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Studies On The Structure And Origin Of The Parasporal Inclusion Of Sporulating Bacilli

James Peter Insell

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L’AVONS RECEUE
STUDIES ON THE STRUCTURE AND ORIGIN OF THE PARASPORAL
INCLUSION OF SPORULATING BACILLI

by

James Peter Insell

Department of Microbiology and Immunology
Faculty of Medicine

Submitted in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
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ABSTRACT

Certain species of *Bacillus*, closely related to *Bacillus cereus*, form a proteinaceous insecticidal inclusion during sporulation. The structural diversity of the parasporal inclusion formed by the various subspecies of *Bacillus cereus* and *Bacillus thuringiensis* has impeded progress in the analysis of both the genetic origin of the parasporal inclusion and its toxicity. Therefore, it was necessary to identify the polypeptide composition of the inclusion, correlate toxicity with these polypeptides and finally locate the genetic loci.

The parasporal inclusions of *B. thuringiensis* subspecies *kurstaki*, subspecies *israelensis*, subspecies *schwetzova*, subspecies *alesti*, subspecies *finitinus*, and *B. cereus* subspecies *medusa* and subspecies *lewinia* were purified and analyzed by one-dimensional slab gel electrophoresis. The inclusion of each of the above subspecies was composed of different polypeptides. The protein composition of the inclusions was correlated with the shape, structure and toxicity of the inclusion. Four types of inclusions were formed by the seven subspecies studied. Three representative subspecies were chosen for further study.

Purified parasporal inclusions of *Bacillus thuringiensis* subspecies *kurstaki*, *Bacillus thuringiensis* subspecies *israelensis* and *Bacillus cereus* subspecies *medusa* were compared by electron microscopy, solubility,
polyacrylamide gel electrophoresis, DEAE-cellulose ion-exchange chromatography, immunological analysis, limited proteolysis and amino acid analysis.

_Bacillus thuringiensis_ subspecies _kurstaki_, which is toxic to lepidopteran larvae, formed a non-crystalline ovoid bead, most often attached to the bipyramidal crystal. The crystal was composed of a glycoprotein with a molecular weight of 135,000 daltons which was soluble at pH 7.5 in an SDS-DTE solvent. The bead was a glycoprotein with a molecular weight of 68,000 daltons but was more insoluble than the crystal, dissolving at pH 9.2. These two proteins (135,000 and 68,000) exhibited different charge characteristics by the criterion of ion-exchange chromatography and showed a partial identity by serology and limited proteolysis. The amino acid and carbohydrate composition of the bead and crystal were markedly different.

The second inclusion-forming bacillus studied, which is toxic to dipteran larvae, was _B. thuringiensis_ subspecies _israelensis_. Electron microscopy revealed that the inclusion was a composite of light and dark stained crystals with different crystalline ultrastructures, enclosed by a thin membrane. In the SDS-DTE solvent, the lighter crystals dissolved at pH 9.2, the darker crystals at pH 10.5. Light crystal proteins had molecular weights of 25,000, 26,000, 47,000, 49,000, 86,000, 90,000, 135,000 and 145,000 daltons. The dark crystal proteins had molecular weights of 35,000 and 70,000 daltons.
The third inclusion forming bacillus studied, which is not toxic to either lepidopteran or dipteran larvae, was *B. cereus* subspecies *medusa*. It formed a spherical or ovoid crystal surrounded by a thick membrane-like structure referred to as the skin. In the SDS-DTE solvent, the crystal dissolved at pH 9.2 whereas the skin dissolved at pH 10.5. The skin was a glycoprotein with a molecular weight of 40,000 daltons which was reduced to 20,000 daltons when heated to 100°C. The crystal was composed of a 135,000 dalton glycoprotein. Amino acid analysis as well as partial proteolytic mapping indicated that the crystal was different than the skin glycoprotein.

Two variants of *B. cereus* subspecies *medusa*, (L07 "donut" and L08-42 "core crystal") were isolated that form aberrant inclusions. The inclusion of strain L07 was shown to contain a series of medium-sized proteins (40,000 to 100,000 daltons), which were absent in the wild-type inclusion. These may be precursor proteins, to the crystal protein, since the addition of sodium sulphite to an inclusion-forming culture of wild-type resulted in the formation of the "donut" type inclusion. L08-42 formed a small cubic crystal which was composed of a 135,000 dalton glycoprotein. Amino acid analysis indicated that there was a similarity between the two 135,000 dalton proteins of wild-type and L08-42.
Two strains of B. cereus and eight subspecies of B. thuringiensis were screened for the presence of plasmid DNA. B. cereus T contained two large plasmids, (17 Md. and 14 Md.), whereas the other strain, B. cereus N, contained no plasmid DNA. All B. thuringiensis subspecies contained plasmid DNA ranging from about 50 Md. to 1.3 Md. A non crystal forming variant of B. thuringiensis subspecies kurstaki contained no plasmid DNA. B. thuringiensis subspecies schwetzova, subspecies M-13 and subspecies B-30-1 contained identical plasmid profiles. The plasmid profiles of B. thuringiensis subspecies sotto and B. thuringiensis subspecies alesi were markedly different than those of the other subspecies. B. cereus subspecies medusa contained three plasmids, (3.1, 6.4, and 12.6 Md.). Endonuclease restriction mapping indicated that the 6.4 and 12.6 Md. plasmids were unique and were not multimers of the 3.1 Md. plasmid. Loss of the plasmid and hence the loss of the inclusion in B. cereus subspecies medusa and B. thuringiensis subspecies kurstaki was achieved by growth of the bacterium at 42°C. Acridine orange and ethidium bromide, known plasmid curing agents, did not result in the loss of the inclusion. When grown at 42°C, B. cereus subspecies medusa formed a small core crystal, whereas B. thuringiensis subspecies kurstaki lost the whole inclusion. This curing phenomenon also occurred spontaneously and as a result of repeated subculturing of the L07 strain on sheep blood agar. These core crystal variants had lost the 12.6
Md. plasmid. Another variant of *B. cereus* subspecies *medusa*, L013, which was isolated on sheep blood agar, formed cells that were smaller than the wild-type cells. The L013 variant was sensitive to penicillin and ampicillin whereas the wild-type was resistant. The L013 variant contained a 7.2 Md. plasmid which was not present in the wild-type.
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Dedicated to Mary
INTRODUCTION

Since Pasteur (1870) first noted that certain spore-forming bacteria are toxic to lepidopteran larvae, it has become apparent that some species of spore-forming bacilli closely related to Bacillus cereus form an insecticidal inclusion (Hannay, 1953; Angus, 1954; Hannay and Fitz-James, 1955). The similarity of Bacillus thuringiensis to Bacillus cereus is so striking that it has been suggested that B. thuringiensis is an inclusion-forming subspecies of B. cereus (Smith, Gordon and Clarke, 1946; 1952; Lysenko, 1964; Krieg, 1969; Lysenko and Kucera, 1971; Seki et al., 1978).

At least 14 different serotypes of Bacillus thuringiensis are toxic to a broad spectrum of insect larvae (Ohba and Aizawa, 1978; reviewed in Forseberg et al., 1976). Toxicity studies have been numerous (reviewed in Heimpel, 1967; Rogoff and Yousten, 1969; Burges and Hussey, 1971; Bulla, 1973; Bulla et al., 1975; Forsberg et al., 1976) and today Bacillus thuringiensis is one of the few bacteria used on a large scale as an insecticide against economically and medically important insects (Bulla and Yousten, 1979).

The formation and morphology of inclusions were first described in Bacillus thuringiensis subspecies alesi by Young and Fitz-James (1959b) and Fitz-James (1962; 1965). The macromolecular structure of the inclusion (Holmes and Munro, 1965), the biochemical composition of the inclusion (Bulla et al., 1977), and the toxic component of the inclusion (Bulla et al., 1979) have now been reported. Yet
numerous reports have furnished evidence suggesting that a great deal of variation exists in the structure, biochemical composition and toxicity of the inclusion (reviewed in Bulla et al., 1980). This enigma may be a result of the use of different dissolution methods (Bulla et al., 1977) or the use of various subspecies that form different inclusions.

The genetics of sporulation (reviewed in Pigott and Coote, 1976) have been studied and some aspects of regulation and control (Linn et al., 1973; Losick and Pero, 1976) have been clarified. Yet, the location of the genetic loci involved in parasporal inclusion formation is not known. Circumstantial evidence suggests that extrachromosomal DNA (plasmid or episome) may be involved in the formation or regulation of inclusion formation (Gillespie, 1969; Debabov et al., 1977; Ermakova et al., 1978).

Thus a program of experiments was initiated to examine the structural and biochemical differences in the inclusion of seven subspecies of Bacillus thuringiensis and Bacillus cereus. Once these differences were established, efforts were made to cause the bacterium to lose the inclusion. Finally, a study of plasmid DNA in several subspecies of Bacillus thuringiensis and Bacillus cereus was undertaken in the hope of establishing a correlation between the development of the inclusion and the presence of extrachromosomal DNA. Clarification of the molecular structure and the genetic loci of the various inclusions may
reveal the relationship between Bacillus cereus and Bacillus thuringiensis.
HISTORICAL REVIEW

Toxicity of *Bacillus thuringiensis* (Sotto-Bacillen) to insects was first described by Ishiwata (1901) and confirmed by (Aoki and Chigasaki 1915). Later, the presence of inclusion bodies or "Restkorper" was described by Berliner (1915) and Mattes (1927). However, the phenomenon was largely forgotten for thirty years until 1951 when Steinhaus published a paper on the possible use of *B. thuringiensis* as an aid in the biological control of the Alfalfa caterpillar. Steinhaus (1951) described the morphology of *B. thuringiensis* subspecies *berliner* but failed to observe the octagonal-shaped inclusions among the free spores. Hannay noted these inclusions in the work of Steinhaus and went on to rediscover the inclusion and show its toxicity to insects (Hannay, 1953). Hannay coined the phrase parasporal body and with Fitz-James described the proteinaceous nature of the inclusion formed during sporulation (Hannay and Fitz-James, 1955).

1.1 Sporulation and Inclusion Development

The ultrastructural changes of *Bacillus cereus* during sporulation were first described by Young and Fitz-James (1959a). These observations were extended to include the inclusion-forming *Bacillus thuringiensis* subspecies *alesti* (Young and Fitz-James, 1959b). Sporulation can be described by seven distinct stages and can be related to a time scale, \( t_0 \) to \( t_q \) (Fitz-James, 1963; Schaeffer et al., 1963). The sporulation process, unlike cell division, proceeds in the
absence of DNA replication. A condensation of the two late vegetative nuclear masses which form an axial filament appears as the initial stage (Stage I) of sporulation (Fitz-James, 1963). Axial filament formation is followed by septum formation (Stage II). Unlike the vegetative cell septum, which is accompanied by transverse wall development, the double membrane septum is formed towards one end of the cell. The septum encloses a small portion of the cell and approximately one half of the filamentous DNA (Hitchins, 1978; Hitchins and Slepecky, 1969).

Parasporal inclusion formation appears at the end of forespore septum formation which marks the initiations of engulfment (Young and Fitz-James, 1959; Fitz-James, 1962; 1965; Betchel and Bulla, 1976). Fitz-James (1962) showed that initiation of parasporal inclusion formation was in late stage II and was associated with the forespore membrane. Sommerville (1971) and Sommerville and James (1970) have indicated that the inclusion is formed in association with the exosporium. However, Betchel and Bulla (1976) reported that inclusion formation begins in stage III and that no apparent association with either the forespore membrane or the exosporium could be seen. In spite of this controversy, it has been shown that the inclusion proteins are synthesized immediately prior to inclusion formation in both stage II and stage III (Fitz-James et al., 1958; Herbert et al., 1971).
Stage III is characterized by the completed forespore membrane surrounding the small portion of the cell previously separated from the sporangium by the septum. Following septal detachment, the double membrane decreases in electron density and splits into an inner and outer membrane (Highton, 1972; Betchel and Bulla, 1976). This development of a "protoplast" surrounded by a double membrane of reversed polarity (Wilkinson et al., 1975) is known as the forespore. From the end of stage III, that is, the process known as engulfment, the cell will no longer respond to nutrient supplements and is committed to complete the process of sporulation (Fitz-James and Young, 1969; Freese, 1972).

Stage IV through to stage VII represents maturation of the forespore. During stage IV, a thin electron opaque layer develops between the inner and outer membranes. A second, less dense, wider layer appears between the membranes, distal to the peptidoglycan layer. This mucoproteptide layer is the spore cortex (Tipper and Gauthier, 1972). Calcium uptake and dipicolinic acid synthesis also occur following completion of the forespore (Hogarth et al., 1977). Simultaneously, the spore nucleoid becomes fibrous and appears to become more crystalline than the vegetative cell DNA (Doi, 1969). The exosporium appears in stage IV and is composed of a unique glycoprotein (Matz et al., 1970). Stage V is devoted to the elaboration of the spore coat. The spore coat appears as three distinct layers, an
undercoat and a "pitted" layer covered by a CP or cross-patched layer (reviewed in Holt and Ledbetter, 1969; Aronson and Fitz-James, 1976). Occasionally, the apparent spore coat material is deposited on the surface of the inclusion (Gillespie, 1969; Aronson and Fitz-James, 1976).

1.2 The Relationship of the Spore coat and the Inclusion

The relationship between the spore coat and the parasporal inclusion has been the subject of a great deal of work and controversy. In 1970, Sommerville and James noted a possible similarity between the two proteins, in spite of their solubility differences (Aronson and Fitz-James, 1968). Sommerville and Pickett (1975) pursued this concept and found toxicity associated with both B. cereus and B. thuringiensis spores. Scherrer and Sommerville (1977) showed identical toxicity in both spore coat and inclusion preparations. Others have shown an immunological similarity between the spore coat protein and the inclusion protein (Decadet and Dedonder, 1971; Sommerville et al., 1968a; 1968b; 1969). Tryptic mapping of the two proteins has shown minor differences that suggest a slight variation in the two proteins which may account for the serological similarity and common antigenic determinants (Gillespie, 1969).

Inclusion formation in B. thuringiensis subspecies kurstaki has been shown to be associated with spore coat formation. Stelma et al., (1978) isolated coat defective mutants that had lost the ability to form inclusions. Electron microscopy of these lysozyme sensitive, coat-
defective spores revealed little spore coat. These defective spores also contained little extractable coat protein (Aronson and Pandy, 1978; Aronson et al., 1982).

Polyacrylamide gel electrophoresis of the spore coat extracts of Bacillus cereus and Bacillus thuringiensis revealed that the coat of B. cereus contained three proteins (68,000; 26,000; 12,600) whereas the spore coat extract of B. thuringiensis contained four (134,000; 68,000; 26,000; 12,600) (Aronson and Fitz-James, 1976; Bulla et al., 1977; Tyrell et al., 1980). However, Aronson and Fitz-James (1976) suggested that the ease of extraction of inclusion-like (134,000) protein from the spores may indicate a superficial association of the protein with the spore either by contamination or a cross-deposition of the protein during development.

1.3 Structure of the Inclusion

Hannay and Fitz-James (1955) showed that the insecticidal activity ascribed to B. thuringiensis by others (Mattes, 1927) resided in the protein crystal, which in B. thuringiensis subspecies berliner was bi-pyramidal with surface striae spaced approximately 29 nm. apart. Crystal preparations examined by electron microscopy of carbon replicas revealed that the crystal of B. thuringiensis subspecies berliner was composed of a lattice which contained two rows of spheres (Labaw, 1964). Labaw postulated that the lattice was composed of four symmetrical
molecules placed cubically in rows of two. Each cube measured 12.3 nm wide and each molecule measured 8.7 nm in diameter.

Holmes and Munro (1965), using X-ray diffraction, examined the crystal of *B. thuringiensis* strain M. The data demonstrated that the protein subunit was not spherical as Labaw (1964) had suggested, but rather a prolate sphere with four molecules forming a tetrahedron. The molecules were packed asymmetrically, with a sheet packing, through a four fold screw axis, eight of them forming a unit cell. The subunits could be most aptly described as two ellipsoids packed together along the longest axis of the crystal with the dimensions of 9.0 nm by 9.0 nm x 26.9 nm. Each of the subunits have a molecular weight of 230,000 and each striae represents the distance of four layers. The space between the rods was found to be 6.15 nm which theoretically should be 6.75 nm if the 8.7 nm spheres were packed as close as possible. Theoretically if four of the prolate spheres compose a striae or row, then the repeating distance of the striae would be 27.0 nm. This correlated well with the X-ray diffraction data.

Norris (1969) examined the internal structure of *B. thuringiensis* subspecies *tolworthii* by thin sectioning and found that this subspecies often produced two or more inclusions. He noted that only one of the inclusions displayed a lattice structure. This striation when viewed in section parallel to the base of the pyramid showed
spherical subunits arranged in a square of 8.3 nm. Sections
perpendicular to the base of the pyramid and parallel to the
long axis of the crystal revealed rod-shaped structures
measuring 11.8 nm by 4.7 wide arranged in a herring bone
pattern.

Norris (1971) and Short et al., (1974) examined B.
thuringiensis subspecies finitimus using negative staining,
freeze-etching as well as thin sectioning and reported the
subunit to be rod-shaped with the dimensions of 5.0 nm by
15.5 nm.

Betchel and Bulla (1976) reported that all B.
thuringiensis subspecies formed an ovoid inclusion attached
to the crystal. Yet, Young and Fitz-James (1958) had not
reported such an inclusion in B. thuringiensis subspecies
alesti. However, Aronson and Fitz-James (1976) showed that
B. thuringiensis subspecies schwetzova formed an ovoid
inclusion attached to the crystal. Baker and Sharpe (1979)
showed that B. thuringiensis subspecies kurstaki formed an
ovoid inclusion attached to the crystal which appeared in a
variety of shapes. The chemical composition and toxicity of
the ovoid inclusion was not established.

1.4 Purification of the Inclusion

Purification of the inclusion has been accomplished
through a variety of procedures (reviewed in Cooksey, 1971;
Bulla et al., 1980). Although many studies in the past were
performed on partially purified material, today purification of both the spore and inclusion is achieved reliably without any notable change in their biological activity.

Biphasic methods, using fluorocarbons, achieve a relatively high (98-100%) purification of the inclusion (Angus, 1959; Batesman, 1965; Pendelton and Morrison, 1966; Goodman et al., 1967; Delafield et al., 1968; Pendelton, 1969). Buoyant density ultracentrifugation using cesium chloride (Fast, 1972; Ang and Nickerson, 1978) or renografin-76 (Tamir and Gilvarg, 1966; Gillespie, 1969; Milne et al., 1977; Sharpe et al., 1975) has become the method of choice for purifying both spores and inclusions.

1.5 Solubility of the Inclusion

Watanabe (1967) showed that the inclusion was insoluble in solvents such as methanol, ethanol, ether, acetone, chloroform and benzene. The insoluble nature of the inclusion has been attributed to either a silicate framework within the crystalline structure (Faust and Estes, 1966), or the combination of disulphide, hydrogen, ionic and hydrophobic bonding (Lecadet, 1967b). Gillespie (1969) could not confirm the findings of Faust and Estes (1966), yet Heimpel (1969) found that crystal formation was impeded by growth in a silicon-free medium.

Three approaches have been taken to solubilize the crystalline inclusion. Reducing agents such as thioglycolic acid (Young and Fitz-James, 1959b), B-mercaptoethanol (Lecadet, 1966; 1967; Cooksey, 1968; 1971; Delafield et al.,
1968) and dithiothreitol, Bulla et al., 1976) are necessary for dissolution of the inclusion. However, these authors also realized that a denaturant such as sodium dodecyl sulphate (SDS) or urea is necessary for the dissociation of the protein. Yet, alkalinity of the buffering system also seemed to be crucial in the dissolution process (Hannay and Fitz-James, 1955; Angus, 1956; Young and Fitz-James, 1959b; Lecadet, 1967b). Bulla et al., (1977) and Andrews et al., (1980) suggested that many of the methods employed were too harsh and may cause hydrolysis of the protein. These workers attempted dissolution of the inclusion with the various dissociation buffers used by other workers and found substantial variability in the results. Although Aronson and Tillinghast (1977) have shown that 1% SDS, 0.15% DTT is insufficient to disperse the inclusion, Bulla et al., (1977) found that 1% SDS, 2% mercaptoethanol in phosphate buffer pH 7.0 or 6 M urea, 0.15% dithiothreitol, pH 7.4 would optimally dissolve the inclusion. Thus, there exists some controversy over the optimum method of dissolution of the inclusion.

1.6 Characterization of the Inclusion Components

The exact number and size of the polypeptide components of the inclusion are not well defined (reviewed in Bulla et al., 1980). The past three decades have seen a wide variety of techniques establish the inclusion to consist of polypeptides ranging from 500 daltons to 230,000 daltons (Table I). Early workers have identified one component
(Young and Mitz-James, 1959), two components (Holmes and Munro, 1965) or multiple components (Lecadet, 1966) in the inclusion, ranging in size from 0.4s to 4.1s using analytical ultracentrifugation. These values for the inclusion components were obtained through alkali-thioglycolate dissolution. Cooksey (1968) and Delafield et al., (1968) performed disc gel electrophoresis on inclusions dissolved in 8M urea, 0.1M mercaptoethanol, pH 9.5 and observed a total of thirteen bands, although only three were reported as major proteins. More recent studies using mercaptoethanol with either sodium hydroxide or urea have also produced a wide range of results (Table 1) (Fast and Angus, 1970; Glatron et al., 1972; Prasad and Shetna, 1974). The polypeptide components were purified by Sephadex gel filtration often followed by polyacrylamide gel electrophoresis.

Bülla et al., (1977; 1979) studied the inclusion of B. thuringiensis subspecies kurstaki using 1% SDS and 2% mercaptoethanol at pH 7.4 as the solvent. Like several other workers (Zalusin et al., 1979; Fast and Milne 1979), Bülla et al., (1977; 1979) found a large (134,000 dalton) major protein. However, Fast and Martin (1980), using 2M KSCN found small (1000 dalton) toxic proteins derived from an 80,000 dalton protein (Fast and Milne, 1979) similar to the 68,000 dalton protein described by Bülla et al., (1979).
Yet, Calabrese and Nickerson (1980) reported a 130,000 dalton protein as the major polypeptide found in all subspecies of B. thuringiensis with the exception of two and also showed very complex gel profiles of the purified inclusions. The subspecies were classified into six types according to the size and number of polypeptides found in the inclusion. All subspecies contain a crystal which contains three major sizes of polypeptides, high, medium and low. B. thuringiensis subspecies thompsoni and subspecies entomocidus have only the low molecular weight proteins (class III) whereas the other subspecies contain a single protein or group of high molecular weight proteins (class Ia and Ib). In some cases, a medium single-sized protein, (class IIa and IIb) was also observed. Within each class several different sized proteins were observed. The two subspecies that only contain the class III (low molecular weight proteins) are unable to form the classical bipyramidal crystal and instead form tetrahedral or pyramidal inclusions.

1.7 Isolation of the Toxin

The toxic molecule has been studied extensively by various methods over the past two decades (Table I). Typically, insect digestive juices or alkali have been used to dissolve the inclusion in order to isolate the toxic moiety (Angus, 1954; Lecadet, 1967; 1972; Pendelton, 1968; Faust et al., 1974). Bulla et al., (1979) have apparently located the toxic molecule (M.W. 68,000) in the inclusion of B. thuringiensis subspecies kurstaki. Although these workers used 1% SDS and 2% mercaptoethanol, pH 7.4 to
<table>
<thead>
<tr>
<th>Subspecies of B. thuringiensis</th>
<th>Characterization (MW in dalton)</th>
<th>Toxicity</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>thuringiensis</td>
<td>40,000</td>
<td>+</td>
<td>Lecadet et al., 1972</td>
</tr>
<tr>
<td></td>
<td>5,000</td>
<td></td>
<td>Lecadet &amp; Martouret, 1967</td>
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<tr>
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<td>40,000</td>
<td>+</td>
<td>Cooksey, 1968</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>thuringiensis</td>
<td>200,000</td>
<td>+</td>
<td>Pendleton, 1968</td>
</tr>
<tr>
<td></td>
<td>5,000-10,000</td>
<td>+</td>
<td></td>
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<td>NR</td>
<td>Sayles et al., 1970</td>
</tr>
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<td>sotto</td>
<td>ND</td>
<td>+</td>
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<tr>
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<td>500-1000</td>
<td>+</td>
<td>Fast &amp; Angus, 1970</td>
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<tr>
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<td>12,000; 140,000</td>
<td>+</td>
<td>Chestukhina et al., 1977</td>
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<td></td>
<td>65,000; 120,000</td>
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<td>190,000; 23,000; 310,000</td>
<td></td>
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</tr>
<tr>
<td>tolworthi</td>
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<td>+</td>
<td>Norris, 1969</td>
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<td>+</td>
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<tr>
<td></td>
<td>10,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aesti</td>
<td>two fractions</td>
<td>NR</td>
<td>Sommerville et al., 1968</td>
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<td>Subspecies of B. thuringiensis</td>
<td>Characterization</td>
<td>Toxicity</td>
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<tr>
<td>-------------------------------</td>
<td>----------------</td>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>kurstaki</td>
<td>235,000; 67,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30,200; 5,000; 1,000</td>
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<td>kurstaki</td>
<td>130,000</td>
<td>+</td>
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</tr>
<tr>
<td>kurstaki</td>
<td>68,000</td>
<td>+</td>
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</tr>
<tr>
<td>dendrolimus</td>
<td>232,000</td>
<td>+</td>
<td>Nagamatsu et al., 1978</td>
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</table>
dissolve the inclusion during protein analysis of the subunit studies (Bulla et al., 1977), 0.135 N sodium hydroxide was used as the solvent for the toxicity studies. Fast and Milne, (1979) and Fast and Martin (1980) have used one to two molar KSCN to obtain an 80,000 dalton toxic protein which further dissociates to small 1000 dalton peptides which are also toxic. Lilley et al., (1980) have shown that similar toxic molecules (135,000 daltons cleaved to 68,000 daltons) can be obtained either by incubation in trypsin or by incubating the crystal in the gut juice of Pieris brassicae larvae.

1.8 Other Toxins of B. thuringiensis

The vegetative cell of B. thuringiensis produces three exotoxins besides the delta endotoxin during sporulation. Smirnoff and Berlinguet, (1966) observed a heat labile substance which was toxic to saw fly larvae. Heimpel (1967) referred to this as the alpha exotoxin which he suggested may be phospholipase C. Toumenoff (1953) observed that B. thuringiensis subspecies alesti cleared egg yolk agar and Heimpel (1955) ascribed this phenomenon to a lecithinase C which he called the gamma toxin. However, this exotoxin is also produced by most strains of B. cereus (Krieg, 1971a; 1971b; Slein and Logan, 1963).

McConnell and Richards (1959) and Burgerjon and de Barjac (1960) detected a heat stable exotoxin which was toxic to Galleria mellonella larvae. This substance, now referred to as the beta exotoxin, was purified (de Barjac
and Dedonder, 1965; Benz, 1966) and shown to be toxic to a wide range of both vertebrates and invertebrates (reviewed in Ignoffo, 1973; Forsberg et al., 1976). The structure of the toxin has been suggested (Farkas et al., 1969) to be a cyclic nucleotide which inhibits adenyl cyclase, (Lysenko and Kucero, 1971), DNA dependent RNA polymerase (Vankova, 1978) and protein synthesis (Grahme-Smith et al., 1975).

Mutagenic and teratoogenic properties have also been associated with the beta exotoxin (Linnainman et al., 1977).

1.9 Taxonomy of Sporulating bacilli

Bacilli appear to fall into three classes according to the G-C content of their DNA. A large number of species have a G-C content similar to B. subtilis, which are in the range of 42 to 46 percent. The B. megaterium group, including B. pumilus and B. sphaericus, have a G-C content of 37 to 40 percent, whereas the B. cereus group have G-C content of 32 to 38 percent (Marmur and Doty, 1962).

Bacillus anthracis, B. mycoides, B. thuringiensis and other inclusion-forming bacilli contain DNA which is similar in C-G. content to B. cereus (Lee et al., 1956; Marmur and Doty, 1962; McDonald et al., 1963), and exhibit extensive homology (Sommerville and Jones, 1977; Seki et al., 1978). Smith, Gordon and Clark (1946; 1952) have placed the vertebrate pathogen, B. anthracis, the rhizoid-forming B. mycoides and the inclusion-forming B. thuringiensis as subspecies of B. cereus, because of their similar physiology and nutrition. Furthermore, the unique characteristics
associated with each of the above bacillus can be readily lost (Smith, Gordon and Clarke, 1952; Stern, 1937; 1937b; Chu; 1952) and these strains become morphologically indistinguishable from _B. cereus_.

Steinhaus (1951) and Heimpel (1967) recognized the similarity between _B. cereus_ and _B. thuringiensis_ but suggested that _B. thuringiensis_ should hold species status on the basis of toxicity. Norris and Burgess (1963) classified _B. thuringiensis_ as a distinct species on the basis of a comparison of electrophoretic patterns of esterases from vegetative cell extracts of _B. cereus_ and _B. thuringiensis_. Recently O'Donnell et al., (1980) using pyrolysis liquid gas chromatography, have likewise placed _B. thuringiensis_ and _B. mycoides_ as distinct species separate from _B. cereus_.

In the absence of a clearly defined distinction between _B. cereus_ and _B. thuringiensis_, other than the formation of a toxic inclusion, most inclusion-forming bacilli are currently classified as either subspecies of _B. cereus_ or varieties of _B. thuringiensis_.

1.10 Classification of Bacillus thuringiensis

Subspecies of _Bacillus thuringiensis_ fall into twelve major categories based on the immunological cross-reactivity of their flagellar antigens (de Barjac and Bonnefoi, 1963; Bonnefoi and de Barjac, 1963) and their vegetative cell esterase types (Norris, 1964). Since the initial serological identification of the inclusion forming bacilli,
further analysis has shown a partial identity among several subspecies of \textit{B. thuringiensis} and no identity with \textit{B. cereus}, \textit{B. pompilliae} and \textit{B. lentimorbus} (de Barjac and Bonnefoi, 1972; 1973). Moreover, new subspecies have been isolated, namely, \textit{B. thuringiensis} subspecies \textit{israelensis} (Goldberg and Margalit, 1977), subspecies \textit{kyushuensis} (Ohba and Aizawa, 1978), and subspecies \textit{dakota} and \textit{indiana} (DeLuca et al., 1979) which have extended the serotypes to sixteen. There also appears to be a relationship between the flagellar antigens and the relative toxicity of the inclusion (Angus and Norris, 1968; Padua et al., 1980). Therefore, the spectrum of toxicity and the number of serotypes seems to be increasing as further isolation and classification studies are pursued. There is doubtful value in the current serotyping of the subspecies (Lysenko and Kucera, 1971) since it does not reflect the important differences in the inclusion and the relationship of similar subspecies.

1.1.1 \textbf{Plasmids in Sporulating Bacilli}

Rolf (1963) first indicated the presence of satellite DNA in \textit{B. subtilis}, which he ascribed to changes in the physical condition of the chromosome during replication. Carlton and Helinski (1969) found extrachromosomal elements in the vegetative cells of \textit{Bacillus megaterium}. The characteristics were unknown except that some 30 to 40\% of the total extractable nucleic acid from these cells existed as small heterogeneously sized minicircles that have
extensive homology with the chromosome. Carlton and Henneberry (1973) and others characterized the plasmids of *B. megaterium* and showed it to contain unusually large amounts of polydispersed plasmid DNA (Carlton, 1976; Gonzales and Carlton, 1980). Gonzales and Carlton (1980) have reported the isolation of twelve unique plasmids from vegetative cells of *B. megaterium*. The significance of these plasmids remains unknown.

Lovett (1973) examined *B. pumilus* and found that the vegetative cells contained approximately 3% plasmid DNA. Lovett and Bramucci (1974) further characterized the plasmids of several strains of *B. pumilus* and found a 28 million dalton plasmid in the oligosporogenous strain of NRS 576. This plasmid was lost in spontaneously derived sporulating strains.

Bernhard et al., (1978) showed a relationship between bacteriocin production and the presence of a plasmid in *B. subtilis*. Bernhard et al., (1978) also examined several strains of *B. cereus* and found several plasmids which code for antibiotic resistance. Miteva (1978), likewise found plasmids in *B. cereus*, but failed to associate a biological function to any of the elements.

Debabov et al., (1977) have shown the presence of plasmids in *B. thuringiensis*. Ermakova et al., (1978) continued this study and showed a correlation between the presence of plasmids and the parasporal inclusion. A unique nutritional effect on the plasmid DNA and the parasporal
inclusion was observed by these workers. The inclusion and plasmid DNA were simultaneously and permanently lost when the bacteria were grown on complex media followed by growth on minimal media.

Stahly et al. (1978a; 1978b), pursued these studies and found that B. thuringiensis subspecies kurstaki contained six plasmids ranging from 47 million daltons to 1.3 million daltons. These workers were unable to repeat the nutritional effect noted by Ermakova (1978) but did cure subspecies kurstaki of the parasporal inclusion and all six plasmids by heating the spores to 60°C for approximately 20 minutes just prior to germination. Gonzales et al. (1981) screened five subspecies of B. thuringiensis and showed their plasmids to be of many sizes, similar to the findings of Stahly et al (1978b). The workers noted a relationship between the loss of the large molecular weight plasmids in the various subspecies and the loss of the parasporal inclusion. The plasmid pattern for the various subspecies was also different. Hence, the large plasmid implicated in inclusion formation was not the same for each of the subspecies; (Gonzales et al., 1981).

On the other hand, Lecadet et al. (1980) showed a diverse plasmid pattern for the various subspecies of B. thuringiensis, but were unable to find any correlation between the loss of the plasmid and the loss of inclusion formation. In support of these findings, Klier et al., (1980) found that messenger RNA coding for the inclusion
protein hybridized to the chromosome. Yet, Schnepf and Whiteley (1981) isolated a large 47 Mdal. plasmid from *B. thuringiensis* subspecies *kurstaki* and cloned several fragments into pBR322. One of the clones produced a toxic 134,000 dalton protein. Therefore, conflicting evidence has left the question of plasmid involvement in parasporal inclusion formation unanswered.

1.12 Studies of *Bacillus cereus* subspecies *medusa*

*B. cereus* subspecies *medusa* isolated by C.F. Robinow (1960), was chosen for further studies because of several unique characteristics which other inclusion-forming bacilli lack. The inclusions of subspecies of *B. thuringiensis* are formed during sporulation beginning at late stage II or early stage III. The one exception to this rule, is the filamentous *B. cereus* subspecies *medusa* which begins inclusion formation during late vegetative growth, about two hours prior to the onset of sporulation (Robinow, 1960; Gillespie, 1969; Fitz-James, 1962; Hendry *et al.*, 1976; Fitz-James and Young, 1959). By phase microscopy, a small bead appears at one end of the cell opposite to the end destined to become the forespore. By electron microscopy, this small inclusion is formed adjacent to the interior surface of the cell membrane. The bead increases in size taking on a cuboidal shape and migrates from the membrane into the cell cytoplasm. As sporulation proceeds, the inclusion continues to grow in size until the cell filaments appear as closely packed strings of refractile spores and
inclusions. The latter are ovoid crystals covered by a thin multi-layered fibrous membrane or "skin" (Gillespie, 1969; Robinow, 1960). The "skin" is composed of fibres which were seen on the surface of the inclusion. The skin is insoluble and keratinase resistant. The skin seems to be sporulation dependent (Gillespie, 1969) and is associated with virus-like particles.

Gillespie (1969) during his studies of the parasporal inclusion of B. cereus subspecies medusa encountered a inducible phage-like particle. This observation was amplified by Hendry (1974) who found that this particular subspecies of B. cereus contained at least three inducible phage-like particles. These were an RNA-containing small phage, 0-1, expressed at stage II-III and often found attached to the inclusion "skin" secondly, an inducible DNA-containing temperate phage, and 0-2 and thirdly an inducible defective DNA type phage, 0-3 (Hendry et al., 1976).

Several morphological variants of the inclusion have been isolated (Gillespie, 1969; Hendry, 1974; Hendry et al., 1976) which are unique among inclusion-forming bacilli. Acrysraliferous variants have been isolated (Stahly, 1978; Meenakshi and Jayaranam, 1979) of B. thuringiensis, but aberrant inclusions have not been reported (reviewed in Bulla et al., 1980). These aberrant inclusion-forming strains present an ideal opportunity for studying the composition of the inclusion.
Some other subspecies of \textit{B. thuringiensis} have been shown to form an ovoid inclusion (Fowler and Harrison, 1952, Hannay, 1961; Aronson and Fitz-James, 1976). The two known subspecies are \textit{B. cereus} subspecies \textit{lewinia} and \textit{B. cereus} subspecies \textit{fowler}. Like \textit{B. cereus} subspecies \textit{medusa} a target insect is not known and therefore their toxicity remains questionable. Gillespie (1969) tested \textit{B. cereus} subspecies \textit{medusa} on both lepidopteran and dipteran larvae and showed no toxicity. Recently, however, the inclusions isolated from \textit{B. cereus} subspecies \textit{medusa} have been shown to have low to moderate toxicity to mosquito larvae in this laboratory.

Thus a great deal of controversy surrounds the structure composition, toxicity and genetic loci of the parasporal inclusion. Until these problems are resolved, the exact relationship of the various subspecies to each other and to \textit{B. cereus} will go unanswered. Therefore a further study of the inclusion of various subspecies of \textit{B. thuringiensis} and \textit{B. cereus} seemed necessary.
METHODS AND MATERIALS

2.1 Bacterial Strains

Strains of *B. thuringiensis* and *B. cereus*, subspecies were obtained from the laboratory collection of Dr. P.C. Fitz-James. The original source where applicable, and the phenotype of these strains are outlined in table II.

Cultures were stored on grelet salts-blood base medium (Gillespie, 1969) (GBBM) slopes at room temperature and were subcultured on either GBBM agar (NA) or nutrient agar bimonthly. Asporogenous strains were stored as a freeze-dried lyophil and were subcultured weekly on nutrient agar.

2.2 Culture Methods

(a) Complex Media

Nutrient agar, nutrient broth, and Mueller-Hinton agar were supplied by Difco Laboratories, (Detroit, Michigan). Yeast-glucose agar was prepared by adding 0.05 gm of yeast extract (Difco) and 20 gm of dextrose (Fishér) to 1 litre of distilled water. Sheep blood agar (SBA) was prepared by adding 150 ml of sheep blood (Sylab, Petrolia, Ontario) to 12 gm of Oxoid blood agar no. 2 (Oxoid Ltd., London, England), which had been rehydrated with 1 litre of distilled water.

L-broth was prepared by adding 18.0 gm of tryptone, (Difco) 5.0 gm of yeast extract (Difco) and 8.0 gm of NaCl to 1 litre of distilled water (Kado and Liu, 1981).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Inclusion Type</th>
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</tr>
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<tbody>
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<td>B. cereus N</td>
<td>cry&lt;sup&gt;+&lt;/sup&gt; sp&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>U.W.O.</td>
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<td>Fitz-James</td>
</tr>
<tr>
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<td>cry&lt;sup&gt;+&lt;/sup&gt; sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>bipyramidal</td>
<td>Fitz-James</td>
</tr>
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<td>cry&lt;sup&gt;+&lt;/sup&gt; sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>bipyramidal</td>
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</tr>
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<td>A. Aronson</td>
</tr>
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<td>Phenotype</td>
<td>Inclusion Type</td>
<td>Source</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-----------</td>
<td>-------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>B. cereus subsp. medusa 9B</td>
<td>cry&lt;sup&gt;+&lt;/sup&gt; sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>spherical to ovoid</td>
<td>C. Robinow</td>
</tr>
<tr>
<td>B. cereus subsp. medusa L06</td>
<td>cry&lt;sup&gt;+&lt;/sup&gt; sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>spherical to ovoid</td>
<td>Gillespie</td>
</tr>
<tr>
<td>B. cereus subsp. medusa L07</td>
<td>Ø&lt;sup&gt;-2&lt;/sup&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>spherical to ovoid</td>
<td>Fitz-James</td>
</tr>
<tr>
<td>B. cereus subsp. medusa L014</td>
<td>Bd&lt;sup&gt;+&lt;/sup&gt; sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>spherical with anular ring</td>
<td>Fitz-James</td>
</tr>
<tr>
<td>B. cereus subsp. medusa L014</td>
<td>Bb&lt;sup&gt;-&lt;/sup&gt; sp&lt;sup&gt;-&lt;/sup&gt;</td>
<td>small bead</td>
<td>Fitz-James</td>
</tr>
</tbody>
</table>
Blood base medium (BBM), (Young, 1958) was prepared by
adding 3.2 gm of nutrient broth, 5.0 gm of protease peptone
no. 2, (Difco) and 5.0 gm of protease peptone no. 3 (Difco)
to 1 litre of distilled water.

(b) **Semi-Synthetic Media**

Grelet salts were prepared according to Grelet (1951).
The salts were added to 1 litre of distilled water. The pH
was adjusted to 6.9 with 10 N KOH. A light precipitate
remained in the solution after autoclaving. The supernatant
was decanted and mixed with BBM and CaCl₂ according to

(c) **Growth Studies**

A loopful of vegetative cells (approx. 10⁶ cells) was
inoculated into 30 ml of GBBM and grown at 30°C on a
platform reciprocating shaker. A 300 ml side-arm Erlenmyer
flask was used to ensure proper aeration, since it was found
that the volume of the flask must be ten times the volume of
the media (Gillespie, 1969). Growth was observed by phase
contrast light microscopy. The absorbance of the culture
was monitored with a Coleman 14 spectrophotometer at a
wavelength of 650 nm.

(d) **Antibiotic Sensitivity Tests**

Susceptibility of the various subspecies of B._
thuringiensis and B. cereus subspecies medusa to antibiotics
was tested using the methods of Kirby and Bauer (1966). A
loopful of inoculum was standardized to an optical density
of 0.01 absorbance units on a Coleman 14 spectrophotometer.
The bacterial suspension was spread on Mueller-Hinton Agar poured to a depth of 4 mm. Bacto-sensitivity discs were applied to the plates and incubated at 30°C for 12 hr. Interpretation of the zone of inhibition was done according to a protocol supplied by Difco Laboratories, Detroit, Michigan.

Alternatively, the various strains of *B. cereus* subspecies *médusa* were analyzed for antibiotic sensitivity by the clinical automated resistance equipment at University Hospital (courtesy of Dr. R. Behme).

2.3 Structural Studies

(a) **Light Microscopy**

Cells were observed as living material by phase contrast microscopy. A small drop of cells suspended in media or distilled water was applied to a grease free (No. 1) coverslip which was mounted on a clean slide and sealed with molten wax. Capsular material was visualized using the India ink method of negative staining. A Zeiss microscopic with an attached 35 mm Pentax camera or a 9 cm by 12 cm large format plate camera was used to take all photographs on Kodak Pan X film.

(b) **Electron Microscopy**

(i) **Negative Staining**

Preparations of inclusions, partially solubilized in 2% to 5% SDS for varying periods of time, were suspended as a drop on a carbon stabilized formvar coated grid (200 mesh) and left for 30 seconds and then blotted. A drop of 2%
phosphotungstic acid, pH 5.6, was suspended on the grid and left for 60 to 90 seconds, and then blotted. Alternatively, the preparation was mixed with the PTA, and left for 60 seconds on a coverslip. A grid was floated on the drop for 60 seconds and then blotted dry.

(ii) Sectioned Material

Preparations were chilled on ice and fixed with cold 4% glutaraldehyde for 4 to 6 hours, washed four times in cold phosphate buffer (0.1 M, pH 6.7) and then suspended in 1% OsO4 in Veronal-Acetate buffer (Kellenberger et al., 1958) for 4 to 12 hours at 4°C. The samples were washed in Veronal-Acetate buffer and embedded in molten agar (45°C) (Pearce and Fitz-James, 1971). The pieces were stained in uranyl acetate, dehydrated through a graded series of acetone, embedded in Vestopal W (Polysciences) and sectioned on a Riechert ultramicrotome. Sections were stained in uranyl acetate and lead citrate and examined on a Phillips 300 electron microscope.

(iii) DNA visualization

DNA preparations obtained through the CsCl-ethidium bromide isopycnic density ultracentrifugation procedure were visualized by a modified method of Lang and Mitani (1970).

Ten to twenty μl of DNA (concentration approximately 15-20 μg/ml.) was mixed with an equal volume of 0.5 M ammonium acetate, pH 8.0. After stirring with a tooth-pick for approximately 30 seconds, 50 μl of this mixture was placed on a sheet of parafilm and allowed to stand for 1 to
2 minutes. A parlodium-coated grid was touched to the drop for 2 seconds and then immediately touched to a drop of 0.25 M ammonium acetate, pH 8.0 for 5 seconds. The grid was stained in a drop of 0.01% of 0.1 M (in 0.5% 10 NHCl) uranyl acetate in 95% ethanol. The grid was washed in 95% ethanol for 30 seconds and then in distilled water for 1 minute. The grid was blotted dry, shadowed with platinum-palladium in an Edwards Piranni-Penning Model 4 rotary shadow caster and examined in a Phillips 300 or Phillips 200 EM at 40 KV.

(iv) Carbon Relics

Purified whole inclusions suspended in distilled water, or partially dissolved purified inclusions in 10% SDS were spread on a formvar coated, 200 mesh grid and allowed to dry. The preparation was platinum shadowed and carbon coated in a Balzer's apparatus (Model BA510M, Balzers AG Lichtenstein). The formvar was dissolved with a drop of chloroform and the inclusions were dissolved in a fresh solution of 12 N concentrated sulfuric acid containing 10% (w/v) potassium dichromate and 10% (w/v) potassium permanganate. The replicas were rinsed briefly in 12 N sulfuric acid, washed in distilled water and allowed to dry before examination. The grid was recycled through the digestion if the biological material had not been sufficiently removed.
(v) **Freeze-etch**

Samples were rapidly frozen as a small peak of material on a scored copper grid which was submerged in liquid Freon cooled to -160°C by liquid nitrogen. Freeze-etching was performed on the pre-chilled (-150°C) stage of a Balzers freeze-etch apparatus (Model BA510M, Balzers AG Lichtenstein). The sample was cleaved with a cold (-150°C) knife while under vacuum. Following etching of the sample using the warmed (-130°C) knife, the sample was shadowed with platinum. The sample was shadowed with carbon followed by digestion of the sample using the same method as the carbon replica procedure.

2.4 **Inhibition and Loss of Inclusion Formation**

(a) **Acridine Orange and Ethidium Bromide**

Thirty ml of nutrient broth or GBBM was inoculated with a vegetative cell suspension (approx. $10^6$ cells/ml) and incubated at 30°C on a reciprocating shaker. Growth was followed by phase contrast microscopy and spectrophotometry. At varying times, either acridine orange or ethidium bromide, (25 μg/ml, 50 μg/ml and 100 μg/ml) was added to the culture. After several hours of growth, the cells were serially diluted and plated onto GBBM agar. The cultures were incubated at 30°C for 24 hours and examined for differences in colony and cell morphology. Two of the treated cultures were aseptically removed from the erlenmeyer flask and placed in a sterile petri dish. The cells were irradiated with ultra-violet light for 25 seconds
at a distance of 35.5 cm (Hendry, 1974) prior to and following the addition of either acridine orange or ethidium bromide. Following ultra-violet radiation, the culture was replaced in the original flask and incubated at 30°C for varying periods of time. Vegetative cells were also plated onto 1.5% nutrient agar containing 25 μg/ml, 50 μg/ml, 75 μg/ml and 100 μg/ml of either acridine orange or ethidium bromide. The plates were incubated at 30°C for 24 hours and then examined, as above.

(b) Temperature
Vegetative cells were inoculated on 1% nutrient agar and incubated at 30°C, 37°C, 42°C and 55°C for 24 to 72 hours (Youngsten, 1978). Colonies that showed abnormal growth were examined by phase contrast microscopy.

(c) Variation of Media
A vegetative cell suspension (approx. 10^6 cells/ml) was plated onto yeast-glucose agar, sheep blood agar and nutrient agar and incubated at 30°C for 24 to 48 hours. Colonies were selected at random and replated in the same manner. This procedure was repeated up to five successive passages. At each passage, 50 colonies were randomly selected and the cells examined by phase contrast microscopy.

(d) Cysteine Starvation
Cells of *B. cereus* subspecies *medusa* were grown at 30°C, in 30 mls of Grelet salts containing 1% glucose, 0.5% (w/v) each of all common amino acids, (except cysteine),
thiamin and riboflavin. Cysteine (0.0%, 0.1%, 0.2% and 0.5%) was added to the cultures at the time of inoculation (Rajalakshmi and Shetna 1977). Growth and sporulation were monitored by phase contrast microscopy and spectrophotometry.

(e) Addition of Sodium Sulphite

Cells of *B. cereus* subspecies medusa 9B and L07 (donut variant) were grown at 30°C in 30 mls. of CBBM sporulation media on a reciprocating shaker. The growth of the cultures was monitored by phase contrast microscopy and spectrophotometry. Following 12.5 hours of growth (stage V of sporulation), 0.9 mls. of 1.0M NaSO₃ were added to both cultures of 9B and L07 (Aronson and Fitz-James, 1968). The sporulating cells were harvested after a further 5 hours of growth and embedded for electron microscopic studies.

(f) Dehydration

Weighed petri dishes were filled with exactly 20 ml of nutrient agar, solidified and weighed again. The petri dishes were stored at 42°C and allowed to dehydrate. The petri dishes were removed and weighed at various intervals of time over a 14 day period. When 4gm, 8gm, 8.5gm, 9.0gm, 9.5gm, 10gm, 10.5gm and 11.0gm loss in the water content of the media was achieved, the petri dishes were inoculated with a vegetative cell suspension (approx. 10⁶/ml). Duplicate plates were incubated at 30°C and 42°C. Cells were taken from 25 randomly selected colonies and examined by phase contrast microscopy.
(g) Variation of Nutrients

Nutrient broth was prepared in varying concentrations, (2%, 4%, 6% and 8%). Agar was added (1.5%) to solidify each of the prepared media. Six petri dishes were prepared for each concentration. The six replicate plates were inoculated with a vegetative cell suspension (10^6/ml) and incubated at 30°C or 42°C for 24 hours. Cells were taken from 25 randomly selected colonies and examined by phase contrast microscopy.

2.5 Preparation of Inclusions

A loopful of vegetative cells was inoculated onto 250 mls of 6GBM agar in Povitsky bottles. Cultures were incubated at 30°C for 72 hrs until the culture had reached sporulation lysis (hereafter, referred to as the sporulate). The sporulate was scraped from the agar surface with a glass hockey stick and washed in 0.14 M NaCl. The suspension was centrifuged at 10,000 x g in an SS34 rotor and resuspended in 0.14M NaCl.

2.6 Purification of Intact Inclusion

Inclusions were separated from spores and cell debris by Renografin-76 (Squibb) density gradient ultracentrifugation. Discontinuous density gradients were made by a modification of the method of Milne et al., (1977). A 60% aqueous solution (6.5 ml) of Renografin-76 (Squibb) was added to a 12 ml cellulose nitrate tube (Beckman). Four ml of 70% aqueous Renografin-76 was pipetted slowly down the side of the tube and allowed to
form a partition at the bottom of the tube. The sporulate suspension (1.5 ml) was overlayed on the gradient and centrifuged for 1.3 hours at 29,000 x g in an Beckman L8-55 ultracentrifuge at 5°C using a SW-27 rotor. The bands that formed in the gradient, were removed and examined by phase contrast microscopy.

The inclusion fraction, determined by phase contrast microscopy, was diluted with an equal volume of 0.14 M NaCl and centrifuged at 10,000 x g RPM for 15 minutes. The pellet was resuspended in 0.14 NaCl and repurified by Renografin-76 (Squibb) continuous gradient ultracentrifugation (Milne et al., 1977). Linear gradients (11.5 ml) of 50% to 80% (v/v) Renografin-76 (Squibb) were prepared on a Buchler gradient maker and pipetted into 12 ml cellulose nitrate tubes with an LKB peristaltic pump. The inclusion suspension (1.0 ml) was overlayed on the gradient, centrifuged, collected and examined, as above.

The refractive index of each fraction was ascertained with an Abbé refractometer. The density of the fraction was obtained using a density-refractive index graph of Renografin-76 established by Gillespie (1969).

2.7 Isotopic Labelling of the Inclusion

A vegetative cell suspension (10⁶/ml) was inoculated into 300 ml of GBBM broth and incubated at 30°C. A ¹⁴C amino acid mixture (New England Nuclear) was added to the cells (final conc. 0.5 μCi/ml) when the culture had attained a density of 0.10 absorbance units on a Coleman 14
spectrophotometer. The culture resumed growth through to sporulation lysis. The sporulate was fractionated, as outlined above.

2.8 Solubility of the Inclusion

The effects of the denaturant sodium dodecyl sulfate (SDS), the reducing agent B-mercaptoethanol and the chelating agent ethylenediamine tetracetic acid (EDTA) on the dissolution of inclusions were observed by phase contrast microscopy and isotopic labelling. The alkalinity and temperature of the dissociation buffer was also tested for its effect on solubility of the inclusion. Typically, 1.0 mg of inclusions was suspended in 1.0 ml of 1%, 5%, or 10% SDS. The pH was monitored and the inclusions incubated at 21°C. At certain intervals of time the suspension was observed as above. The other chaotropic agents were tested similarly.

Radioactively labelled inclusions were suspended in 1.0% SDS, 0.01 M DTE, .05 M Tris-HCl, pH 7.0. The pH was elevated with 0.1 N NaOH, in increments of 0.5 pH units. The suspension was centrifuged at 13,000 x g for 10 minutes and the pellet resuspended in dissolution buffer, as above. Ten µl aliquots of supernatant were mixed with 1.0 ml of aquasol and counted on a Beckman LS-250 liquid scintillation counter. The procedure was also monitored by phase contrast microscopy.
2.9 Purification of the Inclusion Components (Crystal, Bead, Skin, Light and Dark Segments)

Purified intact inclusions were suspended in 
(dissociation buffer) 1% SDS, 0.01 M DTE, 0.5 M Tris-HCl, pH 7.0. Sodium hydroxide (0.1 N) was added to the suspension to raise the pH in 0.5 unit increments. At each increment, the inclusion suspension was observed by phase contrast microscopy. As dissolution of the inclusion proceeded the residual components of the inclusion were collected as a pellet by centrifugation at 13,000 x g for 10 minutes. The pellet was washed several times in 0.14 M NaCl followed by purification on a Renograffin-76 density gradient when required as outlined above.

2.10 Preparation of Samples for Electrophoresis

Intact inclusions or inclusion components were centrifuged for 5 minutes at 13,000 x g into pellets in 1.5 ml eppendorf microtubes using an eppendorf microfuge (Brinkman). The pellet was resuspended in 0.1 ml of 1% SDS, 0.01 M DTE, 0.5 M Tris-HCl. The pH was adjusted with 0.1 N NaOH and monitored with a microelectrode. Ten μl aliquots were pipetted from the microtube and examined by phase contrast microscopy when required. Fifty μl of 10% glycerol and 0.1% bromophenol blue was pipetted into the solution and the solution boiled for 2-3 minutes. B-galactosidase (m.w. 130,000), RNA polymerase (m.w. 165,000, 155,000, 39,000), bovine serum albumin (m.w. 68,000), ovalalbumin (m.w. 43,000), trypsin inhibitor (m.w. 21,700) and cytochrome C
(m.w. 12,700), (all purchased from Sigma), were solubilized, similarly, and electrophoresed concomitantly with the inclusion proteins.

2.11 SDS-Polyacrylamide Gel Electrophoresis

Samples for electrophoresis were prepared as described earlier. Slab gels were prepared as described by Laemmli (1970). The stacking gel consisted of 4.5% acrylamide (acrylamide-bisacrylamide ratio = 30/0.8) in 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS. The lower or separating gel contained the desired concentration of acrylamide (as indicated in figure legends) in 0.375 M Tris-HCl, pH 8.8, and 0.1% SDS. The polymerization of gels was initiated by adding 200 µl of 10% ammonium persulfate and 30 µl of N, N, N', N'-tetramethylethylenediamine (TEMED) per gel. The concentration of ammonium persulfate was reduced to 100 µl and 10 µl TEMED per gel when gradient gels were poured. A vertical slab gel cell (Bio-Rad Laboratories, Model 200) was used throughout these studies and 22.0 ml of lower gel was poured to prepare each gel, unless otherwise indicated. The running buffer was composed of 0.025 M Tris-base, 0.192 M glycine and 0.1% SDS. A constant current of 30 mA was applied until the dye marker reached the bottom of the gel (usually 3-4 hrs.).
Following electrophoresis, gels were fixed and stained overnight in a solution consisting of 50% methanol, 7% acetic acid and 0.10% Coomassie brilliant blue R, whenever necessary. Destaining of gels was carried out in a solution containing 50% methanol and 7% acetic acid.

The gels were photographed using a transilluminating source of visible light and 35 mm pan X film at an exposure of 1/60 sec at f-5.6.

2.12 Peptide Mapping

For a comparison of the peptide composition of two polypeptides (the crystal and the skin or bead), the purified inclusion components were subjected to peptide mapping. A modified version of the limited proteolysis technique of Cleveland et al., (1977), was used which basically employ Laemmli's (1970) SDS-polyacrylamide gel electrophoresis as described earlier. The enzyme _S. aureus_ V-8 protease (Miles Laboratory) was dissolved in 1x running buffer at a final concentration of 200 µg/ml. Purified inclusion components were dissolved in 1% SDS, 0.01 M DTE, pH 7.0 to 10.5. Seventy to eighty µg of protein were incubated with 100 µl of _S. aureus_ V-8 protease at 37°C for 12 hours in a 1.5 ml eppendorf microtube. Samples were then analyzed on 5% to 20% gradient gels or on 20% gels as described above (Weber and Osborn, 1969; Weber and Kuter, 1971).
2.13 Ion-Exchange Chromatography

A 10.0 x 1.5 cm column of preswollen, microgranular anion DE-52 Diethylaminoethyl cellulose (Whatman) was used in the ion-exchange studies. The DEAE-cellulose was washed several times with 150 ml of starting buffer, 0.05 M Tris-HCl (pH 8.0), 6M urea. Samples were dissolved in 6 M urea, 0.01 M DTE at pH 7.2 to pH 10.5 and boiled for 5 minutes. These samples were dialyzed for 48 hours following S-carboxymethylation in 0.05 M iodacetate in 1 N NaOH. Three to four samples were loaded onto the column using a Buchler polystaltastic pump at a flow rate of 80 ml/hr. The direction of flow was from the bottom to top of the column. The ionic gradient was formed of 0.0 M to 0.4 M NaCl in 0.05 M Tris-HCl, pH 8.0, 6M urea in a volume of 250 ml. Five ml fractions were collected and analyzed on a Gilson on line Holochrome model 1200 spectrophotometer at a wavelength of 270 nm. (Instrument Specialists Inc.) and recorded. Radioactively labelled fractions were analyzed by adding 25 µl aliquots of the sample to 1.0 ml of Aquasol (New England Nuclear) and counting radioactivity in a Beckman LS-250 liquid scintillation counter.

2.14 Determination of Hexoses in Inclusions

The amount of total hexoses found in the inclusion of B. cereus subspecies medusa and B. thuringiensis subspecies kurstaki was determined using the anthrone reaction according to Roe (1955).
To 1.0 ml aliquots of the sample in 18 x 150 mm pyrex tubes, 5.0 ml of cold anthrone reagent were added. (10 g thiourea, 500 mg anthrone, 720 ml conc. \( \text{H}_2\text{SO}_4 \) and 280 ml distilled \( \text{H}_2\text{O} \)). The tubes were immersed in boiling water for 15 minutes followed by cooling in a water bath at 20°C. Standard solutions of glucose were run concurrently. The samples were examined on a Gilford spectrophotometer at a wavelength of 620 nm.

2.15 Determination of Glycoprotein

Glycoproteins were detected by the methods of Fairbanks et al. (1971). Separated polypeptides were analyzed for glycoprotein by staining a polyacrylamide gel with periodic acid-schiff's reagent.

2.16 Amino Acid Analysis

Purified fractions of the inclusions were subjected to acid hydrolysis in 6 N HCl under vacuum for 12 hours. The samples were analyzed on a Beckman Spinco Model 1200 Analyzer using the accelerated system, manual injection of 0.5 ml samples and dimethyl sulfoxide ninhydrin reagent. A standard 62.5 nmol of each amino acid was run concurrently with the sample.

2.17 Serology

Antisera against inclusion fractions obtained by inoculating a New Zealand white albino rabbit with 1 mg doses of the protein intramuscularly in two sites, twice, two weeks apart. After a two week interval, a booster injection was given of 1 mg. The protein was suspended in
an equal volume (2 ml) of Freund's incomplete adjuvant. The animal was bled 1 week following the booster. The serum was collected by allowing clotting of the blood and centrifuging at 10,000 x g RPM for 10 minutes.

Ouchterlony gels were prepared with 1% agarose, 2% polyethylene glycol 6,000, 0.089 M boric acid, 0.089 M Tris-HCl, pH 8.3, and 0.001 M EDTA, pH 8.3 (Tyrell et al., 1981).

Antigen (1 mg) and antisera were placed into the wells and allowed to incubate overnight at 4°C. This was followed by several days of incubation at room temperature to enhance the precipitin bands.

2.18 Protein Determination

Protein concentrations were determined by the method of Lowry et al., (1951) using BSA as a standard. Alternatively, the absorbance of the protein solution was obtained using a Beckman model 2400 spectrophotometer at a wavelength of 280 nm and the amount of protein estimated using an extinction coefficient of 1.1=1.0 mg/ml (Bulla et al., 1977).

2.19 Spore Coat Extraction

Spores purified using the methods outlined above for inclusion purification, were extracted three times with 1% SDS, 0.05 DTE pH 9.8 with or without 6M urea (Aronson and Fitz-James, 1976). Both preparations were then subjected to SDS polyacrylamide gel electrophoresis as described above.
2.20 Preparation of Plasmid DNA (Cleared lysate)

Cells (10^8/ml) grown in 30 ml of BBM at 30°C were harvested at late log-phase or early stationary phase and centrifuged at 10,000 x g in a Sorval SS-34 rotor. The washed cells (STE lysis buffer, 20% sucrose, 0.05 M Tris-HCl, pH 8.5, 0.002 M EDTA) (Guerry et al., 1973) were suspended in 1.5 ml of STE lysis buffer and mixed thoroughly by vortexing. Lysozyme was added to a concentration of 20 mg/ml and incubated at 37°C for 4 hours. One ml of 10% SDS was added to the mixture and the cells incubated until the solution became clear and viscous. Five molar NaCl (0.8 ml) was added (final concentration of 0.5M NaCl) and the solution placed at 4°C for 12 hours. The solution was centrifuged in a Sorvall SS-34 rotor at 13,000 x g for 30 minutes at 4°C, and the viscous supernatant decanted. RNAse (Type I pancreatic Sigma); pre-heated at 90°C for 10 minutes, was added to the supernatant (final concentration of 1 µg/ml) and incubated for 1 hour at 37°C. The volume of the solution was doubled with distilled H_2O and purified by one of the following procedures.

1) Phenol Extraction (Meyers et al., 1976)

An equal volume of phenol saturated with 0.05M Tris-HCl (pH 8.0) was added to the cleared lysate. The solution was mixed by inversion for approximately 30 seconds and centrifuged in an IEC centrifuge model PR-2, at 2,000 x g for 20 minutes. The aqueous phase was drawn off with a pasteur pipette and the phenol extraction repeated until the
aqueous phase became clear. Sodium acetate (1.5 M, pH 5.5) was added to the clear aqueous phase to a final concentration of 0.5 M. Three volumes of 95% ethanol were added to the solution and the solution was stored at -20°C for 8 hours. The solution was centrifuged in a refrigerated Sorvall superspeed centrifuge using an HB-4 rotor at 13,000 x g for 30 minutes at 4°C. The pellet was resuspended in 0.1 ml of TES buffer, (0.05 M Tris-HCl, pH 8.0, 0.002 M EDTA, 0.05 M NaCl, pH 8.0) and used immediately or stored at -20°C (Meyers et al., 1976).

(2) CsCl-ethidium bromide Isopycnic Density

Ultracentrifugation (Bauer and Vinograd, 1968)

Cesium chloride was added to the cleared lysate, (final concentration approximately 1gm/ml) until the refractive index of the solution was 1.3965 on an Abbé refractometer. The solution was thoroughly mixed in a 50 ml erlenmeyer flask and ethidium bromide added to a final concentration of 2.5 mg/ml. Occasionally a slight precipitate formed which did not interfere with the gradients. The mixture was poured into 12 ml polypropylene tubes (Beckman) and centrifuged at 60,000 x g, at 5°C, for 40 hours using a Ti-50 rotor. The DNA bands that formed in the gradients were removed with a pasteur pipette. The DNA was cleared of ethidium bromide by adding an equal volume of n-butanol, mixed by inversion and allowed to stand for 20 minutes. This was repeated until the ethidium bromide was in the alcohol phase. Sodium acetate (1.5M, pH 5.5) was added to
the DNA solution (final concentration of 0.5 M sodium acetate) and the DNA precipitated and resuspended as above.

(3) **Ethanol Precipitation**

The cleared lysate (obtained as above) was cooled to 4°C. The supernatant was decanted and vacuum filtered through a 0.22 μm millipore filter (Millipore). The DNA was then ethanol precipitated as above.

### 2.21 Restriction Enzyme Digestion

The reaction mixture in a 30 μl volume contained 10-20 μg of plasmid DNA in EcoRI buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl and 10 mM MgCl₂) and a 2-3 fold excess of EcoRI enzyme (Boehringer) (i.e. 2-3 units of enzyme/1.0 μg DNA). Following incubation at 37°C in a water bath for 3 hrs, 2-3 units of enzyme/μg of DNA were again added and incubation was continued overnight. The enzymatic digestion of DNA was stopped by adding 15 μl of 50% sucrose, 50 mM EDTA, 10 mM Tris-HCl (pH 8.0), and 0.125% bromophenol blue. Digested plasmid DNA was loaded onto an agarose gel immediately or stored at 4°C.

### 2.22 Agarose Gel Electrophoresis

A horizontal gel apparatus (Iceberg Inc.) was used according to McDonnell et al., (1977). Agarose (0.7%), (Seakem) was suspended in electrophoresis buffer consisting of 40 mM Tris-acetate (pH 8.0), 20 mM sodium acetate and 1 mM EDTA, and autoclaved before pouring. About 10.0 μg of DNA was added to each well and electrophoresis was carried out at 50 V for 12 hrs. at room tempature. Following
electrophoresis, the gels were stained with 1 ug/ml ethidium bromide. DNA bands were visualized with Black-Ray ultraviolet transilluminator (Ultra Violet Products) and photographed through a Kodak No. 23 yellow filter with Kodak high-contrast copy film. The plasmids pGM4, pGM5, pBR 322 and pBR 325 (Mackie, 1980) were electrophoresed concomitantly to provide markers for the plasmid DNA agarose gels. Lambda plac5 and lambda GR4 DNA was digested with EcoRI and HindIII respectively and electrophoresed concomitantly to provide a marker for the restriction enzyme agarose gels (Robinson and Landy, 1977).

2.23 DNA Determinations

Aliquots of plasmid solutions were examined in a Gilford model 2400 Spectrophotometer at a wavelength of 260 nm and 280 nm. The $A_{260}/A_{280}$ absorbance ratio was examine to ensure that the ratio was above 2.0. The absorbance was calibrated by using a known amount of calf thymus DNA and the formula 1.0 O.D. unit is equal to 50 μg of DNA.
RESULTS

3.1 Characterization of the Inclusion of B. thuringiensis kurstaki

Previous studies of B. thuringiensis subspecies kurstaki (Betchel and Bulla, 1976; Sharpe and Baker, 1979) have not reported a detailed electron microscopic analysis of the crystal and bead. To confirm and elaborate on these findings, the inclusion of B. thuringiensis subspecies kurstaki was purified and characterized. The inclusion of B. thuringiensis subspecies kurstaki was the typical bi-pyramidal crystal of most other subspecies B. thuringiensis, accompanied by a spherical or ovoid inclusion (Fig. 1-4). The ovoid inclusion, hereafter referred to as the bead, appeared in a variety of shapes (Fig. 3-7) and developed concurrently with the crystal during stage II of sporulation (Fig. 8). The bead stained lighter (electron density) than the crystal (Fig. 2, 7) and most often was attached to the side of the crystal (Fig. 4, 6, 8). Occasionally, the bead was not affixed to the crystal, which allowed the bead to be released freely from the cell and the crystal during sporulation lysis (Fig. 5, 7).

3.1.1 Structure of the Ovoid Inclusion (bead)

Each cell produced only one bead and sometimes two crystals (Fig. 2, 6). Growth of the cells in rich undefined media (NA), sporadically resulted in loss of bead formation in a small percentage of the cells (Fig. 11).
Electron microscopy of thin sections of the purified beads revealed a thin membrane-like layer surrounding the dense non-crystalline mass (Fig. 9, 10). However, in sections of sporulating cells (Fig. 3, 7), this membrane-like layer was not apparent, suggesting that it may have detached from the bead allowing easy visualization.

Electron microscopy of platinum shadowed, carbon replicas of the surface of free crystals and beads revealed striae on the surface of the crystal and a rough granular non-striated surface on the bead (Fig. 4), 11, 17a). The results of the carbon replicas and thin sections indicate that the bead is in the shape of a prolate disc with approximate dimensions of 1.8 \( \mu \) long by 0.68 \( \mu \) wide by 0.3 \( \mu \) thick.

3.1.2 Structure of the Crystal

The crystal displayed surface repeating striae with a centre to centre spacing of 29.5 nm (Fig. 4, 11, 14). The space between the striae was 13.5 nm wide. In figure 14, each striae was composed of a series of repeating subunits or rods 3.7 nm wide by 19.0 nm long and spaced 7.0 nm from centre to centre. Two rods were 9.5 nm wide with the space between them included. At the apex of the crystal depicted in figure 14, the striae diminished and the rod appearance of the subunits became more like stacked sheets, which were flat rather than round. This was probably caused by a accumulation of metal on the top of the rod (Sjostrand, 1979). Figure 16 a,b revealed a new structure in the centre of the rows of subunits. A globular structure 5.2 nm in
diameter and spaced 7.8 nm centre to centre was seen as the shadowing became more parallel to the crystal surface. As the shadowing became more oblique, the rods developed a doublet structure (Fig. 16d) with a very distinct hole in the centre of the rod. The doublet measured 8.7 nm centre to centre with each unit being 6.0 nm in diameter. Figure 15 is a drawing illustrating the structure of the inclusion. Figure 15a illustrates the surface structure served in figure 14 and figure 15b illustrates the structure of the crystal observed in figures 16a-d. It is concluded that the subunit structure and molecular packing of the crystal in B. thuringiensis subspecies kurstaki is similar to the findings of Norris, (1971) and Short et al., (1974) and not those of Labaw (1964) and Holmes and Munro (1965). Holmes and Munro (1965) using X-ray diffraction found that the subunit was 9.0 nm by 9.0 nm by 13.5 nm. Labaw (1964) using carbon replicas found that the subunit was 8.7 nm by 12.3 nm. The rods seen in figure 14, a freeze-etch micrograph, correlate to one-half of the subunit described by these authors (Holmes and Munro, 1964). Since they assigned a molecular weight of 230,000 to the subunit protein then the rods might represent two molecules of 135,000 daltons each.

3.1.3 Separation of Beads and Crystals

To biochemically analyze the two components of the inclusion of B. thuringiensis subspecies kurstaki it was necessary to separate and purify them. Initially in the study, Renografin-76 density gradient centrifugation,
described in the materials and methods, was used to separate the beads, crystals and spores. However, during the course of solubility studies, it was noted that the crystal dissolved at pH 7.5 in 1% SDS, 0.05 M DTE and the bead dissolved at pH 9.2 in the same solvent. Thus, this difference in solubility was used in conjunction with density centrifugation to further separate and purify the beads and crystals.

Prior to centrifugation the bead-crystal complex and spores were sonicated for 1 minute. This resulted in the release of approximately 80% of the beads from the side of the crystal. Figure 17b which shows the effect of 10% SDS on the crystal is a micrograph of a crystal preparation that was sonicated and centrifuged prior to the addition of 10% SDS.

Centrifugation of the inclusions and spores on a Renografin-76 density gradient resulted in the formation of five bands. The top band consisted of the usual cell debris (cell walls). The second band (density 1.280) contained pure beads. The third band (density 1.292) contained the crystal-bead complex (the inclusion) and the fourth band (density 1.296) contained pure crystals. The spores formed a band at the bottom of the gradient. Inevitably, there was some cross contamination and this was overcome by dissolving the crystal in 1% SDS, 0.05 M DTE pH 7.5 and pelleting any
beads still remaining in the pure crystal fraction.
Biochemical analysis was then performed on the bead fraction
and the crystal fraction devoid of beads.

3.1.4 Solubility

When purified inclusion's crystal and bead were
suspended in 5% SDS for 30 minutes, at 20°C, the crystals
enlarged to three to four times their normal size (compare
Fig. 5b to Fig 17a,b). Although the crystals lost the
lattice structure (Fig. 12, 13) they maintained the bi-
pyramidal shape and did not further dissolve until a
reducing agent, (dithiothreitol or B-mercaptoethanol) was
added. The bead was released from the majority of the
crystals. The denaturing solution of 5% SDS had no apparent
effect on the structure of the beads (Fig. 13). Following
this observation, it was found that the crystal dissolved
totally in 1% SDS, 0.05 M DTE when the pH was elevated to
7.5 with 0.1 N NaOH. The beads dissolved at pH 9.2 (Fig.
18).

An analysis of the distribution of radioactivity in
dissolved ¹⁴C-amino acid labelled inclusions indicated that
approximately 80% of the total protein was soluble at a
lower pH 7.5 while 15% was soluble at pH 9.2. As the pH was
elevated from 9.5 to 10 a further 5% increase in soluble
protein was noted. By phase contrast microscopy these
fractions corresponded to the crystal (pH 7.5), the bead (pH
9.2) and residual bead at pH 10.0.
3.1.5 Polyacrylamide Gel Electrophoresis

The purified fractions were analyzed by polyacrylamide gel electrophoresis. Figure 19 shows that the purified crystal is composed of a single 135,000 dalton protein. After overnight storage at room temperature, the crystal solution contained decreased amounts of this major protein but also contained small polypeptides at the dye front, indicative of proteolysis. The bead fraction was composed of a single 68,000 dalton polypeptide (Fig. 19). Unlike the crystal solution, this was stable at room temperature. The minor bands in both crystal and bead gel profiles (Fig. 19) could be removed by further gradient and differential solubility purification (Fig. 19, lanes A and B). It was concluded, therefore, that the minor bands were due to cross-contamination of the protein fractions.

3.1.6 Carbohydrate Analysis

A periodic acid-Schiff stained gel along with a Coomassie blue stained control gel indicated that both the crystal and bead were glycoproteins (Fig. 20). The bead protein was stained (PAS) relatively more intense than the crystal when compared to the intensities of each protein stained with Coomassie blue.

The anthrone reaction showed that the total inclusion (crystal-bead complex) contained 3.8 percent hexoses confirming the results of Bulla et al., (1977). The same test indicated that 2.9 percent of the protein weight of the beads was hexoses. Thus, based on the ratio of crystal
protein to bead protein found by radioactivity analysis (see above), one can calculate that some 4% of the protein weight of the crystals are hexoses. Therefore, most of the carbohydrate is found in the crystal. This is in contrast to the staining characteristics of the bead and crystal by Periodic-Acid Schiff reagent.

The gel depicted in figure 20 also revealed that total inclusion preparations contained a doublet band at 130,000 daltons. A possible explanation for this 130,000 dalton protein, is that the bead contained the 68,000 dalton protein as a dimer which unreduced gave rise to a band slightly less in molecular weight than the 135,000 dalton crystal protein.

3.1.7 Ion-Exchange Chromotography

A total inclusion (crystal-bead complex) preparation was dissolved in 6M urea, 0.05 M DTE, 0.05 M Tris-HCl pH 9.2 and analyzed on a DEAE-cellulose ion exchange column. Two fractions were obtained (Fig. 21). Secondly, a purified (described above) bead preparation dissolved and run similarly on the column gave rise to only the second, more acidic fraction, seen in the whole inclusion preparation. Lastly, after incubation of the whole inclusion dissolved at room temperature for 24 hr. the dissolved protein was run protein at 20°C for 24 hr., the preparation was re-run over the column again. The two peaks remained at the same.
positions but the first peak decreased and the second peak remained constant. This observation is consistent with the results reported by polyacrylamide gel electrophoresis.

3.1.8 Amino Acid Analysis

Amino acid analysis of the purified bead and crystal revealed slight differences in their amino acid composition (Table III). Both proteins are highly acidic with the aspartic and glutamic residues being 28% in the crystal and 27.9% in the bead. Methionine was detected in the bead whereas, it could not be found in the crystal. Aspartic acid was the most prevalent amino acid residue in the bead protein whereas glutamic acid was most prevalent in the crystal.

3.1.9 Immunological Analysis

Ouchterlony diffusion showed a possible partial identity in the two proteins of the crystal and bead (Fig. 22). Both the crystal and bead antigen cross reacted with the antisera prepared against purified crystal and bead.

3.1.10 Limited Proteolysis

Partial proteolysis of the bead and crystal proteins (with S. aureus V-8 protease) revealed that the two polypeptides exhibit partial homology. The crystal contained two to three peptides in common with the bead and contained at least four unique peptides (Fig. 23). A 16,000 dalton fragment, found in large amounts in the crystal was also seen in the bead, but to a lesser degree.
### TABLE III

Amino Acid Composition of the Inclusion of *B. thuringiensis* subspecies kurstaki

<table>
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<th>Amino Acids</th>
<th>inclusion</th>
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<th>bead</th>
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<tr>
<td><strong>% moles</strong></td>
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<tr>
<td>glycine</td>
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<td>8.6</td>
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<tr>
<td>alanine</td>
<td>6.7</td>
<td>5.8</td>
<td>4.5</td>
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<tr>
<td>valine</td>
<td>5.6</td>
<td>5.7</td>
<td>5.3</td>
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<td>isoleucine</td>
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<td>leucine</td>
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<td>phenylalanine</td>
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<td>4.4</td>
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<tr>
<td>proline</td>
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<td>4.6</td>
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<tr>
<td><strong>Total neutral</strong></td>
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<td>43.5</td>
<td>39.6</td>
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<tr>
<td>serine</td>
<td>8.3</td>
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<tr>
<td>threonine</td>
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<td>7.7</td>
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<tr>
<td>tyrosine</td>
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<tr>
<td>aspartic</td>
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<tr>
<td>cysteine**</td>
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<td>0.0</td>
<td>1.0</td>
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** Cysteine and tryptophan were not determined.
3.1.11 Spore Coat Extraction

Spore coat extracts were compared to the inclusion by polyacrylamide gel electrophoresis (Fig. 24). The spore coat contained a major protein at 135,000, as reported by others (Tyrell et al., 1981). However, the gel profile showed the major proteins to be 68,000, 26,000, and 12,600 daltons as well as the presumptive crystal 135,000 dalton protein. The crystal protein was not of the same intensity as the others and, therefore, may be a misplaced protein on the spore coat, or it may be contaminating crystal protein collected during the extraction procedure. There was also a large molecular weight protein (200,000 daltons) extracted from the spore. The significance of this protein is not clear, but it may be the aggregated form of the 68,000 dalton protein or it may be a unique spore coat protein, hitherto not described.

As reported by others (Tyrell et al., 1981), the presence of urea in the extraction solvent had no effect on the number of polypeptides in the spore coat but increased the amount of the 68,000 dalton protein.
reveal its crystallinity. The striae are 29.5nm apart, center to center. The lattice is partially disintegrated on the one side of the crystal. (mag. 59,457X)
Figure 1. A light micrograph of sporulating cells of *B. thuringiensis* subspecies *kurstaki* grown on nutrient agar for 48 h. The cell forms a crystal and an ovoid inclusion (arrow). The free inclusions have a high-refractile bead attached to the crystal. (mag. 5000X)

Figure 2. An electron micrograph of a thin section of a sporulating cell of *B. thuringiensis* subspecies *kurstaki*. Frequently, the cell forms two (C) crystals but only a single bead (OI) along with the spore (S). (mag. 38,034X)

Figure 3. An electron micrograph of a thin section of a bead and crystal in a sporulating cell of *B. thuringiensis* subspecies *kurstaki*. The crystal is stained darker than the bead and has a lattice structure. The lighter stained bead has a granular amorphous substructure. The bead has separated from the crystal during processing. (mag. 98,175X)

Figure 4. A carbon replica electron micrograph of an inclusion from *B. thuringiensis* subspecies *kurstaki*. The bead (OI) or ovoid inclusion is firmly embedded in the side of the crystal (C). The striae on the surface of the crystal
Figure 5a and b. A light micrograph of sporulating cells of *B. thuringiensis* subspecies *kurstaki* grown on nutrient agar for 48 h. Frequently, the ovoid inclusion is free of the crystal and the cell (arrows). The ovoid inclusion, at times, was less refractile than the crystal by phase contrast microscopy. (mag. 4000X)

Figure 6 and 7. Electron micrographs of sporulating cells of *B. thuringiensis* subspecies *kurstaki* showing the bead attached (Fig. 6) and separate (Fig. 7). Figure 7 shows that by stage IV, the inclusion is well developed and the crystal and bead appear to develop concurrently. Figure 6 illustrates the different shapes of the bead. (mag. 57,960X) Figure 6 (mag. 66,240X) Figure 7
Figure 8. An electron micrograph of sporulating cells of *B. thuringiensis* subspecies *kurstaki*, showing the development of the crystal and bead at stage II to III of sporulation. Both the bead (O1) and crystal (C) are developing concurrently, early in sporulation. The difference in staining intensity of the bead and crystal is evident in this micrograph. The inclusion does not appear to be developing on the membrane or on the exosporium. (mag. 56,595X)

Figure 9. An electron micrograph of a thin section of a crystal within a cell of *B. thuringiensis* subspecies *kurstaki*. The striae and the subunit "rodlets" are evident. (mag. 98,175X)

Figure 10. An electron micrograph of a thin section of purified ovoid inclusions showing no internal structure. The fissures are due to embedding artifact. (mag. 86,625X)
Figure 11. A carbon replica electron micrograph of the inclusions of *B. thuringiensis* subspecies *kurstaki* showing the variation in shapes of the ovoid inclusion and the lattice of the crystal. (mag. 42,054X)

Figure 12a and b. Electron micrographs of thin sections of the intact inclusion within a sporulating cell (12a) and following partial dissolution in 5% SDS (12b). The bead remained intact while the crystal lost the lattice structure but remained insoluble. The staining intensities are now reversed and the bead appears darker than the crystal. (mag. 61,200X) Figure 12a
(mag. 86,250X) Figure 12b

Figure 13a and b. Electron micrographs of 2% phosphotungstic acid negative stain of the same preparations as shown in Figure 12. Both components were subjected to 5% SDS treatment. The crystal lost its crystallinity whereas the bead remained unaltered.
(mag. 58,054X) Figure 13a
(mag. 64,322X) Figure 13b
Figure 14. A freeze-etch electron micrograph of *B. thuringiensis* subspecies *kurstaki* showing the striae of the crystal with the subunit rodlets appearing as discs on the one surface of the crystal. This crystal does not have an associated bead. The subunits on one of the faces of the crystal appear as flat discs rather than rodlets.

(mag. 142,300X)
Figure 15. Illustration of the macromolecular structure of the crystal of *B. thuringiensis* subspecies *kurstaki*. (A) represents the structures seen in Figure 14 and (B) represents the structures seen in Figures 16 a-d. All dimensions are given in nanometers.
Figure 16a to d. Freeze-etch electron micrographs of the inclusion of *B. thuringiensis* subspecies *kurstaki* shadowed and viewed at different angles. Figure 16a reveals the globular subunit between the rows of subunits. Figure 16b shows a crystal structure similar to Figure 14. Figure 16c reveals a globular subunit between the rows as viewed from the bottom of the row. Figure 16d shows the previously defined rodlets as doublets. (mag. 115,500x)
Figure 17a. A carbon replica electron micrograph of the inclusions of *B. thuringiensis* subspecies *kurstaki* revealing the variation in size and type of inclusion. One of the inclusions has a bead attached whereas another bead lies free (B). The surface of one of the inclusions appears to be contaminated with granular material. (mag. 39,250X)

Figure 17b. A light micrograph of the inclusions of *B. thuringiensis* subspecies *kurstaki* subjected to dissolution in 5% SDS. The crystals have enlarged but not dismantled whereas the beads remain intact. Crystal (arrowhead), bead (B). (mag. 5000X)
Figure 18: The effect of pH on the dissolution of the inclusion of *B. thuringiensis* subspecies *kurstaki*. The crystals and beads were suspended in 1% SDS, 0.05 M DTE, 0.05 M Tris·HCl, pH 7.0, buffer. The solubility of the inclusions was monitored by phase contrast microscopy as well as isotopic label with $\text{C}^{14}$ amino acids. The pH was adjusted with 0.1 N NaOH and monitored with a micro-electrode in the microtube. The inclusion preparation was centrifuged following dissolution and the soluble labelled protein counted.
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<table>
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<td>JAMES PETER INSELL</td>
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| Date of Birth — Date de naissance           |
| 1947                                         |

| Country of Birth — Lieu de naissance        |
| CANADA                                       |

| Permanent Address — Résidence fixe           |
| 353 Riverside Drive,                         |
| London, Ontario N6H 1G3                      |

| Title of Thesis — Titre de la thèse          |
| STUDIES ON THE STRUCTURE AND ORIGIN OF THE PARASPORAL INCLUSION OF SPORULATING BACILLI |

| University — Université                     |
| THE UNIVERSITY OF WESTERN ONTARIO           |

| Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée |
| DOCTOR OF PHILOSOPHY                        |

| Year this degree conferred — Année d'obtention de ce grade |
| Spring 1983                                               |

| Name of Supervisor — Nom du directeur de these          |
| Dr. P.C. Fitz-James                                      |

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Figure 19. Polypeptides of purified crystal and bead (ovoid inclusion). The crystal and bead were purified as described in the Method and Materials, (sonification and differential solubility). The crystals were dissolved in 1% SDS, 0.5M DTE, 0.05 M Tris-HCl, pH 7.5 (lane C). The ovoid inclusions or beads were dissolved in the same buffer at pH 9.2 (lanes D and E). The preparations in lanes D and E were repurified by the above procedure and the ovoid inclusion redissolved as above, (lane A and B). The proteins were electrophoresed on a 12.5% SDS-polyacrylamide gel at 25 ma for 3 h. The gels were stained with 0.1% Coomaissie Blue and destained as in the Method and Materials.
Figure 20. Polypeptides of the purified intact inclusion of *B. thuringiensis* subspecies *kurstaki* solubilized in 1% SDS, 0.05M DTE, 0.05 M Tris-HCl, pH 9.2. The 12.5% polyacrylamide gel electrophoresed at 25 ma for 3 h. and stained with periodic acid-schiff reagent (PAS) and 0.1% Coomassie Blue. Figure A lanes 1 and 2 are the inclusion polypeptides stained with PAS and Figure B lanes 1 and 2 are the polypeptides stained with 0.1% Coomassie Blue. Figure C lanes 1 and 2 are the inclusion polypeptides stained with 0.1% Coomassie Blue for 72 h.
Figure 21. DEAE-cellulose ion exchange chromatography of the whole inclusion protein and the purified bead protein of *B. thuringiensis* subspecies kurstaki. The purified bead and inclusion were dissolved in 6 M urea, 0.05 M DTE, 0.05 M Tris-HCl, pH 9.2 and dialyzed against 6 M urea, 0.5 M Tris-HCl, pH 8.5. The proteins were carboxymethylated with 0.2 M iodoacetic acid for 2 h. and re-dialyzed against 6 M urea, 0.5 M Tris-HCl, pH 8.5 for 48 h. The column was eluted with 350 ml of 0.0 M to 0.4 M NaCl in 6 M urea, 0.5 M Tris-HCl, pH 8.5.
Figure 22. Ouchterlony double diffusion analysis of purified crystal and bead protein of *B. thuringiensis* subspecies *kurstaki*. The agarose gel was prepared as in the Methods and Materials. The antigen was loaded into the agarose wells at a concentration of 1 mg/ml. The bead and crystal were dissolved in 0.1 N NaOH. C<sub>ab</sub> is crystal antibody, C<sub>ag</sub> is crystal antigen, B<sub>ab</sub> is bead antibody and B<sub>ab</sub> is bead antigen.
Figure 24. Polypeptides of the spore coat of *B. thuringiensis* subspecies *kurstaki*. The spores were extracted in 1% SDS, 0.05 M DTE in 0.05 M Tris-HCl, pH 9.8 with and without 6 M urea. Lane A is the 6 M urea and lane B is without 6 M urea. Approximately 50 μg of protein were loaded onto a 12.5% SDS-polyacrylamide gel and electrophoresed at 25 ma. for 3 h. The gel was stained and destained as per the Methods and Materials.
3.2 Characterization of the Inclusion of *B. thuringiensis* subsp. *israelensis*

To further describe the inclusion of *B. thuringiensis* subsp. *israelensis*, and compare it to the inclusion of *B. thuringiensis* subsp. *kurstaki*, inclusions were purified by Renografin-76 density gradient centrifugation and characterized. The inclusion of *B. thuringiensis* subsp. *israelensis* is a spherical multi-segmented crystal which is formed during stage II to III of sporulation (Fig. 25). The inclusion is formed outside the exosporium but is most often in close proximity to it (Fig. 25b).

3.2.1 Structure of the Crystal

The inclusion was composed of contrasting light (electron density) and dark crystalline regions when seen in stained sections and was enveloped by a thin membrane "skin" (Figures 25b, and 28). Freeze-etch preparations of the inclusion showed a lattice within the inclusion (Fig. 26) which was not obvious in carbon replica preparations, since the membrane masked the surface striae. Each of the segments within the inclusion were oriented in a different direction (Fig. 26). The centre to centre spacing of the lattice striae is approximately 19.5 nm, which was substantially smaller than that of *B. thuringiensis* subsp. *kurstaki* crystals. The subunit rods of the lattice are approximately 14.9 nm long with an electron-lucent region between the rows of rods which measured 7.0 nm
wide. The subunit rods composed the darker regions of the lattice. These rods were approximately 4.0 nm wide and were spaced 5.6 nm center to center. An interesting feature of these rods (Fig. 27) was that they were not oriented vertically as in the crystal of *B. thuringiensis* subspecies kurstaki but were aligned at approximately 75° relative to the horizontal axis of the lattice. Figure 27 illustrates the structure of crystal.

### 3.2.2 Solubility

Denaturation of the inclusion protein by incubation in 5%-20% SDS did not occur. When the inclusions were subjected to dissolution in 1% SDS, 0.05 M DTT, 0.05 M Tris-HCl, pH 9.2, the light crystalline segments dissolved, leaving the dark segments as an insoluble residue (Fig. 29). Therefore, dismantling of the crystal by a denaturant (SDS) was not observed in this case, unlike the positive effect of SDS on the crystal of *B. thuringiensis* subspecies kurstaki. The dark crystal segments dissolved in 1% SDS, 0.05M DTE, 0.05M Tris-HCl as the pH was increased to 10.5.

To observe the fine structural changes during dissolution, thin sections of purified inclusion before and after dissolution were prepared. The striae of the dark segment varied from 7.6 to 8.3 nm. The striae of the light segment varied from 4.3 to 4.5 nm. As dissolution occurred, the crystallinity of the light segments disappeared. The
lattice spacing of the dark segment remained constant (Fig. 29 and Fig. 30) whereas the lattice spacing increased in the light segment as the crystallinity diminished.

3.2.3 Polyacrylamide Gel Electrophoresis

Light and dark segments separated by their differential solubility and analyzed by polyacrylamide gel electrophoresis revealed two distinct protein fractions. Thirteen distinct proteins were in the light crystals, whereas only two (35,000 and 70,000) were found in the dark segments (Fig. 31). Presumably, the 35,000 and the 70,000 dalton protein in the light crystal preparation is due to some cross-contamination of the dark crystals protein during solubilization. That is, some of the dark crystals had begun to dissolve at pH 9.2. It is suggested by the data that the light crystal may be composed of four major proteins, the 25,000 and 26,000 dalton protein complex and the 145,000, 135,000 dalton protein complex. It is possible that the large polypeptides (90K, 86K, 49K, 47K) (see Fig. 31) are concatamers of the 25,000 and 26,000 dalton complex, since the molecular weights of the large proteins are close to being multiples of the small 25,000 and 26,000 dalton protein complex. The skin surrounding the light segment may be composed of either the 36,000 or the 31,000 dalton protein.

The effect of another chaotropic agent, KSCN was examined to see if similar dissolution would result. Several workers (Fast and Milne, 1979; Fast and Martin,
1980) have used 1M to 2M KSCN to dissolve the inclusion of B. thuringiensis subspecies kurstaki. Figure 32 shows that the proteins obtained by the two different solubilizing buffers were not the same. However, in all three cases, 2.0 M KSCN, 2.0 M KSCN with 1% SDS, (lane B) and 1% SDS, 0.05 M DTT (lane A), the 26,000 dalton protein was prominent whereas the 134,000 and 145,000 dalton protein complex was not apparent. Moreover, there are substantial amounts of different polypeptides generated by 2M KSCN dissolution that are not seen by SDS-DTE dissolution.
Figure 25a and b. Electron micrograph of a thin section of a B. thuringiensis subspecies israelensis sporulating cell. In Figure 25a, the inclusion is lightly stained with a thin membrane skin surrounding the crystal. In this particular cell, the inclusion seems to have no relationship with the exosporium. In Figure 25b, the inclusion is composed of alternating light and dark regions with a thin skin surrounding both regions (small arrows).

(mag. 32,450X) Figure 25a
(mag. 34,560X) Figure 25b
Figure 26. A freeze-etch electron micrograph of the inclusion of *B. thuringiensis* subsp. *israelensis*. The striae which form the lattice of the crystal and the subunits of the striae can be clearly seen. In this inclusion, unlike that of *B. thuringiensis* subsp. *kurstaki*, the rodlets are positioned at an angle of $75^\circ$ relative to the lattice of the crystal. The inclusion seems to be composed of segments which may correspond to the different latticed portions of the crystal. (mag. 125,895X)
Figure 27. An illustration of the macromolecular substructure of the inclusion of \textit{B. thuringiensis} subspecies \textit{israelensis}. The drawing represents three rows of striae with the subunits being drawn in one of the rows. All of the dimensions are in nanometers.
Figure 28a and b. Electron micrographs of thin sections of the purified inclusion of B. thuringiensis subspecies israelensis. The inclusion is composed of light and dark crystalline regions. The light segment has striations measuring 4.3 to 4.5 nm. wide whereas the dark segment has striations measuring 7.6 to 8.3 nm. wide (arrows).

(mag. 57,600X) Figure 28a

(mag. 92,400X) Figure 28b
Figure 29. An electron micrograph of a thin section of purified inclusions of *B. thuringiensis* subspecies *israelensis*. The inclusion was dissolved at pH 9.2 in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl. The light segment has partially dissolved whereas the dark segment still maintains its crystallinity. The lattice spacing of the light segment has increased to 5.6 nm. from 4.3 nm. in one of the fragments. (mag. 90,090X)

Figure 30. The same preparation as above, after dissolution has been completed at pH 9.2. The light segments have solubilized leaving the partially dissolved dark segments as a residue. Note the density of the dark segments. (mag. 90,090X)
Figure 31. Polypeptides of the purified and differentially solubilized segments of the inclusion of *B. thuringiensis* subspecies *israelensis*. The purified inclusions were dissolved in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl, pH 9.2. The solution was centrifuged at 13,000xg for 5 min. and approximately 50 μg loaded onto the gel. The residual pellet was resuspended and dissolved at pH 10.5 in the SDS-DTE solvent as as above. The protein was loaded onto a 12.5% SDS-polyacrylamide gel and electrophoresed for 3 h. at 25 ma. Lane B shows the soluble protein at pH 10.5 which corresponds to the dark segment. Lane A shows the soluble protein at pH 9.2 which corresponds to the light segment.
Figure 32. Polypeptides of the whole inclusion of *B. thuringiensis* subspecies *israelensis* dissolved in 2 M KSCN.

Lane A, 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl, pH 9.2; lane B, 1% SDS, 2 M KSCN; and lane C, 2 M KSCN. Approximately 70 μg of protein was loaded onto a 12.5% polyacrylamide gel and electrophoresed at 25 mA. for 3 h. The gel was stained with 0.1% Coomassie Blue.
3.3 Characterization of the Inclusion of \textit{B. cereus} subspecies \textit{medusa}

3.3.1 Structure

The inclusion of \textit{B. cereus} subspecies \textit{medusa} was an ovoidal to spherical inclusion (Fig. 34) which was formed during the later stages of vegetative growth (Fig. 33). Unlike the other inclusion formers, the inclusion was partially sporulation dependent. The outer fibrous skin (Fig. 35), although deposited later in sporulation, (stage VI) is dependent on the cell achieving stage III (Hendry, et al., 1976). Thus the inclusion will be larger and skinless when grown in non-sporulating media (Fig. 33).

The skin appeared in some sections as loose lamellar layers around the inclusion crystal (Fig. 35). The crystal appeared more electron dense than the skin and in many sections it was difficult to visualize the crystalline lattice (Fig. 35). However, the lattice was seen quite clearly in surface replicas (Fig. 37). The centre to centre spacing was 27.5 nm with each row in the lattice being about 14.3 nm wide. The interstriate space was about 4.2 nm. As in the other inclusions described above, each striae contained subunits. The subunit rods were perpendicular to the axis of the lattice. This subunit arrangement was similar to the lattice of \textit{B. thuringiensis} subspecies \textit{kurstaki} but dissimilar to the lattice of the ovoidal inclusion of \textit{B. thuringiensis} subspecies \textit{israelensis} (Fig. 36). Each subunit was approximately 3.4 nm wide with a
space of 2.5 nm in between them. The centre-to-centre spacing of the rodlets or subunits was 6.8 nm. The width of two of the subunits with the space in between included was 10.3 nm. This is slightly larger than the size of two subunits of \textit{B. thuringiensis} subspecies \textit{kurstaki}.

Virus-like particles and fibrils adhered to the skin when examined by negative staining and carbon replicas (Fig. 38 and 39). This confirmed the findings of Hendry et al., (1976). The virus-like particles were approximately 19.3 nm in diameter, whereas the fibrils were 9 nm in diameter with a periodicity of 7 nm (Fig. 38). Because the fibrils readily condense onto the skin and may form the skin structure (Hendry et al., 1976), they were best seen as they were laid down on the inclusion surface or following partial dissolution in 1% SDS, 0.05 DTE, 0.05 M Tris-HCl pH 9.2 (Fig. 38 and 39).

3.3.2 Solubility

Table IV shows the effects of various chaotrophic agents on the inclusion of \textit{B. cereus} subspecies \textit{medusa}. There appeared to be three phases of dissolution of the inclusion as seen in the-phase contrast microscope, depending on the type of chaotrophic agent and the pH of the solution. The first phase of dissolution was a rupturing of the skin of the ovoid inclusion. This was achieved by using either the denaturant, SDS, or the chelating agent, EDTA. After 48 hr. in 5% SDS, the crystalline inclusion was dismantled leaving behind what appeared, to be a residual skin (Fig. 40a,b).
Complete dissolution of the skin was accomplished by boiling the sample at pH 10.5 for 5 minutes. Eighty-five to ninety percent of the total labelled protein was soluble at pH 9.5 when the sample was boiled. At pH 10.5, a further increase of 10 to 15% in total labelled protein was soluble. This increase in labelled soluble protein did not occur unless the sample was boiled for 5 minutes (Fig. 42). Incubation of the inclusion in 0.01 M EDTA ruptured the skin but did not appear to dissolve the inside crystal as did the denaturant. There is a possibility of osmolarity of the solution affecting the dissolution, but this is unlikely since differing concentrations of EDTA had the same effect.

The rate of crystal dispersal as observed by phase contrast microscopy was linear, depending on the concentration of SDS (Fig. 41).

Reducing agents such as B-mercaptoethanol and dithiothreitol were ineffective in either disrupting the skin or dismantling the inclusion at pH 7.5 to 9.5. The addition of 2% SDS to 1% B-mercaptoethanol had little immediate effect on inclusion solubility in the pH range of 7.5-9.0. Above pH 9.0, at room temperature, the protein began noticeable dissolution by both phase contrast microscopy and $^{14}$C-amino acid label analysis. At pH 10.5, the inclusions were completely dissolved. Thus, at pH 9.2, the crystal dissolved leaving a residual skin. At pH 10.5 in the same solvent, the skin began to dissolve when the suspension of inclusions in 1% SDS, 0.05 M DTE, 0.05M Tris-
HCl was heated to 100°C for 5 minutes, the total amount of soluble protein increased by about 30 percent to 40 percent over the unheated solvent system (Fig. 42). Time and temperature are not critical for dissolution but rather affect the rate and efficiency of the dissolving system. This differential solubility was used to separate the two fractions, the skin and crystal protein (Fig. 40a,b).

3.3.3 Polyacrylamide Gel Electrophoresis

The inclusion was found to contain two major components, the crystalline ovoid mass surrounded by a dense fibrous skin. The crystal was composed of a 135,000 dalton protein along with a minor component at 120,000 daltons. The skin was composed of a major protein of 40,000 dalton (Fig. 44) as well as minor 68,000 dalton protein. Both these skin proteins can be seen as major proteins in the sporulating lysate of the cell along with numerous other cell and spore products (Fig. 43). The 68,000 dalton protein was observed using B-mercaptoethanol as a reducing agent in the solvent but when dithiothreitol was used, the 68,000 dalton protein was not observed (Compare Figures 44 and 45). However, the 40,000 dalton protein of the skin was seen when solvents with either reducing agents were used. Thus, the 40,000 dalton protein probably represents the major protein of the fibrous skin. When the dissolved skin fraction was heated to 100°C for 5 minutes, the 40,000 dalton polypeptide became a 20,000 dalton polypeptide, a possible monomer of the 40,000 dalton protein (Fig. 45).
Inclusions that were formed during sporulation were compared to those that were formed in a non-sporulating cell. In both cases, figure 46 shows a 135,000 dalton protein as the major protein in the crystalline ovoid mass. The inclusion formed during sporulation contained a 40,000 dalton skin protein. This inclusion also contained several other proteins, mostly minor ones, that were not seen in the inclusion formed in the asporogenous cell. The 120,000 dalton protein was more prominent in the inclusion of asporogenous cell than in the inclusion produced by the sporulating cells (Fig. 46). The sporogenous cell produced an inclusion which contained a 70,000 and a 90,000 dalton protein not seen in the inclusion of the asporogenous cell. Both asporogenous and sporogenous cells produced inclusions which contained a 230,000 dalton protein as well as the 135,000 dalton protein. The inclusion of the sporogenous cell also contained a 200,000 dalton protein, which was not evident in the asporogenous inclusion. Therefore, it is evident that sporulation does influence the composition of the inclusion.

3.3.4 Carbohydrate Analysis

Inclusions composed of the skin and crystal contained 9.8% hexoses, as determined by the anthrone reaction. The purified crystal, however, contained 4.9% hexoses (both expressed as percent of protein). Since the skin accounts for 15% of the total inclusion protein (Fig. 42), one can calculate that the skin must contain 36.6% hexoses.
The inclusion of B. thuringiensis subspecies kurstaki contained only 3.9% hexoses and the inclusion of B. thuringiensis subspecies israelensis contains 12.9% hexoses (Tyrell et al., 1981). Thus, ovoid inclusions contain substantially larger amounts of hexoses than octrahedral inclusions. Furthermore, in the case of B. cereus subspecies medusa most of the carbohydrates are concentrated in the skin.

3.3.5 Ion Exchange Chromatography

Ion-exchange chromatography of the inclusion extracted at pH 10.5 in the SDS-DTT solvent produced four protein species, which separated well (Fig 47). When the crystal was extracted differentially from the total inclusion, it gave rise to fractions 2, 3 and 4. When the residual skin was dissolved at pH 9.2, it gave rise to the more basic fraction 1. It would appear that the skin is composed of three proteins which are more acidic than the skin protein. There is the possibility, however, that the two minor fractions (fractions 3 and 4) represent proteins that are derivatives of the major fraction fraction. These may be unreduced forms of the major 135,000 dalton protein or these fractions may be the major 135,000 dalton protein with a varying amount of carbohydrates.

3.3.6 Amino Acid Analysis

The amino acid composition of the whole inclusion (skin and crystal) was found to be acidic with the aspartic and glutamic acid residues being 25.3% of the total residues.
The methionine residue was almost 2% of the total (Table V). This is greater than the amount of methionine found in *B. thuringiensis* subspecies *kurstaki*.

Inclusions formed in non-sporulating cells had an amino acid composition similar to that of the inclusions from sporulating cells, with the exception that no sulphur residues could not be found in the inclusion of the asporogenous cell. This is consistent with the results of Aronson and Fitz-James, (1976), which showed that during sporulation the proteins of the spore coat were modified by a disulphide exchange. It is possible that this system also modifies the protein of the inclusion.

The crystal protein which was dissolved at pH 9.2 in SDS-DTE contained different residues than either the whole inclusion or the skin, both extracted at pH 10.5 in SDS-DTE. The more soluble crystal protein contained substantially less neutral amino acids and more basic amino acids than the whole inclusion. The more soluble, (pH 9.2), crystal protein also contained 2.3% methionine, whereas the skin (pH 10.5) contained 1.3% methionine.

The skin contained less acidic amino acid residues than the crystal suggesting that the proteins of the skin and crystal are different.

3.3.7 Limited Proteolysis

Partial proteolysis of the crystal and skin proteins (with *S. aureus* V-8 protease) revealed virtually no homology between the two proteins (Fig. 48). The crystal protein was
digested into two major peptide fragments (21,000 and 33,000 daltons) with many minor fragments (ranging in molecular weights between 33,000 and 16,000 daltons). The skin, however, was digested into five fragments, two of which were common with the crystal major peptide fragments. It is probable that these two peptides in the skin preparation are due to contamination.

It is interesting that a common profile was generated from the crystal protein of *B. thuringiensis* subspecies *kurstaki* and *B. cereus* subspecies *medusa*. Both crystal proteins were digested into three major peptides of approximately 30,000, 21,000 and 16,000 daltons. The 16,000 dalton protein was rather heterogeneous and produced a smear on the polyacrylamide gels.
### TABLE IV

Solubility of the Inclusion of *B. cereus* subspecies medusa

<table>
<thead>
<tr>
<th>Dissociation Buffer</th>
<th>pH</th>
<th>Temp.</th>
<th>Time</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% SDS in H₂O</td>
<td>8.5</td>
<td>21°C</td>
<td>24 hr.</td>
<td>approx. 50% ruptured</td>
</tr>
<tr>
<td>0.2 M β mercaptoethanol</td>
<td></td>
<td>21°C</td>
<td>72 hr.</td>
<td>no effect</td>
</tr>
<tr>
<td>0.1 M EDTA in H₂O</td>
<td>10.8</td>
<td>21°C</td>
<td>12 hr.</td>
<td>approx. 50% ruptured</td>
</tr>
<tr>
<td>2.5% SDS, 0.1 M β mercaptoethanol</td>
<td>7.6</td>
<td>21°C</td>
<td>10 min.</td>
<td>no effect</td>
</tr>
<tr>
<td>0.05 M Tris</td>
<td>9.8</td>
<td>21°C</td>
<td>10 min.</td>
<td>ruptured</td>
</tr>
<tr>
<td>0.05 M Tris</td>
<td>11.0</td>
<td>21°C</td>
<td>10 min.</td>
<td>100% dissolution some skin residue</td>
</tr>
<tr>
<td>0.05 M Tris</td>
<td>12.2</td>
<td>21°C</td>
<td>10 min.</td>
<td>100% dissolution of inclusion and skin</td>
</tr>
<tr>
<td>0.05 M Tris</td>
<td>7.6</td>
<td>21°C</td>
<td>1 hr.</td>
<td>ruptured</td>
</tr>
<tr>
<td>0.05 M Tris</td>
<td>9.8</td>
<td>21°C</td>
<td>1 hr.</td>
<td>100% dissolution, 70% skins dissolved</td>
</tr>
<tr>
<td>0.05 M Tris</td>
<td>7.6</td>
<td>37°C</td>
<td>10 min.</td>
<td>no effect</td>
</tr>
<tr>
<td>0.05 M Tris</td>
<td>9.2</td>
<td>37°C</td>
<td>10 min.</td>
<td>100% dissolved inclusions, skins remain</td>
</tr>
<tr>
<td>0.05 M Tris</td>
<td>10.5</td>
<td>37°C</td>
<td>10 min.</td>
<td>100% dissolved</td>
</tr>
</tbody>
</table>
TABLE V

* Inclusion type and preparation

A. Purified whole inclusions from the lysate of a sporulating culture of *B. cereus* subspecies *medusa* 9B (wt).

B. Purified inclusions from a non-sporulating culture of *B. cereus* subspecies *medusa* 9B lysed in lysozyme (10 mg/ml) and 5% SDS

C. Purified crystals prepared from the lysate of a sporulating culture of *B. cereus* subspecies *medusa* 9B. The inclusions were dissolved in 2% SDS, 0.20 M DTE, pH 9.2 at 37°C for 20 min. The resulting supernatant after centrifugation at 12,000 RPM for 2 min. was analyzed.

D. Purified skins prepared from the pellet in C. The pellet was subjected to a further two extractions as above and washed twice in distilled water.

E. Purified core crystals from the lysate of a sporulating culture of *B. cereus* subspecies *medusa* L08 (Bc).
TABLE V

Amino Acid Composition of the Inclusion of *B. cereus*
subspecies *medusa*

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Inclusion type and preparation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% moles</td>
</tr>
<tr>
<td>glycine</td>
<td>7.3</td>
</tr>
<tr>
<td>alanine</td>
<td>5.0</td>
</tr>
<tr>
<td>valine</td>
<td>5.8</td>
</tr>
<tr>
<td>isoleucine</td>
<td>5.9</td>
</tr>
<tr>
<td>leucine</td>
<td>9.2</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>4.2</td>
</tr>
<tr>
<td>proline</td>
<td>3.7</td>
</tr>
<tr>
<td>Total neutral</td>
<td>41.1</td>
</tr>
<tr>
<td>serine</td>
<td>9.1</td>
</tr>
<tr>
<td>threonine</td>
<td>7.7</td>
</tr>
<tr>
<td>tyrosine</td>
<td>5.7</td>
</tr>
<tr>
<td>Total &quot;OH&quot;</td>
<td>22.5</td>
</tr>
<tr>
<td>aspartic</td>
<td>14.5</td>
</tr>
<tr>
<td>glutamic</td>
<td>10.8</td>
</tr>
<tr>
<td>Total acidic</td>
<td>25.3</td>
</tr>
<tr>
<td>lysine</td>
<td>4.7</td>
</tr>
<tr>
<td>arginine</td>
<td>4.0</td>
</tr>
<tr>
<td>histidine</td>
<td>0.4</td>
</tr>
<tr>
<td>Total basic</td>
<td>9.1</td>
</tr>
<tr>
<td>cysteine**</td>
<td>0.0</td>
</tr>
<tr>
<td>methionine</td>
<td>1.9</td>
</tr>
<tr>
<td>Total sulphur</td>
<td>1.9</td>
</tr>
</tbody>
</table>

** Cysteine and tryptophan were not determined.
Figure 33. A light micrograph of non-sporulating cells of *B. cereus* subspecies *medusa* grown on non-sporulating (BBM) media. The inclusion becomes larger than that of the wild type. (mag. 2500X)

Figure 34. A light micrograph of purified inclusions from a renograffin gradient as described in Methods and Materials. The purity is approximately 100% after two cycles of centrifugation. (mag. 2000X)

Figure 35. An electron micrograph of sporulated cells of *B. cereus* subspecies *medusa* showing the fibrous skin of the inclusion. Note the size, shape and density of the inclusion. (mag. 48,026X)
Figure 36. Illustration of the macromolecular structure of the crystal of *B. cereus* subspecies *medusa*. All dimensions are given in nanometers.
Figure 37. A carbon replica electron micrograph of a purified inclusion of \textit{B. cereus} subspecies \textit{medusa} without the outer fibrous skin. The striae are visible along with the subunits (S) which form the crystal striae. (mag. 118,800X)
Figure 38. Carbon replica electron micrograph of the purified skins of the inclusion of *B. cereus* subspecies *medusa*. The inner crystal has been dismantled in 10% SDS for 2 h. The striated fibers can be seen on the surface of the skin. (mag. 56,890X)
Figure 39. An electron micrograph of a 2% PTA negative stained skin fragment of the inclusion of *B. cereus* subspecies *medusa* which has been partially dismantled in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl, pH 9.2. The skin fragment is composed of three layers; a homogeneous layer, a hexagonal arrayed layer, and fibers and virus-like particles adhered to the hexagonal layer. The hexagonal layer may be spore coat material, since the structure and dimensions of the hexagonal array are similar to those of the spore coat. (mag. 140,000X)
Figure 40a. A light micrograph of purified skins derived by differential solubility of the inclusion of *B. cereus* subspecies *medusa* in the SDS-DTE solvent at pH 9.2. (mag. 2000X)

Figure 40b. An electron micrograph of the above skin preparation slightly stained in 2% PTA. The skin appears as a ruptured "sac". (mag. 28,650X)
Figure 41. The effect of concentration of SDS on the dissolution of the inclusions of *B. cereus* subspecies *medusa*. The skin apparently ruptured allowing the crystal to be dismantled.

- • represents 10% SDS
- ▲ represents 20% SDS

The inclusions were suspended in 10% to 20% SDS and observed by phase contrast microscopy.
Figure 42. The effect of pH and temperature on the dissolution of purified inclusions of B. cereus subspecies medusa. The inclusions were suspended in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl and the pH adjusted with 0.1 N NaOH in increments of 0.5 pH units. The solubility was observed by phase contrast microscopy and isotopic labelling with C\textsuperscript{14} amino acid. The dissolution was repeated followed by heating the sample at 100°C, for 5 min. The suspension was centrifuged at 13,000xg for 5 min. and the soluble labelled protein counted. The counts were normalized to the total insoluble protein at pH 7.0.

- O represents dissolution without heat
- ■ represents dissolution with 100°C for 5 min.
Figure 43. Polypeptides of the crude sporulate of B. cereus subspecies medusa. The crude sporulate, lane B, was dissolved in 1% SDS, 2% β-mercaptoethanol, 0.05 M Tris-HCl, pH 8.5. The purified inclusion was dissolved in the same buffer (lane A). The soluble protein of the inclusion was centrifuged at 13,000xg for 5 min. and dissolved as before (lane C). The samples (50 µg) were applied to an SDS-PAGE and electrophoresed at 25 ma. for 3 h. The gel was stained in 0.1% Coomassie Blue and destained as per the Methods and Materials.
Figure 44. Polypeptides of the skin and crystal of the inclusion of *B. cereus* subspecies *medusa*.

Purified inclusions were dissolved in 1% SDS, 2% β-mercaptoethanol, 0.05 M Tris-HCl, pH 9.2 and centrifuged at 13,000xg for 5 min. The pellet was resuspended and dissolved in the same buffer at pH 10.5. The samples were electrophoresed on an SDS-PAGE at 25 mA. for 3 h. The gel was stained in 0.1% Coomassie Blue and destained as per the Methods and Materials. Lane A contains the soluble protein dissolved at pH 9.2 and lane B contains the soluble protein dissolved at pH 10.5.
Figure 45. Polypeptides of the skin and crystal of the inclusion of \textit{B. cereus} subspecies \textit{medusa}.

Purified inclusions were dissolved in 1% SDS, 0.05 M DTE, pH 9.2 and centrifuged at 13,000xg for 5 min. The pellet was resuspended and dissolved in the same solvent at pH 10.5.

The samples (50 µg) were electrophoresed on an SDS-PAGE at 25 mA for 3 h. The gel was stained in 0.1% Coomassie Blue as per the Methods and Materials. Lane D contains the soluble protein dissolved at pH 9.2 which corresponds to the crystal, lane C contains the soluble protein dissolved at pH 10.5 which corresponds to the skin and residual crystal protein and lane B contains the soluble protein dissolved at pH 10.5 and heated at 100°C for 5-10 min.
Figure 46. An SDS-PAGE of the purified inclusions extracted from sporulating and non-sporulating cells of *B. cereus* subspecies *medusa*. The inclusions were dissolved in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl, pH 9.2. The soluble protein was centrifuged at 13,000xg for 5 min. and the pellet dissolved at pH 10.5 in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl. Lane A, pH 10.5, sporulation inclusion; lane B, pH 10.5, non-sporulation inclusion; lane C, pH 9.2, sporulation inclusion and lane D, pH 9.2, non-sporulation inclusion.
Figure 47. DEAE-cellulose ion-exchange chromatography of the inclusion of B. cereus subspecies medusa. Purified whole inclusions \( \bullet \), and crystal \( \Delta \), were dissolved in 6 M urea, 0.05 M DTE pH 11.0. The samples were boiled for 5 min, then dialyzed against 0.5 M pH 7.5 Tris-HCl, 6 M urea for 48 h. The protein was carboxymethylated with 0.2 M iodoacetic acid and dialyzed again. Protein (3 to 5 mg) was loaded on the column and run for 6 h. Fractions were collected and analyzed as per Methods and Materials. A 0.0 to 0.4 M NaCl gradient was used as the elution buffer.
Figure 48. Limited proteolysis of purified crystal and skin of *B. cereus* subspecies *medusa* by *S. aureus* V-8 protease. The purified proteins were incubated with V-8 protease at 37°C for 8 h. These were electrophoresed on a 20% SDS-PAGE at 25 ma. for 3 h. and stained with Coomassie Blue as per the Methods and Materials. Lane B contains the skin protein, lane D contains the molecular weight standards, ovalbumin and trypsin inhibitor, lane C contains the crystal protein, lane F contains the bead protein of subspecies *kurstaki*, and lane E contains the crystal protein of subspecies *kurstaki*. 
3.4 Characterization of the Morphological Variants of B. cereus medusa

3.4.1 Structure

Two morphological variants of B. cereus subspecies medusa were obtained from either curing experiments or by spontaneous mutation. The first variant, L07, forms a bipartite donut-shaped inclusion (Fig. 49b) that was isolated spontaneously by Dr. P.C. Fitz-James in this laboratory in 1961. It was briefly reported in the description of the virus-like particles found in B. cereus subspecies medusa, (Hendry, Gillespie and Fitz-James, 1976). The second variant, L08-42, formed a cubic core crystal which appeared to be the initial stage of inclusion formation during the early stages of sporulation (Fig. 49c, 50). This variant was isolated by growth of wild-type cells at 42°C. Other strains forming the core crystal were isolated spontaneously (Table VI).

The "donut" inclusion consisted of a bipartite enlarged annular ring surrounding an amorphous medullary area, which in turn surrounded a cubic inner core crystal (Fig. 50b). The overall size of this aberrant inclusion was approximately the same as that of the wild-type. The crystalline inner core was approximately one-ninth the size of the entire "donut" inclusion. The outer lamellar toroid portion of the "donut" consists of an amorphous granular substance, which may be uncrystallized protein, a precursor to that which forms the core. Surrounding this material in
which the core was embedded was a thick fibrous skin somewhat similar to that seen in the wild-type inclusion (Fig. 50a,b). The amorphous granular material was less dense than either the skin or the core crystal. The skin seemed to be thicker than that of the wild-type inclusion. Abnormal variations of the "donut" inclusion appeared during the study. In figures 51a and b, a further variation of the inclusion, referred to as "disorganized donut" (Gillespie, 1969; Hendry et al., 1976) was seen. Here, the core crystal of the inclusion developed separately from the skin and amorphous substance. The core crystal was located on the opposite side of the spore. In figure 51b, the fibrous nature of the skin was very evident with no granular matrix appearing within it. Infrequently, a cell could be observed with no apparent core crystal (Fig. c). However, this may be due to the section not traversing through the core crystal. In other variations, the core crystal was deposited to one side of the inclusion between the granular matrix and the thick outer skin (Fig. 52). Occasionally, an over abundance of skin appeared as whirls surrounding the inclusion. Beneath the skin layer were several layers of fibrous membranes surrounding the granular matrix and the core inclusion (Fig. 52).

The "core" crystal variant, L08-42, formed a small cubic inclusion equivalent in size and shape to the core crystal of the "donut" inclusion. It contained a crystalline substructure similar to that observed in the
wild-type inclusion. The striae of both crystals were identical, with a width of 3.4 nm and a centre to centre spacing of 27.5 nm (Fig. 53). In thin sections, the core crystal subunit array was clearly evident (Fig. 54a, b, c). At high magnification, the matrix appeared to be made of spheres spaced 6.5 nm apart, equidistantly. The core crystal contrary to the ovoid wild-type inclusion has a distinct cuboidal shape.

As expected from the early origin of B. cereus subspecies medusa inclusions, the formation of this early core crystal is independent of sporulation (Fig. 54a). An example of an asporenous isolate of the core crystal variant that is frequently isolated spontaneously from the wild-type or from L07, donut, is shown in figure 54.

3.4.2 Solubility

The "donut" and "core" inclusions exhibited solubilities similar to that of their parent "9B" inclusion, when suspended in 1% SDS, 0.05 M dithiothreitol over the range of pH from 9.2 to 11.0. However, unlike the inclusion of wild-type, the "donut" inclusion suspended in 1% SDS, 0.05 DTE, 0.05M Tris HCl, pH 9.2 did not rupture (Fig. 55a). Rather, when observed in the phase contrast microscope, at pH 9.8 in SDS-DTE, the inclusion became faint and the inner core crystal dissolved, leaving a ruptured thick outer skin (Fig. 55b). At pH 10.5 in SDS-DTE, the thickness of skin was reduced, leaving a skin similar to the skin of the wild-type inclusion (Fig. 55b). Incubation at 37°C for 30
minutes a pH 10.5, pH followed by heating at 100°C for 5 to 10 minutes dissolved the thin outer skin. The skins could also be dissolved by elevating the pH to 11.0 and incubating at 37°C for 1 hour (Fig. 55c).

The core crystal inclusion of variant L08-42 was less soluble than either the wild-type inclusion or the inner core crystal of the "donut" inclusion. It did not dissolve in any of the chaotropic agents listed in Table IV. However, in 5% SDS, it was partially dismantled but did not dissolve (Fig. 56a,b). The extent to which these small core crystal inclusions dismantled was a function of time and temperature of incubation. Complete dissolution of the L08-42 core crystals was achieved by suspension of the crystals in 1% SDS 0.05M DTE, 0.05M Tris-HCl, pH 10.5 incubated at 37°C for 15 min. followed by incubation at 100°C for 5 to 10 min. Approximately 90% of the inclusions were dissolved.

3.4.3 Polyacrylamide gel Electrophoresis

The L08-42 core crystal, when dissolved, was composed of two proteins, 135,000 and 130,000 daltons (Fig. 58). These doublet proteins were not seen in the wild-type inclusion. Possibly, the intensity and amount of protein loaded onto the gel masked the visualization of the doublet bands. The core crystal inclusion, like that of wild-type also contained a protein of about 230,000 daltons suspected of being a dimer of the 135,000 dalton protein.
The bead-like inclusion harvested from vegetative cells of wild-type, prior to the onset of sporulation contained several major proteins, the most prominent one having a molecular weight of 90,000 daltons. There was a slight amount of the 135,000 dalton protein present, but it represented a minor component. Possibly, these polypeptides compose the immature core crystal in the initial stages of development (Fig. 58).

Initially, the "donut" inclusion appeared to be compared of the same major protein (135,000 daltons) as the wild-type inclusion when dissolved in 1% SDS, 2% B mercaptoethanol, 0.05M Tris-HCl, pH 7.5 (Fig. 57). However, as the effects of various solvents and alkalinity were investigated, it became evident that these inclusions were not composed of a single polypeptide (Fig. 59 and 60).

The L07 "donut" inclusion contained proteins which corresponded to the three structural parts of the inclusion described above. The crystalline core and amorphous granular substance, both of which dissolved at pH 9.8 in SDS-DTE. The lamellar fibrous mass which dissolved at pH 10.5 surround by and attached to the outer skin. The thin outer skin which dissolved at pH 11.0 (Fig. 59 and 60). The 1% SDS, 0.05M DTE, 0.05M Tris-HCl, pH 9.8 extract contained the major 130,000 and 135,000 dalton protein doublet, as well as three other major proteins (120,000, 57,000, and 49,000 daltons). Various minor proteins were also visible in the crystal extract (Fig. 59 and 60).
The 1% SDS, 0.05M DTE, 0.05M Tris-HCl, pH 10.5 extract contained two major proteins (43,000 and 120,000 daltons) when the sample was not subjected to 100°C for 5 to 10 minutes and three proteins when it was boiled. When the sample was heated to 100°C, the 40,000 dalton protein was partially reduced to 20,000 daltons. A similar reduction in molecular weight of polypeptides occurred when the skin extract of the wild-type inclusion was heated to 100°C (Fig. 60).

The 1% SDS, 0.05M DTE, 0.05 M Tris-HCl, pH 11.0 extract of the thin skin contained two proteins which were also further denatured by boiling. The expected 40,000 dalton protein of the skin was evident, which became a 20,000 dalton protein when boiled. Surprisingly, the 135,000 dalton protein was also present and was unaffected by heating. The multiplicity of the molecular weights of these proteins suggest that the 20,000 dalton monomer forms a 40,000 dalton dimer, which is reduced by boiling (Fig. 59, 60).

The smearing on the gel of the small molecular weight protein (20,000 daltons) found in the sample dissolved at pH 10.5 and heated to 100°C may be a result of incomplete dissolution of the skin protein (Fig. 60, 61).
Figure 49a. A light micrograph of the sporulating cell of *B. cereus* subspecies *medusa* 9B, wild type, grown on GGBM for 18 h. revealing the spore and ovoid inclusion. The cells of subspecies *medusa* form long filamentous chains.

Figure 49b. A light micrograph of the sporulating cell of *B. cereus* subspecies *medusa* L07 "donut" variant grown on GGBM for 18 h. The inclusion consists of a bipartite lamellar outer toroid with a refractile body in the center of the inclusion.

Figure 49c. A light micrograph of the sporulating cell of *B. cereus* subspecies *medusa* L08-42 "core crystal" variant grown as above. The inclusion is a small refractile cube. (mag. 5000X)
Figure 50a to c. Electron micrographs of cells of the variants shown in Figure 49. Note the density of the wild-type inclusion as compared to the density of the D07 "donut" and L08-42 "core crystal" inclusions. The thickness of the skin of "donut" is greater than the skin of the wild-type inclusion. The "core crystal" is associated with some membranous material. (I) inclusion, (S) spore, (CI) "core crystal". (mag. 52,400X) Figure 50a (mag. 41,040X) Figure 50b (mag. 33,120X) Figure 50c
Figure 51 a to c. Electron micrographs of thin sections of sporulating cells of the L07 "donut" variant showing aberrant inclusion development.

Figure (a) The core crystal is placed outside of the skin and medullary area of the inclusion.

Figure (b) The same variation in inclusion morphology is seen along with the extremely fibrous nature of the skin. Also, the medullary proteins are not present.

Figure (c) An infrequent variation in the inclusion morphology is shown. The core crystal is completely absent, leaving only the skin and medullary proteins of the inclusion in the cell. Note the granular appearance of the medullary area.

(mag. 41,040X) Figures (a) and (b)
(mag. 40,870X) Figure (c)
Figure 52 a and b. Electron micrographs of thin sections of sporulating cells of *B. cereus* subspecies *medusa* LO7 again showing the morphological variation in the structure of the inclusion. The inclusion in (a) formed the "core crystal" (C) outside of the medullary area, between the skin and the medullary matrix (large arrow). The matrix appears to be surrounded by a thin membrane (small arrow). The thick skin (arrowhead) is seen separated from the fibers and membrane. Likewise, in Figure (b), the skin appears to be over produced. The fibers are associated with the side of the thick skin (arrow).

(mag. 86,250X) Figures (a) and (b)
Figure 53 a and b. Carpsoplica electron micrographs of the wild-type inclusion of *B. cereus* subspecies medusa (a) and the "core crystal" inclusion (b) of L08-42. Note the shape change from ovoid to cuboidal in the variant L08-42. The striae of both types of inclusion are the same distance apart, 27.5 nm.

(mag. 36,300X) Figure 53 a
(mag. 54,545X) Figure 53 b
Figure 54 a to c. Electron micrographs of thin sections of cells of an asporogenous mutant of L08-42, (a), and the "core crystal", (b) and (c). The "core crystal" is surrounded by fibrous granular material and contained a lattice spacing similar to that of the wild-type inclusion. The lattice spacing is 9.0 nm, center to center.

(mag. 25,920X) Figure 54 a
(mag. 83,160X) Figure 54 b
(mag. 100,000X) Figure 54 c
Figure 55. Phase contrast microscopy of the purified inclusion of L07 variant showing the effects of pH of the solvent (1% SDS, 0.05 M DTE, 0.05 M Tris-HCl) on the solubility of the inclusion. Figure (a) The inclusions suspended in 0.05 M Tris-HCl. Figure (b) The inclusions partially dissolved in 1% SDS, 0.05 M DTE, pH 9.8. Figure (c) The inclusions dissolved in the same buffer at pH 10.5. Figure (d) The inclusions dissolved in the same buffer at pH 9.2. (mag. 2500X) Figure 55 (a) and (b) (mag. 4000X) Figure 55 (c) and (d)
Figure 56 a and b. An electron micrograph of the purified "core crystal" suspended in 5% SDS followed by negative staining with 2% PTA. The inclusion has been partially dissolved with the crystalline lattice disrupted. It appears that the globular proteins are in the process of dispersing from the edge of the crystal.

(mag. 138,350X) Figure 56 a
(mag. 183,250X) Figure 56 b
Figure 57. Polypeptides of the purified inclusions of B. cereus subspecies medusa 9B, L07 "donut" and L08-42 "core crystal" dissolved in 1% SDS, 2% β-mercaptoethanol, 0.05 M Tris-HCl, pH 7.5. Lane A, 9B; lane B, L08-42 and lane C; L07. Soluble proteins were electrophoresed on a 12.5% polyacrylamide gel for 3 h. at 25 ma. and stained with 0.1% Coomassie Blue and destained as per the Method and Materials.
Figure 58. Polypeptides of the inclusions of wild-type, gel B, "core crystal" and bead inclusions dissolved in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl at pH 9.2 and pH 11.0. Lanes 1 and 2 of gel A are the dissolved proteins of the "core crystal" of L08-42 and the inclusion of 9B respectively. The purified inclusions were dissolved at pH 11.0 and heated at 100°C for 5 min. Lanes 1 and 2 of gel B contain the soluble protein of wild-type inclusions dissolved at pH 9.2. Lane 3 contains the soluble protein of immature bead-like inclusions extracted from the cell, purified by Renografin-76 density centrifugation and dissolved at pH 9.2 in the same solvent as above.
Figure 59. Polypeptides of the purified inclusion of L07 "donut" inclusion dissolved at increasing pH in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl. Lanes 1, 2 and 3 of Figure B represent dissolution of "donut" at pH 9.8 in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl. Lanes 1, 2 and 3 of Figure A contain soluble proteins in the dissociation buffer as above and heated to 100°C for 5 min. Soluble proteins were electrophoresed on a 12.5% polyacrylamide gel at 25 mA for 3 h. and stained in 0.1% Coomassie Blue and destained as per the Methods and Materials.
Figure 24. Polypeptides of the spore coat of *B. thuringiensis* subspecies *kurstaki*. The spores were extracted in 1% SDS, 0.05 M DTE in 0.05 M Tris-HCl, pH 9.8 with and without 6 M urea. Lane A is the 6 M urea and lane B is without 6 M urea. Approximately 50 μg of protein were loaded onto a 12.5% SDS-polyacrylamide gel and electrophoresed at 25 ma for 3 h. The gel was stained and destained as per the Methods and Materials.
Figure 60. Polypeptides of the purified inclusion of L07 "donut" variant extracted and electrophoresed as in Figure 59. Lanes 1 and 2 of Figure B contain soluble protein extracted from the inclusions at pH 10.5 and heated to 100°C for 5 min. in dissociation buffer. Lanes 3 and 4 of Figure B contain soluble protein extracted from the inclusion in the dissociation buffer at pH 9.8 and heated to 100°C for 5 min. Lane 1 contains soluble protein extracted from the inclusion at pH 11.0 and not heated. Lanes 2 and 3 of Figure A contain soluble protein extracted from the inclusion at pH 10.5 and not heated as above. Lane 5 contains the protein markers as outlined in the Methods and Materials.
Figure 61. Glycoproteins of the inclusions of L07 "donut" variant extracted in dissociation buffer and electrophoresed as in Figures 59 and 60. Lanes 1 and 2 contained soluble protein extracted at pH 9.8. Lanes 3, 4 and 5 contained soluble protein extracted from the inclusions at pH 10.5 in dissociation buffer, as above. The protein was electrophoresed in a 12.5% poly-acrylamide gel at 25 ma. for 3 h. and stained with periodic acid-Schiff reagent as per the Methods and Materials.
3.5 Comparison of the Inclusions of Several Subspecies of *B. thuringiensis*

Inclusions of *B. thuringiensis* subspecies *alesti*, subspecies *finitimus*, subspecies *schwetzova* and *B. cereus* subspecies *lewinia* were purified, characterized and compared to each other and to the inclusions of *B. thuringiensis* subspecies *kurstaki*, subspecies *israelensis* and *B. cereus* subspecies *medusa* already studied in detail (Fig. 68).

*B. thuringiensis* subspecies *alesti* and subspecies *finitimus* formed a bipyramidal crystal with no associated inclusion (Fig. 62c,64). The inclusions of *B. thuringiensis* subspecies *alesti* dissolved completely at pH 8.0 in 1% SDS, 0.05M DTT in 0.05 M Tris-HCl whereas the inclusion of *B. thuringiensis* subspecies *finitimus* dissolved at pH 7.6 in the same solvent. The inclusion of *B. thuringiensis* subspecies *alesti* was composed of a single major protein of 135,000 daltons with a minor protein of 130,000 daltons (Fig. 66). The inclusion of *B. thuringiensis* subspecies *finitimus* contained eight proteins, three major and five minor. The inclusion lacked the 135,000 dalton protein but had major proteins of molecular weight 130,000, 116,000 and 107,000 daltons. The five minor proteins ranged in size from 23,000 to 107,000 daltons. These proteins except for the 130,000 dalton protein are unique to the inclusion of *B. thuringiensis* subspecies *finitimus*. The small molecular weight proteins, (23,000 dalton and 29,000 daltons) may be
components of viruses or fibres associated with the inclusion similar to the inclusion of *B. thuringiensis* subspecies *israelensis* (Fig. 62b).

*B. thuringiensis* subspecies *schwetzova* formed an ovoid inclusion associated with the crystal similar to that found in *B. thuringiensis* subspecies *kurstaki* (Fig. 65). The bead was associated with the bipyramidal crystal, less intensely stained (compared to the crystal), non-crystalline and the same shape and size as the bead found in *B. thuringiensis* subspecies *kurstaki*. As observed with the inclusion of *B. thuringiensis* subspecies *kurstaki*, the crystal of *B. thuringiensis* subspecies *schwetzova* dissolved at pH 8.5 whereas the bead dissolved at pH 9.5 in the SDS-DTT solvent. However, unlike the inclusion of *B. thuringiensis* subspecies *kurstaki*, the inclusion of *B. thuringiensis* subspecies *schwetzova* was surrounded by whirls of membrane-like material (Fig. 65). Frequently, the whirls of material seemed to connect to the spore, suggestive that the spore coat had continued to be synthesized filling the cell and surrounding the inclusion. This membrane-like material had more of an affinity for the bead (ovoid inclusion) than for the crystal.

The inclusion of *B. thuringiensis* subspecies *schwetzova* was composed of two major proteins, 135,000 and 68,000 daltons. A minor protein (130,000) was also present (Fig. 66). The protein composition of the inclusion of *B. thuringiensis* subspecies *schwetzova* is identical to that of
whole inclusions (crystal-bead) of *B. thuringiensis* subspecies *kurstaki* with the exception of two minor protein (105,000 and 110,000 daltons) which may comprise the membrane surrounding the inclusion.

*B. cereus* subspecies *lewinia* formed an ovoid to spherical inclusions similar to that found in *B. cereus* subspecies *medusa* (Fig. 62a). The same procedure used to separate the inclusion components *B. cereus* subspecies *medusa* was used for the inclusion of *B. cereus* subspecies *lewinia*. The inclusion was composed of a crystalline body which was soluble at pH 9.2 in SDS-DTE, surrounded by a skin which was soluble at pH 10.5 in SDS-DTE. However, unlike the inclusion of *B. cereus* subspecies *medusa*, the inclusion of *B. cereus* subspecies *lewinia* is not formed during late vegetative growth but is totally sporulation dependent.

At pH 9.2, SDS-DTE extracts of the inclusion of *B. cereus* subspecies *lewinia* revealed seven proteins by polyacrylamide gel electrophoresis. The 135,000 dalton protein was the major protein. Two other major proteins (138,000 and 130,000 daltons) accompanied by four minor proteins (121,000, 115,000, 107,000, 104,000 daltons) were also components of the crystalline inclusion (Fig. 66). The residual skin (obtained by dissolving the crystal at pH 9.2 in SDS-DTE) was washed several times in 0.9% NaCl and dissolved in 1% SDS, 0.05 M DTE, 0.05M Tris-HCl at pH 10.5. The skin was composed of a single 20,000 dalton protein similar to that of *B. cereus* subspecies *medusa*. Also, both
skins were extremely insoluble and required heating to 100°C for 5 minutes for complete dissolution (Fig. 66).
Figure 62. Phase contrast light micrographs of sporulating cells of B. cereus subspecies medusa (a), B. thuringiensis subspecies israelensis (b), and B. thuringiensis subspecies alesti (c). Compare the different shapes of the inclusions representing types I, III and IV (arrow). (mag 2000X)

Figure 63. An electron micrograph of a thin section of a sporulating cell of B. thuringiensis subspecies israelensis revealing the ovoid double component inclusion. (mag. 29,375X)

Figure 64. An electron micrograph of a thin section of a sporulating cell of B. thuringiensis subspecies alesti revealing the crystal without an associated bead or ovoid inclusion. (mag. 21,475X)
Figure 65. An electron micrograph of a thin section of a free inclusion of *B. thuringiensis* subspecies *schwetzova* (Type III). The crystal is attached to a non-crystal ovoid inclusion which is surrounded by whorls of membrane-like material. (mag. 121,428.)
Figure 66. Polypeptides of the various subspecies of _thuringiensis_ and _cereus_, _alesti_, _finitimus_, _lewinia_, _schwetzova_. The inclusions were dissolved in 1% SDS, 0.05 M DTE, 0.05 M Tris-HCl at the appropriate pH to dissolve the inclusion (as observed by phase contrast microscopy). Lanes A and B, _B. thuringiensis_ subspecies _alesti_ at pH 8.0; lane C, _B. thuringiensis_ subspecies _finitimus_ at pH 7.6; lane D, crystal of _B. cereus_ subspecies _lewinia_ at pH 9.2; lane E, skin of subspecies _lewinia_ at pH 10.5; lane F, inclusion of _B. thuringiensis_ subspecies _schwetzova_ at pH 8.5 including the crystal and bead; and lane G, V-8 protease limited proteolytic products of _B. thuringiensis_ subspecies _kurstaki_. The soluble protein was electrophoresed on a 12.5% SDS-PAGE for 3 h. at 25 ma. and stained with 1% Coomassie Blue and destained as per the Methods and Materials.
Figure 67. Comparison of the Structure and Composition of the inclusion of *B. thuringiensis* subspecies *kurstaki* and subspecies *israelensis* and *B. cereus* subspecies *medusa*. The drawing illustrates the subunit arrangement of two striae in the lattice of the crystal. The number given each dimension is the average of ten measurements.
Comparison of subunit structure of three subspecies

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<th>Y</th>
<th>Z</th>
<th>α</th>
<th>x</th>
<th>y</th>
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<td>7.0</td>
<td>3.5</td>
<td>135,000</td>
</tr>
<tr>
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<td>14.9</td>
<td>7.0</td>
<td>75°</td>
<td>5.6</td>
<td>4.0</td>
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<td>4.2</td>
<td>14.3</td>
<td>90°</td>
<td>6.8</td>
<td>3.4</td>
<td>135,000</td>
</tr>
</tbody>
</table>
3.6 Curing and Inhibition Studies

3.6.1 Growth of Variants of B. cereus subspecies medusa

*B. cereus* subspecies *medusa* is the only inclusion forming bacillus that was induced to form morphological variants of its inclusion. Although *B. thuringiensis* subspecies *kurstaki* was cured of the entire inclusion, at no time could *B. thuringiensis* subspecies *kurstaki* be induced to form a morphological variant of the inclusion.

Table VI tabulates the inclusions variants and several asporogenous and cell-shape variants isolated during this study. Two of the inclusion variants, L07 and L08-42 have been described above with respect to the structure and biochemical composition of their inclusion. Strains L09, L010 and L011-nut c formed inclusions, similar to the core crystal inclusion of L08-42 described above.

The two strains L013 and "nut-c" formed small bead-like inclusions approximately 0.2 μ in diameter. The bead was similar to the bead formed early during inclusion development and therefore, probably contains the same proteins. These variants are probable inclusion and spore mutants blocked at stage 0.

A comparison of the growth curves of wild-type and its variants in GBEM broth (Fig. 68) revealed that the core crystal variants grew slightly slower than the wild-type (at a maximum log-phase rate of approximately 0.45 OD units/hr. compared to 0.57 OD units/hr for wild-type). The bead variants L013 and L014 grew at a rate of 0.13 OD units/hr.,
almost half the rate of the wild-type. After 8 hr. of growth, the wild-type and the core crystal variants began to form the primordial bead inclusion. This was 2 to 3 hr. prior to the onset of sporulation. The bead variants, however, began their inclusion formation 1 to 2 hr. later (Fig. 68).

3.6.2 Acridine Orange and Ethidium Bromide Treatment

The addition of 50 µg/ml of either acridine orange or ethidium bromide to a growing culture of B. cereus subspecies medusa resulted in approximately 50% asporogeny. However, inclusion formation was not seemingly affected. The addition of either agent at a lesser concentration (25 µg/ml) had no effect except for a temporary decrease in growth immediately following the addition of the curing agent. Normal exponential growth resumed following a 2 to 3 hr. lag period (Fig. 69). Concentrations of 100 µg/ml of both curing agents were lethal to the culture.

Ultraviolet radiation following the addition of either curing agent resulted in similar effects as those described above (Fig. 70). The difference was that the culture showed an extended recovery period following the treatment. The culture recovered from the treatment after approximately 12 h of growth. The induction of the lysogenic lytic phage $\phi$-2 and the defective $\phi$-3 described by Hendry et al., (1976) may account for the extended recovery period. These cells, when
diluted and plated out following the treatment, showed approximately 30 to 40 per cent asporogeny but no apparent effect on inclusion formation.

3.6.3 Effect of Growth Temperature on B. cereus subspecies medusa

Cultures grown at 37°C resulted in approximately 5% of the culture becoming asporogenous. There was no apparent effect on the formation of the inclusion. The colonies of asporogenous cells appeared as small, round, raised colonies that were rough and dull. Similar colonies appeared in cultures grown at 55°C and the frequency of asporogeny increased to approximately 70% of the cell population. The variant L06-55 was isolated in this fashion and used for further studies.

Growth of cultures at 42°C for 24 hr. followed by reversion to the normal 30°C growth temperature produced a high frequency of acrystalliferous cells in the various subspecies of B. thuringiensis and yielded the core crystal variant in B. cereus subspecies medusa. The variant L08-42 was isolated in this manner and used for most of the core crystal studies.

The two other core crystal inclusion variants, L09 and L010, were spontaneously isolated from B. cereus subspecies medusa wild type 9B and the variant L07, respectively, during the early stages of this study. Although not quantititated, it was noted that if the wild-type 9B and L07 strain were continuously subcultured on nutrient agar,
rather than GBBM sporulation media, the wild-type 9B and L07 donut variant became unstable and would frequently give rise to the "core" type variant. These strains formed similar core crystal inclusions but each grew at markedly different rates (Fig. 71). The rate of growth of the strains 9B, L010 and L08-42 were initially similar, however, the rate of growth of 9B decreased whereas the others did not (L08-42, L09 and L010). The variant L09 grew slower than the other strains. The decrease in the optical density of strain 9B may represent the auto-induction of the temperate phages $\phi$-2 and $\phi$-3 noted by others in wild-type B. cereus subspecies medusa (Hendry, 1974; Hendry, et al., 1976). These phages are apparently not active in the derived strains (Fig. 71). However, one of the salient characteristics of the L08-42 strain was its ability to lyse earlier than either the other core crystal variants or the wild-type. Induced phage were suspected as the agent, but negative staining of the sporulate failed to reveal more phage in L08-42 than in the wild-type.

The spores of the variant L09 were of a different shape and refractility than those of wild-type and the other two variants L08-42 and L010. The spores of the variant L09 were more curved and oblong than those of the other strains (Fig. 72). They also appeared less refractile than those of wild-type. Yet, the spores of the other variants L08 and L010 appeared more refractile than those of wild-type
(Fig. 73). Interestingly, the comparative amounts of spore coat of these spores were not different, when examined by electron microscopy.

The core crystal variants readily gave rise to asporogenous strains that first formed the primordial bead inclusion followed by the crystalization of the core (Fig. 73b). When the core crystal had enlarged to approximately three times the size of the bead, cell lysis ensued (Fig. 73). The core crystal was resistant to proteolysis similar to the wild-type inclusion inspite of the absence of the outer skin on the core crystal. Therefore, the core crystal inclusions could be harvested and separated from the spores, similar to the methods used with the wild-type inclusions.

In an effort to establish the parameters of loss of the inclusion during growth at 42°C, the amount of hydration of the media was investigated study was instigated by the observation that the age of the media seemed to effect the curing of the bacterium at 42°C (Figure 75).

The results of varying amounts of dehydration on the frequency of asporogeny in colonies of wild-type is shown in Fig. 75. Medium that had lost 40% of water content showed a 90% incidence of survivors lacking the ability to form spore. Inclusion formation, however, was unaffected by the degree of hydration of the media.

Therefore, the concentration of the nutrients in the media rather than the hydration of the media was examined next as the critical factor in the heat-curing of the
inclusion. Figure 76 shows the results of increasing the percentage nutrient agar (NA) used in the heat curing procedure. Like the hydration experiment, the frequency of asporogeny increased and as expected, was a function of nutrition of the media. At 8% NA, the frequency of curing the cells of spore formation was in excess of 90%, but again no apparent effect was seen on the formation of the inclusion.

The dehydration of the media by 20% had no effect on either sporulation or inclusion formation. At higher amounts of dehydration, the percentage of asporoegenous colonies increased dramatically, and then remained at approximately 90% curing of spore formation. Therefore, increased nutrient concentration seemed to produce a gradual effect on the degree of asporogeny, which was maximal at 8% nutrient agar.

3.6.4 The Effect of Variation of Media on Inclusion Formation in subspecies of B. thuringiensis and B. cereus

In B. cereus subspecies medusa the frequency of core and bead forming cells could be increased by repeated subculturing of the cells on sheep blood agar (SBA)(Fig. 74). After three successive platings, 70% of the cells were core and bead variants. On the first plating no response (abnormal inclusion formation) was observed. Likewise, the second plating produced no noticeable change in the formation of the inclusion. This peculiar induction of the
core and bead type inclusion could only be induced in the L07 donut variant. The wild-type 9B strain did not respond to growth on sheep blood agar. Likewise, the heat-induced L08-42 core inclusion did not produce the bead variant when grown on SBA. Further subculturing (fourth passage) of the L07 variant did not increase the frequency of core and bead variants.

Variants L011-nut c and L013-nut c were isolated by this procedure and further studied. The bead variant, L013-nut c produced small smooth convex colonies (Fig. 77b,c,d) that were different than the large rough flat colonies of wild-type. The core variant, L011-nut c produced colonies somewhat intermediate to both the wild type and the L013-nut c bead variant (Fig. 77b). These colonies were rougher, whiter and smaller than those of the wild-type. The bead variant formed cells which were approximately 1.75 times longer and approximately 0.70 times as wide as the wild type cells (Fig. 79 and 80). These narrower cells formed helices which twisted around each other in the opposite direction, (Fig. 78a and b). The cells were unencapsulated and lysed earlier than the cells of either the wild-type or the core variant (Fig. 81).

Growth of the *B. cereus*, *B. cereus* subspecies *medusa* and several subspecies of *B. thuringiensis* on sheep blood agar was pursued in an attempt to observe differences in the ability of the bacteria to form hemolysins. *B. cereus* produced a hemolysin as did most of the *B. thuringiensis*
subspecies with the exception of *B. thuringiensis* subspecies *kurstaki* (Tables VII and VIII). *B. cereus* subspecies *medusa* and subspecies *lewinia* did not produce a hemolysin whereas *B. cereus* subspecies *fowler* slowly produced a hemolysin. The texture of the colony of the various subspecies on SBA also varied. Some of the strains produced a smooth colony, whereas others formed the typical rough colony. Unfortunately, no correlation between inclusion formation and hemolysin production could be made except in the case of *B. thuringiensis* subspecies *kurstaki*.

Growth of *B. cereus* subspecies *medusa* and the bead variants on yeast glucose agar revealed colony differences between the bead variant L013 and wild-type 9B. The growth rate of the bead variants was slower than that of wild-type. The bead variants formed small, rough colonies whereas the wild-type formed larger smooth colonies (Table IX). Cells of both strains were examined for capsule formation. Cells of the bead variants L013, lacked a capsule whereas cells of wild-type formed abundant capsular material.

*B. cereus* N formed rough large colonies that formed a distinguishing pink pigment. *B. cereus* T like *B. cereus* subspecies *medusa* formed the usual white colonies. The same pink pigment was seen in colonies of some of the *B. thuringiensis* subspecies (Table X). Colonies of *B. thuringiensis* subspecies *alesti*, *B. thuringiensis* subspecies *israelensis* and *B. cereus* subspecies *fowler* formed the distinctive pink pigment. Several acrystaliferous strains
of *B. thuringiensis* were also grown on YGA in an effort to show a correlation between inclusion formation and pigment production. *B. thuringiensis* subspecies *kurstaki* showed remarkable variation in growth on YGA. The acrystaliferous strain grew extremely slowly and formed donut-like colonies while the wild type grew quite vigorously (Fig. 82a, and b). *B. thuringiensis* subspecies *euxoae* showed substantial colony variation between cry<sup>+</sup> and cry<sup>-</sup> strains, which as it turned out were also asporogenous (Fig. 82c). Also *B. thuringiensis* subspecies *sotto* seemed to be effected in its ability to form the typical colony when it had lost its inclusion (Fig. 82d). The most dramatic effect of growth on yeast-glucose agar was observed in *B. thuringiensis* subspecies *alesti*. The pink pigment described above was not observed in colonies formed by acrystaliferous cells. This suggested a positive correlation between pink pigment formation and inclusion formation. However, for the most part, a common colony characteristic could not be correlated with the formation of the inclusion.

3.6.5 **Effect of Cystine Starvation on Inclusion Formation**

Although it has been reported by others (Rajalkashini and Shetna, 1977) that cystine starvation impedes inclusion formation, this study was unable to confirm this effect in *B. cereus* subspecies *medusa* (Fig. 83).
3.6.6 Effect of Addition of Sodium Sulphite on Inclusion Formation

The addition of sodium sulphite to a growing culture of *B. cereus* subspecies *medusa* 9B resulted in abnormal inclusion formation. The incomplete formation of the skin surrounding the wild type inclusion was observed (Fig. 84a and b). Annular rings of fibers were seen between the crystal and the thickened skin (Fig. 84b). This suggested that the skin was composed of these fibers. Figure 84c shows the donut inclusion of L07 for comparison to the sodium sulphite treated wild-type. The addition of sodium sulphite, to the wild-type cells resulted in the inclusion becoming remarkably similar to the donut inclusion, of variant L07. The inner amorphous matrix surrounding the inner core, however, was absent in the treated cells.

The addition of sodium sulphite to the L07 "donut" variant during late vegetative growth resulted in thickening of the outer skin. The fibers were either not formed or were incompletely integrated into the dense outer skin (Fig. 85). The amorphous matrix and the inner core were unaffected by the addition of sodium sulphite.

3.6.7 Antibiotic Sensitivity of *B. thuringiensis* and *B. cereus* subspecies *medusa*

In an effort to establish a possible linkage between the inclusion gene(s) and the genes for antibiotic resistance, a battery of antibiotics were tested against all of the strains of *B. cereus* subspecies *medusa* and *B.*
**thuringiensis.** Tables XI and XII tabulate the results. All strains of *B. thuringiensis*, were sensitive to all of the antibiotics except penicillin and ampicillin. Likewise, *B. cereus* subspecies *medusa* and its variants were similar. However, L013 was also sensitive to ampicillin and penicillin (Fig. 86). Although the zone of inhibition of penicillin was in the range of intermediate inhibition, the wild-type and other variants did not show the same amount of inhibition. Thus, antibiotic tests could be used on a comparison basis for screening of the variants.

To confirm these findings, a clinical antibiotic sensitivity assay was used to screen for antibiotic resistance. Figure 87 shows that *B. cereus* subspecies *medusa* was sensitive to all the antibiotics tested, including penicillin and ampicillin. Yet, this method did discern sensitivity differences between the core crystal variants. The variant L010 showed an initial sensitivity to penicillin (2.5 µg/ml), whereupon, after approximately 9 hr. of growth, the cells began to develop a resistance to penicillin, (Fig. 88). The final optical density was almost equivalent to that of the wild-type culture. Both sporogenous and asporogenous strains exhibited this late growth phenomenon, therefore, obviating sporulation as the cause of the rise in optical density of the culture.
# TABLE VI

## Isolation of variants of *B. cereus* subspecies *medusa*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>9B</td>
<td>$B^+S^+$ (wild type)</td>
<td>original isolate from Cambridge England by C.F. Robinow</td>
</tr>
<tr>
<td>LO6</td>
<td>$B^+S^+\phi-2^-$</td>
<td>spontaneous cured of phage Gillespie, Hendry (1976)</td>
</tr>
<tr>
<td>LO7</td>
<td>$Bd^+S^+$</td>
<td>spontaneous inclusion variant Fitz-James (1963)</td>
</tr>
<tr>
<td>LO8-42</td>
<td>$Bc^+S^+-lyt^+\phi-2^-$</td>
<td>heat cured inclusion variant at 42°C Insell</td>
</tr>
<tr>
<td>LO9</td>
<td>$Bc^+S^+wh^+$</td>
<td>spontaneous inclusion variant of 9B Insell</td>
</tr>
<tr>
<td>LO10</td>
<td>$Bc^+S^+wh^+$</td>
<td>spontaneous inclusion variant of LO7 Insell</td>
</tr>
<tr>
<td>Loll-nutc</td>
<td>$Bc^+S^+(-)$</td>
<td>nutritionally cured inclusion variant of LO7 Insell</td>
</tr>
<tr>
<td>L012-55</td>
<td>$B^+S^-$</td>
<td>heat cured spore variant at 55°C of 9B Insell</td>
</tr>
<tr>
<td>L013</td>
<td>$Bb^+S^+-cap^-th^+gr^+$</td>
<td>spontaneous inclusion and cell variant of 9B Insell</td>
</tr>
<tr>
<td>L013-nutc</td>
<td>$Bb^+S^+-cap^-th^+$</td>
<td>nutritionally cured inclusion and cell variant of LO7 Insell</td>
</tr>
</tbody>
</table>
Figure 68. Growth of the variants of *B. cereus* subspecies *medusa* in GGBM sporulating media. (Arrow represents *t₀* established by phase contrast microscopy.)

- ■ strains 9B, L06, L07
- ○ strains L08-42, L09, L010
- ▲ strains L013, L013-nutc
Figure 69. The effect of the addition of acridine orange and ethidium bromide on growth and inclusion formation in B. cereus subspecies medusa 9B. The curing agents were added to the culture after 5 h. of growth.

△ represents 50 µg/ml of acridine orange

○ represents 50 µg/ml of ethidium bromide
Figure 70. The effect of the addition of acridine orange and ethidium bromide to B. cereus subspecies medusa followed by irradiation with UV radiation.

○ represents acridine orange (50 μg/ml)
● represents acridine orange (50 μg/ml) followed by UV radiation
□ represents ethidium bromide (50 μg/ml)
■ represents ethidium bromide (50 μg/ml) followed by UV radiation

The curing agents were added to the culture after 5 hr. of growth, followed by ultra-violet irradiation of the cells after 7 hr. of growth.
Figure 71. The growth of the variants LO8-42, LO9, LO10 and 9B B. cereus subspecies medusa in tryptone-soy broth, a non-sporulating media.

○ represents 9B culture
▲ represents LO8-42 culture
■ represents LO9 culture
■ represents LO10 culture
Figure 72a. A light micrograph of sporulated cells of *B. cereus* subspecies *medusa* 9B, showing the cellular capsule. Both the inclusion (dark ovoid body) and spore (white refractile body) can be seen. (India ink) (mag. 4000X)

Figure 72b. A light micrograph of the sporulated cells of the variant L09 showing the oblong spore shape and core crystal. (mag. 4000X)
Figure 73a. A light micrograph of the sporulating cells of the variant LO8-42 showing the uniform crystal formation on nutrient agar after 48 hr. of growth. Note the variation in the size of the core crystal. (mag. 4000X)

Figure 73b. A light micrograph of the late vegetative cells of the variant LO10 showing the beginning of the formation of the core crystal at one end of the cell. Note the size of the inclusion compared to Figure 73c. (mag. 3900X)

Figure 73c. A light micrograph of the sporulating cells of the variant LO10 showing the different growth as compared to LO8-42. Some of the cells form two core crystals, usually in asporogenous cell. Note the length of these cells during vegetative growth. (mag. 4000X)
Figure 74. The frequency of transformation of \textit{B. cereus} subspecies \textit{medusa} 9B and LO7 "donut" to "core crystal" variant LO13 by repeated growth on Sheep blood agar at 30°C.

- represents \textit{LO7}
- represents 9B
Figure 75. The frequency of asporogeny and crystal loss of B. cereus subspecies medusa 9B induced by growth on dehydrated nutrient agar at 30°C.

- represents asporogeny
- represents crystal loss
Figure 76. The frequency of asporogeny and crystal loss of *B. cereus* subspecies *medusa* 9B by growth on varying concentrations of nutrient agar at 30°C.

- ● represents asporogeny
- ■ represents crystal loss
Figure 77. Photographs illustrating the differences in colony morphology of the variants of *B. cereus* subspecies *medusa* grown on nutrient agar at 30°C.

(a) 9B (WT)
(b) LO11-nutc and LO13-nutc
(c) LO11-nutc
(d) LO13-nutc
Figure 78. Light micrographs showing the cell morphology of the variant L013-nutc derived from L07. Note the helixed-type growth and the size and shape of the cells. The cells have lost the ability of sporulation and inclusion formation. (mag. 3000X)
Figure 79. Electron micrographs of the variant LO13-nutc (b) and the wild-type cell (9B) of *B. cereus* subspecies *medusa* (a). Note the reduction in the size of the cell and the loss of inclusion formation in the cells of LO13-nutc. The initial stages of inclusion formation can be seen as a dense region at the end of the cell (a). The cell septum appears to have thickened in the variant LO13-nutc (b).

(mag. 51,200X) (a)
(mag. 52,560X) (b)
Figure 80. Light micrographs of the variant L013 and 9B grown on (BBM) non-sporulating media. Note the difference in the size of the cells and the ability of wild-type to produce large inclusions on non-sporulating media (a) and (b). The L013 cell has lost the ability to form inclusions and has become somewhat smaller in size than the wild-type cell (c). (mag. 3000X)

Figure 81. Light micrographs of the same variant stained in India ink showing the capsular formation in the wild-type (b) and no capsule in the variant (a). (mag. 3000X)
Figure 82. Photographs illustrating the effect of yeast-glucose agar on the colony morphology of
*B. thuringiensis* subspecies *kustaki* (a) and (b), (c) subspecies *alesti* and (d) subspecies *euxoe*. 
Figure 83. The effect of cysteine on growth and sporulation of B. cereus subspecies medusa 9B.

- △ represents growth with 0.0% cysteine
- ▲ represents growth with 0.1% cysteine
- ◇ represents growth with 0.2% cysteine
- ■ represents growth with 0.5% cysteine
Figure 84a to d. Electron micrographs showing the effect of the addition of sodium sulphite to the media on inclusion formation in the variant LO6 and LO7. (a) reveals the structure of the wild-type inclusion. (b) shows the effect of sodium sulphite addition to the media on inclusion formation, resulting in the formation of the fibrous medullary layer between the crystal and the skin. (c) shows the bipartite formation of the donut inclusion. (d) shows the spore structure of LO7.

(mag. 69,000X) Figure 84a
(mag. 70,000X) Figure 84b
(mag. 105,800X) Figure 84c
(mag. 142,600X) Figure 84d
Figure 85. Electron micrographs showing the effect of the addition of sodium sulphite to the media during growth of the variant L07 "donut". Figure (a) shows the untreated inclusion and Figure (b) the spore of L07. Figure (c) shows the sodium sulphite treated inclusion which has the same bipartite structure as the untreated.

(mag. 44,450X) Figure 85a and b
(mag. 43,850X) Figure 85c
Figure 86. Photograph of the effect of penicillin and ampicillin, (10 units) on the wild-type '98 of B. cereus subspecies medusa and variant LO13 using the sensi-disc method. The variant LO13 is sensitive to the antibiotics whereas the wild-type is not.
Figure 87. The effect of antibiotics listed below on the growth of *B. cereus* subspecies *medusa 9B*.

1. Cephalothin 10.0 μg
2. Clindomycin 1.0 μg
3. Erythromycin 3.0 μg
4. Gentomycin 4.0 μg
5. Methicillin 5.0 μg
6. Penicillin 2.5 μg
7. Tetracycline 5.0 μg
8. Kanamycin 8.0 μg
9. Chloroamphenicol 9.0 μg
10. Ampicillin 2.5 μg
Figure 88. The effect of penicillin (2 units) on the growth of the "core crystal" of variants of B. cereus subspecies medusa in tryptone-soy broth using the clinical antibiotic resistance assay.

- ■ represents growth of L010
- ● represents growth of L010 spo-
- ○ represents growth of L010 with penicillin
- ▲ represents growth of L010 spo- with penicillin
### TABLE VII

Growth of *B. thuringiensis* on 1.5% Sheep Blood Agar

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemolysis</th>
<th>Growth</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>berliner</td>
<td>+</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>aesti (cry⁺)</td>
<td>++</td>
<td>++</td>
<td>S</td>
</tr>
<tr>
<td>aesti (cry⁻)</td>
<td>++</td>
<td>++</td>
<td>S</td>
</tr>
<tr>
<td>kurstaki (cry⁺)</td>
<td>+</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>kurstaki (cry⁻)</td>
<td>−</td>
<td>−</td>
<td>R</td>
</tr>
<tr>
<td>aizawa</td>
<td>+</td>
<td>++</td>
<td>S</td>
</tr>
<tr>
<td>sotto</td>
<td>+</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>schwetzova</td>
<td>+</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>M-13</td>
<td>+</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>B-30-1</td>
<td>+</td>
<td>++</td>
<td>S</td>
</tr>
<tr>
<td>fowler</td>
<td>(+)</td>
<td>++</td>
<td>S</td>
</tr>
<tr>
<td>lewinia</td>
<td>−</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>israelensis</td>
<td>+</td>
<td>++</td>
<td>S</td>
</tr>
</tbody>
</table>

--; no hemolysis, +; partial hemolysis; (+) partial hemolysis after 72 hr; ++; good hemolysis after 12 hr.

++; good growth, R; rough colony, S; smooth colony appearance.
**TABLE VIII**

Growth of Strains of *B. cereus* subspecies *medusa* on 1.5% Sheep Blood Agar

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemolysis</th>
<th>Growth</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. medusa 9B</td>
<td>-</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>L06 Ø-2</td>
<td>-</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>L07 Bd</td>
<td>-</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>L08 Bc</td>
<td>-</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>L09 Bc</td>
<td>-</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>L010 Bc</td>
<td>-</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>L013 Bb</td>
<td>-</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>L014 Bb</td>
<td>-</td>
<td>++</td>
<td>R</td>
</tr>
<tr>
<td>B. cereus N</td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>B. cereus T</td>
<td>+</td>
<td>+</td>
<td>S</td>
</tr>
</tbody>
</table>

- : no growth, + : growth, − : poor growth, ++ : good growth
R : rough, S : smooth
− : no hemolysis
TABLE IX

Growth of Strains of *B. cereus* subspecies *medusa* on
Yeast-glucose Agar

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pigment</th>
<th>Growth</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. medusa 9B</td>
<td>-</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>LO6</td>
<td>-</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>LO7</td>
<td>-</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>LO8</td>
<td>-</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>LO9</td>
<td>-</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>LO10</td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>LO13</td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>LO14</td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
</tbody>
</table>

B. cereus N  
B. cereus T

--; no pink pigment
--; no growth, +; growth, +; slow growth, +++; extremely good growth
R; rough colony appearance, S; smooth colony appearance
<table>
<thead>
<tr>
<th>Strain</th>
<th>Pigment</th>
<th>Growth</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>berliner</td>
<td>-</td>
<td>++</td>
<td>S</td>
</tr>
<tr>
<td>alesi (cry+)</td>
<td>+</td>
<td>++</td>
<td>S</td>
</tr>
<tr>
<td>alesi (cry-)</td>
<td>-</td>
<td>++</td>
<td>S</td>
</tr>
<tr>
<td>kurtaki (cry+)</td>
<td>-</td>
<td>+++</td>
<td>R</td>
</tr>
<tr>
<td>kurtaki (cry-)</td>
<td>-</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>aizawa</td>
<td>-</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>sotto</td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>schwetsova</td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>M-13</td>
<td>-</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>B-30-1</td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>fowler</td>
<td>+</td>
<td>+++</td>
<td>R</td>
</tr>
<tr>
<td>lewinia</td>
<td>-</td>
<td>+++</td>
<td>R</td>
</tr>
<tr>
<td>israelensis</td>
<td>+</td>
<td>+++</td>
<td>R</td>
</tr>
</tbody>
</table>

-; no pink pigment production, +; pink pigment production
+; good growth, ++; very good growth, +++; extremely rapid growth
++; slow growth
R; rough colony appearance, S; smooth colony appearance
TABLE XI

Antibiotic Sensitivity of Strains of *B. cereus subspecies medusa*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>9B</th>
<th>L07</th>
<th>L08</th>
<th>L09</th>
<th>L010</th>
<th>L011</th>
<th>L012</th>
<th>L013</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin (10 u)</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r(?)</td>
<td>r</td>
<td>r</td>
<td>s</td>
</tr>
<tr>
<td>Ampicillin (10 mg)</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Tetracycline (30 mg)</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Erythromycin (15 mg)</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Novobiocin (30 mg)</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Kanamycin (30 mg)</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Chloramphenicol (30 mg)</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Dihydrostreptomycin (30 mg)</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
</tbody>
</table>

r; resistant
s; sensitive
**TABLE XII**

Antibiotic Sensitivity of Strains of *Bacillus thuringiensis*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin (10 units)</td>
<td>resistant</td>
</tr>
<tr>
<td>Ampicillin (10 mg)</td>
<td>resistant</td>
</tr>
<tr>
<td>Tetracycline (30 mg)</td>
<td>sensitive</td>
</tr>
<tr>
<td>Erythromycin (15 mg)</td>
<td>sensitive</td>
</tr>
<tr>
<td>Novobiocin (30 mg)</td>
<td>sensitive</td>
</tr>
<tr>
<td>Kanamycin (30 mg)</td>
<td>sensitive</td>
</tr>
<tr>
<td>Chloramphenicol (30 mg)</td>
<td>sensitive</td>
</tr>
<tr>
<td>Dihydrostreptomycin (10 mg)</td>
<td>sensitive</td>
</tr>
</tbody>
</table>

*strains listed in Table VII.*
3.7 Plasmid DNA Studies

3.7.1 Comparison of Methodology

Before characterizing the plasmid DNA of *B. cereus* and *B. thuringiensis*, the reliability of current methods used for plasmid extraction was ascertained. Extraction methods have two basic aims: first, to obtain cellular contents in an undisturbed condition, the so-called cleared lysate, and secondly, to select from the contents, the closed covalently circular DNA free of protein, lipid, and degraded linear DNA. Also, since almost all of the current methods of lysis were designed for gram negative bacteria, it was necessary to modify them so that *B. cereus* and *B. thuringiensis* could be lysed.

The most reliable method of lysis was a modified version of the procedure by Guerry et al., (1973). Although the cells were lysozyme resistant, lysis was achieved by incubation of log phase cells with an increased concentration of lysozyme, 20 mg to 50 mg/ml., at 37°C for 4 to 6 h. Cells of some subspecies (*B. thuringiensis* subspecies *israelensis*) formed protoplasts. Whereas, cells of *B. cereus* subspecies *medusa* remained as rods, until SDS was added, whereupon immediate lysis would ensue.

Following lysis, the Hirt procedure (1967) was employed to remove the chromosomal DNA from the lysate. Guerry et al., (1973) found that close to 98% of the chromosome was removed by overnight incubation at 4°C with 5M NaCl and 1% SDS. The efficiency of chromosome precipitation stated by
Guerry et al., (1973) could not be obtained and most often a substantial amount of residual chromosomal DNA accompanied the plasmid DNA through the procedure.

The cleared lysate was subjected to a number of purification procedures to isolate and characterize the plasmid DNA by agarose gel electrophoresis. The most reliable of these was cesium chloride-ethidium bromide dye buoyant density ultracentrifugation (Bauer and Vinograd 1967). The residual chromosomal DNA along with open circular and linear DNA were separated from the closed circular plasmid DNA.

A more rapid, but less reliable method of isolating plasmid DNA was the procedure by Meyers et al., (1976). These workers suggested phenol as a reagent to remove contaminating protein, followed by the selective precipitation of DNA with 95% ethanol. However, this method also isolated open circular forms and replicative intermediates of plasmid DNA. The rapid ethanol precipitation method was also unreliable in isolating all of the plasmid DNA. Large molecular weight DNA was lost during this extraction and therefore was not used for characterization studies. However, this procedure was advantageous in the selection of small molecular weight plasmid DNA for restriction endonuclease mapping.

In agreement with the report of Gonzales and Carlton (1980), it was found that stationary phase cells yielded a greater amount of plasmid DNA than did log-phase cells.
Moreover, growth of the cells to stationary phase in either blood-base media, nutrient broth, or yeast extract-glucose resulted in identical plasmid profiles of *B. cereus* subspecies medusa.

3.7.2 Plasmid DNA of *B. thuringiensis* subspecies

*israelensis* and of *B. cereus* subspecies medusa

*B. thuringiensis* subspecies *israelensis* contained at least 7 plasmids of molecular weights 15.2, 9.8, 8.9, 7.5, 5.1, 4.6 and 3.8 Mdal. (Fig. 89a). The seven plasmids were isolated by the phenol extraction procedure of Myers et al., (1976) and also by CsCl-ethidium bromide density gradient ultracentrifugation. The plasmid DNA was separated and observed by agarose gel electrophoresis. Thus, barring alternate forms of the same plasmid, the bands in the gel possibly represent unique plasmids.

In contrast, *B. cereus* subspecies medusa contained only three plasmids of molecular weights 12.6, 6.4 and 3.4 Mdal. (Fig. 89a and 89b). However, this plasmid DNA profile varied, unlike that of *B. thuringiensis* subspecies *israelensis*. Occasionally, a fourth band was observed in the gel, above the 12.6 Mdal. plasmid and below the chromosomal DNA. Since the large molecular weight band was not visualized in the CsCl ethidium bromide density gradient ultracentrifugation preparation (Fig. 89b) it may represent replicate or alternate forms of one of the smaller plasmids. Also, there was at least one and possibly two high molecular weight circular DNAs which were not present in L06 (compare...
Fig. 89a to Fig. 89b). Since a large molecular weight plasmid marker was not available, the sizes of these bands were not established. Variants such as L06-55 which were isolated from L06, did not contain one of the high molecular weight band (Fig. 89b).

3.7.3 Plasmid DNA of the Variants of B. cereus subspecies medusa.

In section 3.5 the isolation of core crystal variants L08-42, L09 and L010 was described. These core crystal variants consistently lacked the 12.6 Mdal. plasmid (Fig. 89b). The two variants L09 and L010 which lacked the phage 0-2, also lacked one of the large molecular weight band. L08-42 did contain the high molecular weight plasmid very close to the origin (Fig. 89b).

Repeated growth of B. cereus subspecies medusa (L07) on sheep blood agar resulted in the generation at a high frequency of two types of variants. One, L011-nutc, belonged to the core crystal variant series, whereas the other was a variant blocked at the initial (bead) stage of inclusion formation. The core crystal variant L011-nutc lacked the 12.6 Mdal. plasmid. The bead variant (L013) contained the same plasmids (12.6, 6.4 and 3.1 Mdal.) as the wild type, but surprisingly also contained a new plasmid with a molecular weight of 7.2 Mdal. This type of bead variant was frequently spontaneously isolated over the course of the study and always contained the new 7.2 Mdal. plasmid.
The donut variant, (L07) contained the same plasmid compliment as the wild type (Figure 89b). In figure 89b the 3.4 Mdal. plasmid is not shown, however, an intense 10 Mdal. band arose in the position of the open circular form and it is possible that the 3.4 Mdal. plasmid was nicked during this extraction and therefore migrated slower than the closed circular form. Other plasmid DNA extractions yielded a plasmid profile identical to that of the wild type.

The relationship of plasmid DNA with the process of sporulation was examined. An asporogenous variant L06-55, isolated by growth of the wild-type culture at 55°C contained the 12.6, 6.1 and the presumptive open circular form of the 3.4 Mdal plasmids. It also contained the large molecular weight band corresponding to the phage \( \Phi - 2 \), but did not contain the largest plasmid which migrated close to the origin.

3.7.3 Sucrose Gradient Ultracentrifugation

The plasmid DNA of *B. cereus* subspecies *medusa* was purified by sucrose gradient ultracentrifugation using plasmid pBR325, as a marker. Five fractions were isolated from the gradient (Fig. 91) and checked by agarose gel electrophoresis. The largest DNA corresponded to circular DNA with a molecular weight of approximately 42 Mdal. This presumably, was phage \( \Phi - 2 \) DNA. Fraction 4 which was a broad peak, corresponded to chromosomal DNA. Fractions 1, 2 and 3
corresponded, in molecular weight, to the three plasmids (12.9, 6.1 and 3.4 Mdal.) shown by agarose gel electrophoresis.

The number and size of the plasmid DNA observed by agarose gel electrophoresis were confirmed by sucrose gradient ultracentrifugation. Although the largest band seen agarose gel electrophoresis was not found in the sucrose gradient, presumably, it had migrated through the gradient into the cushion at the bottom (Fig. 91).

3.7.4 **Restriction Endonuclease Mapping**

The three plasmids of *B. cereus* subspecies *medusa* were purified by sucrose gradient ultracentrifugation. The separated plasmids were digested by three enzymes, EcoRI, Bam HI and Hind III. The two smallest plasmids, 3.4 and 6.1 Mdal., contained no restriction sites (Fig. 92A) when digested with EcoRI, Bam HI or Hind III. However, all three plasmids isolated together when digested with Hind III produced seven fragments (Fig. 92B). The undigested circular 6.1 Mdal. plasmid was seen among the fragments. The 3.4 Mdal. plasmid was also visible among the restricted fragments, but it was extremely faint.

These results would indicate that the 12.9 Mdal. plasmid is not a multimer of the two smallest plasmids.

3.7.5 **Plasmid DNA of *B. thuringiensis* and *B. cereus N, T**

Two strains of *B. cereus*, N and T, were examined for the presence of plasmid DNA. *B. cereus* N contained no detectable plasmid DNA. Both the phenol extraction and
cesium chloride-ethidium bromide density ultracentrifugation failed to isolate any plasmid DNA. *B. cereus* T contained two and possibly three, plasmid bands (Fig. 93a) at molecular weights 14 and 17 Mdal. and approximately 50 Mdal.

Eight subspecies of *B. thuringiensis* were examined for the presence of plasmid DNA *B. thuringiensis*. Subspecies *berliner* contained five, possibly six plasmid bands, corresponding to molecular weights of 13.2, 11.6, 9.8, 1.5 and 0.5 Mdal. Note that one of the plasmids was the same size as the 9.8 Mdal plasmid in *B. thuringiensis* subspecies *israelensis*. *B. thuringiensis* subspecies *alesti* contained three plasmids of medium size, none of which corresponded to any of the plasmids in the other strains. *B. thuringiensis* subspecies *sotto* contained three plasmids, two high molecular weight plasmids and a small one with a molecular weight of 5.8 Mdal. The molecular weights of the large plasmids of *B. thuringiensis* subspecies *sotto* were not established but are probably about 40 to 50 Mdal. *B. thuringiensis* subspecies *schwetzova* contained three plasmids (50, 9.8 and 4.7 Mdal.) which were identical to the plasmids of *B. thuringiensis* subspecies M-13 and *B. thuringiensis* subspecies B-30-1. These two strains were isolated by Toumanoff (1953) and may in fact, be isolates of *B. thuringiensis* subspecies *schwetzova*. *B. thuringiensis* subspecies M-13 lacked the giant 50 Mdal plasmid and *B. thuringiensis* subspecies B-30-1 contained a fourth plasmid with a molecular weight of about 13 Mdal. *B. cereus*
subspecies *fowler* contained only the single giant plasmid at approximately 50 Mdal. It is interesting that all of the strains contained the giant 50 Mdal. plasmid, except *B. cereus* subspecies *medusa*, *B. thuringiensis* subspecies M-13 and *B. thuringiensis* subspecies *berliner*.

The plasmid compliment of *B. thuringiensis* subspecies *kurstaki* was identical to that reported by others (Stahly et al., 1978). A spontaneously derived acrystaliferous strain of *B. thuringiensis* subspecies *israelensis* contained five plasmids, 9.8, 8.9, 7.5, 5.1, 4.6 and 3.8 Mdal. which were similar to those of the wild-type. But the largest plasmid (15.2 Mdal.) was lost. This was consistent with the finding that the largest plasmid (12.6 Mdal.) of *B. cereus* subspecies *medusa* was correlated with the presence of the wild-type inclusion. Thus, the correlation of the large plasmids with inclusion formation was found in *B. cereus* subspecies *medusa* and *B. thuringiensis* subspecies *israelensis*. 
Figure 89. Agarose gel electrophoresis (0.7%) of plasmid DNA of *B. thuringiensis* subspecies *israelensis* and of *B. cereus* subspecies *medusa*. Cleared lysates were purified by phenol extraction (gel 89 A) and by CsCl-ethidium bromide buoyant density gradients (89 B).

Gel A, lanes 1 and 2, contain approximately 20 µg of plasmid DNA from *B. thuringiensis* subspecies *israelensis*, lane 3 10 µg of pGM-4, pGM-5, lane 4 15 µg of pBR-322, pGM-4, pGM-5, and Lambda, and lane 5 20 µg of plasmid DNA from *B. cereus* subspecies *medusa* LO6.

Gel B contains plasmid DNA purified by cesium chloride ethidium-bromide dye buoyant density ultracentrifugation. Lane 1 L013, lane 2 L8-42, lane 3 9B, lane 4 asporogenous strain of 9H, lane 5 L07 and lane 6 L06-55.
Figure 90. Electron micrographs of CsCl-ethidium bromide dye buoyant density purified circular plasmid DNA of B. cereus subspecies medusa. Figure 90 (a) shows the 12.6 Mdal. and the 6.1 Mdal. plasmids. The CCC DNA has been partially opened. Figure 90 (b) shows the 3.4 Mdal. plasmid and Figure 90 (c) shows the 75 Mdal. circular DNA. Figure 90 (d) shows the plasmid DNA electrophoresed on a 0.7% agarose gel. Five distinct bands can be visualized on the gel.

(mag. 1,220X) Figure 90 a
(mag. 175,660X) Figure 90 b
(mag. 28,320X) Figure 90 c
Figure 91. Neutral 5% to 20% sucrose gradient ultracentrifugation of the CsCl ethidium bromide buoyant density ultracentrifugation plasmid DNA of B. cereus subspecies medusa. PBR-325 (3.6 Mdal.) was used as a molecular weight marker. The five fractions were collected after fractionation on an isco UV analyzer, fraction collector and electrophoresed on a 0.7% agarose gel. Fraction 1 is the 3.4 Mdal., fraction 2 is 6.1 Mdal., fraction 3 is 12.9 Mdal., fraction 4 the chromosomal fragments and fraction 5 is phase ø-2 genome.
Figure 92. Restriction endonuclease analysis of the isolated plasmids of *B. cereus* subspecies *medusa*.

Figure A; lane 1 contains lambda DNA digested with EcoRI, lane 2 lambda DNA digested with Bam HI, lane 3 the 0.6 form of the 3.4 Mdal. plasmid, lane 4 the 6.1 Mdal. plasmid, lane 5 the 3.4 Mdal. plasmid digested with EcoRI, lane 6 the 6.1 Mdal. plasmid digested with EcoRI, lane 7 the 3.4 Mdal. plasmid digested with Bam HI, lane 8 the 6.1 Mdal. plasmid digested with Hind III and lane 10 the 6.1 Mdal. plasmid digested with Hind III.

Figure B; the restriction endonuclease analysis of isolated plasmids of *B. cereus* subspecies *medusa* digested with Hind III. Lane 1 contains lambda-GR4 digested with Hind III, lane 2 the purified plasmids of *B. cereus* subspecies *medusa*, lane 3 the three plasmids (12.6, 6.4, 3.1 Mdal.) digested with Hind III, lane 4 the 3.4 Mdal. and the 6.1 Mdal. plasmid digested with Hind III and lane 5 the 6.1 Mdal. plasmid digested with Hind III.
Figure 93. Agarose gel electrophoresis (0.7%) of the plasmid DNA of *B. cereus* strain N and T and *B. thuringiensis*.

Figure A, lane 1 contains *B. cereus* subspecies *medusa*, lane 2 is *B. cereus* N, lane 3 is subspecies B-30-1, lane 4 is subspecies *israelensis*, lane 5 is subspecies M-13, lane 6 is subspecies *schwetzova*, lane 7 is subspecies *sotto*, lane 9 is subspecies *alesti*, lane 10 is subspecies *kurstaki* and lane 11 is *B. cereus* T.

Figure B, an agarose gel electrophoresis (0.7%) of the plasmid DNA of *B. thuringiensis* subspecies *israelensis* (lane 3) and a spontaneously derived acrystalliferous variant of *B. thuringiensis* subspecies *israelensis* (lane 1).
DISCUSSION

The efforts described in this thesis have attempted to first elucidate, the structure and composition of inclusions of different subspecies of B. cereus and B. thuringiensis and, secondly, to correlate the presence of plasmid DNA to the structure of the inclusions.

4.1 Comparative Structure and Composition of the Parasporal Inclusion of Various Subspecies

4.1.1 Subunit Size and Molecular Packing

Previous studies of the crystalline inclusion of various subspecies (Labaw, 1964; Holmes and Munro, 1965, Norris, 1971; Short et al., 1974) have provided a generalized model for the molecular packing and size of the subunit in the parasporal inclusion. The current model of the subunit is a tetrahedral cell (9.0 nm x 9.0 nm x 26.9 nm) which is composed of eight proteins each with a molecular weight of 230,000 daltons. Although Norris (1971) and Short et al., (1974) reported variations in the size of the subunits, these workers generally supported the model described by Holmes and Munro (1965).

The structural studies of the inclusions of B. thuringiensis subspecies kurstaki and subspecies israelensis and B. cereus subspecies medusa reported here indicate that a general model for the subunit and its crystalline array may not be viable. Although the measurements of the lattice spacing in the inclusion of B. thuringiensis subspecies kurstaki are in good agreement with past studies, the size
of the subunit is substantially (3.5 nm) smaller than the dimensions given by Holmes and Munro (1965) and Labaw (1964) and are more in agreement with the results of Norris (1969; 1971). The results reported here show that the subunit is rod-shaped for the crystal of all three organisms, but the size of the subunit varies among differing subspecies (Table XIV). Likewise, the spacing between the subunits and the angle at which they are positioned varies from 75° to 90° in the different subspecies. This would suggest that the tetrahedral model proposed by Holmes and Munro (1965) may only be applicable to subspecies which form the typical octahedral inclusion. The inclusions of *B. thuringiensis* subspecies *israelensis* and *B. cereus* subspecies *medusa* are ovoid and are packed in a different array than the octahedral inclusion of *B. thuringiensis* subspecies *kurstaki*.

The apparent discrepancy in the structure of the subunit and the description of a new spherical structure of the inclusion of *B. thuringiensis* subspecies *kurstaki* observed in figures 14 and 16 a-d may be explained by assuming that the subunit consists of eight proteins, packed in rows of two (Labaw, 1964). Therefore, figures 16 a-d show the end view of the protein subunit directly behind the protein subunit forming the face of the crystal. Notice that figure 14 is shadowed from the side of the crystal whereas figures 16 a-d are shadowed obliquely from the apex of the crystal. The view is always from the apex of the
crystal in these figures (Fig. 16a-d). The apparent spherical subunit was never observed in the ovoid inclusions of *B. cereus* subspecies *medusa* or *B. thuringiensis* subspecies *israelensis*.

4.1.2 Solubility

Solubilization of the inclusion has posed a major problem for workers studying the protein composition of the inclusion. Careful microscopic observation of the solubilization process may help overcome much of the problem. The solubilization process occurs in two phases. First, the dismantling of the crystal and secondly, the solubilizing of the native polypeptide components. Although the inclusion suspension may clear during dissolution, when the suspension is examined by phase contrast microscopy, there are still insoluble components. These residual structures (bead, skin and dark segment) only dissolve when the pH is further elevated. Also, the results show that solubility of the inclusion of the several subspecies examined differs. The alkalinity of the dissolution buffer (SDS-DTE) varied from pH 7.5 to 10.5 for the crystalline inclusions of the various subspecies.

The results reported here are not consistent with those of Huber et al., (1981). These workers suggested that six disulphide bonds hold the crystal subunits together and these disulphide bonds are only accessible to a reducing agent when preceded by a denaturant. However, in this study, when the crystals of the various subspecies were
subjected to both the denaturant SDS and the reducing agent DTE, the crystals swelled and lost their crystalline array but did not dismantle until the pH of the solvent was elevated. These results would preclude only disulphide bonds and hydrophobic interaction holding the subunits together. Ionic or covalent bonding may also be involved in the linking of the crystal subunits. Dastidar and Nickerson (1978) reported the release of the carbohydrate from the glycoprotein by B-elimination in alkali. Possibly, the carbohydrate decoration of the protein is responsible for linking the subunits together. The results here as well as those of others (Bateson and Stainsby, 1970; Bulla et al., 1977; Tyrell et al., 1981) show a substantial (4.0%) amounts of hexoses associated with the crystal protein of *B. thuringiensis* subspecies *kurstaki* and 9.8% hexoses in the inclusion of *B. cereus* subspecies *medusa*. Additional evidence for this suggestion lies in the relationship between the pH at which the inclusion dissolves and the amount of sugars found in that inclusion. The crystal of *B. thuringiensis* subspecies *kurstaki* contained the least amount of carbohydrates and dissolved at pH 7.5 in 1% SDS, 0.05 M DTE. The bead which is relatively richer in sugars dissolved at pH 9.5 in the same solvent. Likewise, the crystal of *B. cereus* subspecies *medusa* dissolved at pH 9.2 and contained 9.8% carbohydrates. The skin of this inclusion contains 37% carbohydrates and partially dissolved at pH 10.5. Completely dissolution required the addition of
heat. Although only circumstantial, this evidence does suggest the possibility of carbohydrate involvement in the subunit linkage.

4.1.3 Polypeptide Composition of the Inclusions

The morphology and protein composition of the different inclusions (Table XV) suggest that the various subspecies fall into four main categories and these categories can be correlated to toxicity.

Type I describes *B. thuringiensis* subspecies *alesti* and subspecies *finitimus* which form a simple bipyramidal crystal with no associated structure. The crystal is composed of a 135,000 dalton protein which is toxic to lepidopteran larva and a 130,000 dalton protein which is not toxic.

Type II describes the *B. thuringiensis* subspecies *kurstaki* and subspecies *schwetzova* which contain the bipyramidal crystal found in type I, as well as an associated ovoid inclusion. The crystal is composed of a 135,000 dalton protein and the bead is composed of a 68,000 dalton protein. Both of these subspecies are toxic to lepidopteran larva.

Bullá et al., (1979) have suggested that the 134,000 dalton glycoprotein is a prototoxic molecule which gives rise to a 68,000 dalton toxic molecule when subjected to alkaline (pH 11.8) conditions. More detailed studies reported in this dissertation show that the inclusion of *B. thuringiensis* subspecies *kurstaki* was composed of a crystalline tetrahedron with a non-crystalline accessory
body referred to as the bead or ovoid inclusion. The crystal and bead were separable by sonification, gradient centrifugation and differential solubility (pH 7.5 and 9.2 respectively). The purified bead was composed of a single glycoprotein, 68,000 daltons, which was partially similar to the crystal subunit glycoprotein (135,000 daltons). Following separation and purification of these two bodies of the inclusion, the protein profile of the purified crystal contained only a single 135,000 dalton protein that was degraded to small peptide fragments either on storage or on dissolution at a high pH. This is probably due to the action of endogenous inherent proteases present in the inclusion (Chestukhina et al., 1977). Thus, the breakdown of the crystal protoxin to a smaller 68,000 dalton toxin may not be biologically significant in contrast to the suggestion of Bulla et al., (1979). Instead, the 68,000 dalton toxic protein may reside exclusively in the bead.

Further evidence for this hypothesis is furnished by the fact that a single 68,000 dalton protein was generated from the whole inclusion (crystal and bead) preparation when the inclusion was dissolved at pH 9.2 (SDS-DTE) and allowed to stand at 20°C for 12 hr before analysis. Since the crystal is more labile than the bead and dissolves at a lesser pH (7.5), the differential solubility of the crystal and bead may explain the findings of Bulla et al., (1977; 1979). Also, since the bead is relatively more toxic than the crystal (Fitz-James, inpublished), the 68,000 dalton
toxin observed by Bulla et al., (1979) may have been the residual bead protein which was not solubilized during their protein analysis of the inclusion (Bulla et al., 1977). The flaw in this explanation resides in the fact that the type I inclusion, e.g. B. thuringiensis subspecies alesti is toxic to lepidopteran larvae and does not contain a bead.

Therefore, a more likely explanation of the composite inclusion is that the bead may be the result of a genetic lesion which causes excessive formation of the toxic portion (68,000 daltons) of the 134,000 dalton crystal molecule. The abundant 68,000 dalton protein is aggregated, but not crystallized on, the surface of the crystal. Evidence for this postulate resides in the fact that first, Dulmage (1970) isolated B. thuringiensis subspecies kurstaki as a more toxic mutant of B. thuringiensis subspecies alesti which does not form the bead. Secondly, the two proteins (135,000 and 68,000) of the crystal and bead exhibit partial homology. Therefore, it seems likely that the bead protein is in fact a portion of the crystal protein which is formed excessively.

Type III describes subspecies which contain spherical or ovoid inclusions that have a different molecular packing. The inclusion is composed of a crystal which is surrounded by a skin. The skin seems to be associated only with spherical inclusions. The crystal is composed of a 135,000 dalton protein and the skin is composed of a small protein approximately 20,000 daltons. These inclusions are not
toxic to either lepidopteran or dipteran larva (Gillespie, 1969). Examples of this type are B. cereus subspecies lewinia and subspecies medusa.

The results reported here confirm and elaborate on the findings of Gillespie, (1969), Hendry (1974) and Hendry et al., (1976). The inclusion of B. cereus subspecies medusa was composed of three distinct components; the skin, the crystal, the virus-like particles and fibers. The skin component appeared to be composed of several layers which was not consistent with the findings of Gillespie (1969). In the studies reported here, the skin appeared as a sac with no apparent substructure with fibers and virus-like particles adhered to it. The fibers described by Gillespie (1969) may in fact only cover the external surface of the skin rather than form it. The skin did not dissolve in the SDS-DTE solvent until the pH was elevated to alkaline conditions (pH 10.5). Under these conditions small amounts of a 40,000 dalton protein were observed by polyacrylamides gel electrophoresis. This may represent the initiation of dissociation of the skin. Incubation of the skin preparation (SDS-DTE, pH 10.5) at 100°C for 10 minutes resulted in the observation of a 20,000 dalton protein rather than the 40,000 dalton protein by polyacrylamide gel electrophoresis. These data would indicate that the skin is composed of a 20,000 molecular weight protein which is probably in the form of a dimer (40,000 daltons), held together by either strong covalent bonding. Excessive heat under alkaline
conditions will break strong disulphide bonding in the form of carbohydrate linkages (see section 5.4.2) or strong hydrophobic bonding (Thompson and Murray, 1982).

The crystal subunit was composed of a major 135,000 dalton glycoprotein and several minor proteins. The source of the minor proteins observed by polyacrylamide gel electrophoresis is not known. Contamination during extraction procedures, protein aggregation, (Aronson and Tillinghast, 1976) or the action of endogenous proteases (Chestukhina et al., 1980) could all account for these minor proteins seen by polyacrylamide gel electrophoresis. Frequently, a major protein 130,000 daltons was observed which seemed to be dependent on the amount of protein loaded onto the gel. A 90,000 dalton protein was also seen accompanying the major 135,000 dalton protein. It may be that these represent alternate forms of the major 135,000 dalton protein as described in section 5.1.4.

The different proteins of the inclusion formed in sporulating and non-sporulating cells may represent a change in the inclusion structure during maturation of the spore. Possibly, the cystine exchange reaction postulated by Aronson and Fitz-James (1976) as the protein-altering factor in spore coat development may also be involved in the final stages of inclusion formation. Exactly what phenomenon could account for the generation of new proteins in the inclusion of sporulating cells remains unknown.
Studies of the inclusion variants L07 "donut" and L08-42 "core crystal" were pursued with the aim of biochemically dissecting inclusion formation. The results here indicate that the core crystal formed during late vegetative growth was composed of a 135,000 dalton protein which was similar if not identical to the 135,000 dalton wild-type crystal protein. However, the solubility differences of the two inclusions indicate structure differences between the core crystal and the wild-type crystal. The 135,000 dalton protein of the mature wild-type crystal may be altered by the same cystine exchange reaction during sporulation, since the core crystal is formed during late vegetative growth prior to sporulation. The core crystal of the bipartite inclusion of L07 "donut" was also composed of a similar 135,000 dalton protein and therefore, is probably the same protein formed as the first event in inclusion development. However, as well as the major 135,000 dalton protein, a variety of medium-sized proteins were also observed, which probably correspond to the medullary region of the donut inclusion. From both the structural analysis and the polyacrylamide gel electrophoresis, the medullary region appears to contain precursor proteins which were not incorporated into the crystal. It is unlikely that the medium-sized proteins are precursors of the skin protein, since a small 20,000 dalton protein was observed as the major polypeptide in the skin preparation. The effect on inclusion formation of sodium sulphite furnishes further
evidence for this precursor hypothesis. The L07 "donut" variant may have a genetic lesion linked to sporulation which blocks the polymerization of the medium-sized proteins into the 135,000 dalton major protein of the mature crystal.

The morphological and biochemical analysis of the inclusion variants, L07 and L08-42, presented here suggests that there are two genetic loci used for the synthesis of the inclusion in *B. cereus* subspecies *medusa*. One is vegetative located on the chromosome and the other is sporulative located on the plasmid. The sporulative pathway includes first, the maturation of the 135,000 dalton crystal protein by an unknown polymerization process possibly similar to the cysteine exchange reaction in spore coat formation (Aronson and Fitz-James, 1976). Secondly, the final laying down of the fibrous skin around the outside of the crystal. Evidence for this dual loci hypothesis is furnished by the fact that the crystal of wild-type was composed of a 135,000 dalton glycoprotein which had the same molecular weight as the core crystal protein but differed in solubility. Unusual disulphide bridging could alter the structure of the 135,000 dalton protein of the cuboidal core crystal which formed during late vegetative growth to form a mature crystal with a different tertiary structure. This presents a phenomenon in inclusion formation not previously described. Unlike *B. cereus* subspecies *medusa*, all *B. thuringiensis* subspecies form an inclusion entirely during sporulation (Aronson and Fitz-James, 1976). Yet, it has
confirmed this observation and both groups showed a heterogeneity of the major proteins of *B. thuringiensis* subspecies *kurstaki* and subspecies *israelensis*.

Tyrell et al., (1981) showed that the major component of the inclusion was a 26,000 dalton protein. The results shown here report a more detailed structural analysis on both the light and electron microscopic level. The inclusion was found to be unique and more complex than previously reported (Tyrell et al., 1981; Huber et al., 1981). Solubility studies, observed by phase contrast microscopy and electron microscopy, revealed two different segments of the inclusion as well as a thin skin. The dark segment, soluble at pH 10.5, was composed of two polypeptides (70,000 and 35,000 dalton) which do not contain the toxic moiety (Fitz-James, unpublished). The 70,000 dalton polypeptide may be a dimer of the 35,000 dalton polypeptide. The function of this non-toxic segment of the crystal is unknown. However, it may be that the dark segments are portions of the whole crystal in which the toxic molecule has not yet been inserted into the (145,000, 135,000 dalton) major protein subunit. This explanation would account for the dark segment (35,000-70,000 dalton proteins) being more osmiophilic and more heavily stained by uranyl acetate and lead citrate than the light segment.

The lighter stained segment has a different macromolecular structure, solubility, protein composition and contains the toxic moiety (Fitz-James, unpublished).
been shown that the two processes are partially independent and that the inclusion can be formed in a stage II blocked asporogenous cell (Meenakshi and Jayarman, 1979). The exact relationship between crystal and spore formation remains obscure. However, there does seems to be a regulatory system involved in crystal formation which is sporulation dependent.

Type IV describes B. thuringiensis subspecies israelensis which contain an ovoid inclusion which has two components. The crystal is surrounded by a thin skin and is toxic to dipteran larva. The crystal is differentially soluble into at least two components which have a different macromolecular structure as well as different protein composition. The one component is composed of a 35,000 dalton protein, and the other component is composed of a 25,000 and 26,000 dalton complex, which are probably the monomers of the 135,000 proteins and 145,000 dalton. A recently identified (Ohba and Aizawa, 1979) subspecies within this type may be B. thuringiensis subspecies kyushuensis.

Since B. thuringiensis subspecies israelensis has been only recently isolated (Goldberg and Margalit, 1977), the inclusion has been studied by only a few previous workers. Previous electron microscopic studies of the cells of B. thuringiensis subspecies israelensis revealed an ovoid inclusion accompanying the spore (déBarjac, 1978a; Bulla et al., 1980; Tyrell et al., 1981). Huber et al., (1981)
The light segment which dissolved at pH 9.2 in the SDS-DTE solvent is composed of multiple polypeptides (145,000; 135,000; 90,000; 86,000; 49,000; 47,000; 26,000; 25,000).

The higher molecular weight polypeptides have an arithmetic relationship to the smaller proteins. Therefore, the 145,000; 135,000; 90,000; 86,000; 49,000; and 47,000 dalton proteins may be concatomers of the smaller 25,000, 26,000, dalton proteins due to aggregation or incomplete dissociation. Since the 25,000, 26,000, dalton are the most abundant proteins in the inclusion, these may either be the toxin or contain the toxic moiety.

Fast and Milne (1979) reported that the toxic molecule was 80,000 daltons in the inclusion of B. thuringiensis subspecies kurstaki when the chaotropic agent 1 M KSCN was used as the solvent. Therefore, to compare the proteins produced by 1 M KSCN and SDS-DTE, the inclusion of B. thuringiensis subspecies israelensis was also subjected to the dissolution procedure of Fast and Milne (1979). The results reported here show that this chaotropic agent dissolved the inclusion in a different manner than the SDS-DTE solvent. The KSCN solvent reduced the amount of high molecular protein weight molecules and produced more polypeptides. The 145,000 and 135,000 dalton proteins were virtually absent in the presence of 1 M KSCN. Yet the 25,000 and 26,000 dalton proteins were still present as major proteins along with a substantial number of minor
### TABLE XIII

Comparison of the Proteins in the Inclusions of *B. thuringiensis kurstaki, finitimus, schwetzova, alesti, israelensis* and *B. cereus* subspecies *lewinia, medusa.*

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Toxicity</th>
<th>Shape</th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>alesti</td>
<td>lepidopteran</td>
<td>bipyramidal</td>
<td>135K, 130K pH 8.0</td>
</tr>
<tr>
<td></td>
<td>(Tumanoff and Vago 1951)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>finitimus</td>
<td>none</td>
<td>bipyramidal</td>
<td>pH 7.6 130K, 116K, 107K, minor proteins 107K - 23K</td>
</tr>
<tr>
<td></td>
<td>(Heimpel and Angus 1958)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kurstaki</td>
<td>lepidopteran</td>
<td>bipyramidal with bead</td>
<td>135K - crystal pH 7.5, 68K - bead pH 9.2</td>
</tr>
<tr>
<td></td>
<td>(Dulmage 1970)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>schwetzova</td>
<td>lepidopteran</td>
<td>bipyramidal with bead</td>
<td>135K, 110K, 105K pH 8.5, 68K pH 9.2</td>
</tr>
<tr>
<td></td>
<td>(Tumanoff and Vago, 1951)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Aronson and Fitz-James, 1976)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>medusa</td>
<td>none</td>
<td>spherical with skin</td>
<td>135K, 130K pH 9.2, 23K- skin pH 10.5</td>
</tr>
<tr>
<td></td>
<td>(Gillespie 1969)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(de Barjac 1978)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
polypeptides in the presence of 1 M KSCN. This would support the postulate of the toxic moiety being located on the 25,000 and/or 26,000 dalton proteins.

4.1.4 Model for the Subunit Protein

Chestukhina et al., (1982) have recently shown that limited proteolysis of the inclusion protein of _B. thuringiensis_ subspecies _alesi_, subspecies _schwetzova_, subspecies _tolworthi_, and subspecies _insectus_ by certain proteases (subtilisin, trypsin and chymotrysin) results in the cleavage of the 135,000 dalton protein into a stable N-terminal peptide fragment (85,000 to 65,000 daltons depending on subspecies) and a C-terminal labile fragment (65,000 daltons) which is stepwise cleaved into 15,000 dalton fragments by further proteolysis with trypsin. The N-terminal domain contains the toxic moiety. Similar cleavage of the proteins was observed in this study by limited proteolysis of the inclusion of _B. thuringiensis_ subspecies _kurstaki_ and _B. cereus_ subspecies _medusa_ by V-8 protease).

Although not conclusive, the evidence presented in this study along with the work of Chestukhina et al., (1982) suggests a working model for the protein subunit of the various parasporal inclusions. The octahedron-type (bipyramidal) inclusions are composed of a 135,000 dalton glycoprotein which consists of two domains, each of which serves a particular purpose (Fig. 9). The 135,000 dalton protein molecule consists of a variable (subspecies
dependent), C-terminal domain, used as a protector moiety of the molecule through tertiary structure and a constant, N-terminal domain that contains a small peptide (5,000 daltons) in the primary structure which is the toxic moiety. The toxic moiety is released from the 68,000 N-terminal domain either by proteolysis active at pH 11.0 or by alkaline hydrolysis. The small 5,000 dalton peptide toxin concept is suggested by the protein composition of *B. thuringiensis* subspecies *finitimus* as well as the presence of a 130,000 dalton minor protein in the other toxic inclusion. *B. thuringiensis* subspecies *finitimus* which is non-toxic, formed an inclusion composed only of a 130,000 dalton protein devoid of any 135,000 dalton protein. The inclusion of the other subspecies contained two proteins at 130,000 and 135,000 daltons. These two proteins could be due to all of the crystal-forming proteins (135,000 daltons) not containing the toxic moiety. Those that do are 135,000 daltons and the non-toxic proteins that do not are 130,000 daltons. Furthermore, this hypothesis could also account for the variation in the characterization of the subunit proteins observed by other workers. The C-terminal variable domain of the protein could be susceptible to endogenous proteases which produce multiple polypeptides.

This model could also account for the formation of accessory bodies in *B. thuringiensis* subspecies *kurstaki* and subspecies *schwetzbeva* (Fig. 94). The 68,000 dalton N-terminal domain containing the toxic moiety may be
Figure 94. A working model of the protein structure of the subunit in the crystal and bead of *B. thuringiensis* subspecies *kurstaki*. The bead is composed of a peptide segment (68,000 daltons) of the crystal-forming protein (135,000 daltons). The toxin is a small (5,000 daltons) peptide located in the primary structure of the N-terminal (68,000 daltons) peptide fragment.
overproduced, caused by a mutation in the gene for the 135,000 dalton protein. This polypeptide (68,000) by itself is unable to form the bipyramidal crystal and therefore forms an ovoid inclusion affixed to the side of the crystal.
4.2 Plasmids in Inclusion-Forming Bacilli

*B. cereus* subspecies *medusa* contained at least three plasmids and a fourth large circular piece of DNA which may be the \( \phi -2 \) genome described by Hendry *et al.* (1976). The three plasmids were compared by restriction endonuclease mapping and shown to be unique. The two smaller plasmids (3.4 and 6.1 Mdal.) remain cryptic and their biological significance remains unknown. A search for antibiotic resistance and other phenotypic markers (e.g. colony pigment, colony morphology) proved unfruitful. However, the results do indicate that the gene coding for the 20,000 dalton skin protein resides on the large 12.9 Mdal. plasmid. Since the skin is only formed during sporulation, it would appear that the regulation of this plasmid may be sporulation dependent. Also, circumstantial evidence presented here suggests that the core crystal is coded for by a gene located on the chromosome. If these findings are confirmed through future studies, then this hypothesis would readily explain the current dilemma of the location of genetic loci (Klier *et al.*, 1980) for the parasporal inclusion. The gene(s) for the inclusion may reside on both a plasmid and the chromosome and spore dependent regulatory systems may be involved in expression of these genes.

Although there are numerous reports of asporogeny produced by treatment of the cells by acridine orange (Rogolsky and Slepecky, 1964; 1968; Bott and Davidoff-Abelson, 1966; Fitz-James, 1955), the results here show no
apparent relationship between plasmid DNA and sporulation. The variant L06-55 which was induced through growth at 55°C to become asporogenous contained the same plasmids as the wild type. Likewise, the asporogenous mutants produced by acridine orange or nutritional variation showed no change in the plasmid DNA content. Thus, the results presented here are not consistent with the hypothesis of Jacob, Schaeffer and Wollman (1960) that the spore genome is episomal.

The L013 "bead" variant, however, was shown to contain a new plasmid of 7.0 Mdal molecular weight. Its origin is puzzling since the size of the other plasmids did not decrease, therefore, eliminating the possibility of a mini-plasmid phenomenon similar to that found by Round (Round et al., 1966; 1975) in Proteus. The shape and size of the cell may also be effected by the presence of the new 7.0 Mdal. plasmid. The size of the cells of L013 was dramatically reduced from that of the wild-type. The cell wall of the L013 variant was altered (as indicated by its sensitivity to penicillin and lysozyme) as was capsule formation. The relationship between the plasmid DNA and the alterations in the structure of the cell is obscure but perhaps these alterations are conducive to transformation of the cell by plasmid DNA.

The results presented here suggest that the L07 "donut" inclusion is the result of a mutation of the gene coding for the crystal and is not the result of a change in the plasmid DNA. The "core crystal" variants (L08-42, L09, L010) lost
the largest 12.9 Mdal. plasmid. One of these variants (L09) contained the presumptive phage DNA and two (L08, L010) variants did not contain the phage. The phage DNA, therefore, does not appear to be associated with inclusion formation.

The plasmid DNA studies presented in this study are consistent with the notion that _Bacillus thuringiensis_ is a pathogenic variant of _Bacillus cereus_ (Smith, Gordon and Clarke, 1952). The two strains of _B. cereus_ which were examined for plasmid DNA contained two plasmids in the case of strain T and no plasmid DNA in the case of strain N. The large plasmid, approximately 40-70 Mdal., was also observed in several of the subspecies of _B. thuringiensis_. These results are also consistent with the findings of Klier et al., (1980) Gonzales and Carelton, (1980) and Gonzales et al., (1981) who also found large amounts of plasmid DNA in _B. thuringiensis_, and several of the subspecies contained extremely (40-70 Mdal.) large plasmids.

What is the significance of large amounts of plasmid DNA _B. thuringiensis_? There are very few examples of bacteria which contain large amounts of plasmid DNA. _Erwinia stewartii_ contains at least twelve plasmids (Kado et al., 1981) and _Bacillus megaterium_ contains eleven different plasmids (Gonzales and Carelton, 1980). It is plausible that plasmid DNA may confer the ability to form the insecticidal inclusion on _Bacillus cereus_ which would give a selective advantage to the recipient over those species
which only sporulate. Therefore, assuming this hypothesis to be correct, *Bacillus anthracis* must also be a pathogenic variant of *B. cereus*. Several similarities were found between the results in this dissertation and previous work on *B. anthracis* which are consistent with this hypothesis. Both *B. cereus* subspecies medusa and *B. anthracis* are unstable when grown on sheep blood agar. Blood increases the virulence of *B. anthracis* (Smith and Keppie, 1955). Growth of *B. anthracis* (Chu, 1952), *B. cereus* subspecies medusa and *B. thuringiensis* at 42°C results in the loss of pathogenicity with a high frequency. *B. thuringiensis* and *B. anthracis* are morphologically indistinguishable from *B. cereus* when cured of their pathogenicity. Finally, the non-toxic strains of *B. anthracis* which are cured by growth at 42°C have lost a single plasmid (10-15 Mdal) (Insell and Fitz-James, unpublished results; Mikesell et al., 1982).

In conclusion, the inclusions of the various subspecies of *B. thuringiensis* exhibit marked differences in structure and composition. These differences can be used to categorize the various subspecies instead of serological differences. The gene for the inclusion protein is probably on a plasmid but may also exist on the chromosome. The regulation of these genes is complex but appears to be sporulation dependent. Plasmid DNA may confer the ability to form the inclusion on *Bacillus cereus* consistent with the notion set forth by Smith Clarke and Gordon (1952).
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