found them to have an inhibitory action.

Some *B. thuringiensis* and *B. cereus* strains produce a linear aminopolyol antibiotic called zwittermicin A (Stabb, *et al.*, 1994). Zwittermicin A produced by *B. cereus* strain UW85 is able to suppress some diseases of plants such as alfalfa damping-off caused by *Phytophthora medicaginis* (Silo-Suh *et al.*, 1994), and inhibit bacterial and fungal growth (Kevany, *et al.*, 2009). *B. cereus* strain UW85 produces two antibiotics zwittermicin A and Kanosamine (Silo-Suh, *et al.*, 1998). Zwittermicin A has a wide range of activity against plant disease agents, and this may indicate the potential for using it in the biological control of such diseases (Silo-Suh, *et al.*, 1998). It has also been reported that Zwittermicin A enhances the insecticidal activity of the *B. thuringiensis* endotoxin proteins (Kevany, *et al.*, 2009). The structure of Zwittermicin A is shown in figure 4.1.1.

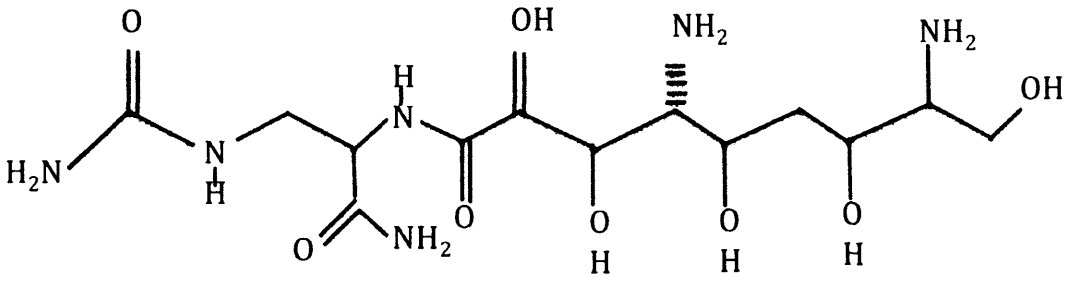


Figure 4.1.1. The structure of Zwittermicin A

Adapted from (Silo-suh *et al.*, 1998).

4.2 Plasmid pBtoxis may encode antibiotics

The pBtoxis plasmid of *B. thuringiensis* subsp. *israelensis* appears to encode proteins related to peptide antibiotics (Berry, *et al.*, 2002). The genes pBt136, pBt137 and pBt138 encode proteins that are related to the gram-positive circular bacteriocins, and maybe involved in peptide antibiotic production and export. In a subsequent study, Stein *et al.* (2006) showed these genes to be transcriptionally active. Using pBt136 and pBt137 specific primers, these genes have been shown to be co-transcribed. No product was obtained with primers spanning pBt137 and pBt138, indicating that pBt138 may not form part of the same operon with pBt136 and pBt137 (Stein *et al.*, 2006).

These genes are similar in orientation and order to those in the *Enterococcus faecalis* subsp. *liquefaciens* AS-48 operon (as-48A, as-48B, as-48C, as-48C1, as-48D and as-48D1) that are responsible for the production and secretion of the ribosomally synthesized circular peptide antibiotic AS-48 (Martinez-Bueno *et al.*, 1998). The re-analysis of the pBtoxis plasmid done in this study found that genes pBt136, pBt137 and pBt138 might be related to the production of bacteriocins (section 3.3).

The antibiotic AS-48 is a non-lantibiotic produced by *Enterococcus faecalis* AS-48; it has a unique structure and is encoded by the 68 kb plasmid pMB2, which carries all the information required for AS-48 production and immunity (Martinez-Bueno *et al.*, 1998). AS-48 has a broad antimicrobial spectrum against both gram-positive and gram-negative bacteria (Galvez *et al.*, 1989 a, b), such as *Escherichia coli, Myxococcus, Agrobacterium* and *Rhizobium* (Galvez *et al.*, 1989a; Abriouel, *et al.*, 1998). Some pathogenic bacteria are sensitive to AS-48, including *Etaphylococcus, Enterococcus* and *Salmonella* species (Galvez *et al.*, 1989c), whereas sensitive gram-negative bacteria (and especially Enterobacteriaceae family) require at least 10-times-higher concentrations of AS-48 for inhibition (Galvez *et al.*, 1989c).

AS-48 is not included in the lantibiotic group because AS-48 does not have lanthionine or other unusual amino acids (Galvez *et al.*, 1989b). AS-48 is rapidly absorbed to cytoplasmic membranes of target cells (Joosten *et al.*, 1996) and its antimicrobial effect is due to its ability to form pores by insertion of the polypeptide into the cytoplasmic membrane of target bacteria (Galvez *et al.*, 1991), leading to the

efflux of small molecules and loss of cytoplasmic material and ultimately leading to cell death (Martinez-Bueno *et al.*, 1994).

The pBt136 CDS encodes a protein similar in sequence and length to the processed propeptide of AS-48. Whereas genes pBt137 and pBt138 encode predicted integral membrane proteins similar to AS-48B and AS-48C, which have been suggested to be involved in the antibiotic maturation and secretion (Berry *et al.*, 2002). AS-48 is produced by the circularisation of a pro-peptide, from which, a 35 amino acid signal sequence was removed (Martinez-Bueno *et al.*, 1994).

Table 4.2.1 The pBtoxis plasmid genes with possible peptide antibiotic activity

Genes identified by Berry *et al* 2002; transcriptional activity determined by Stein *et al* 2006.

Name	Predicted product	Database similarity EMBL % identity	Transcriptionally active
pBt136	Possible peptide antibiotic precursor	Very weak similarity to *E. faecalis* peptide antibiotic AS-48 TR: Q47765 (x79542) (27-14 in 70 aa)	Yes
pBt137	Integrated membrane protein (possible peptide antibiotic maturation and biosynthesis protein)	Similar to *E. faecalis* BacB protein TR:052964 (D b5752) AS-48 B TR: 053024	Yes
pBt138	Integrated membrane protein (possible accessory factor in peptide antibiotic)	Similar to *E. faecalis* AS-48 C protein putative accessory factor in AS-48 secretion	Yes
pBt139	Putative bacteriocin transporter	Similar to *Clostridium beijerinckii* (*Clostridium* MP); cirD; ATP-binding protein.	Not assessed

4.3 The aim

The aim of the following study was to detect any antibiotic production by *B. thuringiensis* subsp. *israelensis* strain 4Q5 (also known as 4Q2-72) by using its conditioned medium against different *B. thuringiensis* strains and other bacterial species and assessing effects on growth.

4.4 Bacterial and fungal species tested

Several bacterial strains were used in this experiment and are shown in Table 4.4.1.

Table 4.4.1 Bacterial and fungal strains

No	Strains	Notes
1	*Bacillus thuringiensis* subsp.*israelensis* strain 4Q5 (also known as 4Q2-72)	Contains pBtoxis plasmid
2	*Bacillus thuringiensis* subsp. *israelensis* strain 4Q7 (also known as 4Q2-81)	Lacks pBtoxis plasmid
3	*Bacillus subtilis* strain IA2	
4	*Bacillus thuringiensis* subsp.*israelensis* vectobac®	
5	*Bacillus sphaericus* strain 1593	
6	*E. coli K12*	
7	*Enterobacter sp.*	Gut bacterium of our laboratory reared *Aede aegypti*
8	*Microbacterium sp. (oxydans)*	Gut bacterium of our laboratory reared *Aede aegypti*
9	*Brevundimonas diminuta*	Gut bacterium of our laboratory reared *Aede aegypti*
10	*Bacillus sp.*	Gut bacterium of our laboratory reared *Aede aegypti*
11	*Bacillus circulans*	Gut bacterium of our laboratory reared *Aede aegypti*
12	*Nitrosococcus sp.*	Gut bacterium of our laboratory reared *Aede aegypti*
13	*Pseudomonas aeruginosa PAO1*	Provided by Dr. Mahenthiralingam, Cardiff University
14	*Burkholderia multivorans ATCC 17616*	Provided by Dr. Mahenthiralingam, Cardiff University
15	*Saccharomyces cerevisiae* strain *BY4741*	Provided by Prof. Richard Dickinson Cardiff University

4.5 Bacterial culture preparation

All *B. thuringiensis* strains, *B. sphaericus* and *B. subtilis* cultures were prepared using nutrient broth medium. All other *Bacillus sp.*, *Enterobacter sp.*, *Microbacterium sp.*, *Nitrosococcus sp.*, *Brevundimonas diminuta*, *Pseudomonas aeruginosa PAO1*, *Burkholderia multivorans ATCC 17616* and *E. coli* K12 cultures were prepared in Luria Bertani (LB) medium, whereas *Saccharomyces cerevisiae* strain *BY4741* (fungal strain) culture was prepared in 1 l of a yeast growth medium (10 g yeast extract, 20 g Bacteriological peptone and 20 g glucose). The cultures were grown overnight and then 5 µl of the cultures were added to 5 ml of fresh medium to make a 1:1000 dilution, which was incubated for 2 h in an incubator at 37°C for *Pseudomonas aeruginosa PAO1*, *Burkholderia multivorans ATCC 17616*, *Enterobacter sp.*, *Microbacterium sp.*, *Nitrosococcus sp.*, *E. coli* K12 and *Brevundimonas diminuta*, cultures and 30°C for *Saccharomyces cerevisiae strain BY4741* and *Bacillus* species cultures. The cultures were then diluted 1:10, 1:100 and 1:1000 in fresh medium. The three dilutions were used to make bacterial (or yeast) lawns by spreading 200 µl on plates for use in the experiment.

4.6 Conditioned medium

Conditioned medium from *B. thuringiensis* was prepared using overnight cultures, which were produced by the vegetative stages in which no spores had developed. Separate conditioned media were also prepared from the late stage of vegetative growth and sporulation (three day cultures). The cells were pelleted by centrifugation at 13,000 rpm in a microfuge for 5 minutes and the resuling supernatant was made sterile by filtration through a 0.2 µM pore size membrane into a new tube to remove all cells. The supernatants of *B. thuringiensis* subsp. *israelensis* 4Q5,

B. thuringiensis subsp. *israelensis* 4Q7, *B. sphaericus* strain 1593 and *B. thuringiensis* subsp. *israelensis* Vectobac, were used for the experiment. In addition, the supernatant of *B. thuringiensis* subsp. *israelensis* 4Q5 was used either (i) neat, (ii) heated in a water bath at 100°C for 5 minutes and (iii) concentrated by lyophilization in a freeze dryer for 3 hours before it was re-dissolved in 1 µl of sterile water. An antibiotic mixture of 500 µg/ml each of streptomycin, rifampicilin, penicillin and gentamicin was used as a positive control for antibiotic activity against bacterial strains, whereas the anti-fungal 10 mM $CuSO_4$ was used as the positive control for *Saccharomyces cerevisiae* strain *BY4741* in the experiment.

4.7 Disc assay

Small paper discs were made from 3MM paper using a hole punch and autoclaved in a glass tube and then oven dried. These were used to absorb 10 µl of culture supernatant and were then placed on the bacterial lawns on the plates to localise the samples. Paper discs were placed apart from each other to allow a clear reading of their effects. The plates were incubated overnight at 30°C for *Bacillus* species and *Saccharomyces cerevisiae* strain *BY4741* and at 37°C for *E.coli K12*, *Pseudomonas aeruginosa PAO1, Burkholderia multivorans ATCC 17616*, *Enterobacter sp., Microbacterium sp., Nitrosococcus sp.,* and *Brevundimonas diminuta.*

4.8 Results

Discs impregnated with the positive control (antibiotic mixture or antifungal) gave zones of clearance in the bacterial and fungal lawns, whereas none of the conditioned medium discs produced zones of clearance. Thus, no evidence of antibiotic production was detected in this experiment.

For further confirmation that *Bacillus thuringiensis* subsp. *israelensis* does not appear to produce AS - 48–like antibiotics, strains *B. thuringiensis* subsp. *israelensis* 4Q5 and *B. thuringiensis* subsp. *israelensis* 4Q7 were sent to the laboratory of Professor Maqueda, Granada, Spain – an expert in AS - 48 and related antibiotics. Experiments in her laboratory (data not shown) confirmed that no antibiotic activity was produced and also showed both *B. thuringiensis* subsp. *israelensis* strains to be sensitive to AS - 48 from *Enterococcus faecalis.*

4.9 Discussion

The growth inhibition assay was carried out to test the possibility of antibiotic production by the *B. thuringiensis* subsp. *israelensis* 4Q5 suggested by the presence on pBtoxis of pBt136, pBt137 and pBt138 that may encode proteins that are similar to those necessary for the production of peptide antibiotic AS-48 in *Enterococcus faecalis* (Martinez-Bueno *et al.*, 1998). The effect of AS-48 on *Escherichia coli* and *Salmonella* is well documented (Martinez-Bueno *et al.*, 1998).

However, despite the similarity of pBt136 and pBt137 to the *Enterococcus faecalis* AS-48 operon, no evidence of antibiotic production in *B. thuringiensis* subsp. *israelensis* (or other species) was found. Different *B. thuringiensis* subsp. *israelensis* strains and other bacterial cultures were used to test the effect of the conditioned media of *B. thuringiensis* subsp. *israelensis.* The cultures of all tested species were shown to grow without any effect by *B. thuringiensis* subsp. *israelensis* conditioned medium, whereas there were clear zones around all antibiotic paper- discs in all culture plates. *B. thuringiensis* strains may produce a range of bacteriocins as described above (4.1). It has also been reported that *B. thuringiensis* subsp. *israelensis* produces crystal-associated proteins Bti34 and Bti36 with antimicrobial activity for *Streptomyces chrysomallus* and *Micrococcus luteus* (Revina *et al.*, 2005).

However, our assay revealed no antibiotic activity, but this may be because we were assessing the ability of our strains to produce soluble antibiotics similar to AS - 48, whereas Revina *et al.* (2005) studied the activity of alkaline-solubilised proteins, unrelated to AS-48 and not encoded on the pBtoxis plasmid.

The pBtoxis plasmid of *B. thuringiensis* subsp. *israelensis* (Berry, *et al.*, 2002) appears to encode proteins that beside their killing ability to a range of insects may also show antimicrobial activity, which works in different way (Yudina, *et al.*, 2003). Some Cry proteins have been reported to show less specific antimicrobial activity than their insecticidal activity (Yudina, *et al.*, 2003), and Cyt proteins may show antimicrobial activity that is higher than Cry proteins (Yudina *et al.*, 2007).

Cahan *et al.* (2008) in their recent study have shown that Cyt1Aa produced by *B. thuringiensis* subsp. *israelensis* has an antibacterial effect on gram-negative and gram-positive bacteria. Cyt1Aa has an antimicrobial activity against *Micrococcus luteus* that is higher than Cry11A and Cry4B (Yudina, *et al.*, 2003). Also, it has been reported that *B. thuringiensis* strains produce parasporal proteins with antimicrobial activity against two species of anaerobic archaea, *Methanobrevibacter arboriphilus* and *M. barkeri* (Yudina *et al.*, 2004). As discussed above, our experiment did not assess antimicrobial activities of crystal proteins that would require alkaline solublisation.

The production of antibiotics may be expected to provide a selection advantage for the host strain in the competitive environment of insect gut. However we found no evidence for the activity of AS - 48-like antibiotics in our experiments. The possibility that antibiotic may be produced under other growth conditions or be active against other bacterial species cannot be ruled out.

CHAPTER 5

Bacterial flora and *Bacillus thuringiensis* subsp. *israelensis* insecticidal activity against larvae of *Aedes aegypti* mosquitoes

5.1 The aim:

- To investigate the role of the midgut bacterial community of mosquito larvae in *Bacillus thuringiensis* subsp. *israelensis* (Bti) insecticidal activity.

5.1.1 Objectives;

The experiment has two objectives: first to identify the culturable midgut bacterial community of laboratory reared *Aedes aegypti* mosquito larvae; second to investigate the role of this flora in *B. thuringiensis* subsp. *israelensis* toxicity.

5.2 Introduction

The insecticidal activity of *B. thuringiensis* subsp. *israelensis* against *Aedes aegypti* larvae is not fully understood. When *B. thuringiensis* is eaten by insect larvae, the insecticidal proteins produced during sporulation are solubilised in the midgut due to the alkaline environment. Solubilised protoxins will be activated by midgut enzymes (proteinases) and bind to specific receptors on the midgut epithelial surface. Then the toxins are inserted into the gut cell membranes and this leads to cell lysis by pore formation and eventually death occurs (Schnepf *et al.*, 1998). To initiate its toxic action, *Bacillus thuringiensis* subsp. *israelensis* must be taken into the midgut of larvae, where a community of other midgut bacteria is already present. Such midgut bacterial species exist naturally in wild and laboratory reared mosquitoes and have been analysed by Toure *et al.* (2000).

The most common bacteria found in the mosquito midgut include *Pseudomonas aeruginosa* and *Enterobacter agglomerans* (Toure *et al.*, 2000). *Pseudomonas* species have been found in the midgut of mosquitoes found in the

Democratic Republic of the Congo (Jadin *et al.*, 1966, cited by Lindh *et al.*, 2005). Straif *et al.*, (1998) have found that apart from *Escherichia coli, Pantoea agglomerans* (*Enterobacter agglomerans*) is the most common species identified from field-caught *Aedes gambiae sensu lato* and *Aedes funestus* mosquito midgut from Kenya and Mali. They identified twenty different bacterial genera, including *Bacillus* species, by gas chromatography from 73 bacterial isolates.

Pumpuni *et al.* (1996) have found *Enterobacter agglomerans* and *Flavobacterium spp* in midguts of three laboratory reared *anopheline* species (*An. stephensi, An. gambiae,* and *An. albimanus*). *Enterobacter amnigenus, Enterobacter cloacae, Enterobacter sp, Serratia marcescens,* and *Serratia sp* have been isolated from *Anopheles albimanus* mosquitoes caught in southern Mexico (Gonzalez-Ceron *et al.*, 2003). The most commonly isolated midgut bacterial flora from wild-caught *Aedes triseriatus, Culex pipiens* and *Psorophora columbiae* mosquitoes in the USA, were *Serratia marcescens, Klebsiella ozonae, Pseudomonas aeruginosa,* and *Enterobacter agglomerans* (Demaio *et al.*, 1996). Fouda *et al.*, (2001) have isolated *Bacillus, Streptococcus, Staphylococcus* and also *Salmonella* and *Shigella* from the midgut of female *C. pipiens*.

The midgut is the normal place where the pathogen infection starts, and a diverse population of culturable bacterial species can be isolated from mosquito midgut but little is known about the functional role of the mosquito midgut bacterial community (Pumpuni *et al.*, 1996). Fouda *et al.*, (2001) have stated that the presence of *Bacillus* and *Staphylococcus* in the midgut of female *C. pipiens* is very related to, and essential for, normal and high fecundity.

Broderick *et al.* (2006) have found *E. coli* and *Enterobacter sp.* in the midgut of gypsy moth larvae and they have proposed that by eliminating the midgut bacteria of these larvae, using an antibiotic mixture consisting of 500 µg/ml each of penicillin, gentamicin, streptomycin and rifampicin, the *B. thuringiensis* subsp. *kurstaki*-induced mortality is abolished. They have restored the *B. thuringiensis* insecticidal activity by re-establishing midgut bacterial species such as *Enterobacter sp.* and also they stated that the larvae can be killed by using an *E. coli* strain alone that produces the *B. thuringiensis* toxin in recombinant form. Their results suggested that flora such as *E. coli* and *Enterobacter sp.* are required for *B. thuringiensis* subsp. *kurstaki* insecticidal activity to kill the larvae of gypsy moth. This indicated that the belief that *B. thuringiensis* causes mortality by itself should be reviewed, as they concluded that *B. thuringiensis* toxicity depends on an interaction with normal larval midgut flora.

In the following experiments, the midgut bacterial community in a laboratory reared colony of *Aedes aegypti* larvae was studied to characterize the culturable species and all of the bacterial isolates from this study were evaluated for their functional role in growth development after reintroduction into the mosquito midgut. The midgut bacteria of the larvae of *A. aegypti* were eliminated using antibiotic treatment following the methods described by Broderick *et al.* (2006) and the insecticidal activity of *B. thuringiensis* subsp. *israelensis* against mosquitoes was studied.

5.3 Isolation and characterization of bacterial species in the larval midgut

Aedes aegypti eggs were obtained from Keele University and were hatched under non-sterile conditions (tap water) and grown to third instar. Then all larvae were

washed with sterile distilled water and then with ethanol to eliminate surface bacteria, followed by further wash with sterile water. Five larvae were then homogenised in 200 µl of ¼ strength Ringer's solution and after vigorous shaking the suspensions were spread on Luria Bertani (LB) agar plates to detect bacterial species. The plates were incubated at 30°C for 24 h. Bacteria from non-sterile larvae were initially differentiated by the morphology of bacterial colonies into 12 types and every type was inoculated onto a new LB plate for colony purification.

The isolated bacteria were further identified by PCR amplification of the16S rRNA gene using two PCR primers: forward primer 63f (5`-CAG GCC TAA CAC ATG CAA GTC- 3`) and reverse primer 1387r (5`- GGG CGG WGT GTA CAA GGC-3`) (Operon) (Marchesi *et al.*, 1998) as described in section 2.6.

Amplified bands of approximately 1,300bp were seen for most colonies. The products were separated by agarose gel electrophoresis (Section 2.8) to determine the size of the DNA fragments, estimate DNA concentration and to purify the DNA. Products were submitted to Lark Technologies for direct sequencing using the 63f primer and the DNA sequences were analysed as described below.

5.3.1 16S rRNA analysis

To analyse the 16S rRNA taxonomy of the samples, the sequences of the 16S rRNA genes were compared to the non-redundant database at the NCBI homepage (http://www.ncbi.nih.gov/BLAST/Blast.cgi) and also submitted to expasy homepage (www.expasy.org) to determine the most similar database sequence.

The results of this experiment show a complex flora with up to six genera isolated from 3rd instar larvae. They are; *Pantoea sp =(Pantoea agglomerans = Enterobacter agglomerans), Microbacterium sp., Brevundimonas diminuta, Nitrosococcus sp., Bacillus circulans* and *Bacillus cereus* and the most frequently isolated species are *Enterobacter sp.* and *Microbacterium sp.* Isolated gut flora from mosquito larvae are shown in table. 5.3.1.1

Table 5.3.1.1 Isolated gut flora from mosquito larvae

Isolates No	Best match in blast search	Accession number
1	*Enterobacter sp*	EF429007.1
2	*Enterobacter sp*	EF429007.1
3	*Microbacterium sp*	DQ852355.1
4	*Brevundimonas diminuta*	DQ979376.1
5	*Microbacterium sp*	DQ852355.1
6	*Enterobacter sp*	EF429007.1
7	*Bacillus circulans*	DQ74636
8	*Microbacterium sp*	DQ852355.1
9	*Bacillus cereus*	GU391511.1
10	*Pantoea sp =(Pantoea agglomerans = Enterobacter agglomerans)*	EF429007.1 and DQ356903.1
11	*Nitrosococcus sp*	GQ451698
12	*Pantoea sp =(Pantoea agglomerans = Enterobacter agglomerans)*	EF429007.1 and DQ356903.1

5 .4 Growth in sterile/ non- sterile conditions

To investigate the effect of the gut flora in *B. thuringiensis* subsp. *israelensis* toxicity, the elimination of bacteria was undertaken. Initial attempts to hatch eggs in sterile water containing 500 μg/ml each of penicillin, gentamicin, streptomycin and rifampicin (Broderick *et al.*, 2006) were unsuccessful since the eggs failed to hatch in these conditions, although eggs in sterile water alone hatched. As a result, eggs were hatched in sterile water, and then larvae were fed sterile larval food and antibiotics were added after 2-3 days once larvae reached 1st instar and larvae were maintained in these conditions for 24 h. After 24 h, larvae were transferred to fresh sterile water with no antibiotics. In parallel, eggs were hatched in non-sterile tap water and fed non-sterile larval food.

Comparing the larvae reared on sterile diet to larvae reared on non-sterile diet showed that eggs hatched faster in non-sterile conditions than in sterile conditions if other factors were the same such as, temperature, light and water volume. Also, it was notable that larvae grow faster in non-sterile conditions than in sterile conditions (especially when midgut bacteria are eliminated by antibiotic treatment). The larval growth in sterile conditions appeared to be slow and it took a slightly longer time to develop from each instar to the next stage of development and to proceed to the adult stage compared to those reared in non-sterile conditions (table 5.4.1). This effect was further quantified as described in section 5.7. The data in table 5.4.1 show a significant reduction in size when antibiotic treatment was used in comparison to non-sterile conditions. Size reduction was also seen when larvae were reared in sterile conditions without antibiotics but to a smaller extent than less that seen with antibiotic treatment.

5.5 Re- introduction of midgut bacterial species

To investigate the role of the microflora further, bacteria isolated from larvae grown in non-sterile conditions (section 5.3; isolate no: 1, 3, 4,7,9, 11) were re-introduced to larvae reared under sterile conditions. The midgut flora was eliminated using autoclaved water and antibiotic treatment as above (sterile conditions). Antibiotic treatment was continued for 24 h before larvae were transferred to fresh sterile water. Bacteria were re-introduced to the larval midgut by adding 250µl (at D_{600} = 1) of overnight culture for each bacterium alone to investigate the capability of individual bacteria to restore the growth rate. In addition a mixture of an equal volume of an overnight culture of each of the 6 isolates (total volume 250µl) was used to investigate the capability of all bacteria together to restore the growth rate of the larvae. The 250µl of culture (individual or all isolates) were added to 10 larvae in 10 ml sterile water to allow the larvae to feed on the bacterial species.

When all six isolated bacterial species were re-introduced together, either to antibiotic treated 3rd instar larvae or to earlier instars reared in sterile water, they showed the ability to restore the higher rate of larval growth. In addition, when *Microbacterium sp.* and/or *Enterobacter sp.* were re-introduced to larvae singly, they were also able to enhance the larval growth compared to those maintained in sterile conditions but did not reach the normal growth rate as in non-sterile conditions. This was not surprising, as the larvae had been reared in sterile conditions for 9 days, so that early phases of growth had already been affected. However, *Brevundimonas diminuta, Nitrosococcus sp., B. circulans* and *B. cereus* did not increase the rate of growth, indicating that the beneficial effects of midgut bacteria are species specific.

5.6 Introduction of *Microbacterium oxydans* NRRL-B24236

Since an uncharacterized *Microbacterium* from laboratory-reared *A. aegypti* larvae (section 5.3) was able to restore larval growth, the ability of a well-characterised strain was also examined. The midgut flora was eliminated using autoclaved water and antibiotic treatment as above (sterile conditions) and then *Microbacterium oxydans* NRRL-B24236 (a well- characterized strain obtained from Agricultural Research Service Culture Collection National Centre for Agricultural utilization Research Peoria, Illinois USA) was re-introduced to the larval midgut by adding 125 μl (at D_{600} =1) of overnight culture to 5 tubes each containing 5 larvae in 5 ml sterile water. When *Microbacterium oxydans* NRRL-B24236 was re-introduced, it showed the ability to enhance the rate of larval growth compared to sterile conditions. This result confirms the above results (section 5.5) and supports the suggestion that the microbial community of the larval midgut may play a role in larval growth and development.

Table 5.4.1 development of *Aedes aegypti* larvae in sterile and non-sterile conditions

Day	Non-sterile conditions	Sterile conditions	Antibiotics after 1st instar	Re-introduce *Microbacterium oxydans* NRRL-B24236
1	Eggs	Eggs	--------	
2	Hatch	Hatch	--------	
3	1st instar	1st instar	1st instar in Antibiotic for 24h	
4	1st/2nd instar	1st/2nd instar	1st instar Transferred to sterile water	
5	Mostly 2nd instar	Mostly 1st instar	Mostly 1st instar some 2nd instar	
6	3rd instar but mostly 2nd instar	1st instar but mostly 2nd instar	1st instar more of 2nd instar	
7	Mainly 3rd instar	Mainly 2nd instar few 3rd	Mainly 2rd instar	
8	Almost all 3rd instar 90%	Mainly 2nd instar few 3rd	Mainly 2rd instar few 3rd	
9	Few 2nd and mostly 3rd instar	Mainly 2nd instar few 3rd	Mainly 2nd instar few 3rd	Mainly 2nd instar few 3rd
10	Few 2nd and mostly 3rd instar	Mainly 2nd + more 3rd	Mainly 2nd instar some 3rd	Mainly 2nd + more 3rd
11	Few 2nd and mostly 3rd instar and few 4th instar	Half 2nd instar half 3rd	Mainly 2nd instar and more of 3rd	2nd and More 3rd instar
12	Mostly 3rd instar + some 4th instar and few pupa	Few 2nd More 3rd instar	Mainly 2nd + More 3rd instar	Almost all 3rd instar 90% + few 4th instar
13	Mostly 3rd instar + some 4th instar and few pupa	More 3rd instar	2nd + a lot of 3rd instar	Almost 3rd instar 90% + some 4th instar
14	Mostly 3rd instar + some 4th instar and some pupae	Almost all 3rd instar 75% + few 4th instar	Almost all 3rd instar +2nd instar	Almost all 3rd instar 90% + some 4th instar + few pupa
15	Mostly 3rd instar + some 4th instar and few pupae and adults emerging	Almost 3rd instar 75% + 4th instar and few pupae	Almost 3rd instar + 2nd instar + few 4th instar	Mainly 3rd instar + some 4th instar + few pupae
16	Same as 15 days more adults coming out	Almost 3rd instar + 4th instar + pupae	Almost 3rd instar + some 2nd instar + some 4th instar + pupae	Mainly 3rd instar + some 4th instar + some pupae+ adults staring to emerge

As seen in the table 5.4.1 the antibiotics were added at day 3 for 24 h then larvae transferred to fresh sterile water. *Microbacterium* was re-introduced at day 9 to larvae in sterile water that were antibiotic treated at day 3.

5.7 Larval growth measurement

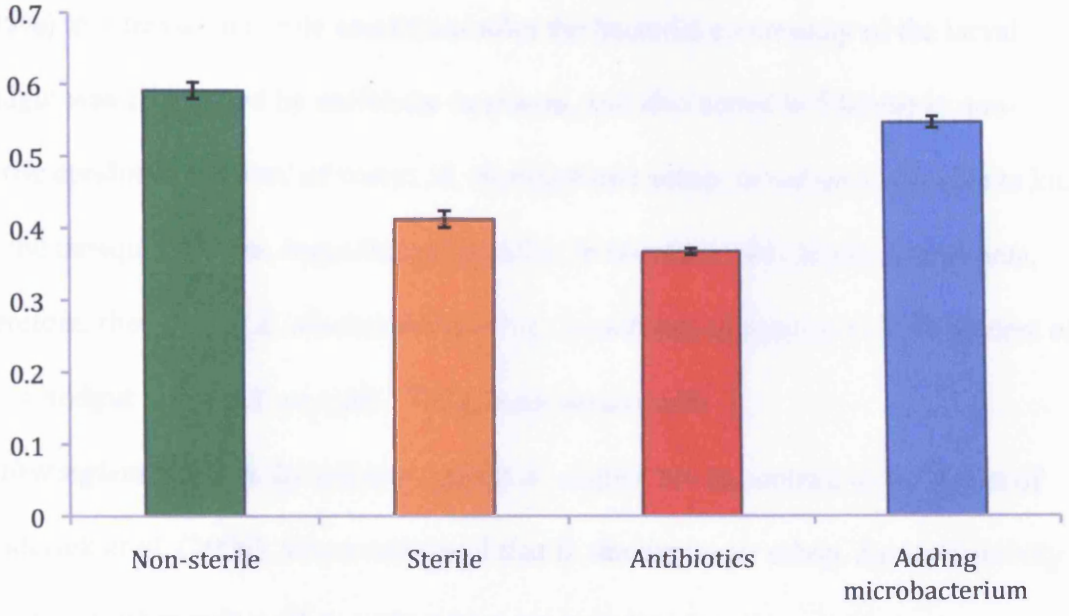
The larvae were grown under different conditions: non-sterile, sterile and antibiotic treated, as above. *Microbacterium oxydans* NRRL-B24236 was added to antibiotic treated larvae after 9 days. At day 13 larvae that appeared to be in 3rd instar were measured using a vernier (39 larvae were measured for each treatment). At this time the mean larval length in mm (± SE) were; Non-sterile 0.591 (±0.011); sterile 0.413 (±0.011); antibiotic treated 0.367 (±0.004); *M. oxidans* NRRL- B24236 – fed at day 9, 0.545 (0.007).

5.7.1 Statistical analysis of measurements of larval lengths

Using a 1-way ANOVA test showed that there was a significant difference in mean larval length between the all the different experimental conditions ($F_{3,152} = 130.53$, $P < 0.001$), also the adjusted R-squared value for this ANOVA showed that 71.5% of the variance in larval length was accounted for by the experimental treatment.

A post-hoc Tukey test revealed that all 2-way comparisons between treatment groups were significant ($p<0.05$), i.e. each treatment group was significantly different from each other treatment group.

Figure 5.7.1.1 larval growth in different conditions



The graph shows that all groups are different in length (mm) according to the growth conditions they been treated with. Larvae in non-sterile conditions grew faster than other conditions and the antibiotic treated larvae showed the lowest level of growth, but when they treated with *Microbacterium* treated larvae growth was enhanced and they grew faster than those in sterile conditions and near to the level of those grown in non-sterile conditions.

5.8 The role of midgut flora in *Bacillus thuringiensis* subsp. *israelensis* insecticidal activity

B. thuringiensis subsp. *israelensis* was introduced at an inoculation of 250µl (D_{600} = 1.8) of a 4 day culture in embrapa sporulation medium at 30°C, (sporulation >95%) to 5 larvae in sterile conditions after the bacterial community of the larval midgut was eliminated by antibiotic treatment, and also added to 5 larvae in non-sterile conditions in 5 ml of water. *B. thuringiensis* subsp. *israelensis* was able to kill all the mosquito larvae, regardless of sterility, in less than 24 h. In this experiment, therefore, the toxicity *B. thuringiensis* subsp. *israelensis* appears to be independent of other midgut flora in *A. aegypti*. Thus, these results with *B. thuringiensis* subsp. *israelensis* against *A. aegypti* are in contrast to the report of Broderick *et al.* (2006), which indicated that *B. thuringiensis* subsp. *kurstaki* activity in gypsy moth requires other midgut bacteria to induce larval mortality.

5.9 Discussion

In the characterisation of the mosquito midgut community in this study, similar bacteria to those reported by others were discovered. For instance, Straif *et al.* (1998) who found *Pantoea agglomerans (Enterobacter agglomerans)* to be the most common species identified from field- caught *Anopheles gambiae* and *An. funestus* from Kenya, and Mali, and Pumpuni *et al.*, (1996) who found *Enterobacter agglomerans* and *Flavobacterium spp* in the midgut of three laboratory reared *anopheline* species (*An. stephensi, An. gambiae,* and *An. albimanus*). *Enterobacter agglomerans* is amongst the most commonly isolated midgut bacterial flora from wild-caught *Aedes triseriatus*, *Culex pipiens* and *Psorophora columbiae* mosquitoes in USA (Demaio *et al.*, 1996).

Further, we show that growth is inhibited by elimination of the larval midgut flora and that re-introduction of *Microbacterium sp* and *Enterobacter sp* restores the normal growth rate. When *Microbacterium sp* and *Enterobacter sp* were re-introduced alone or together, they were able to enhance the rate of growth of larvae close to the normal rate. As discussed above, *Microbacterium sp.* and/or *Enterobacter sp.* are the most frequently isolated species found in the larval midgut and appear, therefore, to be important constituents of the gut flora. The results suggest that the microbial community of the larval midgut plays a role in larval growth and development. The role of bacteria in adult mosquitoes is not known (Pumpuni *et al.*, 1996) but it can be postulated that in larvae their role in facilitating growth, may be for example, by playing a role in digestion and/or induction of the larval appetite since larval growth appeared to be faster in non-sterile conditions where gut flora are present.

It is very difficult to describe the complex relationships between insects and their gut bacteria because many of the gut microbial species are not easily culturable (Broderick *et al.*, 2004). However, endosymbionts contribute to insect reproduction, digestion, nutrition, and pheromone production (Broderick *et al.*, 2004), and gut bacterial species may play very important roles in the growth and development of many insect species such as bark beetles (Brand *et al*, 1975) and in termites (Okhuma and Kudo, 1996). The nature of the symbiotic community in the termite gut is a sophisticated system in which diverse microorganisms of a wide variety of functions are coordinated and integrated to support the biological activity of termites (Ohkuma, 2001).

More than 200 bacterial species are found in termites and 95% of cellulose is converted within 24 h by the bacterial community to simple sugars and the gut bacteria also synthesise a range of wood-digesting enzymes. These enzymes enable the termite to extract nutrients and energy and the termite provides an oxygen-free environment inside its gut for the gut bacteria to survive. Gut microbiota have also been shown to contribute to the health of their insect hosts, and any alterations in the composition of the gut microbiota could lead to disease (Broderick *et al.*, 2009).

The result of insecticidal bioassays shows that *B. thuringiensis* subsp. *israelensis* is able to kill the larvae of *A. aegypti* independent of the presence of midgut bacteria in contrast to a report that *B. thuringiensis* subsp. *kurstaki* was not able to kill gypsy moth larvae in the absence of midgut bacteria (Broderick *et al.*, 2006). This insecticidal effect may be seen due to the use of high dose to cause death in less than 24 h. However, the bacterial community in *A. aegypti* larval midgut may have a role in growth but does not induce larval mortality by *B. thuringiensis* subsp. *israelensis*. Since this experiment was carried out, several other groups have conducted similar studies using different lepidopteran species to question the interaction between midgut bacteria with *B. thuringiensis* larval killing capability.

Another study by Broderick *et al.* (2009) using six species of Lepidoptera has concluded that midgut bacteria contribute to *B. thuringiensis* susceptibility in some larvae of some species. Whereas Johnston and Crickmore (2009) using *M. sexta* showed that midgut bacterial presence in the larvae does not contribute to the insecticidal activity of *B. thuringiensis.*

The findings of Broderick *et al.* (2006) and (2009), which claimed that B. *thuringiensis* requires midgut bacteria for toxicity to kill host larvae using gypsy moth, was questioned by Raymond *et al.* (2009) and van Frankenhuyzen *et al.* (2010). Raymond *et al.* (2009) argued that the study did not take into account the effect of antibiotics on *B. thuringiensis*, and they have studied this effect in their work using diamondback moth. Their results showed that *B. thuringiensis* is capable of killing the larvae in the absence of culturable midgut bacteria. Van Frankenhuyzen *et al.* (2010) argued that the Broderick study did not look at the growth of bacteria in both treated and untreated larvae to find out *B. thuringiensis* killing mode of action in both. Van Frankenhuyzen *et al.* (2010) examined the abundance of bacteria from inoculation to larval death using the same insect, gypsy moth and concluded that gut bacteria do not play a role in *B. thuringiensis* toxicity towards larvae of this insect as larval death occurred in the presence and the absence of gut bacteria (van Frankenhuyzen *et al.*, 2010).

There are two modes of action suggested for *B. thuringiensis* killing its hosts; either by starvation caused by toxemia, or septicemia (Angus, 1962). The results of many studies (Johnson and Crickmore, 2009 and Raymond *et al.*, 2009) support *B. thuringiensis* mode of action to kill its host by septicemia caused by spore outgrowth, as proposed before, and Raymond *et al.* (2009) supported this conclusion by finding that when the *B. thuringiensis* growth was inhibited using antibiotics this results in a reduction of its pathogencity. It has also been suggested that when Cry toxin proteins damage the epithelial cells of larval midgut and the gut contents mix with haemolymph liquids, this may generate favourable conditions for the *B. thuringiensis* spores to germinate. The resulting vegetative cells of

B. thuringiensis and other bacteria that are found in the gut proliferate in the haemocoel causing septicaemia, and thus they may contribute to the mortality of the insect larvae (WHO, 1999; http://www.inchem.org).

Some studies suggest that the mode of action depends on several factors such as the dose of the bacteria and the strain as well as the host species and larval stage (van Frankenhuyzen *et al.*, 2010). It also depends on the inoculation method, which affects the number of pathogens taken up by the host (eg: droplet -feeding or disk-feeding). Van Frankenhuyzen *et al.*, (2010) suggested that some midgut bacterial species might become pathogens by taking advantage of the damage to midgut cells caused by *B. thuringiensis* crystal proteins, which facilitates their multiplication in the midgut. However, these authors argued that it would not be an advantageous adaptation for *B. thuringiensis* and other bacteria to depend on their competitors for full pathogenicity (van Frankenhuyzen *et al.*, 2010).

CHAPTER 6

Germination

6.1 Introduction

Germination of spores is an integral part of the life cycle of sporulating bacterial species, which involves a series of reactions between chemical germinants in the surrounding environment and specific receptors in the spore (Moir *et al.*, 2002). Among these germinants are amino acids and sugars (Moir, 2003). This leads to loss of dormancy and resistance, which are very important properties of spores for survival in unpleasant environments, but spores, in order to germinate and re-establish their vegetative stage under the right conditions, must be able to monitor changes in the surroundings (Moir *et al.*, 2002). Spores, in order to return to active vegetative stage need to go through three phases; activation, germination and outgrowth. The activation process is not fully defined and little is known about it, and no activation associated genes have been identified (Moir and Smith, 1990). Activation of germination receptors makes spores committed to germinate and bacterial spores depend upon some molecules in the environment such as amino acids, sugars and ribosides to trigger germination at the right conditions (Christie and Lowe, 2007). The effectiveness of particular chemicals to trigger germination differ widely between species (Moir and Smith, 1990).

6.1.1 Activation

Some spores can be activated by conditions such as heat or storage at low temperature for long periods to increase their response to germinants (Moir and Smith, 1990). Activation can be achieved by exposure of spores to low and high pH, heat treatment, ionising and strong oxidising agents. Heat activation can increase the percentage of germinated spores, but the mechanism of this process is still unknown (Zhang *et al.*, 2009). Heat activation is the simplest way to activate spores of

B. thuringiensis, but by using alkaline pH 10, eg in potassium carbonate or sodium carbonate buffers to induce germination of crystalliferous strains, there is no need for heat activation (Wilson and Benoit, 1990), also NH_4Cl can be used to activate *B. cereus* spores to germinate (Preston and Douthit, 1988). Benoit *et al.*, (1995) have used 0.1 M postassium carbonate (pH 10) to activate *B. thuringiensis* subsp. *kurstaki* spores from crystal forming and non-crystal forming strains and also *B. cereus* strains. Another study has found that only the spores of crystal-forming strains can be activated by alkaline pH. Another study of crystalliferous (Cry+) and acrystalliferous (Cry-) strains of *B. thuringiensis* subsp. *israelensis*, wild-type *B. cereus* and its transcipient crystalliferous derivatives and wild type *B. subtilis* to establish the possibility of alkaline activation by 0.1 M potassium carbonate (pH 10) and in the midgut of *Aedes aegypti* larvae was carried out (Bhattacharya, 1999). The findings suggested that only crystalliferous strains could be activated by alkaline pH 10 and in the alkaline environment of the midgut. In both studies it was suggested that the protoxin in the coat of the spore may be responsible for the alkaline-activation phenotype and also may have an ecological function.

6.1.2 Germination

Germination is a rapid process associated with changes including, alteration in the permeability properties and loss of heat resistance (Moir *et al.*, 2002; Moir, 2003). The core will swell through water uptake and the activity of cortex lytic enzymes will lead to degradation of the peptidoglycan cortex layer, then metabolic processes will resume and DNA transcription and protein synthesis will be started (Hornstra *et al.*, 2006a). Spores also lose turbidity and release dipicolinic acid from their core as germination progresses (Moir and Smith, 1990).

Prior to germination, spores appear phase-bright, but when the germination process begins they change to phase-grey and finally they become phase-dark (Moir and Smith, 1990) and the size of the spore increases to as much as twice that of a dormant spore due to water uptake. The process of germination can be monitored in several ways such as loss of heat resistance, change of attenuance and phase-dark changes (Moir and Smith, 1990). Outgrowth is a phase, in which synthetic processes occur to prepare the cell for the release from the spore wall and for first cell division and, thus, vegetative growth resumes (Santo and Doi, 1974; Bulla *et al.*, 1980). It is characterised by sensitivity to inhibitors of macromolecular synthesis and it completely relies on the success of germination (Moir and Smith, 1990).

Spore populations are a mix of individual spores that may have different properties to germinate, as some may need a higher germination signal to germinate than other spores in the same population (Ghosh and Setlow, 2009). Dormant spores have several protective layers each layer may contribute to the spore resistance in the unpleasant environments (Setlow, 2006; Ball *et al.*, 2008; Abee *et al.*, 2010). Also dormant spores have sensors to initiate the process of germination, and these sensors are called germinant receptors and are found in the inner membrane of the spore (Hudson *et al.*, 2001). The first step is that the germinant must permeate the outer coat and the layers of the cortex to be able to interact with the receptor in the inner membrane (Moir, 2006). The interaction between germination receptors and germinants has not been fully described (Hornstra *et al.*, 2006a). The process of germination appears to involve membrane changes and activation of some enzymes, and the membrane receptors may have a role in the ion movement during germination (Moir, 2003; Setlow, 2003).

Nutrient germinants such as L-alanine (a simple amino-acid used to trigger *Bacillus* species to germinate), complex nutrient germinants (such as sugars and amino acids) and non-nutrient germinants such as calcium dipicolinate, can be used to trigger germination and growth (Paidhungat and Setlow, 1999). Germination can also be triggered by physical factors such as hydrostatic pressure (Wuytack *et al.*, 2000), and other germinants include salts, lysozyme and Ca^{2+}-DPA (Setlow, 2003). Spores can germinate in the presence of single germinants such as inosine, or more complex germinants such as a mixture of asparagine, glucose, fructose and potassium ions (AGFK). In *Bacillus* species, germinant receptors are encoded by tricistronic operons, which have been found to be involved in germination response initiated by L-alanine and inosine (Hornstra *et al.*, 2005). However, tetracistronic operons have been reported in some *Bacillus* species such as *Bacillus megaterium* (Christie *et al.*, 2008).

6.1.3 Germination receptors

Many germinant receptors have been identified in the inner membrane of *Bacillus* group members, the *gerA* family in *B. subtilis* (*gerA, gerB* and *gerK* operons) and in *B. cereus* (*gerI, gerQ* and *gerL* operons) that has shown to be functional (Barlass *et al.*, 2002; Moir *et al.*, 2002: Setlow, 2003; Abee *et al.*, 2010). The germinant specificity of the *gerL* operon of *B. cereus* is very close to the *gerA* operon in *B. subtilis,* however, they show considerable primary sequence divergence, e.g. only 23% of amino acids are identical between GerLB and GerAB (Barlass *et al.*, 2002). The germinant receptors recognise specific molecules. Whereas some receptors recognise more than one germinant (GerB and GerK for the recognition of AGFK in *B. subtilis*), others recognise only one single germinant (GerA for the recognition of alanine in *B. subtilis*). On the other hand some germinants are

recognised by more than one receptor such as *gerI* and *gerQ* for inosine in *B. cereus* 569 (Moir and Smith, 1990; Moir *et al.*, 2002). Spores of *B. megaterium* QM B1551 germinate very quickly in response to many germinants, including glucose, leucine, proline, and a variety of inorganic salts (Christie and Lowe, 2007). Most germinant receptors act in concert to trigger germination, and the interaction between two or more receptors can occur in response to single germinants, such as the inosine germination response in *B. cereus* 569, which requires the products of the *gerI* and *gerQ* operons (Barlass *et al.*, 2002).

Many of the proteins are germinant-specific such as those encoded by the *gerA*, *gerB* and ger*K* operons in *B. subtilis,* whilst others are involved in spore morphogenesis such as GerE, which is a regulator of spore-coat gene expression (Robinson *et al.*, 1998). For *B. cereus,* responses to single amino acids are mediated by the GerR receptor and a strong germination can be induced by using inosine as the sole germinant in *B. cereus* ATCC14579. Concentration of germinant does not affect inosine -induced germination while the amino acid-induced germination is strongly affected (Hornstra *et al.*, 2005) and the germination response of *B. cereus* ATCC 14579 to inosine differs from the L-alanine response (Hornstra *et al.*, 2005).

In *B. subtilis*, the germination process in response to AGFK differs from that of L-alanine, as the *ger*B, *gerK* and *fruB* products are important for the response to AGFK but not required in L-alanine germination pathway. Furthermore, there are two other loci (*gerD* and *gerF*) identified with a minor role in the L-alanine route but which are essential for AGFK pathway (Moir and Smith, 1990). The *ger*D gene appears to be expressed under the control of fore-spore sigma factor sigma^{G} (Kemp

et al., 1991). Mutants of *ger*D were still able to germinate by non-nutrient germinants such as CaDPA, which does not involve the membrane receptors (Pelczar *et al.*, 2007). GerD has been found to be located in the inner membrane of *B. subtilis* (Mongkolthanaruk *et al.*, 2009). Mutants of *gerB, gerK* and *fruB* do not germinate in AGFK but can germinate in L-alanine. The difference in germination response from one germinant to another suggests that spores have two systems for sensing the alternative germinants (Moir and Smith, 1990). Receptors of the same germination pathway cannot complement the function of a defective receptor and this indicates that the receptors act in concert with the gene products of other *ger* operons (Hornstra *et al.*, 2005). An alteration of this complex by disrupting one or more of its proteins will reduce or inhibit the spore germination in response to nutrients. The receptor complex can be disturbed by disruption of the *gerR* locus and *gerI* and that will affect both L-alanine and inosine-induced germination (Hornstra *et al.*, 2005).

Some germination receptors are proven to have germinant specificities, but the precise activation conditions are not known for most of these receptors (Hornstra *et al.*, 2006a). Receptors of the same group may differ in the specificity of the binding site in the inner membrane of the spores, for example, in *B. subtilis,* alteration of a few amino acids within the germination proteins GerBA and GerBB change the nutrient specificity from L-alanine to D-alanine (Paidhungat and Setlow, 1999).

D-alanine is a competitive inhibitor of L-alanine, which inhibits germination mediated by *gerA*, but does not affect *gerB/K* - dependent germination (Moir *et al.*, 2002). Western blot analysis was used to locate *ger* receptors in *B. subtilis* and suggested that GerAA, GerAB, GerAC and GerBA proteins are located in the inner

membrane of the dormant spore as well as the GerB receptor (Moir *et al.*, 1994; Hudson *et al.*, 2001; Paidhungat and Setlow, 2001). The *gerA* family members (*gerA, gerB* and *gerK*) in *B. cereus* are each tricistronic operons sharing significant homology, but these open reading sequences can vary between species (Paidhungat and Setlow, 2000). Each receptor contains three proteins and mutation of any cistron within the operon will result in inactivation of the respective receptor (Paidhungat and Setlow, 2000; Moir *et al.*, 2002). The process of signal transduction from the spore membrane receptor to other components is still unclear (Mongkolthanaruk *et al.*, 2009).

6.1.4 Germination genes

Smith, Moir and co-workers in the early 1980s started genetic studies of germination and identified the germinant receptors (Gould, 2006). The first described operon was *gerA*, which is required in *Bacillus subtilis* for alanine-dependent germination (Moir *et al.*, 1994; 2002). The *gerA* family in *B. subtilis* includes *gerA, gerB*, and *gerK* encoding spore receptors, which are activated by germinant binding (Paidhungat and Setlow, 2001) and when the members of *gerA* family are deleted from the spore, the nutrient induced germination is severely reduced (Paidhungat and Setlow, 2000). Mutation in the *ger* genes of *B. subtilis* have defined a number of gene products required for the germination of spores in response to chemical germinants (Moir and Smith, 1990).

In *B. subtilis,* the spore receptor proteins, which recognise nutrient germinants are encoded by three loci (*gerA, gerB* and *gerK*) and the deletion of these three operons abolishes the ability of spores to respond to nutrient germinants, indicating

that these three operons contain the genes responsible for germinant sensing (Paidhungat and Setlow, 2000). Spores with mutations in any one of these loci fail to germinate in response to specific germinants (Moir and Smith, 1990). In *B. cereus*, the *gerA* family members have been identified (Clements and Moir, 1998) and the *gerA, gerB* and *gerK* are each tricistronic operons sharing significant homology (Corfe *et al.*, 1994).

Clements and Moir (1998) have identified another *gerA* operon family member (*gerI* operon), which is required for the response to inosine as sole germinant and contributes to alanine-induced germination in *B. cereus*. Thackray *et al.*, (2001) have shown that *gerN* and a Na^+/K^+-H^+ ion antiporter are required for germinations by inosine. Behravan *et al.*, (2000) have stated that the permeability of spore coats to germinants is affected by *gerP*. In *B. cereus*, at least two separate receptors (GerI and GerL) mediate the germination using L-alanine as sole germinant and GerL is the main contributor, and germination in inosine requires two receptors of *gerI* and *gerQ* operons (Barlass *et al.*, 2002). Multiple receptors can be involved in germination by combination of inosine and L-alanine in *B. cereus* (Barlass *et al.*, 2002), but the GerA receptor in *B. subtilis* is able to act independently to trigger L-alanine induced germination (Christie and Lowe, 2007). *B. subtilis* requires GerB and GerK to germinate in response to a mixture of asparagine, glucose, fructose and potassium (AGFK) (Corfe *et al.*, 1994).

B. anthracis, B. thuringiensis and *B. cereus* are closely related and could be considered as one species and all of them germinate in inosine and alanine but their germination responses are related but not identical (Barlass *et al.*, 2002). The

B. cereus ATCC14579 strain contains seven putative *ger* operons in its genome, whereas the genome of *B. subtilis* has five *ger* operons (Hornstra *et al.*, 2006b), and the *ger* operons are transcribed in the forespore by sigma G-dependent RNA polymerase (Paidhungat and Setlow, 2001). Three germinant receptors have been described in *B. anthracis* and the *B. anthracis* Sterne 7702 strain harbours the *gerX* operon on plasmid pXO1. The disruption of this operon results in diminished germination within phagocytic cells (Guidi-Rontani *et al.*, 1999).

B. megaterium QM B1551 spore germination response to a single germinant (such as glucose, proline, or leucine) is mediated by a plasmid-borne GerA-type receptor operon (Christie and Lowe, 2007). In *B. megaterium* QM B1551, about 11% of its genome is carried on seven discrete plasmids, ranging in size from 5.4 kb to 165 kb (Kieselburg *et al.*, 1984). When this strain was cured of the 165 kb plasmid, it failed to germinate in response to any single germinants, suggesting that this plasmid contains a gene or genes encoding essential components of the germination apparatus (Stevenson *et al.*, 1993 cited in Christie and Lowe, 2007). The germination apparatus must already be present as an integral part of the mature spore and is most likely synthesised and assembled during sporulation (Moir and Smith, 1990).

The full sequence of *B. thuringiensis* subsp. *israelensis* pBtoxis 128 kb plasmid was elucidated and 125 coding sequences were found on it (Berry *et al.*, 2002). Stein *et al.* (2006) have detected transcripts for the *cry11Aa* (pBt023) and *cyt1Aa* (pBt021) genes, which is consistent with the fact that *B. thuringiensis* subsp. *israelensis* produces corresponding proteins during sporulation. A number of pBtoxis plasmid genes with possible effect on the host phenotype were identified (Berry *et al.*, 2002),

and some of these genes such as pBt145 and pBt031 are transcribed and may be associated with sporulation.

Other genes that may also be involved in germination are transcribed such as pBt084, which encodes similar protein to the germination protein A3 precursor GerAC of *B. subtilis*. The pBt085 CDS encodes a similar protein to germination protein GerIB of *B. cereus* and pBt086 encodes a protein similar to germination protein GerIA of *B. cereus* and these three genes form one operon similar to many operons encoding germination-complex genes.

This suggests that the germination of *B. thuringiensis* may be affected by plasmid-borne *ger* genes (Berry *et al.*, 2002). Another pBtoxis plasmid gene pBt156 was transcribed with a possible role in cell division, and this gene encodes protein similar to cell division protein FtsZ (Stein *et al.*, 2006). Delta-endotoxins can be embedded in the wall of the spore, and has been found that spores of *B. thuringiensis* subsp. *kurstaki* HD-73 with the Cry1Ac embedded in the spore coat can be activated by alkaline conditions (Du and Nickerson, 1996), and this may give an explanation of the fast germination of *B. thuringiensis*, and provide it with a good chance for dominating the insect gut (Jensen *et al.*, 2003).

6.2 Materials and Methods

6.2.1 Bacterial strains

Table 6.2.1.1 *Bacillus* strains used in this study

Strains	Notes	Source
B. thuringiensis subsp. *israelensis* strain 4Q5 (also known as 4Q2-72)	Cured of all plasmids except pBtoxis plasmid	Collection of Entomopathogenic Bacilli, Institute Pasteur, Paris, France
B. thuringiensis subsp. *israelensis* strain 4Q7 (also known as 4Q2-81)	Cured of all plasmids including pBtoxis: Crystal minus strain	Collection of Entomopathogenic Bacilli, Institute Pasteur, Paris, France
B. thuringiensis subsp. *israelensis* strain IPS 78/11	Cured of all plasmids including pBtoxis: Crystal minus strain	Prof: David Ellar Cambridge University
B. thuringiensis subsp. *israelensis* Vectobac ®	Contains all plasmids of wild-type *B. thuringiensis* subsp. *israelensis* including pBtoxis	Valent Bioscience Corp, USA
B. thuringiensis subsp. *israelensis* 4Q7 *ger*	Strain 4Q7 transformed with pHT304-*ger* (6.3.3)	Dr K. Gammon Cardiff University

Strain stock are maintained as spore preparations on paper strips and used to make fresh cultures as required.

6.2.2 Growth media and conditions

All *Bacillus* strains were cultured at 30°C in Embrapa medium to enhance germination, for 1 l volume: 8 g Nutrient broth, 1 g yeast extract, 1 g sodium hydrogen phosphate and 10 ml of mineral salts (10 g/l $CaCO_3$: $7H_2O$, 10 g/l $MgSO_4$: $7H_2O$, 1 g/l $FeSO_4$: $7H_2O$, 1 g/l $MnSO_4$: $7H_2O$, $ZnSO_4$: $7H_2O$) and adjusted to pH 7, prior to autoclaving (20 minutes at 120 °C).

6.2.3 Spore preparations

Cultures grown in Embrapa medium were checked by phase-contrast microscopy for sporulation. Spores were harvested between 4 to 7 days after inoculation, and in every experiment, fresh spores were used that had been prepared on the same day.

Sporulated cultures in the Empraba medium (1 ml) were centrifuged in a microfuge at 1300 rpm before the supernatant was removed as completely as possible. Then the spores were washed six times in ¼ strength Ringer's solution (Oxoid®) by repeated centrifugation. The upper layer was discarded on each wash and finally the spores were resuspended in 50 µl of the same solution.

6.2.4 Spore activation

6.2.4.1 Heat activation

After spore preparation as described above, they were transferred into PCR tubes and placed in a water bath at 70°C for 20 minutes to heat-activate the spores. This acts

to synchronise germination within the spore population (Harwood and Archibald, 1990).

6.2.4.2 Alkaline activation

The spores were harvested and washed twice by centrifugation with sterile water to minimise the effect of the medium on spore activation and germination buffers. Then the pellet of spores was suspended (at approximate attenuance of 0.5 at 600 nm in most experiments) in 0.1 M sodium carbonate (pH 10) for 30 minutes with gentle rocking using a shaking platform. The spores were then centrifuged and washed three times in ¼ strength Ringer's solution before adding the germinants (see below).

6.2.5 Germinants

Three germinants were used in this study to trigger germination of spores after the activation stage was completed. There were the single germinants, inosine (5 mM), L-alanine (10 mM) and the complex germinant nutrient broth (Sigma). With L-alanine, 13 mM D-cycloserine (Sigma) was added to prevent conversion of L-alanine to D-alanine by the activity of alanine racemase on the coat of the spores, which acts to inhibit germination (Clements and Moir, 1998).

6.2.6 Sample preparation

Germinant solutions (5 µl) were added to an equal volume of the spore sample in PCR tubes and pipetted to mix them thoroughly. Then 5 µl of the mixture was pipetted onto a microscope slide, which had been incubated at 35°C over a water bath, and cover slip was added to allow examination of the sample.

6.2.7 Germination measurement

Germination was measured using a Leitz Diaplan phase-contrast microscope at 10,000 times magnification. Prior to germination, all spores appear phase-bright, but when the germination process begins they rapidly become phase-dark. On this basis, germination can be measured by counting the proportion of phase-dark spores out of a sample of 300 spores in the field of view. Counting can be repeated at different time intervals. The experiment was repeated at least three times with each strain, for each germinant.

6.2.8 Data plotting

Graphs were plotted using excel software, using the mean of repeated experiments. Error bars are shown as standard error of the means after calculation of standard deviation.

6.3 Results

Different *B. thuringiensis* strains can be activated by heat and alkaline treatments and germinate in response to L-alanine, inosine and nutrient broth.

6.3.1 Heat activation

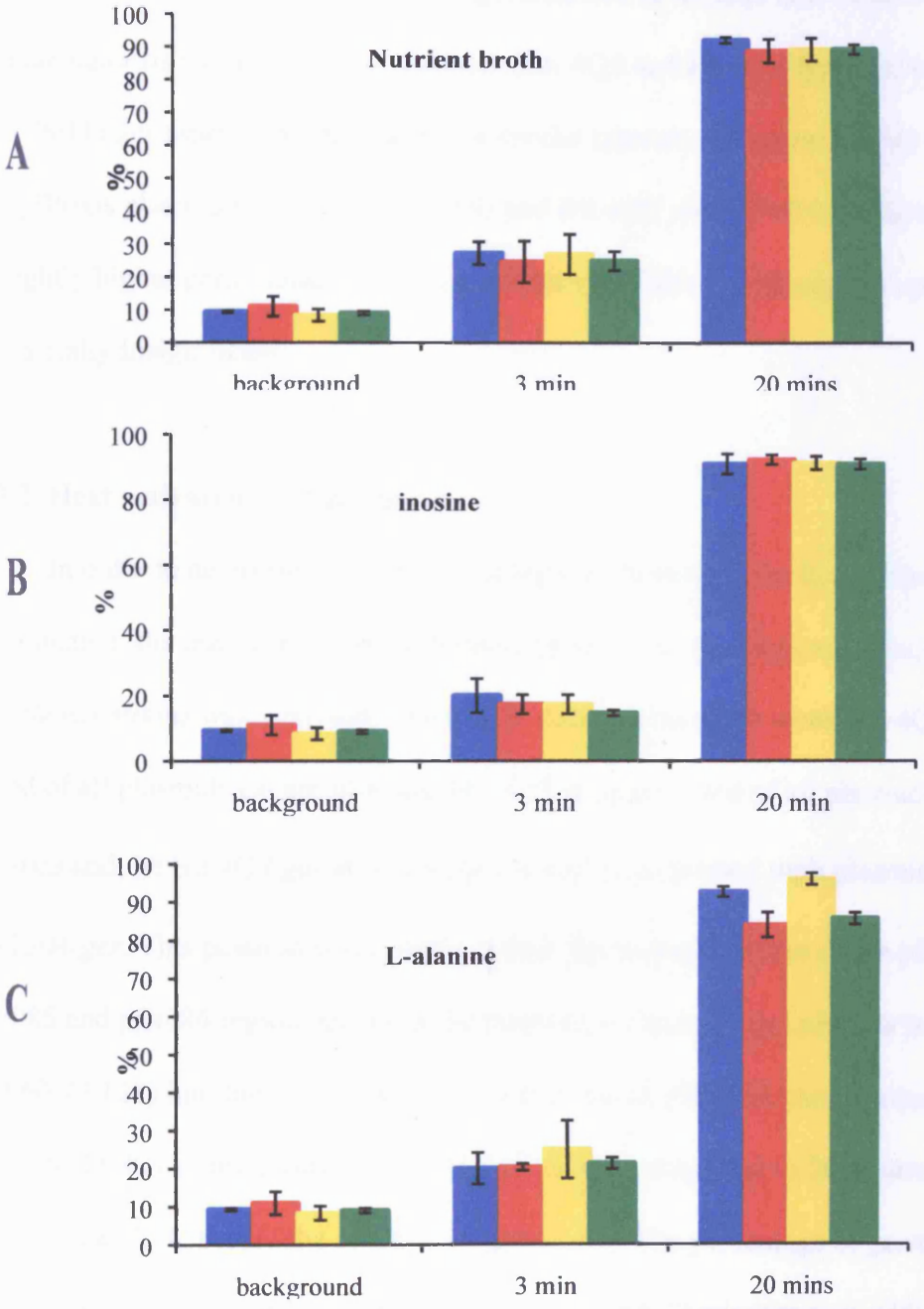
The response of heat-activated spores of *B. thuringiensis* subsp. *israelensis* strains was analysed. The strains used in this study were, a wild-type *B. thuringiensis* subsp. *israelensis* strain from the commercial formulation Vectobac (Valent Bioscience Corp); *B. thuringiensis* subsp. *israelensis* 4Q5, a strain cured of all plasmids except pBtoxis; *B. thuringiensis* subsp. *israelensis* 4Q7, a strain cured of all

plasmids including pBoxis and, therefore, an acrystalliferous strain; and *B. thuringiensis* subsp. *israelensis* IPS 78/11, a further plasmid-cured strain produced independently.

Spores were subjected to 20 minutes activation at 70°C before the addition of germinant. The percentage of germination recorded prior to exposure to germinant (background), and at 3 and 20 minutes post-addition. The experiments were repeated 3 times and the results are presented graphically in the following figures. The error bars are shown as standard error of the means after calculation of standard deviation.

Figure: 6.3.1.1 The germination responses of *B. thuringiensis* subsp. *israelensis* (Bti) strains after heat activation. Panel A, nutrient broth, Panel B, 5 mM inosine, or Panel C, 10 mM L-alanine (containing 13 mM D-cycloserine to prevent conversion of L-alanine to D-alanine by the activity of alanine racemase on the coat of the spores, which acts to inhibit germination).

Bti Vectobac ; Bti 4Q5 ; Bti 4Q7 ; Bti IPS 78/11 .



The results in figure 6.3.1.1.A and B show that the rate of germination of all the *B. thuringiensis* subsp. *israelensis* strains (Bti 4Q5 and Bti wild type, Bti 4Q7 and Bti IPS 78/11) was high and very similar in response to both nutrient broth and the single germinant inosine. The germination of all *B. thuringiensis* subsp. *israelensis* strains (regardless of the presence of the pBtoxis plasmid) was comparable throughout the experiment from the moment of adding the germinant, at 3 minutes and at 20 minutes. Figure 6.3.1.1.C shows that the rate of germination in respone to L-alanine, where all *B. thuringiensis* subsp. *israelensis* strains (Bti 4Q5 and Bti wild type, Bti 4Q7 and Bti IPS 78/11) germinated strongly and in a similar manner. However, strains containing the pBtoxis plasmid (Bti 4Q5 (P= 0.065) and Bti wild type (P= 0.080) showed a slightly higher germination rate at 20 minutes with this germinant that appears to be statistically insignificant.

6.3.2 Heat activation and *ger* genes

In order to determine whether the *ger* genes have a role in heat activation of germination, the response of heat-activated spores of *B. thuringiensis* subsp. *israelensis* strains was analysed. The strains used in this study were, Bti 4Q5, a strain cured of all plasmids except pBtoxis; Bti 4Q7, a strain cured of all plasmids including pBtoxis and the Bti 4Q7 *ger* strain, which is 4Q7 transformed with plasmid pHT304-*ger*. This plasmid was constructed by the insertion of the entire pBt084, pBt085 and pBt086 region encoding the putative *ger* genes from pBtoxis (nucleotides 69,060-73,121) into the *E. coli-Bacillus* shuttle vector pHT304 (Arantes and Lereclus, 1991) by Dr K Gammon (unpublished). Spores were subjected to 20 minutes activation at 70°C before the addition of germinant. The percentage of germination prior to exposure to germinant (background), at 3 and 20 minutes post-addition

was recorded. The experiments were repeated 3 times and the results are presented graphically in the following figures. The error bars are shown as standard error of the means after calculation of standard deviation.

Figure: 6.3.2.1 The germination responses of *B. thuringiensis* subsp. *israelensis* strains after heat activation with 10 mM L-alanine (containing 13 mM D-cycloserine). Bti 4Q5 ; Bti 4Q7 ; Bti 4Q7 *ger* .

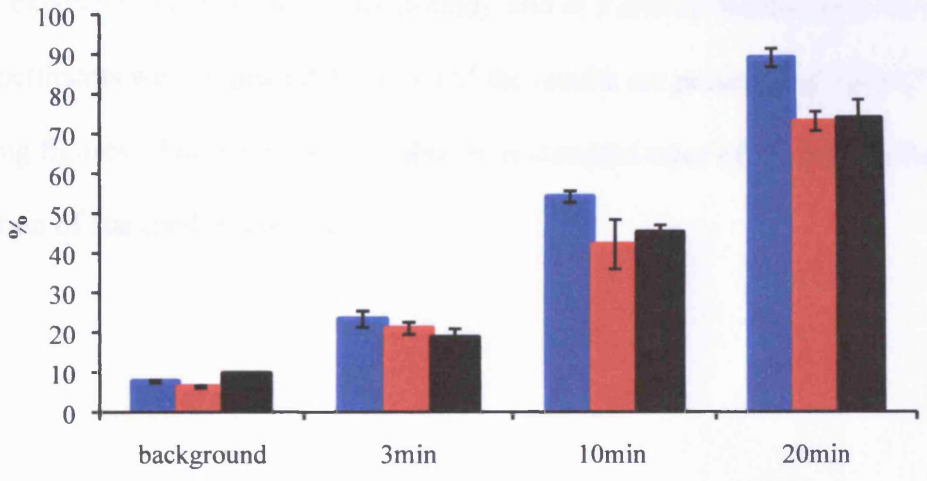


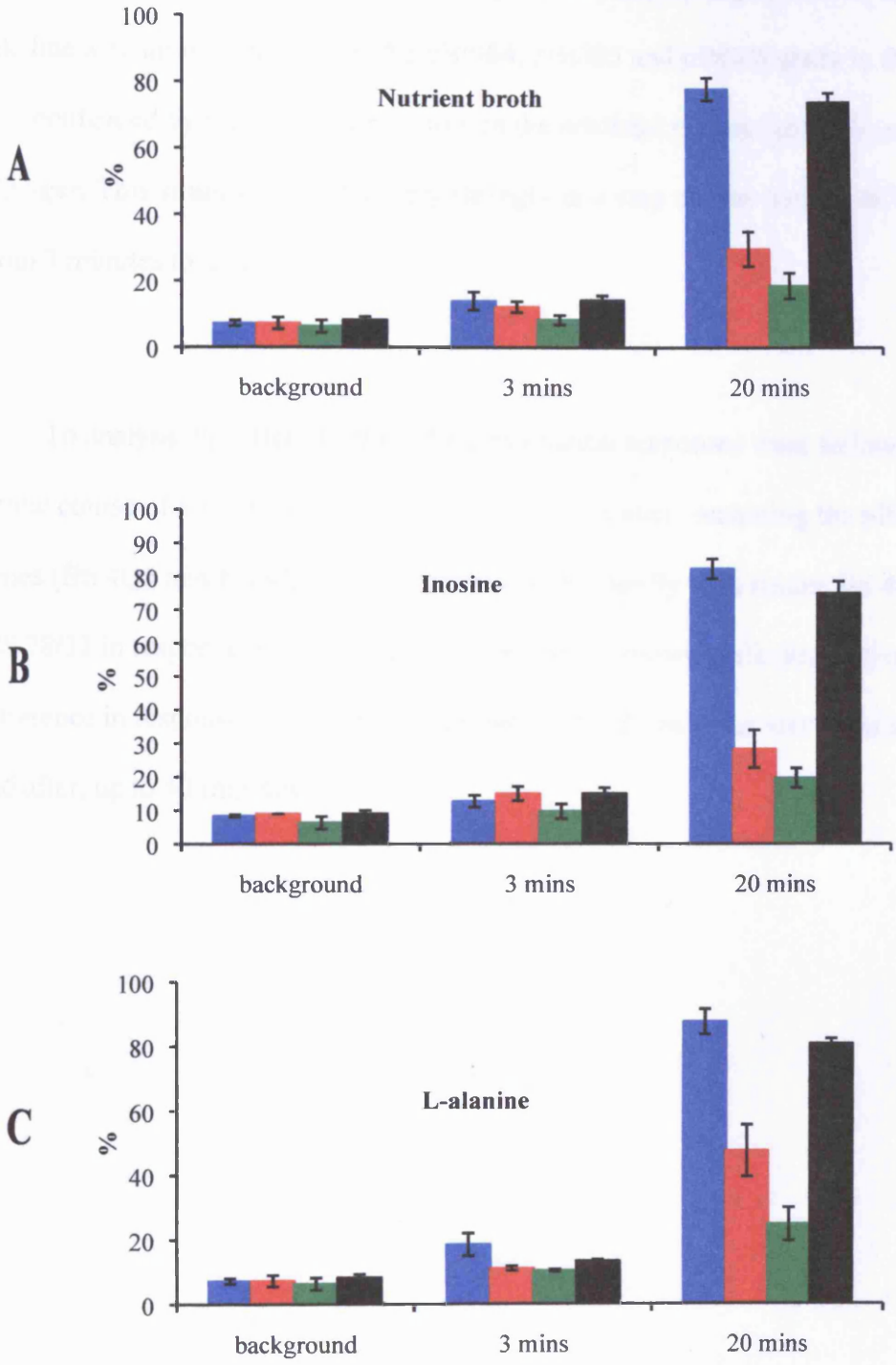
Figure: 6.3.2.1 shows that the germination of all *B. thuringiensis* subsp. *israelensis* strains (regardless of the presence of the pBtoxis plasmid) was comparable after heat activation. Bti 4Q5 showed a slightly higher germination rate, but strains Bti 4Q7 and Bti 4Q7*ger* were indistinguishable, indicating that *ger* genes play no role in the heat activation response.

6.3.3 Alkaline activation

The response of alkaline-activated spores of *B. thuringiensis* subsp. *israelensis* strains was analysed. The strains used in this study were, Bti 4Q5; Bti 4Q7; Bti IPS 78/11 as before and the Bti 4Q7 *ger* strain, which is 4Q7 transformed with plasmid pHT304-*ger*.

Spores were incubated in 1 ml of 0.1 M sodium carbonate (pH 10) for 30 minutes with gentle rocking before being washed three times in ¼ strength Ringer's solution and the addition of germinant. The percentage of germination was recorded prior to exposure to germinant (background), and at 3 and 20 minutes post-addition. The experiments were repeated 3 times and the results are presented graphically in the following figures. The error bars are shown as standard error of the means after calculation of standard deviation.

Figure: 6.3.3.1 The germination responses of *B. thuringiensis* subsp. *israelensis* strains after alkaline activation. Panel A, nutrient broth, Panel B, 5 mM inosine, or Panel C, 10 mM L-alanine (containing 13 mM D-cycloserine). Bti 4Q5 ; Bti 4Q7 ; Bti IPS 78/11 ;Bti 4Q7*ger*

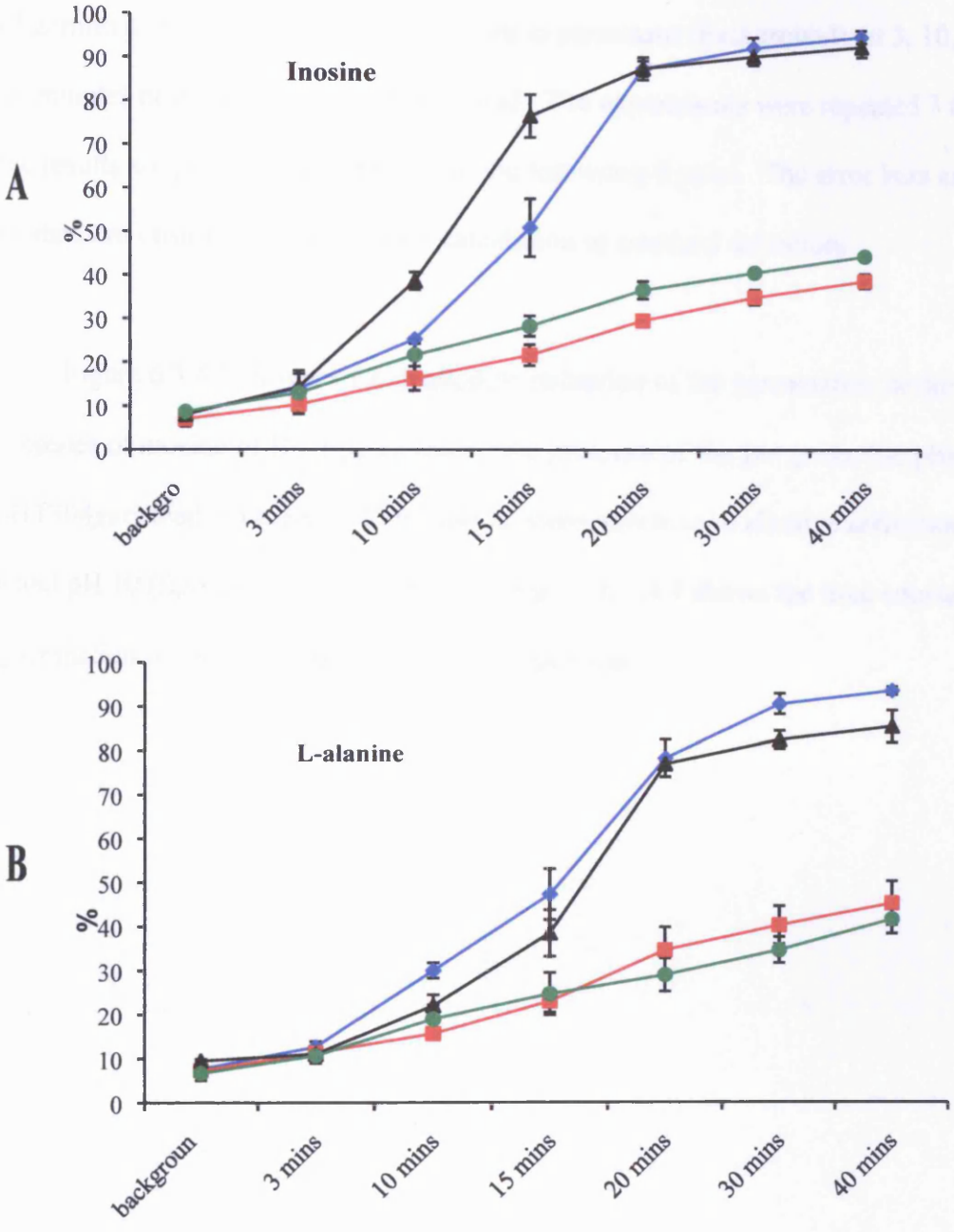


Figures 5.3.3.1 shows that following alkaline activation the Bti 4Q5 (strain containing the pBtoxis plasmid) germinated at high rate equivalent to that seen after heat activation in response to all germinants. However Bti 4Q7 and Bti IPS 78/11, both lacking pBtoxis, have a much lower germination rate in response to all three germinants. This indicates a role for the pBtoxis plasmid in germination following alkaline activation. The role of the pBt084, pBt085 and pBt086 genes in this effect was confirmed by replacing this region, in the artificial plasmid pHT304 *ger* in strain 4Q7-*ger*. This strain germinated very strongly in a very similar manner to Bti 4Q5 from 3 minutes to 20 minutes.

To analyse this effect further, the germination responses were followed over a time course of 40 min (Figures 6.3.3.2). Again, strains containing the pBtoxis *ger* genes (Bti 4Q5 and Bti 4Q7-ger) germinate more rapidly than strains Bti 4Q7 and Bti IPS 78/11 in response to both inosine and alanine, following alkaline activation. The difference in response was already noticeable at 5 min and was very clear at 10 min and after, up to 40 minutes.

Figures: 6.3.3.2 (A and B) Time-course of germination responses of *B. thuringiensis* subsp. *israelensis* strains after alkaline activation. Germinants used were Panel A, 5 mM inosine and Panel B, 10 mM L-alanine (containing 13 mM D-cycloserine).

Bti 4Q5 ; Bti 4Q7 ; Bti IPS 78/11 ; Bti 4Q7*ger* .



6.3.4 Alkaline activation and different pH

The response of alkaline-activated spores was seen at pH 10 and, therefore, in this experiment the pH threshold for the activation effect of pH on spore germination was investigated. The previous experiments were repeated with Bti 4Q5, Bti 4Q7 and Bti 4Q7ger but using a range of different pH of Tris HCl buffer: pH 8, pH 8.5 and pH 9: Sodium carbonate buffer for pH 10, to attempt spore activation. The percentages of germination recorded prior to exposure to germinant (background), at 3, 10, 15 and 20 minutes post-addition were determined. The experiments were repeated 3 times and the results are presented graphically in the following figures. The error bars are shown as standard error of the means after calculation of standard deviation.

Figure 6.3.4.1 shows a weak alkaline induction of the germination in the presence of inosine of Bti 4Q7 however, the presence of the *ger* genes (on pBtoxis or pHT304*ger*) leads to a marked increase in responsiveness to alkaline activation at pH 9 and pH 10 (figures 6.3.4.2 and 6.3.4.3). Figure 6.3.4.4 shows the time course of germination for all 3 strains at pH 9 for comparison.

Figure: 6.3.4.1 The germination responses of Bti 4Q7 strain after alkaline activation at a range of pH with inosine germinant.

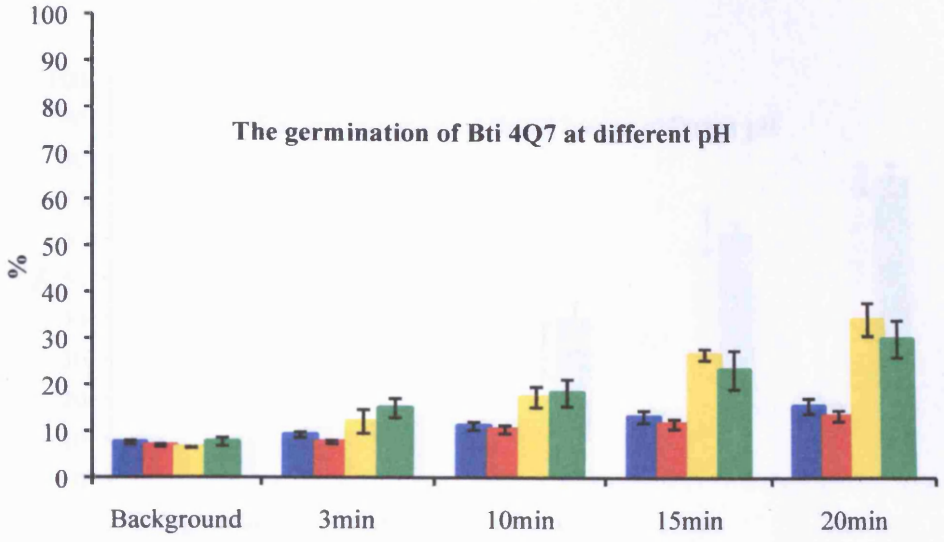
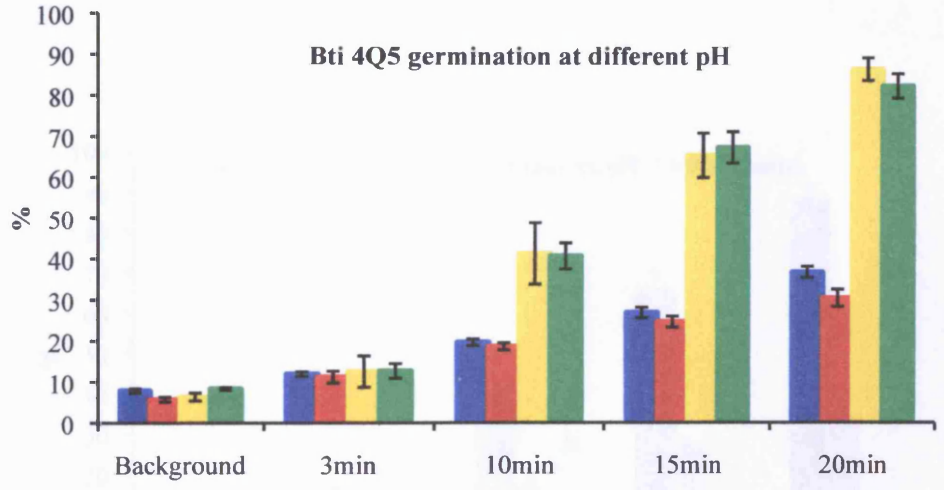


Figure: 6.3.4.2 The germination responses of Bti 4Q5 strain after alkaline activation at a range of pH with inosine germinant.



Figures legends: pH 8 ; pH 8.5 ; pH 9 ; pH 10 .

Figure: 6.3.4.3 The germination responses of Bti 4Q7*ger* strain after alkaline activation at a range of pH with inosine germinant.

pH 8 ; pH 8.5 ; pH 9 ; pH 10 .

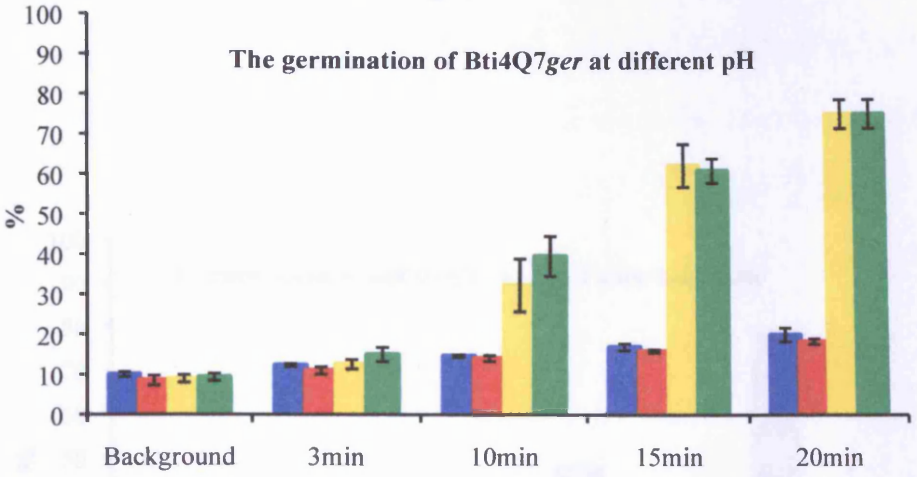


Figure: 6.3.4.4 The germination responses of *B. thuringiensis* subsp. *israelensis* strains after alkaline activation at pH 9 with inosine germinant.

Bti 4Q5 ; Bti 4Q7 ; Bti 4Q7 *ger* .

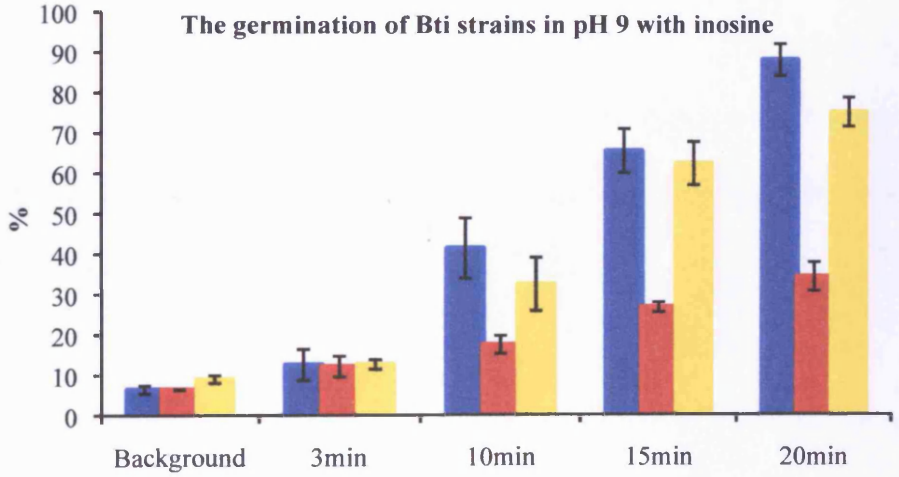
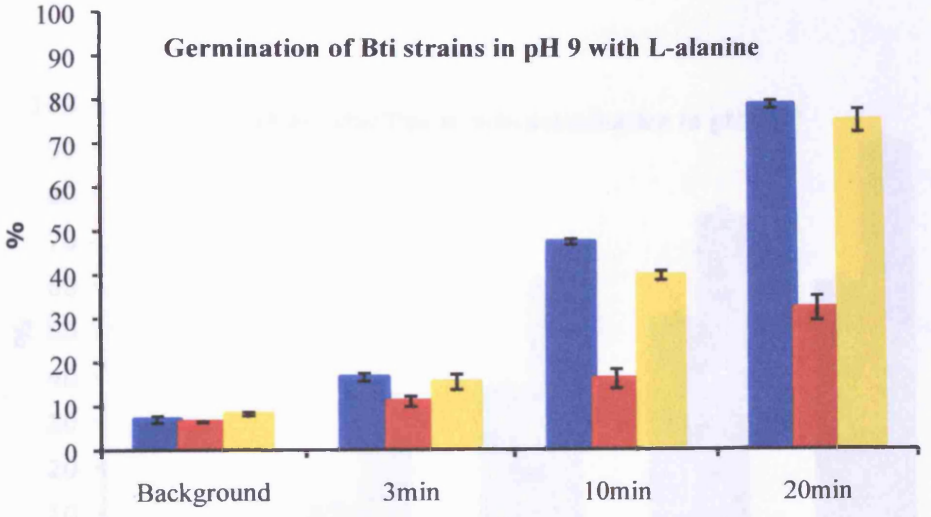


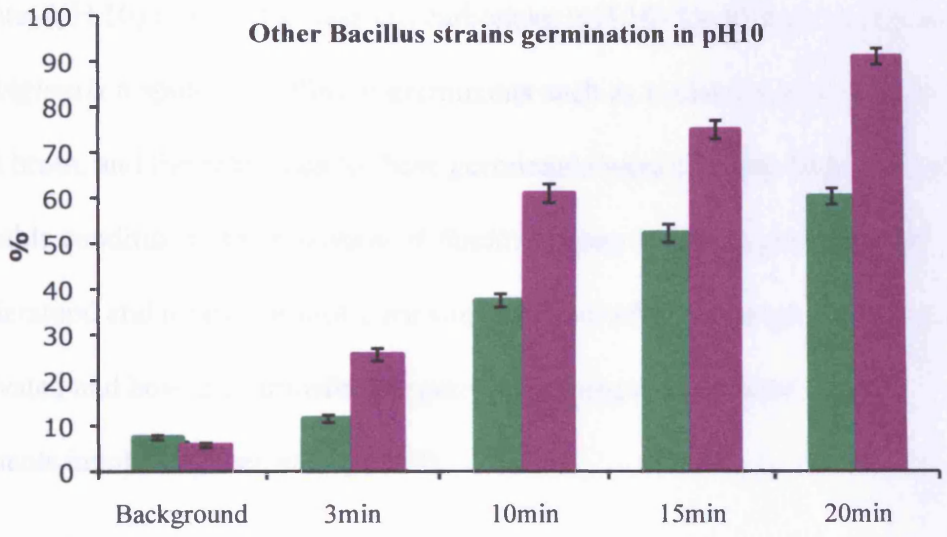
Figure: 6.3.4.5 The germination responses of *B. thuringiensis* subsp. *israelensis* strains after alkaline activation at pH 9 with L–alanine germinant (containing 13 mM D-cycloserine). Bti 4Q5 ; Bti 4Q7 ; Bti 4Q7 *ger* .



6.3.5 Germination of other *Bacillus* strains

The other *Bacillus* strains (*B. thuringiensis* subsp. *kurstaki* and *B. subtilis* IA2) were tested for alkaline activation at pH 10 using nutrient broth to assess their response.

Figure: 6.3.5.1 The germination responses of *B. thuringiensis* subsp. *kurstaki* (Btk) and *B. subtilis* strain IA2 after alkaline activation at pH 10 with nutrient broth germinant. *B. subtilis* ; Btk .



The graph shows that *B. thuringiensis* subsp. *kurstaki* germinates in higher rates than *B. subtilis* strain IA2 up to 95% germination at 20 min while *B. subtilis* strain IA2 germinates up to 60% at this time. The rate of germination of *B. thuringiensis* subsp. *kurstaki* in this experiment is comparable to that of *B. thuringiensis* subsp. *israelensis* 4Q5 following alkaline activation (figure 6.3.2.1).

6.6 Germination discussion

Endospore forming bacteria return to vegetative growth in the right conditions by germination, which is triggered by the presence of germinant in the surrounding environment. Germination is induced by the activation of spores and triggered by the binding of germinants to the spore receptors.

Activation of *B. thuringiensis* subsp. *israelensis* spores can be conducted by heat treatment at 70°C for 20 minutes, and also by alkaline treatment in 0.1 M sodium carbonate, (pH 10) or 0.1 M potassium carbonate, (pH 10) for 30 minutes. Spores of *B. thuringiensis* respond to different germinants such as L-alanine, inosine and nutrient broth, and the responses to these germinants were found to be similar under comparable conditions. Germination of *Bacillus* group spores in general is still not full understood and many questions are still unanswered, for example, how receptors are activated and how they transfer the germination signal, and what other components involved (Abee *et al.*, 2010).

The *B. thuringiensis* subsp. *israelensis* vectobac wild-type and *B. thuringiensis* subsp. *israelensis* 4Q5 strain containing pBtoxis showed no significant differences in germination from *B. thuringiensis* subsp. *israelensis* 4Q7 and *B. thuringiensis* subsp. *israelensis* IPS 78/11 strains lacking the plasmid, following heat activation, except when alanine was used as the germinant. The features responsible for heat activation in these strains must, therefore, be located on the chromosome of *B. thuringiensis* subsp. *israelensis*. In contrast, following alkaline activation significant differences were observed in the germination rates with all the germinants between strains *B. thuringiensis* subsp. *israelensis* 4Q5 containing pBtoxis and

B. thuringiensis subsp. *israelensis* 4Q7 and *B. thuringiensis* subsp. *israelensis* IPS 78/11 strains lacking this plasmid. This effect could be reversed by complementation with the pBt084, pBt085 and pBt086 genes in strain *B. thuringiensis* subsp. *israelensis* 4Q7 *ger*. This clearly indicates a role for these genes in the alkaline activation of spores.

This response of alkaline–activated spores was seen at pH 10 and, when the pH threshold for the activation effect of pH on spore germination was assessed using a range of different pH (8, 8.5, 9 and 10), *B. thuringiensis* subsp. *israelensis* 4Q7 showed a weak alkaline induction of the germination in the presence of inosine at pH 9 and above, however, the presence of the *ger* genes (on pBtoxis or pHT304ger) leads to a marked increase in responsiveness to alkaline activation at pH 9 and pH 10. Interestingly, the germination of all *B. thuringiensis* subsp. *israelensis* strains (regardless of the presence of the pBtoxis plasmid) was similar after heat activation. *B. thuringiensis* subsp. *israelensis* 4Q5 showed a higher germination rate than *B. thuringiensis* subsp. *israelensis* 4Q7 and *B. thuringiensis* subsp. *israelensis* 4Q7*ger*, but strains *B. thuringiensis* subsp. *israelensis* 4Q7 and *B. thuringiensis* subsp. *israelensis* 4Q7*ger* were indistinguishable. The result of this experiment indicates that *ger* genes do not play a significant role in the heat activation response, although other pBtoxis factors may have a role.

B. subtilis strain IA2 germinates at lower rate than *B. thuringiensis* subsp. *israelensis* 4Q5 and *B. thuringiensis* subsp. *kurstaki* following alkaline activation, and further work is needed to assess the response of *B. subtilis* when the *ger* genes of pBtoxis are transferred onto it to find out if they can increase its germination rates to

a level similar to *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *kurstaki*.

The findings are similar to those of Benoit *et al.*, (1995) who used 0.1 M potasium carbonate pH 10 to activate *B. thuringiensis* subsp. *kurstaki* spores from crystal-forming and non-crystal-forming strains and also *B. cereus* and found that only the spores of crystal-forming strains activated. In another study of crystalliferous (Cry+) and acrystalliferous (Cry-) strains of *B. thuringiensis* subsp. *israelensis*; wild type *B. cereus* and its transcipient crystalliferous derivatives; and wild type *B. subtilis*, Bhattacharya (1999) showed that only crystalliferous strains can be activated by alkaline *in vitro* and in the alkaline environment of the *Aedes aegypti* midgut. Both studies along with work by Du and Nickerson (1996) were interpreted as indicating the protoxin in the coat of the spore may be responsible for the alkaline-activation phenotype. However, since toxin production is plasmid associated, it seems likely that loss of crystal production may reflect curing of the toxin-coding plasmid, which may, like pBtoxis, encode genes responsible for alkaline activation.

The complementation of the alkaline activation response seen in the experiments presented here indicates that the *ger* genes pBt084, pBt085 and pBt086 can produce this effect in the absence of any toxin production in strain *B. thuringiensis* subsp. *israelensis* 4Q7 *ger*. The other possible *ger*-related CDSs encoded by pBtoxis (pBt060 and pBt063) were identified as probable pseudogenes (Berry *et al.*, 2002) and were transcriptionally inactive under the conditions tested by Stein *et al.* (2006). The present study also indicates that these genes do not have an essential role in the alkaline

activation response. The pBtoxis *ger* genes appear to encode a GerA receptor type system but the nature of the alkaline activation response is unknown and little studied.

The alkaline conditions may be causing an effect such as a change in permeability of the spore or stripping of the spore coat but even if this occurs, the pBtoxis *ger* genes appear to offer a specific alkaline activation response that is not available to strains lacking these genes. The midgut of many insects including mosquitoes, are alkaline and a selective advantage may be gained by spores able to germinate at the appropriate time in the gut (Jensen *et al.*, 2003).

Also has been suggested that when Cry toxin proteins damage the epithelial cells of the larval midgut and the gut contents mix with haemolymph liquids, this may become a favourable condition for the *B. thuringiensis* spores to germinate (WHO, 1999).

Combination of *ger* genes encoding this function with toxin genes to damage the gut and kill the host insect may provide a double advantage to the bacteria.

CHAPTER 7

Co-feeding of *Bacillus thuringiensis* subsp. *israelensis* strains

7.1 Co-feeding of *B. thuringiensis* subsp.*israelensis* strain (4Q5kan and 4Q7rif)

The germination experiments showed that *B. thuringiensis* subsp.*israelensis* 4Q5kan (Bti 4Q5kan) germinates faster than *B. thuringiensis* subsp.*israelensis* 4Q7rif (Bti 4Q7rif) in alkaline conditions, which are similar to the alkaline condition of the midgut of mosquito larvae. In an attempt to assess whether fast germination provides an advantage to Bti 4Q5 in alkaline conditions over Bti 4Q7, a co-feeding experiment was carried out.

Also, to assess whether any advantage for of Bti 4Q5kan is due to the *ger* genes on the pBtoxis plasmid, Bti 4Q7*ger* (erythromycin resistant) was fed to mosquito larvae along with rifampicin resistant Bti 4Q7.

7.2 Materials and methods

7.2.1 Production of Bti 4Q5 kanamycin and 4Q7 rifampicin antibiotic resistance

The antibiotic resistant strains of Bti were produced by selection to facilitate identification after co-feeding. Bti 4Q5kan was selected using kanamycin while Bti 4Q7rif was selected with rifampicin, as follows.

From an overnight culture of Bti, 100 µl was inoculated into 10 ml of fresh LB containing 0.1 µg/ml antibiotic and incubated over night in a shaking incubator at 30°C. Then 100 µl of this culture was inoculated into 10 ml of fresh LB medium containing 1 µg/ml antibiotic and incubated overnight in a shaking incubator at 30°C. Finally 100 µl of this culture was inoculated into fresh LB medium containing

10 μg/ml antibiotic and incubated overnight in a shaking incubator at 30°C. These cultures were plated onto LB agar for the isolation of individual, antibiotic- resistant colonies.

7.2.2 Co-feeding experiments

Individual colonies of the Bti 4Q5kan and Bti 4Q7rif strains were grown in embrapa medium with antibiotic for 4-5 days (to > 95% sporulation). The D_{600} of the cultures were measured and the spores were harvested by centrifugation at 1300 rpm for 5 min in a microfuge, before resuspension to approximentaly the same density ($D_{600} \approx 1$). Five 3rd instar *Aedes aegypti* mosquito larvae were placed in 5 ml water in universal tubes and 250 μl of each resuspended culture was added. Exact bacterial counts were also obtained either by acridine orange staining or drop counting (section 2.3).

The dose used was sufficient to cause 100% larval mortality (approximately $9x10^8$ spores per ml in the assay), typically within 2 h but the larvae were left for 24 h from the start of the experiment to allow colonisation, after larvae were transferred to a fresh 5 ml of water in universal tubes. After this period the larvae were washed once using 100% ethanol to eliminate the surface bacteria, followed by two washes in distilled water and then homogenised in an eppendoff tube with ¼ strength Ringer's solution and serial dilutions were made (neat, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) into eppendoff tubes with ¼ strength Ringer's solution. The drop plate technique was used for counting the bacterial cells after feeding (section 2.3).

7.3 Results

7.3.1 Co-feeding Bti 4Q5kan and Bti 4Q7rif

Co-feeding Bti 4Q5kan and Bti 4Q7rif at different input ratios produced the final ratios of each strain shown in table 7.3.1.1.

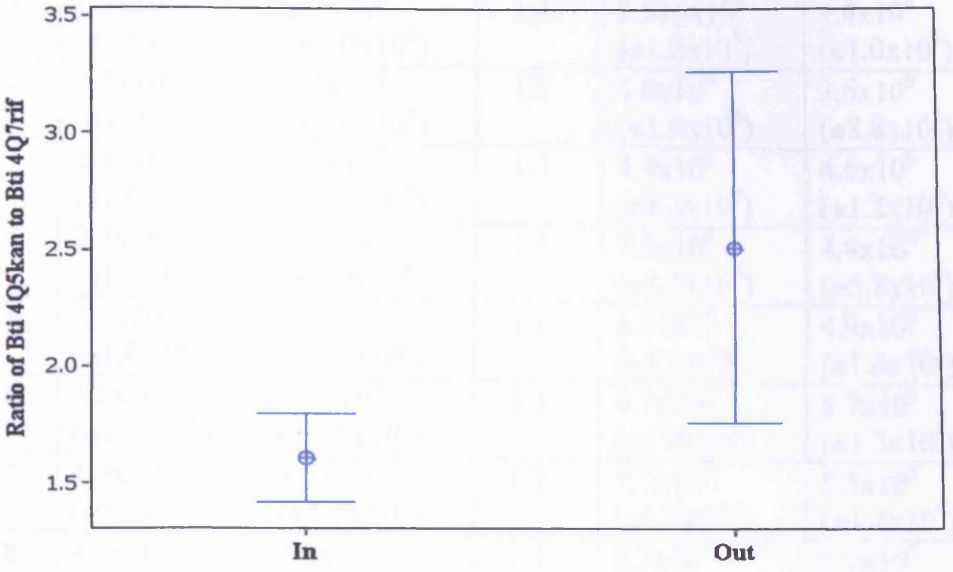
Table 7.3.1.1 The number of bacterial cells Bti 4Q5kan and Bti 4Q7rif before and after 24 h of feeding

No	Bti 4Q5kan added	Bti 4Q7rif added	Ratio-in	Bti 4Q5kan isolated	Bti 4Q7rif isolated	Ratio-out
1	$7.0x10^9$ ($\pm 2.0x10^8$)	$6.2x10^9$ ($\pm 1.8x10^8$)	1.13:1	$9.5x10^6$ ($\pm 7.7x10^5$)	$7x10^6$ ($\pm 2.7x10^5$)	1.35: 1
2	$5.5x10^9$ ($\pm 2.2x10^8$)	$4.42x10^9$ ($\pm 1.6x10^8$)	1.23: 1	$3.8x10^6$ ($\pm 3.2x10^5$)	$3x10^6$ ($\pm 3.8x10^5$)	1.27: 1
3	$6.5x10^9$ ($\pm 2.3x10^8$)	$5.2x10^9$ ($\pm 1.8x10^8$)	1.25: 1	$5.6x10^6$ ($\pm 3.8x10^5$)	$3.6x10^6$ ($\pm 2.8x10^5$)	1.56: 1
4	$4.2x10^7$ ($\pm 2.1x10^6$)	$2.7x10^7$ ($\pm 3.5x10^6$)	1.59: 1	$3.3x10^6$ ($\pm 6.8x10^5$)	$2.3x10^6$ ($\pm 1.0x10^5$)	1.45: 1
5	$4.2x10^7$ ($\pm 2.1x10^6$)	$2.7x10^7$ ($\pm 3.5x10^6$)	1.59: 1	$2.3x10^6$ ($\pm 3.4x10^5$)	$2.1x10^6$ ($\pm 2.7x10^5$)	1.07: 1
6	$4.2x10^7$ ($\pm 2.1x10^6$)	$2.7x10^7$ ($\pm 3.5x10^6$)	1.59: 1	$2.6x10^6$ ($\pm 3.9x10^5$)	$1.9x10^6$ ($\pm 4.5x10^5$)	1.30: 1
7	$5.4x10^7$ ($\pm 2.0x10^6$)	$2.7x10^7$ ($\pm 2.3x10^6$)	1.96: 1	$4.6x10^6$ ($\pm 2.0x10^5$)	$2.0x10^6$ ($\pm 1.3x10^5$)	2.20: 1
8	$5.3x10^7$ ($\pm 2.0x10^6$)	$2.7x10^7$ ($\pm 2.3x10^6$)	1.96: 1	$4.3x10^6$ ($3.5x10^5$)	$1.6x10^6$ ($\pm 1.0x10^5$)	2.80: 1
9	$5.3x10^7$ ($\pm 2.0x10^6$)	$2.7x10^7$ ($\pm 2.3x10^6$)	1.96: 1	$3.8x10^6$ ($\pm 1.9x10^5$)	$1.7x10^6$ ($\pm 1.2x10^5$)	2.30: 1
10	$4.3x10^7$ ($\pm 2.0x10^6$)	$2.7x10^7$ ($\pm 3.6x10^6$)	1.60: 1	$1.7x10^7$ ($\pm 1.3x10^6$)	$6.2x10^6$ ($\pm 2.1x10^5$)	2.6: 1
11	$4.3x10^7$ ($\pm 2.0x10^6$)	$2.7x10^7$ ($\pm 3.6x10^6$)	1.60: 1	$1.9x10^7$ ($\pm 1.0x10^6$)	$5.2 x10^6$ ($\pm 1.6x10^5$)	3.6: 1
12	$5.3x10^7$ ($\pm 2.127x10^6$)	$2.7x10^7$ ($\pm 2.4x10^6$)	1.95: 1	$1.9x10^7$ ($\pm 1.3x10^6$)	$4.5x10^6$ ($\pm 4.7x10^6$)	4.25: 1

13	$5.3x10^7$ ($\pm 2.1x10^6$)	$2.7x10^7$ ($\pm 2.4x10^6$)	1.95: 1	$1.8x10^7$ ($\pm 4.2x10^5$)	$4.0x10^6$ ($\pm 3.4x10^5$)	4.20: 1
14	$5.3x10^7$ ($\pm 3.2x10^6$)	$3.7x10^7$ ($\pm 2.7x10^6$)	1.40: 1	$3.4x10^7$ ($\pm 4.0x10^6$)	$3.0x10^6$ ($\pm 2.8x10^5$)	1.12: 1
15	$5.3x10^7$ ($\pm 3.2x10^6$)	$3.7x10^7$ ($\pm 2.7x10^6$)	1.40: 1	$3.4x10^7$ ($\pm 1.6x10^6$)	$1.7 x10^6$ ($\pm 2.9x10^5$)	1.90: 1
16	$9.3x10^7$ ($\pm 2.2x10^6$)	$7.4x10^7$ ($\pm 4.5x10^6$)	1.25: 1	$8.5x10^6$ ($\pm 3.1x10^5$)	$4.8x10^6$ ($\pm 4.0x10^5$)	1.76: 1
17	$9.3x10^7$ ($\pm 2.2x10^6$)	$7.4x10^7$ ($\pm 4.5x10^6$)	1.25: 1	$6.7x10^6$ ($\pm 5.7x10^5$)	$2.8x10^6$ ($\pm 2.3x10^5$)	2.4: 1

Statistical analysis of these data showed the ratio of Bti 4Q5kan to Bti 4Q7rif was significantly higher at the end of the experiment than at the start, i.e. Bti 4Q5kan grew more rapidly than Bti 4Q7rif (matched-pairs t-test: $t=2.91$, $N = 17$, $p = 0.010$). There was also a significant positive correlation between the ratio at the start of the experiment (fixed in the experiment) and the ratio at the end of the experiment (Pearson's correlation: $r = +0.617$, $N = 17$, $p = 0.008$). The assumptions for t-test were checked and they were satisfied, and the equality of variances in the two samples. The results are shown graphically in figure 7.3.1.1.1.

Figure 7.3.1.1.1 The increase of Bti 4Q5kan relative to Bti 4Q7rif in mosquito larvae



The graph shows that the ratio of bacterial cells of Bti 4Q5kan has increased relative to Bti 4Q7rif after 24 h of feeding to mosquito larvae. Error bars show 95% confidence intervals.

7.3.2 Co-feeding of Bti 4Q7*ger* and Bti 4Q7rif

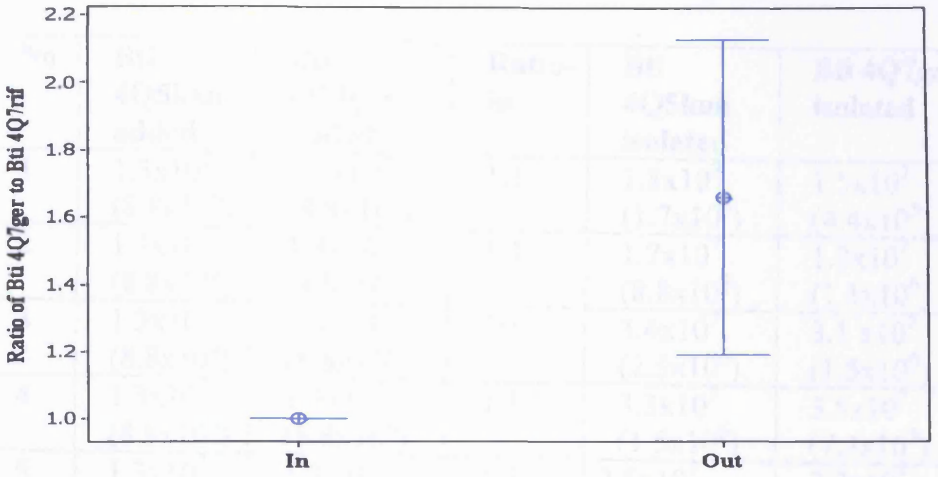
The co-feeding experiment was then repeated using Bti 4Q7*ger* and Bti 4Q7rif. These strains produce no toxins so in order to cause larval death, without the interference of the pBtoxis plasmid and its effect; the crystal proteins of Bti 4Q5 were used after being purified as described in the general materials and methods (section 2.5). The dose used was sufficient to cause death within approximately 2 h and the experiment was continued as before. The number of bacterial cells of each strain were counted 24 h after feeding to mosquito larvae and the results are shown in table 7.3.2.1.

Table 7.3.2.1 Number of Bti 4Q7*ger* and Bti 4Q7rif cells before and after 24 h

No	Bti 4Q7*ger* added	Bti 4Q7rif added	Ratio-in	Bti 4Q7*ger* isolated	Bti 4Q7rif isolated	Ratio-out
1	$2.3x10^8$ ($\pm1.0x10^6$)	$2.3x10^8$ ($\pm1.0x10^6$)	1:1	$5.516x10^6$ ($\pm1.2x10^5$)	$4.4x10^6$ ($\pm1.0x10^5$)	1.20:1
2	$2.3x10^8$ ($\pm1.0x10^6$)	$2.3x10^8$ ($\pm1.0x10^6$)	1:1	$5.0x10^6$ ($\pm1.6x10^5$)	$3.6x10^6$ ($\pm8.8x10^4$)	1.38:1
3	$2.3x10^8$ ($\pm1.0x10^6$)	$2.3x10^8$ ($\pm1.0x10^6$)	1:1	$4.9x10^6$ ($\pm1.3x10^5$)	$4.6x10^6$ ($\pm1.2x10^5$)	1.01:1
4	$2.3x10^8$ ($\pm1.0x10^6$)	$2.3x10^8$ ($\pm1.0x10^6$)	1:1	$5.4x10^6$ ($\pm1.1x10^5$)	$4.4x10^6$ ($\pm5.8x10^4$)	1.20:1
5	$2.3x10^8$ ($\pm1.0x10^6$)	$2.3x10^8$ ($\pm1.0x10^6$)	1:1	$6.2x10^6$ ($\pm1.1x10^5$)	$4.9x10^6$ ($\pm1.6x10^5$)	1.20:1
6	$4.5x10^7$ ($\pm1.5x10^6$)	$4.5x10^7$ ($\pm1.5x10^6$)	1:1	$6.7x10^5$ ($\pm1.0x10^4$)	$5.7x10^5$ ($\pm1.3x10^4$)	1.17:1
7	$4.5x10^7$ ($\pm1.5x10^6$)	$4.5x10^7$ ($\pm1.5x10^6$)	1:1	$7.7x10^5$ ($\pm1.9x10^4$)	$5.5x10^5$ ($\pm1.4x10^4$)	1.40:1
8	$4.5x10^7$ ($\pm1.5x10^6$)	$4.5x10^7$ ($\pm1.5x10^6$)	1:1	$5.7x10^5$ ($\pm1.6x10^4$)	$5.1x10^5$ ($\pm1.2x10^4$)	1.14:1
9	$4.5x10^7$ ($\pm1.5x10^6$)	$4.5x10^7$ ($\pm1.5x10^6$)	1:1	$6.2x10^5$ ($\pm1.8x10^4$)	$4.9x10^5$ ($\pm1.6x10^4$)	1.26:1
10	$4.5x10^7$ ($\pm1.5x10^6$)	$4.5x10^7$ ($\pm1.5x10^6$)	1:1	$7.2x10^5$ ($\pm1.6x10^4$)	$3.7x10^5$ ($\pm1.2x10^4$)	1.90:1
11	$4.5x10^7$ ($\pm1.5x10^6$)	$4.5x10^7$ ($\pm1.5x10^6$)	1:1	$7.7x10^5$ ($\pm1.8x10^4$)	$5.6x10^5$ ($\pm9.3x10^3$)	1.37:1
12	$4.5x10^7$ ($\pm1.5x10^6$)	$4.5x10^7$ ($\pm1.5x10^6$)	1:1	$3.1x10^5$ ($\pm1.3x10^4$)	$8.2x10^4$ ($\pm1.0x10^3$)	3.80:1
13	$3.9x10^7$ ($\pm1.5x10^6$)	$3.9x10^7$ ($\pm1.5x10^6$)	1:1	$3.7x10^5$ ($\pm1.5x10^4$)	$7.8x10^4$ ($\pm2.2x10^3$)	4.70:1
14	$3.9x10^7$ ($\pm1.5x10^6$)	$3.9x10^7$ ($\pm1.5x10^6$)	1:1	$8.6x10^4$ ($\pm1.7x10^3$)	$4.5x10^4$ ($\pm1.8x10^3$)	1.90:1
15	$3.9x10^7$ ($\pm1.5x10^6$)	$3.9x10^7$ ($\pm1.5x10^6$)	1:1	$1.4x10^6$ ($\pm5.7x10^4$)	$3.7x10^5$ ($\pm1.3x10^3$)	3.50:1
16	$3.9x10^7$ ($\pm1.5x10^6$)	$3.9x10^7$ ($\pm1.5x10^6$)	1:1	$1.1x10^6$ ($\pm2.2x10^4$)	$6.1x10^5$ ($\pm1.9x10^4$)	1.60: 1
17	$3.9x10^7$ ($\pm1.5x10^6$)	$3.9x10^7$ ($\pm1.5x10^6$)	1:1	$8.5x10^5$ ($\pm2.1x10^4$)	$9.3x10^5$ ($\pm1.5x10^4$)	0.9: 1
18	$5.3x10^7$ ($\pm1.7x10^6$)	$5.3x10^7$ ($\pm1.7x10^6$)	1:1	$1.2x10^6$ ($\pm2.6x10^4$)	$1.1x10^6$ ($\pm1.2x10^4$)	1: 1
19	$5.3x10^7$ ($\pm1.7x10^6$)	$5.3x10^7$ ($\pm1.7x10^6$)	1:1	$2.8x10^6$ ($\pm1.2x10^5$)	$2.5x10^6$ ($\pm1.3x10^5$)	1.1:1
20	$5.3x10^7$ ($\pm1.7x10^6$)	$5.3x10^7$ ($\pm1.7x10^6$)	1:1	$1.9x10^6$ ($\pm1.2x10^5$)	$2.2x10^6$ ($\pm1.0x10^5$)	0.89:1
21	$5.3x10^7$ ($\pm1.7x10^6$)	$5.3x10^7$ ($\pm1.7x10^6$)	1:1	$6.5x10^5$ ($\pm1.8x10^4$)	$4.9x10^5$ ($\pm1.0x10^4$)	1.3:1

In this experiment the bacterial cells of both strains were fed to larvae at the same ratio of 1:1 and when recounted after 24 h, the ratio of Bti 4Q7*ger* to Bti 4Q7rif was increased. This is shown graphically in figure 7.3.2.1.

Figure 7.3.2.1.1 Increase of Bti 4Q7*ger* relative to Bti 4Q7rif after mosquito feeding



The graph shows that the ratio of bacterial cells of Bti 4Q7*ger* has increased relative to Bti 4Q7rif, 24 h after feeding to mosquito larvae despite the fact that both bacterial strains were fed to larvae at a ratio of 1:1. Error bars show 95% confidence intervals.

Statistical analysis of these data using the Wilcoxon test showed that the ratio of Bti 4Q7*ger* after 24 h was significantly greater than the starting ratio of 1:1. (Wilcoxon 1- sample test, w=203.5, N=21, p<0.001, estimated median = 1.355). A 1-sample t-test using Box-Cox-transformed data showed that the ratio [of strain Bti 4Q7*ger* to strain Bti 4Q7rif] at the end of the experiments was significantly higher than the ratio of 1.0 [or 1:1] at the start of the experiments ($t = 5.16$, $n = 21$, $P < 0.001$).

7.3.3 Co culture of cells in LB medium

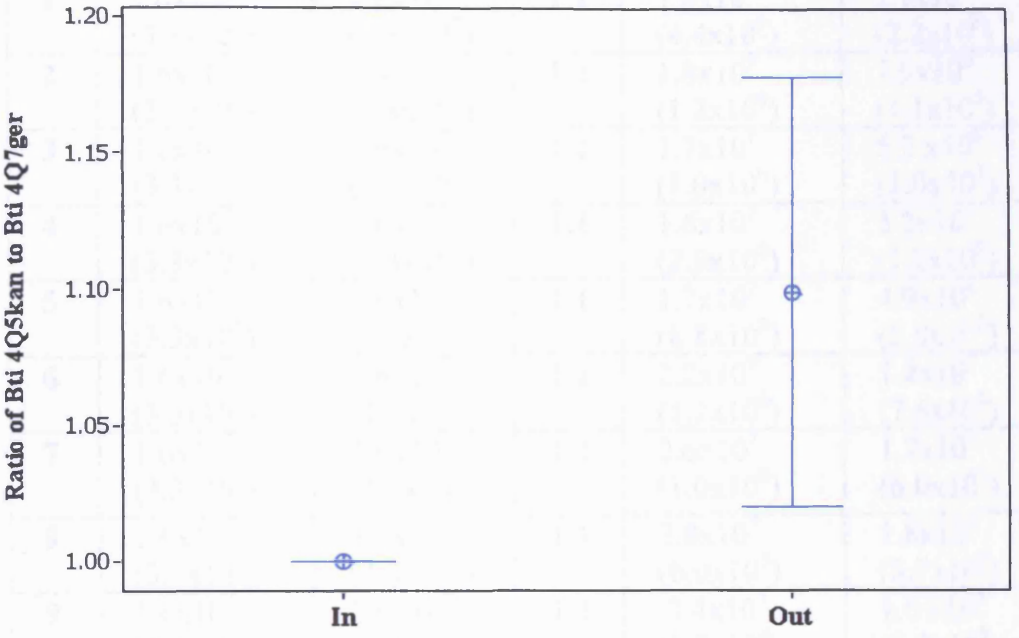
To assess the relative growth rates in rich medium, spores of Bti 4Q5kan and Bti 4Q7*ger* were inoculated into LB medium and table 7.3.3.1 shows bacterial counts before and after culture.

Table 7.3.3.1 Bti 4Q5kan and Bti 4Q7*ger* numbers and ratio after growth in co-culture in LB

No	Bti 4Q5kan added	Bti 4Q7*ger* added	Ratio-in	Bti 4Q5kan isolated	Bti 4Q7*ger* isolated	Ratio - out
1	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$1.8x10^7$ ($1.7x10^6$)	$1.5x10^7$ ($4.4x10^5$)	1.26:1
2	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$1.7x10^7$ ($8.8x10^6$)	$1.2x10^7$ ($1.1x10^6$)	1.38:1
3	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$3.4x10^7$ ($2.5x10^6$)	$3.1 x10^7$ ($1.5x10^6$)	1.08:1
4	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$3.3x10^7$ ($1.5x10^6$)	$3.5x10^7$ ($7.3x10^5$)	0.96:1
5	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$2.5x10^7$ ($1.0x10^6$)	$2.2x10^7$ ($6.0x10^5$)	1.08:1
6	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$2.7x10^7$ ($1.0x10^6$)	$3.1x10^7$ ($1.1x10^6$)	0.90:1
7	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$3.4x10^7$ ($2.5 x10^6$)	$3.2x10^7$ ($1.7x10^6$)	1.06:1
8	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$3.5x10^7$ ($1.3x10^6$)	$3.3x10^7$ ($2.5x10^6$)	1.08:1
9	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$3.6x10^7$ ($1.2x10^6$)	$3.2x10^7$ ($1.0x10^6$)	1.11:1
10	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$3.5x10^7$ ($1.7x10^5$)	$3.3x10^7$ ($1.3x10^6$)	1.06:1
11	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$4.2x10^7$ ($5.8x10^5$)	$3.1x10^7$ ($1.2x10^6$)	1.36:1
12	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$3.2x10^7$ ($1.0x10^6$)	$3.0x10^7$ ($8.7x10^5$)	1.049:1
13	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$2.2x10^7$ ($1.1x10^6$)	$2.5x10^7$ ($8.8x10^5$)	0.86:1
14	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$3.9x10^7$ ($9.3x10^6$)	$3.2x10^7$ ($1.3x10^6$)	1.22:1
15	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$2.2x10^7$ ($1.0x10^6$)	$2.x10^7$ ($1.0x10^6$)	0.99:1
16	$1.3x10^7$ ($8.8x10^6$)	$1.3x10^7$ ($8.8x10^6$)	1:1	$3.1x10^7$ ($1.0x10^6$)	$2.5x10^7$ ($2.9x10^5$)	1.26:1

The table 7.3.3.1 shows that the ratio of Bti 4Q5kan relative to Bti 4Q7*ger* increased after growth in the same tube (mixed together), the ratio of Bti 4Q5kan and Bti 4Q7*ger* was significantly greater than 1:1. This is shown graphically in figure 7.3.3.1.1.

Figure 7.3.3.1.1 Increase of Bti 4Q5kan relative to Bti 4Q7*ger* after inoculation at a 1:1 ratio in LB



The graph shows that the ratio of bacterial cells of Bti 4Q5kan relative to Bti 4Q7*ger* after growth in LB has increased despite starting at a ratio of 1:1. Error bars show 95% confidence intervals.

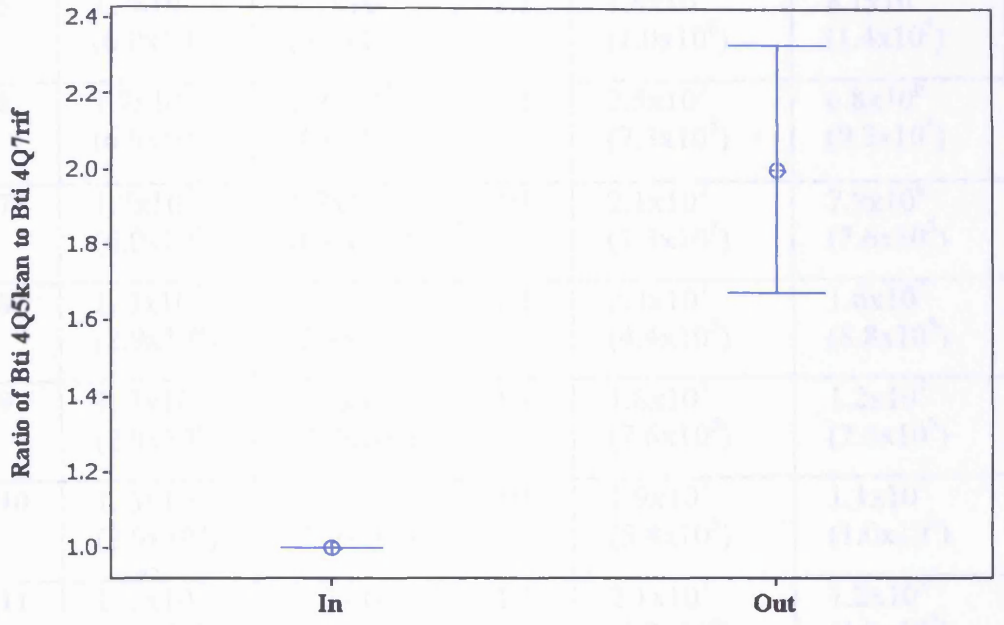
Statistical analysis of these data shows that Bti 4Q5kan grew more rapidly than Bti 4Q7*ger* (mean ratio ± SE = 1.100 ± 0.037, 1-sample t-test: t=2.688, d.f. =15, p=0.017). The experiment was then repeated with spores of strains Bti 4Q5kan and Bti 4Q7rif. The results are shown in table 7.3.3.2 and figure 7.3.3.2.1.

Table 7.3.3.2 Bti 4Q5kan and Bti 4Q7rif numbers after growth in co-culture in LB

No	Bti 4Q5kan inoculated	Bti 4Q7rif inoculated	Ratio -in	Bti 4Q5kan isolated	Bti 4Q7rif isolated	Ratio - out
1	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$1.8x10^7$ ($4.4x10^5$)	$1.1x10^7$ ($2.2x10^6$)	1.61:1
2	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$1.8x10^7$ ($1.2x10^6$)	$7.9x10^6$ ($1.1x10^5$)	2.24:1
3	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$1.7x10^7$ ($1.0x10^6$)	$5.2 x10^6$ ($1.0x10^5$)	3.10:1
4	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$1.6x10^7$ ($2.9x10^5$)	$5.2x10^6$ ($1.2x10^5$)	2.97:1
5	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$1.7x10^7$ ($8.8x10^5$)	$4.9x10^6$ ($5.8x10^5$)	3.57:1
6	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$2.2x10^7$ ($1.2x10^6$)	$1.4x10^7$ ($7.6x10^5$)	1.53:1
7	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$2.6x10^7$ ($1.0x10^6$)	$1.7x10^7$ ($6.0x10^5$)	1.49:1
8	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$2.8x10^7$ ($6.0x10^5$)	$1.8x10^7$ ($8.7x10^5$)	1.61:1
9	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$3.4x10^7$ ($1.2x10^6$)	$1.6 x10^7$ ($6.0x10^5$)	2.09:1
10	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$3.3x10^7$ ($2.0x10^6$)	$2.1x10^7$ ($1.0x10^6$)	1.58:1
11	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$3.4x10^7$ ($8.8x10^5$)	$1.8x10^7$ ($4.4x10^5$)	1.90:1
12	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$3.1x10^7$ ($2.8x10^6$)	$2.1x10^7$ ($1.7x10^6$)	1.51:1
13	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$2.9 x10^7$ ($3.5x10^6$)	$1.7 x10^7$ ($3.3x10^5$)	1.81:1
14	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$2.9x10^7$ ($2.3x10^6$)	$1.4x10^7$ ($1.7x10^5$)	2.14:1
15	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$2.9x10^7$ ($2.5x10^6$)	$1.8x10^7$ ($2.9x10^5$)	1.63:1
16	$1.6x10^7$ ($3.3x10^5$)	$1.6x10^7$ ($3.3x10^5$)	1:1	$2.7x10^7$ ($1.3x10^6$)	$1.7x10^7$ ($5x10^5$)	1.60:1

The table 7.3.3.2 shows that when grown in the same LB culture (mixed together), the ratio of Bti 4Q5kan and Bti 4Q7rif was significantly greater than 1:1. i.e. Bti 4Q5kan grew more rapidly than Bti 4Q7rif, (mean ratio ± SE = 2.006 ± 0.155, 1-sample t-test: $t=6.501$, d.f.=15, $p<0.001$). This is shown graphically in figure 7.3.3.2.1.

Figure 7.3.3.2.1 Increase of Bti 4Q5kan relative to Bti 4Q7rif after inoculation at a 1:1 ratio in LB



The graph shows that the ratio of bacterial cells of Bti 4Q5kan has increased relative to Bti 4Q7rif after grow in LB despite starting at the same ratio of 1:1. Error bars show 95% confidence intervals.

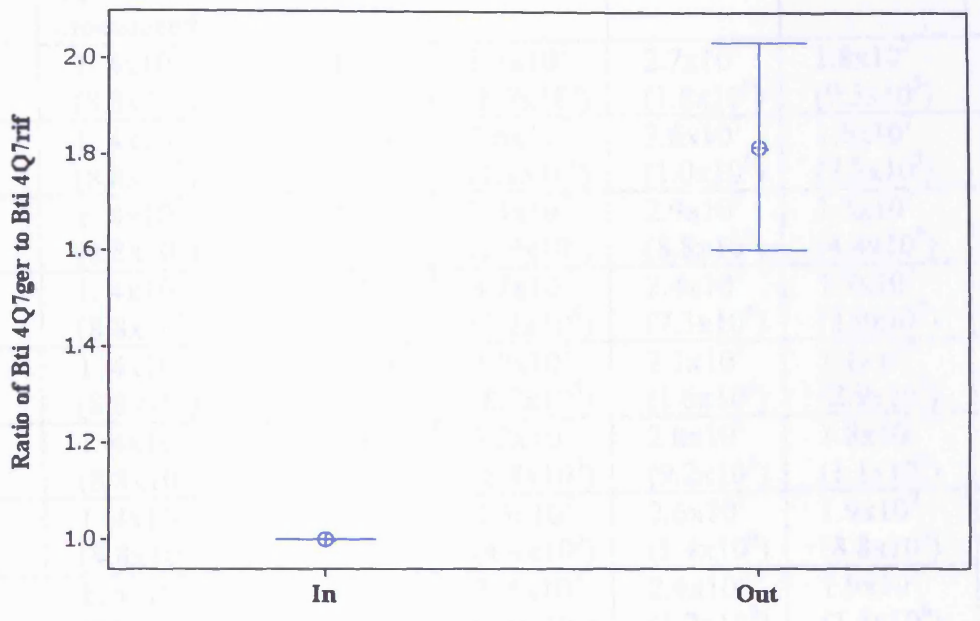
Finally, the experiment was repeated with strains Bti 4Q7ger and Bti 4Q7rif. The results are shown in table 7.3.3.3 and figure 7.3.3.3.1.

Table 7.3.3.3 Bti 4Q7*ger* and Bti 4Q7rif numbers and ratios after growth in co-culture

No	Bti 4Q7*ger* inoculated	Bti 4Q7rif inoculated	Ratio -in	Bti 4Q7*ger* isolated	Bti 4Q7rif isolated	Ratio – out
1	$1.7x10^7$ ($6.0x10^5$)	$1.7x10^7$ ($6.0x10^5$)	1:1	$2.3x10^7$ ($8.7x10^5$)	$1.5x10^7$ ($4.4x10^5$)	1.48:1
2	$1.7x10^7$ ($6.0x10^5$)	$1.7x10^7$ ($6.0x10^5$)	1:1	$1.6x10^7$ ($2.9x10^5$)	$9.1x10^6$ ($2.0x10^5$)	1.76: 1
3	$1.7x10^7$ ($6.0x10^5$)	$1.7x10^7$ ($6.0x10^5$)	1:1	$2.5x10^7$ ($9.3x10^5$)	$7.5x10^6$ ($9.3x10^5$)	3.33: 1
4	$1.7x10^7$ ($6.0x10^5$)	$1.7x10^7$ ($6.0x10^5$	1:1	$2.2x10^7$ ($1.2x10^6$)	$8.7x10^6$ ($1.2x10^5$)	2.51: 1
5	$1.7x10^7$ ($6.0x10^5$)	$1.7x10^7$ ($6.0x10^5$)	1:1	$1.8x10^7$ ($1.0x10^6$)	$8.1x10^6$ ($1.4x10^5$)	2.20:1
6	$1.7x10^7$ ($6.0x10^5$)	$1.7x10^7$ ($6.0x10^5$)	1:1	$2.5x10^7$ ($7.3x10^5$)	$6.8x10^6$ ($9.3x10^5$)	3.17:1
7	$1.7x10^7$ ($6.0x10^5$)	$1.7x10^7$ ($6.0x10^5$)	1:1	$2.1x10^7$ ($7.3x10^5$)	$7.9x10^6$ ($7.6x10^5$)	2.66:1
8	$1.3x10^7$ ($2.9x10^6$)	$1.3x10^7$ ($2.9x10^6$)	1:1	$2.3x10^7$ ($4.4x10^5$)	$1.6x10^7$ ($8.8x10^5$)	1.43:1
9	$1.3x10^7$ ($2.9x10^6$)	$1.3x10^7$ ($2.9x10^6$)	1:1	$1.8x10^7$ ($7.6x10^5$)	$1.2x10^7$ ($7.6x10^5$)	1.41:1
10	$1.3x10^7$ ($2.9x10^6$)	$1.3x10^7$ ($2.9x10^6$)	1:1	$1.9x10^7$ ($5.8x10^5$)	$1.1x10^7$ ($1.0x10^5$)	1.71:1
11	$1.3x10^7$ ($2.9x10^6$)	$1.3x10^7$ ($2.9x10^6$)	1:1	$2.1x10^7$ ($1.3x10^6$)	$1.2x10^7$ ($1.2x10^5$)	1.68:1
12	$1.3x10^7$ ($2.9x10^6$)	$1.3x10^7$ ($2.9x10^6$)	1:1	$2.2x10^7$ ($9.3x10^5$)	$1.3x10^7$ ($9.8x10^5$)	1.71:1
13	$1.3x10^7$ ($2.9x10^6$)	$1.3x10^7$ ($2.9x10^6$)	1:1	$1.6x10^7$ ($6.0x10^5$)	$1.1x10^7$ ($5.8x10^5$)	1.43:1
14	$1.6x10^7$ ($8.3x10^5$)	$1.6x10^7$ ($8.3x10^5$)	1:1	$2.6x10^7$ ($8.8x10^5$)	$1.1x10^7$ ($6.0x10^5$)	2.27:1
15	$1.6x10^7$ ($8.3x10^5$)	$1.6x10^7$ ($8.3x10^5$)	1:1	$1.7x10^7$ ($4.4x10^5$)	$8.7x10^6$ ($2.5x10^5$)	2.12:1
16	$1.6x10^7$ ($8.3x10^5$)	$1.6x10^7$ ($8.3x10^5$)	1:1	$2.0x10^7$ ($1.1x10^6$)	$1.6x10^7$ ($2.9x10^5$)	1.24:1

When grown in the same tube (mixed together), the ratio of Bti 4Q7*ger* and Bti 4Q7rif was significantly greater than 1:1, i.e. Bti 4Q5*ger* grew more rapidly than Bti 4Q7rif (mean ratio ± SE = 2.034 ± 0.157, 1-sample t-test: t=6.600, d.f.=15, $p<0.001$). The results are shown graphically in figure 7.3.3.3.1.

Figure 7.3.3.3.1 Increase of Bti 4Q7*ger* relative to Bti 4Q7rif after inoculation at a 1:1 ratio in LB



The graph shows that the ratio of bacterial cells of Bti 4Q7*ger* relative to Bti 4Q7rif after growth in LB has increased. Error bars show 95% confidence intervals.

7.3.4 Individual culture of all 3 strains

The 3 strains Bti 4Q5kan, Bti 4Q7*ger* and Bti 4Q7rif were inoculated at the same spore densities into separate tubes of LB and the yield of cells after 24 h was

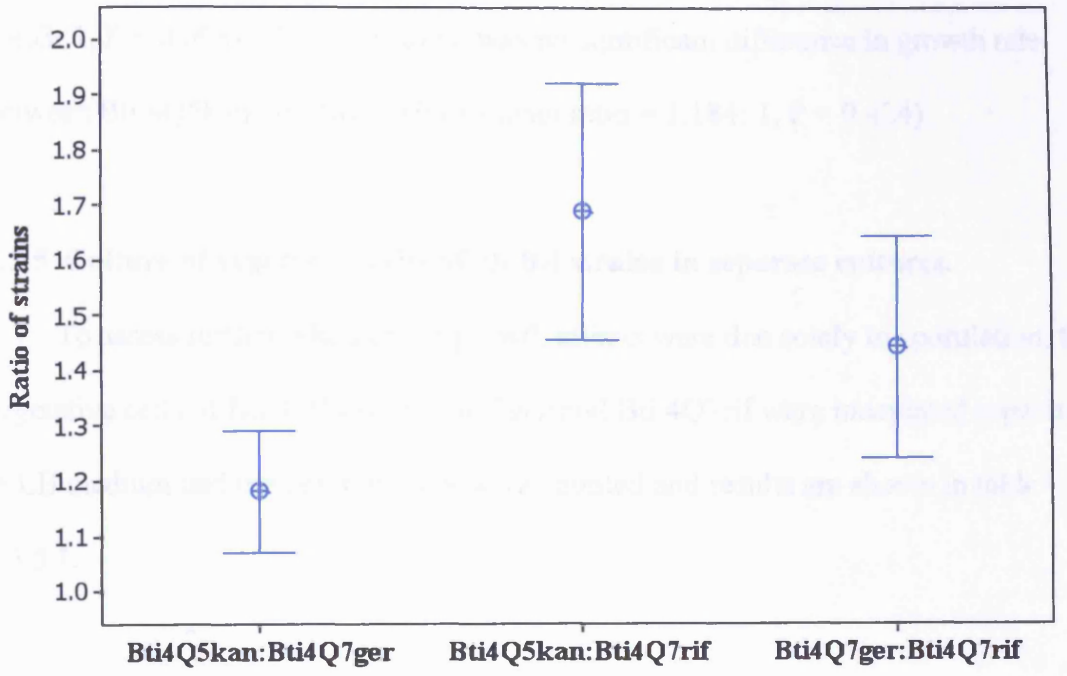
determined in order to assess whether changes in strain ratios were due to general differences in growth rates or a more complex competition. The results are shown in table 7.3.4.1 and graph 7.3.4.1.1.

Table. 7.3.4.1 Bti 4Q5kan, Bti 4Q7*ger* and Bti 4Q7rif numbers after growth in separate cultures in LB

No	Bti 4Q5k, Bti 4Q7*ger* and Bti 4Q7rif inoculated	Ratio - in	Bti 4Q5k isolated	Bti 4Q7*ger* isolated	Bti 4Q7rif isolated	Ratio – out
1	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$3.5x10^7$ ($8.7x10^5$)	$2.7x10^7$ ($1.8x10^6$)	$1.8x10^7$ ($9.3x10^5$)	1.90 :1.50:1
2	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$3.6x10^7$ ($1.1x10^6$)	$3.6x10^7$ ($1.0x10^6$)	$1.6x10^7$ ($7.3x10^5$)	2.19 : 2.20: 1
3	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$3.3x10^7$ ($2.9x10^6$)	$2.9x10^7$ ($8.8x10^5$)	$1.3x10^7$ ($4.4x10^5$)	2.40: 2.20 : 1
4	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$3.7x10^7$ ($3.2x10^6$)	$2.4x10^7$ ($7.3x10^5$)	$1.7x10^7$ ($2.9x10^5$)	2.18: 1.40 :1
5	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.9x10^7$ ($8.7x10^5$)	$2.3x10^7$ ($1.6x10^6$)	$1.1x10^7$ ($2.9x10^5$)	2.48: 2 : 1
6	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$3.2x10^7$ ($8.8x10^5$)	$2.8x10^7$ ($9.2x10^5$)	$1.8x10^7$ ($1.1x10^6$)	1.72: 1.53 :1
7	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$3.3x10^7$ ($4.4x10^5$)	$2.6x10^7$ ($1.4x10^6$)	$1.9x10^7$ ($8.8x10^5$)	1.86:1.46:1
8	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.5x10^7$ ($6.0x10^5$)	$2.4x10^7$ ($1.2x10^5$)	$1.9x10^7$ ($1.1x10^6$)	1.28:1.20:1
9	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.7x10^7$ ($1.2x10^6$)	$2.2x10^7$ ($1.2x10^5$)	$1.8x10^7$ ($1.2x10^6$)	1.50:1.26:1
10	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.5x10^7$ ($9.3x10^5$)	$1.5x10^7$ ($1.7x10^5$)	$1.7x10^7$ ($7.6x10^5$)	1.49:0.89:1
11	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.8x10^7$ ($6.0x10^5$)	$2.2x10^7$ ($1.3x10^6$)	$1.9x10^7$ ($1.3x10^6$)	1.47:1.16:1
12	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.7x10^7$ ($1.0x10^6$)	$2.6x10^7$ ($9.3x10^5$)	$2.1x10^7$ ($9.3x10^5$)	1.26:1.25:1
13	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.8x10^7$ ($9.3x10^5$)	$2.5x10^7$ ($1.0x10^6$)	$1.8x10^7$ ($1.3x10^6$)	1.6:1.44:1
14	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.4x10^7$ ($1.2x10^6$)	$1.9x10^7$ ($7.3x10^5$)	$1.7x10^7$ ($1.0x10^6$)	1.44:1.15:1
15	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.0x10^7$ ($1.5x10^6$)	$2.1x10^7$ ($4.4x10^5$)	$1.833x10^7$ ($1.2x10^6$)	1.09:1.15:1
16	$1.4x10^7$ ($8.8x10^5$)	1:1:1	$2.3x10^7$ ($1.5x10^6$)	$2.5x10^7$ ($6.0x10^5$)	$1.9x10^7$ ($7.6x10^5$)	1.18:1.32:1

Table. 7.3.3.4 shows a higher level of growth for Bti 4Q5kan relative to Bti 4Q7*ger* and Bti 4Q7rif and also shows that Bti 4Q7*ger* grows to a higher level than Bti 4Q7rif, after growth in LB medium in separate tubes and start ratio at a 1:1:1.

Figure 7.3.4.1.1 The increase of Bti 4Q5kan and Bti 4Q7*ger* relative to Bti4Q7rif after growth separately from the same rate of inoculation in LB



The graph shows that the ratio of cells of Bti 4Q5kan and Bti 4Q7*ger* have increased relative to Bti 4Q7rif after growth in LB despite starting at the same inoculation (1:1:1). Also it shows that the ratio of Bti 4Q5kan relative to Bti 4Q7*ger*. Error bars show 95% confidence intervals.

Statistical analysis of these data shows that the ratios of Bti 4Q5kan: Bti 4Q7*ger*: Bti 4Q7rif grown in separate tubes of LB were significantly different from 1:1:1 (Mean ratios ±SE = 1.731±0.140 Bti 4Q5kan: 1.463±0.129 Bti 4Q7*ger*: 1 Bti 4Q7rif, 1-way ANOVA: $F_{2,47} = 11.359$, $P<0.001$). Post-hoc pairwise comparisons between ratio values (using Tamhane's method, suitable for unequal variances) showed that Bti 4Q5kan grew more rapidly than Bti 4Q7rif (mean ratio = 1.731: 1, $P < 0.001$). Similarly, Bti 4Q7*ger* grew more rapidly than Bti 4Q7rif (mean ratio = 1.463: 1, $P < 0.008$). However, there was no significant difference in growth rate between Bti 4Q5kan and Bti 4Q7*ger* (mean ratio = 1.184: 1, $P = 0.424$).

7.3.5 Culture of vegetative cells of all Bti strains in separate cultures.

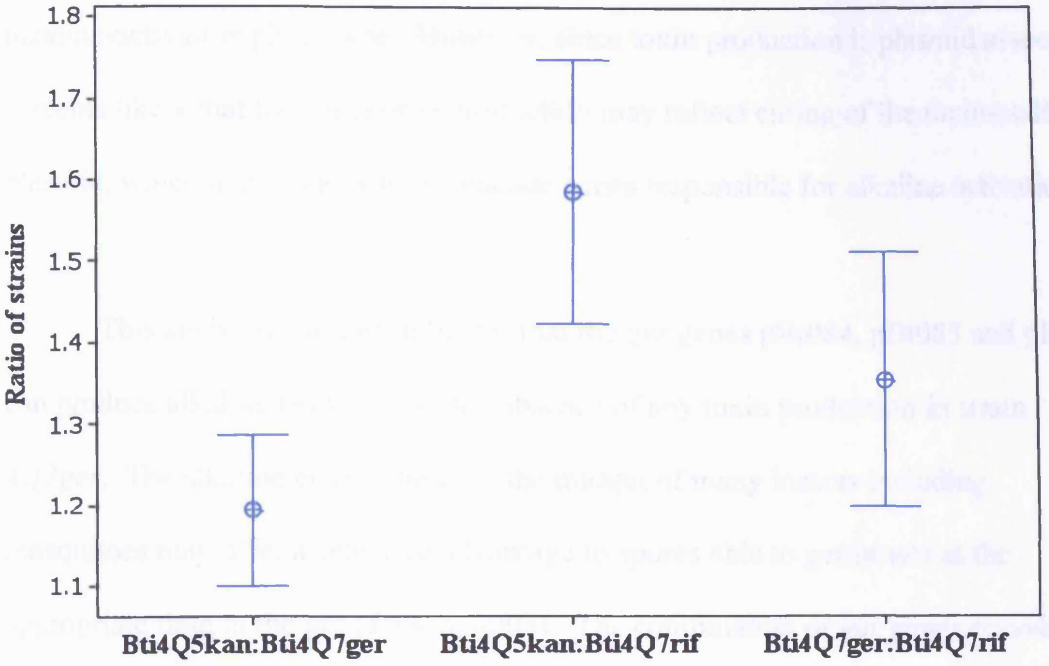
To assess further whether the growth effects were due solely to sporulation, the vegetative cells of Bti 4Q5kan, Bti 4Q7*ger* and Bti 4Q7rif were inoculated separately in LB medium and the cell numbers were counted and results are shown in table 7.3.5.1.

Table 7.3.5.1 The growth of vegetative cells of all Bti strains in separate LB culture

No	Bti 4Q5Kan, Bti 4Q7*ger* and Bti 4Q7rif vegetative cells inoculated	Ratio -in	Bti 4Q5kan isolated	Bti 4Q7*ger* isolated	Bti 4Q7rif isolated	Ratio – out to 4Q7rif
1	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.5x10^7$ ($\pm7.6x10^5$)	$2.1x10^7$ ($\pm9.3x10^5$)	$1.5x10^7$ ($\pm1.7x10^5$)	1.6: 1.4:1
2	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.6x10^7$ ($\pm1.3x10^6$)	$2.3x10^7$ ($\pm1.0x10^6$)	$1.3x10^7$ ($\pm1.6x10^5$)	1.90:1.70: 1
3	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.3x10^7$ ($\pm1.2x10^6$)	$1.8x10^7$ ($\pm6.0x10^5$)	$9.5x10^6$ ($\pm7.3x10^4$)	2.30: 1.90: 1
4	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.5x10^7$ ($\pm8.8x10^5$)	$2.1x10^7$ ($\pm1.3x10^6$)	$1.5x10^7$ ($\pm2.9x10^5$)	1.70: 1.40:1
5	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.8x10^7$ ($\pm6.7x10^5$)	$2.5x10^7$ ($\pm7.3x10^5$)	$1.3x10^7$ ($\pm2.9x10^5$)	2: 1.90:1
6	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.1x10^7$ ($\pm1.2x10^6$)	$1.8x10^7$ ($\pm8.8x10^5$)	$1.4x10^7$ ($\pm1.1x10^5$)	1.50: 1.30:1
7	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.9x10^7$ ($\pm9.279x10^5$)	$2.2x10^7$ ($\pm8.8x10^5$)	$1.5x10^7$ ($\pm7.3x10^5$)	1.90: 1.50:1
8	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.5x10^7$ ($\pm9.3x10^5$)	$2.1x10^7$ ($\pm9.3x10^5$)	$1.9x10^7$ ($\pm8.3x10^5$)	1.30:1.10:1
9	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.4x10^7$ ($\pm1.4x10^6$)	$2.1x10^7$ ($\pm1.1x10^6$)	$1.7x10^7$ ($\pm3.3x10^5$)	1.40:1.20:1
10	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.3x10^7$ ($\pm1.0x10^5$)	$1.3x10^7$ ($\pm2.0x10^6$)	$1.6x10^7$ ($\pm4.4x10^5$)	1.40:0.80:1
11	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.2x10^7$ ($\pm8.8x10^5$)	$2.3x10^7$ ($\pm1.0x10^6$)	$1.7x10^7$ ($\pm1.0x10^6$)	1.30:1.30:1
12	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.6x10^7$ ($\pm1.2x10^6$)	$2.3x10^7$ ($\pm1.3x10^6$)	$1.6 x10^7$ ($\pm4.4x10^5$)	1.60:1.40:1
13	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.7x10^7$ ($\pm6.0x10^5$)	$2.3x10^7$ ($\pm7.6x10^5$)	$1.6x10^7$ ($\pm2.9x10^5$)	1.60:1.40:1
14	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.5x10^7$ ($\pm1.0x10^6$)	$1.9x10^7$ ($\pm1.0x0^5$)	$1.7x10^7$ ($\pm4.4x10^5$)	1.40:1.12:1
15	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.2x10^7$ ($\pm1.2x10^6$)	$2.1x10^7$ ($\pm8.8x10^5$)	$1.8x10^7$ ($\pm1.0x10^6$)	1.20:1.18:1
16	$1.5x10^7$ ($\pm1.7x10^5$)	1:1:1	$2.6x10^7$ ($\pm1x10^6$)	$2.1x10^7$ ($\pm9.3x10^5$)	$1.9x10^7$ ($\pm8.9x10^5$)	1.30:1.10:1

The table 7.3.5.1 shows the increase of the vegetative cells of Bti 4Q5kan relative to Bti 4Q7rif and this is shown graphically in figure 7.3.5.1.1.

Figure 7.3.5.1.1 The increase of Bti 4Q5kan relative to Bti 4Q7rif after growth separately from the same rate of inoculation in LB



The graph shows the ratio of cells after 24 h of growth in LB medium with a ratio at a1:1:1. Error bars show 95% confidence intervals.

7.4 Discussion

Previous studies (Benoit *et al.*, 1995) (Bhattacharya, 1999) and (Du and Nickerson, 1996) showed that only crystalliferous strains can be activated in alkaline conditions and suggested that the protoxin in the spore coat may be responsible for the alkaline-activation phenotype. However, since toxin production is plasmid associated, it seems likely that loss of crystal production may reflect curing of the toxin-coding plasmid, which may, like pBtoxis, encode genes responsible for alkaline activation.

This study (section 6) indicates that the *ger* genes pBt084, pBt085 and pBt086 can produce alkaline responses in the absence of any toxin production in strain 4Q7*ger*. The alkaline environment in the midgut of many insects including mosquitoes may offer a selective advantage to spores able to germinate at the appropriate time in the gut (Jensen, 2003). The combination of *ger* genes encoding this function with toxin genes to damage the gut and kill the host insect may provide a double advantage to the bacteria.

The results presented in this chapter show that in competition to colonise intoxicated *A. aegypti* larvae, strain Bti 4Q5kan, carrying the pBtoxis plasmid, is able to outgrow the plasmid cured strain Bti 4Q7rif. This indicates a selective advantage for the presence of pBtoxis. Strain Bti 4Q7*ger* was also able to outgrow Bti 4Q7rif, indicating that the *ger* genes may be responsible for this effect, perhaps by allowing Bti 4Q5kan and Bti 4Q7*ger* a "head-start" by germinating more rapidly in the insect gut.

The exact mechanism, however, is unclear. These two strains were also able to outgrow Bti 4Q7rif where spores were inoculated into LB medium in which more rapid germination might not be expected and the results in table 7.3.5, indicate that outgrowth occurs even when vegetative cells are inoculated. Nevertheless, whatever the precise mechanism, these results indicate a competitive advantage for the strains that carry pBtoxis plasmid and its *ger* genes - not only in LB but of particular interest, in intoxicated mosquito larvae.

CHAPTER 8

General discussion

B. thuringiensis is a gram-positive spore forming bacterium in the complex group *Bacillus cereus sensu lato*. The capacity to form endospores is essential for survival in difficult environmental conditions such as shortage of nutrients. When conditions become favourable, spores return to the vegetative state by the process of germination (Moir *et al.*, 1994). Germination occurs in response to certain molecules in the environment, which indicate the right time for re-establishing the vegetative form (Moir *et al*, 2002).

The *B. cereus* group has a large number of plasmids that carry genes, which may produce strain phenotypes (Helgason *et al.*, 2000). These plasmids are exchangeable between *B. cereus* group members (Jensen *et al.*, 2003). *B. thuringiensis* is phenotypically similar to *B. cereus*, but with the ability to form parasporal crystal protein inclusions during sporulation, and these inclusions display toxic activity against many orders of insects and are used in insect control programmes worldwide (Whiteley and Schnepf., 1986; Delecluse *et al.*, 1991; Sorokin *et al.*, 2006). The insect pathogen *B. thuringiensis* produces insecticidal crystal inclusions (δ-endotoxins) inside the cell during sporulation (Whiteley and Schnepf., 1986). There are two main families of toxins, Cry toxins and Cyt toxins.

B. thuringiensis subsp. *israelensis* has two large plasmids: pXO16, which is able to mobilise other plasmids; and the toxin-encoding plasmid pBtoxis, which encodes many genes involved in determining the phenotype of *B. thuringiensis* subsp. *israelensis*. This plasmid has been sequenced and found to carry coding sequences other than toxin genes (Berry *et al.*, 2002) and many of these genes are transcribed (Stein *et al.*, 2006).

A number of transcriptionally active genes were identified with possible roles in germination (pBt084, pBt085 and pBt086) and others in antibiotic production (pBt136, pBt137 and pBt138) (Stein *et al.*, 2006). These genes were investigated to determine their possible roles, and also the role of midgut bacteria in mosquito larvae in *B. thuringiensis* subsp. *israelensis* insecticidal activity was studied.

The pBtoxis plasmid sequence was analysed again and revealed new information on several coding sequences (CDS). For instance CDS pBt001 now appears to be related to a putative transcriptional regulator but was originally annotated as "unknown function" (Berry, *et al;* 2002). Similarly the pBtoxis coding sequences pBt010, pBt013, pBt020, pBt030, pBt032, pBt034, pBt065, pBt066, pBt113, pBt114, pBt115, pBt116, pBt127, pBt146 and pBt150 were considered as hypothetical proteins and now changed to conserved hypothetical proteins as there are similar genes now present in the database.

The pBtoxis plasmid genes pBt136, pBt137 and pBt138 previously shown to be related to genes involved in the production of an antibiotic peptide AS - 48 were, in the re-analysis of the pBtoxis plasmid found to be related to production of bacteriocin (pBt136), bacteriocin maturation (pBt137) and bacteriocin secretion (pBt138). Also pBt139 may be related to bacteriocin transportation, whereas it was annotated as an ATP-binding protein in the previous analysis (Berry, *et al* 2002).

On the plasmid pBtoxis of *B. thuringiensis* subsp. *israelensis* CDSs pBt136, pBt137 and pBt138 may encode proteins with similarity to those necessary for the production of peptide antibiotic AS - 48 by *Enterococcus faecalis*, which has an effect on *Escherichia coli* and *Salmonella* (Martinez-Bueno *et al.*, 1998), or to bacteriocins. However no evidence of antibiotic production in *B. thuringiensis* subsp. *israelensis* was found. The possibility of antibiotic production under other growth conditions or against other bacterial species cannot, however, be ruled out. Our experiments only assessed the ability of *B. thuringiensis* subsp. *israelensis* to produce soluble antibiotics similar to AS - 48, whereas Revina *et al.* (2005) studied the activity of alkaline-solubilised proteins, unrelated to AS - 48 and not encoded on the pBtoxis plasmid. The antibiotic production of *B. thuringiensis* subsp. *israelensis* could affect other members of the midgut bacterial community in mosquito larvae, since several genera of bacteria can be found in the midgut.

Midgut bacteria were analysed from laboratory reared *Aedes aegypti* larvae and found to be similar to those reported by others (Pumpuni *et al.*, 1996; Demaio *et al.*, 1996; Straif *et al.*, 1998). The present study showed that growth is inhibited by elimination of the larval midgut flora and that re-introduction alone or together of *Microbacterium sp.* and/or *Enterobacter sp.* isolated from larvae restores the normal growth rate. This effect can be duplicated using the laboratory strain *Microbacterium oxydans* NRRL-B24236.

Midgut flora have been shown to contribute to the health of their insect hosts, as any alterations in the composition of the gut community may cause disease (Broderick *et al.*, 2009). It is difficult to explore the relation and role of gut bacteria with their host due to the fact that many of the gut flora may be difficult to grow in culture.

The role of midgut bacteria is not known but the midgut bacteria obviously have no role in the insecticidal activity of *B. thuringiensis* subsp. *israelensis*, since this bacterium is able to kill the larvae of *A. aegypti* independent of the presence of other midgut bacteria in contrast to reports for *B. thuringiensis* subsp *kurstaki* (Broderick *et al.*, 2006). Although the latter findings have been widely questioned (Johnston and Crickmore, 2009; Raymond *et al.* 2009; van Frankenhuyzen *et al.* 2010). *B. thuringiensis* subsp. *israelensis* kills the larvae and then uses the cadavers as source of nutrients for growth.

B. thuringiensis has been proposed to kill its hosts either by starvation caused by toxaemia, or septicaemia (Angus, 1962; van Frankenhuyzen *et al.* 2010). In the septicaemia model Cry toxin proteins damage the epithelial cells of the larval midgut leading to the mixture of midgut and haemolymph contents. This may generate favourable conditions for the *B. thuringiensis* spores to germinate. Then *B. thuringiensis* and other bacteria that are found in the gut in the haemocoel profilrate causing septicaemia (WHO, 1999; http://www.inchem.org). In this way, although *B. thuringiensis* is the primary cause of larval mortality, other bacteria may contribute to the process in *vivo* under normal, non-sterile conditions (Johnson and Crickmore, 2009 and Raymond *et al.*, 2009).

Germination is induced by the activation of spores and triggered by the binding of germinants to spore receptors. In this work activation was conducted by heat treatment at 70°C for 20 minutes or by alkaline treatment in 0.1 M sodium carbonate (pH 10) for 30 minutes. Three different germinants (L-alanine, inosine and nutrient broth) were used and the responses to these germinants were found to be similar under comparable conditions.

Heat activation showed no significant differences in germination between *B. thuringiensis* subsp. *israelensis* strains containing pBtoxis or pHT304*ger* and *B. thuringiensis* subsp. *israelensis* strains lacking the plasmid, except when alanine was used as the germinant the latter strains showed a slightly lower rate of germination. It appears that the features responsible for heat activation in these strains must be located on the chromosome of *B. thuringiensis* subsp. *israelensis*, although pBtoxis factors other than the *ger* genes may influence response to alanine.

In contrast, alkaline activation showed significant differences in the germination rates with all the germinants between strains containing pBtoxis and strains lacking this plasmid. This effect could be reversed by complementation with the pBt084, pBt085 and pBt086 genes in *B. thuringiensis* subsp. *israelensis* strain 4Q7 *ger*. This clearly indicates a role for these genes in the alkaline activation of spores.

The alkaline activation occured from pH 9 and above, whereas *B. thuringiensis* subsp. *israelensis* strains did not germinate in significant rate after alkaline activation at pH 8 and 8.5. These results provide the first evidence of a

contribution of pBtoxis to host phenotype, other than production of toxins and, thus, represents the first report to illustrate the potential importance of genes that are co-inherited with the toxins.

Previous studies (Benoit *et al.*, 1995) (Bhattacharya, 1999) and (Du and Nickerson, 1996) showed that only crystalliferous strains can be activated in alkaline conditions and suggested that the protoxin in the spore coat may be responsible for the alkaline-activation phenotype. However, since toxin production is plasmid associated, it seems likely that loss of crystal production may reflect curing of the toxin-coding plasmid, which may, like pBtoxis, encode genes responsible for alkaline activation.

This study indicates that the *ger* genes pBt084, pBt085 and pBt086 can produce alkaline responses in the absence of any toxin production in *B. thuringiensis* subsp. *israelensis* strain 4Q7 *ger*. The alkaline environment in the midgut of many insects including mosquitoes may offer a selective advantage to spores able to germinate at the appropriate time in the gut (Jensen, 2003). The combination of *ger* genes encoding this function with toxin genes to damage the gut and kill the host insect may provide a double advantage to the bacteria.

The results of co-feeding of *B. thuringiensis* subsp. *israelensis* 4Q5 and *B. thuringiensis* subsp. *israelensis* 4Q7 to *A. aegypti* larvae have shown that the Bti 4Q5kan, strain carrying the pBtoxis plasmid, and strain Bti 4Q7*ger* (strain with *ger* genes only) are able to outgrow the plasmid-cured strain Bti 4Q7rif. This result suggests a selective advantage for the presence of pBtoxis, and also indicates that the *ger* genes may be responsible for this effect, perhaps by allowing Bti 4Q5kan and Bti 4Q7*ger* a

"head-start" by germinating more rapidly in the insect gut. The precise mechanism is not clear but these results indicate a competitive advantage for the strains that carry pBtoxis plasmid and its *ger* genes - not only in LB but also of particular interest, in intoxicated mosquito larvae.

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Genetic Basis for Alkaline Activation of Germination in *Bacillus thuringiensis* subsp. *israelensis*[▽]

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Differences in activation between spores from strains of *Bacillus thuringiensis* subsp. *israelensis* with and without the toxin-encoding plasmid pBtoxis are demonstrated. Following alkaline activation, the strain bearing pBtoxis shows a significantly greater germination rate. Expression of just three genes constituting a previously identified, putative *ger* operon from this plasmid is sufficient to produce the same phenotype and characterizes this operon as a genetic determinant of alkaline activation.

Bacillus thuringiensis is a member of the *Bacillus cereus* sensu lato complex of bacilli and is distinguished by its ability to produce parasporal crystalline inclusions that may be toxic to invertebrates, usually insects (13). Spores and crystals are ingested by susceptible larvae, and the constituent Cry and/or Cyt crystal proteins are solubilized and proteolytically processed in the gut to release the active toxins (7). *B. thuringiensis* spores are able to germinate in the intoxicated larva and exploit the rich nutrient resources of the insect cadaver. Germination is triggered in response to germinants, usually nutrients that signal favorable conditions for growth (15). Like other *B. cereus* group bacteria, *B. thuringiensis* will germinate in response to nonspecific germinants, such as nutrient broth, and to specific germinants including L-alanine or ribonucleosides, such as inosine, and the process can be visualized by a loss of phase brightness (2).

In the laboratory, spores can be "activated" (induced to relatively synchronous germination) by conditions such as brief sublethal heat treatment or preincubation in an alkaline environment. The latter treatment is used most often in laboratories studying *B. thuringiensis* since such a treatment is more physiologically relevant than heat activation because most insects have alkaline guts in which the pH stimulates germination (18). Despite this interesting and relevant phenomenon, the genetic basis for alkaline activation has not been elucidated, although an association between alkaline activation and crystal production in strains of *Bacillus thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* has been noted (2, 4).

The genes encoding the crystal toxins are usually extrachromosomally borne on high-molecular-weight, stringent plasmids (14), and to date the sequence of only one such plasmid, pBtoxis from *B. thuringiensis* subsp. *israelensis*, has been reported (3). In addition to genes encoding four Cry proteins and three Cyt proteins, pBtoxis appears to encode a range of other proteins, including factors that may be involved in spore germination. The pBtoxis genes pBt084, pBt085, and pBt086 show similarities to previously reported *ger* genes (encoding proteins similar to the *Bacillus subtilis* germination protein GerAC and *B. cereus* proteins GerIB and GerIA, respectively). In addition, the plasmid bears two apparent pseudogenes, pBt060 and pBt063, that are similar to pBt086 and pBt085, respectively (3). Transcriptional analysis showed no mRNA production from the apparent pseudogenes but confirmed transcription from pBt084, pBt085, and pBt086 as a single operon (17). Related operons appear to exist in the genomes of *B. cereus* G9842 and *Bacillus weihenstephanensis* KBAB4 (EMBL accession numbers CP001186 and CP000903, respectively), but only distantly related genes appear in the completed genomes of *B. thuringiensis* strains. A close homolog (>70% identity at the level of the three proteins) has, however, recently been reported from *B. thuringiensis* subsp. *kurstaki* HD73 (accession number EF618567), but it is not clear whether this is a genomic or plasmid-borne operon in this strain.

In this study, we demonstrate that *B. thuringiensis* subsp. *israelensis* strains with and without the pBtoxis plasmid show different germination responses. In particular, different rates of germination are seen following an alkaline activation step, and the pBt084-pBt085-pBt086 operon alone is responsible for this phenomenon.

Bacterial strains, plasmids, and growth conditions. The plasmid pHT304 (1) was kindly provided by D. Lereclus of the Institut Pasteur, Paris, France. *Bacillus thuringiensis* subsp. *israelensis* strain 4Q5, cured of all plasmids except pBtoxis and an approximately 15-kb linear DNA element, and strain 4Q7, which is cured of all plasmids, including pBtoxis (5), were obtained from the *Bacillus* Genetic Stock Center, Ohio State University. All strains were grown at 30°C in Embrapa medium (16) for 4 to 7 days until sporulation, as assessed by phase-contrast microscopy, was in excess of 95%. Sporulated cultures (1 ml) were centrifuged in a microcentrifuge at 1,300 rpm and washed six times in a concentration of 1/4 strength Ringer's solution (Oxoid) by repeated centrifugation before resuspension in 50 μl of the same solution.

Activation of spores. Spores were activated by two alternative methods. Incubation of spore resuspensions in a water bath at 70°C for 20 min was used to achieve heat activation without a loss of spore viability (12). To induce alkaline activation, spores were harvested and washed twice by centrifugation with sterile water. Spore pellets were then resuspended (to

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an approximate attenuance of 0.5 at 600 nm) in 0.1 M sodium carbonate (pH 10) and incubated at room temperature for 30 min with gentle rocking using a shaking platform. The spores were then centrifuged and washed three times in 1/4-strength Ringer's solution before use in germination assays.

Germination. The following three germinants were used in this study: (i) the complex germinant nutrient broth (Sigma); (ii) the single germinant inosine (5 mM); and (iii) the single germinant L-alanine (10 mM), to which 13 mM D-cycloserine (Sigma) was added to prevent the activity of alanine racemase, which converts L-alanine to D-alanine, an inhibitor of germination (6).

Germinant solutions (5 μl) were added to equal volumes of the spore suspensions in PCR tubes and mixed thoroughly. Then, 5 μl of the mixture was pipetted onto a microscope slide, which had been incubated at 35°C over a water bath, and a coverslip was added to allow examination of the sample. Germination was measured using a Leitz Diaplan phase-contrast microscope at a magnification of ×1,250. Prior to germination, all spores appear phase bright, but when the germination process begins they rapidly become phase dark. On this basis, germination can be measured by counting the proportion of phase-dark spores out of a sample of 300 spores in the field of view at different time points. The experiment was repeated at least three times with each strain for each germinant. We used general linear models to explain the variance in the response, using strain, replicate, and time as candidate predictor variables. We considered models including the main effects and each of the possible two-way interaction terms. Analyses were carried out using the SPSS v16 program (SPSS Inc., Chicago, IL).

Cloning of pBtoxis *ger* operon. The germination genes of the pBtoxis plasmid (nucleotides 69,060 to 73,121 [3]) were amplified from vegetative cells of *B. thuringiensis* subsp. *israelensis* strain 4Q5 by using the following primers: 5′-CATTGAAAC GAGCAAATGTCA-3′ and 5′-CCTTTGCACCAAATCCTT TT-3′. The PCR analysis using *Taq* polymerase (Promega) was carried out with an annealing temperature of 49°C and an extension time of 4 min at 72°C for the first 10 cycles, followed by a further 20 cycles with a 1-min extension period. The approximately 4-kb amplicon was cloned using the TOPO TA cloning kit (Invitrogen). The region was then excised using EcoRI, and the fragment was recloned into pHT304 (1) and transformed into *Escherichia coli* strain DH5α. The resultant pHT304-ger plasmid DNA was isolated using the Qiagen Midi kit following the manufacturer's recommendations, and the inserted DNA was sequenced prior to its introduction into strain 4Q7 by electroporation as previously described (11) to produce the strain 4Q7-ger.

Germination of strains. In the absence of any prior activation step, *B. thuringiensis* strain 4Q5 containing pBtoxis or strain 4Q7 cured of this plasmid was incubated with inosine as the germinant, and the percent germination was followed over 20 min. Germination for strain 4Q5 reached approximately 28%, a level that was significantly higher ($P = 0.002$) than that for 4Q7 and 4Q7-ger (approximately 23%) (data not shown).

Following heat activation, germination rates were higher than those without heat treatment, with no significant differences between strains when either nutrient broth or inosine was used as a germinant (not shown). In response to L-alanine, strain 4Q5 showed a slightly higher germination rate ($P = 0.004$) at 20 min than 4Q7 (Fig. 1), but strain 4Q7-ger was indistinguishable from 4Q7 ($P = 0.862$), indicating that the *ger* genes play no role in the heat activation response to L-alanine. Both strains 4Q5 and 4Q7 were produced from the same parental *B. thuringiensis* strain 4Q2 (5), so the difference in germination phenotype between 4Q5 and 4Q7 could be due to factors present on elements found in the former strain but cured from the latter, including the pBtoxis plasmid and the linear DNA element first described in *B. thuringiensis* subsp. *israelensis* by González and Carlton (9). Although no other transcriptionally active *ger*-like genes were identified on pBtoxis (3, 17), there are numerous coding sequences of unknown function and many putative regulator genes that could, in principle, help to produce these responses by their action on other plasmid or chromosomal genes.

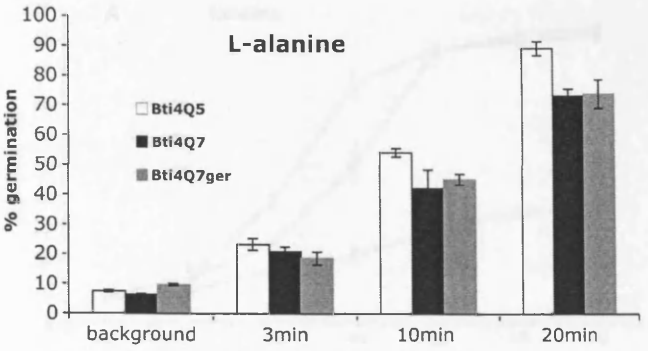

FIG. 1. Germination responses of *Bacillus thuringiensis* subsp. *israelensis* (Bti) strains after heat activation with 10 mM L-alanine. Experiments were repeated three times. Error bars are shown as standard errors of the means after calculation of standard deviation.

Following alkaline treatment, strain 4Q5 germinated at a high rate in response to all germinants, while strain 4Q7 showed a much lower germination rate (a P value of 0.043 for the inosine germinant) (Fig. 2), indicating a role for the pBtoxis plasmid in enhancing germination following alkaline activation. Strain 4Q7-ger germinated very strongly in a manner statistically indistinguishable from strain 4Q5 (a P value of 0.973 for the inosine germinant) (Fig. 2). To analyze this effect further, the germination responses were followed over a time course of 40 min. Again, strains containing the pBtoxis *ger* genes (4Q5 and 4Q7-ger) germinate more rapidly than strain 4Q7 in response to both inosine and alanine after alkaline activation, with differences that are noticeable at 10 min and very clear from 15 min onwards (Fig. 3).

Alkaline activation and different pH. We then investigated the effect of the pH of preincubation on the ability to promote alkaline activation. Spores of strains 4Q5, 4Q7, and 4Q7-ger were incubated in either Tris-HCl buffer (pH 8 and pH 8.5) or sodium carbonate buffer (pH 9 and pH 10) to attempt spore activation. At pH values of 8 and 8.5, germination rates were comparable to those of unactivated spores. At pH values of 9 and 10, a weak alkaline induction of germination in response to inosine was seen with strain 4Q7; however, the presence of the *ger* genes (on pBtoxis or pHT304-ger) leads to a marked increase in responsiveness to alkaline activation at these pH values (Fig. 4A to C).

Following alkaline activation, significant differences were

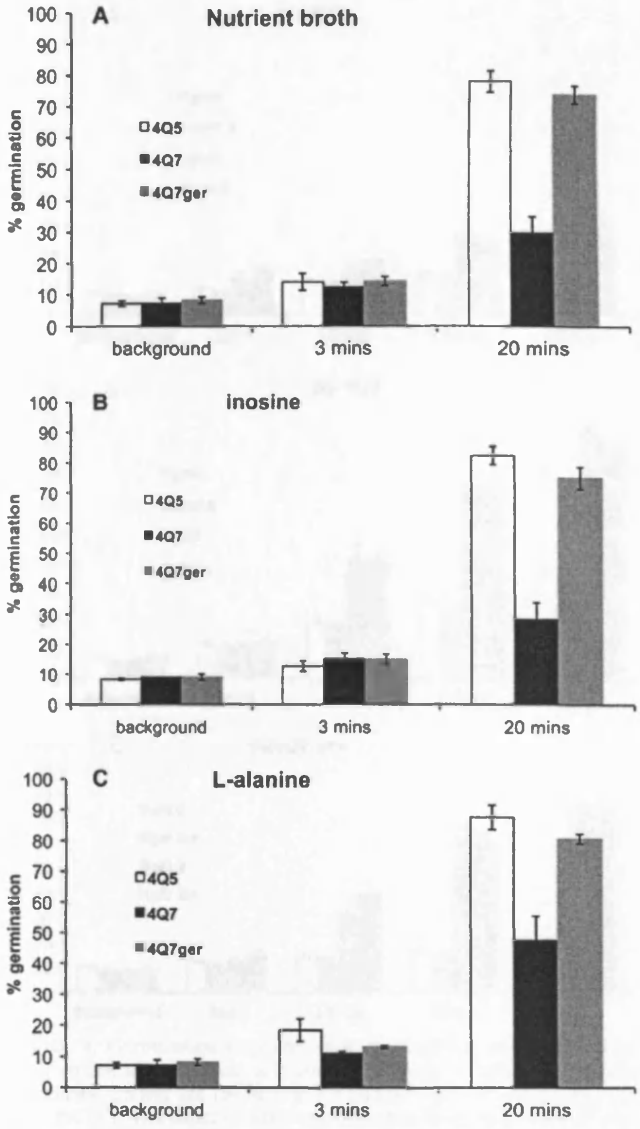

FIG. 2. Germination responses of *B. thuringiensis* subsp. *israelensis* strains after alkaline activation at pH 10. Shown are results for nutrient broth (A), 5 mM inosine (B), or 10 mM L-alanine (containing 13 mM D-cycloserine) (C). Experiments were repeated three times. Error bars represent the standard errors of the means after calculation of the standard deviation.

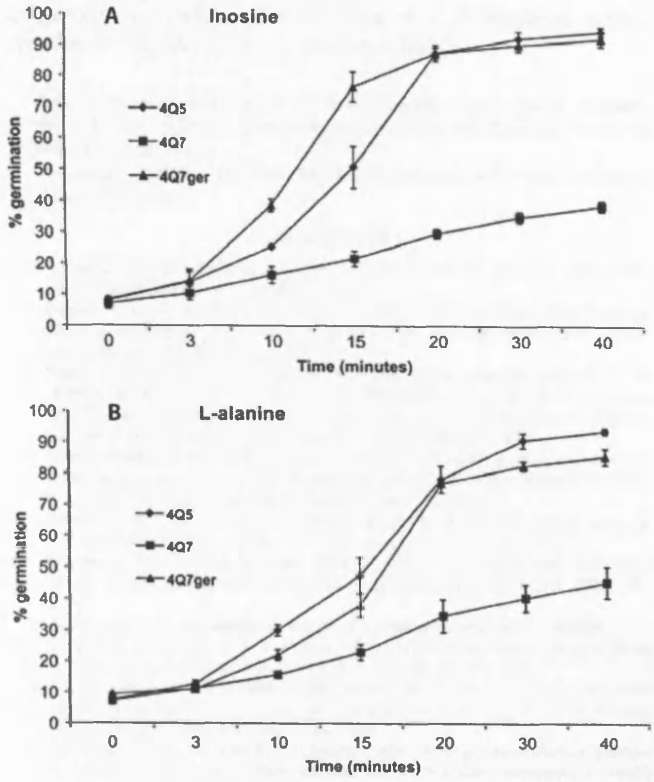

FIG. 3. Time course of germination responses of *B. thuringiensis* subsp. *israelensis* (Bti) strains after alkaline activation. Germinants used were 5 mM inosine (A) and 10 mM L-alanine (containing 13 mM D-cycloserine) (B). Experiments were repeated three times. Error bars represent the standard errors of the means after calculation of the standard deviation.

observed in the germination rates with all the germinants between strains 4Q5 and 4Q7. In contrast to the unactivated and heat-activated responses to L-alanine (as described above), the loss of the alkaline activation response in strain 4Q7 could be reversed by complementation with the pBt084, pBt085, and pBt086 genes in strain 4Q7-ger. This clearly indicates a role for these genes in the alkaline activation of spores.

The nature of the alkaline activation response is unknown and little studied. In contrast to heat activation, alkaline activation is irreversible and sensitive to ethanol (8), perhaps suggesting some permanent change as a result of alkaline treatment. We might speculate that the alkaline conditions may be causing an effect such as a change in permeability of the spore, structural changes in spore proteins, or stripping of the spore coat. Whatever the mechanism, the pBtoxis *ger* genes appear to mediate this effect in some way to offer a specific alkaline activation response that is not available to strains lacking these genes. The midguts of many insects, including mosquitoes, are alkaline and it is possible that a selective advantage may be gained by spores able to germinate at the appropriate time in the gut (10).

Our findings significantly extend those of several other authors (2, 4, 8) who have shown in vitro and using insect gut extracts that alkaline activation is a feature of crystal-forming strains that is lacking from acrystalliferous strains. These previous results have been interpreted to suggest a role for the protoxin in the coat of the spore in the alkaline activation phenotype. However, since the *ger* genes described in our study colocalize with the crystal toxin genes on the pBtoxis plasmid, a similar situation may prevail in the *B. thuringiensis* strains used in previous studies, and the loss of crystal production may reflect curing of the toxin-coding plasmid along with *ger* genes of the type found on pBtoxis. The complementation of the alkaline activation response by pHT304-ger indicates that the *ger* genes pBt084, pBt085, and pBt086 can produce this effect in the absence of any toxin production in strain 4Q7-ger.

The combination on the pBtoxis plasmid of toxin genes with

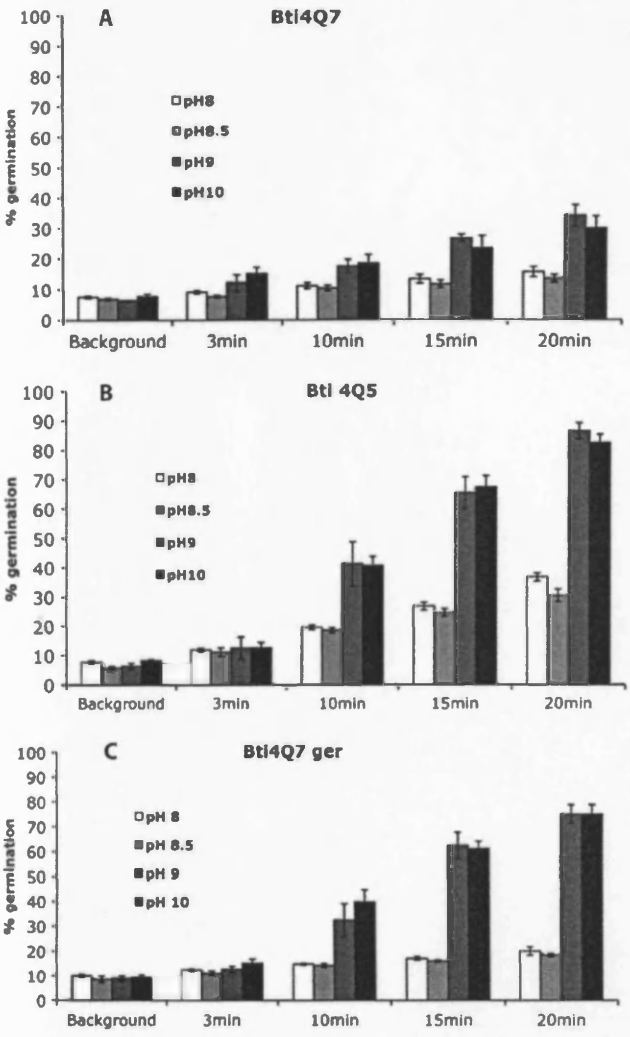

FIG. 4. Germination responses of *B. thuringiensis* subsp. *israelensis* (Bti) strains after alkaline activation at a range of pH with inosine germinant. Shown are the results for strains 4Q7 (A), 4Q5 (B), and 4Q7-ger (C). The experiments were repeated three times. Error bars represent the standard errors of the means after calculation of the standard deviation.

ger genes may exert a multifactorial influence on the phenotype of the host bacterium to coordinate germination under the alkaline conditions of the gut with toxin damage to the gut and death of the host insect. The potential significance of the effect of germination rate on the virulence of *B. thuringiensis* subsp. *israelensis* is currently under investigation.

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