

COMPARATIVE BIOCHEMISTRY OF SELECTED STRAINS OF
BACILLUS THURINGIENSIS

by

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PREFACE

The research data in this thesis is presented in the form in which it was submitted for publication. Section II was published in the October 1979 issue of Applied and Environmental Microbiology, Section III will be submitted for publication in the Journal of Bacteriology, and Section IV will be submitted to Archives of Biochemistry and Biophysics. Section I is an introduction and review of the literature which was designed to give the reader background information on practical applications and molecular biology of Bacillus thuringiensis. Section V, the appendix, contains additional pertinent data.

The work of some other investigators is included in Section III: The electron micrograph of the B. thuringiensis subspecies kurstaki cell and the sectioning of the subspecies israelensis cell was done by Donald B. Bechtel, the alkali solubilization of crystals, column chromatography, and immunological work was done by Dr. Robert Andrews; and the carbohydrate analysis was done by Dr. Phillip Nordin.

I would like to thank Dr. Lee A. Bulla, Jr., my major professor, for his instruction, advice, and encouragement throughout this study. I thank Dr. John Iandolo and Dr. George Marchin for their advice and encouragement. The use of the facilities of Dr. W. A. Ramoska's laboratory and his advice is appreciated. The advice and assistance of Loren I. Davidson, Karl J. Kramer, Donald B. Bechtel, and Berne Jones is especially appreciated. The expert technical assistance of Elizabeth Cauthorn, John Schesser, John Hubbard, and Barbara Campbell is acknowledged.

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Dana Jo Tyrell

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SECTION I
INTRODUCTION

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INTRODUCTION

Bacillus thuringiensis is an aerobic, gram-positive, sporeforming bacillus that forms one or more glycoproteinaceous parasporal crystals during sporulation. The crystals are generally toxic to lepidopteran larvae and crystals from several strains are used to control agriculturally important pests in various parts of the world. Recently, a new subspecies of this bacterium was isolated which is being developed as a control agent for medically important species of mosquitoes and black flies.

Biochemical investigations of toxic subunits from the crystals of the lepidopteran toxic subspecies of B. thuringiensis have been made by many different investigators using different methods and different strains of these bacteria. Toxic subunits ranging from peptides ($MW_{app} = 500$) to large proteins ($MW_{app} = 310,000$) have been described.

Biochemical comparison of the properties and structure of crystals from strains used to control the lepidopteran pests using the same methods for each strain could clarify some of the confusion regarding their crystal subunits. A biochemical characterization of the new, dipteran toxic subspecies's crystals would be useful in its development as a safe insecticide. A comparison of the biochemical properties of these toxic proteins is a prerequisite to the understanding of the mechanism(s) of insect pathology and the genetics of crystal production.

This study was undertaken as a comparison of the biochemistry, morphology, and insect toxicity of parasporal crystals from agriculturally important subspecies of B. thuringiensis and the new dipteran toxic subspecies.

It represents the first comprehensive comparative study of purified crystals of different strains of B. thuringiensis.

Spores of many of these bacteria are also toxic and contain the crystal protein in their spore coats. Spore coat extracts of the subspecies were compared with each other and with a closely related species, Bacillus cereus. The crystals and spore coats of the lepidopteran toxic subspecies are similar whereas the dipteran toxic subspecies has a unique crystal protein and a spore coat unlike either the other B. thuringiensis strains or B. cereus.

These results and future investigations should increase knowledge of the biochemical makeup of these useful and unique bacterial products. This could lead to more efficient insecticidal crystal production and assure the safety of their use as well as increasing our knowledge of the crystal as a sporulation product.

REVIEW OF LITERATURE

Although numerous attempts have been made to use bacterial insect pathogens as insecticides, Bacillus thuringiensis is the only micro-organism which has been successfully developed as a commercial insecticide. An active insecticidal material is produced commercially on a very large scale and used in many countries against economically important pests. Most of the insecticidal activity of these bacteria is due to the production of a glycoprotein crystal (Bulla et al., 1977a) during sporulation. Besides being insecticidal, this crystal protein is also a spore coat component (Bulla et al., 1977b; Aronson and Fox, 1978). The crystal is about one third of the spore's dry weight and so represents a large percentage of the sporulated cell's protein. Because of the abundance of the crystal protein, this organism is a good model for the study of sporulation using the crystal as a sporulation specific protein. It would also be ideal for the study of the formation and regulation of a protoxin and its conversion to a toxin. The study of the molecular biology of B. thuringiensis is providing a better understanding of the biological significance of the crystal to the bacterium as well as the beginnings of an elucidation of its insectidal activity.

Historical Background

Insect toxicity of B. thuringiensis was first described by Ishiwata (Aoki and Chigasaki, 1915a) in 1902 who isolated it from dying silkworm larvae and named it "Sotto-Bacillen". Aoki and Chigasaki (1915a, b) described this silkworm disease and the bacterium that caused it. They

noted that only sporulated cultures were toxic. The presence of inclusion bodies on "Restkörper" was described by Berliner (1911, 1915) and later by Mattes (1927), but the observation attracted little attention and was almost forgotten for 25 years.

In 1951, Steinhaus published a paper containing a micrograph (magnification X 2000) which showed diamond shaped crystals. Hannay (1953) published micrographs of these inclusions and described the unique morphogenesis of sporulating B. thuringiensis cells. He called the inclusion a "parasporal body" and speculated as to its possible association with pathogenicity. Angus (1954) proved the toxicity of the inclusions and Hannay and Fitz James (1955) showed their proteinaceous nature. The crystals then became the center of considerable attention and were actively studied by many investigators, both in the laboratory and in the field.

Toxicity

Extensive field and laboratory testing of different strains of B. thuringiensis against many different insects has been done for a long time (Tanada, 1967; Bulla et al., 1975) and I will not attempt to cover the voluminous literature that has been published. Over 130 susceptible insect species have been found, mostly in the order Lepidoptera, and these include some of the most important pests in the world (Bulla et al., 1975).

Besides the crystal toxin or δ -endotoxin, B. thuringiensis produces several other substances which may have some role in toxicity (Heimpel, 1967). The β -exotoxin or fly factor produced by some strains is the most important

of those substances. It is an ATP analog and competes with ATP in RNA polymerization (Vankova, 1978). Since it is toxic to mammals as well as insects it is no longer considered as an insecticide in this country. Subspecies kurstaki, which is used in Dipel® and Thuricide®, produces no β -exotoxin (Bassand and Carpy, 1977). Dendrobacillin, which is used in the USSR, contains small amounts of β -exotoxin (Bulla et al., 1975). This review considers only the crystal toxin produced by these bacteria.

Insect pests of some orders besides Lepidoptera have been reported to be susceptible to the crystal toxin. These include Coleoptera (Japanese beetle larvae: Sharpe, 1976; Sharpe and Detroy, 1979; and weevils: Naidenov, 1977), Mallophaga (chicken lice: Hoffman and Gingrich 1968) Homoptera (scale insects: Arutyunova, 1977) and Diptera. More extensive studies have been done with the dipteran larvae and will be described below. New subspecies have also been isolated which appear to be specific for dipteran larvae.

B. thuringiensis has been used to control lepidopteran pests in forests and on field crops and has been shown to be effective against stored-product insects. Field crop pests controlled include the imported cabbage worm (Jaques and Laing, 1978; Couch, 1978) several soybean defoliating caterpillars (Ignoffo et al., 1977), cotton bollworms and tobacco budworm (Couch 1978). Some of the important forest defoliators include tent caterpillars, cankerworms, currant spanworms, gypsy moths, Douglas fir tussock moths and spruce budworms (Couch, 1978). Stored product insects include angoumois grain moth (McGaughey and Kinsinger, 1978), Indian meal

moth, and almond moth (McGaughey, 1978a,b,c; Schesser and Bulla, 1979). B. thuringiensis is even more effective when combined with chemical insecticides (Herfs, 1965; Pristavko, 1967; Jaques and Laing, 1978) and is compatible with fungicides and herbicides. It can also be combined with entomopathogenic fungi (Kalyuga, 1968) and nuclear polyhedrosis viruses (Steinhaus, 1951; Stelzer, 1965 and 1967). It has low toxicity to most insect predators and parasites (Bulla et al., 1975; Marchal-Segault, 1975; Franz, 1976; and Workman, 1977).

The pathological symptoms caused by the crystal toxin to lepidopteran larvae have been described by Angus and Heimpel (1959) and Heimpel and Angus (1959). They divided susceptible insects into three groups. Type I insects exhibit general paralysis on ingesting crystals. Most Lepidoptera are type II insects which stop feeding a few minutes after ingesting crystals because of paralysis of the digestive tract and never feed again. The insects then become dehydrated and die 2-4 days later. Type III insects require both crystals or dissolved toxic material and spores in order to be killed. In Heimpel and Angus's study (1959), toxic material was extracted from the spores so any toxic spore coat protein would be removed. Somerville et al. (1970) showed that highly purified spores, which were not extracted with alkali, killed insects that were only slightly affected by spore free crystals.

After ingestion, the crystal dissolves in the highly alkaline ($\text{pH} \approx 10$) midgut of the larvae, the protoxin is activated and the toxin interacts with the midgut epithelial cells to cause necrosis. Changes induced by the toxin have been described using both light and electron microscopy

of insect guts (Atwa and Abel-Rahman, 1974; Hoopingarner and Materu, 1964; Sutter and Raun, 1967; Ebersold et al., 1977) and on insect tissue culture cells (Leuthy et al., 1976). Pathological disorders ascribed to the crystal include (1) separation of gut cells and detachment from the basement membrane, (2) enhancement of secretory ability of gut epithelial cells, (3) increase in permeability of the gut wall to sodium ions with a slower rate of glucose uptake into the hemolymph, and (4) elevated level of potassium ion concentration in the hemolymph (Bulla et al., 1980). Several hypotheses have been proposed to explain the toxic action of the crystal. These include activity as a neurotoxin (Cooksey et al., 1969) or a respiratory uncoupler (Faust et al., 1974 and Travers et al., 1976 a,b; Faust and Travers, 1979) or by impairment of glucose and ion transport (Fast and Donaghue, 1971; Fast and Morrison, 1972; Griego, et al., 1979) or by enzymic activity (Somerville and Pockett, 1976). The recent purification and characterization of the toxin from B. thuringiensis subsp. kurstaki (Bulla et al., 1979) should facilitate mode of action studies.

Crystals from certain isolates of lepidopteran toxic B. thuringiensis have shown toxicity to mosquitoes (Reeves and Garcia, 1971; Singer, 1974; and Hall et al., 1977) and to black flies (Lacey and Mulla, 1977; Lacey, et al., 1978). These isolates were never developed commercially. Activity against black flies did not compare favorably with existing larvicides (Lacey and Mulla, 1977) and another bacterium, Bacillus sphaericus, was more active against mosquitoes (Singer, 1974). A recent lepidopteran toxic isolate (deBarjac et al., 1977) B. thuringiensis var. pakistanicus produces crystals which are somewhat more toxic than the older strains (Saubenova,

1979; W. A. Ramoska, personal communication). Crystals from another new subspecies, B. thuringiensis subsp. israelensis, (deBarjac, 1978c) are very toxic to many species of mosquitoes (Goldberg and Margalt, 1977; deBarjac and Coz, 1979; Garcia and Desrochers, 1979; Tyrell et al., 1979[Section II]) and black flies (Undeen and Nagel, 1978; Undeen and Berl, 1979). Larvicidal powder produced from these bacteria is at least as toxic as B. sphaericus (deBarjac, 1978d) and a patent application (patent application 030-961 by L. J. Goldberg) has been filed with the U.S. Department of Commerce describing a method of controlling mosquito larvae using these bacteria. However, unlike the other B. thuringiensis subspecies, israelensis is not toxic to lepidopteran larvae (deBarjac, 1978d; Tyrell et al., 1979 [Section II] Ohba and Aizawa (1979) have described another isolate, subsp. kyushuensis, which is also toxic to Diptera and nontoxic to Lepidoptera. These could form a second group of B. thuringiensis strains which will be useful for controlling Dipteran pests.

The mode of action of subsp. israelensis on mosquito larvae appears to be similar to that of the other subspecies on lepidopteran larvae (deBarjac, 1978a). Loss of integrity of the gut epithelium occurred and gut cells became swollen and distorted and finally burst. Black fly larvae showed a similar effect when treated with high doses of one of the older strains of B. thuringiensis (Lacey and Federici, 1979). Mosquito and black fly larvae have a gut pH ≥ 10 like lepidopteran larvae (Dadd, 1975; Lacey and Federici, 1979). A similar dissolution and activation of protoxin molecules could occur in these insects.

The crystal toxin of lepidopteran toxic B. thuringiensis strains and insecticides using this toxin have been tested on various laboratory animals and humans by several types of exposure and no serious hazards have been revealed (Heimpel, 1967; Cooksey, 1971; Shea, 1977). The lack of toxicity to mammals could be because of the low pH of the mammalian stomach which allows the crystal to be degraded to an atoxic state. B. thuringiensis subsp. israelensis is innocuous "per os" for mammals (deBarjac, 1978c) and is non-toxic for almost all non-target aquatic organisms (Sinegre et al., 1979). The safety of these bacterial insecticides make them especially attractive for pest control where the natural ecosystem must be protected.

Classification

In the eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) Bacillus thuringiensis is distinguished from B. cereus by its production of the toxic parasporal crystal. Although the inclusion is not a stable property of these bacteria and acrySTALLiferous mutants are easily produced, it is the most important property in most investigations. The biochemical and genetic analyses performed by Somerville and Jones (1972) possibly place B. thuringiensis as a separate species, but it is very similar to B. cereus. Methods involving zone electrophoresis of enzymes (Baptist et al., 1978) or DNA base composition (Candeli et al., 1978) do not distinguish B. thuringiensis from B. cereus. Stahly et al., (1978) have produced acrySTALLiferous mutants of B. thuringiensis whose germination properties and spore coats are similar to B. cereus. A

generalized transducing phage (Thorne, 1978) carries out generalized transduction in both B. cereus and B. thuringiensis.

The various strains of B. thuringiensis are presently classified by serological analysis of flagellar (H) antigens, esterase types, and various biochemical criteria (deBarjac and Bonnefoi, 1962, 1968, 1973; Norris and Burges, 1963). More than 20 strains and 14 serotypes have been differentiated by these techniques and new ones are being described (Delucca et al., 1979). Although this is an easy and useful method for classifying these bacteria, it has little relation to the toxicity of the organisms. Activity differences are as great within the strains of bacteria as between the strains (Angus and Norris, 1968; Dulmage, 1971). Classification by crystal serotype has been suggested (Krywienczyk et al., 1978) but it has not become widely used.

Growth, Sporulation and Crystal Biosynthesis

Like that of other bacilli, the life cycle of B. thuringiensis has two stages: vegetative cell division and sporulation (Ribier and Lecadet, 1973). The nutritional requirements of this and other sporeformers has received limited attention (Bulla et al., 1980). The first chemically defined synthetic medium for growth, sporulation and parasporal crystal formation was that of Nickerson and Bulla (1974). A glucose-salts medium must contain certain growth factors in order to support growth of B. thuringiensis (Nickerson and Bulla, 1974, 1975). The obligate growth factors are glutamic acid and either citric or aspartic acid. L-cystine, EDTA and sodium thiosulfate can replace these dicarboxylic acids, but at certain concen-

trations, L-cystine inhibits sporulation and crystal synthesis

(Rajalakshmi and Shethna, 1977). Apparently, the bacteria require a stimulator of fatty acid synthesis in order to grow in a defined medium (Bulla et al., 1980). Elucidation of the functions of these compounds could help to clarify regulation of growth, sporulation and crystal biosynthesis in these bacteria. The sequence of sporulation and germination has been described in detail for subspecies kurstaki (Bechtel and Bulla 1976; Bulla et al., 1980). Sporulation is generally similar to that of other bacilli except for the formation of the crystal and ovoid inclusion. Bechtel and Bulla (1976) described it according to the conventional stages: stage I -- axial filament formation; stage II -- forespore septum formation; stage III -- engulfment, first appearance of ovoid inclusion and parasporal crystal, change in stainability of membranes and cytoplasm, formation of forespore; stages IV - VI: formation of exosporium, primordial cell wall, cortex, and spore coats accompanied by transformation of the spore nucleoid; stage VII -- spore maturation and sporangial lysis. Crystal antigens have been detected and the start of crystal formation has been observed microscopically during the latter part of stage II (Somerville, 1971; Lecadet and Dedonder, 1971, Ribier and Lecadet, 1973). Mutants which produce crystals but no spores are usually blocked at stage II of sporulation (Somerville, 1971, Lecadet and Dedonder, 1971) although one blocked at stage 0-1 has been described (Meenakshi and Jayaraman, 1979). It has been suggested that the crystal is formed in close association with the exosporium membrane (Somerville and James, 1970; Somerville, 1971) but Bechtel and Bulla (1976) and Bulla et al. (1980) have shown that the

crystal can form in any part of the sporangium. They and Ribier and Lecadet (1973) suggest that the inclusion is associated with the forespore septum during the early part of its development.

Activation, germination and outgrowth of B. thuringiensis spores has been described in detail by Bulla et al. (1980). These spores germinate more slowly than B. cereus spores (Aronson and Pandey, 1978). The crystal appears to have little effect on the germinating spore but some intra-sporangial crystals lose lattice fringes and their matrix becomes globular during germination (Bulla, et al., 1980).

Crystal Shape

Most B. thuringiensis strains produce a bipyramidal or diamond-shaped crystal (Bulla et al., 1975) but crystals which are square, rhomboid, wedge-shaped or have rounded edges have been reported (Hannay, 1955; Heimpel and Angus, 1963; Pendleton, 1969). Subspecies israelensis and kyushuensis which are toxic to dipteran larvae and non-toxic to Lepidoptera produce irregular crystals having several sizes and shapes (deBarjac, 1978c; Ohba and Aizawa, 1978, 1979; Section III). As shown in Section III, subsp. israelensis produces small cubic or bipyramidal, oval shaped, and irregular inclusions, sometimes all within one cell. The oval and irregular crystals are often enclosed in net-like structures (Section III; Bulla et al., 1980). Net-like structures have been observed in a Lepidopteran toxic strain after the crystals were reduced with catechol and ascorbic acid and digested with trypsin (Bulla et al., 1980) and appear to be similar to exosporia of some *Bacillus* and *Clostridium* species,

including B. thuringiensis (Gerhardt and Ribí, 1964; Beaman et al., 1971; Mackey and Morris, 1972; Scherrer, and Somerville, 1977; Lund et al., 1978). The nets could be exosporial membrane which has been selectively assembled around the crystals (Bulla et al., 1980).

Crystal Biochemistry

Before a cellular component can be characterized, it must be isolated in relatively pure form. One of the best methods for separating parasporal crystals from spores and cellular debris is isopycnic density gradient centrifugation using certain x-ray contrasting agents (Sharpe et al., 1975; Milne, et al., 1977). No ultracentrifugation is required and the x-ray contrasting agents are less expensive than cesium chloride and are non-toxic to spores (Bulla, et al., 1980). A method developed recently with potential value for large-scale purification is isopycnic zonal centrifugation in sodium bromide gradients (Ang and Nickerson, 1978).

The native crystals are insoluble in aqueous solution at neutral or acidic pH and severe treatments such as the use of buffers at alkaline pH and/or elevated temperatures have been used to solubilize them (Cooksey, 1971). Such treatments often lead to degradation and erroneous molecular weight values of the material. The glycoprotein also loses its toxicity and cannot be used to study the mode of action. Bulla et al. (1976) compared several solvents for dissolving the crystals. In a solvent containing urea and mercaptoethanol, the crystals were soluble at pH 8.4 but partial aggregation occurred during electrophoresis. A solvent containing 6 M urea, 2% SDS, and 2% mercaptoethanol solubilized the crystals at pH 7 and 28°C

(Bulla et al., 1977a) and prevented reaggregation. The crystals can also be solubilized by titration with alkali but sulfhydryl, serine, and metalloproteases are associated with the crystals and are activated under alkaline conditions (Bulla et al., 1977a; Chestukhina et al., 1978). These could be involved in the activation of the protoxin. A mild alkali titration procedure and removal of contaminating proteases by anion exchange chromatography (Bulla et al., submitted) generates a solubilized protoxin which has chemical and biological properties similar to those of the crystal. It must be used within several hours of preparation or stabilized by placing the protein in a denaturing solvent and alkalating the disulfide linkages to prevent degradation. This indicates that a protease co-fractionates with the crystal subunit. Solubilization and toxicity is pH- and time-dependent (Nishiitsutsuji-Uwa et al., 1977; Bulla et al., 1980) and reaggregation can occur if the pH is lowered to neutrality.

Most of the evidence reported in the literature indicates that several distinct polypeptide components make up the crystal although their exact number and properties is not well defined. Molecular weights calculated for major crystal components range from 500 to 310,000 (Bulla et al., 1980). The results have been ascribed to differences in the strains used and the methods of solubilization (Somerville, 1973) as well as to differences in the methods of molecular weight determination. Table 1 from Bulla et al. (1980) is a summary of results from various investigations of toxic subunits obtained from B. thuringiensis crystals. Most of the solubilization methods

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Table 1. Summary of investigations on toxic subunits obtained from Bacillus thuringiensis.

Investigator(s)	Variety of <u>B. thuringiensis</u>	Method for extraction of toxin from crystal	Characterization of subunit	Toxicity
Angus, 1956	<u>sotto</u>	0.05N NaOH, 1 hr. dialysis of toxic supernatant	ND ^a	+
Lecadet and Dedonder, 1967	<u>thuringiensis</u>	<u>Pieris brassicae</u> gut juice, 0.1M bicarbonate buffer (pH 10.5) + 10 ⁻³ M EDTA	(a) Six protein fractions of MW 50,000, 13,000 (b) Peptide fraction, MW 5,000 (c) 4 peptides are toxic	+
Lecadet and Martouret, 1967				+
Watanabe et al., 1967	<u>sotto</u> and <u>strain T84A1</u>	<u>Bombyx mori</u> gut juice in alkaline solution gives crude toxin	One toxic component	+
		Glycine-NaOH buffer (pH 11.5) extraction of crude toxin	Fast and slow components, on Sephadex One toxic fraction on DEAE cellulose Molecular weights not determined	+

Table 1. (Continued)

Cooksey, 1968	<u>thuringiensis</u>	Extraction with 0.04N NaOH, precipitation at pH 4.4 with acetate buffer	3 bands on disc gel electrophoresis; 2 antigens	+
		Purified toxin + <u>P. brassicae</u> gut juice, chymotrypsin, trypsin, or subtilisin	Antigenic fractions of MW 40,000. Non-antigenic fraction, MW 10,000	+
Pendelton, 1968	<u>thuringiensis</u>	Gut juice of <u>P. brassicae</u> in 0.2M carbonate-bicarbonate buffer (pH 9.5) + 2 X 10 ⁻³ M EDTA	MW 20,000 and MW 5,000 - 10,000 fractions. Low mol. wt. fraction has 3 antigens.	+
Sommerville et al., 1968	<u>alesti</u>	Dissolved in 8M urea containing 10% 2-mercaptoethanol dialysed to pH 8.3 - 8.5	Fractions I and II	NR ^b
Fast and Angus, 1970	<u>sotto</u>	0.01N NaOH + 0.2M mercaptoethanol. Filtered through milipore and incubated with trypsin (1 mg/ml)	Toxic fragment with mol. wt. between 500-1000	+

Table 1. (Continued)

Sayles et al., 1970	<u>thuringiensis</u>	Solubilized in 8M urea + 0.5% dithiothietol	2 small peptides of mol. wt. 1,000 - 1,500	NR
Norris, 1968	<u>tolworthi</u>	NaOH solubilized	3 major antigens. One toxic, two non-toxic	+
Herbert et al., 1971	<u>tolworthi</u>	Extracted with Ellis buffer containing 0.08M Na ₂ CO ₃ and 0.1M mercaptoethanol	2 polypeptide chains. A (MW 55,000) and B (MW 120,000). A is toxic.	+
Cooksey, 1968	<u>thuringiensis</u>	0.05M glycine-NaOH (pH 9.0) containing 10 ⁻³ M 2-mercaptoethanol	2 major antigens. Both toxic.	+
Clatron et al., 1972	<u>thuringiensis</u>	0.1M 2-mercaptoethanol + 6M guanidine HCl at pH 9.5	One repetitive subunit, MW 80,000	NR
		0.1N and 1 N NaOH	2 to several components with sedimentation constants of 0.87S to 3.4S, 4.6S, and 10S	NR
Faust et al., 1974	<u>alesti</u>	Gut juice of Bombyx mori larvae	3 protein fractions, MW 235,000, 67,000, 30,200	+

Table 1. (Continued)

Prasad and Shethna, 1974	<u>thuringiensis</u>	Dissolved in 1.0N NaOH and dialysed against 0.02M phosphate buffer, pH 7.0	2 protein fractions. I, MW 100,000, and II, 13,000. Both toxic.	+
Bulla et al., 1977a	<u>kurstaki</u>	Sodium dodecyl sulfate, 2-mercaptoethanol, urea, phosphate buffer at pH 7.0; or SDS, Tris-glycine buffer, pH 8.4, 70°C 20 min; or 0.1N NaOH.	Glycoprotein with 95% protein and 5% carbohydrate and MW 130,000. Also a dialysable chromophore produced by alkaline hydrolysis.	+
Chestukhina et al., 1977	<u>galleriae</u>	Guanidine · HCl + dithiothreitol, pH 8.5; urea + DTT, pH 8.5; guanidine. HCl + 2 mercaptoethanol + SDS + DTT pH 8.5; NaOH to pH 12.0 and above.	Main component, MW 120,000 to 140,000; several components of MW 65,000 - 120,000 and even high MW range 190,000 to 230,000 and 260,000 to 310,000	+
Nagamatsu et al., 1978	<u>dendrolimus</u>	0.1M DTT at pH 10.0	Five components. One toxic component, MW 232,000	+

^aND = not determined^bNR = not reported

produce two toxic complexes, one with a high molecular weight and one with a low molecular weight. Possibly the small-molecular-weight complex is generated from the larger proteins. Evidence that the crystal is composed of a single subunit that is converted to smaller components by the conditions of dissolution has been presented by Bulla et al. (1977a) and Chestukhina et al. (1977). Bulla et al. (submitted) established that the molecular weight of this subunit is 134,000 in subspecies kurstaki. Section III describes subunits of approximately this molecular weight from crystals of several B. thuringiensis strains. Some strains contain two of these subunits (Section III, Zalunin et al., 1979) which can be separated by SDS polyacrylamide gel electrophoresis. Zalunin et al. (1979) suggest that all strains synthesize one common crystal protein which undergoes secondary modification by intracellular proteinases in some strains during crystal biosynthesis. Bulla et al. (submitted) have isolated the 134,000-dalton crystal subunit from subspecies kurstaki and have shown it to be a protoxin. They also have isolated a 68,000-dalton toxin which is produced from this protoxin (Bulla et al., 1979). This is the smallest toxic component produced from the subspecies kurstaki crystals.

The 134,000-dalton subunit probably occurs as a dimer in the native crystals since Holmes and Munrow (1965) have found that the basic crystal molecular unit is asymmetric and has a molecular weight of approximately 230,000.

Amino acid analyses of crystals from several strains of B. thuringiensis have been reported (Cooksey, 1971; Section III). Aspartic acid and glutamic acid are the most abundant and methionine is the least abundant. Crystals

from most strains have similar amino acid compositions. Most of the amino acids are present; no unusual amino acids have been detected. The minimal molecular weight calculated from the amino acid composition is about 13,000.

Crystals from many stains have been found to contain carbohydrate. Holmes and Munro (1965) reported 0.5%, Bateson and Stainsby (1970) found 12% including glucose, mannose, xylose, and arabinose, and Bulla et al. (submitted) have found that the purified protoxin contains glucose (3.8%) and mannose (1.8%). No amino sugars have been reported. No lipids, nucleic acids, or sialic acid derivatives have been found in the crystals. The structure of the carbohydrate chains and their sites of attachment are unknown. Dastidar and Nickerson (1978) have shown that carbohydrate is released via β -elimination in alkali along with the formation of lysinoalanine which indicates that the sugars are attached to serine or threonine. They suggest that this lysinoalanine formation may have some role in toxicity. An unusual ultraviolet-absorbing ($\lambda_{\text{max}}=240$ nm) chromophore was reported (Bulla et al., 1977a; Dastidar and Nickerson, 1978) which is associated with the crystal or is produced during solubilization. Metals have been detected in crystal preparations (Faust et al., 1973). Estes and Faust (1966) detected silicon and hypothesized that it forms a lattice upon which the protein molecules are fixed.

The use of impure crystal preparations which may contain contaminating components of spores or vegetative cells associated with the crystals makes describing the presence of metals and carbohydrates difficult to interpret. The use of extensively purified crystal protein subunits (Bulla et al., submitted) eliminates most of this confusion.

Spore Coat Structure

Extensive chemical and immunological studies indicate that the crystal protoxin is also a major spore coat protein in B. thuringiensis. (Delafield, et al. 1968; Somerville, et al. 1968; Somerville, et al., 1970; Lecadet et al., 1972; Short et al., 1974; Scherrer and Somerville, 1977; Aronson and Pandey, 1978; Somerville, 1978). Bulla et al. (1977b) has shown the identity of the major spore and crystal proteins in subspecies kurstaki by gel electrophoresis and by the identical toxicity (specific activity) of the two preparations (Schesser et al., 1977; Schesser and Bulla, 1978). Sections III and IV show by gel electrophoresis that crystal proteins are found in spore coat extracts of several subspecies of B. thuringiensis, including the new subspecies israelensis. Aronson and Pandey (1978) and Bulla et al. (1980) did not detect the low molecular weight, keratin-like proteins, characteristic of B. cereus spore coats, in spore coats of B. thuringiensis. The polyacrylamide gradient gels shown in sections III and IV reveal that low molecular weight proteins ($MW_{app} = 13,000$) are found in B. thuringiensis spore coats along with the crystal proteins. These proteins, however, were not quite the same size as those found in B. cereus coats (Section IV). B. thuringiensis spores also lack part of a spore coat layer which is found on B. cereus spores (Aronson and Fitz-James, 1976).

An anaerobic sporeformer, Clostridium perfringens, type A, produces an enterotoxin which causes human food poisoning and has biochemical and immunological homology with a spore coat protein (Friebe and Duncan 1973, 1975). The spore coat extract also has enterotoxin-like biological

activity (Friebe and Duncan, 1973). Several enterotoxin A producing strains of these bacteria also form paracrystalline inclusions during sporulation which could consist of enterotoxin (Duncan et al., 1973). These studies indicate that a toxin which is also a spore coat protein is not unique to B. thuringiensis.

Function of Crystal Protein

The functions of B. cereus spore coat proteins include protection of the spore and a role in the spore's response to germinants (Aronson and Fitz-James, 1975, 1976; Stelma, et al., 1978). B. thuringiensis spore coats retain the protective function since spores of both species are equally resistant to lysozyme (Bulla et al., 1980), but B. thuringiensis spores respond more slowly to germinants than B. cereus (Aronson and Pandey, 1978). Lysozyme sensitive mutants have the same germination time as the wild-type whereas lysozyme sensitive B. cereus mutants germinate more slowly than wild type (Aronson and Pandey, 1978). Spore coats of lysozyme sensitive mutants always contain little or no protoxin (Aronson and Pandey, 1978; Stahley et al., 1978) but acrySTALLIFEROUS mutants which have protoxin deficient, lysozyme resistant spores have been isolated (Stahley et al., 1978). This indicates that other spore components can provide lysozyme resistance. Lysozyme resistant "revertants" have been isolated from lysozyme sensitive, acrySTALLIFEROUS mutants. These revertants have B. cereus-like coat profiles and respond rapidly to B. cereus germinants (Stahley et al., 1978). The inter-relationship among crystal-forming, lysozyme sensitive, and rapid germinating

strains implies that B. cereus somehow has acquired the ability to produce crystals and has become insecticidal (Bulla et al., 1980). The function of the crystal protein could be to provide a protected, nutrient-rich environment in which the spore can germinate by permitting entry into the hemolymph of insect larvae. Since the crystal is formed outside the exosporium, the crystal protein in the spore coat allows the spore alone to penetrate the haemolymph. Rapid response to germinants would be dispensable in such a protected environment (Aronson and Pandey, 1978; Stahly et al., 1978; Bulla et al., 1980). If the crystal protein is lost by mutation, double mutants which germinate rapidly would be selected for in a more competitive soil environment (Stahly et al., 1978)

Possible Origin

There is some evidence that extrachromosomal DNA has a role in crystal production in B. thuringiensis (Galushka and Azizbekyan, 1977; Ermakova et al., 1978; Stahly et al., 1978). Three groups using different methods have found a correlation between existence of extrachromosomal DNA in a strain of B. thuringiensis and the formation of crystals. Galushka and Azizbekyan (1977) isolated acrySTALLiferous mutants from several strains by ethidium bromide treatment or culturing under severe conditions. Stahly et al. (1978) used heat treatment of spores to enrich for acrySTALLiferous mutants. Ermakova et al. (1978) used a strain which forms crystals in rich medium and fails to produce them in a minimal medium. Plasmids, which were present in all the

strains used, were lost when the ability to form crystals was lost. The high frequency of acrySTALLIFEROUS mutants (1 per 500 - 1000 colonies) also suggests involvement of an unstable genetic element, i.e. a plasmid (Stahly et al., 1978). This correlation between crystal formation and presence of plasmids also occurs in a B. subtilis crystal forming mutant (Ermakova et al., 1978). Santo and Doi (1973) have isolated a temperature sensitive RNA polymerase mutant of B. subtilis which forms crystals in 3% of the cells and Kaneko and Matsushima (1975) have detected a crystal frequency (0.1%) in wild type B. subtilis. Crystal formation in B. thuringiensis could be stabilized by the selective advantage of using the insect haemolymph as a niche. Faust et al. (1979) have discussed the potential for genetic engineering in B. thuringiensis and Thorne (1978) has isolated phages which carry out generalized transduction in these bacteria. Since the toxicity of these bacteria to man and his environment is so low, these techniques could be used to produce ideal strains for insect control and to determine the genetics of crystal formation.

SECTION II

TOXICITY OF PARASPORAL CRYSTALS OF BACILLUS
THURINGIENSIS SUBSP. ISRAELENIS TO MOSQUITOES

ABSTRACT

Toxicity of Bacillus thuringiensis subsp. israelensis (ONR-60A/WHO 1897) parasporal crystals to three medically important mosquito larvae is described. The numbers of larvae killed are in relation to crystal dry weight. The crystals are lethally toxic to Aedes aegypti Linnaeus (mean 50% lethal concentration $[LC_{50}] = 1.9 \times 10^{-4}$ $\mu\text{g/ml}$), Culex pipiens var. quinquefasciatus Say ($LC_{50} = 3.7 \times 10^{-4}$ $\mu\text{g/ml}$), and Anopheles albimanus Wiedemann ($LC_{50} = 8.0 \times 10^{-3}$ $\mu\text{g/ml}$). Purified crystals of B. thuringiensis subsp. kurstaki, which are toxic to lepidopteran insects, are ineffective against the mosquito larvae. Likewise, B. thuringiensis subsp. israelensis parasporal crystals are not efficacious for larvae of the lepidopteran, Manduca sexta.

INTRODUCTION

Bacillus thuringiensis is a gram-positive sporeforming bacterium that synthesizes a discrete intracellular glycoprotein crystal during sporulation (Bechtel and Bulla, 1976; Bulla et al., 1977). The crystalline inclusion is the basis for commercial insecticidal formulations of B. thuringiensis because it is toxic to lepidopteran insect larvae (Cooksey, 1971; Schesser et al., 1977). Recently, a new strain of B. thuringiensis (designated ONR-60A/WHO 1897; Goldbert and Margalt, 1977) was isolated and given the subspecific epithet israelensis and serotype number 14 according to its flagellar antigenicity (deBarjac, 1978). Spore and crystal mixtures of this bacterium are toxic to mosquitoes and to black flies (deBarjac, 1978; deBarjac, 1978; Goldberg et al., 1977; Undeen et al., 1978), and a patent application (patent application no. 888-083 by L. J. Goldberg) has been filed with the U.S. Department of Commerce describing a method of controlling mosquito larvae by using B. thuringiensis subsp. israelensis ONR-60A.

In our laboratory, we are comparing the insecticidal properties and biochemical mode of action of parasporal crystals isolated from B. thuringiensis subsp. israelensis and from B. thuringiensis subsp. kurstaki, the latter of which are toxic to lepidopteran insects. Earlier, we developed a sensitive and reproducible bioassay for homogeneous crystal preparations of B. thuringiensis subsp. kurstaki by using neonate larvae of the tobacco hornworm, Manduca sexta (Schesser et al., 1977). The present report describes a quantitative determination of the lethality

of purified parasporal inclusions of B. thuringiensis subsp. israelensis to three medically important mosquitoes: Aedes aegypti Linnaeus, vector of yellow fever; Culex pipiens var. quinquefasciatus Say, a serious pest as well as a vector of encephalitis; and Anopheles albimanus Wiedemann, a carrier of malaria. Also compared are toxicities of B. thuringiensis subsp. kurstaki and israelensis crystals to both tobacco hornworm and mosquito larvae.

MATERIALS AND METHODS

Test organisms. B. thuringiensis subsp. israelensis used in this study was a gift from David Rockhold, Stauffer Chemical Co., San Jose, Calif. It originally was isolated by Goldberg and Margalt (1977) from soil samples near mosquito larvae breeding sites and is called strain 60A. Stock cultures of the organism were maintained on MD (Bulla et al., 1976) agar slants. Cells for experimental use were cultured in the manner described previously for B. thuringiensis subsp. kurstaki (Schesser et al., 1977). Parasporal crystals were separated from spores and cellular debris by buoyant density centrifugation in Renografin gradients (Sharpe et al., 1975). Crystals isolated in this manner were washed at least three times in water and lyophilized to constant weight. Purity of preparations was monitored at each step of the separation procedure by phase-contrast microscopy.

Experimental treatments. Cultures of Aedes aegypti, C. pipiens var. quinquefasciatus, and Anopheles albimanus were obtained originally from the Gulf Coast Mosquito Research Laboratories, U.S. Department of Agriculture, Lake Charles, La., and have been maintained in our laboratory for the past several years. Second-instar larvae of each mosquito species were used in all experiments. Parasporal crystal suspensions were made by homogenizing a certain dry weight of crystals with distilled water and diluting to the desired concentration. For each experiment, 10-fold serial dilutions of the test preparation were made to a final volume of 50 ml by adding 5 ml of the crystal suspension to 45 ml of distilled water

contained in 3.5-ounce (99.2-g) plastic cups. A 5-ml portion of this dilution was then transferred to 45 ml of water in a second cup, and the process was repeated. Final dilutions ranged from 10^{-8} to 1 μg of crystals per ml. Each dilution was replicated at least three times.

Before making the serial dilutions and adding the crystals, 10 second-instar mosquito larvae were placed in each cup containing water to which had been added 1.5 mg of dry artificial diet consisting of 66.7% (wt/wt) of a 1:1 mixture of finely ground rabbit and hog chow, 13.3% Tetra Min fish food powder, and 6.7% each of dried blood powder, brewers' yeast, and dried liver powder. The cups holding larvae and crystals were maintained in a 10-h light period at 25°C for 72 h. Mortality counts were made at 1, 4, 24, 48, and 72 h after the crystals were placed into the cups. Control larvae were incubated in cups containing diet without parasporal crystals. This bioassay procedure is a modification of the method described by Ramoska et al (1978). Bioassays against the tobacco hornworm, M. sexta, were done as described previously (Schesser and Bulla, 1978; Schesser et al., 1977). All data were subjected to probit analysis (Finney, 1952).

RESULTS AND DISCUSSION

Mortalities of the three mosquitoes tested, Aedes aegypti, C. pipiens var. quinquefasciatus, and Anopheles albimanus, are listed in Table 1. Only those mortalities recorded after 2 days are given because there was no significant increase in larval deaths after this time. Parasporal crystals of B. thuringiensis subsp. israelensis exhibited toxicity to all three mosquito larvae at a concentration 10^{-4} $\mu\text{g/ml}$. Approximately 90% of Aedes aegypti and C. pipiens larvae were killed at a dosage rate of 10^{-2} $\mu\text{g/ml}$, whereas only 50% of the Anopheles albimanus larvae died at this concentration. Mean lethal endpoints and 95% confidence limits are provided in Table 2. Based on mean 50% lethal concentration values (LC_{50}), relative sensitivities of the mosquitoes to the crystals were: Aedes aegypti ($\text{LC}_{50}=1.9 \times 10^{-4}$ $\mu\text{g/ml}$) > C. pipiens var. quinquefasciatus ($\text{LC}_{50}=3.7 \times 10^{-4}$ $\mu\text{g/ml}$) > Anopheles albimanus ($\text{LC}_{50}=8.0 \times 10^{-3}$ $\mu\text{g/ml}$). Under our experimental conditions, the crystal was about 20 to 40 times less toxic to the Anopheles larvae than to either Aedes or Culex larvae. Other investigators have noted similar results using spore-crystal formulations (Goldberg and Margalt, 1977). The variable toxicities may be due to the feeding habits of the mosquitoes because Anopheles mosquito larvae are mainly surface feeders whereas the Aedes and Culex larvae feed beneath the surface and consequently may ingest more crystal.

Interestingly, the parasporay crystals of B. thuringiensis subsp. kurstaki, normally toxic to lepidopteran insects, exhibited at least 10^4

times less toxicity toward mosquitoes. Only about 50% of the Aedes larvae died at a crystal concentration of 10 µg/ml, and about 30% of the Culex larvae died at a concentration of 100 µg/ml. No Anopheles larvae were killed at these high concentrations. Likewise, crystalline inclusions of B. thuringiensis subsp. israelensis were not toxic to neonate larvae of the tobacco hornworm, M. sexta; crystals administered at a concentration as high as 300 µg/ml were ineffective (data not shown). deBarjac (1978) also observed that B. thuringiensis subsp. israelensis was ineffective against lepidopteran larvae.

Previously, we characterized the parasporal crystal of B. thuringiensis subsp. kurstaki (Bulla et al., 1977) and found it to be a glycoprotein. The glycoprotein is composed of a single subunit with an apparent molecular weight of 1.34×10^5 and is a protoxin that becomes activated after ingestion by an insect susceptible to the toxic product. Preliminary data in our laboratory (unpublished) indicate that the crystalline inclusion of B. thuringiensis subsp. israelensis also is a protoxin composed of a single subunit, the molecular weight of which is approximately 134,000, but that upon solubilization and activation, the protoxic molecule of B. thuringiensis subsp. israelensis is converted to small-molecular weight, toxic material more rapidly than is the B. thuringiensis subsp. kurstaki protoxin. Also, the amino acid compositions of the two crystals are significantly different. For example, the B. thuringiensis subsp. israelensis crystal contains three times more lysine than the B. thuringiensis subsp. kurstaki crystal and three-and-a-half times less arginine. Possibly, such variation in activation and amino acid composition could account for the differences in toxicity and host specificity.

Whether there are distinct sequences of amino acids in the active site of the two protoxins or whether there are activating proteases associated with the isolated crystals that render dissimilar toxins is not known. A more detailed investigation of the biochemical nature of the B. thuringiensis subsp. israelensis crystal and how it differs from that of B. thuringiensis subsp. kurstaki should provide a better understanding of the toxic mode of action and host specificity.

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Table 1. Mortality of mosquito larvae after 2 days in water containing B. thuringiensis subsp. israelensis parasporal crystals.

Crystal concentration ($\mu\text{g/ml}$)	% Mortality ^a		
	<u>Aedes aegypti</u>	<u>Culex pipiens</u>	<u>Anopheles albimanus</u>
Control	0	0	0
10^{-5}	0	15	0
10^{-4}	25	18	10
10^{-3}	84	68	10
10^{-2}	93	90	50
10^{-1}		100	85
10^0			95

^aValues corrected by subtracting percent mortality of control mosquito larvae.

Table 2. Lethal doses and confidence limits of B. thuringiensis subsp. israelensis parasporal crystal for mosquito larvae.

Species	Endpoint (%)	Lethal concentration $\mu\text{g/ml}$	95% Confidence limits ($\mu\text{g/ml}$)	
			Upper	Lower
<u>Aedes aegypti</u>	50 ^a	1.9×10^{-4}	2.5×10^{-4}	1.4×10^{-4}
	95	1.7×10^{-3}	3.3×10^{-3}	1.1×10^{-3}
<u>Culex pipiens</u> var. <u>quiquefasciatus</u>	50 ^b	3.7×10^{-4}	6.6×10^{-4}	2×10^{-4}
	95	2.5×10^{-2}	9.6×10^{-2}	1×10^{-2}
<u>Anopheles albimanus</u>	50 ^c	8.0×10^{-3}	1.4×10^{-2}	4.6×10^{-3}
	95	2.9×10^{-1}	1.0×10^0	1.3×10^{-1}

^aSlope, 1.72; intercept, 11.40.

^bSlope, 0.90; intercept, 8.10.

^cSlope, 1.05; intercept, 7.20.

SECTION III

COMPARATIVE BIOCHEMISTRY OF ENTOMOCIDAL PARASPORAL CRYSTALS OF SELECTED STRAINS OF BACILLUS THURINGIENSIS

ABSTRACT

Parasporal crystals of Bacillus thuringiensis subsp. kurstaki tolworthi, alesti, berliner, and israelensis were compared by electron microscopy, polyacrylamide gel electrophoresis (PAGE), amino acid analysis, tryptic peptide mapping, immunological analysis, and insecticidal activity. Spore coats also were compared by PAGE. Subspecies israelensis crystals were lethally toxic to mosquito larvae and non-toxic to tobacco hornworm larvae. Conversely, crystals from the other subspecies killed tobacco hornworm larvae but were ineffective against mosquitoes. Crystalline inclusion bodies of all subspecies contained a protoxic subunit that has an apparent molecular weight of approximately 1.35×10^5 . However, PAGE patterns of solubilized crystals revealed a small molecular weight component ($MW_{app} = 26,000$) in israelensis that was absent in the other subspecies. Also, differences were noted in amino acid composition and tryptic peptide fingerprints. Crystal proteins were found in spore coats of all subspecies. The results suggest that insecticidal specificity is due to unique polypeptide toxins.

Previously, we characterized the entomocidal parasporal crystal of Bacillus thuringiensis subsp. kurstaki (Bulla et al., 1977a). The crystal contains a single glycoprotein subunit that has an apparent molecular weight of 1.35×10^5 . The glycoprotein is a protoxin that, under appropriate conditions, is converted to a toxic molecule whose apparent molecular weight is 6.8×10^4 (Bulla et al., 1979).

The subspecies kurstaki is an agriculturally important organism because it kills lepidopteran insects (moths). Recently, another potentially useful strain called subspecies israelensis has been isolated that produces a toxin effective against mosquitoes and black flies (deBarjac, 1978b; deBarjac, 1978c; deBarjac and Coz, 1979; Garcia and Desrochers, 1979; Goldberg and Margalt, 1977; Tyrell et al., 1979; Undeen and Berl, 1979; Undeen and Nagel, 1978). We are interested in comparing the properties of the mosquito toxic crystal of subspecies israelensis with those of other strains which synthesize crystals that kill moths in order to determine whether there are differences in the polypeptide composition of these various strains. Therefore, we have compared some of the biochemical, immunological, and toxicological properties of parasporal crystals isolated from five strains of B. thuringiensis. The strains selected include some of the more important subspecies that have been isolated from different parts of the world. Four of the strains that we investigated produce crystals that are toxic to lepidopterans; the fifth one is subspecies israelensis whose crystals kill dipteran insects.

MATERIALS AND METHODS

Organisms and cultural conditions. B. thuringiensis subsp. kurstaki used in this study was isolated from a commercial insecticidal formulation called Dipel (Abbott Laboratories, North Chicago, IL); B. thuringiensis subsp. alesti was a gift from P. C. Fitz-James, University of Western Ontario Medical School, London, Ontario, Canada; subspecies berliner was given to us by M. M. Lecadet, Centre National de la Recherche Scientifique et Universite, Paris, France; subspecies tolworthi was a gift from B. N. Herbert, Dept. of Biochemistry, Imperial College, London, England; and B. thuringiensis subsp. israelensis was isolated from an experimental preparation provided by Abbott Laboratories, North Chicago, IL. Originally, it was isolated by Goldberg and Margalt (1977) from soil samples near mosquito breeding sites and is designated strain 60A. All cultures were maintained on MD agar (Bulla et al., 1970) slants. Cells for experimental use were cultured in the manner described previously for B. thuringiensis subsp. kurstaki (Schesser et al., 1977).

Determination of growth characteristics. Cells maintained on MD agar slants were inoculated into 100 ml of liquid MD medium contained in 300 ml-Erlenmeyer flasks and aerated by rotary agitation at 200 rpm overnight at 28°C. The cells were phased by transferring a 10% inoculum into 100 ml of modified GYS (Nickerson et al., 1974) medium also contained in 300 ml-Erlenmeyer flasks and incubating at 28°C. Midexponential cells (6% inoculum) were transferred into 300 ml-

nephelometer flasks and were shaken then at 200 rpm and 28°C for an extended period. Absorbance and pH readings were taken at 1-2 hour intervals throughout the growth and sporulation cycle of each strain. Morphological changes were monitored by phase-contrast microscopy. Duplicate growth curves were plotted for each B. thuringiensis strain.

Electron microscopy. Sporulated but unlysed cultures of each B. thuringiensis strain were centrifuged at 4°C and the pellets were suspended in 4% glutaraldehyde in 0.01 M phosphate-buffered saline at pH 7.2 for 5 min at 4°C. The cells were pelleted at 2,000 rpm and suspended in warm (55°C) 2% water agar. The agar was immediately cooled to 4°C, cut into 1-mm cubes, and placed into fresh cold fixative for 1 h. Samples were washed four times in cold buffer for a total of 80 min. Postfixation, embedding in Epon 812 (Luft, 1961), sectioning, and electron microscopy were done as previously described (Bechtel and Bulla, 1976).

Isolation, purification and solubilization of parasporal crystals. Parasporal crystals were separated from spores and cellular debris by bouyant density centrifugation in Renografin gradients (Sharpe et al., 1975). Crystals isolated in this manner were washed at least three times in water and lyophilized to constant weight. Purity of preparations was monitored at each step of the separation procedure by phase contrast microscopy. Solubilization was accomplished by incubating the crystals in sodium dodecyl sulfate (SDS), 1% (wt/vol); beta-mercaptoethanol (ME), 2% (vol/vol); urea, 6M; equimolar (0.01M) ratio of NaH_2PO_4 and Na_2HPO_4

at pH 7.2 for 1 hour at 28°C. Soluble toxic material was obtained in the following manner: A suspension of wet crystals (0.4% wt/vol) was titrated to pH 12 with aliquots of 1 M NaOH at 28°C. The crystal suspension was allowed to stay at this pH for 5 hr and then dialyzed against 0.02M phosphate at pH 7.5. Protein was determined by absorbance at 280 nm using an extinction coefficient of 1.1 absorbance units equal to 1 mg of protein per ml. After various times of incubation, samples were subjected to either bioassays or gel filtration as described below.

Spore coat protein extraction. Spores purified on Renografin gradients were extracted three times with 6M guanidinium chloride and 0.1M ME at pH 8.6. The extracts were subsequently dialyzed against 0.1M Tris-hydrochloride buffer containing 9M urea (pH 8.6) for 24 hr and then against water for 48 hr (40-44 liters) before they were lyophilized.

Polyacrylamide gel electrophoresis. Parasporal crystals solubilized by the methods described above and spore extracts solubilized with SDS, urea and ME as described above were subjected to electrophoresis on SDS polyacrylamide slab gels (1.5 x 140 x 170 cm; Buchler Polyslab polyacrylamide gel electrophoresis system) using the method of Weber and Osborn (1969, 1972). Gradient gels containing 3-10%, 5-15%, 5-20%, and 10-20% acrylamide were used to characterize spore and crystal polypeptides. Electrophoresis was carried out at 40-90 volts. Gels were stained 3-12 h in 0.25% (wt/vol) Coomassie brilliant blue (Eastman R250) in methanol-acetic acid-water (25:7.5:62.5, vol/vol/vol) and

destained in methanol-acetic acid-water (25:7.5:62.5, vol/vol/vol) for 16-48 hr. Molecular weight standards for the 3-10% acrylamide gels were myosin (200,000) β -galactosidase (135,000) phosphorylase B (92,000), bovine serum albumin (68,000) and ovalbumin (43,000) which were prepared by Bio-Rad Laboratories. Cross-linked bovine serum albumin (68,000, 136,000, etc.) from Sigma and bovine albumin (65,000) ovalbumin (45,000), beef pancreas chymotrypsinogen A (25,000), sperm whale myoglobin (17,000) from Schwarz/Mann, ribonuclease A (13,600) from Pharmacia, and bovine pancreas insulin (5,700) from Sigma were used as standards in the other gels. Destained gels were photographed on 35 mm high contrast copy film that was developed in Kodak D-19. Gels were dried using a Bio-Rad Model 224 slab gel dryer.

Reduction and S-alkylation. Purified crystals were reduced and S-carboxymethylated according to the procedure of Crestfield et al. (1963) in 6 M guanidinium chloride, 0.25 M Tris-HCl, 0.003 M EDTA, pH 8.5 S-carboxymethylation was done using iodoacetate in 1M NaOH (Heinrikson, 1971). The material was then dialyzed against distilled water (40 liters) for 48 hrs.

Protein determination. Protein was determined by the Lowry et al. procedure (1951) and by absorbance at 280 nm using an extinction coefficient of 1.1 absorbance unit equal to 1 mg of protein per ml for all crystal preparations.

Amino acid analysis. Samples were analyzed on a Beckman 120C analyzer after hydrolysis for 25 h in 6 N HCl. Labile amino acids were quantitated by extrapolation to zero time. Cystine and cysteine

were determined as cysteic acid and methionine as the sulfone after performic acid oxidation (Hirs, 1967). Tryptophan was quantitated in samples hydrolyzed in 3M p-toluenesulfonic acid containing a small amount of tryptamine. The hydrolysis was carried out for 24 h at 110°C in vacuo (Liu and Chang, 1971).

Tryptic digestion and peptide mapping. S-alkylated crystals were incubated for 24 hrs at 37°C with diphenylcarbamyl chloride treated trypsin as previously described (Kramer et al., 1973). Two aliquots of trypsin (each 2%, wt/wt) were used. One was added at the beginning of incubation, the other after 2-4 hrs of incubation.

Maps of tryptic digests of 300-500 µg of S-alkylated crystals were prepared on thin-layer chromatography sheets (20 x 20 cm). Material was dissolved in 80% methanol, centrifuged for 15 min at 5,000 rpm and spotted onto a corner of the sheet, 5 λ at a time. Two dimensional separation was obtained by electrophoresis at pH 2 (8% acetic acid, 2% formic acid, vol/vol) at 200 V per plate for 2 hours followed by chromatography with butanol, pyridine, acetic acid, and water (15:10:3:12, vol/vol/vol/vol) for 3-4 hrs. The peptides were visualized by staining with ninhydrin-cadmium acetate (Heilmann, 1957). Plates were photographed using high contrast copy film which was developed using Kodak D19.

Carbohydrate analysis. Monosaccharides were determined by high performance liquid chromatography (Mopper, 1978) after hydrolysis with 2M HCl for 2 hrs or 4N HCl for 3 hrs, and evaporation of the acid under vacuum at 0°C. Sugars also were chromatographed on the same column as [³H] alditols, after reduction with NaB₃H₄ (Takesaki and Kobata,

1974). Retention times and elution volumes were compared with those of standard sugars and alditols.

Preparation of antisera. Antisera were obtained by inoculating New Zealand white albino rabbits subcutaneously in four sites with a total of 1 mg of solubilized crystal toxin suspended in complete Freund's adjuvant. After four weeks, the rabbits were reinoculated with another 1-mg dose of solubilized crystal in incomplete Freund's adjuvant. The animals were bled at the end of two weeks and each subsequent week thereafter.

Ouchterlony assays. Ouchterlony gels were prepared in 1% agarose, 2% polyethylene glycol 6000, 0.089M boric acid, 0.089M Tris base, and 0.001M ethylenediaminetetraacetic acid at pH 8.3. Antigen and anti-serum were placed into the gels and allowed to incubate overnight at room temperature. Precipitin bands were visible within 10-12 h.

Gel filtration chromatography. Sepharose CL-4B (100-200 mesh; Pharmacia) was equilibrated in 0.02M NaH_2PO_4 at pH 7.5. The gel slurry was poured to a column bed height of 50 cm in a glass column (Pharmacia, 1.5-cm inner diameter by 90 cm). The Sepharose bed was further equilibrated by passing two column volumes of the above buffer through the column. The void and inclusion volumes were determined by using blue dextran 2000 and ^3H -labeled diisopropyl phosphorofluoridate. The elution behavior of alkali solubilized (see below) parasporal crystals was compared under identical conditions with that of protein molecular weight standards (2 mg/0.4 ml) including aldolase (1.58×10^5), egg albumin (45,000), and chymotrypsinogen A (25,000).

Insect bioassays. Bioassays were done as previously described against the tobacco hornworm Manduca sexta (Schesser et al., 1977) and the mosquitoes Aedes aegypti, Culex pipiens var. quinquefasciatus, and Anopheles albimanus (Tyrell et al., 1979). Alkali solubilized crystals were bioassayed using the same techniques after the preparations were dialyzed against 0.02N NaH_2PO_4 (pH 7.5). Gel filtration chromatographic fractions also were bioassayed exactly as were the alkali solubilized crystals.

RESULTS

Growth characteristics. All five strains grew at almost the same rate. The doubling times were: subsp. kurstaki, 50 min; subsp. berliner, 70 min; subsp. alesti, 65 min; subsp. tolworthi, 80 min; and subsp. israelensis, 65 min. Sporulation was complete (beginning of lysis) at 16 hours in subsp. berliner and at 10 hours in subsp. kurstaki, alesti, tolworthi, and israelensis. Parasporal crystals formed at about stage III of sporulation in all of the strains. The pH of the culture medium during exponential growth decreased from 7.0 to about 6.6 at the beginning of sporulation and then increased to about 8.0 during the early stages of sporulation. The pH remained constant during the later stages of sporulation. All five subspecies exhibited this pattern although the pH during the later stages of sporulation varied somewhat among the subspecies.

Morphology. Thin sections of B. thuringiensis subspecies kurstaki and israelensis cells are shown in Fig. 1. The four subspecies toxic to lepidopteran insects had bipyramidal crystals and ovoid inclusions in sporulating cells as is depicted in Fig. 1A. Each cell usually contained only one crystal and one ovoid inclusion. Subspecies israelensis cells contained inclusions that varied in size and shape (Fig. 1B). The crystals were either cubic, bipyramidal, ovoid, or amorphous. Also, there were complexes of several inclusion bodies enclosed in net-like structures. Generally, cells of subspecies israelensis contained two or three inclusion bodies as shown in Fig. 1B.

Insect toxicity. The effects of parasporal crystals from the four lepidopteran toxic crystals on the tobacco hornworm are presented in Table 1 as lethal concentrations (50% endpoints) along with 95% confidence limits. As can be seen, crystals from all four strains were extremely toxic to the hornworm. B. thuringiensis subsp. berliner was about 5 times as toxic as the other three strains that had nearly equal LC_{50} values. Approximately 90-100% of the larvae were killed at a concentration of 25.1 ng/cm^2 for subsp. kurstaki, 5.5 ng/cm^2 for subsp. berliner, 27.2 ng/cm^2 for subsp. alesti, and 34.1 ng/cm^2 for tolworthi. B. thuringiensis subsp. israelensis crystals, however, exhibited no toxicity to M. sexta larvae at concentrations as high as $300 \text{ } \mu\text{g/ml}$ ($2.0 \times 10^4 \text{ ng/cm}^2$). deBarjac (1978b) also observed that B. thuringiensis subsp. israelensis was ineffective against lepidopteran larvae.

The toxicity of B. thuringiensis subsp. israelensis parasporal crystals on larvae of three species of mosquitoes is shown in Table 2. The crystals were very effective against these particular insects. Parasporal crystals from the other four subspecies affected the mosquito larvae only at very high concentrations. Their toxicity to mosquitoes was 10^3 - 10^6 times less than that of israelensis crystals.

Polyacrylamide gel electrophoresis of crystals. Crystals of all strains are extremely insoluble and require denaturants such as SDS, urea, or guanidine hydrochloride plus a reducing agent such as 2-mercaptoethanol to render them soluble (Cooksey, 1971). Reduced and S-carboxymethylated crystal proteins in reducing and denaturing solvents

exhibited the same electrophoretic profile on SDS-polyacrylamide gels as proteins dissolved in the reducing and denaturing solvent without alkylation. Fig. 2 is an electrophoretogram of B. thuringiensis crystals solubilized in 6M urea, 1% SDS, 1% 2-mercaptoethanol, 0.01M NaH_2PO_4 , Na_2HPO_4 , pH 7.2. Electrophoresis of the solubilized crystals produced a major band (band 1) in the SDS polyacrylamide gel with an apparent molecular weight of approximately 1.35×10^5 in subspecies kurstaki (track B), tolworthi (track E), alesti (track F), and a double band with the same apparent molecular weight for subsp. berliner (track G). This protein has been shown to be the protoxin in subspecies kurstaki (Bulla et al., unpublished data) and is a single repeating subunit in the crystal. The smallest protein produced from subsp. kurstaki, berliner, alesti, and tolworthi had an apparent molecular weight of 6.8×10^4 (band 2). This polypeptide is the insecticidal toxin in subsp. kurstaki (Bulla et al., 1979). Subspecies israelensis crystals produced only minor amounts of material with apparent molecular weights of approximately 1.35×10^5 and 6.8×10^4 (bands 1 and 2, track D) as well as several lower molecular weight polypeptides. The molecular weights of these bands were determined by electrophoresis on a gel having a 5-20% acrylamide gradient which is shown in Fig. 3. The major component of israelensis crystals had an apparent molecular weight of 2.6×10^4 (band 1, tracks B and C; also band 3, tracks C and D of Fig. 2).

Crystals from subsp. kurstaki and israelensis also were solubilized together and the electrophoretic profile produced is shown in Fig. 2 (track C). The pattern obtained overlapped with the pattern for

crystals solubilized separately and electrophoresed (tracks B and D). Apparently, the unique electrophoretic pattern produced by subspecies *israelensis* crystals is not due to proteases of different specificity bound to the crystals.

Crystals can be solubilized without reducing and denaturing agents by increasing the pH of the solvent (Bulla et al., 1979). This was accomplished by titration of the crystals with 1M NaOH to pH 12 and dialysis against 0.02N phosphate at pH 7.5. These solubilized preparations had LC_{50} values similar to those of whole crystals. Electrophoresis of such preparations (Fig. 4) produced patterns similar to those shown in Fig. 2 except that there were more "conversion products" of molecular weights 6.8×10^4 - 1.3×10^5 produced from the lepidopteran toxic crystals (tracks C-F, Fig. 4) and greater amounts of components ranging in molecular weight from 6.8×10^4 to 2.6×10^4 from subspecies *israelensis* (track G).

Gel filtration chromatography. Gel filtration of alkali solubilized crystals in Sepharose CL-4B and phosphate buffer at pH 7.5 yielded single peaks suggesting the presence of only one subunit in all of the crystals. Molecular weight estimation, based on elution volumes (Fig. 5) for the lepidopteran crystal subunits was 1.35×10^5 whereas that for subspecies *israelensis* was 2.5×10^4 .

Amino acid analysis. The amino acid compositions of *B. thuringiensis* crystals are given in Table 3. Glutamic acid and aspartic acid residues were the most abundant in all five strains. Subspecies *kurstaki*, *berliner*, *alesti*, and *tolworthi* had very similar compositions and

closely resemble amino acid analyses reported previously for crystals of other strains of B. thuringiensis (Cooksey, 1971). Subspecies israelensis, however, contained 4 mole % less arginine, 3 mole % less glycine, and 3 mole % more valine. There were smaller differences in the amounts of other amino acids also.

Carbohydrate content. The amount of sugar released by 4M HCl was several fold greater for subspecies israelensis than for the other subspecies. Israelensis crystals contained glucose, mannose, fucose rhamnose, xylose, and galactosamine whereas crystals from the other strains contained only glucose and mannose.

Immunological characterization. The antigenicity of alkali solubilized crystal proteins was examined by means of Ouchterlony double diffusion procedures. The results are presented in Fig. 6. The top assay in each gel (A-E) shows the reaction of each crystal antigen with its homologous antiserum; the bottom portion of each gel shows reactions of all five crystal antigens with a single antiserum. Subspecies israelensis crystal antigen did not react with antisera from the other subspecies. Likewise, crystal antigens from the four lepidopteran toxic strains did not react with israelensis antiserum: however, the former did cross react with each other on unstained Ouchterlony gels.

Tryptic peptide analysis. Crystals were approximately 90% solubilized by trypsin treatment after reduction and S-alkylation. Polyacrylamide gel electrophoretic patterns of insoluble residue from

subspecies kurstaki and israelensis are shown in Fig. 7. Insoluble peptides with apparent molecular weights of 2×10^3 or less were produced from subspecies kurstaki (track D) and from israelensis (track C). However, the israelensis insoluble peptides were slightly larger than those from subspecies kurstaki. The 80% methanol soluble peptides were separated by two dimensional chromatography and electrophoresis. Maps of tryptic peptides are shown in Fig. 8 and a reconstruction of subspecies kurstaki and israelensis maps is shown in Fig. 9. Fingerprints of subspecies kurstaki, berliner, alesti, and tolworthi all were very similar (Fig. 8A-D). All five maps contained the major peptides numbered 1-6. Subspecies israelensis contained four major peptides (numbered 8, 11, 13, and 14) either absent or present in extremely low amounts on the other four maps. These are shaded in Fig. 9B. A comparison of the peptides in Fig. 9 indicates that the crystal of subspecies israelensis is only partially homologous with the other B. thuringiensis crystals.

Spore extracts. Because spore extracts reportedly contain crystal protein (Aronson and Fox, 1978; Bulla et al., 1977; Lecadet et al., 1972; Somerville et al., 1968) and we have shown that purified subspecies kurstaki spores are insecticidal (Schesser and Bulla, 1978), we were interested in examining the extractable spore proteins from these bacteria. An electrophoretic profile of guanidine-hydrochloride, β -ME extracts is displayed in Fig. 10. All spores contained their respective crystal proteins (bands 1, 2, and 3) as shown in Fig. 2 along with an array of lower molecular weight proteins. Subspecies berliner, alesti, tolworthi,

and kurstaki extracts produced similar profiles (tracks C-F), differing mainly in the amount of each protein extracted. Subspecies israelensis spore extract (track B) contained many proteins the same size as those in the other subspecies, including a 68,000-dalton protein (band 2) that is the presumed toxin of subspecies kurstaki (track F). Interestingly, the 26,000-dalton crystal polypeptide (see arrow, track B) was not present in the other spore extracts (tracks C-F).

DISCUSSION

The results of these experiments show that subspecies israelensis, which is toxic to dipteran insects, produced crystals that are structurally, biochemically, and immunologically different from lepidopteran toxic B. thuringiensis parasporal crystals. The closely related lepidopteran toxic subspecies kurstaki, berliner, alesti, and tolworthi produced crystals that are similar structurally, biochemically, immunologically, and functionally.

The four lepidopteran toxic subspecies synthesized bipyramidal crystals and usually each sporangium contained only one such crystal. Subspecies israelensis formed crystals of various shapes and each sporangium contained two or three crystals. Electrophoretic profiles of all five subspecies revealed a protein ($MW_{app} = 1.35 \times 10^5$) which is the major component in the lepidopteran toxic crystals and has been identified as the protoxic subunit from subspecies kurstaki (unpublished data). This component is converted to a toxin ($MW_{app} = 6.8 \times 10^4$) in subspecies kurstaki (Bulla et al., 1979). Proteins of approximately this molecular weight were the smallest found in electrophoretic profiles of the lepidopteran toxic crystals. Profiles of subspecies israelensis crystals showed a smaller major component ($MW_{app} = 26,000$) which may also be a conversion product produced upon solubilization and activation of a 135,000-dalton protoxic molecule.

All five subspecies have six major tryptic peptides in common and peptide maps of the lepidopteran toxic subspecies were almost identical.

However, peptide fingerprints of subspecies israelensis crystals showed four major peptides not present in the other four subspecies. Differences in the number and amounts of minor peptides also occurred.

The insecticidal specificity of B. thuringiensis may be due to toxins with unique amino acid sequences, different carbohydrate content, or both. The four lepidopteran toxic crystals were similar immunologically whereas the dipteran toxic crystal did not cross react immunologically with any of the others. The amino acid composition of subspecies israelensis crystals was slightly different from the other crystals. Particularly, there was more lysine and valine but less arginine and glycine. Small differences in the amounts of other amino acids were observed also. Another interesting feature was that israelensis crystals contained more carbohydrate and a greater variety of sugars than did the other crystals.

Crystal protein is a major spore coat component in subspecies israelensis as well as in spores of the other subspecies. However, the differences in subspecies israelensis extractable coat protein profile to that of the other strains was not due entirely to variation in crystal proteins. Differences in small molecular weight protein components of the coat also occurred. What significance this aspect has on the biology of subspecies israelensis is not known.

Like the other B. thuringiensis subspecies, subspecies israelensis is nontoxic to mammals (deBarjac, 1978). The toxicity spectrum with regard to insects, however, is completely different since purified subspecies israelensis crystals are toxic to dipterans but do not kill lepidopterans

whereas crystals from the other subspecies kill lepidopteran larvae and have only a minimal effect on dipteran larvae. Recently, Ohba and Aizawa (Ohba and Aizawa, 1979) isolated a new subspecies, B. thuringiensis subspecies kyushuensis, that produces crystals having a toxicity spectrum similar to that of subspecies israelensis (Ohba and Aizawa, 1979; Ohba and Aizawa, 1978). Those findings along with the data reported here indicate that two groups exist within B. thuringiensis: one group that synthesizes lepidopteran toxic crystals and a new group that produces dipteran toxic crystals. Subspecies kurstaki, berliner, alesti, and tolworthi currently are used to control agriculturally important lepidopteran insect pests in various parts of the world (Cooksey, 1971). B. thuringiensis subsp. israelensis now is being developed as a control agent for several medically important mosquitoes and black flies (deBarjac, 1978; Garcia and Desrochers, 1979; Goldberg and Margalt, 1977; Undeen and Nagel, 1978). Further characterization of the dipteran toxic crystals and elucidation of the genetics of both crystal types is now being conducted in our laboratory.

ACKNOWLEDGMENTS

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Table 1. Lethal concentrations and confidence limits of B. thuringiensis parasporal crystal for M. sexta.

Subspecies	Lethal concentration (ng/cm ²)	95% confidence limits (ng/cm ²)	
	50% endpoint	Upper	Lower
<u>kurstaki</u> ^a	6.88	8.50	5.50
<u>berliner</u> ^b	1.36	2.25	1.16
<u>alesti</u> ^c	6.14	13.10	3.41
<u>tolworthi</u> ^d	6.82	9.48	5.53
<u>israelensis</u>	>100		

^aSlope = 2.52, intercept = 7.40

^bSlope = 1.94, intercept = 8.18

^cSlope = 1.27, intercept = 6.34

^dSlope = 3.59, intercept = 8.56

Table 2. Lethal concentrations and confidence limits of B. thuringiensis subsp. israelensis parasporal crystal for mosquito larvae.

Species	Lethal concentration ($\mu\text{g/ml}$) 50% endpoint	95% confidence limits ($\mu\text{g/ml}$)	
		Upper	Lower
<u>Aedes aegypti</u> ^a	1.9×10^{-4}	2.5×10^{-4}	1.4×10^{-4}
<u>Culex pipiens</u> var. <u>quinquefasciatus</u> ^b	3.7×10^{-4}	6.6×10^{-4}	2×10^{-4}
<u>Anopheles albimanus</u> ^c	8.0×10^{-3}	1.4×10^{-2}	4.6×10^{-3}

^aSlope, 1.72; intercept, 11.40.

^bSlope, 0.90; intercept, 8.10.

^cSlope, 1.05; intercept, 7.20.

Table 3. Amino acid composition of *B. thuringiensis* crystals.

Amino acid	Moles per 100 moles of crystals ^a				
	kurstaki	berliner	alesti	tolworthi	israelensis
Tryptophan	0	0	0	0	0
Lysine	2.8	2.5	2.6	2.9	4.2
Histidine	1.8	1.7	1.5	1.7	1.3
Arginine	6.7	5.5	5.3	6.3	2.3
Aspartic acid	13.5	15.1	14.0	13.7	14.2
Threonine	6.2	6.9	6.3	6.6	8.0
Serine	8.3	9.1	10.2	9.0	6.9
Glutamic acid	12.2	12.9	13.1	12.6	10.7
Proline	4.4	3.1	4.1	3.2	5.0
Half-cystine ^b	2.0	1.3	1.8	1.2	2.1
Glycine	7.2	9.0	9.1	8.2	5.4
Ananine	5.4	6.5	6.4	6.4	7.3
Valine	6.2	4.3	4.6	5.0	7.7
Methionine ^b	1.0	0.8	0.7	0.9	1.9
Isoleucine	4.9	3.0	3.2	4.3	6.1
Leucine	8.2	8.7	8.6	8.5	8.2
Tyrosine	4.9	4.5	4.4	4.6	3.8
Phenylalanine	5.2	4.6	4.7	4.9	4.8

^aDetermined by hydrolysis in p-toluenesulfonic acid (Liu and Chang, 1971).
Half cystine and methionine were not stable to hydrolysis.

^bDetermined as cysteic acid and methionine sulfone after performic acid oxidation (Hirs, 1967).

Fig. 1. Electron micrographs of thin-sectioned, sporulated cells of Bacillus thuringiensis subsp. kurstaki (A, X 44,000) and israelensis (B, X 37,000).

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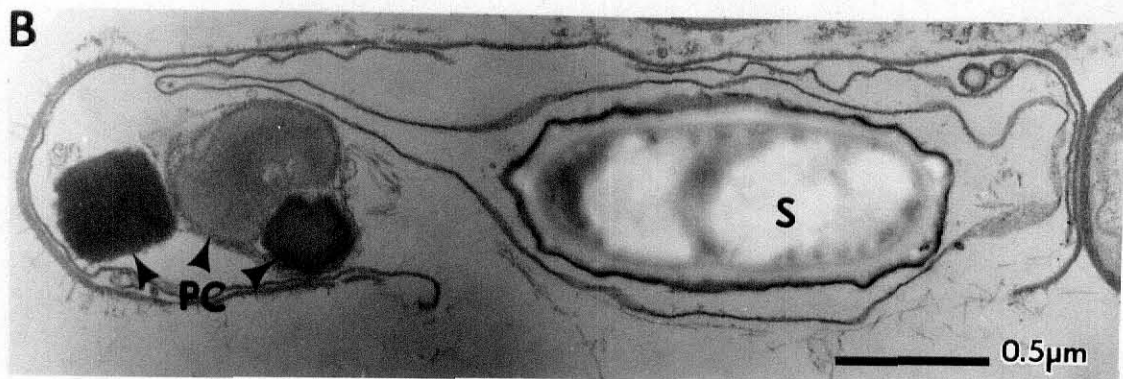
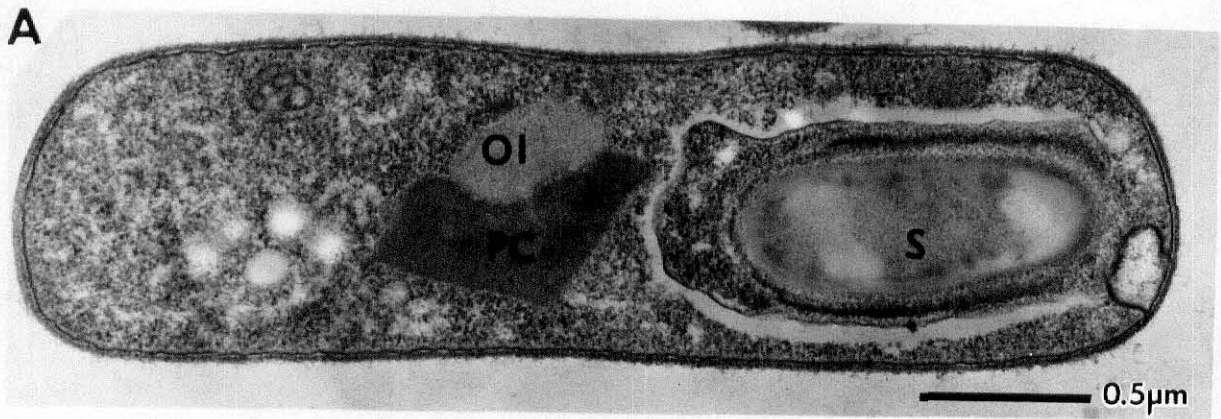


Fig. 2. Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of B. thuringiensis paraporal crystals solubilized in 1% SDS, 1% ME, 6 M urea, 0.01 N $\text{NaH}_2\text{PO}_4:\text{Na}_2\text{HPO}_4$, pH 7.2. This slab contained a continuous gradient of 3-10% acrylamide. (A) Standards: myosin (200,000) β -galactosidase (135,000), phosphorylase B (92,000), bovine serum albumin (68,000) and ovalbumin (43,000); 1 μg of each protein was applied; (B) subspecies kurstaki, 5 μg ; (C) subspecies kurstaki, 5 μg and subspecies israelensis, crystals, 10 μg , solubilized together; (D) subspecies israelensis, 10 μg ; (E) subspecies tolworthi, 7 μg ; (F) subspecies alesti, 5 μg ; (G) subspecies berliner, 5 μg .

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of B. thuringiensis subsp. israelensis crystals solubilized as in Fig. 2 on a slab containing a continuous gradient of 5-20% acrylamide. (A) Standards: bovine albumin (65,000), ovalbumin (45,000), chymotrypsinogen A (25,000), myoglobin (17,000) ribonuclease A (13,600), and insulin (5700), 1-2 μg of each protein was applied; (B) subspecies israelensis crystal, 10 μg ; (C) subspecies israelensis, 15 μg ; (D) bovine albumin, 1 μg ; (E) ovalbumin, 1 μg ; (F) chymotrypsinogen A, 1 μg ; (G) myoglobin, 1 μg ; (H) ribonuclease A, 1 μg ; (I) insulin, 1 μg .

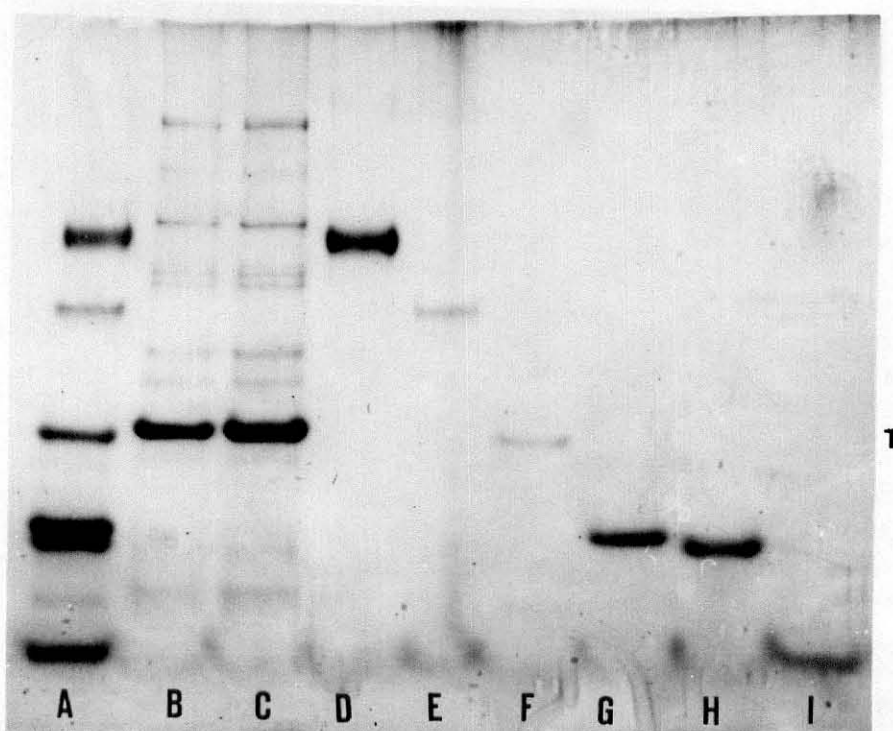
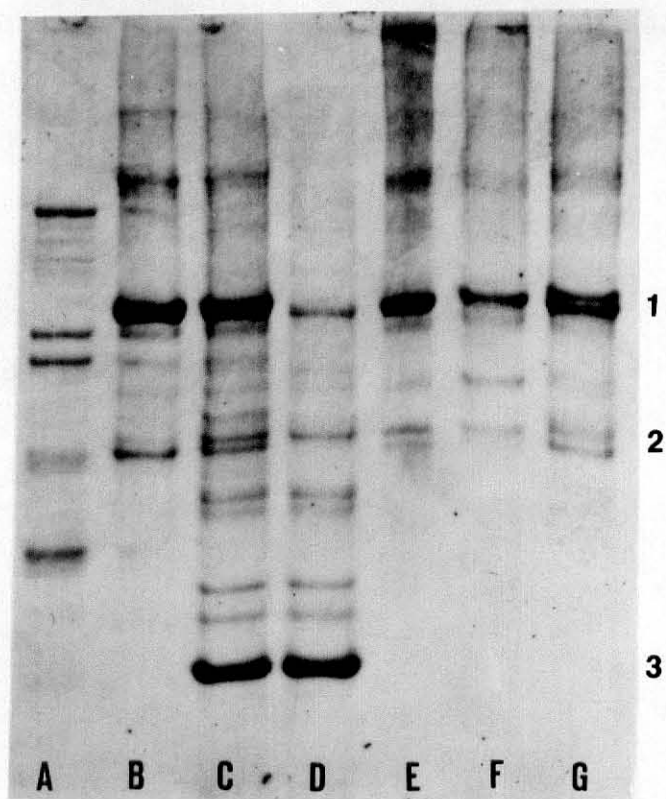


Fig. 4. Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of the B. thuringiensis parasporal crystals solubilized in NaOH and dialyzed overnight against the solubilization buffer described in Fig. 2. The slab contained a continuous gradient of 5-15% acrylamide. (A) Cross linked bovine serum albumin (68,000, 136,000, etc.); (B) Standards as in Fig. 3, 1.5 - 3 μ g per protein applied; (C) subspecies berliner (D) subspecies alesti; (E) subspecies tolworthi; (F) subspecies kurstaki; (G) subspecies israelensis; (H) standards are same as in track B; (I) cross linked bovine albumin, same as track A. 10-20 μ g of each crystal protein was applied in tracks C-G.

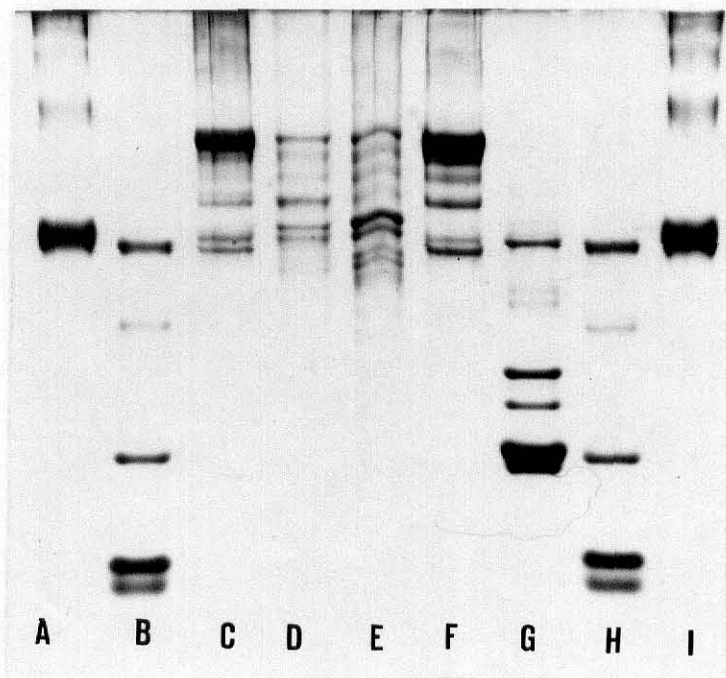


Fig. 5. Determination of molecular weight for major peak from alkali solubilized crystals. Standards left to right: aldolase (158,000), egg albumin (45,000), chymotrypsinogen A (25,000); (■) kurstaki, (Δ) alesti, (○) berliner, (□) tolworthi, (▲) israelensis.

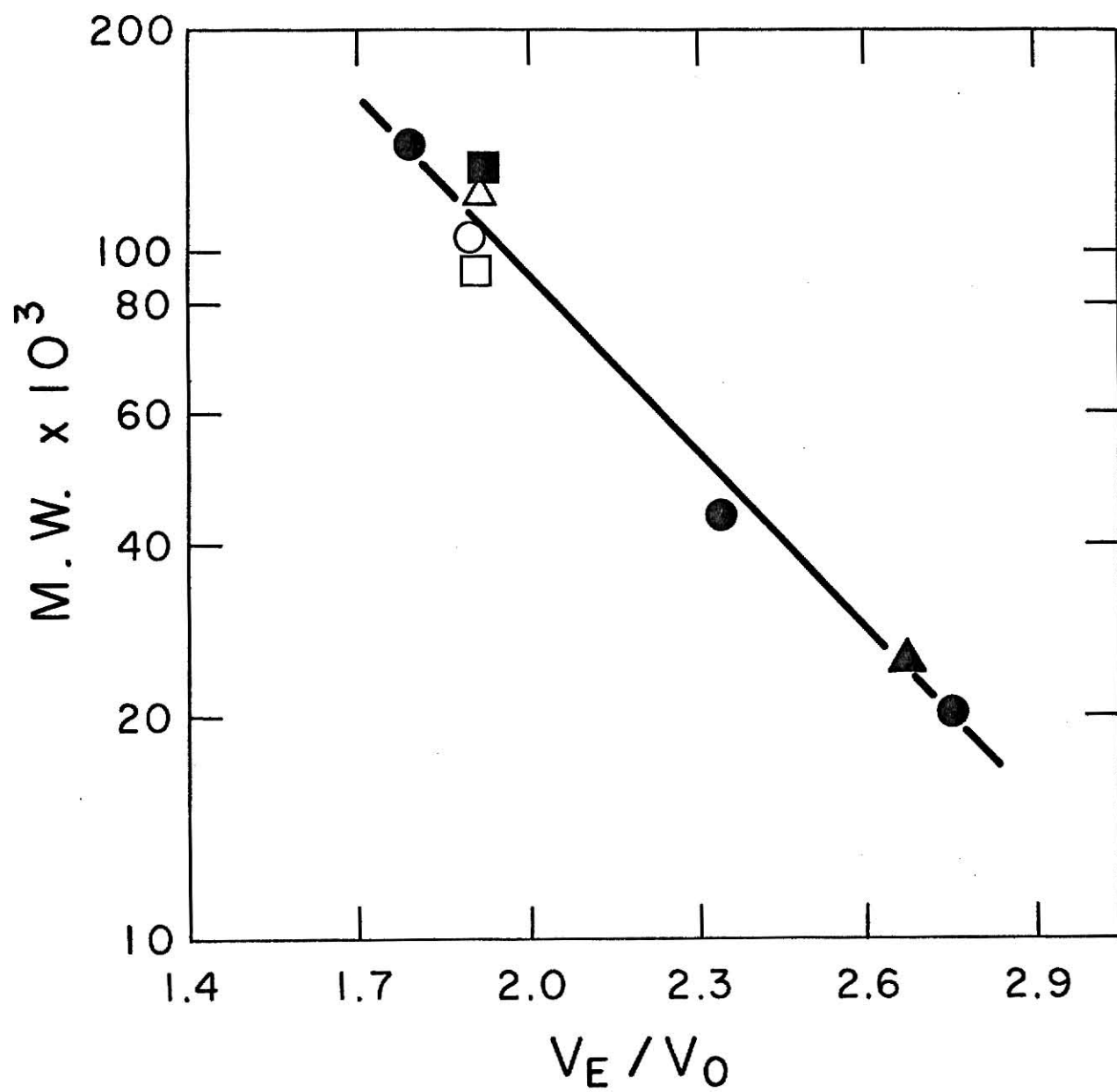


Fig. 6. Ouchterlony double diffusion immunological analysis of parasporal crystals. (A) Center wells contain kurstaki crystal antibody; wells 1-6: kurstaki crystal antigen; well 7: israelensis antigen; wells 8 and 11: kurstaki antigen; well 9: berliner antigen; well 10: tolworthi antigen; well 12: alesti antigen. (B) Center wells contain berliner antibody; wells 1-6: berliner crystal antigen; well 7: israelensis antigen; wells 8 and 11: berliner antigen; well 9: tolworthi antigen; well 10: kurstaki antigen; well 12: alesti antigen. (C) Center wells contain tolworthi crystal antibody; wells 1-6: tolworthi crystal antigen; well 7: israelensis antigen; wells 8 and 11: tolworthi antigen, well 9: berliner antigen; well 10: kurstaki antigen; well 12: alesti antigen. (D) Center wells contain alesti crystal antibody; wells 1-6: alesti crystal antigen; well 7: israelensis antigen; wells 8 and 11: alesti antigen; well 9: kurstaki antigen; well 10: berliner antigen; well 12: tolworthi antigen. (E) Center wells contain israelensis crystal antibody; wells 1-6: israelensis crystal antigen; well 7: tolworthi antigen; wells 8 and 11: israelensis antigen; well 9: kurstaki antigen; well 10: berliner antigen; well 12: alesti antigen.

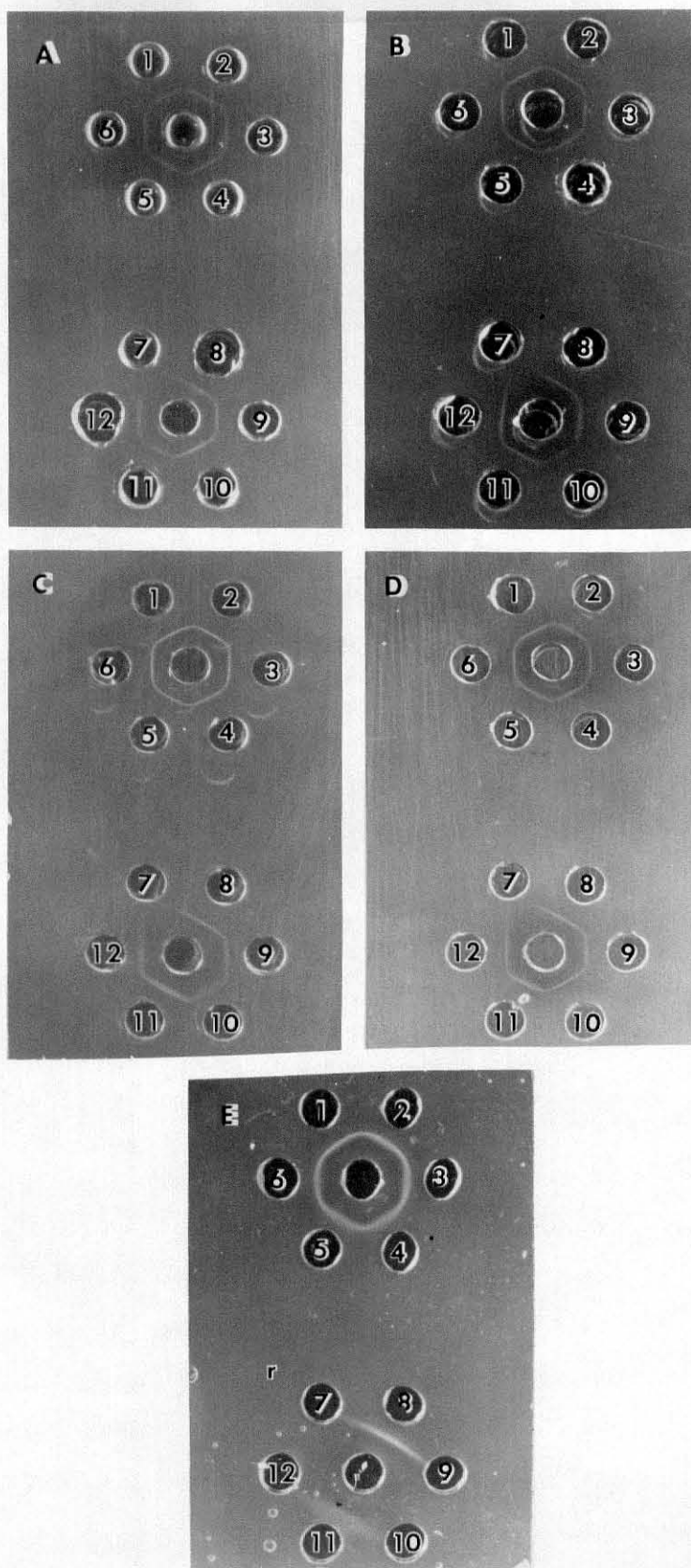


Fig. 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of S-alkylated and trypsin treated B. thuringiensis crystals. The methanol insoluble material was solubilized as in Fig. 2. The slab contained a continuous gradient of 10-20% acrylamide. (A) Standards as in Fig. 3, 1.5-3 μ g of each protein was applied. (B) subspecies kurstaki crystal (C) subspecies israelensis crystal.



Fig. 8. Fingerprints of tryptic digests of B. thuringiensis crystal proteins. (A) subspecies kurstaki, (B) subspecies berliner (C) subspecies alesti, (D) subspecies tolworthi, (E) subspecies israelensis.

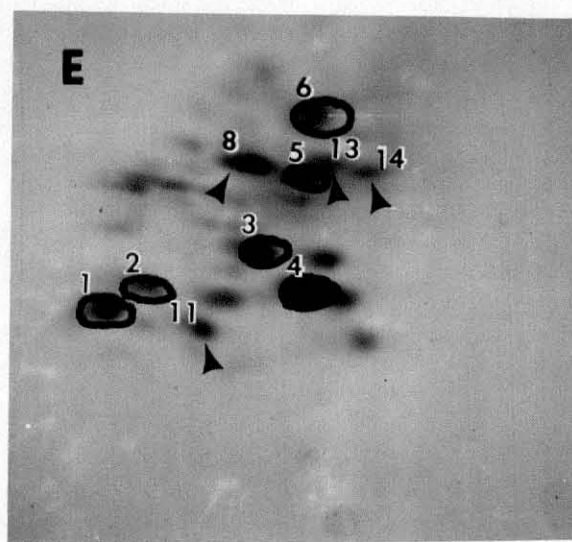
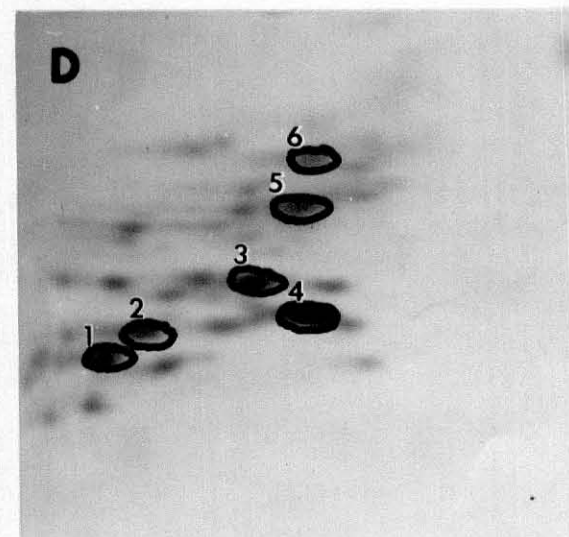
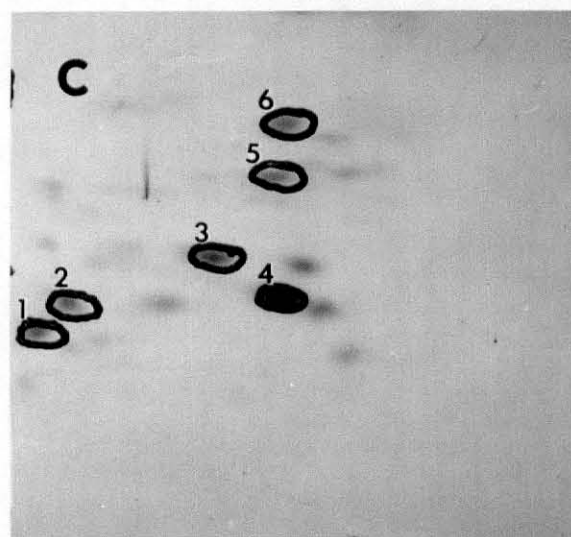
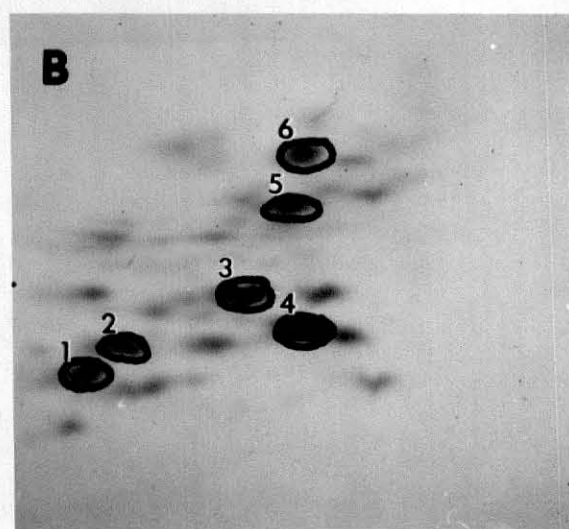
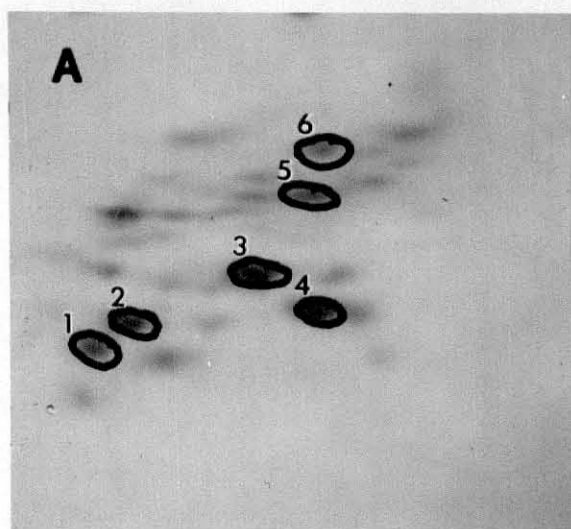


Fig. 9. Reconstructed tryptic peptide maps of parasporal crystals from B. thuringiensis subspecies kurstaki (A) and israelensis (B). Spots 1-6 outlined with solid lines represent major peptides common to both subspecies. Shaded spots in (B) are major peptides unique to subspecies israelensis.

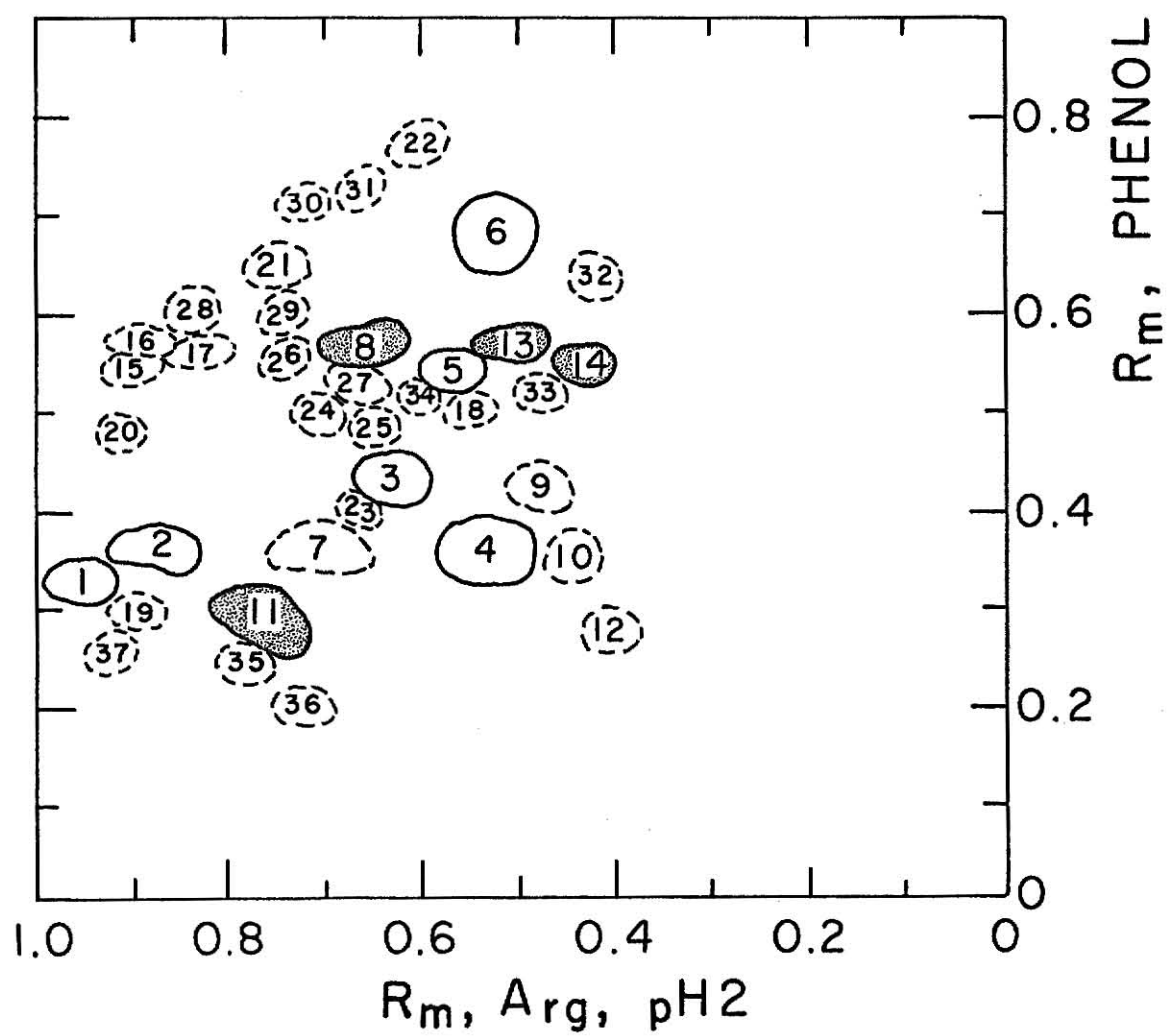
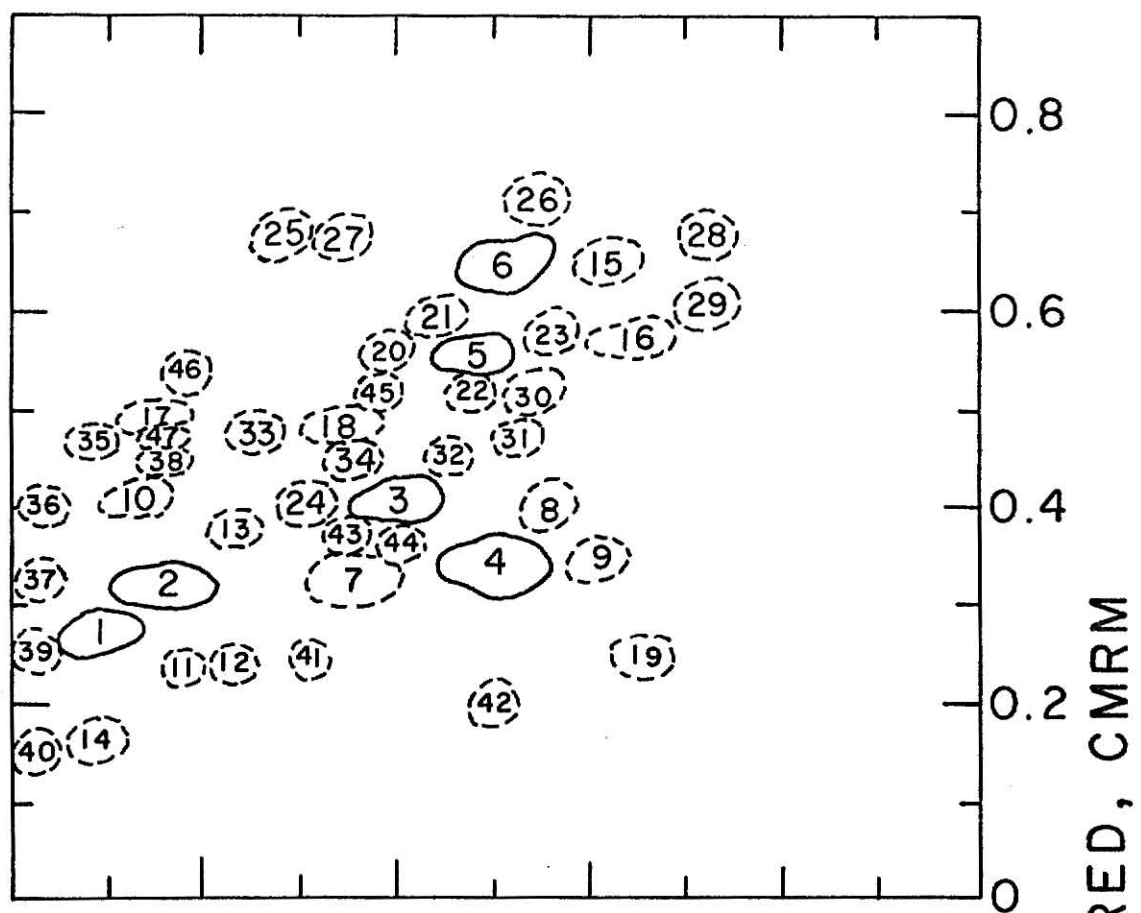
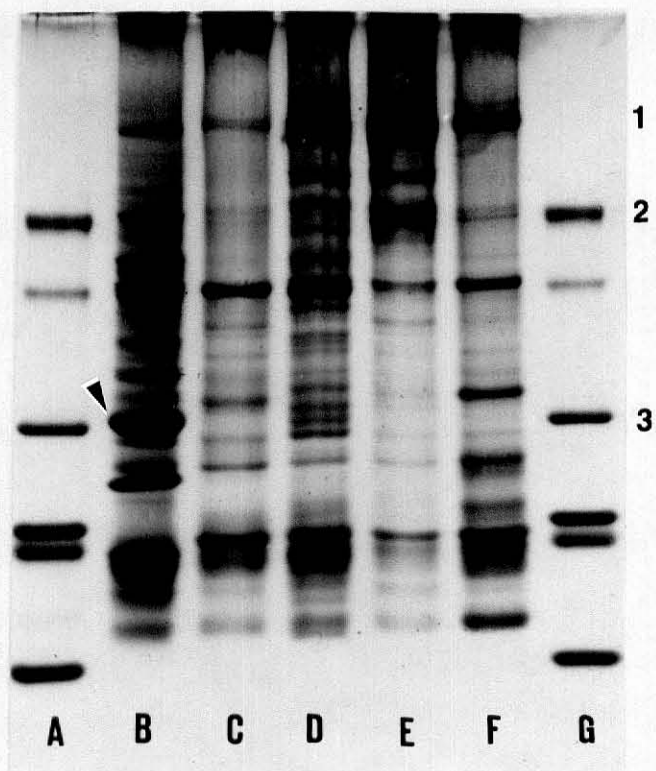


Fig. 10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of B. thuringiensis spore extracts solubilized as in Fig. 2. The slab contained a continuous gradient of 5-20% acrylamide. (A) Standards as in Fig. 3, 1.5-3 μ g of each protein was applied; (B) subspecies israelensis spore extract; (C) subspecies berliner spore extract; (D) subspecies alesti spore extract; (E) subspecies tolworthi spore extract; (F) subspecies kurstaki spore extract; (G) Standards as in track A. 60 μ g of each spore extract was applied.



SECTION IV

CHARACTERIZATION OF SPORE COATS OF BACILLUS THURINGIENSIS

AND BACILLUS CEREUS

ABSTRACT

Spore coat extracts from Bacillus thuringiensis subspecies kurstaki and israelensis and Bacillus cereus T and B. cereus NRRL 569 were characterized by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by amino acid analysis. Both B. cereus spore coats had similar electrophoretic profiles. The B. thuringiensis spore coats contained crystal proteins as major components as well as lower molecular weight proteins. B. thuringiensis subsp. israelensis had a unique coat protein profile which was different from B. cereus and B. thuringiensis subsp. kurstaki coats. Insecticidal activity of spores against the tobacco hornworm, Manduca sexta, and the mosquito, Aedes aegypti, also was determined. B. thuringiensis subsp. kurstaki spores were lethally toxic to the tobacco hornworm (Lepidoptera) larvae whereas spores of the other subspecies were not. Except for minimal toxicity of subspecies israelensis, none of the spores were effective against the mosquito (Diptera) larvae.

INTRODUCTION

Bacillus thuringiensis and B. cereus often are considered as one group of bacteria and their relatedness has been shown by various classification methods (Buchanan and Gibbons, 1974). For example, the two species are indistinguishable by zone electrophoresis of certain enzymes (Baptist et al., 1978); they have a high degree of DNA sequence homology (54-80%; Seki et al., 1978; Somerville and Jones, 1972); and bacteriophages have been isolated that carry out generalized transduction in both organisms (Thorne, 1978). The feature that has been used most to distinguish the two species is the presence of a discrete parasporal crystal that is formed along side the endospore in B. thuringiensis (Buchanan and Gibbons, 1974). The crystal, which is insecticidal to lepidopteran larvae, contains a single glycoprotein subunit that has an apparent molecular weight of 1.35×10^5 (Bulla et al., 1977a). B. cereus does not form such a crystalline inclusion. Interestingly, spore coats of B. thuringiensis contain the crystal protein (Bulla et al., 1977b) and spores of this bacterium also are insecticidal (Schesser and Bulla, 1978).

B. cereus spore coats, on the other hand, contain a 13,000-dalton monomer and a 26,000-dalton dimer (Aronson and Pandey, 1978). Spores of B. cereus do not contain a 135,000-dalton glycoprotein like that of B. thuringiensis subsp. kurstaki (Bulla et al., 1977b), they are not insecticidal (unpublished data), and they germinate more rapidly than do B. thuringiensis spores (Aronson and Pandey, 1978).

Recently, a new strain of B. thuringiensis was isolated, designated subspecies israelensis, that synthesizes parasporal inclusions which are toxic to mosquitoes and blackflies (deBarjac, 1978c; Tyrell et al., 1979; Undeen and Nagel, 1978). Unlike most of the other subspecies of B. thuringiensis, it does not kill lepidopteran insects (moths and butterflies). The major crystal component of this bacterium is a glycoprotein with an apparent molecular weight of 26,000 (unpublished data). Because of the similarity in protein composition of crystals of B. thuringiensis subsp. israelensis to that of spore coats of B. cereus, i.e. the reported 26,000-dalton protein (Aronson and Pandey, 1978), we wanted to investigate the spore coat protein composition of B. thuringiensis subsp. israelensis for comparative analysis. In this study, the spore coat of B. thuringiensis subsp. israelensis was compared to subspecies kurstaki spore coat and to spore coats from B. cereus T and B. cereus NRRL 569.

MATERIALS AND METHODS

Organisms and cultural conditions. B. thuringiensis subsp. kurstaki was isolated from a commercial insecticidal formulation called Dipel[®] (Abbott Laboratories, North Chicago, IL). B. thuringiensis subsp. israelensis was a gift from David Rockhold, Stauffer Chemical Co., San Jose, CA. B. cereus T was a gift from A. I. Aronson, Purdue University, West Lafayette, IN, and B. cereus NRRL 569 was given to us by C. B. Thorne, University of Massachusetts, Amherst, MA.

Stock cultures were maintained on MD agar (Bulla et al., 1970) or glucose yeast extract agar (Nickerson et al., 1974) slants. Cells for experimental use were cultured in the manner previously described for B. thuringiensis subsp. kurstaki (Bulla et al., 1977a).

Isolation of spores and crystals. Spores and parasporal crystals of the B. thuringiensis subspecies were isolated by bouyant density centrifugation in Renografin gradients (Sharpe et al., 1975), washed at least three times in water, and lyophilized. Purity of preparations was monitored at each step of the separation procedure by phase-contrast microscopy. Sporulated B. cereus cultures were centrifuged in a Sorval SS3 centrifuge at 10,000 rpm for 10 min. The pellets were washed three times with distilled water and centrifuged at 3000 rpm for 10 min. after each wash. Purity of these preparations was monitored also by phase-contrast microscopy. The washed spores were then lyophilized.

Insect bioassays. Bioassays against the tobacco hornworm, Manduca sexta, and the mosquito, Aedes aegypti were done as previously described (Schesser and Bulla, 1978; Tyrell, 1979).

Spore coat extractions. All spores were extracted with three aliquots of 6 M guanidinium chloride, 0.1 M beta-mercaptoethanol, pH 8.6, (extraction buffer A). The material was subsequently dialyzed against 0.1 M Tris-HCl and 9 M urea (pH 8.6) for 24 hr and against water for 48 hr (40 - 44 liters) and then lyophilized.

B. cereus T spores also were extracted with three aliquots of the extraction buffer described by Cheng and Aronson (1977) containing 8 M urea, 0.07 M DTT, 1% SDS, 5 mM CHES, pH 9.6 (extraction buffer B). The combined extracts were either used immediately for polyacrylamide gel electrophoresis, refrigerated overnight and then electrophoresed, or dialyzed and lyophilized as described above.

B. cereus T and B. thuringiensis subsp. kurstaki spores were extracted in a third way using buffer B without SDS and containing 2 mM PMSF (extraction buffer C) and dialyzed and lyophilized as described for the buffer A extracts.

Polyacrylamide gel electrophoresis. Spore extracts were solubilized by incubating the lyophilized material in 1% (wt/vol) SDS, 1% (vol/vol) beta-mercaptoethanol, 6 M urea, 0.01 M NaH_2PO_4 : Na_2HPO_4 at pH 7.2 for 1 hour at room temperature. These solubilized extracts as well as the undialyzed buffer B extracts of B. cereus T were subjected to electrophoresis on SDS polyacrylamide gradient slab gels (1.5 x 140 x 170 cm; Buchler Polyslab polyacrylamide gel electrophoresis system) using the method of Weber and Osborn (Weber et al., 1972). Gradient gels contained 5-20% or 10-20% acrylamide. Electrophoresis was carried out at 0-90

volts. Gels were stained 3-12 hr in 0.25% (wt/vol) coomassie brilliant blue (Eastman R250) in methanol:acetic acid:water (25:7.5:62.5; vol/vol/vol) and destained for 16-48 hr in methanol:acetic acid:water (25:7.5:62.5; vol/vol/vol). Molecular weight standards were cross-linked bovine serum albumin (68,000, 136,000, etc.) from Sigma; bovine albumin (65,000), ovalbumin (45,000), beef pancreas chymotrypsinogen A (25,000) and sperm whale myoglobin (17,000) from Schwarz/Mann; ribonuclease A (13,600) from Pharmacia; and bovine pancreas insulin (5700) from Sigma. Destained gels were photographed on 35 mm high contrast copy film which was developed in Kodak D-19. Gels were dried using a Bio-Rad Model 224 slab gel dryer.

Amino acid analysis. Parasporal crystals of B. thuringiensis subsp. kurstaki and israelensis and buffer A spore coat extracts were analyzed on a Beckman 120C analyzer after hydrolysis in 3M p-toluenesulfonic acid containing a small amount of tryptamine. The hydrolysis was carried out for 24 hr at 110°C in vacuo (Liu and Chang, 1971). Labile amino acids were quantitated by extrapolation to zero time. Cystine and cysteine were determined as cysteic acid and methionine as the sulfone after performic acid oxidation (Hirs, 1967).

RESULTS AND DISCUSSION

Insect toxicity. Spores of both B. cereus strains caused no mortality of M. sexta larvae at concentrations as high as 6.8 μg per cm^2 of diet surface. Similarly, B. thuringiensis subsp. israelensis spores caused no mortality of this insect at concentrations as high as 20.5 μg per cm^2 diet surface. Crystals from these bacteria also were nontoxic to M. sexta (Tyrell et al., 1979). Spores of B. thuringiensis subsp. kurstaki did kill tobacco hornworms. The lethal concentrations (50% endpoints) or LC_{50} of B. thuringiensis subsp. kurstaki spores was determined previously (Schesser and Bulla, 1978) and the spore protein concentration at the LC_{50} value (138 mg/ml) was 23% less than that of the crystal protein (112 mg/ml).

Both B. cereus subspecies and B. thuringiensis subsp. kurstaki caused no mortality of the mosquito, A. aegypti, at spore concentrations as high as 100 mg/ml (dry weight). B. thuringiensis subsp. israelensis spores had an LC_{50} value of 4×10^{-2} $\mu\text{g}/\text{ml}$ against this mosquito, two hundred times more than that of the crystal ($\text{LC}_{50} = 1.9 \times 10^{-4}$ $\mu\text{g}/\text{ml}$). Because the spore LC_{50} value was somewhat high, we believe that the mortality observed could be accounted for by the presence of minute quantities of contaminating crystals. The relative nontoxicity of israelensis spores may be due to inefficient extraction of the protein in the mosquito larval gut or to modification of the protein during coat assembly so that it loses its toxicity or the crystal and coat proteins may be different.

Spore coat protein extraction and gradient gel electrophoresis.

Various spore extraction methods were tried using B. cereus T and B. thuringiensis subsp. kurstaki spores. The B. cereus T extracts are shown in Fig. 1. Fig. 1A shows a comparison of buffer B and buffer A extracts. Both methods produced the same protein profiles with buffer B extracting slightly more of a 12,500-dalton polypeptide. Buffer A, containing guanidine and beta-mercaptoethanol, is the buffer we routinely use for extracting B. thuringiensis spores whereas buffer B containing urea, SDS, CHES, and DTT, is the buffer used by Aronson and Pandey (1978) and Cheng and Aronson (1977) to extract B. cereus T spores (Aronson and Pandey, 1978; Cheng and Aronson, 1977). The SDS gels used by these investigators contained urea and were buffered in Tris-glycine whereas ours were phosphate buffered. The investigators (Aronson and Pandey, 1978; Cheng and Aronson, 1977) reported a 13,000-dalton coat protein and a 26,000-dalton dimer as the major extractable proteins. Our 5-20% acrylamide gradient resolves a 13,000-dalton protein into two bands with apparent molecular weights of 12,500 and 14,000. We found no 26,000-dalton dimer under our electrophoresis conditions. Buffer B extracts of B. cereus T, which were not dialyzed or lyophilized are shown in Fig. 1B and C. These extracts produced electrophoretic profiles identical to dialyzed and lyophilized protein. The buffer C extract also is shown in Fig. 1B. This buffer contained urea, DTT, and PMSF but no SDS. The profile was identical to the buffer A extract. Buffer A also contained no SDS. B. thuringiensis subsp. kurstaki spore extracts, however, gave different profiles after extraction with

buffers A and C. These are shown in Fig. 2. The buffer A extract had the 135,000-dalton crystal protein and 68,000-dalton conversion product (Bulla et al., 1977) as its major spore coat proteins along with a 13,000-dalton polypeptide. However, when buffer C was used to extract the B. thuringiensis subsp. kurstaki spores, most of the 135,000 and 68,000 molecular weight proteins were degraded to a polypeptide with an apparent molecular weight of 11,000. The serine protease inhibitor PMSF did not prevent this degradation.

Buffer A, which contains guanidine and mercaptoethanol, but no SDS, was chosen as the extraction buffer for the comparison of coat proteins since it prevents degradation of B. thuringiensis coat proteins without having them bound by SDS and it extracts the same proteins as buffer B from B. cereus T. The coat profiles of the B. cereus strains and the coat and crystal protein profiles of the B. thuringiensis strains are shown in Fig. 3. Apparent molecular weights of the major coat proteins are given in Table I. As can be seen, the B. cereus coat profiles were very similar. The coats of both strains contained proteins with the same molecular weights. Differences occurred in the relative amounts of each protein extracted. Both coats contained major components with apparent molecular weights of 14,000 and 12,500. B. cereus 569 coats also contained major proteins with apparent molecular weights of 21,000 and 15,500. These were present in small amounts in B. cereus T coats.

The B. thuringiensis coats were different from each other as well as from the B. cereus coats. The B. thuringiensis subsp. kurstaki

coat protein composition was similar to those of other lepidopteran toxic B. thuringiensis subspecies (unpublished data). It contained large amounts of the crystal monomer ($MW_{app} = 135,000$) and the crystal conversion product ($MW_{app} = 68,000$). The major low molecular weight coat protein (not a crystal coat component) had an apparent molecular weight of 13,000. B. thuringiensis subsp. israelensis spores contained crystal proteins, the major band having an apparent molecular weight of 26,000. The non crystal coat proteins were a 27,000-dalton protein (possibly, it is a modified crystal protein) and a 13,000-dalton polypeptide. In addition to the crystal proteins, B. thuringiensis subsp. israelensis coat contained several minor components also not found in the B. cereus or B. thuringiensis kurstaki coats.

Amino acid analyses were done using buffer A spore coat extracts and B. thuringiensis crystals. The data are presented in Table II. The amino acid contents of the B. cereus coats were very similar to each other. The major difference was that B. cereus 569 had almost twice as much histidine as B. cereus T. Differences of only one mole/100 moles or less were observed in the other amino acids.

The B. thuringiensis coat extracts were similar. Approximately 1.4 moles/100 moles or less variance occurred between the amounts of each amino acid in each coat. There also was some similarity to both the B. cereus coat amino acid compositions although several small differences were evident. Major differences occurred between the B. thuringiensis crystal and coat amino acid profiles, particularly tryptophan, arginine, and glycine. Probably this was due to the presence of noncrystal coat components rich in certain amino acids.

The results of this investigation indicate that the new B. thuringiensis subsp. israelensis has a unique spore coat protein profile unlike those of lepidopteran toxic B. thuringiensis coats, such as subspecies kurstaki, and B. cereus coats. Previously, we had shown that spores of acrySTALLiferous mutants of B. thuringiensis subsp. kurstaki germinate more rapidly than spores of B. cereus (Stahly et al., 1978). Interestingly, the rapidly germinating spores lacked the 135,000-dalton crystal protein in their coats, and the spores were not toxic to M. sexta. What particular function(s), besides insecticidal activity, that the high molecular weight coat proteins have is not known. Perhaps, survival in nature is dependent upon spore germinability, which, in turn may be determined by certain coat proteins. Certainly, the differences in coat proteins among the organisms that we studied could affect the stability of their spores in certain environments. If so, the unique spore coat protein profile of B. thuringiensis subsp. israelensis may afford that organism a distinct survival advantage over the other bacilli in mosquito breeding sites.

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Table I. Apparent molecular weights of the major spore coat proteins
of B. thuringiensis and B. cereus.

Bacterial species	MW _{app} of spore coat components			
<u>B. thuringiensis</u>				
subsp. <u>kurstaki</u>	135,000			68,000
			13,000	
<u>B. thuringiensis</u>				
subsp. <u>israelensis</u>	27,000			26,000
			13,000	
<u>B. cereus</u> T			14,000	12,500
<u>B. cereus</u> NRRL 569	21,000	15,000	14,000	12,500

Table II. Amino acid composition. ^{a,b}

	<u>B. thuringiensis</u>		<u>B. thuringiensis</u>		<u>B. cereus T</u>		<u>B. cereus 569</u>	
	crystals	spore coats	crystals	spore coats	spore coats	spore coats	spore coats	spore coats
Trp	0	5.3	0	5.2	6.3		5.2	
Lys	2.8	3.5	4.2	3.9	3.5		4.6	
His	1.8	1.8	1.3	2.3	1.9		3.7	
Arg	6.7	3.0	2.3	2.8	2.4		2.8	
Asp	13.5	10.5	14.2	11.9	10.2		10.3	
Thr	6.2	8.9	8.0	8.2	9.1		8.0	
Ser	8.3	7.3	6.9	6.7	7.8		7.0	
Glu	12.2	9.9	10.7	10.3	9.5		8.3	
Pro	4.4	4.8	5.0	5.0	7.0		8.1	
Cys/2 ^c	2.0	2.6	2.1	2.0	1.0		1.9	
Gly	7.2	11.4	5.4	10.3	11.3		12.1	
Ala	5.4	7.6	7.3	6.9	9.3		8.9	
Val	6.2	4.4	7.7	4.9	4.1		3.8	
Met ^c	1.0	0.5	1.9	0.7	0.4		0.3	
Ile	4.9	3.7	6.1	3.8	3.4		2.6	
Leu	8.2	7.5	8.2	7.8	7.5		6.8	
Tyr	4.9	2.9	3.8	3.1	2.0		1.5	
Phe	5.2	4.6	4.8	4.0	3.4		4.1	

^aResults in mol %.^bDetermined by hydrolysis in p-toluenesulfonic acid (Hirs, 1967).^cDetermined as cysteic acid and methionine sulfone after performic acid oxidation (Liu and Chang, 1971).

Fig. 1. SDS polyacrylamide gels of B. cereus T spore extracts.

(A) 5-20% acrylamide gradient. A1: Standards: bovine serum albumin (65,000), ovalbumin (45,000), chymotrypsinogen A (25,000), myoglobin (17,000), ribonuclease A (13,000), and insulin (5,700). A2, 3, and 4: Buffer B extract, dialyzed and lyophilized. A5, 6, and 7: Buffer A extract. A8: Standards: same as A1.

(B) 10-20% acrylamide gel. B1: standards, same as A1. B2 and 3: Buffer B extract, fresh. B4: Buffer B extract, dialyzed. B5: Buffer C extract.

(C) 5-20% acrylamide gel. (1) Buffer B extract refrigerated overnight, (2) Standards, same as A1.

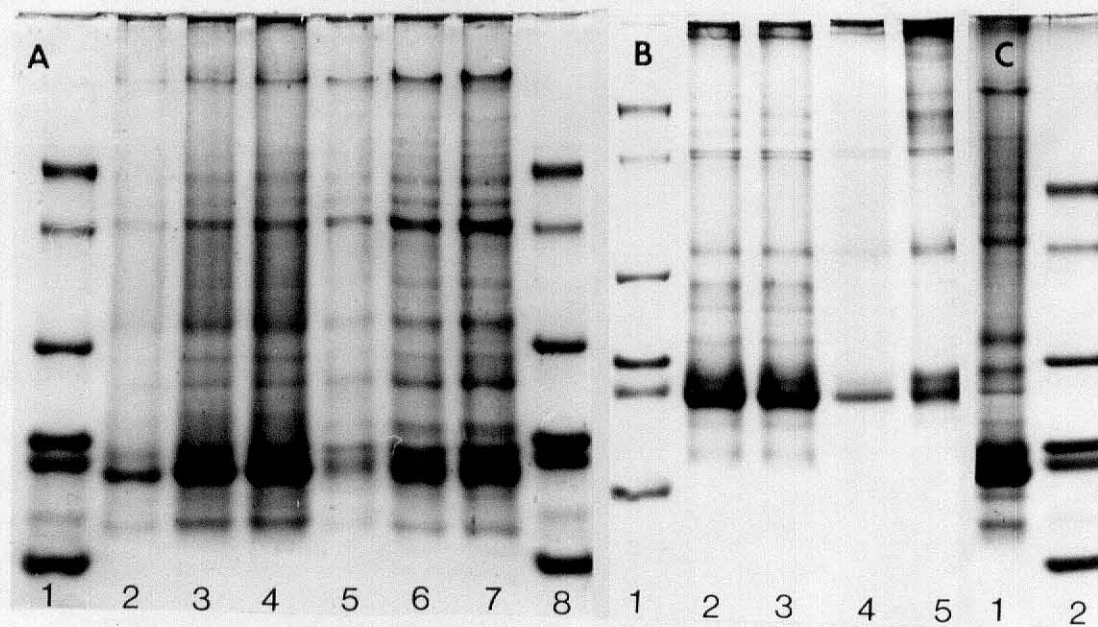
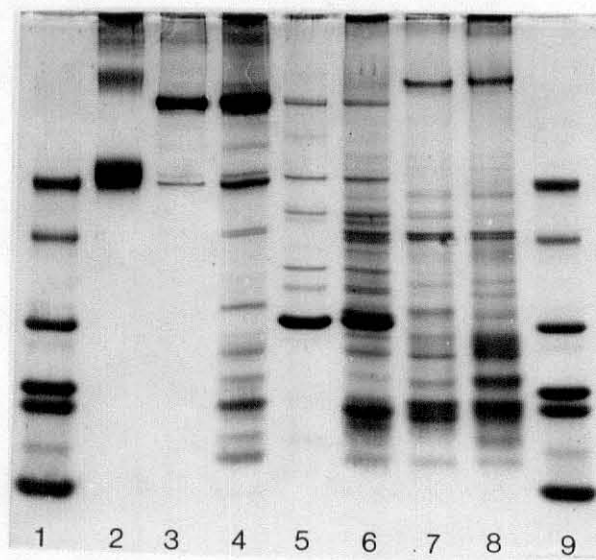
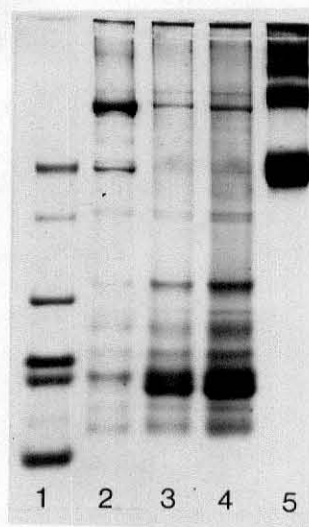


Fig. 2. SDS 5-20% polyacrylamide gel of B. thuringiensis subsp. kurstaki spore extracts. (1) Standards: same as Fig. 1, (2) Buffer A extract, (3 and 4) Buffer C extracts, and (5) Cross-linked bovine serum albumin.

Fig. 3. SDS 5-20% polyacrylamide gel of B. cereus and B. thuringiensis spore coats extracted with Buffer A, and solubilized B. thuringiensis crystals. (1) Standards: same as Fig. 1, (2) Cross linked BSA, (3) B. thuringiensis subsp. kurstaki crystal, (4) B. thuringiensis subsp. kurstaki spore coat, (5) B. thuringiensis subsp. israelensis crystal, (6) B. thuringiensis subsp. israelensis spore coat, (7) B. cereus T spore coat, (8) B. cereus 569 spore coat, and (9) Standards: same as Fig. 1.



SECTION V
LITERATURE CITED

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SECTION VI**APPENDIX**

GROWTH CHARACTERISTICS

To facilitate morphological studies and to determine whether significant differences occur, growth characteristics of six B. thuringiensis subspecies in modified GYS medium were compared. This medium contains glucose, salts, and yeast extract and allows good growth and sporulation of B. thuringiensis. The growth curves were done as described in Section III. The origins of the subspecies is also described in Section III, except for subspecies aizawai which was obtained from Keio Aizawa, Institute of Biological Control, Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan.

Growth curves are shown in Fig. 1-6; generation times and sporulation times are shown in Table 1. Generation times were similar, ranging from 0.8 - 1.3 hours. Two isolates of subspecies kurstaki were used, one which had been isolated from the insecticidal formulation called Dipel and maintained for several years in the laboratory and another which had been recently isolated from Dipel. Crystals formed by the two isolates seem to be identical. The new isolate has a slower generation time as shown in Fig. 1A and Table 1. The two isolates' generation times encompass the range of times observed in the other subspecies so differences in generation times could merely indicate the degree of adaptation of the bacteria to the modified GYS medium.

The times when phase bright spores and crystals are first observed is very similar in all subspecies, ranging from 7.5 - 9 hours after t_0 (the beginning of sporulation). The beginning of lysis occurs at a similar time in all but subsp. berliner, which takes about 6 hours longer to

Table 1. B. thuringiensis growth characteristics in modified GYS medium.

Subspecies	Generation Time*	Time * t_o to phase bright spore formation	Time t_o to lysis
<u>kurstaki</u> (laboratory strain)	0.8	8	10
<u>kurstaki</u> (newly isolated)	1.3	8	10
<u>israelensis</u>	1.1	9	10
<u>berliner</u>	1.2	8	16
<u>alesti</u>	1.1	8	10
<u>tolworthi</u>	1.3	8	10
<u>aizawai</u>	0.9	8	12

*All times are in hours. Generation times are rounded to the nearest 0.1 hr, other times are rounded to the nearest hour.

Fig. 1. Growth of B. thuringiensis subspecies kurstaki in modified
GYS medium. (↓) Phase bright spores and crystals. (↓)
Lysis begins.

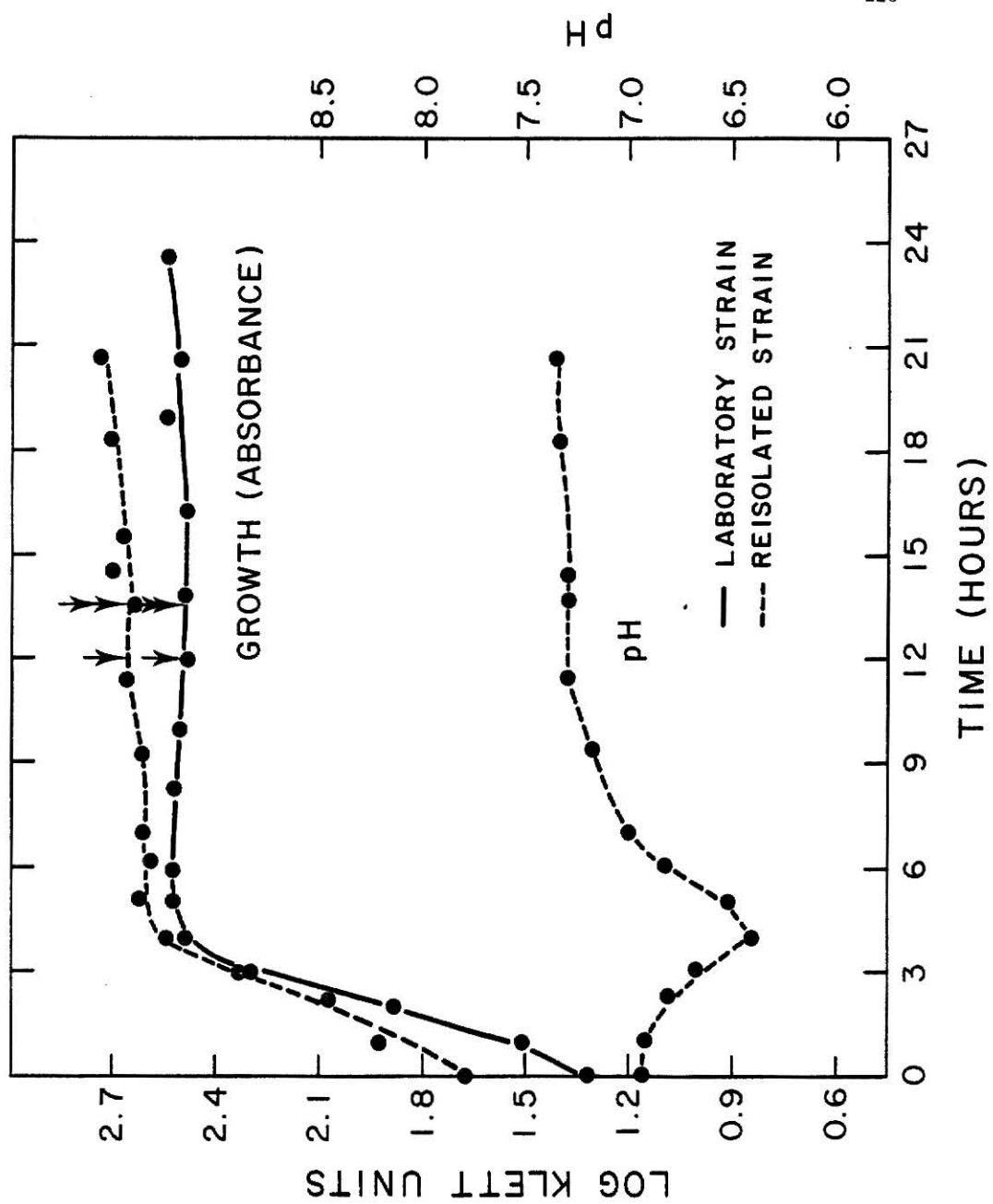


Fig. 2. Growth of B. thuringiensis subspecies israelensis in modified GYS medium. (V) Phase bright spores and crystals.
↓
(V) Lysis begins.

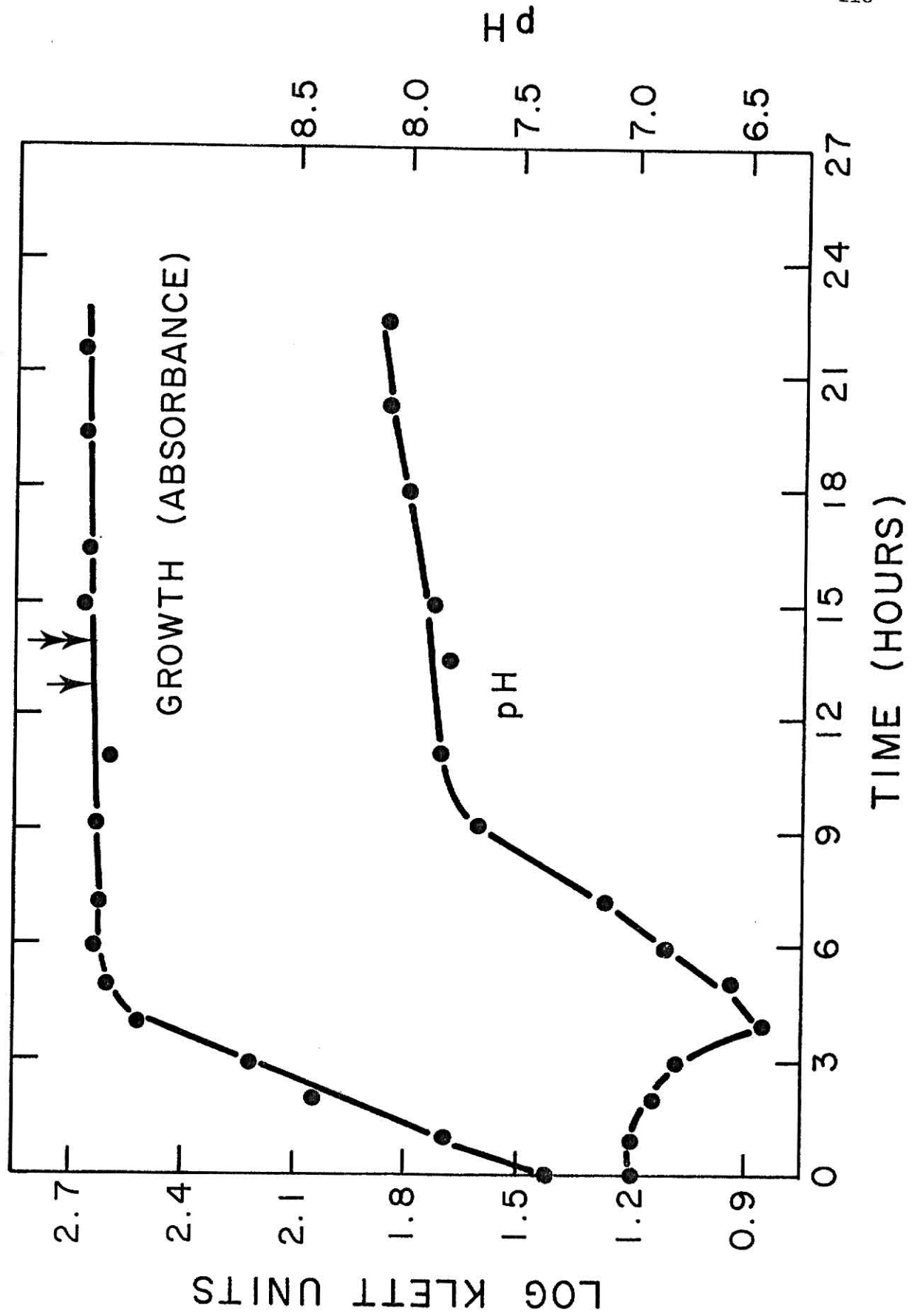


Fig. 3. Growth of B. thuringiensis subspecies berliner in modified GYS medium. (♥) Phase bright spores and crystals. ↓ (♥) Lysis begins.

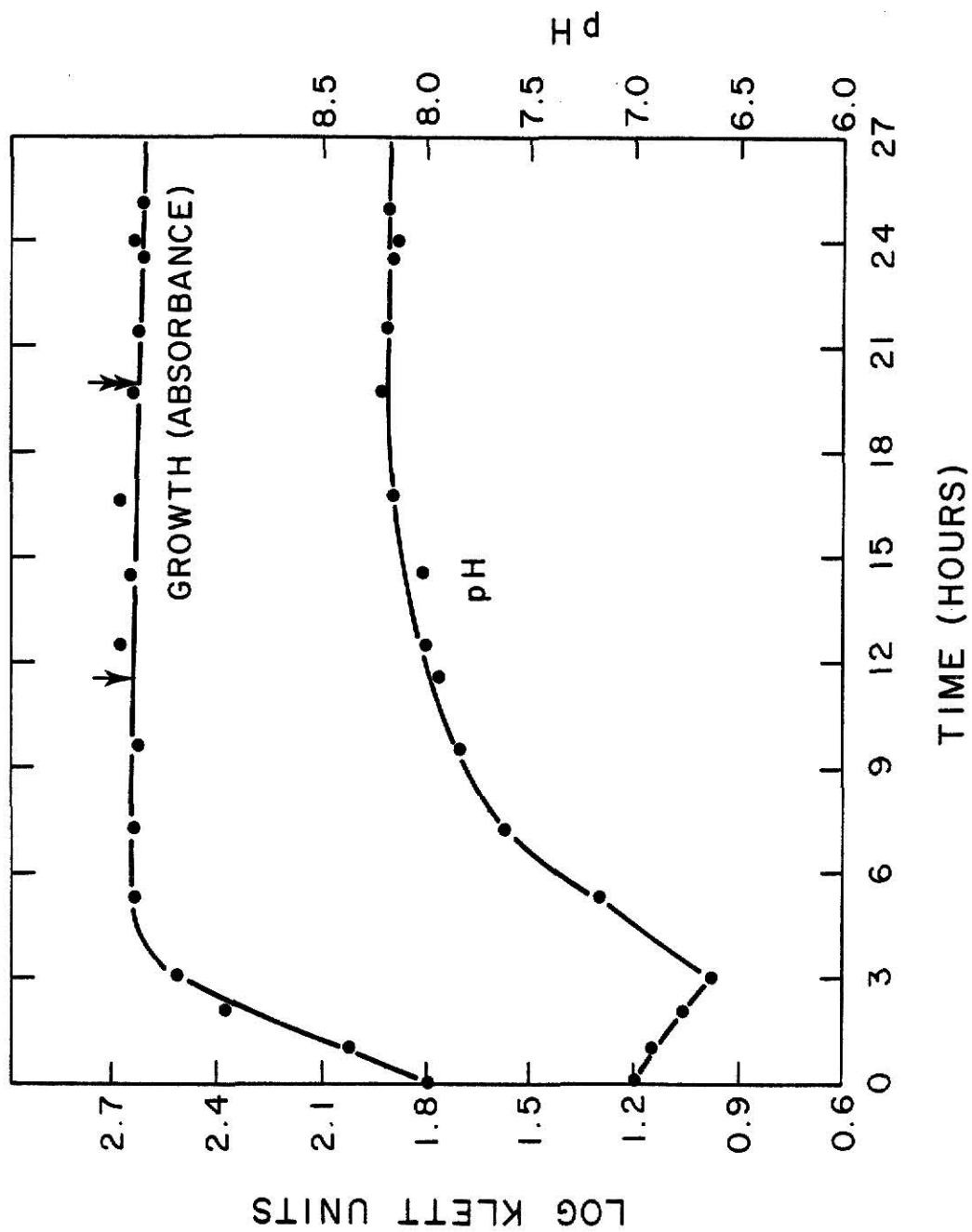


Fig. 4. Growth of B. thuringiensis subspecies alesti in modified
GYS medium. (↓) Phase bright spores and crystals. (↓)
Lysis begins.

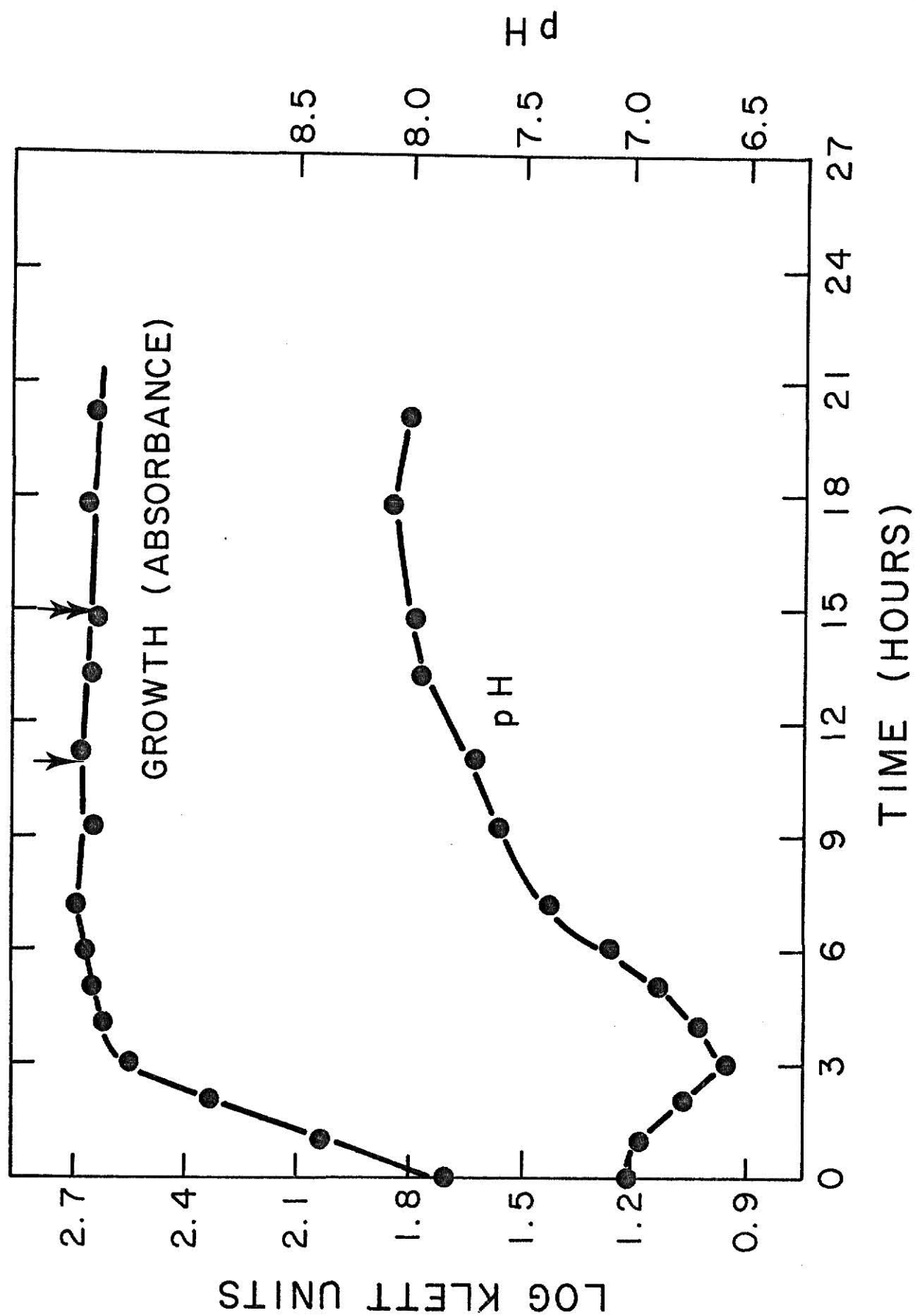


Fig. 5. Growth of B. thuringiensis subspecies tolworthi in modified GYS medium. (V) Phase bright spores and crystals. (V) Lysis begins.

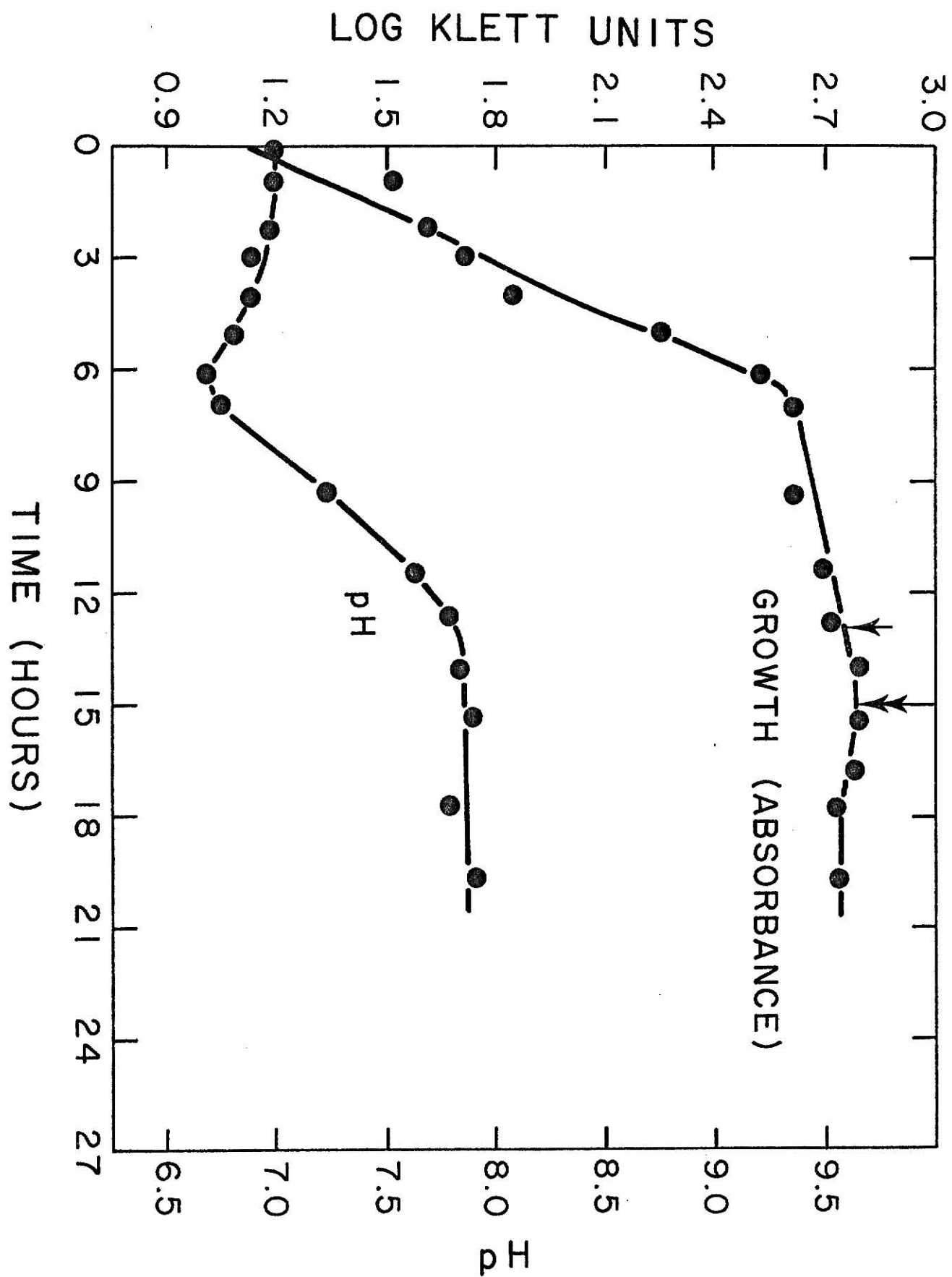
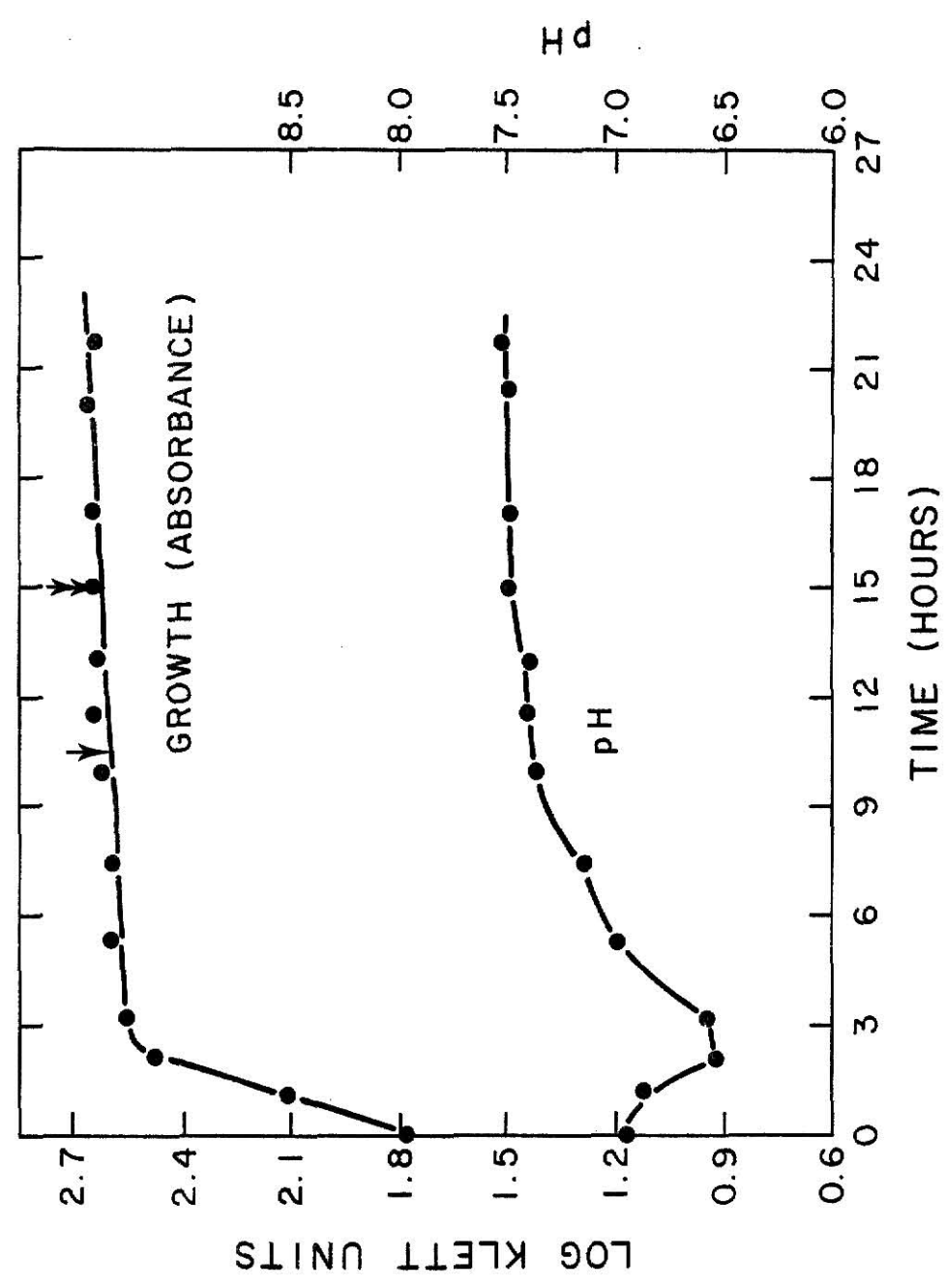


Fig. 6. Growth of B. thuringiensis subspecies aizawai in modified
GYS medium. (V) Phase bright spores and crystals. (V)
Lysis begins.



complete sporulation. pH changes are generally similar in all subspecies although the lowest and highest pH reached varies with the subspecies. The low pH at the beginning of sporulation ranges from 6.4 - 6.7 and the high pH at lysis ranges from 7.35 - 8.2. These are shown in Table 2.

Spores of the six subspecies appeared similar by phase contrast microscopy except that subspecies alesti and aizawai formed smaller spores. Cells and crystals of these subspecies were also smaller. Subspecies aizawai crystals could not be separated by bouyant density centrifugation in Renografin gradients because the tiny crystals became intercalated in clumps of spores. For this reason, this subspecies was not further characterized. The crystal shapes are described in paper II.

Table 2. pH extremes reached by B. thuringiensis in Modified GYS medium.

Subspecies	Lowest pH (t _o)	Highest pH (lysis)
<u>kurstaki</u>	6.4	7.35
<u>israelensis</u>	6.4	8.1
<u>berliner</u>	6.65	8.2
<u>alesti</u>	6.6	8.05
<u>tolworthi</u>	6.7	7.85
<u>aizawai</u>	6.55	7.5

CRYSTAL TOXICITY DATA

The data used to calculate the L.C. 50's of B. thuringiensis crystals bioassayed against the lepidopteran, Manduca sexta, is shown in Table 3. The L.C. 50's and confidence limits are described in Section III. Even though subspecies kurstaki, alesti, and tolworthi had similar L.C. 50's, the effect of lower and higher concentrations of crystals on the insect larvae vary between the species.

Table 3. Mortality of M. sexta after 7 days on a diet surface treated with B. thuringiensis crystals.

Concentration		% Mortality			
$\mu\text{g/ml}$	ng/cm^2	<u>kurstaki</u>	<u>berliner</u>	<u>alesti</u>	<u>tolworthi</u>
Control	0	0	0	0	0
0.002	0.14		0		
0.005	0.34		15		
0.01	0.68		25	10	
0.02	1.4		45	40	
0.04	2.7 (2.5) ^a	0	60	22	
0.05	3.4				0
0.06	4.1			44	20
0.08	5.5 (5.0)	33	90	44	50
0.10	6.8 (6.2)	60	100	30	60
0.20	13.6 (12.5)	80		60	70
0.40	27.2 (25.1)	90		100	
0.50	34.1				100
0.60	40.9 (37.7)	93			
0.80	54.6 (50.3)	96			
1.00	68.2 (62.8)	100			

^aValues in parentheses are for subspecies kurstaki crystals.

SPORE TOXICITY DATA

The spores of three of the Bacillus thuringiensis subspecies were also toxic. Effects of the spores of the four lepidopteran pathogens on the tobacco hornworm are shown in Table 4 and as lethal concentrations (50% endpoints) and 95% confidence limits in Table 5. The spore toxicities are extremely variable among these strains, ranging from the very toxic berliner spores to the essentially nontoxic tolworthi spores. Spores contain 0.01 - 0.1% crystals so the toxicity shown by the subspecies tolworthi spores is due to contaminating parasporal crystals.

The lepidopteran toxic spores are not toxic to mosquitoes and the toxicity shown by subspecies israelensis spore preparations is due to contaminating crystals.

Table 4. Mortality of M. sexta after 7 days on a diet treated with
B. thuringiensis spores.

Concentration		% Mortality			
$\mu\text{g/ml}$	ng/cm^2	kurstaki	berliner	alesti	tolworthi
Control	0	0	0	0	0
0.05	3.4				
0.10	6.8	0	0		
0.20	13.6	15			
0.25	17.1		10		
0.40	27.2	20			
0.50	34.1		60		
0.60	40.9	25			
0.80	54.6	35			
1.00	68.2	55	90		
1.40	95.5			0	
2.00	136.4			20	
2.40	163.7			50	
3.00	204.6			60	
4.00	272.9			80	
5.00	341.1			80	
6.00	682.1			90	
7.00	477.5				10
15.00	1023.2				30
25.00	1705.3				70
40.00	2728.5				90
50.00	3410.6				80

Table 5. Lethal conc. and confidence limits of B. thuringiensis spores
for M. sexta.

Subspecies	Lethal Concentration	95% Confidence	Limits
	50% Endpoint ¹	Upper ¹	Lower ¹
<u>kurstaki</u> ²	73.66	172.58	51.16
<u>berliner</u> ³	32.06	45.02	22.51
<u>alesti</u> ⁴	195.09	233.29	160.30
<u>tolworthi</u> ⁵	1330.15	1846.52	875.17

¹ ng/cm²

² Slope = 1.89 Intercept = 336.29

³ Slope = 4.34 Intercept = 437.93

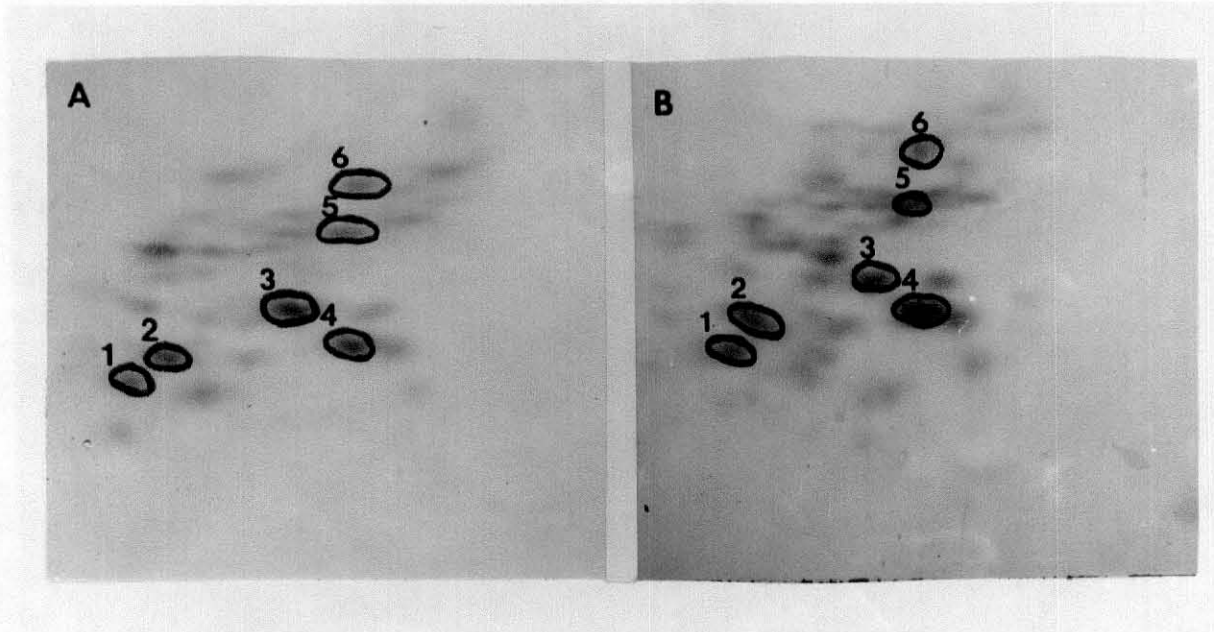
⁴ Slope = 4.65 Intercept = 196.45

⁵ Slope = 2.94 Intercept = 82.53

PEPTIDE MAP OF SPORE COAT EXTRACT

The B. thuringiensis subsp. kurstaki spore coats were extracted as described in Section III. Reduction and alkylation and tryptic peptide mapping was done as described for crystals in Section III except twice as much material (by weight) was used per map since the spore coat extracts contain a significant amount of non-proteinaceous material. The subsp. kurstaki spore extract peptide map is compared with the subsp. kurstaki crystal map in Fig. 7. The maps are similar and differences occur only among the minor peptides. This is additional evidence that the major spore coat protein is the same as the crystal protein.

Fig. 7. Peptide maps of tryptic digests of B. thuringiensis subspecies kurstaki crystals and spore coats. (A) crystals (B) spore coats.



SECTION VII

VITA

VITA

Dana was born on April 29, 1956 to Mr. and Mrs. Duane Orten in Atwood, Kansas. She graduated from McDonald High School, McDonald, Kansas in 1974 as a National Merit Scholar. She received a State of Kansas scholarship at Kansas State University and graduated cum laude in December, 1977 with a Bachelor of Arts Degree in microbiology. She was initiated into Phi Beta Kappa in 1978. She entered graduate school at Kansas State University in the spring of 1978 where she received a research assistantship from the Grain Marketing Research Center, United States Department of Agriculture, Manhattan, Kansas.

COMPARATIVE BIOCHEMISTRY OF SELECTED STRAINS OF
BACILLUS THURINGIENSIS

by

DANA JO TYRELL

B. A., Kansas State University, 1977

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Microbiology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1980

Parasporal crystals of Bacillus thuringiensis subsp. kurstaki, tolworthi, alesti, berliner and israelensis were compared by electron microscopy, polyacrylamide gel electrophoresis, amino acid analysis, tryptic peptide mapping, and insecticidal activity. Spore coats were compared with each other and with Bacillus cereus spore coats. Subspecies israelensis crystals were lethally toxic to mosquito larvae and non-toxic to tobacco hornworm larvae. Conversely, crystals from the other subspecies killed tobacco hornworm larvae and were ineffective against mosquitoes. Crystalline inclusion bodies of all subspecies apparently contain a protoxic subunit that has a molecular weight of approximately 1.35×10^5 . However, PAGE patterns of solubilized crystals revealed a small molecular weight component ($MW_{app} = 26,000$) in israelensis that was absent in the other subspecies. Also, differences were noted in amino acid composition and tryptic peptide fingerprints. Crystal proteins were found in spore coats of all subspecies. The results suggest that insecticidal specificity is due to unique toxins.