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CONTENTS

The Utility of Strains of Morphological Group II *Bacillus*

SAMUEL SINGER

Phytase

RUDY J. WODZINSKI AND A. H. J. ULLAH

The Insecticidal Proteins of *Bacillus thuringiensis*

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I. Introduction

Bacillus thuringiensis (Bt) is a gram-positive, aerobic, endospore-forming bacterium belonging to morphological group I along with *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus laterosporus* (Parry *et al.*, 1983). All these bacteria have endospores. Bt, however, is recognized by its parasporal body (known as the crystal) that is proteinaceous in nature and possesses insecticidal properties. These insecticidal proteins, synthesized during sporulation, are tightly packed by hydrophobic bonds and disulfide bridges. Various forms of true crystals have been observed using phase contrast microscope (Srinivas *et al.*, 1995; Jung *et al.*, 1995). The most common shape is a bipyramidal structure (Fig. 1). A Bt mutant defective in sporulation accumulates insecticidal proteins to form large crystal inclusion (Fig. 2) that remained encapsulated within the ghost

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 42

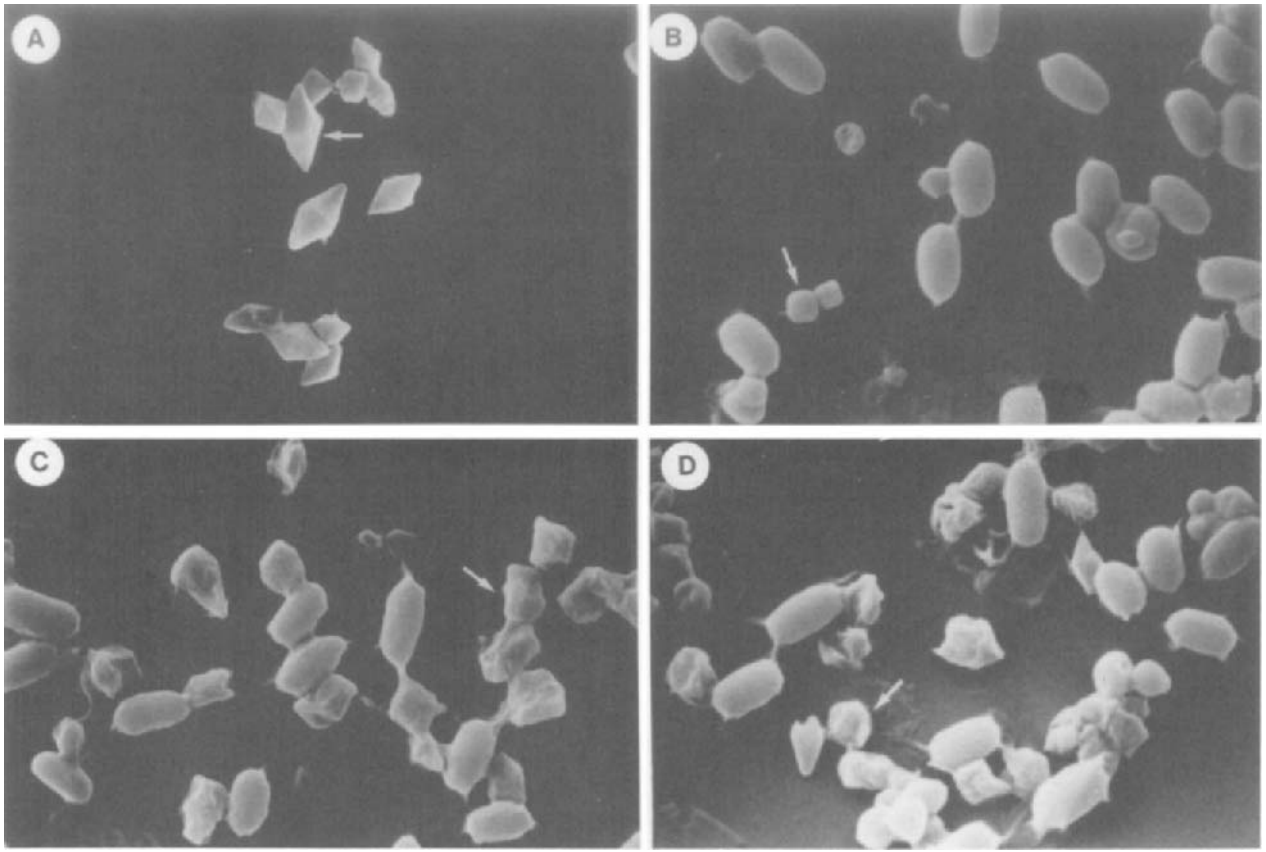


FIG. 1. Scanning electron micrograph of *Bacillus thuringiensis* crystals: (A) bipyramidal crystals produced by a lepidopteranactive strain; (B) spherical crystals produced by a mosquito-active strain; (C and D) irregularly shaped crystals produced by nontoxic strains (arrows indicate crystals). Reproduced with permission from Chilcote, C.N. and Wigley P.J. (1994). *Agric.* Eco systems *Environ.* **49**, 51–57.

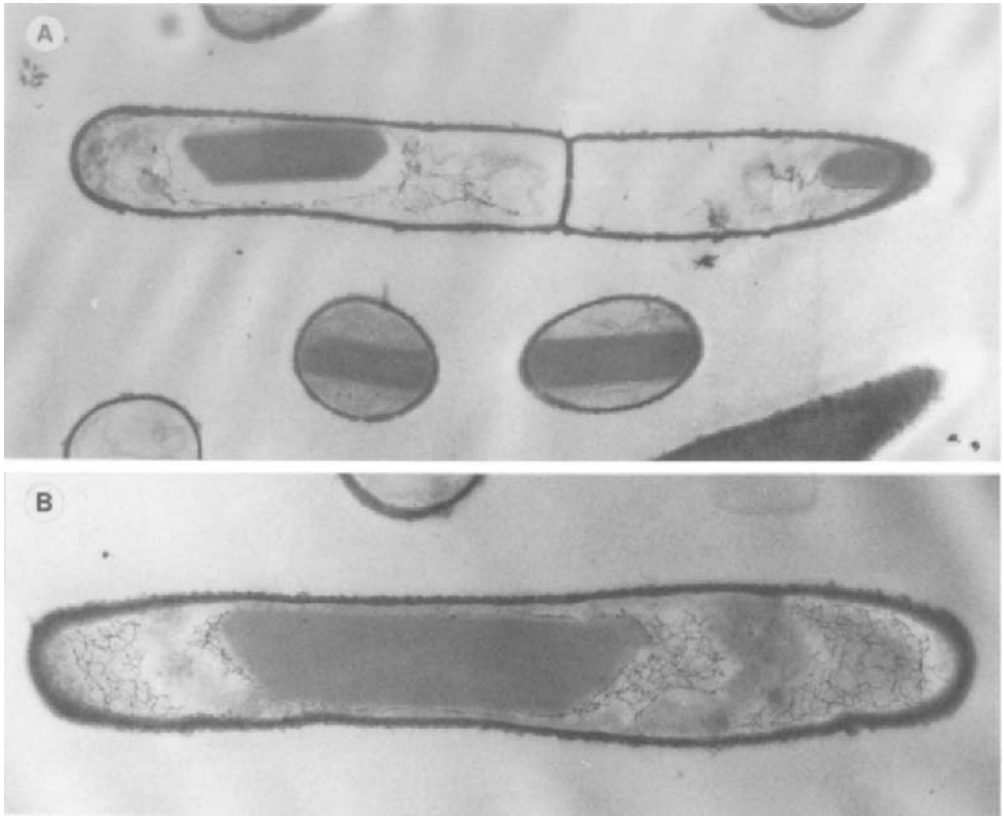
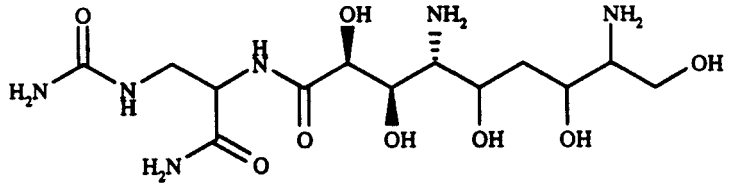


FIG. 2. Electron micrographs of a SpoOA mutant strain overproducing the CryIIIA crystal protein. Reproduced with permission from D. Lereclus, Institut Pasteur, Paris.

cell (Lereclus *et al.*, 1915). The first record on Bt goes back to 1901, when Ishiwata discovered a bacterium from diseased silkworm larvae that he named *Bacillus sotto* (Ishiwata, 1901). Between 1909 and 1912, Berliner (1915), working at a research station for grain processing in Berlin, investigated an infectious disease of the Mediterranean flour moth *(Ephestia kuehniella).* The infected insects were originally obtained from a mill in the district of Thueringen. In a detailed report, Berliner (1915) described a spore-forming bacterium as the causative agent and designated it as *B. thuringiensis.*

The first practical application of Bt was reported by Husz (1928) who isolated a Bt strain from *Ephestia* and tested it on European corn borer. This work eventually led to the first commercial product, Sporeine, which was produced in France in 1938 (Luthy *et al.*, 1982). The development of potent organic insecticides, however, prevented the interest for biological alternatives for pest control to some extent. The pioneering research of Steinhaus (1951) on Bt and a growing realization that organic insecticides are deleterious to the environment and human health spurred a renewed interest in Bt in the 1960s. This led to the introduction of viable Bt biopesticides like Thuricide and Dipel. For many years, the inclusion body protein and spores were generally recognized as the two essential ingredients for most of the insecticidal activity of *B. thuringiensis.* Scientists at the Sandoz company and Asano and Hori (1995) discovered in the supernatant of the *B. thuringiensis* a growth medium potency-enhancing factor, Kurstakolin (Fig. 3), which enhances the insecticidal activity of *B. thuringiensis* cellular preparations by 30%.

There are many subspecies and serotypes of Bt with a range of well-characterized insecticidal proteins or Bt toxins. Known Bt toxins kill subsets of insects among the Lepidoptera, Coleoptera, Diptera (Hofte and Whiteley, 1989), and nematodes (Feitelson *et al.*, 1992). The host range of Bt has expanded considerably in recent years due to extensive



$C_{13}H_{28}N_6O_8$

FIG. 3. Structure of Kurstakolin.

screening programs (Table I). By virtue of the lack of toxicity toward other species of animals, human beings, and plants, there is tremendous potential for exploiting Bt as a biological control agent (Jones and Khachatourians, 1995; Salama *et al.,* 1995; Bradley *et al.,* 1995).

Various aspects of Bt fermentation (Capalbo, 1995; Gangurde and Shethna, 1995), biology and genetics (Bulla *et al.,* 1978; Aronson, 1986), molecular biology (Hofte and Whiteley, 1989; Yoshisue *et al.,* 1995a; Dervyn *et al.,* 1995), mechanism of action (Gill *et al.,* 1992; Knowles, 1994), application as biopesticide (Gawron-Burke and Baum, 1991;

TABLE I

HOST RANGE OF *Bacillus thuringiensis*

		Susceptible families	
Order	Toxin	Family	Example
Insecta			
Lepidoptera	δ-Endotoxin	Most lepidopteran families susceptible examples	
		Spingidae	Hawkmoths
		Pieridae	Cabbage worms
		Lymantriidae	Tussock moths
		Tortricidae	Leafroller moths
		Noctuidae	Cutworms/armyworms
Diptera	δ-Endotoxin	Culicidae	Mosquitoes
		Simuliidae	Blackflies
		Anisopodidae	Gnats
		Chironomidae	Midges
		Psychodiae	Moth flies
		Sciaridae	Black fungus gnats
		Tipulidae	Craneflies
	Thiringiensin	Muscidae	Houseflies
		Calliphoridae	Blowflies
Coleoptera	δ-Endotoxin	Chrysomelidae	Leaf beetles
Phthiraptera		Philopteridae	Bird lice
		Trichodectidae	Mammalian lice
Arachnida			
Acari	Thuringiensin	Dermanyssidae	Animal mites
		Tetranychidae	Phytophagous mites
Nematoda			
Strongylida	?	Trichostrongylidae	Animal endoparasitic nematodes
Tylenchida	?	Tylenchidae	Phytophagous nematodes

Aronson, 1994, Pedersen *et al.*, 1995; Farrar and Ridgway, 1995; Yang *et al.*, 1995; Gibson *et al.*, 1995; Li *et al.*, 1995), and Bt transgenic plants (Peferoen, 1992; Kumar and Sharma, 1994) have been reviewed. Here, the classification and mode of action of Bt toxins are discussed. Strategies to screen new Bt strains/genes, expression of the toxin protein in transgenic microorganisms (Shin *et al.*, 1995), and plants and various resistance management strategies in agricultural systems are examined. The review puts emphasis on agricultural application of Bt.

II. Classification of Bt Toxins

A large number of Bt isolates are now available in laboratories around the world (Schnepf, 1995; Jung *et al.*, 1995; Burtseva *et al.*, 1995; Shin *et al.*, 1995). New strains are being added every year. Bt strains can be characterized by a number of techniques including serotyping, crystal serology, crystal morphology, protein profiles, peptide mapping, DNA probes, and insecticidal activity. De Barjac first attempted to classify Bt toxins based on flagellar (H) agglutination (De Barjac and Bonnefoi, 1962). Recently, the classification of Bt based on H antigen was revised (De Barjac and Franchon, 1990) (Table II). More than 40 H-serotypes are

TABLE II

CLASSIFICATION OF *Bacillus thuringiensis*

H-antigen	Variety	Toxicity[a]
1	*thuringiensis*	L,D
2	*finitimus*	
3a,3c	*alesti*	L
3a,3b,3c	*kurstaki*	L,D
3a,3d	*sumiyoshiensis*	
3a,3d,3e	*fukuokaensis*	D
4a,4b	*sotto*	L
4a,4c	*kenyae*	L,D
5a,5b	*galleriae*	L,C
5a,5c	*canadensis*	L
6	*entomocidus*	L
6a,6c	*oyamensis*	
7	*aizawai*	L,D
8a,8b	*morrisoni*	L,D,C
8a,8c	*ostriniae*	L
8b,8d	*nigeriensis*	

(*continues*)

TABLE II—*Continued*

H-antigen	Variety	Toxicity[a]
9	*tolworthi*	
L,D10a,10b	*darmstadiensis*	L,D
10a,10c	*londrina*11a,11b	
toumanoffi		
11a,11c	*kyushuensis*	L,D
12	*thompsoni*	L,D13
	pakistani	
14	*israelensis*	D
15	*dakota*	
16	*indiana*	
17	*tohokuensis*	
18a,18b	*kumamotoensis*	C
18a,18c	*yosoo*	
19	*tochigiensis*	
20a,20b	*yunnanensis*	L
20a,20c	*pondicheriensis*	L
21	*colmeri*	
22	*shandongiensis*	L
23	*japonensis*	C
24a,24b	*neoleonensis*	
24a,24c	*novosibirsk*	
25	*coreanensis*	
26	*silo*	
27	*mexicanensis*	L
28a,28b	*monterrey*	
28a,28c	*jegathesan*	D
29	amagiensis	
30	*medellin*	D
31	*toguchini*	
32	*cameroun*	
33	*leesis*	
34	*konkukian*	
35	*seoulensis*	
36	*malaysiensis*	D
37	*andalousiensis*	
38	*oswaldocruzi*	
39	*brasiliensis*	
40	*huazhongensis*	
41	*sooncheon*	
42	*jinghongiensis*	
43	*guiyangiensis*	
44	*higo*	
45	*roskildiensis*	

[a] *L, lepidopteran active; D, dipteran active; C, coleopteran active.*

now available and in many of these the array of Bt toxin genes present in isolates from a particular serovar are the same (Rabinovitch *et al.*, 1995). A notable exception is the presence of very different Bt toxin genes in subspecies morrisoni and tenebrionis within serotype 8a,b. Some of the serotypes are divided into subserotypes that can be differentiated by PCR (Bourque *et al.*, 1993; Brousseau *et al.*, 1993). However, a high level of sequence similarity among *B. anthransis, B. cereus, and B. Thuringiensis* does not permit construction of sequence-specific probes to be used in identification (Bourque *et al.*, 1994).

The most useful scheme for classification of Bt toxins is based primarily on homology of toxin gene sequences and the spectrum of insecticidal activity (Hofte and Whiteley, 1989; Ogiwara *et al.*, 1995). A large number of distinct Bt toxin genes have been cloned and sequenced since the first report published in 1981 (Schnepf and Whiteley, 1981). Hofte and Whiteley (1989) have classified 42 Bt genes into 14 distinct types and grouped them into four major classes. The classes are *cryI* (Lepidoptera specific), *cryII* (Lepidoptera and Diptera specific), *cryIII* (Coleoptera specific), and *cryIV* (Diptera specific). Many more Bt genes have since been sequenced and analyzed. Following the analysis of toxin domains of 29 distinct Bt toxin proteins, Feitelson *et al.* (1992) added two new major classes, *cryV* and *cryVI.* Several novel genes were also added within the previously defined classes (Table III). The nomenclature of Hofte and Whiteley (1989), based mainly on insecticidal activity, failed to accommodate genes that were highly homologous to known genes but with a different insecticidal spectrum. *cryIIA* and *IIB* were included in the Diptera-specific class because it is known that *cryIIB* is inactive against Diptera. *cryIC* is toxic to both Diptera and Lepidoptera (Smith and Ellar, 1994). Several genes with differing homology and bioactivity were named *cryV*, the next available Roman number in the original system (Gleave *et al.*, 1992, Tailor *et al.*, 1992).

Based on amino acid identity of full-length gene products, Crickmore *et al.* (1996) have introduced a systematic nomenclature for classifying the *cry* genes and their protein products. Most *cry* genes retain the name assigned by Hofte and Whiteley with a substitution of Arabic for Roman numerals (e.g., *cryl Aa*) to accommodate the newly discovered genes. Fifty genes comprising 16 homology groups are systematically arranged. Their dendrogram depicts the possible evolutionary relationships between the entire set of Bt toxins. Primary through quaternary ranks are based on 45, 75, and 95% level of sequence identity. Eighteen sets at the primary rank, CytA, CytB, and Cry1 through -16, are defined into 4 homology groups. Cry1, -3, -4, -7, -8, -9 and -10 form the largest group. Cry2 and Cry11 are the second group. The third group is Cry5, -12, -13 and -14. The fourth group is the two Cyt proteins. The Cry6, -15, and -16 consist of unique proteins.

TABLE III

Bacillus thuringiensis CRYSTAL PROTEIN GENES

Gene designation	Predicted M_r	Toxicity[a]
cryIA(a),(b),(c)	131–133	L
IB	137	L
IC	134	L
ID	133	L
IE	137	L
IF	134	L
IG	130	L
cryIIA	71	L,D
IIB	71	L
IIC	71	L
cryIIIA	73	C
IIIB	73	C
IIIC(a),(b)	73	C
cryIVA	134	D
IVB	128	D
IVC	77	D
IVD	72	D
cryV	80	L,C
Genes not yet cloned	130	?
	100	?
	40	?

[a] L, Lepidoptera; D, Diptera; C, Coleoptera; Based on Hofte and Whiteley (1989).

Crickmore *et al.* (1996) define *cry* as a gene from *B. thuringiensis* encoding a parasporal inclusion protein that exhibits pesticide activity or is homologous to a known *cry* gene.

1. The mnemonic cry shall remain for the crystal-forming pesticidal genes from B. thuringiensis. The cry gene nomenclature shall be distinguished at all ranks on the basis of comparative amino acid sequence identity of the full-length gene products.
2. The primary rank of the nomenclature shall be Arabic numbers. The *cry* genes whose products share less than 45% amino acid homology shall be characterized by different Arabic numbers.
3. The secondary rank shall be an uppercase letter. The *cry* genes of the same rank whose products show less than 75% homology shall be separated into different secondary ranks.
4. The tertiary rank shall be a lowercase letter without parentheses. The *cry* genes whose products share less than 95% homology shall be given different tertiary ranks.

5. The quaternary rank shall be allele numbers. The *cry* genes whose products differ in amino acid sequence, but are more than 95% identical to each other, shall be given separate quaternary ranks.

Crickmore *et al.* (1996) are the *B. thuringiensis cry* Gene Nomenclature Committee, a standing committee of the Bacillus Genetic Stock Center. They will assist workers in the field of *B. thuringiensis* genetics in assigning names of new *cry* genes and periodically review the literature of the *cry* genes.

III. Structure of Bt Toxin Proteins and Genes

Bt toxin genes are usually plasmid borne (Gonzalez *et al.*, 1995) but also chromosomally located (Carlson and Kolsto, 1993; Klier *et al.*, 1982; Kronstad *et al.*, 1983). The Bt toxin genes are encoded on plasmids of molecular weight 40–150 mDa (Carlton and Gonzalez, 1985; Jensen *et al.*, 1995). Most of the plasmids are of low copy number. In addition to the toxin-encoding plasmids, there are often several other cryptic plasmids of 4–150 mDa whose function is not clearly known. Many of the plasmid-encoded toxin genes are bordered by transposons and/or insertion sequences (Delecluse *et al.*, 1990). Dervyn *et al.* (1995) examined the transcriptional regulation of the *cryIVD* gene operon from *B. thuringiensis* subspecies *israelensis.*

Hofte and Whiteley (1989) compared sequences among a number of toxins with varying specificities and found five well-conserved regions designated blocks 1–5 (Fig. 4). Exceptions to this include CryIVC toxin of Bt subspecies israelensis and a novel toxin from subspecies *thompsoni* (Brown and Whiteley, 1992). Blocks 1 and 2 are very hydrophobic and are present as amphipathic α-helices with membrane-spanning potential. The protoxins designated CryIA–CryIG, CryIVA, and CryIVB contain 1100–1200 amino acids and the toxin is processed from within the amino half as shown in Fig. 4. The CryII, CryIII, and CryIVD protoxins are smaller, with processing to toxins as indicated. The carboxyl halves of the CryI, CryIVA, and CryIVB protoxins are also highly conserved except that there is a deletion of 26 amino acids in CryIA(b) protoxins.

On the basis of the conservation of the defined blocks, it was postulated that all of the Bt toxins probably have a three-dimensional conformation similar to that of a CryIIIA toxin reported by Li *et al.* (1991) (Fig. 5). According to this, the first 285 residues are present as a bundle of seven amphipathic α-helices, wherein six are arranged in a circle, and helix 5 is in the center (domain I). Residues 286–500 are organized as three β-sheets (domain II) and contribute to the toxin specificity. The remaining amino acids are also present as β-sheets and arranged like a