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A Group Of Phage-like-particles In Three Subspecies Of Bacillus Thuringiensis And Bacillus Medusa

Anthony Chee-wah Tam

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A Group of Phage-Like-Particles in Three Subspecies of *Bacillus thuringiensis* and *Bacillus medusa*

Anthony Chee-Wah Tam

Department of Biochemistry

Submitted in partial fulfillment
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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TO WHOM IT MAY CONCERN:

This is to permit the paper "Bacteriophage and bacteriophage-like structures carried by Bacillus medusa and their effect on sporulation" by G.S. Hendry, J.B. Gillespie and P.C. Fitz-James and published in J. Virology Vol. 18, 1051-1062, 1976, to be reprinted as an appendix in the thesis of A. Tam.

G.S. Hendry

November 1, 1985

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J.B. Gillespie

ABSTRACT

Phage-med-1, a small phage-like-particle (approx. 22 nm in diameter) specifically found in stage II sporulating cells of B. medusa and initially reported as RNA containing was studied further. Similar particles, designated as Øisr-1, Økyu-1 and Ø10-2-1, were subsequently found in Bacillus thuringiensis subsp. israelensis (serotype 14), B. thuringiensis subsp. kyushuensis (serotype 11) and B. thuringiensis isolate 73-E-10-2 (serotype 10). These strains share the unique character of producing solely ovoid or round parasporal crystalline inclusion (cry) toxic to mosquito larvae.

Re-examination, with modifications, of the purification method of Ømed-1 developed in the initial study failed to confirm that the particles were RNA-containing. An improved purification enabled PLP close to homogenous to be isolated. Purified phage-like-particles (PLP) of the four Bacilli can be divided, by several properties, into two groups, Ømed-1 and Øisr-1 in one and the remaining two in another. Chemical and electrophoresis analyses on the purified PLP again failed to show a nucleic acid content. By SDS-PAGE analysis the PLP were composed of one major protein of molecular weight around 30 kilodaltons. These proteins were shown to be similar by limited proteolysis peptide mapping. The four PLP also immunologically cross-reacted with each other . Electron microscopic studies showed that all PLP were assembled at early stages of sporulation, while

immunoprecipitation showed that at least in B. medusa and subsp. israelensis, the PLP proteins are synthesized at the time the PLP appeared.

Examination of acrystalliferous (cry^-) variants of subsp. israelensis isolated by a 42°C curing method revealed the presence in the sporulated cells of a satellite inclusion (sat) which was subsequently found in the wild type. Cry^-sat^+ variants were Øisr-1 producing while cry^-sat^- variants were not. The latter strains also lacked a 68MDa plasmid of the wild type. A cry^+sat^- strain was also found to be Øisr-1 producing. Examination of transformed strains of a cry^-sat^- and plasmidless variant revealed that the sat^+ and Øisr-1 producing characters could have been co-transformed in a recipient strain which had acquired the 68 MDa plasmid; likewise, the cry^+ and Øisr-1 producing characters could have been co-transformed in a strain which had acquired the 75 MDa plasmid already known to be associated with the synthesis of the inclusion.

B. thuringiensis isolate 73-E-10-2 produced inclusions of varying size. The size distribution of the inclusions was observed to be influenced by the composition of the medium and the growth temperature. Inclusions prepared from cultures with higher number of small inclusions producing cells were found to have relatively less of a 25.5 K proteins and two smaller proteins. These inclusion preparations were also less toxic than those preparations with more of these three proteins. Variants producing solely small non-toxic inclusions lacking the above three proteins and a 140 K protein were

isolated by the 42°C curing method. These variants were devoid of Ø10-2-1 and at least two plasmids, a ~94 and a 8.8 MDa plasmid of the wild type.

The results of this study indicated that the genes responsible for the syntheses of Øisr-1 and Ø10-2-1 could be located on plasmids which also carried the genes determining the syntheses of components of the inclusions.

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Chapter I

Introduction and Historical Review

A. Introduction

The Bacillus is the most studied genus of bacteria after the Escherichia. Some of the reasons for the scientific popularity of the genus are as follows. The genus Bacillus is one of the two genera of bacteria that sporulate under adverse conditions. The endospores so formed are intriguing in that they are totally dormant cells able to withstand hazardous conditions. Yet to many investigators the sporulation process itself appeals as a model system for studying cellular differentiation. Bacilli are also important in the fermentation industry; some species overproduce a variety of useful enzymes and antibiotics. More to the point of this study, the entomocidal activities of the parasporal inclusions produced by the species Bacillus thuringiensis are being activity exploited in the production of effective and highly specific larvacides.

While most Bacillus studies have been directed to the physiology of the vegetative cells, gene expressions during sporulation and the composition of endospores and parasporal inclusions, other structures in the sporangia have received little attention. In an electron microscopic study of sporulated cell lysates of the peculiar Bacillus medusa, small phage-like-particles, resembling in size and outline the Escherichia coli RNA phage, were observed (Hendry et al., 1976). The small phage-like-particles appeared to be sporulation specific from

observations confirmed by studies of mutants blocked at early stages of sporulation. Subsequently, the phage-like-particles was shown to contain RNA (Hendry et al., 1976).

In continuing this study, this candidate improved the purification method for the phage-like-particles. Chemical and immunological analyses as well as polyacrylamide gel electrophoreses were applied in to these purified preparations (Chapter 2 and 3). While this work was underway, three B. thuringiensis strains were found to be carrying similar looking phage-like-particles and were included in this study. These, namely, B. thuringiensis subsp. israelensis, B. thuringiensis subsp. kyushuensis and B. thuringiensis isolate 73-E-10-2, are all mosquitocidal. Subsequently, inclusions variants were isolated from subsp. israelensis (Chapter 4) and isolate 73-E-10-2 (Chapter 5). Out of these variants, strains not producing the phage-like-particles were found and a relationship between the syntheses of the phage-like-particles and the parasporal inclusion proteins was observed in these two bacilli. Further examination of these variants and similar variants from another laboratory led to the discovery that plasmids could be involved in determining the syntheses of two of the phage-like-particles.

B. Historical Review

Preamble

This review will cover the background materials for this study; beginning in part one with a general description of the sporulation process. In part two, an introduction to the parasporal inclusion forming species, Bacillus thuringiensis will be presented. This will be followed, in part three, by a description of the parasporal inclusions and, in part four, an account of the relation between the parasporal inclusions and the extrachromosomal DNA carried by the inclusion formers. Part five will be devoted to a description of the four Bacillus species which produce the phage-like-particles. A brief description of the previous works on the phage-like-particle of B. medusa will be included. The chapter will conclude in part six with a description of some aspects of the Escherichia coli RNA phages and the Bacillus phages.

1. General features of sporulation

Members of the genus Bacillus are defined as rod shaped bacteria capable of aerobically forming endospores (Gordon et al., 1973a). A spore is a dormant cell highly resistant to heat, ultraviolet and ionizing irradiation, as well as many toxic compounds. A spore retains indefinitely its potential to germinate and develop into a vegetative cell. A healthy vegetative cell is initiated to undergo sporulation when an essential nutrient in the environment is

deficient. The cell then undergoes a series of morphological and biochemical changes resulting in a spore which persists and a sporangium which will soon disintegrate. The process of morphological changes is conventionally divided into seven stages (Fitz-James, 1963) though some of the divisions (stage IV to VII) are only approximations; many of the changes at those stages being continuous processes (Young and Fitz-James, 1959a; Fitz-James, 1963; Ellar and Lundgren, 1966). The following is a brief description of the seven stages of sporulation (see also Fig. 1). At the onset of sporulation (stage I), a mature binucleate vegetative cell fails to form a transverse septum at the middle of the cell and so generate two daughter cells. Instead, the two chromatin bodies condense and then extend longitudionally until the two fuse to form an elongated structure, the axial filament. At stage II, the plasma membrane at a subpolar region of the cell starts to invaginate inward. As the growth of the membrane continues, a complete forespore septum separates the cell into two compartments. The smaller compartment containing a portion of the axial filament is referred as the incipient forespore. Stage III commences as the membrane at the base of forespore septum grows toward the pole of the cell closer to the incipient forespore. As a result, the incipient forespore is engulfed by a newly synthesized membrane which eventually encloses the structure to form a double membraned forespore. At stage IV, material starts to deposit between the outer and inner membrane of the forespore. The material first deposited becomes the primordial germ cell wall and later the cortex is deposited against the inner germ cell wall. The exosporium, (if the spore has one), also starts to

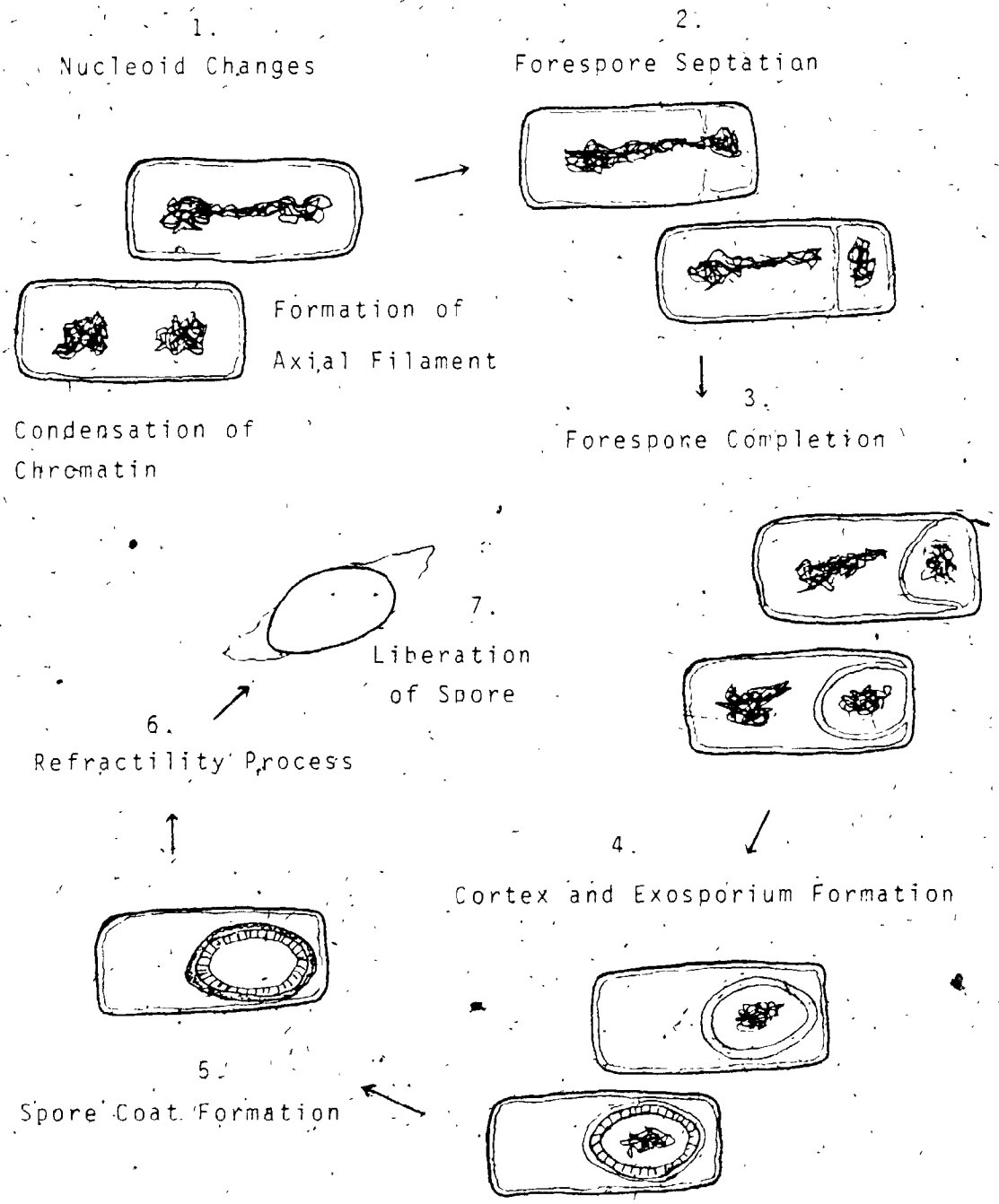


Fig. 1. The stages of spore formation, from Fitz-James (1965).

appear at stage IV. At stage V, spore coat protein is deposited on the outer membrane. Simultaneously, the spores start the refractility process. At stage VI, the cortical material and spore-coat layers complete their development along with the process of refractility. Lysis of the remaining mother cell, the sporangium, and the release of the spore into the environment mark the last stage, stage VII.

The most notable biochemical changes during sporulation are as follows. At stage I of sporulation, massive breakdown of ribosomal RNA and messenger RNA occur (Young and Fitz-James, 1959). Despite this, new messenger RNAs are being synthesized, partly from purine and pyrimidines in the medium and partly from degradation products of pre-existing RNA (Balassa, 1963). Ca^{++} ion accumulation starts to occur at stage III of sporulation (Young and Fitz-James, 1962). The Ca^{++} ions eventually end up in the protoplast of the spore (Stewart et al., 1980) and can reach a level as high as 3% of the total dry weight of the spore. At stage IV, synthesis of Dipicolinic acid (DPA) starts probably, in the developing spore. The DPA in a spore can be as much as 15% of its dry weight (review by Murrell, 1967). Ca^{++} ions and DPA are believed to be the prime factors in determining the heat resistance characteristic of the spores.

The morphological and biochemical changes during sporulation are controlled in B. subtilus by the expression of 30 or more genes scattered in clusters along the chromosome (reviewed by Piggot, 1976). The transcription of these genes is believed to be by RNA polymerases which differ from the RNA polymerases in vegetative cells in certain

enzyme subunits. Cloned sporulation genes are currently used as templates to test the specificity and activities of different types of RNA polymerase (reviewed by Losick, 1982).

2. Bacillus thuringiensis

Members of the species Bacillus thuringiensis differ from the other bacilli in that, upon sporulation, they form one, or in some strains two crystalline protein inclusions adjacent to each spore. Many of these inclusions are toxic to insects and are referred as δ-endotoxin (Heimpel, 1967a) although it has been suggested that only the toxic components of the inclusions should be thus called (Bulmage, 1979). Taxonomically, Bacillus thuringiensis and the much studied Bacillus cereus and Bacillus subtilus are in the group I division of Bacilli because of their similarities in having oval spores located in the central positions of sporangia which walls are not distended by the spores (Gordon et al., 1973b), B. thuringiensis and B. cereus are distinct from B. subtilus by having larger vegetative cell sizes, different nutritional requirements and exosporia. Doubts have been raised whether the parasporal inclusion forming-Bacilli as a group deserve a species status or they should be regarded as a subspecies of B. cereus as suggested by Smith et al., (1952). Investigators favoring the classification of the inclusion forming bacilli under B. cereus argued that the formation of the inclusions was an unstable character; non-inclusion forming variants could readily be isolated (review by Heimpel, 1967b; Krieg, 1969a). In addition, physiological characteristics of the inclusion-producing bacilli and B. cereus were

very similar (Gordon et al., 1973c). However, this view was not generally accepted by the insect pathologists who maintained that the name B. thuringiensis had already been widely adopted by investigators such that a change in the nomenclature would result in confusion (Heimpel and Angus, 1958). Thus in most current literature, the name B. thuringiensis is used. Members within the species thuringiensis having characteristics distinct from the original members are refeired as varieties or subspecies.

In 1963, an extensive study of 24 subspecies of B. thuringiensis showed that these strains could be subdivided into six "biochemical groups" and this division was supported by the presence in the strains of a flagellar protein (H-antigen) which was group specific (review by de Barjac and Bonnefoi, 1968). In another study, electrophoresis analysis of the esterase isolated from the cells of subspecies B. thuringiensis showed that they could be grouped by means of their esterase patterns and such subdivision corresponded closely to that defined by means of flagellar antigens (Norris, 1964). Since then, classifications of subspecies of B. thuringiensis into H-antigen serotypes have been generally adopted by investigators as a means of reflecting their phylogeny. In a course of twenty years, hundreds of new subspecies of B. thuringiensis have been isolated and the number of serotypes has been expanded to 19. Subdivision within the serotypes have also been found necessary.

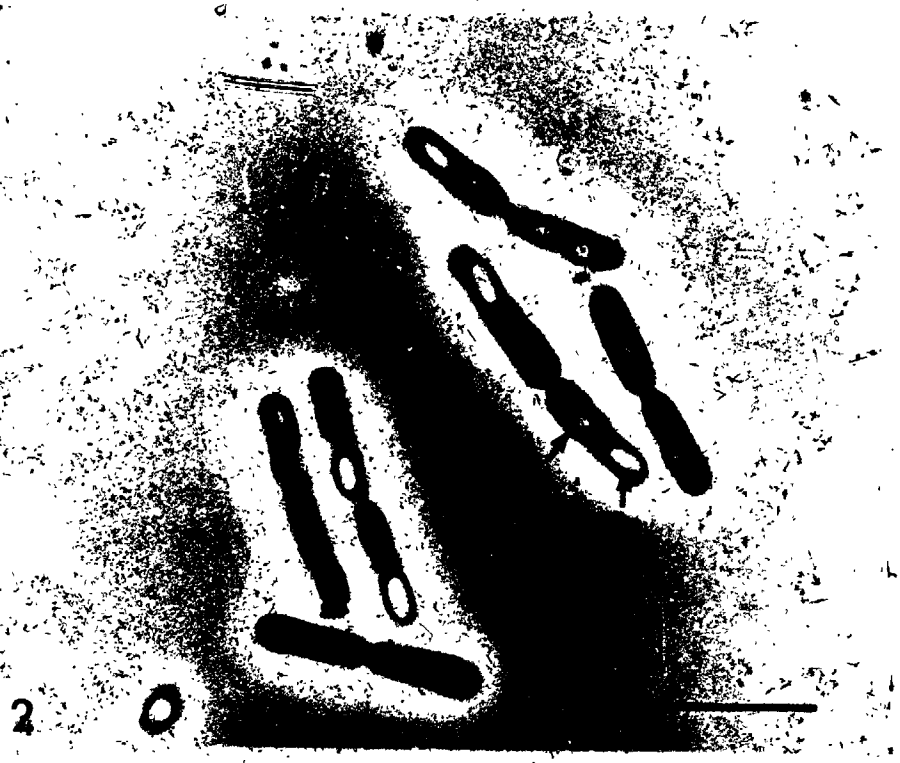
3. General features of parasporal inclusions

Most of the B. thuringiensis produce bipyramidal crystalline inclusions toxic to lepidopteran larvae (Fig. 2). Other subspecies produce tetrahedral, ovoid and ovoid with irregular outline inclusions, the latter two being toxic to mosquito larvae. In some strains which produce bipyramidal inclusions, a small ovoid or cuboidal inclusion is also produced (Fitz-James and Young, 1969; Bechtel and Bulla, 1976). These small inclusions of some strains have been found to be slightly toxic to mosquito larvae (Insell, 1983; Iizuka and Yamamoto, 1983). Electron microscopic study of some subspecies of B. thuringiensis showed that the inclusions were formed at the beginning of stage III when engulfment of the incipient forespores had just started (Young and Fitz-James, 1959b; Bechtel and Bulla, 1976). The inclusions were found to be formed first in close association with the forespore membrane. With the exception of B. thuringiensis subsp. finitimus, the inclusions eventually separate from the developing forespore such that they are not included by the developing exosporium. Kinetic studies on several subspecies revealed that the inclusion proteins were synthesized at the same times the inclusions appeared (Sommerville, 1971; Herbert and Gould, 1973). Though similar kinetics experiments have not been generally carried out on newly isolated subspecies, it can be assumed that the above finding is true for most. Expression of genes coding for inclusion proteins is believed to be under sporulation control. Studies have

Fig. 2 Phase-contrast micrograph of sporulated cells of B. thuringiensis subsp. alesti. Note the phase refractile spores (Sp) and the bipyramidal parasporal inclusions (I) adjacent to the spores.

Fig. 3 Phase-contrast micrographs of B. medusa. (a) Cells at late vegetative stage. Note the primordial inclusions. (b) Cells at late sporulation stage. Note the refractile spores (Sp) and the large inclusions (i).

The magnification is indicated by the 5 μm bar marker in Fig. 2.



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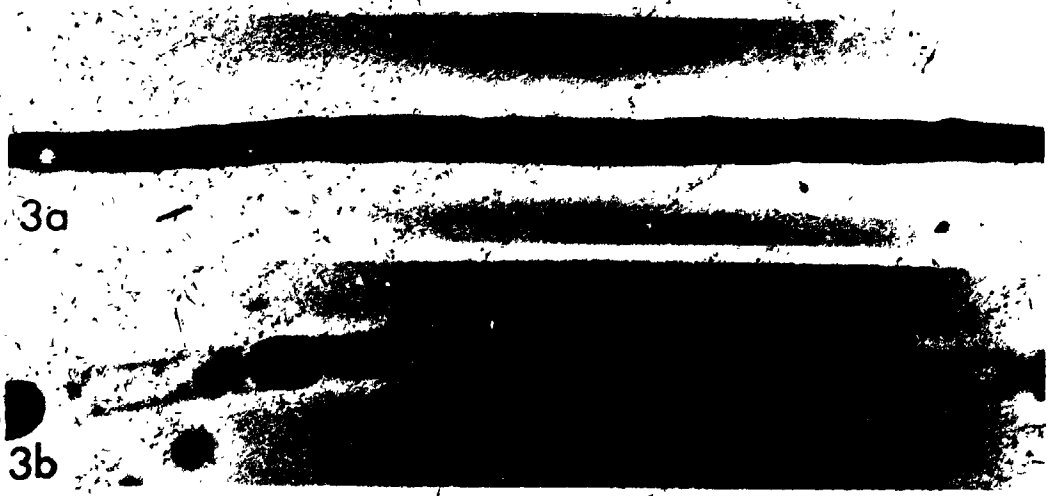

3a

3b

shown that the transcription of the inclusion protein gene of subsp. Berliner was by a form of RNA polymerase not found in the vegetative cells of the strain (Klier et al., 1973; Klier et al., 1983).

The inclusions are composed of proteins or glycoproteins (Hannay and Fitz-James, 1955; Bulla et al., 1977). The most reliable method in determining the number and sizes of protein species making up an inclusion has been shown to be SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis of the inclusion proteins solubilized in SDS plus a sulfhydryl reducing agent, either mercaptoethanol or dithiothreitol. With this method, bipyramidal crystals of a number of subspecies of different serotypes were analyzed and they were shown to contain one major protein of molecular weight of 130,000 daltons in one report (Huber et al., 1981) and of 135,000 daltons in another (Tyrell et al., 1981). However, in another study involving the inclusions of 16 subspecies, smaller proteins of molecular weight ranging from 60,000 to 40,000 daltons were found to be present in the inclusions in considerable magnitude (Calabrese et al., 1980). Based on the protein profiles, the inclusions were classified into six groups. It was shown that inclusions produced by subspecies of the same serotypes did not always fall into the same group.

Similarities among the proteins making up the lepidocidal inclusions of different subspecies have first been demonstrated serologically. Antisera raised against the inclusions from three subspecies of B. thuringiensis were found to cross react with all the inclusion proteins of eleven subspecies. The only inclusion proteins that did not react with the sera in that study was that derived from

subsp. finitimus (Krywienczyk and Angus, 1967). In the study conducted by Tyrell et al., (1981), tryptic peptide fingerprints of the inclusion proteins derived from four subspecies of B. thuringiensis were found to be very similar. In another study, high performance liquid chromatography (HPLC) was used to analyse tryptic peptides generated from the purified 135,000 dalton inclusion proteins (referred in the report as P-1 proteins) derived from twenty strains of B. thuringiensis (Yamamoto, 1983). The study showed that HPLC profiles of the P-1 proteins of some strains were very similar to each other but were less similar to that of other strains. The HPLC profiles of some strains belonging to the same serotypes were found to bear many differences. Hence the nature of the inclusion proteins did not correlate very well with the antigenicities of the flagella among the B. thuringiensis.

The structural stability of the parasporal inclusions has been attributed to intermolecular disulfide linkages and noncovalent intermolecular forces (Huber et al., 1981). Investigators working on the actions of inclusion proteins toward insects encountered difficulties in dissolving the inclusions without denaturing and thus lowering the biological activities of the proteins. In addition, protease activity has been shown to be active in solubilized preparations of inclusion of B. thuringiensis subsp. kurstaki at high pH (Bulla, 1977). It was suggested that protease activity might degrade the proteins during dissolution of the inclusions in mild solvents and in subsequent manipulations. The very small peptides reported in a number of inclusions could, in fact, be the degradation products of proteases (review by Bulla, 1980). Nevertheless, Huber et

al., (1981) reported that soluble active toxins (with 95% of the activity of native inclusions) could be obtained from inclusions of a number of lepidocidal inclusions by using a non-alkaline solvent consisting of high concentration of guanidine, plus dithiothreitol and disodium ethylenediaminetetraacetate (EDTA).

Once in the midguts of insects, the ingested inclusions are solubilized by the high pH and gut proteases. The liberated toxins then cause a disorganization of the midgut epithelium and a loss of regulation of potassium ion transport (Cooksey, 1971). Paralysis of the guts and often the entire larvae eventually brings death to the insects. The biochemical actions of the toxins are just beginning to be understood (Thomas and Ellar, 1983b; Knowles et al., 1984).

4. Relation of inclusions and plasmids.

Most of the Bacillus plasmids are cryptic elements without known functions. The plasmids housed by a strain of B. thuringiensis subsp. gallerie were first suspected to be related to the synthesis of the inclusion in a study which showed that the inclusion formation was much reduced in minimal medium. In such cultures, plasmids that were normally present in an enriched culture could no longer be detected (Ermakova et al., 1978). While this work has not been elaborated, another study showed that acrystalliferous (non-inclusion producing) variants isolated from B. thuringiensis subsp. kurstaki HD-1 by heat treatment of spores prior to germination lost all plasmids of the parental wild type (Stahly et al., 1978). However, other investigators showed that loss of inclusion production was not

necessarily accompanied by the loss of all plasmids (González et al., 1981). An analysis of acrystalliferous variants isolated from strains of B. thuringiensis carrying different complex arrays of plasmids showed that only one comparatively large plasmid was likely to be associated with the crystal production in each strain. Strong evidence showing that a plasmid was indeed responsible for determining inclusion production arose when fragment(s) of a large plasmid of B. thuringiensis subsp. kurstaki HD-1 were inserted into the cloning vector pBR322. After being transformed into a strain of E. coli, the plasmid induced the bacteria to form a 130,000 dalton lepidocidal protein reacting with the antiserum raised again the kurstaki inclusion (Schnepf and Whiteley, 1981). In a similar experiment, a fragment of DNA derived from total cellular DNA of subsp. kurstaki HD-1 and containing the gene determining the synthesis of the inclusion protein was cloned. The cloned DNA hybridized with a 45-kilobase-pair plasmid (approximately 29 megadalton) housed by the strain as well as with chromosomal DNA (Held et al., 1982). This indicated that the gene determining the synthesis of the P-1 (adopting Yamamoto's terminology) inclusion protein was present on both a plasmid and the chromosome of subsp. kurstaki HD-1.

Recently, González and Carlton (1982) discovered that plasmids of B. thuringiensis could be transmitted at a high frequency between two strains when grown in mixed culture. By allowing transfer of plasmids from a crystalliferous strain to an acrystalliferous strain in a mixed culture, they were able to obtain crystalliferous transcipients (recepients which had acquired one or more plasmids). The transformation experiments confirmed their previous work (González

et al., 1981) and showed that a 75 megadaltons (MDa) plasmid was indeed associated with inclusion production in B. thuringiensis subsp. thuringiensis HD-2 and a 50 MDa plasmid with inclusion production in subsp. kurstaki HD-73. When the 75 MDa plasmid of subsp. thuringiensis HD-2 was transformed into an acrystalliferous strain of kurstaki HD-73, the crystal produced by the transcipient was of the type produced by subspecies thuringiensis HD-2 and not that of subspecies kurstaki HD-73. This indicated that the 75 MDa plasmid of subspecies thuringiensis HD-2 carried the structural gene for the inclusion protein rather than a regulatory gene. More recently, a 75 MDa plasmid has been found to be associated with the synthesis of the mosquito killing inclusion of B. thuringiensis subsp. israelensis (González and Carleton, 1984). This will be discussed further in Chapter 4.

In continuing the study of Schnepf and Whitely (1981), Kronstrad et al., (1983) used an intragenic restriction fragment from the cloned crystal gene of subsp. kurstaki HD-1 to hybridize with plasmid preparations from 22 strains of B. thuringiensis. They found that the DNA fragment hybridized to a single plasmid in 8 strains, to more than one plasmid in 7 strains and to one or both of two large unresolved plasmids in two strains. The sizes of the hybridized plasmids ranged from 33 to over 150 MDa. No hybridization was found to plasmids of the mosquitocidal subsp. israelensis and subsp. kyushuensis. This study revealed the diversity in locations of the structural genes coding for the P-1 inclusion proteins among the B. thuringiensis subspecies.

5. The Bacilli which produce phage-like-particles

a. Bacillus thuringiensis subsp. israelensis

B. thuringiensis subsp. israelensis was isolated in Israel in 1977 (Goldberg and Margalit, 1977). This strain has an flagellar H-antigen different from the then existing 13 serotype antigens and was thus assigned a new serotype——serotype 14. The parasporal inclusion of the strain differ from that of most of the B. thuringiensis by being ovoid with irregular outline. The occurrence of two inclusions in a sporulating cell is not unusual (Tyrell et al., 1981). The inclusion is non-toxic to lepidotera but highly toxic to certain dipteran larvae, notably, that of mosquito and blackfly (de Barjac, 1978). A study has shown that the LC_{50} (concentration that will be lethal to 50% of the larvae in a defined time) of purified inclusions toward the mosquito, Aedes aegypti, 3rd instar larvae is 5-50 ng protein/ml (Thomas and Ellar, 1983a). Unlike most of the bipyramidal crystals that are composed of one major protein (P-1 proteins), the inclusion of subsp. israelensis consists of multiple species of protein with molecular weights of 98, 93, 68, 67, 52, 45, 28 and 15 kilodaltons in one report (Thomas et al. 1983a) and 140, 135, 70, 68, 53, 40, 38 and 28 kilodaltons in another study (Pfannenstiel et al., 1984). The inclusion proteins did not cross react with antiserum raised against the crystals of several lepidocidal subspecies B. thuringiensis tested and the tryptic peptide fingerprint of the ovoid inclusion was also different from that of the bipyramidal crystals (Tyrell et al.,

1981). In the study carried out by Thomas and Ellar (1983a), the ovoid inclusions were partially solubilized in 50 mM $NaCO_3$.HCl (pH 10.5), and the solubilized proteins were found to cause rapid cytological and cytopathological changes in several dipteran, lepidopteran and mammalian tissue cell lines. In addition, the solubilized proteins were shown to cause haemolysis of mammalian erythrocytes and at a certain dose rate, can cause death when injected to mice. However, the LC_{50} of the solubilized inclusion preparation toward mosquito larvae was found to be 100 times higher than the native inclusions i.e. much less toxic. A recent study showed that the plasma membrane could be the target of the action of the toxin though it is not known which of the proteins composing the inclusion is the active component (Thomas and Ellar, 1983b).

b. Bacillus thuringiensis subsp. kyushuensis and Bacillus thuringiensis isolate 73-E-10-2

B. thuringiensis subsp. kyushuensis was isolated in Kyushu island, Japan (Ohba, 1979). Its inclusion is also ovoid with irregular outline. Spore and inclusion mixtures were found to be toxic to mosquito larvae tested but not to lepidopteran larvae. Biochemically, the strain is very similar to B. thuringiensis subsp. toumanoffi, serotype 11 which produces a bipyramidal crystalline inclusion toxic to lepidopteran larvae and was isolated in Paris, France (Krieg, 1969b). The flagellar H-antigen of subsp. kyushuensis cross reacts with antiserum against the H-antigen of subsp. toumanoffi yet after saturation of the antiserum with H-

antigen of subspecies kyushuensis, a residual agglutinin to the H-antigen of the toumanoffi strain was found in the serum. This showed that subsp. kyushuensis should be grouped into serotype 11 but should be in a subtype other than that of subsp. toumanoffi.

B. thuringiensis isolate 73-E-10-2 and its close relative 73-E-10-16 were isolated from Shikoku, Japan (Padua et al., 1980). These two strains produce round inclusions. Spore and inclusion mixtures were also found to be toxic to mosquito larvae but not to silkworms. Their flagellar H-antigen was found to be identical to that of B. thuringiensis subsp. darmstadiensis, serotype 10, isolated from Darmstad, Germany (Kreig et al., 1968). The bipyramidal inclusion of the latter strain is toxic to lepidopteran larvae. Biochemical tests showed that the three strains have similar characteristics. So far, isolate 73-E-10-2 and 73-E-10-16 have not been assigned a subspecies name (Ohba, personal communication). Besides subsp. israelensis, subsp. kyushuensis, isolate 73-E-10-2 and isolate, 73-E-10-16, the only B. thuringiensis strain that has so far been reported to produce solely round or ovoid inclusions (with irregular outline) is a strain designated as PG-14 recently isolated from Philippines (Padua et al., 1984). This strain belongs to serotype 8 and the inclusions produced is also mosquitocidal. Strains of B. thuringiensis which have been examined for phage-like-particle production are included in the strain list (Appendix 3).

c. Bacillus medusa and its phage-like-particles

B. medusa was isolated from Cambridgeshire, England by C.F. Robinow in 1945, (Delaporte, 1969). It was distinct from B. thuringiensis in several morphological characteristics. It has a large cell size (a width of 1.6 μ as compared to a width of 1.2 μ of B. thuringiensis) and the square-ended cells do not separate from each other after cell division (Fig. 3). As a result, long chains are found in the culture. Moreover its round inclusion is produced at a late vegetative stage some four hours before stage II of sporulation and the inclusions first appear as a deposit against the membrane of a transverse septum following the final cell division (Fitz-James, 1962). However, the GC content of the DNA of B. medusa was found to be 38%, close to the range of 32-37% found in B. cereus (Gillespie, 1969). Like its group representative, B. cereus, B. medusa also produces an exosporium. The round crystalline inclusion of wild type B. medusa is covered by a net of fibrous material and is not toxic to any insect larva yet tested. The one protein composing the inclusion proper has a molecular weight of 135,000 daltons and that composing the fibers, 20,000 daltons (Insel, 1983).

An extensive study of the temperate phage in B. medusa has been carried out by G. Hendry in his doctorate study (1973) at the University of Western Ontario. The following information is derived in part from his thesis and a publication (Hendry et al., 1976) and in part from some more recent observations.

After being induced by mitomycin C or UV light, a log-phase culture of wild type B. medusa lysed and two phage structures were seen in the cell lysates. One of these, a large empty head structure completely devoid of attached or associated tail structures, was produced in copious quantity by all strains of B. medusa examined and measured some 57 nm in diameter. The other, a slightly larger (60 nm in diameter) complete phage was found only in the original wild type. Sporulation lysates, on the other hand, contained minute polyhedral particles measuring 20-25 nm in diameter. A few of the larger inducible phage elements were also seen. The small phage-like-particle, so called Ømed-1, was found to be sporulation specific as it was not found in vegetative cells nor in the cells of a strain of B. medusa which was genetically blocked at stage I of sporulation. Ømed-1 was partially purified by differential centrifugations and treatment with ribonuclease and deoxyribonuclease. The final pellet was found to contain little DNA but to contain RNA which upon being purified was extremely sensitive to RNAse. It was concluded that Ømed-1 was a minute single-stranded RNA-containing bacteriophage or phage-like-particle. No infective activity was found with Ømed-1 but some signs of inhibition of three variants of B. medusa were found with the particles. One particular feature of Ømed-1 was that quite a number of them were found to be attached to sheet-like structures of unknown origin in the sporulated cell lysates and to fibers covering the inclusions.

6. Escherichia coli RNA phages and some aspects of Bacillus phages

No temperate RNA bacteriophage or RNA containing phage-like-particles except Qbeta-1, has ever been reported. Bacteriophage containing RNA have been found for three genera of eubacteria namely, Escherichia, Pseudomonas and Caulobacter (Shapiro and Bendis, 1975). With only one exception, all the RNA phages are similar in morphology, all being icosahedral and 21-26 nm in diameter. The most studied RNA phages are those of E. coli. A phage particle of this group is made up of a capsid composed of 180 molecules of coat protein, an inner RNA molecule folded up in a well-defined way and a molecule of maturation protein. The molecular weight of the coat protein in most RNA coliphages is 14,000 daltons. In phage R17, the maturation protein was found to have a molecular weight of 38,000 daltons and the RNA molecule 10-12 megadaltons (Boedtker and Gesteland, 1975). Defective phage particles which are sensitive to RNAse have also been isolated. Repeated freezing and thawing renders RNA phages sensitive to RNAse and such treated phage particles could readily be penetrated by negative stain and hence are described as empty shells. Such shells could also be reconstituted from purified coat proteins at certain salt and protein concentrations. At lower salt concentrations and a higher protein concentration, a double shell capsid, rarely penetrated by negative strain, could be formed (review by Hohn, 1970).

No viable RNA phages have ever been found in the genus Bacillus though double stranded and single stranded DNA phage abound. Most of these phages are tailed, but a few are cubic with spikes at the vertices (review by Reanney, 1981). Temperate defective phage particles are also

commonly found in Bacilli (review by Garro and Marmur, 1970). These phage particles either contain host DNA or phage specific DNA and are unable to form plaques on any known host. None of the Bacillus phages, viable or defective, are as small as 25 nm in diameter. However, small round phage-like-particles, 19 nm in diameter, have been seen in sporulated cell lysates of a strain of B. cereus and several strains of B. thuringiensis, but no elaborate studies have been carried out as the particles were reported to be too rare (Ackermann et al., 1974; Ackermann, 1978). The only known temperate RNA virus are the retroviruses found in animals. These particles are enveloped in lipoprotein, roundish and about 100 nm in diameter.

Chapter 2

Purification and Characterization of the Phage-Like-Particles of Four Bacillus cereus - Related Bacilli

1. Introduction

The phage-like-particles of B. medusa, Ømed-1, was previously determined to be a single stranded-RNA containing phage or phage-like-particle (Hendry et al., 1976). The conclusion was based on orcinol tests and $^{32}PO_4^{---}$ labelling of partially purified preparations of Ømed-1. Because of the wide variety of structures present in the sporulated cell lysate of B. medusa, this candidate suspected information derived from assaying the partially purified preparations, including the infectivity assays, might not be too reliable. Therefore, further purification was pursued.

In the course of this study of Ømed-1, similar looking phage-like-particles (PLP) were found in B. thuringiensis subsp. israelensis, (serotype 14), B. thuringiensis subsp. kyushuensis (serotype 11), B. thuringiensis isolate 73-E-10-2 and isolate 73-E-10-16 (serotype 10). Since the latter two were very similar, only isolate 73-E-10-2 was chosen to be examined further. The PLP of these bacilli, designated as Øisr-1, Økyu-1 and Ø10-2-1, to reflect the strains in which they were found, are included in the study. This report describes a re-examination of the partial purification

procedure described by Hendry et al., (1976), an improvement of the purification procedures and the subsequent characterization of the purified PLP so obtained.

2. Materials and Methods

2.1. Bacterial strains

Bacillus medusa L06 was from the culture collection of Dr. Fitz-James, University of Western Ontario. Bacillus thuringiensis subsp. israelensis originated from the Culture Collection of Entomogenous Bacteria, Institute of Entomology, Czechoslovak, Academy of Science, Czechoslovakia as CCEB-950. Bacillus thuringiensis isolate 73-E-10-2 and subsp. kyushuensis were obtained from M. Ohba, Kyushu University, Japan. Escherichia coli K12 strain HfrP and RNA phage R17 were from W. Paranchych, University of Alberta, Canada.

2.2. Growth of bacteria and media

Growth and sporulation were by reciprocal shaking in fluid GBBM at 30°C-32°C for all bacilli except isolate 73-E-10-2 which was shaken at 28°C. GBBM is a BBM-salt mixture. BBM, the blood base medium, contained 5 gm each of protease peptones no. 2 and no. 3 and 3.2 gm of dehydrated nutrient broth per litre of distilled water. The salt solution contained 0.1 M KNO_3, 1.2×10^{-2} M KH_2PO_4, 1.0×10^{-2} M K_2HPO_4, 1.0×10^{-3} M K_2SO_4, 2.5×10^{-4} M $MgSO_4.7H_2O$, 1.3×10^{-5} M $MnSO_4.H_2O$, 2.3×10^{-5} M $FeSO_4$ and 5.0×10^{-5} M $ZnSO_4.7H_2O$ in

distilled water. The pH of the salt solution was adjusted to 6.9 with KOH before autoclaving. The final medium (GBBM) was a mixture of one volume of sterile BBM, four volumes of sterile salt solution and sterile $CaCl_2$ added to a final concentration of 1.0×10^{-3} M. GBBM was also used in a 2% agar form for monthly culture maintenance of the bacilli.

Soft agar used in the inhibition experiment was nutrient broth with 0.5% agar, 2×10^{-3}M $MgCl_2$ and 2×10^{-3}M $CaCl_2$. The base agar was nutrient broth with 1.5% agar and 2×10^{-3}M $MgCl_2$.

Strain E. coli K12 was maintained on agar slants of yeast tryptone agar which is 0.5% yeast extract, 0.5% NaCl and 1% tryptone and 2% agar in distilled water.

E. coli RNA phage R17 was raised by infecting E. coli K12 strain HfrP with the phage as described by Gesteland and Boedker (1964).

All non salt media chemicals were from Difco.

Optical density of cultures was measured on samples diluted 5 fold with saline (0.14 M NaCl) in a Coleman Universal Spectrophotometer (Model 14) at 645 nm.

2.3. Buffers and materials

Three main buffers used in this study were (1) PLP buffer, 0.1 M NaCl, 5×10^{-3} M $MgCl_2$ and 0.01 M Tris(hydroxylmethyl)aminomethane. HCl, pH 7.2; (2) SSC buffer, 0.15MNaCl and 0.015 M sodium citrate; (3)

RNA electrophoresis buffer, 36 mM Tris, 30 mM NaH_2PO_4 and 1 mM Na_2EDTA pH 7.8. A sample of tRNA was from Dr. G. A. Mackie of the Dept. of Biochem. U.W.O.

2.4. Morphological observations

Phase-contrast microscopy of cover-slip smears with oil immersion optics was used for routine examination. Photomicrographs were taken with a bellows camera at 2000 magnification onto tri-X pan film (Kodak). For negative stain electron microscopy, a loopful of sample mixed with a similar volume of 1% phosphotungstic acid (pH 5-6.5, adjusted with KOH) was deposited on the center of a formvar coated grid (100 mesh). After removing excess liquid with a pointed wick of filter paper, the rim of the dried sample was examined in a Phillips EM 300.

2.5. Electron microscopic detection of PLP

Cells from 20 ml of culture, harvested and resuspended in 1 ml of PLP buffer, were disrupted in a French press (American Instrument Co.) or lysed by sporulation. After a centrifugation at 10,000 x g for 30 min., the supernatants were negatively stained and examined under the EM. Alternatively, the harvested cells were resuspended in 10 ml of PLP buffer, lysed and then centrifuged as before at low speed. The resulting supernatants were centrifuged at 100,000 x g in

a Beckman ultracentrifuge (model L8-55) for 1 hr and the pellets, resuspended in 0.5 ml of PLP buffer, were examined by negative-stain electron microscopy.

2.6. Partial purification of Ømed-1

The purification of Ømed-1 as developed by Hendry (1973, and appendix 1 and 2) was followed both with no modification and with some minor modifications as in the following. Fluid media were used instead of the agar form. Cells at stage VII of sporulation were harvested, resuspended in phage buffer at 1/25 the original volume of cultures, and lysed by passing through a French Press at 2000 lb/sq. in. instead of being allowed to autolyse. The subsequent steps were then carried out as exactly described in appendix 2.

2.7. Examination of RNA in the partially purified Ømed-1 preparations

Phage pellet derived from 4 litres of culture was resuspended in 2 ml of SSC buffer. RNA was extracted from the sample by the method of Pace et al., (1968). The suspension was first made to 2% with SDS and left at room temperature for 15 min. The solution was then shaken with an equal volume of SSC buffer-saturated distilled phenol for 10 min. and the mixture was centrifuged in a clinical centrifuge (International Equipment Co. Model PR-2). The upper aqueous layer was removed and extracted again with phenol. One-tenth of a ml of diethylpyrocarbonate (Sigma) was added to the mixture before the

second extraction to inactivate residual nucleases. The aqueous phase of the second extraction was made to 0.3 M sodium acetate at pH 5.2 and two volumes of 95% ethanol was added. The precipitate that appeared after an overnight storage at -20° C was collected by centrifugation and resuspended in SSC. A volume of sample containing 10 µg of RNA was mixed with equal volume of a loading buffer containing 10% sucrose and 0.001% bromophenol blue in RNA electrophoresis buffer. The mixture was loaded on a slab gel of 4% acrylamide, 0.2% bisacrylamide and 3% sucrose in RNA electrophoresis buffer. The dimensions of the gels were 17 cm. x 12 cm. x 0.3 cm. Electrophoresis was at 40 volts for 10 hrs. Gels were either stained with ethidium bromide (1 µg/ml) or with 0.1% methylene blue (in 0.1 M sodium acetate, pH 4.5).

2.8. Purification of PLP

Bacteria grown in fluid GBBM to stage seven of sporulation were harvested by low speed centrifugation and resuspended in PLP buffer at 1/25th the original volume of the culture. Triton X-100 was added to a final concentration of 0.1%. This facilitated sporangial lysis (1-2 hrs) in all cultures except those of B. medusa, whose cells were lysed by 2 passes through a French press at 3000 lb./sq. in. Lysates were then diluted with an equal volume of PLP buffer and then centrifuged at low speed to remove spores and inclusions. The low speed supernatants were centrifuged in a Beckman ultracentrifuge in a type 30 rotor at 19,500 x g for 1 hr. (all centrifugations were at 4°C).

The supernatants of this clarification were supplemented with 2 µg/ml each of ribonuclease A (Sigma) and deoxyribonuclease I (Millipore Corp.) and incubated for 1 hr. at room temperature. The PLP in the suspensions were then pelleted by centrifugation at 55,000 x g for 2 hrs, and resuspended in small volumes of PLP buffer. After a sonification (Measuring and Scientific Equipment Ltd; code 3-62) of 1 min., the suspensions were loaded onto continuous gradients (5 ml) of 0-80% Renografin-76 (Squibb Inc.) in 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.2) containing 0.1% Triton X-100 (scintillation grade, Kodak). Centrifugation was carried out in a SW 50 rotor at 85,000 x g for 3 hrs. The PLP bands were collected, diluted 10 fold in PLP buffer without $MgCl_2$ and centrifugated in a SW 41 rotor at 149,800 x g for 1 hr. The pellet resuspended in PLP buffer was stored at 4°C.

The cell lysate of E. coli strain HFr after the infection of R17 phage was subjected to the same purification procedure as were the sporulated lysates of the Bacillus strains. The R17 phage collected was also stored at 4°C.

2.9. Chemical determinations

Protein contents of PLP suspensions were determined by the Lowry method (Lowry et al., 1951), with bovine serum albumin (Nutritional Biochemical) as standard. For DNA and RNA contents, the preparations were fractionated by the modified Schmidt-Thannhauser method (1945) as described by Merchant et al. (1964). DNA was determined by the Burton Method (1956) and RNA by the orcinol method

(Horecker, 1957). Nucleic acids were expressed as a percent of the protein content. Glycoproteins were examined by staining an SDS-polyacrylamide gel of solubilized protein (see below) by the Schiff method described by Fairbanks et al (1971).

2.10. SDS-Polyacrylamide gel electrophoresis (PAGE) of proteins

SDS-Polyacrylamide gel electrophoresis (PAGE) of proteins was performed in a slab gel apparatus as describe by Laemmli and Favre (1973) using 4% acrylamide stacking gel and 12% acrylamide running gel. Samples were solubilized in dissociation buffer of 2% SDS, 1% 2-mercaptoethanol in 30 mM Tris-HCL, pH 6.8 before loading on a gel. The following proteins were used as molecular weight markers (molecular weight in kilodaltons and source are shown in parenthesis): β-galactosidase, (130, Boehringer) bovine serum albumin (68, Nutritional Biochemical), ovalbumin (43, Sigma), chymotrysinogen (25.7, Sigma), trypsin inhibitor (20.1, Sigma) and cytochrome C (11.7, General biochemical). Protein bands of interest were cut out from gels and loaded into the wells of second gels for peptide mapping by limited proteolysis and electrophoresis as described by Cleveland et al., (1977). The proteases used were Staphylococcus aureus V8 protease (Miles Laboratory) and trypsin (pancreatic, Calbiochem). All protein gels were stained by Coomassie Brilliant Blue R (Sigma).

2.11. Polyacrylamide gel electrophoresis of RNA

To separate the RNA of PLP preparations from proteins, the single step RNA isolation procedure as described by Gopalakrishna et al., (1981) was employed with minor modification. Briefly, PLP preparations were supplemented with sodium dodecyl sulfate to a final concentration of 0.5%, proteinase K (EM Inc.) 0.025 mg/ml and EDTA 10mM. The mixtures were incubated at 37°C for at least 40 min and then loaded on cylindrical gels of 3.3% polyacrylamide in the RNA electrophoresis buffer (Thach and Boedtker, 1967). The dimensions of the gels were 7cm x 3 mm. Electrophoresis was at a constant current of 6 MA per gel. Gels were stained in 0.1% methylene blue in 0.1 M sodium acetate, pH 4.5.

2.12. Isoelectric focusing gel electrophoresis

Isoelectric focusing gel electrophoresis of PLP protein was preformed according to O'Farrell (1975). The dimensions of gel were 2.5 mm x 10 cm. The ampholines were from Bio. Rad.

2.13. Preparation of parasporal inclusions

Inclusions were purified by the method of Sharpe et al., (1975) with some modifications. Briefly, sporulated cell lysates (see section 2.8) of GBBM fluid cultures (50 ml) of the Bacillus strains were harvested by low speed centrifugation. The spore plus inclusion

mixtures were washed once with 1 M NaCl at 0°C and resuspended in 1 ml of PLP buffer. The samples were then loaded in continuous gradients of 50-80% Renografin-76 in the same buffer as that used in preparing the gradients for PLP purifications. Centrifugations were at 18,000 x g and 4°C for 45 min in a SW 50 rotor. The inclusion bands were collected, washed in PLP buffer and then subjected to a second gradient centrifugation. Purified inclusions were stored at -20°C. Protein determination of inclusion preparations was also by the methods of Lowry et al., (1951).

2.14. Immunology

Antisera against PLP preparations were raised in rabbits by intramuscular injections of PLP preparations mixed with equal volume of Complete Freund's adjuvant (Difco Lab). The initial injection for each rabbit was 500 µg of PLP (undissolved) proteins with a booster injection of 300 µg at two week intervals for a period of six weeks. The rabbits were bled ten days after the last injections and the sera stored at -20°C. To raise antisera against inclusions, two injections (1 mg protein), were given to a rabbit at two week intervals. The rabbits were again bled ten days after the final injection. Gamma globulin was isolated from the sera by ammonium sulfate precipitation as described by Campbell et al., (1970). For Ouchterlony assay, PLP and inclusions were incubated in 1 M KCNS, containing 25 mM 1,4 dithioerythreitol and 0.05 M Tris-HCl (pH 11) for 1 hr at 37°C before

loading into the well. Ouchterlony gels were made up of 1% agarose, 2% polyethylene glycol 6000, 0.089 M Boric acid, 0.089 M Tris and 0.001 M EDTA (pH 8.3).

2.15. PLP inhibition assay

0.2 ml of a culture to be tested (OD_{645} of 0.1 x 5) and 4 ml of soft agar at 42°C were mixed and poured on a nutrient agar plate. After incubation at 30°C for 8 hr, 5 µg of PLP in 10 µl of sterile phage buffer was spotted on the lawn and the plate was incubated for another 12 hrs at 30°C. Partially purified phage preparations obtained by the method described by Hendry et al., (1976) were also tested.

3. Results

3.1. Electron microscopic examination of sporulation lysates

PLP could readily be seen among the other spore specific structures in the low speed supernatants and suspensions of high speed pellets as outlined in Methods. The individual PLP of the four Bacillus strains examined looked identical, all were hexagonal and had a diameter of approximately 22 nm. Free PLP of B. medusa and subsp. israelensis very often appeared as clusters of two or more particles while those of isolate 73-E-10-2 and subsp. kyushuensis more often appeared either as individual particles or as groups packed together

in regular arrays (Fig. 1). A small fraction of these particles appeared to be empty or "opened". Those without inward leakage of PTA strain are considered intact. The other cellular structures commonly seen are long fibres, flat and folded sheet-like structures of different sizes, as well as vesicles of different diameters. The sporulated lysate of B. medusa and subsp. israelensis also contained triangular and quadrangular sheets with abundant PLP attached as well as larger phage-like-particles (over 50 nm in diameter). As they were only found in sporulated cell lysates, this mixture is collectively termed parasporal structure. PLP of subsp. israelensis like their counterparts in B. medusa, were also found to attach to the surface of the inclusions (Fig. 2).

3.2. Examinations of the partially purified preparations of Ømed-1

Partially purified preparations of Ømed-1 were first prepared from agar grown cultures as described by Hendry (1973; appendix 2). The preparations were found to contain very few Ømed-1. When liquid grown cultures were used instead, more Ømed-1 was observed in the final phage pellets. Other minute parasporal structures were also present and hence the preparations were far from pure. Those preparations, indeed, contained a significant amount of orcinol-reacting material. After the phenol extraction procedure, an orcinol test showed an RNA preparation derived from a 4 litres culture contained 3.8 mg. of RNA. When 10 µg of this RNA was run in a gel, a single spot having a similar mobility to a t-RNA standard included in

Fig. 1 Electron micrographs of sporulated cell lysates of PLP producing Bacilli. Sporulated cell lysates were centrifuged at 10,000 x g for 30 min and the supernatants were again centrifuged at 100,000 for 1 hr. The pellets, resuspended in PLP buffer, were stained with PTA. The magnification is indicated by the 500 nm bar marker in (c).

a. B. medusa. The arrow points at a cluster of phage-like-particles.

b. B. thuringiensis subsp. israelensis. Note the cluster of PLP and a sheet-like-structure with PLP attached.

c. B. thuringiensis isolate 73-E-10-2. Note that the PLP do not aggregate as in (a) and (b). A sporulated cell lysate of B. thuringiensis subsp. kyushuensis similarly treated as above was identical to C.

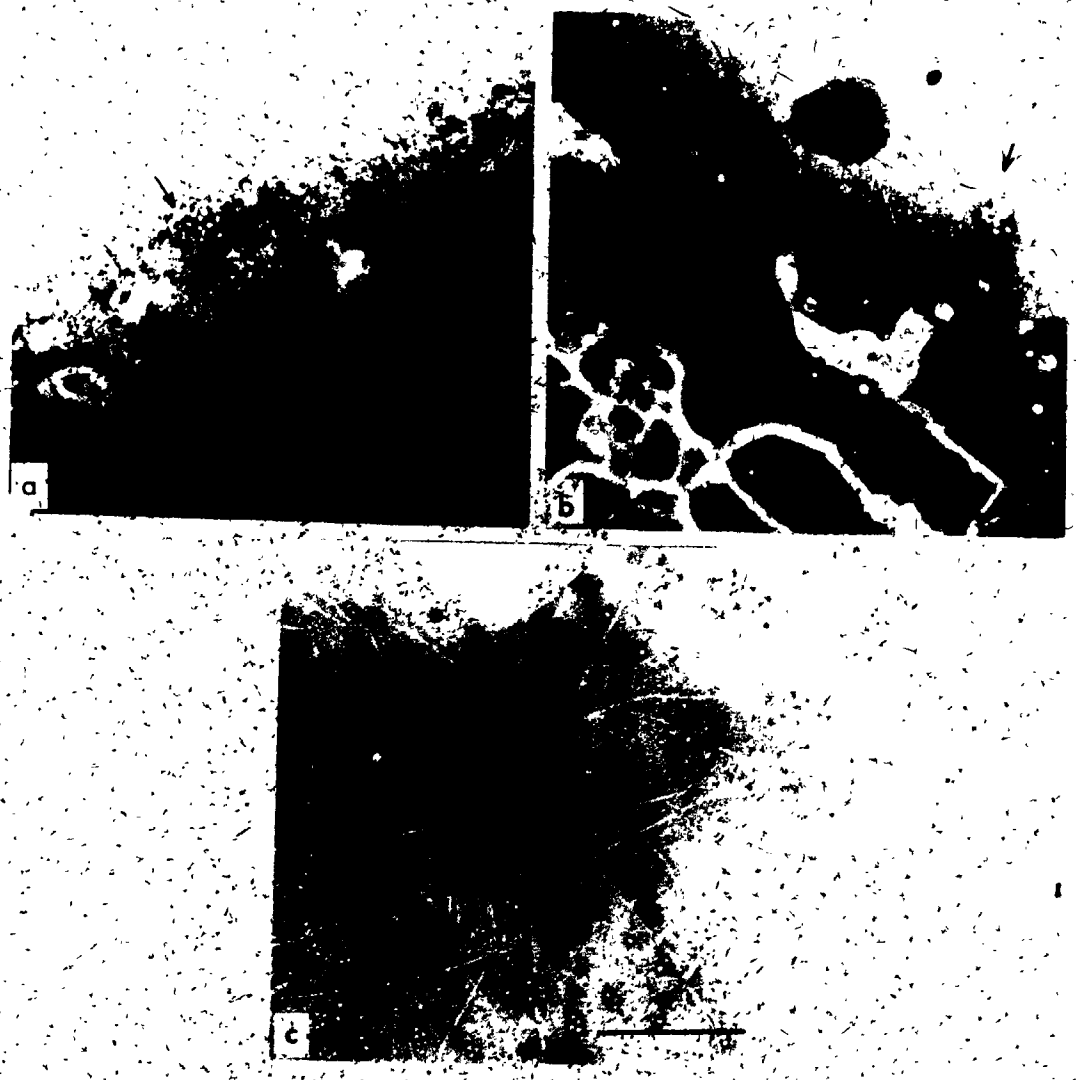
a
b
c

Fig. 2 Electron micrograph of a negatively stained (PTA) inclusion preparation of subsp. israelensis purified by Renografin gradient centrifugation. The arrow points to a phage-like-particle. Sheet-like-structures were also present in the preparation. The magnification is indicated by the 100 nm bar marker.

Fig. 3 Gel electrophoresis of RNA in Ømed-1 preparation. RNA in the partially purified Ømed-1 preparation (Hendry et al., 1976) was extracted twice with phenol and then ethanol precipitated. The resuspended RNA (10µg) was run in a 4% polyacrylamide gel (17 cm x 12 cm x 0.3 cm) at 40 V for 10 hr. The gel was stained with eithidium bromide. Lane 1: purified t-RNA. Lane 2: RNA from sporulated cells of B. medusa. Lane 3: RNA from vegetative cells of B. medusa similarly purified.


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the gel was detected by staining with either ethidium bromide or methylene blue. A vegetative culture subjected to the same purification and RNA extraction procedure, when run in a gel showed a spot also migrating as fast as t-RNA (Fig. 3).

3.3. Purification of PLP

Purification of PLP was rendered difficult by the presence of the minute parasporal structures. In the purification procedure developed in this work, cells other than that of B. medusa were allowed to lyse by their own lytic activities rather than by any mechanical disruption which would have generated membrane vesicles and fractured some of the minute parasporal structures. The sporangia of B. medusa lysed very slowly. Yet their walls were sufficiently delicate that they could be disrupted by passing through the French press at low pressure (3000 lb/sq. in.) which did little damages to the minute parasporal structures. The medium speed centrifugation (19,500 x g) effectively sedimented many of the minute parasporal structures but a considerable amount of PLP was also included in the pellet. After the nuclease treatment, subsequent 55,000 x g centrifugation brought down most of the remaining PLP which then aggregated extensively with each other and with other minute parasporal structures. However a brief sonication effectively dispersed the aggregates. Renografin-76 (Squibb Inc.) was used for the density gradient because unlike caesium chloride, it did not salt out the large amount of hydrophobic proteins in the sporulation lysates.

After 3 hrs of buoyant density centrifugation, PLP of B. medusa and subsp. israelensis banded at a position about 1.4 cm from the bottom of the gradients while PLP of isolate 73-E-10-2 and subsp. kyushuensis banded at a position about 2 cm from the bottom (Fig. 4). Centrifugation for 3 more hr (or longer) brought the bands of the PLP of the latter two cultures to the same position of that of the former two which remained unchanged. At the end of a 3 hr centrifugation, R17 phage formed a light scattering band also at a position 2 cm from the bottom of the gradient. After a final wash, the preparations of PLP derived from B. medusa and subsp. israelensis, appeared in smears to be very pure (Fig. 5 and 11). Those derived from isolate 73-E-10-2 and subsp. kyushuensis are also pure except for the presence of some ribbon-like structures (Fig. 5 and 11). The PLP of B. medusa and subsp. israelensis still tended to aggregate; but after a brief sonification, they were dispersed to reveal some 30% were intact. When these PLP were subjected to a second Renografin gradient centrifugation, they became 100% opened. The PLP of 73-E-10-2 and subspecies kyushuensis lay exclusively close to the margins of the PTA droplets and, if dense enough, formed regular arrays in which some 90% appeared to be intact. This percentage did not change when the centrifugation period was prolonged to 6 hr. The R17 preparation contained sex pili and over 95% were intact (Fig. 6). Infectivity was also retained in the preparations.

Fig. 4 Renografin gradient centrifugation of PLP collected from sporulated cell lysates by high speed centrifugation, resuspended in PLP buffer, and then loaded on to 0-80% continuous gradients of 0-80% Renografin-76. Centrifugations were at 85,000 x g for 3 hr. The arrows point at the PLP bands. (a) PLP from B. medusa. (b) PLP form subsp. israelensis. (c) PLP from subsp. kyushuensis.

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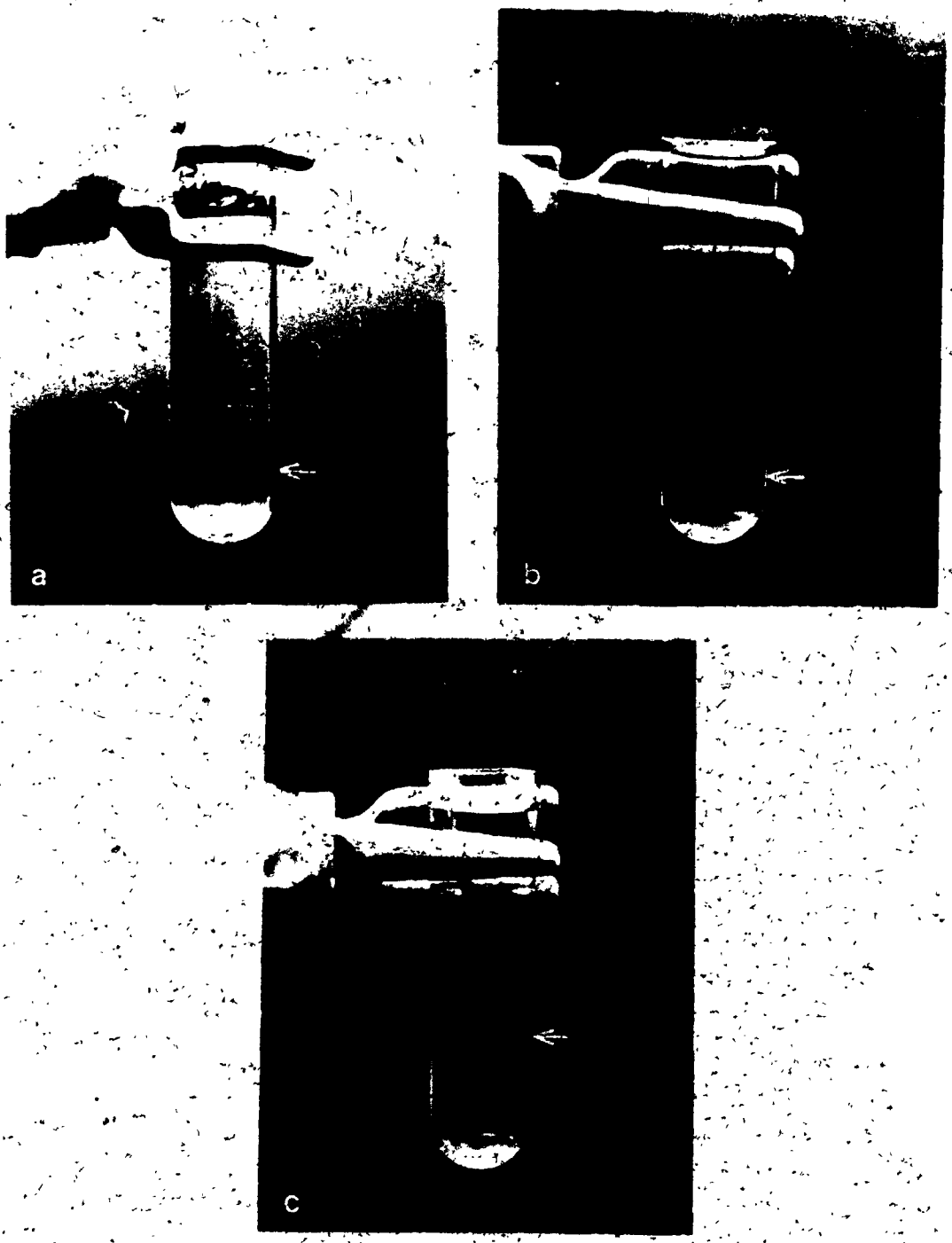

Fig. 5 Electron micrographs of PLP purified by Renografin gradient centrifugation (see section 2.8), resuspended in PLP buffer and stained with PTA. The magnifications are indicated by the 500 nm bar marker in each figure. (a) Ømed-1 (b) Øisr-1, (c) Økyu-1 and (d) Ø10-2-1 after two cycles of Renografin gradient centrifugation (magnification not recorded).

Above pictures with higher magnification are shown in Fig. 11.

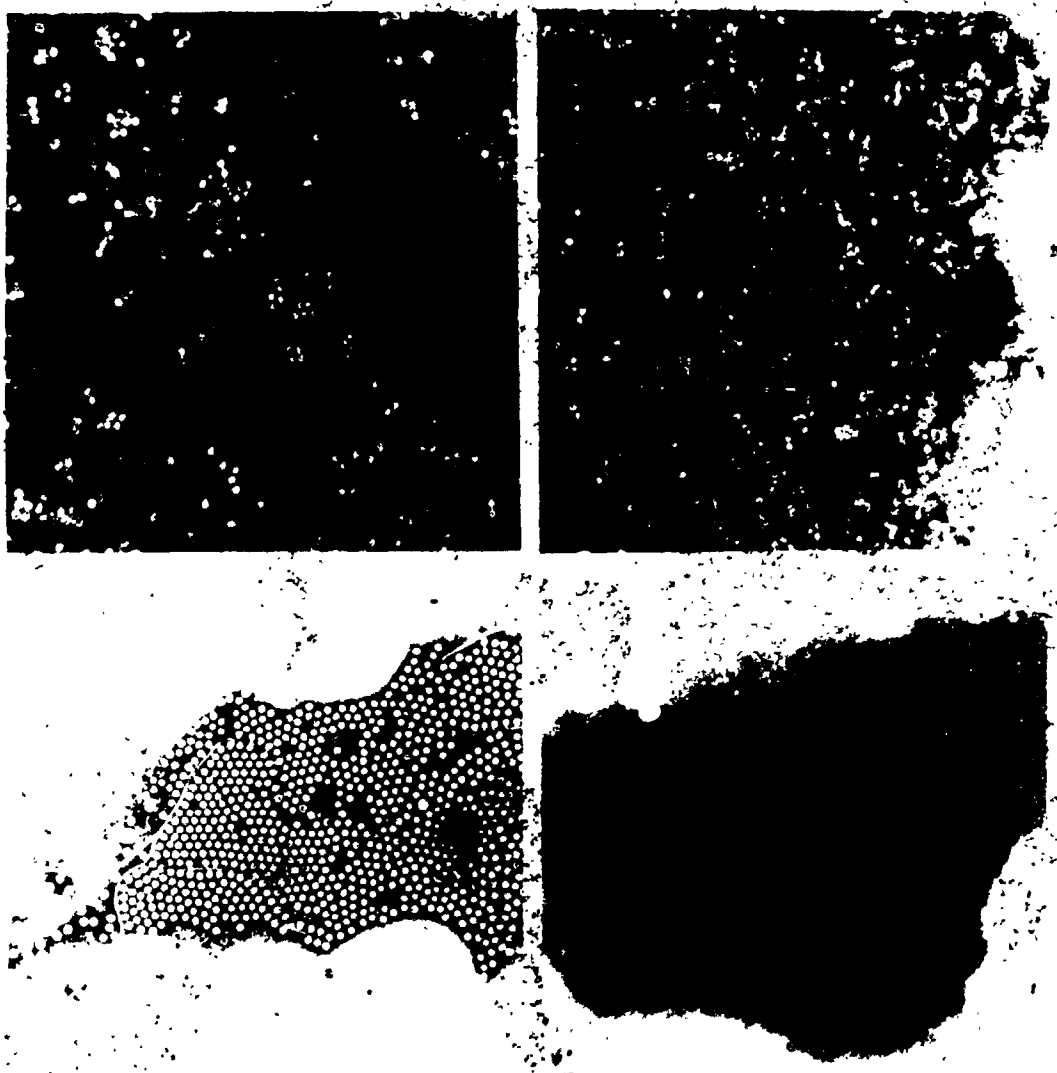

Fig. 6 Electron micrograph of E. coli R17 phage purified by Renografin gradient centrifugation and stained with PTA.

Fig. 7 Polyacrylamide gel electrophoresis of RNA dissociated from RNA phage and phage-like-particles. RNA phage and PLP preparations each containing approximately 4 µg of RNA were loaded on 3.3% acrylamide gels (7 cm x 3 mm) and electrophoresis carried out at 6 mA per tube for 4 hrs. The gels were stained with methylene blue. gel 1: E. coli R17 phage. gel 2: PLP preparation of B. thuringiensis subsp. kyusheunsis. Gel 3: PLP preparation of B. thuringiensis subsp. israelensis. The arrow points at the R17 RNA in gel 1.

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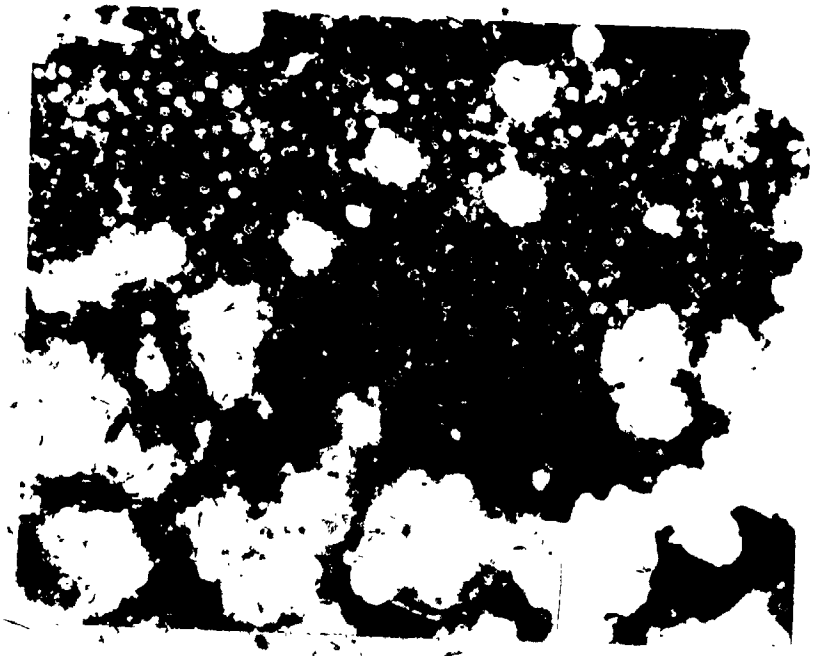

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3.4. Chemical composition of PLP

A PLP preparation derived from 2800 ml culture of any one of the four Bacilli strains contained from 250 µg to 400 µg of protein. Isolate 72-E-10-2 when grown at 30°C or warmer produced considerably less PLP (less than 150 ug). Lowering the culturing temperature to 28°C raised the yield of PLP to around 250 µg. None of the PLP-polypeptide bands generated by SDS-polyacrylamide gel electrophoresis were stained by the periodic acid-Schiff procedure indicating a negligible content of glycoprotein. The Burton reaction (for DNA) on the perchloric acid extracts of all PLP preparations were negative while the oricinol tests for RNA showed slightly positive reactions. The masses of RNA in Ømed-1 and Øisr-1 preparations were calculated to be 15-16% of their protein content while that of Ø10-2-1 and Økyu-1 preparations were 6-8%. An R17 preparation contained RNA at 42% of phage proteins.

3.5. Gel electrophoresis of RNA

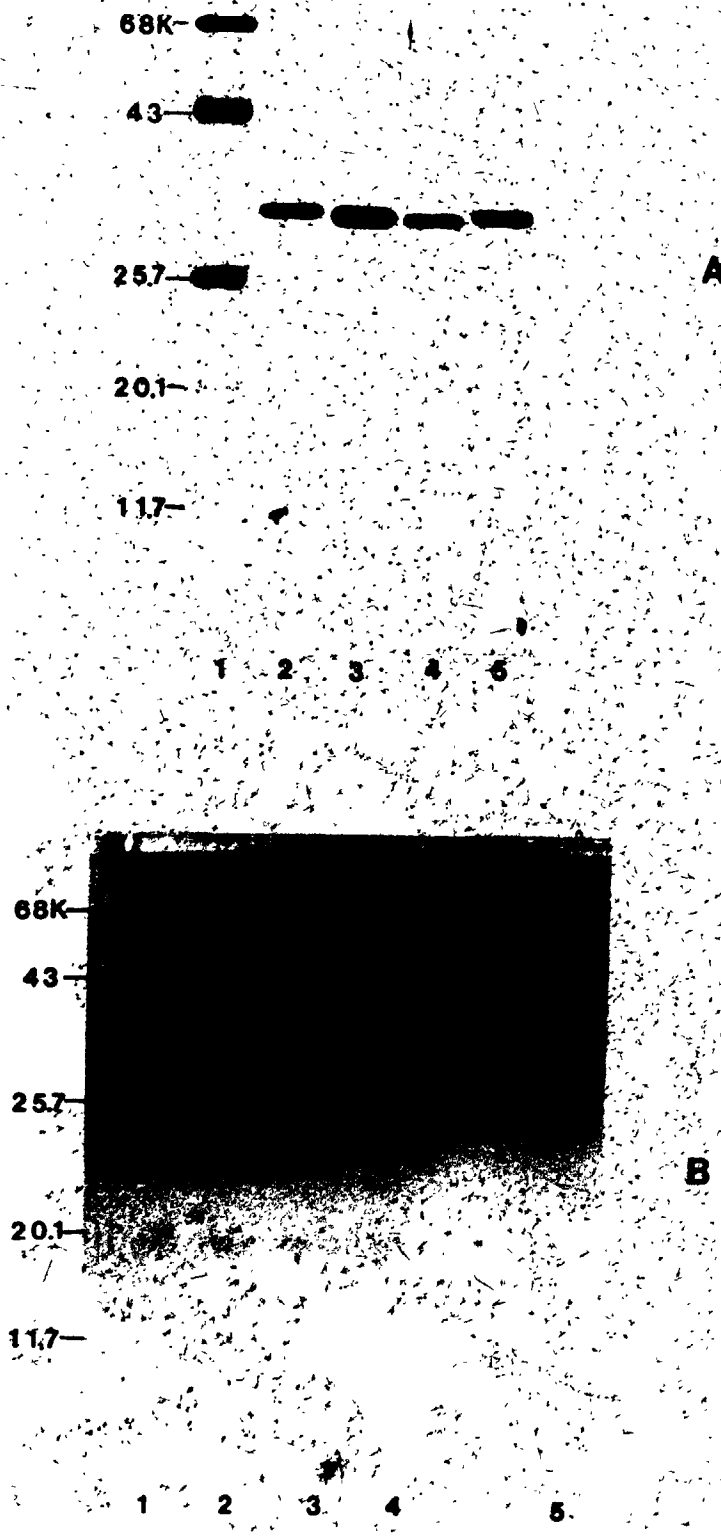
Purified PLP preparations of B. medusa and subsp. israelensis containing approximately 50 µg of RNA when subjected to the phenol extraction procedure yielded negligible amounts of RNA in the ethanol precipitates. Hence a single step RNA isolation procedure (Gopalakrishna et al., 1981) was employed. A sample of R17 phage, containing some 4 µg of RNA by the orcinol test, when subjected to the single step RNA isolation procedure followed by electrophoresis,

showed a single band in the gel stainable by methylene blue and ethidium bromide. Preparations of PLP containing equivalent amounts of orcinol positive material (4 μg) when similarly treated did not show any band irrespective of the period of electrophoresis and the period of incubation of the samples at 37°C before loading the gels (Fig. 7).

3.6. Polyacrylamide gel electrophoresis of PLP proteins

The PLP preparations from all Bacillus strains appeared to be completely solubilized after boiling for 5 min in the dissociation buffer. That is, no pellet was recovered after centrifugation at 10,000 x g for 10 min. When these solutions were analyzed by SDS-polyacrylamide gel electrophoresis, the Ømed-1 preparation gave one band with a relative molecular weight of 30.5 K daltons, and the Øisr-1 preparation a band with a relative molecular weight of 29.7 K daltons. None of the origins stained for residual protein. A very faint band with a relative MW of 37 K daltons was seen in some preparations. The Ø10-2-1 preparation gave three bands, a major band of relative MW of 29.5 K daltons and two fainter bands of 42 K daltons and 45.4 K daltons. The Økyu-1 preparation gave three bands, a 30 Kd major band as well as a thinner 42 Kd and a 44.5 Kd bands (Fig. 8a). A preparation of Ø10-2-1 was further purified by going through a second Renograffin gradient centrifugation. The resulting preparation contained many fewer ribbon-like-structures (Fig. 5d). This preparation again gave three bands as described before. The protein

Fig. 8 Polyacrylamide gel electrophoresis of PLP proteins. (A) Purified preparations containing 7 µg. of protein were mixed with 50 µl of dissociation buffer and boiled for 5 min. before loading on the gel. Lane 1: molecular weight standards. Lane 2: PLP from B. medusa. Lane 3: PLP from subsp. israelensis. Lane 4: PLP from isolate 73-E-10-2. Lane 5: PLP from subsp. kyushuensis. (B) gel electrophoresis of peptides generated by limited proteolysis of the ~30 Kd proteins of the four PLP. Gel slices containing approximately 15 ug of the ~ 30 K protein of each of the PLP were cut from gels and applied to the second gel (15% acrylamide). Staphylococcus aureus V8 protease (0.05 ug) was added to each slot to digest the proteins. Lane 1: 30.5 Kd protein of Ømed-1. Lane 2: 29.7 Kd protein of Øisr-1. Lane 3: 29.5 kd protein of Ø10-2-1. Lane 4: 30 Kd protein of Økyu-1. Lane 5: 29.7 Kd protein of Øisr-1 with no enzyme added. The numbers on the left of each gels indicate the molecular weights (in kilodaltons) of the gel markers. The gels were stained with Coomassie blue.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bands of relative MW around 30 Kd of the four strains were analysed by limited proteolysis and peptide mapping. S. aureus V8 protease (0.05 μg) was used to digest approximately 15-20 μg of proteins in each well. Very similar peptide profiles were generated from the proteins of all four strains (Fig. 8b).

3.7. Immunology

Phage-like-particles did not appear to dissolve in the KCNS solution, but were dispersed in it. When such preparations of PLP (each containing 100 μg of proteins) of the four bacilli were used to challenge antiserum raised against Ømed-1, precipitin bands formed with all samples in two days (Fig. 9a). In another experiment, a sample of Ømed-1 containing 200 μg of proteins was dissolved in 67% acetic acid (10 min at 0°C). The solution was then dialysed against two changes of distilled water and then against 50 mM sodium phosphate, pH 6. The precipitate formed was collected by centrifugation and redissolved in KCNS solution before being used to challenge anti-Ømed-1 antiserum. A precipitin band was formed in the plate in one day (data not shown).

Inclusions were readily soluble in the KCNS solution. The antiserum against Ømed-1 did not react with dissolved inclusions of all four strains. However, antisera against the inclusions of B. medusa and subsp. israelensis, did react with both Ømed-1 and Øisr-1, but the lines of precipitin formed by the antisera and the inclusion proteins crossed over with that formed by the PLP proteins (Fig. 9b),

Fig. 9 Double diffusion immunological analysis of PLP proteins. Purified PLP preparations containing 100 ug of protein and a purified B. medusa inclusion preparation containing 50 µg of proteins were incubated in 1M KCNS, 25mM DTE, 50mM Tris-HCl (pH 11) at 37°C for 1 hr before loading into the wells.

(a) The center well contained antiserum against purified Ømed-1. Well A: Ømed-1. Well B: Øisr-1. Well C: Ø10-2-1. Well D: Økyu-1.

(b) The center well contained antiserum against purified inclusion of B. medusa. Well A: dissolved inclusions of B. medusa. Well B: Ømed-1. Well C: Øisr-1.

Fig. 10 Isoelectric focussing gel electrophoresis of Ømed-1 proteins. Ten µg of Ømed-1 protein, solubilized in 9.5 M urea, 2% NP-40, 2% ampholine and 5% 2-mercaptoethanol, were loaded on an isoelectric focus gel (10 cm x 2 mm). The gel was stained with Commassie blue. To determine the pH gradient of the gel, an unloaded gel was run and cut into 5 mm sections each of which was placed in 2 ml of degassed distilled water. After shaking for 20 min, the pH of the water was then measured.

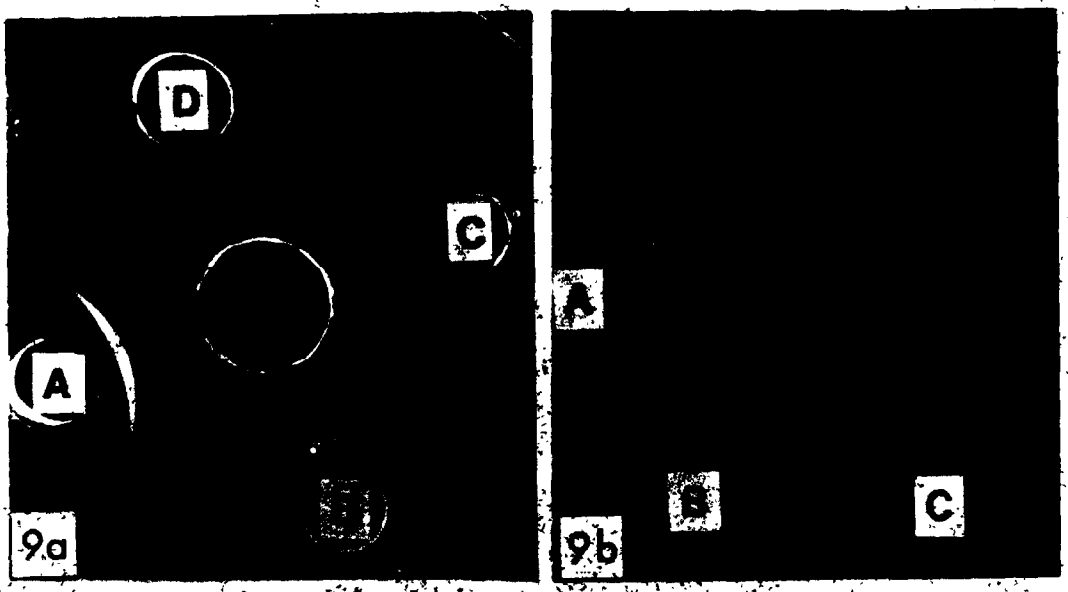

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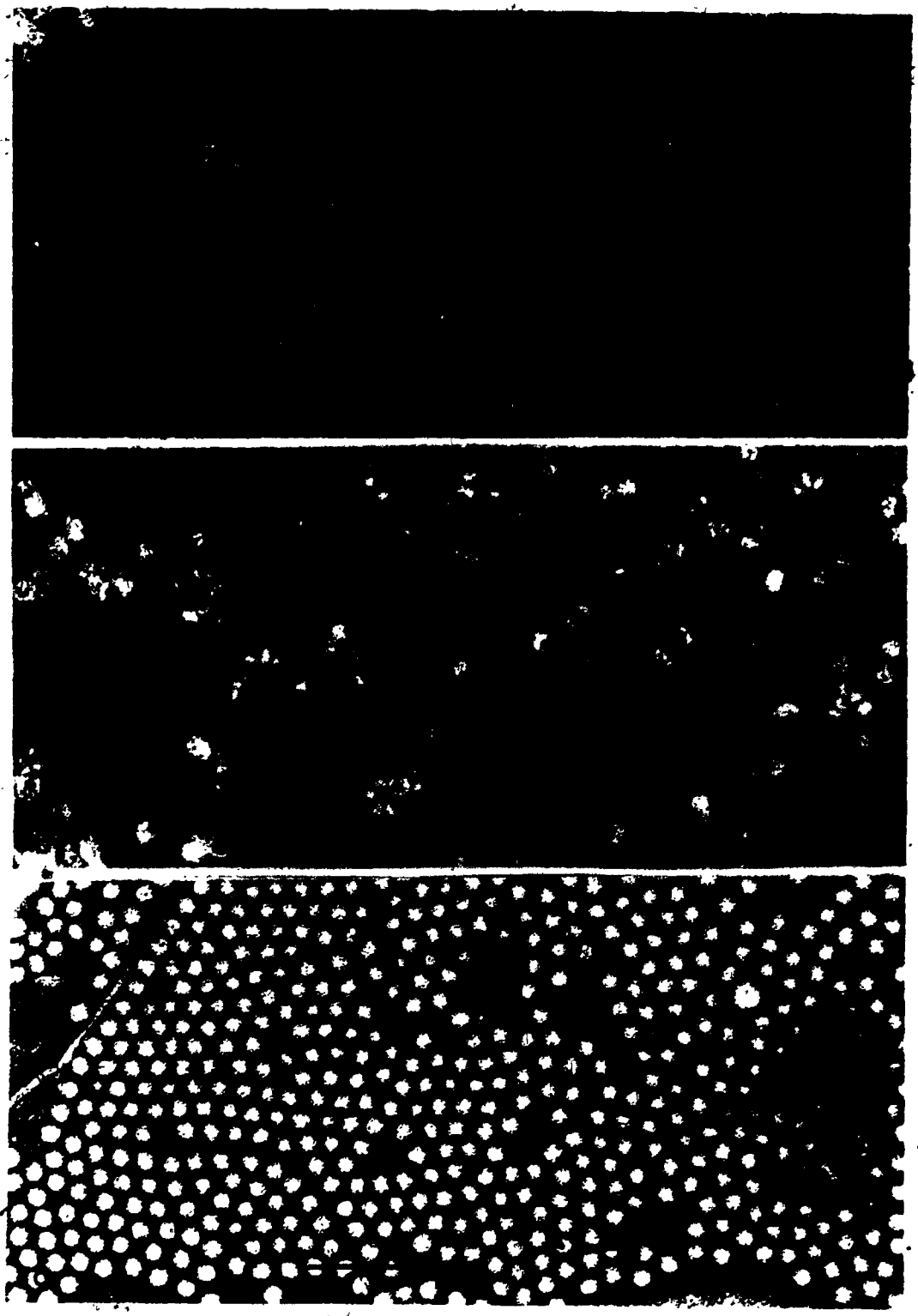
Fig. 11 Electron micrographs of PLP purified by Renografin gradient centrifugation in high magnification. The magnifications are indicated by the 100 nm bar markers in each figure.
(a) Ømed-1, (b) Øisr-1, (c) Økyu-1.



indicating the PLP proteins and the inclusion proteins did not react with the same species of IgG. This supports the structural evidence that inclusions of B. medusa and subsp. israelensis have PLP on their surfaces. Antiserum against the inclusions of subspecies kyushuensis did not react with PLP of the four strains.

3.8. Isoelectric focusing gel electrophoresis

Isoelectric focusing gel electrophoresis of a Ømed-1 preparation resolved the 30.5 Kd protein into two proteins, one with a pI of 5.2 and the other a pI of 5.3 (Fig. 10).

3.9. Phage-like-particles inhibition assays

The growth inhibition of Ømed-1 preparations against log phase cultures of various mutants of B. medusa reported earlier (Hendy et al., 1976) could not be confirmed in this study. Likewise, PLP preparations from any of the four Bacillus species did not appear to inhibit growth of B. medusa variants nor of variants of isolate 73-E-10-2 not producing Ø10-2-1 (see Chapter 5 for their isolation).

4. Discussion

A wide variety of structures are usually found in sporulated cells of B. cereus-related bacilli. They have not aroused much interest probably because their presence is unknown or there are no

apparent biological functions associated with these structures. Among them, the PLP are unique in that they take the form of a small virus somewhat similar to that of an E. coli RNA phage. Yet this work does not confirm what previous investigators (Hendry et al., 1976) have reported: that Ømed-1 is a minute RNA containing bacteriophage or phage-like-particle. In following exactly the partial purification procedure of Hendry (1973), this candidate did not find in the final phage pellets enough Ømed-1 that justified further analyses. Hence two changes were made. Fluid rather than solid medium was used in order to increase the yields of Ømed-1 and cell lysis was promoted with the French press (at very low pressure) rather than aging on agar. Presumably these minor changes should not affect the RNA content or the growth inhibition differences found in this comparison. An interpretation of some of the results of this work is as follows. Polyacrylamide gel electrophoresis of the purified Ømed-1 proteins showed the particles were composed of at least one species of protein which had a relative MW of 30.5 kilodaltons. Such a protein would contain over 200 amino acids and require a gene composed of more than 600 nucleotides to code for it. RNA containing more than 600 nucleotides would be unlikely to have an electrophoretic mobility similar to that of t-RNA. Thus the RNA which was detected in the partially purified preparation of Ømed-1 and had the same electrophoretic mobility as t-RNA is unlikely to be RNA determining the synthesis of a phage. The presence of similar sized RNA in a preparation derived from a vegetative culture not producing Ømed-1 indicates that the RNA detected was likely to be partially digested

cellular RNA of the bacteria. RNA in the partially purified preparation of Ømed-1 has, indeed, been shown to be separable from the minute parasporal structures by sucrose gradient centrifugation (data not shown) in which a significant amount of RNA stayed at the upper portion of the gradient while the minute parasporal structures pelleted.

In the PTA smear, a surface active or hydrophobic nature of the PLP is suggested by the manner in which they lay close to the margins of the dried droplets. Phage-med-1 and Øisr-1 differed from the other two PLP in that the particles tended to aggregate, particularly during high speed centrifugation. On the other hand, two dimensional regular arrays of PLP were frequently seen in smears of Økyu-1 and Ø10-2-1 preparations. It is likely that the regular arrays were formed during the mounting of the preparations on the grid as packed arrays could still be seen in EM specimens made from a preparation of Ø10-2-1 right after a sonication. Phage-Kyu-1 and Ø10-2-1 were also not seen to attach to other sporulation structures as did the other two PLP.

The purification method developed in this work has two advantages over the method used by Hendry et al., (1976). Firstly, RNAse and DNAse digestions of cellular nucleic acids were performed on supernatants of a medium speed centrifugation rather than on the resuspended high speed centrifugation pellets in which some minute parasporal structures still aggregated. Secondly, the Renografin density gradient centrifugations employed effectively separated particles with different sedimentation coefficients as well as

particles with different densities from each other. Thus after a short run, any residual RNA present in the samples would remain at higher positions in the gradient than the PLP. That Ømed-1 and Øisr-1 migrated to the equilibrium positions earlier than the other two PLP was probably due to the fact that the former aggregated in the Renografin and, with increased sedimentation coefficients, moved faster down the gradients than their nonaggregating counterparts.

Purified preparations of Ømed-1 and Øisr-1 differed further from that of Økyu-1 and Ø10-2-1 in the precentages of particles remaining intact among the total particles (30% in the former pair as compared to 90% in the latter). However, Økyu-1 and Ø10-2-1 preparations contained lower amounts of RNA while preparations of Ømed-1 and Øisr-1 contained higher amounts. One explanation for this difference is that the aggregates of Øisr-1 and Ømed-1 in the resuspended high speed pellets had not been completely dispersed by the sonication before gradient centrifugation. It is also possible that some of the PLP had reaggregated immediately after sonication. In this way, partially digested RNA fragments might have been trapped in these aggregates. The results of the polyacrylamide gel electrophoresis of the RNA from purified PLP preparations showed that no discrete RNA was associated with the four PLP. Thus it appears that Økyu-1 and Ø10-2-1 do not contain RNA inside their protein capsids as does R17 and can be compared with the double shell capsids assembled from purified coat proteins of E. coli RNA phages (Holm, 1970). The protein monomers making up the Økyu-1 and Ø10-2-1 could be so tightly packed that penetration of PTA stain was rendered difficult as in the case of the

R17 double shell capsids. However, at this point, the study of the composition of native Ømed-1 and Øisr-1 cannot be regarded as exhaustive; nucleic acid may have been lost during the purification. Hendry et al., (1976) have shown that in a thin section of one of their partially purified preparations of Ømed-1, the core of some structures appearing to be Ømed-1 attached to small pieces of sheet-like-structures were positively stained in osmium-fixed preparations stained with lead citrate and uranyl acetate (Appendix 1, Fig. 6). Thus an electron microscopic study of uranyl-staining of thin sections of sporulated cells of B. medusa and subsp. israelensis would be an appropriate starting point for further studies of the content of PLP.

Phage-Ømed-1 and Øisr-1 are composed of one major protein with relative MW around 30 kiloddaltons. The small amounts of 42 Kd and 45.5 Kd proteins found in Ø10-2-1 and their counterparts in Økyu-1 could be components of PLP or contaminants but these proteins were not further studied. Proteolytic peptide mapping and immunological results (more data to be presented in Chapter 3) indicated that the four major proteins of the PLP are similar. However, the 30.5 Kd protein of Ømed-1 is actually two proteins of slightly different isoelectric points (pI 5.2 and pI 5.3). Whether these are two different proteins or very similar proteins differing from each other by one or two amino acids or by some type of post-translational modification has yet to be determined. However, for the sake of convenience, these proteins will be mentioned as one protein in the rest of this thesis.

Chapter 3

A study of the Times of Synthesis of PLP: Immunological Detection and Electron microscopy.

1. Introduction

Phage-med-1 was described as a spore-specific product not observed in the vegetative cells of B. medusa nor in a mutant blocked at stage I of sporulation. The particles were observed in cell lysates of wild type at late stage II of sporulation and thereafter (Hendry, et al., 1976). The time at which the protein making up ϕmed-1 is first synthesized had not been determined by these investigators and thus became a point of investigation in this study. Included in this study are also the times of synthesis of the other PLP. To detect the ~30 K protein of the PLP in cells, an immunoprecipitation method using antiserum against ϕmed-1 as the probe and Staphylococus aureus cells as the IgG-absorbent was employed. S. aureus cells are capable of absorbing IgG molecules because of the presence on the cell wall of a component, protein A, which has high affinity to the Fc regions of most mammalian IgG molecules (Forgren and Sjoquist, 1966). Upon being added to a solution containing an antigen and its specific antiserum, the S. aureus cells effectively precipitate the IgG molecules and the antigen-antibody complexes from the solution. Since the immunoprecipitation assay developed in this study were used repeatedly (see Chapter 4 and 5), the reliability of the assay will be shown in detail in this Chapter.

In order to determine the times of synthesis of the BLP proteins in relation to the differentiation processs, a method for the precise definition of the time of sporulation was required. Such a method has been developed for fluid culture of Bacillus subtilus (Aubert, et al., 1969). In this method, the time at which the exponential increase in optical density of a culture abruptly ceases and becomes linear is assigned as t_0. Hours after t_0 are referred as t_1, t_2, t_3 and so on. In theory, t_0 is the time the cells in the culture have just sensed the signal inducing sporulation and all cell divisions cease. Young cells formed at t_0 continue to grow to maturity (longer and binucleated) and then go into stage I of sporulation. Thus under one culturing condition, at a particular time after t_0, e.g. t_4, (4 hrs after t_0), most of the cells will be at or close to one particular stage of sporulation provided the culture develops synchronously.

In this study, the timing method mentioned above was employed. This system is much better than the one in which the time of sporulation of a culture is related to the time the culture is inoculated. This is because a slight variation of the culturing conditions would shift the time of onset of sporulation from the time of inoculation of a culture by hours.

2. Materials and Methods

2.1. Bacterial strains

Staphylococcus aureus Cowan I was from the cultural collection of the Department of Microbiology, University of Western Ontario. Bacillus thuringiensis subsp. kurstaki HD-1 was from cultural collection of P.C. Eitz-James, University of Western Ontario. B. thuringiensis subsp. darmstadiensis was from M. Ohba, Kyushu University, Japan.

2.2. Growth of bacteria and medium

S. aureus was maintained on agar slants of yeast tryptone and was grown in BBM at 37°C with reciprocal shaking.

2.3. Buffers

RIA buffer is 40 mM sodium phosphate pH 7.4, 0.15 M NaCl, containing 0.1% triton X-100, 0.1% SDS and 1mM 4-toluene sulfonyl fluoride. Staph A washing buffer is 0.15 M NaCl, plus 0.5% triton X-100 in 10 mM Tris-HCl, pH 8.3.

2.4. Immunoprecipitation assay

S. aureus Cowan I cells (Staph A) were prepared as an IgG adsorbent by the method described by Cullen and Schwartz (1976), except that the cells were grown in BBM. To assay a culture, 20 ml of cells, taken at the stage required, were harvested by centrifugation, resuspended in 1 ml of RIA buffer and lysed by French pressing. The lysate was centrifuged first at 3000 x g for 5 min, then at 18,000 x g for 25 min, both at 4°C. The resulting supernatant (cleared lysate) was then assayed by the immunoprecipitation procedure as described by Kessler (1975) with minor modifications. Briefly, the cleared lysate was first preabsorbed (one hour at 0°C) with 0.2 ml of 10% (W/V) of Staph A cells in Staph A wash buffer. After the Staph A cells have been removed by centrifugation at 5000 x g for 10 min, an appropriate amount of antiserum (usually 1 µl of anti-Gmed-1-serum) was added to the precleared sample and the mixture was left at 0°C for over 10 hrs. Washed Staph A cells (0.2 ml) were then added to absorb the IgG. Following an hour of incubation, the Staph A cells were collected by centrifugation, washed twice with Staph A wash buffer, and boiled for 2 min in 50 µl of dissociation buffer. The supernatant of a low speed centrifugation for sedimentating the cells was loaded on an SDS-polyacrylamide gel. In experiments in which ^{14}C-L-amino acids were used, the quantities of the materials were scaled down by one-half. Fluorographs of the resulting polyacrylamide gels were prepared by the method of Bonner and Laskey (1974).

2.5. Determination of the time of synthesis of the PLP

To time the formation of a PLP and the synthesis of its ~30 Kd protein, the following procedure was followed. A batch of medium was divided into two portions one of which was inoculated with a log phase culture of the PLP producing strain to an OD of 0.25. Growth and differentiation of the culture was followed by OD measurement (of samples diluted 5-fold with saline) and phase contrast microscopy. An OD versus time curve was plotted and the relationship between the OD and the stages of differentiation of the cells as roughly determined by the phase-contrast microscopy was recorded. The other portion of the medium was then similarly inoculated and the OD of the culture was again monitored. At a time before the onset of sporulation (as indicated by the OD of the previous culture), this second culture was divided into two halves. ^{14}C(U)-L-amino acid mixture (New England Nuclear) was added to one of these cultures to a final concentration of 0.2 μCi/ml. Growth of the cultures was then resumed. At hourly intervals, 10 ml portions of the unlabelled culture were sampled for the presence of PLP as described in Chapter 2. Simultaneously, 10 ml portions of the radioactive culture were harvested, frozen and later subjected to the immunoprecipitation assay using anti-Bmed-1 serum. Unlabelled cultures (5 ml) were also sampled at times for thin section electron microscopy.

2.6. Electron microscopy of thin sections

Electron microscopy of thin sections was by the method described by Fitz-James (1971) except the glutaraldehyde prefixation was at 0-1°C and a Phillips EM 400 microscope was used. Micrographs photographed at 9,000-15,000 times were enlarged 5-10 times in printing.

2.7. Radioactive labelling of PLP

^{14}C(U)-L-amino acid mixture was added to cultures of PLP-producing strains to a final concentration of 0.2 μCi/ml at approximately 1 hr prior to onset of sporulation. The cultures were allowed to complete sporulation and the PLP produced were purified as described in Chapter 2. A Beckman liquid scintillation counter (model LS-250) was used to monitor radioactivity.

2.8. Pertinent experimental methods not described here are covered in the preceding Chapter.

3. Results

3.1. Validity of the immunoprecipitation assay for PLP proteins

The fixed Staph A cells prepared for this study were effective in precipitating IgG molecules when added to a serum diluted in 1 ml of RIA buffer. A 50 kilodalton band of the H chain of IgG dominates

the wide variety of protein bands originating from the Staph A cells (Lane 2, Fig. 1A). When added to a cleared sporulated cell lysate of B. medusa, the Staph A cells bound nonspecifically to several proteins which could not be removed by washing in Staph A wash buffer (Lane 3, Fig. 1A). However, a unique 30.5 Kd protein was precipitated by the Staph A cells from the same cleared lysate previously incubated with antiserum against Ømed-1. This band was not observed when preimmune serum was used (Lane 4 and 5, Fig. 1A). Similarly, a 29.7 Kd protein was precipitated from a cleared sporulation cell lysate of subsp. israelensis only when it had been incubated with anti-Ømed-1 serum (Lane 2 and 3, Fig. 3). The immunoprecipitated 30.5 Kd protein from B. medusa was subsequently identified as the 30.5 Kd protein of Ømed-1 by tryptic peptide mapping of the protein from the gels (Fig. 1B).

To test for maximal precipitation of Ømed-1 protein, increasing amounts of anti-Ømed-1 serum were added to B. medusa cleared sporulated cell lysates. The 30.5 Kd protein band increased as the amount of antiserum added was increased up to 1 µl. Further increase of antiserum (up to 5 µl) showed no obvious change in the magnitude of the 30.5 Kd protein band. Hence 1 µl of antiserum was chosen as the amount of antiserum used in a standardized immunoprecipitation assay. Sensitivity of the standardized assay was tested using different amounts of sporulated culture of subsp. israelensis to prepare the cleared lysates (in 1 ml of RIA buffer). It was found that a band of 29.7 Kd could still be discerned in the lane when only 3 ml of sporulated culture was used (Fig. 2).

Fig. 2 Sensitivity of the immunoprecipitation assay for PLP protein. Different volumes of a sporulated culture of subsp. israelensis were harvested, resuspended and lysed in 1 ml of RIA buffer and then subjected to the immunoprecipitation assay using 1 µl of anti-Ømed-1-serum and 200 µl of 10% (W/V) Staph A cells. Lane 1: control with 12 ml of culture and preimmune serum. Lane 2: 3 ml of culture, with antiserum. Lane 3: 6 ml of culture, with antiserum. Lane 4: 12 ml of culture, with antiserum. Lane 5: 24 ml of culture, with antiserum. The gel is stained with Coomassie blue. The arrow indicates the position of the 29.7 Kd immunoprecipitated protein.

Fig. 1 Immunoprecipitation assay for PLP protein.

A. 200 µl of 10% (W/V) Staph A cells were incubated in 1 ml RIA buffer containing the indicated test materials (Lane 2 to 5). Any precipitated proteins were dissolved by boiling the staph A cells in dissociation buffer for 2 min and then loaded on the gel. Lane 1: Ømed-1 protein included in the gel as marker. Lane 2: 1 µl of anti-Ømed-1-serum. Lane 3: sporulated cell lysate of B. medusa. Lane 4: sporulated cell lysate of B. medusa with preimmune serum. Lane 5: sporulated cell lysate of B. medusa with 1 µl of anti-Ø-med-1-serum. The gel was stained with Coomassie blue.

B. Twenty ml B. medusa culture was supplemented with ^{14}C(U)-amino acids mixture to a final conc. of 0.4 µCi/ml. and then allowed to complete sporulation before being subjected to immunoprecipitation assay using anti-Ømed-1-serum. The 30.5 Kd immunoprecipitated protein band was cut out and subjected to the limited proteolysis and gel electrophoresis. Lane 1: Ømed-1 protein from gel (approximately 3000 cpm), no enzyme added. Lane 2: Ømed-1 protein from gel (approximately 3000 cpm), 0.05 µg trypsin added. Lane 3: 30.5 Kd immunoprecipitated protein from gel, 0.05 µg trypsin added. The radioactive peptides were detected by fluorography.

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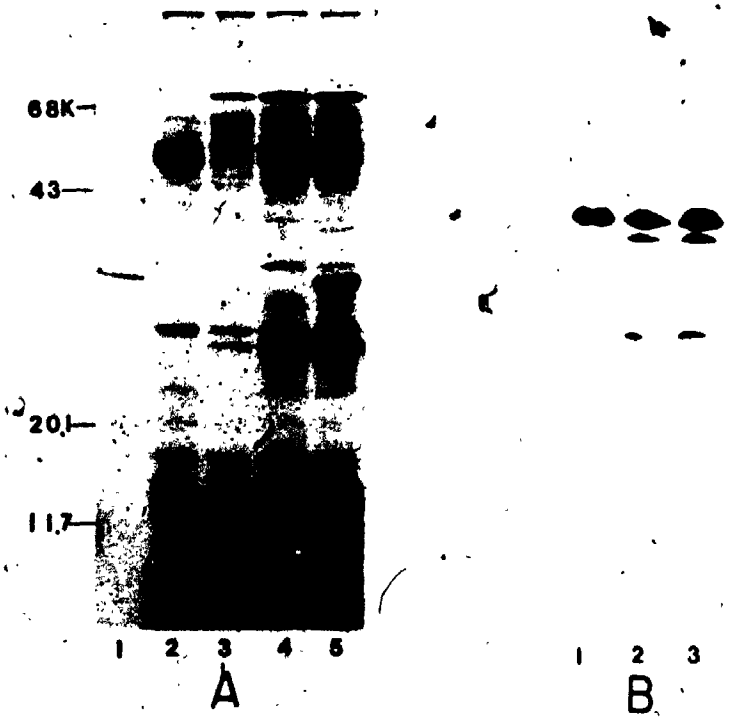

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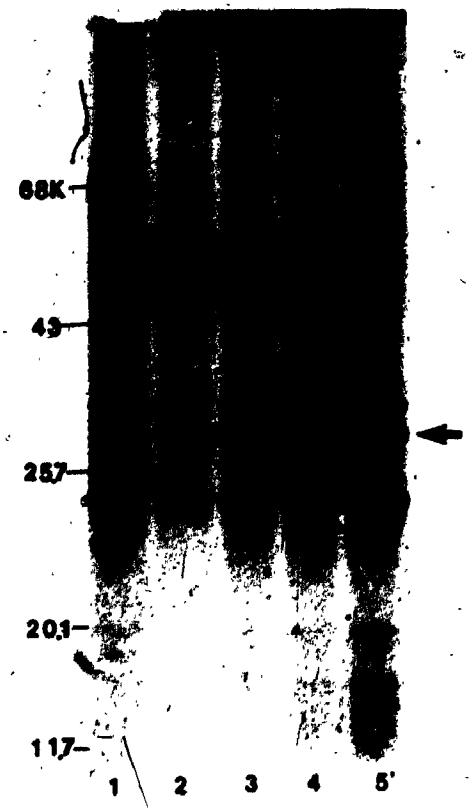

3.2. Detection of PLP proteins in various Bacillus thuringiensis

When cleared sporulated cell lysates of isolate 73-E-10-2 and subsp. kyushuensis were subjected to the immunoprecipitation assay, 29.5 Kd and 30 Kd proteins, respectively, were precipitated. Again, these proteins were not precipitated in control experiments using preimmune serum (Fig. 3). No similar proteins were precipitated when similar lysates of B. thuringiensis subsp. kurstaki, HD-1, a weak mosquitocidal bacillus, and subsp. darmstadiensis, a member of serotype 10, were subjected to the assay.

3.3. Time of synthesis of Bisr-1

When the OD of the first subculture of subsp. israelensis was followed, an abrupt break in the rapid increase of OD was observed (Fig. 4). However, after half an hour, the OD level again showed a drastic increase and then leveled off. This increase proved not to be the result of an increase in cell number. Cultures sampled before and after the drastic rise of OD but washed in saline and resuspended in 5 ml of saline had nearly identical optical densities. The time at which the abrupt break occurred was assigned as t_0. At t_3, parasporal inclusions could definitely be identified under the phase microscope as a refractile structure located at a subcentral position of each cell. At t_4, phase dark spores were first seen in some cells.

The second subculture of subsp. israelensis was divided in half at t_{-1} (1 hr before t_0). ^{14}C-amino acids were added to one of the cultures. Examination of the unlabelled culture for PLP showed that

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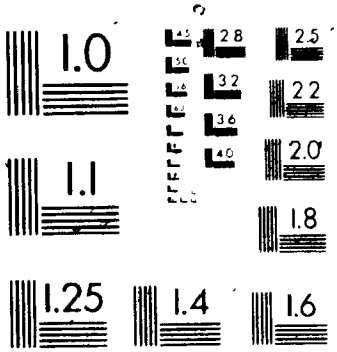

Fig. 3 Immunoprecipitation assay for PLP proteins in sporulated cell lysates of Bacilli. Anti-Ømed-1-serum (1µl) and 200 µl of 10% (W/V) Staph A cells were used to precipitate PLP proteins in 1 ml of 20 x concentrated lysates. Precipitated proteins were solubilized by boiling the Staph A cells in 50 µl of dissociation buffer for 2 min before being loaded on the gel. Lane 1: Ømed-1 protein as marker Lane 2: preimmune serum plus subsp. israelensis lysate. Lane 3: antiserum plus subsp. israelensis lysate. Lane 4: preimmune serum plus subsp. kyushuensis lysate. Lane 5: antiserum plus subsp. kyushuensis lysate. Lane 6: preimmune serum, plus isolate 73-E-10-2 lysate. Lane 7: antiserum plus isolate 73-E-10-2 lysate. Lane 8: preimmune serum, plus subsp. kurstaki lysate. Lane 9: antiserum plus subsp kurstaki lysate. Lane 10: preimmune serum plus B. thuringiensis subsp. darmstadiensis lysate. Lane 11: antiserum plus B. thuringiensis subsp. darmstadiensis lysate. The gel was stained with Coomassie blue.

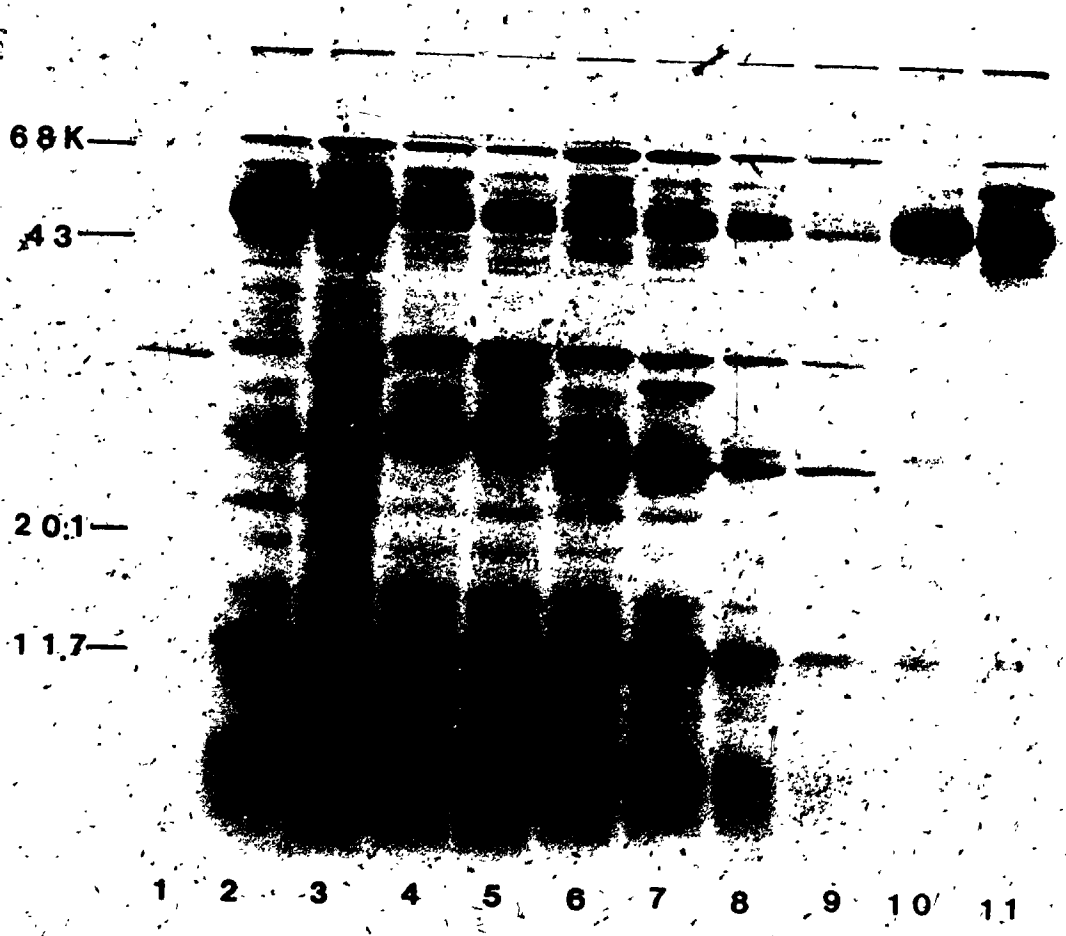
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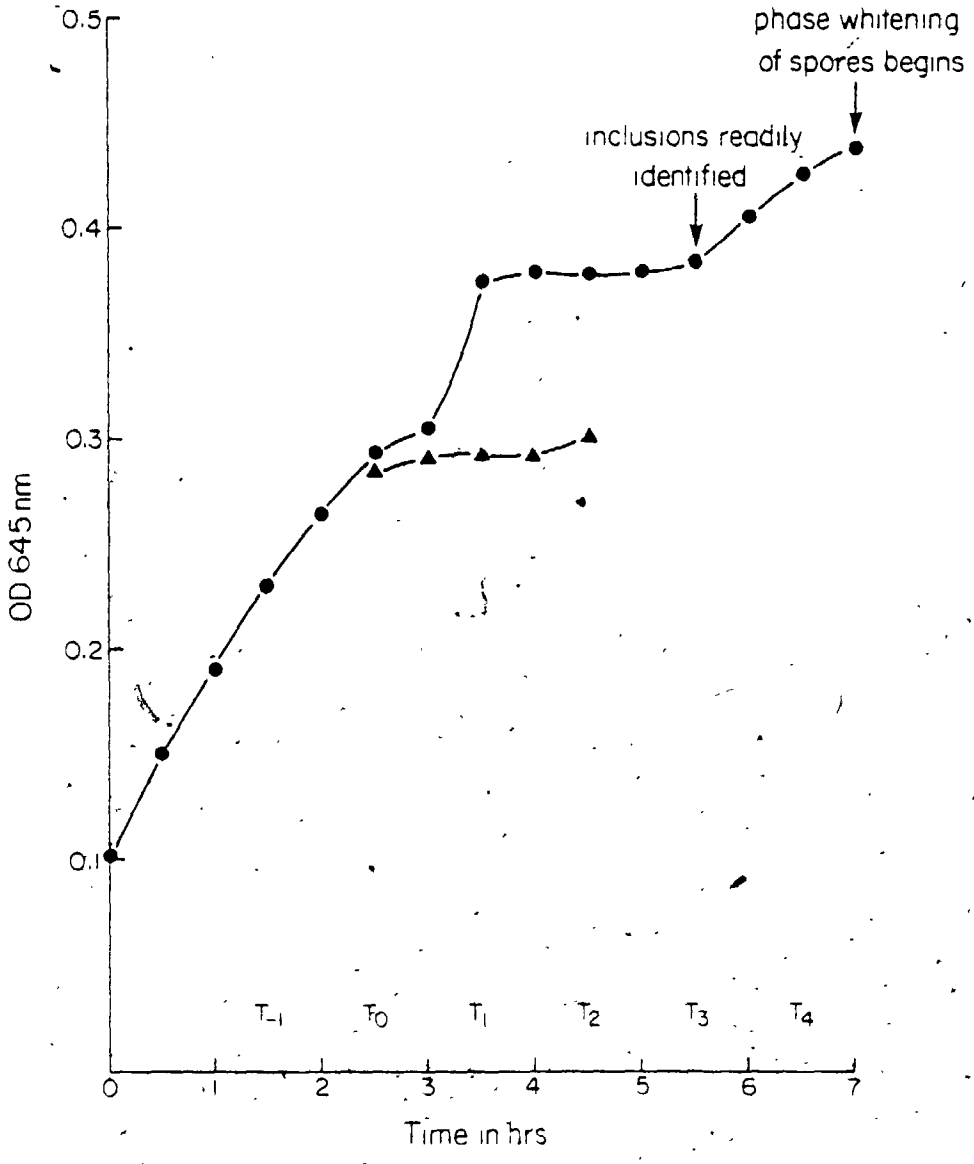
Fig. 4 Optical density versus time curve of B. thuringiensis subsp. israelensis. One ml portions of a culture sampled at half hr intervals were diluted with 4 ml of saline and the ODs measured. The time at which the first reading was taken was arbitarily assigned at time zero. Morphology of the developing cells was monitored by phase-contrast microscopy. From t_0 to t_2, additional 1 ml samples of the culture were washed in saline, resuspended in 5 ml of saline and the ODs measured.
●—● ,OD of unwashed sample. ▲—▲ ,OD of washed sample.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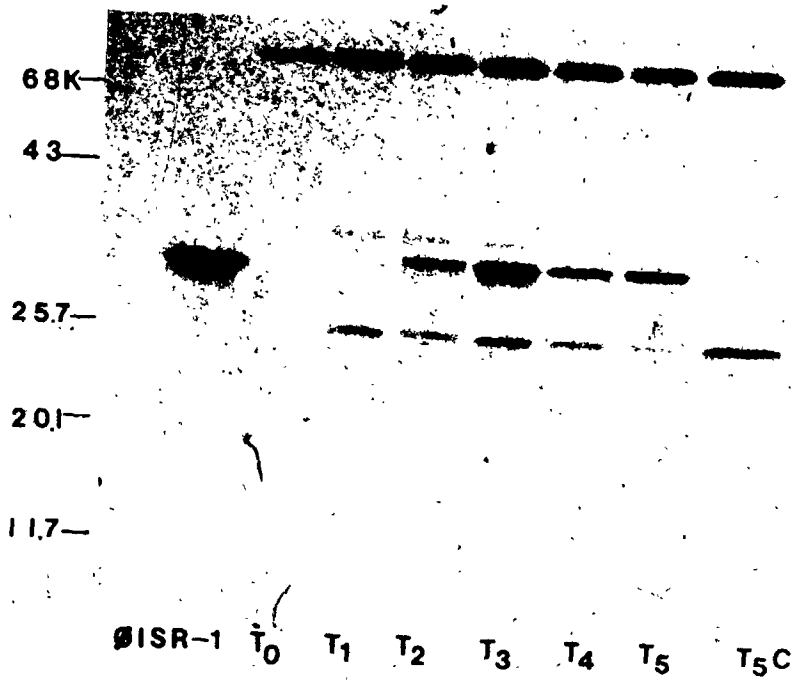
7
Time in hrs

Øisr-1 appeared in the cell lysate sampled at t_2 but was only rarely seen in the one sampled one hour earlier. The resulting fluorograph of the immunoprecipitation experiment showed that the 29.7 Kd protein was first precipitated from the cell lysates at t_2 and no protein band that could be the precursor of the 29.7 Kd protein was detectable (Fig. 5A). Electron microscopic examination of thin sections of cells sampled at t_2 indicated that approximately 50% of the cells were at stage II and the remaining were at stage III. Small inclusions were seen in cells at stage III of sporulation (Fig. 6A).

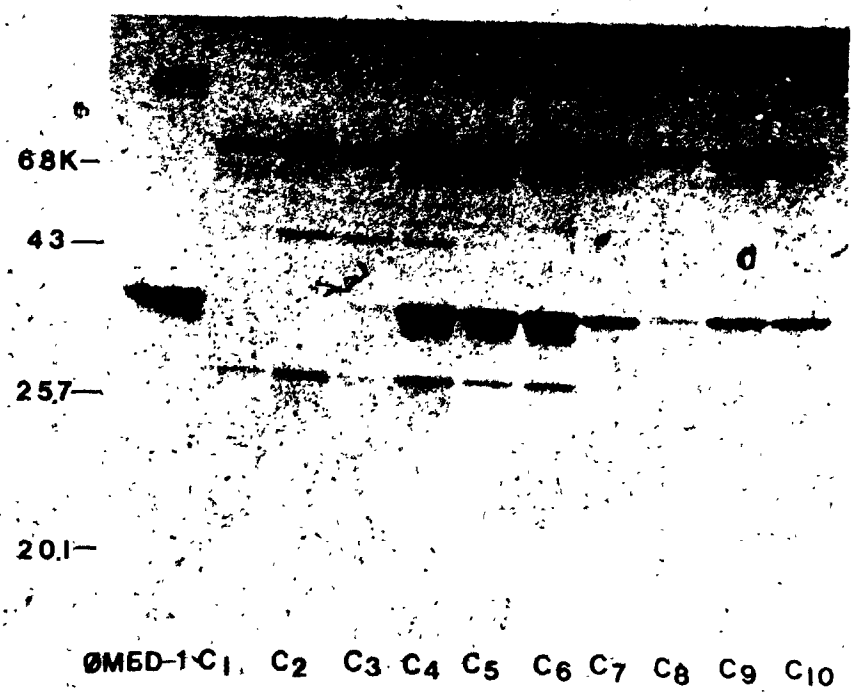
3.4. Time of synthesis of Ømed-1

Cultures of B. medusa did not exhibit an OD versus time curve similar to that of B. subtilus. Instead, the OD of the culture increased steadily until late stages of sporulation (Fig. 7). Washing of the samples before reading the OD did not change the shape of the curve. The time at which the inclusions were first detected by phase microscopy was arbitrarily designated as C_0 (following the t_0 system). This occured when the OD of the culture was approximately 0.215 x 5. For B. medusa, the use of the phase-contrast microscope was adequate for revealing the sporulation stages of cells. It was found that in the culture, stage II of sporulation was well established in some cells at C_4, 4 hr after C_0, and phase dark prespores first appeared in the cells when C_6 was reached. Examination of an unlabelled culture for Ømed-1 showed that the PLP could rarely be seen in cell lysates sampled at C_3 but could be seen in cell lysates sampled at C_4 or later. Immunoprecipitation experiments on the culture supplemented

Fig. 5 Determination of the time of synthesis of PLP proteins in developing cultures of Bacilli. Anti-Ømed-1-serum (0.5 µl) and 100 µl of 10% (W/V) Staph A cells were added to 0.5 ml of 20 x concentrated cell lysates labelled with ^{14}C(U)-L-amino acids. Precipitated proteins were solubilized by boiling the Staph A cells in dissociation buffer for 2 min before being loaded on the gel. Labelled proteins were detected by fluorography. (A) subsp. israelensis culture. The first sample was taken from the culture at T_0 and subsequent samples every hour thereafter. As a control (T_5C), an additional sample was taken at T_5 and supplemented with preimmune serum. Labelled Øisr-1 protein was included in the gel as marker. (B) B. medusa culture. The first sample was taken from the culture at C_1 and subsequent samples every hour thereafter. Labelled Ømed-1 protein was included in the gel as marker.


68K
43
25.7
20.1
11.7
ØISR-1 T0 T1 T2 T3 T4 T5 T5C

A


68K
43
25.7
20.1
ØMED-1 C1 C2 C3 C4 C5 C6 C7 C8 C9 C10

B

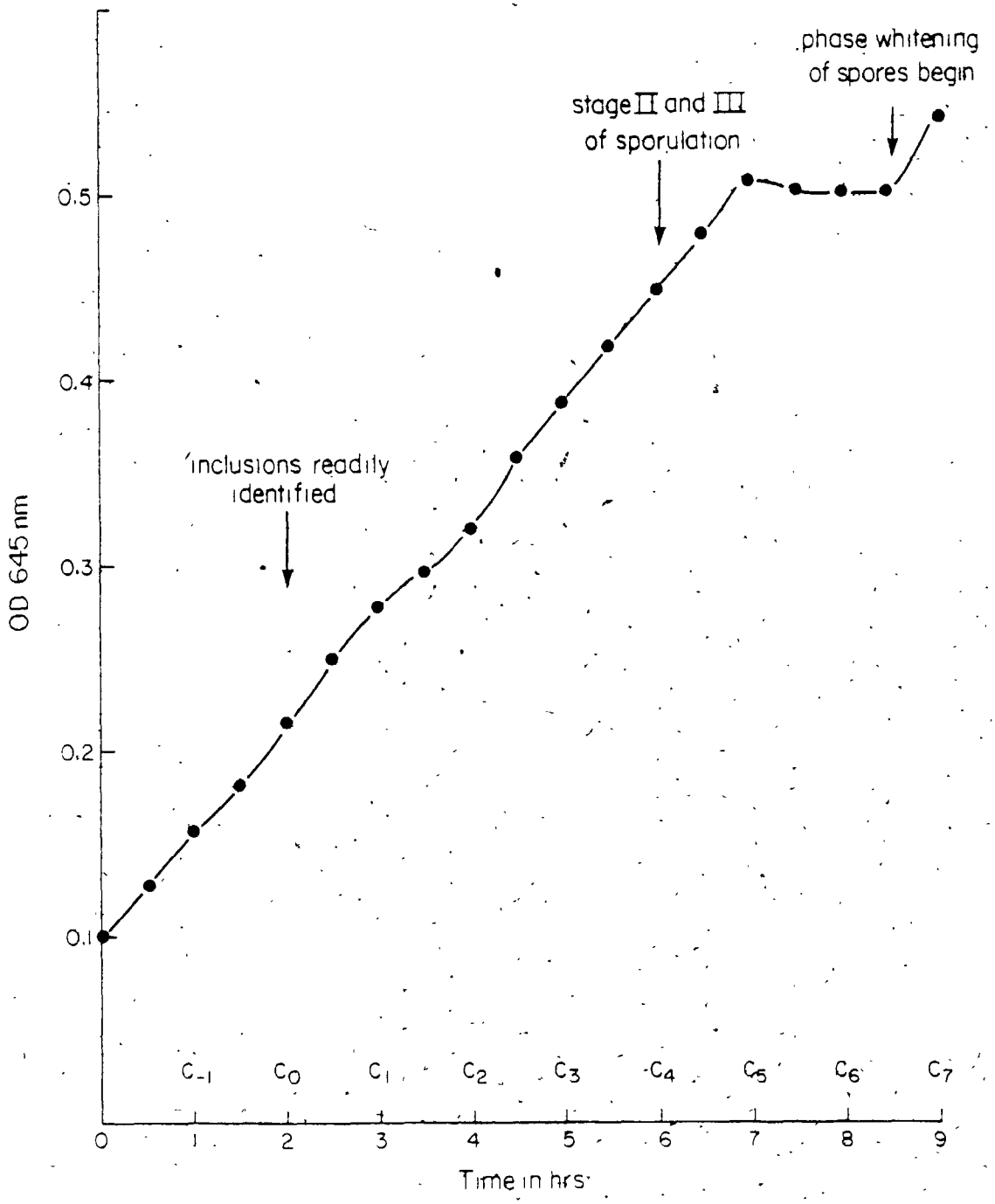
Fig. 6 Thin section electron micrographs of cells sampled when the 30 Kd PLP proteins were first detected.

a. B. thuringinesis subsp israelensis cell at early stage III of sporulation. Note that the primordial inclusion (i) is formed in close association to the forespore membrane (M). FSp is the incipient forespore.

b. Thin section electron micrograph of a B. thuringiensis isolate 73-E-10-2 cell at mid stage III of sporulation. The magnifications are indicated by the 500 nm bar marker in each figure.


6a
F5p
b

Fig. 7 Optical density versus time curve of B. medusa. One ml portions of a culture sampled at half hr intervals were diluted with 4 ml of saline and the ODs measured. The time at which the first reading was taken was arbitarily assigned time zero. Morphology of the developing cells was monitored by a phase-contrast microscope.


phase whitening
of spores begin
stage II and III
of sporulation
inclusions readily
identified
OD 645 nm
0.5
0.4
0.3
0.2
0.1
C-1
C0
C1
C2
C3
C4
C5
C6
C7
0
1
2
3
4
5
6
7
8
9
Time in hrs

with ^{14}C-L-amino acids at C_0 showed that the Ømed-1 30.5 Kd protein was precipitated from the cell lysate samples starting at C_3 and reached a maximum level at C_4 (Fig. 5B). A protein with a relative MW of around 46 Kd was precipitated from the cell lysates samples at C_2, C_3 and C_4. No similar protein was precipitated when antiserum against Øisr-1 was used as the probe though, under such conditions, the 30.5 Kd protein of Ømed-1 was readily precipitated. Therefore, the 46 Kd protein was unlikely to be the precursor of the 30.5 Kd protein. Electronmicroscopic examination of the thin sections of cells sampled at C_4 revealed that approximately 35% of the cells were at stage II, 30% of the cells were at stage III, 8% of the cells were at stage IV and the remaining 18% were at stage I.

3.5. Time of synthesis of Økyu-1 and time of formation of Ø10-2-1

Cultures of isolate 73-E-10-2 and subsp. kyushuensis in GBBM both showed an abrupt break in the exponential increase of OD as cells entered sporulation (Fig. 8 and 9). However, the cells of isolate 73-E-10-2 started to clump approximately an hour after t_0 and hence the OD dropped.

In subsp. kyushuensis, Økyu-1 were found at t_2, at later times and rarely in cells sampled at t_1. The 30.5 Kd protein of Økyu-1 was precipitated from the cell lysate sampled at t_1, reaching a maximum level at t_2. There was no obvious protein that could be the precursor of the 30.5 Kd protein. Yet there were four nonspecifically bound proteins of great abundance that could mask the precursor (Fig. 10). Thin section electron microscopy showed that, at t_2, approximately 84%

Fig. 8 Optical density versus time curve of B. thuringiensis subsp. kyushuensis. One ml portions of a culture sampled at half hr intervals were diluted with 4 ml of saline and the ODs measured. The time at which the first reading was taken was arbitarily assigned time zero. Morphology of the developing cells was monitored by a phase-contrast microscope.

Fig. 1 A plate of *B. thuringiensis* subsp. *israelensis* strain CCEB-950-1 after inoculation with spores and incubation at 42°C for three days. The 2% BBM agar had lost 25% of its weight of water through evaporation before the inoculation.

Fig. 2 Phase-contrast micrographs of *Bacillus thuringiensis* subsp. *israelensis* sporulated on GBBM agar. (a) strain CCEB-950-1, the wild type. (b) a cry⁻ variant. i , the ovoid crystalline inclusion. S, the satellite inclusion. The magnification is indicated by the 5 μm bar marker in (b).

Fig. 9 Optical density versus time curve of B. thuringiensis isolate 73-E-10-2. One ml portions of a culture sampled at half hr intervals were diluted with 4 ml of saline, and the ODs measured. The time at which the first reading was taken is arbitarily assigned time zero. Morphology of the developing cells was monitored by a phase-contrast micrscope.

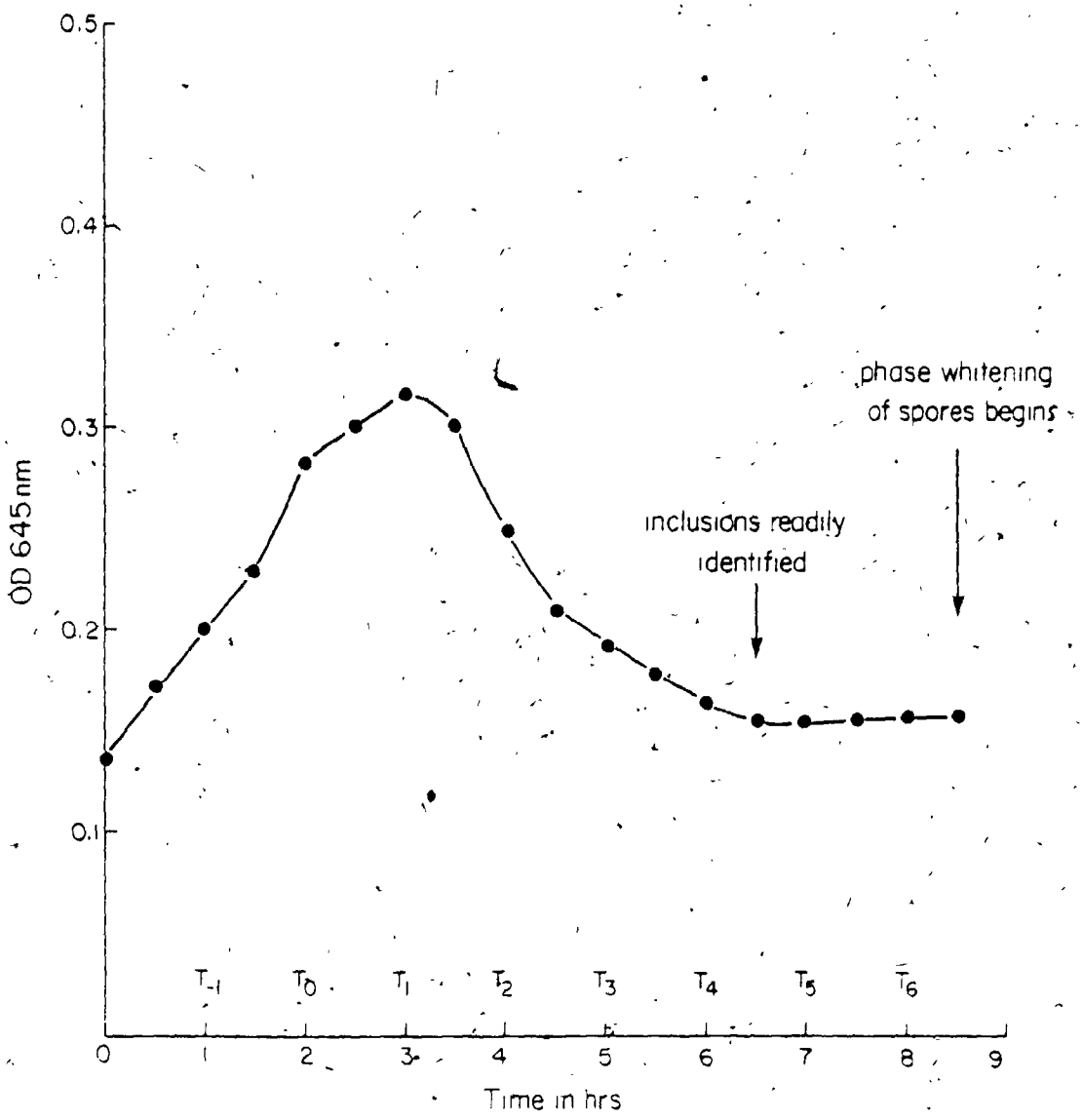
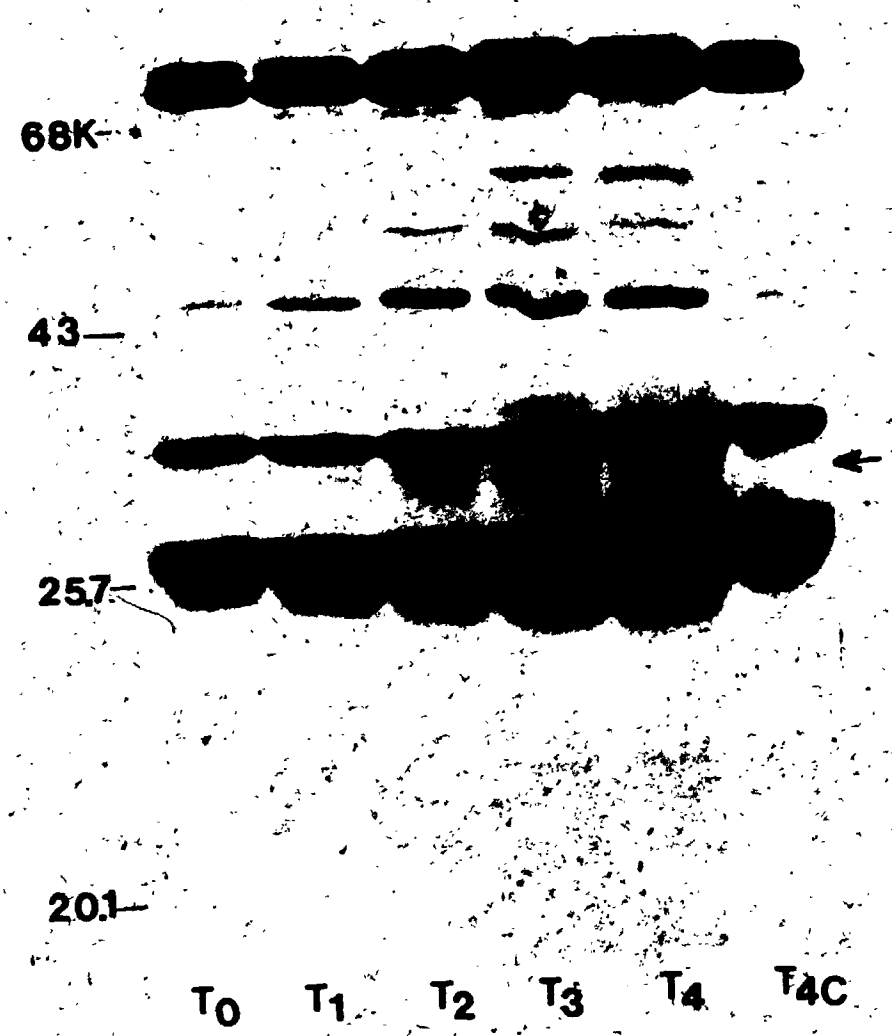

0.5
0.4
0.3
0.2
0.1
OD 645 nm
phase whitening
of spores begins
inclusions readily
identified
T-1
T0
T1
T2
T3
T4
T5
T6
0
1
2
3
4
5
6
7
8
9
Time in hrs

Fig. 10 Determination of the time of synthesis of the 30 Kd Økyu-1 protein in a developing culture of subsp. kyushuensis. Anti-Ømed-1-serum (0.5 µl) and 100 µl of 10% (V/W) Staph A cells were added to 0.5 ml of 20 x concentrated cell lysates labelled with ^{14}C(U)-L-amino acids. Precipitated proteins, dissolved by boiling the Staph A cells in 50 µl of dissociation buffer for 2 min, were applied to the gel. The first sample of cells was taken at t_0 and the following samples every 1 hr thereafter. As a control, (T_4C), an additional sample was taken at T_4 and supplemented with preimmune serum. Labelled protein was detected by fluorography. The arrow indicates the position of the 30 K immunoprecipitated protein.


68K
43
25.7
20.1
T0
T1
T2
T3
T4
T4C

of the cells were at stage II and 16% were at stage III. In isolate 73-E-10-2, the first appearance of Ø10-2-1 in the cells occured at t_4. Electron microscopy of thin sections of cells sampled at t_4 showed that approximately half of the cells were at stage II and the other half were at stage III of sporulation (Fig. 6B).

3.6. Detections of PLP in stationary cultures of B. medusa and subsp. israelensis

B. medusa did not form spores and inclusions in the enriched medium, BBM, even though the formation of inclusions can be regarded as a late vegetative event. A culture of such when sampled at different times of the stationary phase was found to be devoid of Ømed-1 and the 30.5 Kd proteins of Ømed-1. Similarly, no Øisr-1 and the 29.7 Kd proteins of Øisr-1 were detected in a stationary (BBM) culture of subsp. israelensis.

4. Discussion

The immunoprecipitation assay adopted in this work proved to be capable of detecting the ~30 Kd PLP proteins. The method was well able to detect the presence of the 29.7 Kd protein of Øisr-1 when the concentration of the 29.7 Kd protein in a sample was as low as 15% of that in a sporulated culture. One drawback of this method is the chance of losing parts of the samples during the French pressing and

washing steps. Hence the magnitude of the resulting protein bands (~ 30 Kd) did not always reflect the quantities of PLP proteins in the cells assayed.

The immunoprecipitation assays showed that no protein similar to the ~30 Kd PLP protein was detected in strains of B. thuringiensis not producing the PLP, indicating that the absence of PLP in those strains was due to an absence of monomers and not due to their inability to assemble into PLP. Similarly, the absence of PLP in vegetative and nonsporulating stationary cultures of B. medusa and subsp. israelensis were due to an absence of the PLP proteins in the cells.

The B. thuringiensis strains under study grew synchronously in the sporulation medium and their growth curve exhibited an abrupt break as the cells approached sporulation. The cause of the drastic increase in OD of the subsp. israelensis culture after the break is not known. It could be due to an excretion of cellular material into the medium. Nevertheless, the timing system developed first for B. subtilus (Aubert et al., 1969) could be employed with these strains. However, in contrast to the report by Hendry et al., (1976), this study found that the OD of a B. medusa culture increased steadily until the late stages of sporulation. The steady rise in OD was probably due to the rapid growth of the inclusion of the strain which started its formation some 2 hr before the onset of sporulation and continued to grow until the late stages of sporulation (Fitz-James, 1962). The B. medusa culture also differed from that of the thurigiensis subspecies in that the cells did not grow as synchronously as the thuringiensis cells.

In all four PLP producing strains, the initial appearance of the PLP occured at early stages of sporulation (Stage II or III). Though the B. medusa culture did not develop too synchronously at C_4, it could be deduced from the experimental results that the first appearance of Ømed-1 did not occur late at stage IV. In the three strains examined by the immunoprecipitation assay, the initial appearances of the ~30 Kd PLP proteins were concomitant with the initial appearances of the PLP, indicating a rapid assembly of PLP proteins into particles. In B. medusa and subsp. israelensis, no proteins that were likely to be the precursors of the PLP proteins were detected. Thus Ømed-1 and Øisr-1 proteins were apparently synthesized de novo at the time the PLP started to appear. Due to the nonspecific precipitation of other cellular proteins in the assays of subsp. kyushuensis, similar conclusions that the 30 Kd proteins of Økyu-1 were synthesized de novo at early stages of sporulation cannot be drawn. Such problems could be circumvented in the future by running proteins from whole cells sampled at different times of sporulation in SDS-polyacrylamide gels followed by Western Blot transfer and radiographic detection of the 30 Kd PLP proteins with antiserum and radioiodinated protein A. In any events, the similarities among the PLP suggest that the 30 Kd protein of Økyu-1 and the 29.5 Kd protein of Ø10-2-1 were synthesized at the time the PLP appeared.

It has been reported that the inclusion proteins of some B. thuringiensis species are synthesized at stage III of sporulation (Sommerville, 1971). Spore coat proteins of B. cereus and B. subtilis were also found to be synthesized at early stages of sporulation

(Aronson and Fitz-James, 1968; Pandey and Aronson, 1979). Are there any relations between the synthesis of these proteins and that of the PLP? The following chapter will consider the relationship between the syntheses of Bisr-1 and the inclusion proteins.

Chapter 4

A Study of PLP in Variants of Bacillus thuringiensis subsp. israelensis.

1. Introduction

The parasporal ovoid inclusions of B. thuringiensis subsp israelensis are extremely toxic to mosquito larvae (Goldberg and Margalit, 1977) and hence the products of this bacteria sporulation have become an important insecticide for mosquito control. The location of the gene determining the synthesis of the ovoid crystalline inclusions has been under much study (Ward and Ellar, 1983; Clark and Dean, 1983; Faust et al., 1983) and just recently a conclusive study has been reported (González and Carlton, 1984). In that study, a strain of subsp. israelensis designated as HD-567 was found to house eight plasmids with rel. mol. wt. ranging from 3.3 to ~135 MDa plus a plasmid-like linear DNA element of ~10 MDa. Acrystalliferous variants (not producing the ovoid crystalline inclusion) arose spontaneously or derived from fluid culture grown at 42°C were found to have lost a 75 MDa plasmid. Through sequential loss of all plasmids, a plasmidless acrystalliferous strain designated as HD567-61, was constructed. This strain was then transformed by the mixed culture method (González et al., 1981) to become

crystalliferous. Independently transformed strains were found to have acquired the 75 MDa plasmid while transcipients having acquired other plasmids of the donor remained acrystalliferous.

In the course of studying Øisr-1 by this candidate, hundreds of acrystalliferous variants were isolated from wild type subsp. israelensis by a 42°C heat curing method. The morphology of the variants was studied and their production of Øisr-1 examined. Plasmid patterns of particular variants were analyzed by the modified Eckhardt lysate-electrophoresis method which involved lysing protoplasts directly in the slots of a vertical agarose gel followed by immediate electrophoresis of the plasmids released (González et al., 1981; Eckhardt, 1978). The method, beside time saving, is capable of detecting small (less that 10 MDa) and very large (over 100 MDa) plasmids. In addition, since the plasmids are immediately run in a gel after releasing from the cells, there is little chance for them to be degraded. Hence open circular forms (OC) of plasmids do not usually appear.

In this report, a characterization of several acrystalliferous variants of subsp. israelensis isolated in this laboratory and a re-examination of several variant strains of HD-567, including the plasmidless HD567-61 and some transcipients, from González and Carlton, are described. The results revealed a relationship between Øisr-1 and the ovoid crystalline inclusion as well as a relationship between Øisr-1 and a small phase-dark structure not previously described.

2. Materials and Methods

2.1. Bacterial strains

Bacteria B. thuringiensis subsp. israelensis strain HD567-1, HD567-54, HD567-61, HD567-61-6, HD567-61-9 and B. thuringiensis subsp. thuringiensis strain HD-2 were obtained from Dr. Bruce C. Carlton of Ecogen Incorporated, Princeton, N.J. The israelensis strain obtained from Culture Collection of Entomogenous Bacteria, Czechoslavakia (used in Chapter 2 and 3) was designated as CCEB-950 in this chapter to differentiate it from HD-567.

2.2. Growth of Bacteria

For isolation of inclusion mutants by heat treatment, bacteria were grown on solid BBM with 2% agar. For plasmid extraction, cells were grown with reciprocal shaking at 30°C in minimal phosphate medium (Spizizen, 1958) supplemented with 0.5% glucose and 0.1% casamino acids. Preculturing was done on the same medium solidified with 1.5% agar.

2.3. Isolation of parasporal inclusion mutants

Agar plates of BBM were incubated at 55°C for one or two days until the medium lost over 20% of its weight through evaporation of water. Spores from single colonies were spread on the dehydrated agar

and the plates were incubated at 42°C for three days. Colonies that arose were streaked on GBBM agar plates and examined for parasporal inclusion mutants (after 24 hours at 28°C). In later experiments, it was found that BBM agar made to contain as little water as the dehydrated BBM agar and incubation at 42°C served equally well for the isolation of inclusion variants.

2.4. Buffers

The protoplasting medium was lysozyme (2 mg/ml, Sigma), 20% sucrose plus 100 µg/ml preboiled RNAse A, all in TES buffer (5 mM disodium EDTA, 50 mMNaCl and 30 mM Tris-HCl, pH 8.0). The tris-borate electrophoresis buffer was 25 mM disodium EDTA, 89 mM boric acid and 89 mM Tris-HCl, pH 8.3. SDS buffer contained SDS (2%), sucrose (5%) and bromophenol blue (0.05%) in the Tris-borate electrophoresis buffer.

2.5. Examination of plasmid patterns

The modified Eckhardt's lysate electrophoresis method as described by González et al., (Eckhardt, 1978; González, et al., 1981) was employed for examining plasmids of inclusion variants. The method was further modified in that cells grown overnight on agar were first rejuvenated in liquid medium for 3 hr (OD_{645} around 0.25) before protoplast generation. Three ml cultures were then harvested and resuspended in 50 µl of protoplasting medium at 37°C. The samples

were ready to be loaded on gels when 80% of the cells became protoplasts (approximately 1 hr). The dimensions of the vertical agarose gels were 16 x 14 x 0.3 cm and the wells were 0.5 cm in width. Twenty µl of the SDS buffer was first loaded on the gels and 10 µl of the protoplast preparations were loaded underneath by a Hamilton syringe. Electrophoresis was carried out at 3 mA for 1 hr, then at 7 mA for half an hour and then at 28 mA for 3 hrs (according to Gonzalez et al., 1982). Bacillus thuringiensis subsp. thuringiensis strain HD-2 was included in the experiments as a source of known molecular weight plasmids for calibration of relative molecular weights. After electrophoresis, gels were stained in 1 µg/ml ethidium bromide for 2 hr and photographed on a ultraviolet light box. The relative molecular weights of plasmids were determined by the method described by Meyers et al., (1976). Since only the 75 MDa plasmid and those with smaller masses of B. thuringiensis subsp. thuringiensis HD-2 have been analysed by electron microscopy for mass determination (González and Carlton, 1980), the values of relative molecular weights of those plasmids determined to be larger than 75 MDa by the method of Meyer et al., (1976) were only approximations. These values will be preceded by the sign (≈).

2.6. Pertinent experimental details not described here are covered in Chapter 2 and 3.

3. Results

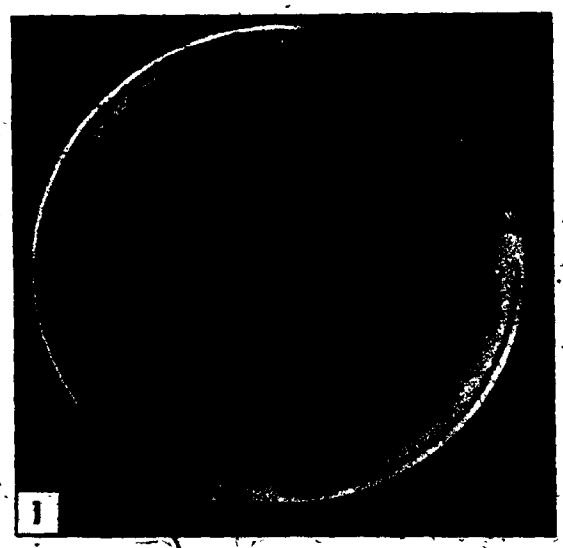
3.1. Isolation of mutants and their characteristics

Spores of B. thuringiensis subsp. israelensis strain CCEB-950-1 (this candidate's working strain of CCEB-950) derived from a single colony when spread on 2% BBM agar and incubated at 42°C gave rise, in 2 days, to a thick lawn of distorted vegetative cells. On a similar plate which had been dehydrated and lost around 20% of its weight, the lawn of cells derived from a spore inoculum was much thinner but in 2 to 3 days contained many scattered colonies of heavier growth (Fig. 1). After subsequent growth and sporulation at 30°C on GBBM agar, the subcultures of the colonies were found to be either crystalliferous or acrystalliferous (not producing the crystalline inclusion). The frequency of the occurrence of acrystalliferous variants among the heat resistant colonies appeared to depend on the degree of dehydration of the agar, i.e. the greater the degree of dehydration, the higher the occurrence. In one experiment, on a plate having lost 25% of the water content of the solid medium, around 50% of the examined colonies were acrystalliferous variants. One acrystalliferous isolate from each experiment was collected and designated.

Heat resistant variants, crystalliferous or not, lost the wild type mobility and cell division morphology, forming long chains of cells rather than separate cells. However, the acrystalliferous variants were nontoxic to mosquito larvae. When the acrystalliferous strains reached stage VII of sporulation, a small phase dark

Fig. 1 A plate of *B. thuringiensis* subsp. *israelensis* strain CCEB-950-1 after inoculation with spores and incubation at 42°C for three days. The 2% BBM agar had lost 25% of its weight of water through evaporation before the inoculation.

Fig. 2 Phase-contrast micrographs of *Bacillus thuringiensis* subsp. *israelensis* sporulated on GBBM agar. (a) strain CCEB-950-1, the wild type. (b) a cry⁻ variant. i , the ovoid crystalline inclusion. S, the satellite inclusion. The magnification is indicated by the 5 μm bar marker in (b).


1

structure, 0.4 μm (or less) in diameter, was observed in the sporangia (Fig. 2). A similar structure could not be seen in the wild type sporangia by phase-contrast microscopy but could be detected by thin section electron microscopy as a satellite to the larger crystalline ovoid inclusion (Fig. 3a). This cryptic structure, designated as satellite inclusion hereafter, when released from the sporangia, could not be differentiated from its counterpart in the acrystalliferous variants. Thus they were initially assumed to be identical.

3.2. Characterization of the satellite inclusion

In the purification of the crystalline ovoid inclusions (see methods, Chapter 2), the satellite inclusions co-migrated with the larger inclusions in Renografin density gradients. No attempt was made to separate the two. After a similar gradient centrifugation, the small inclusions of the acrystalliferous variants formed a band at the position in the gradients at which the ovoid inclusions would have migrated. These purified small inclusions were found to be non-toxic to mosquito larvae at a dose of 10 μg/larva.

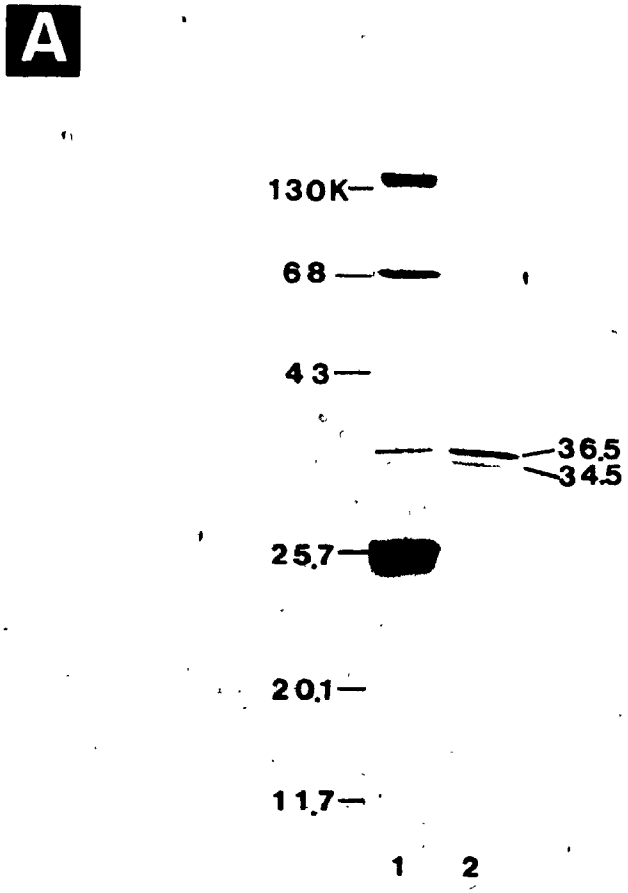
Both ovoid inclusions and the smaller inclusions could be solubilized by boiling in dissociation buffer. SDS-polyacrylamide gel electrophoresis showed that the small inclusions of acrystalliferous variants were composed of two polypeptides, one with a relative molecular weight of 36.5 and another of 34.5 Kd (Fig. 4). Purified inclusion preparations (containing ovoid and satellite inclusions) of the wild type, upon gel electrophoresis, generated more than ten

Fig. 3 Thin section electron micrographs of sporulated cells of (a) B. thuringiensis subsp. israelensis strain CCEB-950-1 showing the presence of the ovoid crystalline inclusion (i) and the satellite inclusion (S), and (b) a cry⁻ variant showing the persistence of the satellite inclusion (S). (Sp) is the spore. The magnifications are indicated by the 500 nm bar marker in each figure.

3a

b

Fig. 4 A. SDS-PAGE of Renografin gradient-purified inclusion preparations from B. thuringiensis subsp. israelensis. Purified inclusions were boiled in dissociation buffer for 5 min and then loaded on the gel. Lane 1: from CCEB-950-1, 50 µg of protein. Lane 2: from 950-2, a cry⁻ strain, 10 µg of protein. The gel is stained with Coomassie blue. B. Phase-contrast micrograph of a purified preparation of satellite inclusions from 950-2. The magnification is indicated by the 5 µm bar marker.


A
130K
68
43
36.5
34.5
25.7
20.1
11.7
1
2
B

protein bands including two that matched the small inclusion bands in mobility. Moreover, antiserum raised against the small inclusions reacted with a KCNS extract of purified inclusion preparations of the wild type and vice versa (data not shown). Hence the identity of the two small structures was confirmed.

3.3. Isolation and characterization of variants without satellite inclusions

A screening of over 400 acrystalliferous isolates, turned up, from separate experiments, two isolates lacking the satellite inclusion. They were designated 950-6 and 950-7. In a similar attempt, spores of an acrystalliferous variant, designated 950-4 and still carrying the satellite inclusion, gave rise to two identical satellite-minus variants after incubation on a dehydrated BBM plate at 43°C. These were designated 950-5. In the following discussion, cry^+, sat^+ and $\emptyset$-1^+ are used to denote respectively the phenotype of crystalline inclusion, satellite and PLP production. A schematic representation of the derivations of variants was shown in Fig. 5. Vegetative cultures of the cry^-sat^- strains showed no difference from the cry^-sat^+ variants. Electron microscopy of the sporulation lysate of the acrystalliferous variants, however, revealed all except the sat^- strain carried $\emptyset$isr-1. Immunoprecipitation assay of the sporulation lysates of sat^- strains using anti-$\emptyset$med-1-serum indicated that the strains did not produce the 29.7 Kd protein of $\emptyset$isr-1 (Fig. 6).

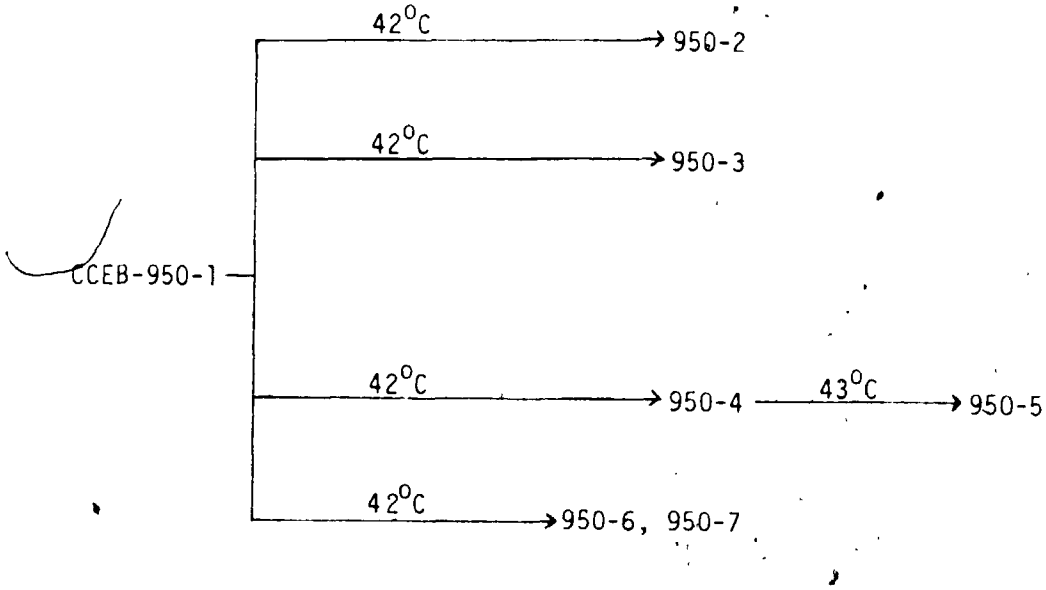
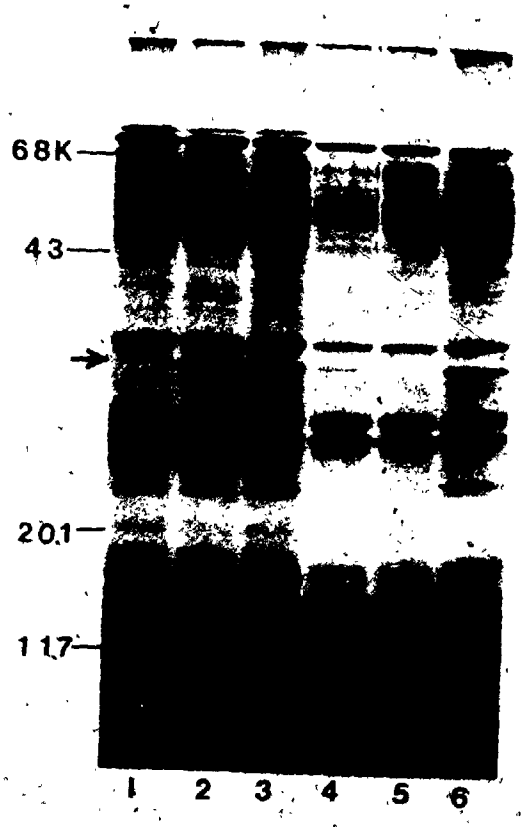


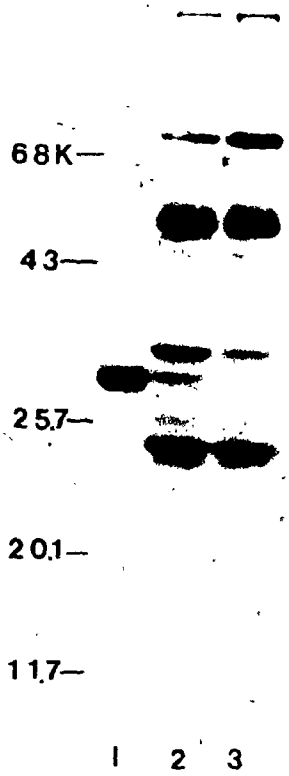
Fig. 5. Derivation of variants of B. thuringiensis subsp. israelensis (strain CCEB-950). The method of derivation is given above the arrow preceding each variant: 42°C, the variant arose after germination and growth at 42°C. 43°C. the variant arose after germination and growth at 43°C.

Fig. 6 Immunoprecipitation assay for PLP protein from B. thuringiensis subsp. israelensis strain CCEB950-1 and its variants. One µl of anti-Ømed-1-serum and 200 µl of 10% (W/V) Staph A cells were added to 1 ml of 20 x concentrated sporulated cell lysates of strains of subsp. israelensis and the precipitated proteins were solubilized by boiling the Staph A cells in 50 µl of dissociation buffer before being loaded on the gel.

Gel A. Lane 1: a control with preimmune serum added to strain CCEB-950-1. Lane 2: CCEB-950-1. Lane 3: strain 950-2, a cry^- sat^+ variant. Lane 4: strain 950-4, a cry^- sat^+ variant. Lane 5: strain 950-6, a cry^-sat^- variant. Lane 6: strain 950-3, a cry^-sat^+ variant. The arrow on the left of the gel indicates the position of the 29.7 K protein of Øisr-1. GelB. Lane 1: Øisr-1 protein as marker. Lane 2: strain CCEB-950-1. Lane 3: 950-5, a cry^-sat^- variant. The gels were stained with Coomassie blue.



A



B

3.4. Analysis of the plasmid patterns of acrystalliferous variants

The modified Eckhardt-lysate electrophoresis revealed the presence in CCEB-950-1 of the eight covalent closed circular (CCC) plasmids (Lane 2, Fig. 7) already reported in B. thuringiensis subsp. israelensis strain HD567 by González and Carlton (1984). However, the plasmid-like linear DNA element (LDE) carried by HD567 was not seen in CCEB-950-1. One cry^-sat^+ strain, 950-2, was found to have lost the 75, 10.6, 4.9 and 3.3 megadaltons (MDa) plasmids. In addition, a faint band which migrated as an 8.4 MDa CCC plasmid was detected (Lane 4, Fig. 7). Another cry^-sat^+ strain, 960-3, had lost the same plasmids missing in 950-2 plus the 4.2 MDa plasmid (Lane 3, Fig. 7). No new band was detected. The cry^-sat^+ strain, 905-4, had lost the 135, 75, 4.9 and 3.3 MDa plasmids and had also gained the 8.4 MDa plasmid-like band. The $cry^-sat^-\emptyset\text{-}1^-$ 950-5 derived from 950-4 further lost the 68 MDa plasmid but unlike its parent, retained the ~135 MDa plasmid. Moreover, it had also acquired the 8.4 MDa plasmid-like band which was more intensified than that present in 950-4. The two independently isolated $cry^-sat^-\emptyset\text{-}1^-$ strains, 950-6 and 950-7 had identical plasmid patterns, both had only the ~135, ~105 and 10.6 MDa plasmids. The phenotypes and plasmid patterns of these strains are collected in Table I.

Fig. 7 Modified Eckhardt's lysate electrophoresis of plasmids of B. thuringiensis subsp. israelensis strain CCEB-950-1 and its variants. Twenty μl of lysozyme mixtures containing spheroplasts derived from approx. 1.2 ml of early log phase cultures of strains of subsp israelensis were loaded to the 5% agarose gel. Lane 1: Plasmids of B. thuringiensis subsp. thuringiensis HD-2 included as molecular weight standards. Lane 2: CCEB-950-1. Lane 3: the $cry^-sat^+\emptyset\text{-}1^+$ variant, 950-3. Lane 4: the $cry^-sat^+\emptyset\text{-}1^+$ variant, 950-2. Lane 5: the $cry^-sat^+\emptyset\text{-}1^+$ variant, 950-4. Lane 6: the $cry^-sat^-\emptyset\text{-}1^-$ variant, 950-5. Lane 7: the $cry^-sat^-\emptyset\text{-}1^-$ variant, 950-6. Lane 8: the $cry^-sat^-\emptyset\text{-}1^-$ variant, 950-7. The gel was stained with ethidium bromide. The numbers on the left of the gel indicate the molecular weights (in megadaltons) of the plasmid standards. The numbers on the right indicate the relative molecular weights of the plasmids of CCEB-950-1. The white arrows indicate the positions of the plasmids which appeared as faint bands on the original photographic negative. The bracket ([) indicates the region in which linear fragments of large plasmids and chromosomal DNA are found.

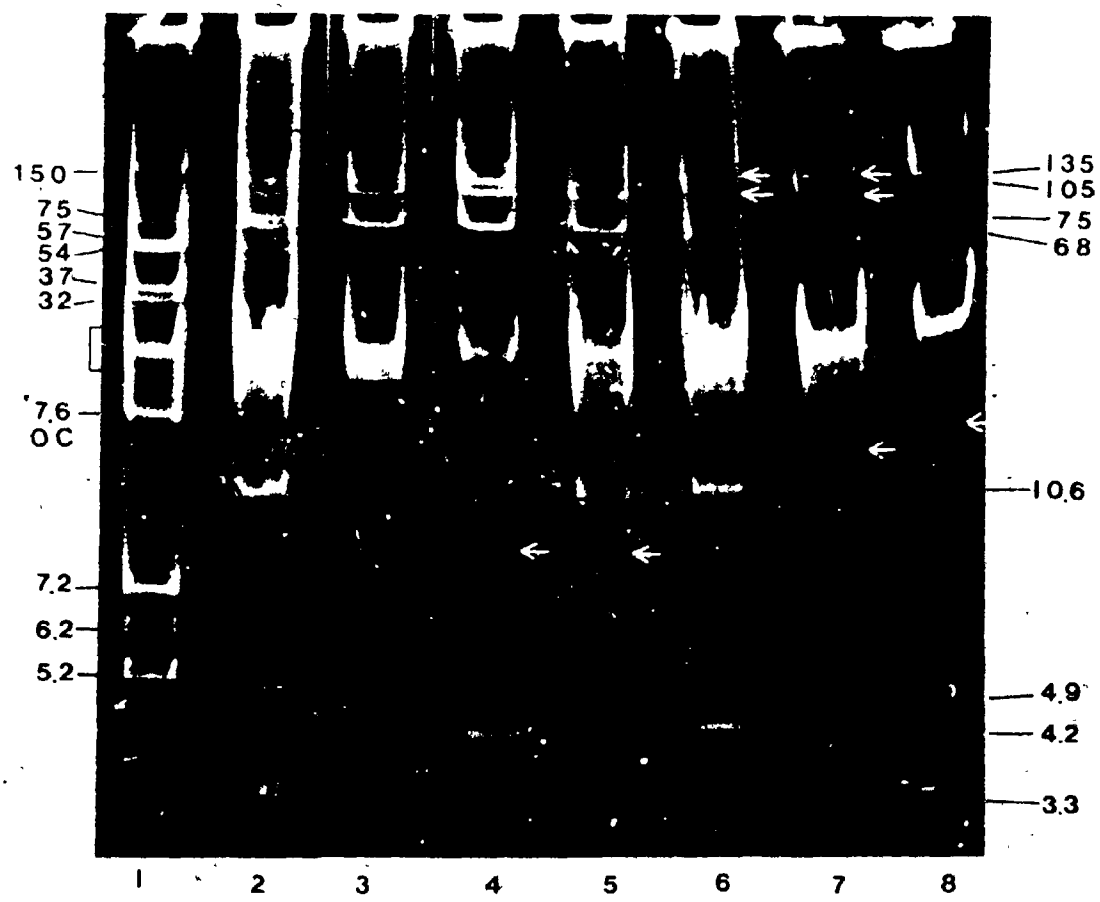

150
75
57
54
37
32
7.6
OC
7.2
6.2
5.2
135
105
75
68
10.6
4.9
4.2
3.3
1
2
3
4
5
6
7
8

Table I

Variants of Bacillus thuringiensis subsp. israelensis (strain CCEB950)

Strain	Origin	phenotype	plasmid present in the wild type (in Megadalton)									New plasmid
		*cry	sat	Ø-1	3.3	4.2	4.9	10.6	68	75	~105	~135	
CCEB950-1		+	+	+	+	+	+	+	+	+	+	+	-
950-2	CCEB-950-1	-	+	+	-	+	-	-	+	-	+	+	(8.4)
950-3	as above	-	+	+	-	-	-	-	+	-	+	+	-
950-4	as above	-	+	+	-	+	-	+	+	-	+	-	(8.4)
950-5	950-4	-	-	-	-	+	-	+	-	-	+	+	(8.4)
950-6	CCEB-950-1	-	-	-	-	-	-	+	-	-	+	+	-
950-7	as above	as above											

(+) in the phenotype column indicates producing the structures, (-) indicates not producing. In the plasmid columns, (+) indicates carrying, and (-) not carrying, the plasmid. In the 'new plasmid' column (8.4) indicates housing an 8.4 MDa plasmid-like molecule.

* The mosquito-larvacidal toxins resides in the crystal (cry). Strains without the crystal were nontoxic.

3.5. Analysis of derivative strains of HD 567 B. thuringiensis subsp. israelensis

The plasmid distribution of the HD567 series from the collection of Dr. Bruce Carlton was examined and the results are in close agreement with the published findings of González and Carleton (1984) (Fig. 8 and Table II). These strains, HD567-1, the wild type; HD567-54, a cry⁻ variant missing the 68 MDa and some other plasmids; HD567-61, the plasmidless recipient; HD567-61-6, and HD567-61-9, the transcipients having acquired the 68 and 75 MDa plasmids respectively were analyzed for their phenotype regarding satellite inclusion and Øisr-1 production (Fig. 9). The phenotypes are collected in Table II. Phase-contrast microscopy of the sporulated cell lysates of HD567-1 and thin section electron microscopy of its sporulating cells confirmed that, like CCEB-950-1, it also carried the satellite inclusions. Such a structure was not found in similar lysates of HD567-54, HD567-61-9 and HD567-61, but was present in HD567-61-6, though its size appeared to be smaller. The structure could be seen with good phase microscopy in some of the sporangia of this strain at stage VII of sporulation (Fig. 9c). Thin section electron microscopy helped to confirm its absence in HD567-54 and HD567-61-9 (Fig. 10). Electron microscopy on negatively stained smears of the sporulated cell lysates revealed that all these strains except HD567-61 produced Øisr-1. Immunoprecipitation assay of the sporulation lysates using Ømed-1 antiserum as the probe confirmed that HD567-61 did not produce the 29.7 K Øisr-1 protein (Fig. 11).

Fig. 8 Modified Eckhart's lysate electrophoresis of plasmids of variants of B. thuringiensis subsp. israelensis strain HD567-1 and its variants. Gel A. Lane 1: HD567-1. Lane 2: HD567-54. Lane 3: HD567-61. Lane 4: HD567-61-6. Lane 5: HD567-61-9. Gel B. Lane 1: HD567-61-9. Lane 2: HD567-61-9A., Lane 3: B. thuringiensis subsp. thuringiensis HD-2 plasmids included as molecular weight standards. The gels are stained with ethidium bromide. The number on the left of gel (A) indicate the relative molecular weights of the plasmids HD567-1. The number on the right of gel (B) indicate the molecular weights of the plasmid standards. The white arrows indicate the positions of the plasmids which appeared as faint bands on the original photographic negative. The bracket ([) indicates the region in which linear fragments of large plasmids and chromosomal DNA are found.

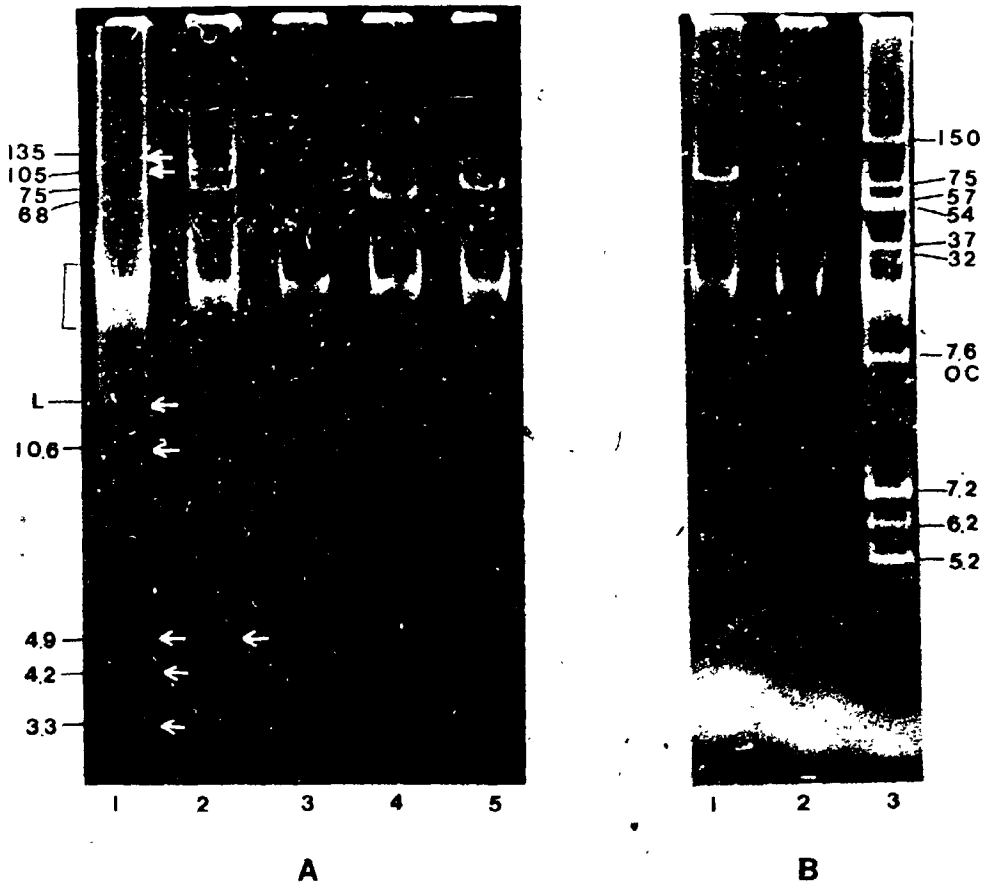

135
105
75
68
L
10.6
4.9
4.2
3.3
1
2
3
4
5
A
150
75
57
54
37
32
7.6
OC
7.2
6.2
5.2
1
2
3
B

Table II

Variants of Bacillus thuringiensis subsp. israelensis (strain HD567)

Strain	Phenotype			plasmid present (in megadaltons)
	cry	sat	β-l	
HD567-1	+	+	+	3.3, 4.2, 4.9, 10.6, L.D.E., 68, 75, ~105, ~135
HD567-54	+	-	+	4.9, 75, ~105
HD567-61	-	-	-	none
HD567-61-6	-	+	+	68
HD567-61-9	+	-	+	75
HD567-61-9A	-	-	-	none

(+) in the phenotype columns indicates producing the structure. (-) indicates not producing. L.D.E. is a plasmid-like linear DNA element.

Fig. 9 Phase-contrast micrographs of sporulated cells of variants of B. thuringinesis subsp. israelensis strain HD567-1. (a) HD567-54. (b) HD567-61. (c) HD567-61-6. (d) HD567-61-9. The magnification is indicated by the 5 μm bar marker in (a).

Fig. 10 Thin section electron micrographs of sporulated cells of variants of B. thuringiensis subsp. israelensis strain HD567-1. (a) HD567-61-9. (b) HD567-54. Note that only ovoid crystalline inclusions (i) are seen in both strains. The satellite inclusions is absent. The magnifications are indicated by the 500 nm bar markers.

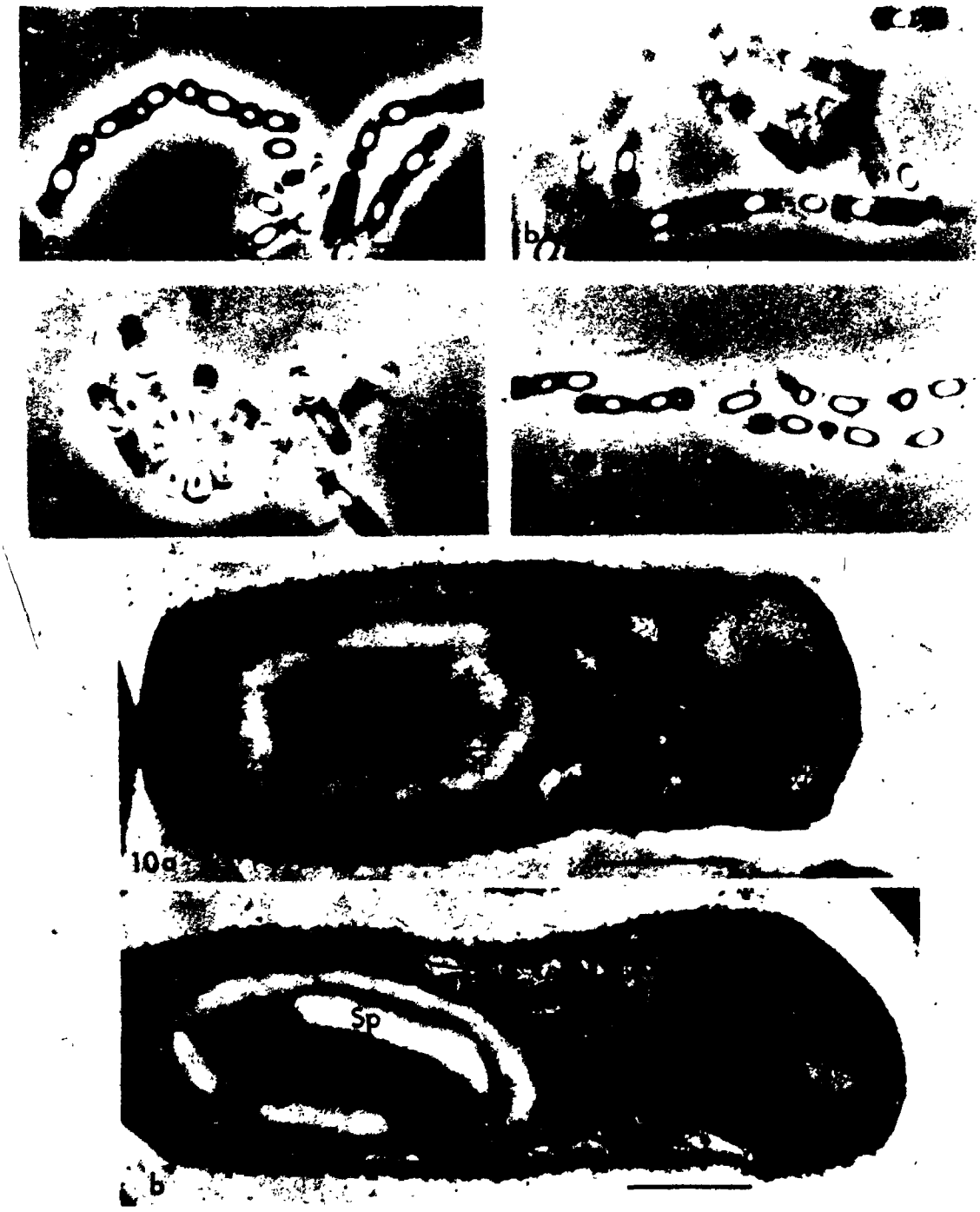
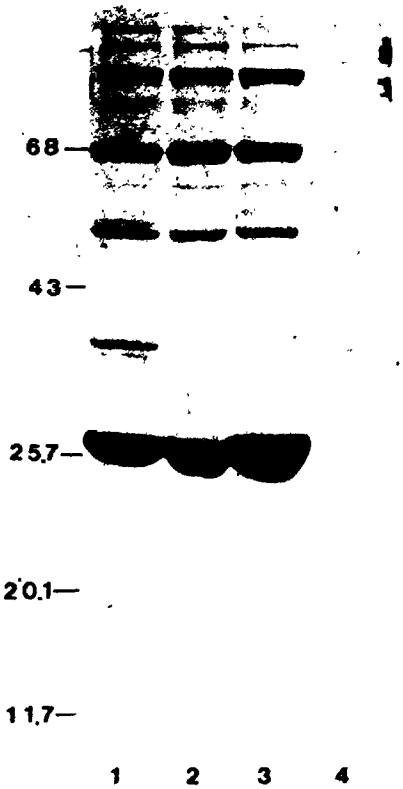

b
10a
Sp
b

Fig. 11 Immunoprecipitation assay for PLP protein in B. thuringiensis subsp. israelensis strain HD567-1 and its variants. One µl of anti-Ømed-1-serum and 200 µl of 10% (W/V) Staph A cells were added to 1 ml 20 x concentrated sporulated cell lysates of the various strains. Precipitated proteins were solubilized by boiling the Staph A cells in dissociation buffer for 2 min before being applied to the gel. Lane 1. Øisr-1 protein as marker. Lane 2: HD567-1. Lane 3: HD567-54. Lane 4: HD567-61. Lane 5: HD567-61-6. Lane 6: HD567-61-9. Lane 7: HD567-61-9A. The gel was stained with coomassie blue.

Fig. 12 SDS-PAGE of Renografin gradient-purified inclusions from strain HD567-1 and its variants. Purified inclusions of cry^+ strains of HD567 (50 µg of protein) and purified satellite inclusions of HD567-61-6 (8 µg) were boiled in dissociation buffer for 5 min and then loaded in the gel. Lane 1: HD567-1. Lane 2: HD567-54. Lane 3: HD567-61-9. Lane 4: satellite inclusions of HD567-61-6. The gel was stained with Coomassie blue.

(11)

(12)



4. Discussion

Initial examination of cry$^-$ variants of B. thuringiensis subsp. israelensis strain CCEB-950-1 indicated that they were $\emptyset$-1$^+$. Thus the production of the PLP and the toxic ovoid inclusion appeared to be unrelated. However, phase microscopy examination of the cry$^-$ variants revealed the presence in the sporangia of a small additional inclusion. These satellite inclusions were subsequently found also in the wild type sporangia. The inability of phase contrast microscopy to detect the satelites in the wild type sporangia was simply due to the presence of the larger ovoid inclusion. The 42^{o}C heat curing of plasmids on cells growing on agar enabled an unrestricted number of cry$^-$ variants to be isolated. These variants were either sat$^+$$\emptyset$-1$^+$ or sat$^-$$\emptyset$-1$^-$. Electrophoresis revealed that the comparatively small plasmids (those migrating faster than the linear chromosomal DNA) were not responsible for the sat and $\emptyset$-1 markers. For example, 950-3 which housed only the ~135 MDa, ~105 MDa and 68 MDa was sat$^+$$\emptyset$-1$^+$. The occurrence of sat$^-$$\emptyset$-1$^-$ strains 950-6 and 950-7 having only the ~135, ~105 and 10.6 MDa plasmids suggests that the 68 MDa plasmid is responsible for determining both the sat$^+$ and $\emptyset$-1$^+$ characteristics. The change of phenotype to sat$^-$$\emptyset$-1$^-$ of 940-4 after losing the 68 MDa also supports this conclusion. The fact that the ~135 MDa plasmid was present in 950-5 but not in 950-4 is probably due to a loss of the plasmid in 950-4 after 950-5 was generated. The appearance of new plasmids in B. thuringiensis strains without

conferring any apparent change in phenotypehas been reported (González et al., 1981). Thus the significance of the 8.4 MDa plasmid-like DNA found in 950-2, 950-4 and 950-5 has not been pursued in this study.

The plasmidless HD567-61 was found, as expected, to be $sat^-\emptyset\text{-}1^-$. On the other hand, the small inclusion produced by the transcipient HD567-61-6 was shown to be of the same composition as the wild type satellite inclusion. The strain was also found to be PLP producing. Since the sat^+ and $\emptyset\text{-}1^+$ characteristics are very stable, it is safe to assume that HD567-61 did not become $sat^-\emptyset\text{-}1^-$ on subsequent storage after the transcipient HD567-61-6 was generated. Thus the acquisition of the 68 MDa plasmid in HD567-61-6 is likely to be accompanied by a change from $sat^-\emptyset\text{-}1^-$ to $sat^+\emptyset\text{-}1^+$. This observation ruled out the possibility that the simultaneous loss of the 68 MDa plasmid, satellite inclusion and PLP productivity in 950-6, 950-7 and 950-5 was due to the pleiotropic effects of a single mutation. The supposition that the satellite inclusion and PLP productivity are genetically determined by the 68 MDa plasmid is thus further substantiated.

From the above findings, it was initially surprising to discover that HD567-54 lacking the 68 MDa plasmid and strain HD567-61-9 housing only the 75 MDa plasmid were also $\emptyset\text{-}1^+$. Following the same argument as applied to the 68 MDa plasmid, an acquisition of the 75 MDa plasmid seemed to have converted HD567-61 from $cry^-sat^-\emptyset\text{-}1^-$ into $cry^+sat^-\emptyset\text{-}1^+$. Thus the 75 MDa, besides being responsible for determining the production of the major inclusion, is also likely to be responsible for determining the synthesis of $\emptyset$isr-1. The isolation from HD567-61-9 of a cry^- strain which had simultaneously lost the 75 MDa plasmid

Acrystalliferous strains were readily obtained from HD567-61-9 when spores of the strain were incubated at 43°C on dehydrated BBM plates (HD567-61-9 itself was 42°C heat resistant). These cry⁻ strains did not produce the PLP. One such strain designated as HD567-61-9A (Table II) was assayed for the 29.7 Kd Ø-isr-1 protein by immunoprecipitation and was found to be nonproducing (Lane 7, Fig 11). It had also lost the 75 MDa plasmid of the parental strain (Fig. 8). Those 43°C heat resistant strains which retained toxic crystals still carried Øisr-1.

Purified preparations of crystalline inclusions of HD567-1, HD567-54 and HD567-61-9 as well as purified small inclusions of HD567-61-6 similarly prepared were analysed by SDS-polyacrylamide gel electrophoresis (Fig. 12). The protein profile of HD567-1 was very similar to that of CCEB-950-1 except three minor bands of relative molecular weights of around 32 Kd to 30 Kd in HD567-1 were not present in CCEB-950-1. The protein profile of HD567-54 inclusions was similar to that of HD567-1 but the 36.5 Kd and 34.5 Kd bands were missing. This was also true for the inclusion profiles of transcipient HD567-61-9 except that the bands of approximately 32 to 30 Kd were less prominent, as in HD567-1. The small inclusions of HD567-61-6 only generated protein bands of 36.5 and 34.5 Kd.

In an Ouchterlony double diffusion plate, antiserum against pure satellite inclusions from 950-2 reacted with a KCNS extract of purified small inclusions of HD567-61-6 and also with an extract of a crystalline inclusion preparation of HD567-1 but not with preparations derived from strains HD567-54 and strain HD567-61-9. (data not shown)

and PLP productivity further support this supposition. Thus the gene responsible for the production of PLP can be in both the 75 MDa and the 68 MDa plasmids and both copies of the gene can be independently functioning genes. González and Carlton (1984) reported that the 75 MDa plasmid was partially homologous to the 68 MDa plasmid. Probably the copies of genes responsible for the production of PLP lie within these homologous regions.

Crystalline inclusion preparations from strains HD567-54 and HD567-61-9 analyzed by SDS-PAGE and immunology indicated that they were composed of proteins different from the 36.5 Kd and 34.5 Kd proteins of the satellite inclusions. Therefore these two structures are unrelated. The biological significance of the satellite inclusions is still not known. The SDS-PAGE protein profiles of the purified inclusion preparations of CCEB-950-1 and HD567-1 in this study are similar to those reported by Pfarnenstiel et al., (1984; see Chapter 1). More information about the inclusion proteins of subsp. israelensis will be presented in Chapter 5.

Chapter 5

The Association of PLP with the Inclusions in B. thuringiensis isolate 73-E-10-2.

1. Introduction

B. thuringiensis subsp. kyushuensis and Bacillus thuringiensis isolate 73-E-10-2 are similar to B. thuringiensis subsp. israelensis, in being toxic to mosquito larvae and in having ovoid or round inclusions (Padua et al., 1980; Ohba and Aizawa, 1979, Tyrell, et al., 1981). The toxicity of the sporulation products of these two Japanese strains were found to be much lower than the value published by Goldberg and Margalit (1977) for subsp. israelensis. Thus these strains are not suitable candidates for use in mosquito control. At the time of this writing, no studies on the inclusion components of these strains has been reported in available journals. However, studies on the plasmids have been published (Iizuka et al., 1981; Iizuka et al., 1983).

Subsp. kyushuensis and isolate 73-E-10-2 were found to be PLP producing (Chapter 2), adding one more characteristic common among the three mosquitocidal thuringiensis strains. In light of their similarities, a comparison of the inclusions produced by the three subspecies were undertaken. Isolation of inclusion variants of the two Japanese subspecies were also attempted.

Strain 73-E-10-2 produces inclusions varying greatly in size. It was found that the inclusion size distribution varied with culture conditions (this work). .This size shift was not observed in the other two mosquito larvicidal strains. However, it has been reported (Scherrer et al., 1973) that an increase in media glucose concentration resulted in production of larger inclusions with a greater insecticidal activity in B. thuringiensis subsp. thuringiensis.

In this report, studies on the effects of culturing conditions on the composition and toxicity of the inclusions of isolate 73-E-10-2 are included along with a description of some inclusion variants of isolate 73-E-10-2 and a comparison of the inclusions of the three mosquitocidal B. thuringiensis strains. The results show what appears to be the toxic component or components of the inclusions of the two Japanese strains and suggested which plasmid(s) could carry the genes determining the synthesis of these toxic components and the PLP in isolate 73-E-10-2.

2. Materials and Methods

2.1. Scoring the distribution of inclusion size in sporulating cells

Even smears, free of clumps, of stage VII sporulated cells of isolate 73-E-10-2 were made by spreading a loopful of culture on a clean slide with a bent platinum wire and allowing it to air dry. A small amount (5 μl) of water was deposited on the smear, a cover slip applied and the preparation examined by oil immersion phase optics.

In each smear some two hundred sporulating cells were scored subjectively for large or small inclusions and the percentage of large inclusions recorded.

2.2. Preparation of parasporal inclusions and spores

Free inclusions and spores were prepared as described in Chapter 2 with the addition of the two-phase separation method (Delafield et al., 1968) as follows: a spore and inclusion mixture was suspended in two volumes of 15% (W/V) of sodium dextran sulfate 500 (Pharmacia Inc.) and one volume of a solution of 50mM $NaPO_4$ (pH 7.2), 60mM NaCl and 6% (W/V) polyethlene glycol 6000 (Union carbide). The mixture was then shaken vigorously and allowed to separate into its two phases (30 min at 0^oC). The upper phase rich in polyethylene glycol and spores was removed and a volume of flesh polyethylene glycol solution was added to repeat the extraction procedure. After 5 extractions, the lower phase was diluted with an equal volume of distilled water and the inclusions and any remaining spores were sedimented by centrifugation. The preparation was then subjected to Renografin gradient centrifugation as described before (Chapter 2).

2.3. Two dimensional gel electrophoresis

Two dimensional gel electrophoresis of inclusion proteins was carried out according to the method of O'Farrell (1975).

2.4. Determination of the protein content of SDS-polyacrylamide gel bands of inclusion proteins

Inclusion preparations containing 50 µg of proteins were analyzed by SDS-polyacrylamide gel electrophoresis and the resulting gel was stained with Coomassie blue. Scanning densitometry of slices of the gel was conducted at 565 nm on a Beckman DU-8 spectrophotometer equipped with a chart recorder. The total areas under the entire densitometry tracing for each slice (a lane) were cut out and weighed to give a value of total protein in the lane. The area under the peak corresponding to the protein band of interest was also similarily manipulated. The ratio of the amount of band protein to that of total protein in each sample was so calculated.

2,5. Comparison of the production of Ø10-2 under different culture conditions

Fifty ml of cultures of isolate 73-E-10-2 grown under different culture conditions were harvested and allowed to lyse in 10 ml of phage buffer supplemented with 0.1% triton X-100. Spores, inclusions and other large structures were removed by centrifugation at 19,500 x g for 30 min. Phage-10-2-1 and other minute parasporal structures were pelleted at 55,000 x g for 1 hrs and resuspended in 0.1 ml. of phage buffer. Suspensions containing 60 µg of protein were analyzed by gel electrophoresis. The ratio of the amount of the 29.5 K protein of 010-2-1 to that of total protein in each high speed pellet was determined by scanning densitometry as described in the above section.

2.6. Analysis of low molecular weight plasmids

Low molecular weight plasmids of isolate 73-E-10-2 were purified from mid-log cells by the clear lysate technique as described by Guerry et al., (1973) followed by CsCl-EtBr gradient centrifugation as described by Crosa and Falkow (1981). To differentiate closed covalent circular (CCC) plasmids from open circular (OC) DNA, the method as described by Van den Hondel et al., (1979) was employed. Briefly, plasmid samples in 0.1% Sarkosyl were heated at 100°C for 2 min before being loaded onto a 0.6% agarose vertical slab gel (of the same dimensions as that employed in the modified Eckhardt lysate electrophoresis, Chapter 4). Electrophoresis was at 90 V for 4 hrs. The plasmid patterns were then compared with that of an unboiled sample. Comparison of low molecular weight plasmids derived from wild type and variants of isolate 73-E-10-2 were carried out by electrophoresis of the DNA in 0.7% agarose horizontal slab gels, 16 cm x 15 cm x 0.6 cm, at 60 V for 10 hrs.

2.7. Assay of Toxicity

Five 3rd instar larvae (Aedes egypti) were introduced to each of a series of test tubes (2.5 cm x 15 cm) containing five ml of water and, with these tubes, a serial dilution of an inclusion suspension with known protein concentration was made. The mortality was recorded after 24 hr and the LD_{50} (µg of protein/ml) was calculated according to the method of Reed and Muench (1938). Purified spores of subsp. kyushuensis and isolate 73-E-10-2 were also tested.

2.8. Pertinent experimental details not described here were covered in previous chapters.

3. Results

3.1. Morphological observations by phase contrast microscopy

Bacillus thuringiensis subsp. kyushuensis when grown in GBBM generally produced one inclusion per cell. Some cells produced two or more (Fig. 1a). The size of the inclusions varied only slightly. On the other hand, isolate 73-E-10-2 produced only one inclusion per cell but the size of inclusion varied from 0.45-1.7 μm in diameter. The majority of inclusions, in fact, fell into two populations, one approximately 0.7 μm, the other 1.2 μm. The proportion of cells forming large inclusions among the whole population in a liquid culture was found to vary according to the growth conditions (Table 1 and Fig. 1b). When grown at 32°C in GBBM medium, isolate 73-E-10-2 formed comparatively less (12%) large inclusion bearing cells whereas at 28°C their number was some 3.5 fold higher (Table 1). The proportion of large inclusion producers was further increased (6.5 fold) over that at 32°C if the spores were first germinated and grown to OD 0.45 (7 hrs) in nutrient broth and then subcultured to the GBBM fluid sporulation medium at 28°C. Cultures growing on solid medium also gave rise to a greater proportion of large inclusion-bearing cells than did cultures grown in fluid medium at the same temperature.

Fig. 1 Phase-contrast micrographs of B. thuringiensis subsp. kyushuensis and B. thuringiensis isolate 73-E-10-2 after growth and sporulation at 28°C. The magnification is indicated by the 5 μm bar marker in (a).

a. B. thuringiensis subsp. kyushuensis on GBBM agar. The parasporal inclusions (i) are of moderate size and double inclusions are found in some cells.

b. B. thuringiensis isolate 73-E-10-2 in fluid GBBM. The culture contained cells producing large inclusions (L) and cells producing small inclusions (S).

c. A culture of small inclusion variant of B. thuringiensis isolate 73-E-10-2 in fluid GBBM.

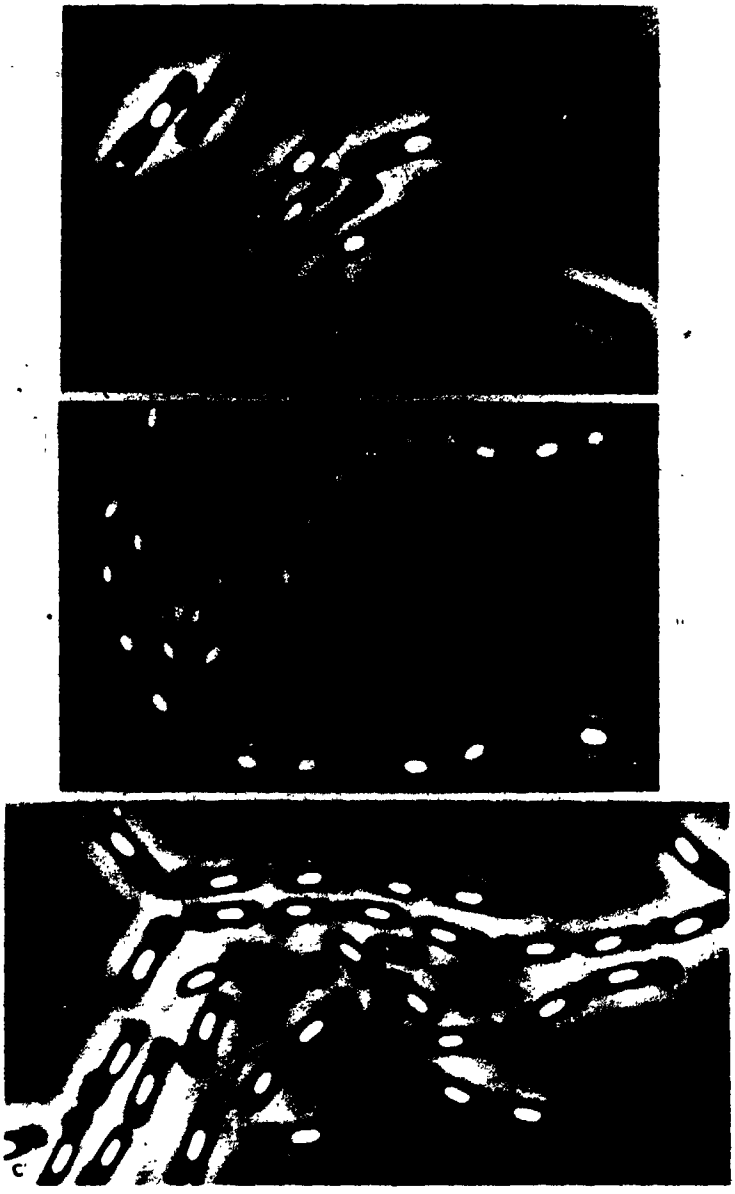


TABLE I

Inclusion Sizes and Toxicity of Strains of *Bacillus thuringiensis* at Different Growing Conditions

Strains	Growing Conditions	% of large Inclusions	LD_{50} (in µg protein/ml)
73-E-10-2 wild type	1. Spores germinated and grown in GBBM at 32°C.	12 ± 2%	1.13 ± 0.12
	2. Spores germinated and grown in GBBM at 28°C.	42 ± 4%	0.285 ± 0.027
	3. Spores germinated and grown in nutrient broth until OD reached 0.45, then 5 ml subcultured to 100 ml of GBBM at 28°C.	80 ± 5%	0.178 ± 0.022
Type I and II variants	as in (3)	zero	no toxic effect at 10 µg/ml.
subsp. *kyushuensis*	as in (3)	N/A	1.125 ± 0.035

The data expressed is the mean of data obtained from two separate experiments

Since cultures did not sporulate synchronously on agar medium, the number of large inclusion producers among the whole population could not be scored.

3.2. Isolation of mutants and their characteristics

Like subsp. israelensis, isolate 73-E-10-2 grew poorly at 42°C on 2% BBM agar which had lost 20% of its weight of water. Again, after 2 to 3 days of incubation, scattered colonies of heavier growth were found. When these colonies were subcultured on GBBM agar at 28°C, a number of them produced, on sporulation, only small inclusions of approximately 0.6 μm in diameter (Fig. 1c). The frequency of the occurrence of these small inclusion variants also appeared to depend on the degree of dehydration of the plates. In one experiment, on a plate which lost 28% of the water content of the solid medium, over 50% of the examined colonies (22 of them) were variants with small inclusions. This same treatment when applied to subsp. kyushuensis did not yield any inclusion mutants. Instead, those colonies which did come up on dehydrated plates incubated at 42°C produced only wild type inclusions.

3.3. Assays of toxicity

Bioassays showed that the toxic component of the sporulating cells of isolate 73-E-10-2 and subsp. kyushuensis resided exclusively in the inclusions. Inclusion preparations produced by isolate 73-E-10-2 grown at 32°C were only one fourth as toxic as those derived fom

the cultures grown at 28°C (Table I). The toxicities of the inclusion preparations derived from cultures subjected to nutrient broth preculture were 1.5 times higher than cultures not so treated. The inclusions produced by the small inclusion producing variants were not toxic for mosquito larvae at a concentration as high as 10 µg per larvae for a period of two days. Inclusions produced by variants surviving the 42°C treatment and retaining the wild type morphology were found to be as toxic as the original wild type inclusions. The toxicity of the inclusions of subsp. kyshuensis grown at 28°C was as low as that of the inclusions of isolate 73-E-10-2 grown at 32°C.

3.4. Polyacrylamide gel electrophoresis of inclusion proteins

The inclusions of Bacillus thuringiensis subsp. kyushuensis and isolate 73-E-10-2, like that of subsp. israelensis (Chapter 4), could be completely solubilized by boiling for five minutes in 2% SDS plus 1% 2-mercaptoethanol at pH 6.8. SDS-polyacrylamide gel electrophoresis of the solubilized inclusion proteins revealed that the inclusions of all these subspecies were composed of a number of polypeptides (Fig. 2A). Isolate 73-E-10-2 displayed eleven discrete bands, subsp. kyushuensis twelve and subsp. israelensis ten (there were also some minor bands). The molecular weights of the proteins are listed in Table II. All three strains had proteins of molecular weights at around 130 Kd, 68 Kd, 51 Kd and 26 Kd. The 51 Kd proteins in the inclusions of isolate 73-E-10-2 and subsp. kyushuensis

Fig. 2 SDS-PAGE of inclusion proteins of strains of B. thuringiensis. Fifty µg of inclusion proteins solubilized in dissociation buffer by heating for 5 min at 100°C were run in the gel. All cultures if not specified were grown at 28°C. Gel A. Lane 1: inclusions of B. thuringiensis isolate 73-E-10-2. Lane 2: B. thuringiensis subsp. kyushiensis. Lane 3: B. thuringiensis susp. israelensis. Gel B. Lane 1: B. thuringiensis subsp. kyushuensis. Lane 2: B. thuringiensis isolate 73-E-10-2. Lane 2: inclusions of isolate 73-E-10-2 grown at 32°C. Lane 4: inclusions of isolate 73-E-10-2 germinated and grown in nutrient broth then subcultured to GBBM. Lane 5: small inclusions of the type I heat resistance variant, 10-2-2. Lane 6: small inclusions of a type II heat resistance variant, 10-2-3. The numbers on the right of gel (B) indicate the estimated molecular weights of the inclusions of wild type isolate 73-E-10-2. The gels were stained with Coomassie blue.

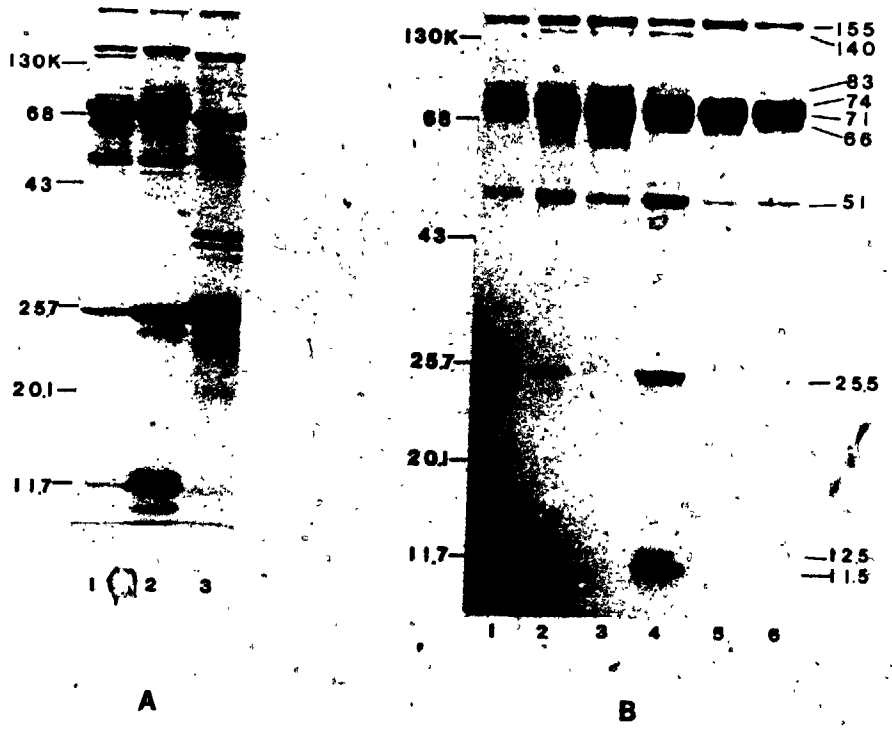

130K
68
43
25.7
20.1
11.7
1
2
3
A
130K
68
43
25.7
20.1
11.7
155
140
83
74
71
66
51
25.5
12.5
11.5
1
2
3
4
5
6
B

TABLE II

Molecular Weights of Major Proteins in the Inclusions of Strains of *Bacillus thuringiensis* and their variants.

Strains	Molecular Weights of Proteins (in Kilodaltons)
B. thuringiensis subsp. *israelensis*	140, 87, 68, 62, 55, 51, 36.5, 34.5, 26, 11.2
B. thuringiensis subsp. *kyushuensis*	155, 90, 83, 77, 71 (51), 25.5, 23, 12.5, 11.5, 10
B. thuringiensis isolate 73-E-10-2	155, 140, 83, 74, 71, 66, (51), 25.5, 12.5, 11.5
Isolate 73-E-10-2 Type I variant	155, 74, 71, 66, (51)
Isolate 73-E-10-2 Type II variant	155, 83, 74, 71, 66, (51)

Underlined values are those of proteins present in much smaller amounts than the other proteins. The 51 Kd proteins in parenthesis sometimes appeared as one band and sometimes as two bands in SDS-polyacrylamide gels. They are counted as two proteins.

sometimes appeared as a duplex and sometimes as a single band. A 23 K protein appeared in the protein profile of isolate 73-E-10-2 inclusions on prolonged storage.

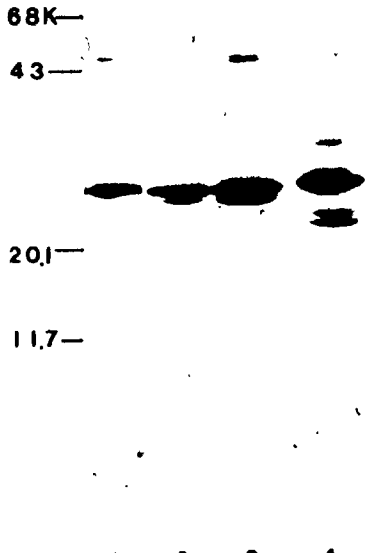
Peptide mapping by limited proteolysis and gel electrophoresis of the individual proteins of the inclusions separated by SDS-polyacrylamide gel electrophoresis were carried out using Staphylococcus aureus V8 protease. The proteins of molecular weights about 26 Kd, 51 Kd and 68 Kd of the three strains were analyzed. Only the 25.5 Kd proteins of subsp. kyushuensis and isolate 73-E-10-2 were found to have identical peptide profiles (Fig. 3). Attempts were made to analyze the 12.5 Kd and 11.5 Kd proteins of isolate 73-E-10-2 and that of subsp. kyushuensis by peptide mapping but without success; no discrete peptides could be seen in the gel after the limited proteolysis. The inclusion preparations of these two subspecies were then subjected to two dimensional gel electrophoresis. The result (Fig. 5) indicated that the 12.5 Kd protein and 11.5 Kd proteins of both strains had isoelectric points (pI) of 5.95 and 5.5 respectively. The 25.5 Kd proteins of both strains had a pI of 6.25.

The inclusions prepared from cultures of isolate 73-E-10-2 grown under different conditions (Table I) showed differences in protein profiles (Fig. 2B). Inclusions derived from cultures grown at 32°C had much less protein of 25.5 Kd, 12.5 Kd and 11.5 Kd than those derived from culture grown at 28°C (lanes 2 and 3, Fig. 2B). A culture grown in GBBM at 28°C but from spores germinated and pregrown in nutrient broth produced inclusions that had much more of the 25.5 Kd, 12.5 Kd and 11.5 Kd proteins, the 51 Kd protein band was also slightly intensified (lane 4, Fig. 2B). In a similar experiment, the

Fig. 3 SDS-PAGE analysis of peptides generated by limited proteolysis of the 26-25.5 Kd proteins of inclusions of B. thuringiensis subsp. israelensis, subsp. kyushuensis and isolate 73-E-10-2. Gel slices containing 10-15 µg of each of the proteins were cut from an SDS gel which had been loaded with inclusions proteins of subsp. israelensis, kyushuensis and isolate 73-E-10-2. The gel slices were then applied to the second SDS gel (15% acrylamide) in the presence of 0.05 µg of Staphylococcus aureus V8 protease for each lane. Lane 1: 25.5 Kd protein of the inclusions of subsp. kyushuensis with no enzyme added. Lane 2: 25.5 Kd protein of isolate 73-E-10-2 with enzyme. Lane 3: 25.5 Kd protein of subsp. kyushuensis with enzyme. Lane 4: 26 Kd proteins of subsp. israelensis with enzyme. The gel was stained with Commassie blue.

Fig. 4 Oucheterlony double diffusion immunological analysis of inclusions of Bacillus thuringiensis subsp. kyushuensis and isolate 73-E-10-2. The center well contained antiserum against intact inclusions of subsp. kyushuensis. Well A contained dissolved inclusions of subsp. kyushuensis. Well B contained dissolved inclusions of isolate 73-E-10-2 grown at 32°C. Well C contained inclusions of type II small inclusion variants of isolate 73-E-10-2. Well D contained dissolved inclusions of isolate 73-E-10-2 germinated and grown in nutrient broth and then subcultured to GBBM. All dissolution was done in 1 M KCNS, 25 mDTE and 0.05 M Tris-HCl (pH 11).

③



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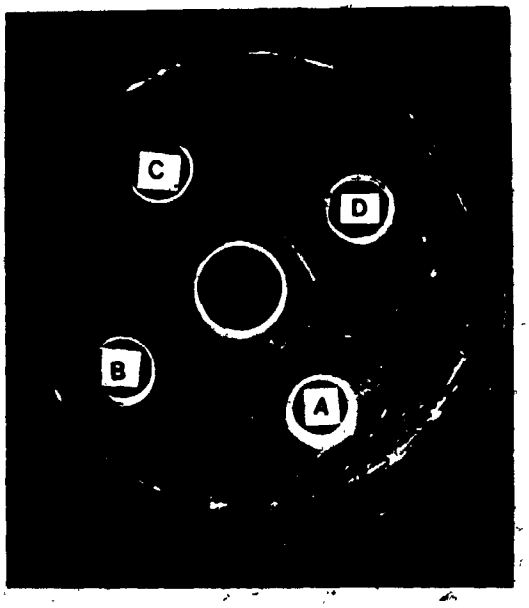
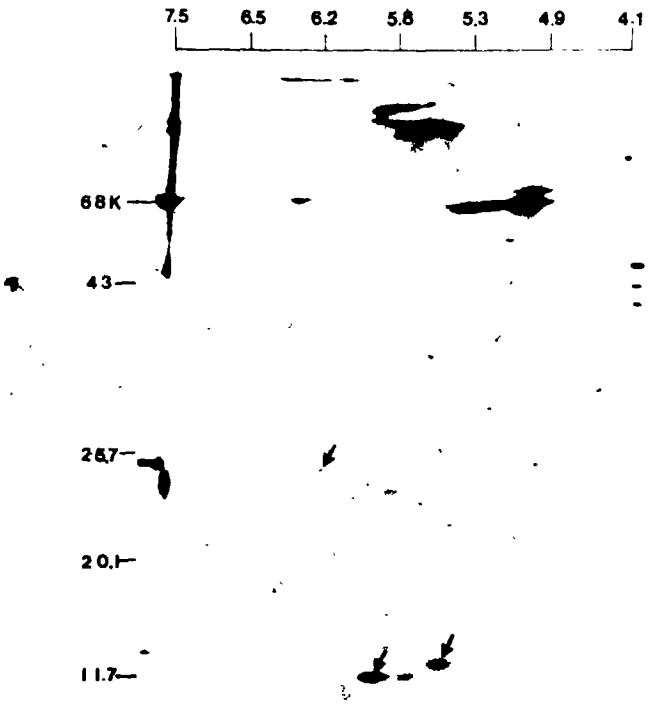
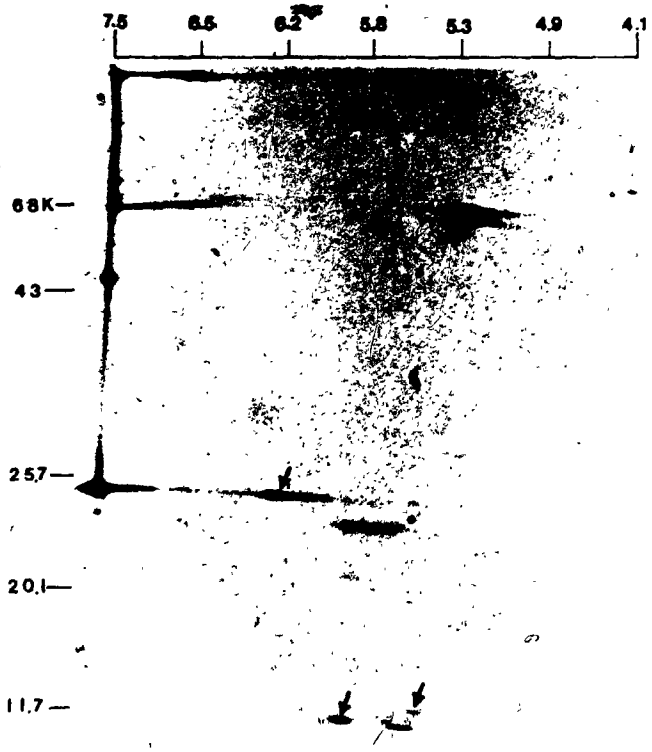


Fig. 5 Two-dimensional gel electrophoresis of inclusion proteins of B. thuringiensis subsp. kyushuensis and isolate 73-E-10-2. Fifty µg of ^{14}C labelled inclusion protein (approximately 25,000 cpm) solubilized in 9.5 M urea, 2% NP-40, 2% ampholines and 5% 2-mercaptoethanol were loaded on the isoelectric focussing (IEF) gel. Labelled proteins on the second gel were detected by fluorography. A: inclusions proteins of B. thuringiensis isolate 73-E-10-2. B: inclusion proteins of B. thuringiensis subsp. kyushuensis. The numbers on the left of each gel indicate the molecular weights and positions of migration of each gel markers and the numbers on top of each gel indicate the pH gradient of the IEF gel. The arrows indicate the positions of the 11.5 Kd, 12.5 Kd and the 25.5 Kd proteins.


7.5
6.5
6.2
5.8
5.3
4.9
4.1
68K
43
25.7
20.1
11.7

A


7.5
6.5
6.2
5.8
5.3
4.9
4.1
68K
43
25.7
20.1
11.7

B

resulting gel was analyzed by scanning densitometry. The amount of the 25.5 Kd protein was found to be 10.0% of that of total protein for the preparations derived from the culture with a preculture phase, 6.2% for the preparation from the regular culture and 3.2% for the preparation from the culture grown at 32°C.

The inclusions of the small inclusion variants of isolate 73-E-10-2 also showed differences in protein composition from the inclusions of the wild type strain grown under the same condition (lane 5 and 6, Fig. 2B). Two types of variants were found. The inclusions from the only type I variant, designated as 10-2-2, lacked the 140 Kd, 83 Kd, 25.5 Kd, 12.5 Kd, and 11.5 Kd proteins of the wild type. Inclusions produced by type II variants had a protein profile similar to those of the type I except that the 83 Kd protein of the wild type was retained. The heat resistant variants which retained the wild type characteristic in respect to inclusion size showed inclusion protein profiles identical to those of the wild type.

3.5. Immunological characterization

Immunoassays carried out on Ouchterlony plates (Fig. 4) indicated that antiserum raised against intact inclusions of subsp. kyushuensis reacted not only to the homologous antigen but also to that of isolate 73-E-10-2 inclusions derived from a culture which was first precultured in nutrient broth. No precipitin line was seen when the antiserum was challenged by inclusion proteins derived from a culture grown at 32°C or by the inclusion proteins of a type II variant. The same antiserum did not react with inclusion proteins of

subsp. israelensis. Likewise, antiserum against the inclusions of subsp. israelensis did not react with inclusions of wild type strain 73-E-10-2 nor those of subsp. kyushuensis (data not shown).

3.6. Examination of small inclusion variants for PLP

Small inclusion variants of isolate 73-E-10-2 were morphologically similar to the wild type except for the inclusion characteristic. However, electron microscopy and immunoprecipitation assays indicated that Ø10-2-1 and its 29.5 Kd protein were not present in the small inclusion variants, irrespective of whether they were of the type I or type II (Fig. 6).

3.7. Comparison of the production of Ø10-2-1 under different culture conditions

Protein determination on the high speed pellets of sporulated lysates of isolate 73-E-10-2 grown under the three conditions as described in the previous section (Table I) showed that the amounts of measurable proteins in the samples derived from cultures grown at 28°C with or without the nutrient broth preculturing were roughly the same while that derived from cultures grown at 32°C were only about 70% of the former. After 60 µg of proteins of each samples were run in a gel, a band comigrating with the 29.5 Kd protein of Ø10-2-1 was seen for all samples, but the band derived from the culture grown at 28°C with a preculture phase had the highest intensity while that from the culture grown at 32°C had the lowest (Fig. 7). The amount of the

Fig. 6 Immunoprecipitation assays for PLP protein in sporulated cell lysates of inclusion variants of isolate 73-E-10-2. One µl of anti-Ømed-1-serum and 200 µl of 10%(W/V) Staph A cells were added to 1 ml of 20 x concentrated sporulated cell lysates of strains of isolate 73-E-10-2. Precipitated proteins were solubilized by boiling in dissociation buffer for 2 min before loading in the gel. Lane 1: wild type isolate 73-E-10-2. Lane 2: strain 10-2-2, the type I small inclusion variant. Lane 3: strain 10-2-3, a type II small inclusion variant. The arrow on the left of the gel indicates the position of the 29.5 Kd protein. The gel was stained with Coomassie blue.

Fig. 7 Comparison of the production of Ø10-2-1 under different culture conditions. Sporulated cell lysates of isolate 73-E-10-2 grown under different conditions were centrifuged at 19,500 x g for 30 min and the supernatants were centrifuged at 55,000 x g for 1½ hr. Sixty µg of proteins from the resuspended pellets were solubilized in dissociation buffer and run in the gel. Lane 1: purified Ø10-2-1 as marker. Lane 2: proteins from cells germinated and grown in BBM and then subcultured to GBBM at 28°C. Lane 3: proteins from cells germinated and grown in GBBM at 28°C. Lane 4: proteins from cells germinated and grown in GBBM at 32°C. The gel was stained with Coomassie blue.

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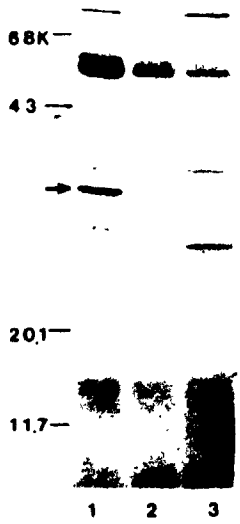

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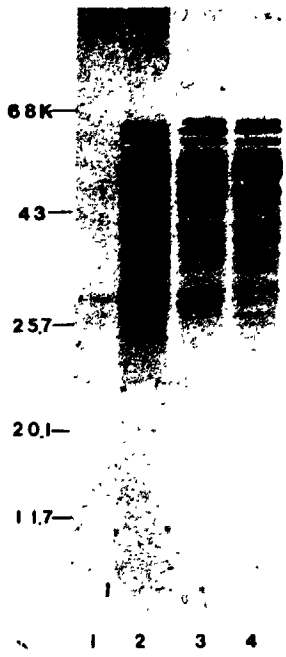


29.5 Kd as deduced from scanning densitometry was 18.8% of total sample proteins for the culture grown with a preculture phase, 8.6% for the regular culture at 28^{0} and 3.3% for the culture grown at 32^{0}C.

3.8. Examination of plasmids

The Eckhardt lysate electrophoresis procedure revealed 8 distinct plasmid bands in isolate 73-E-10-2 (Lane 2, Fig. 8). The molecular weights of these plasmids were estimated to be ~130, ~94, 63, 59, 29, 8.8, 8.4 and 4.9 Megadaltons (MDa). There were some faint bands between the linear fragment of chromosomal DNA and the 8.8 MDa plasmid band.

Three small inclusion producing variants of isolate 73-E-10-2 were also examined for their plasmid patterns. The type I variant, 10-2-2 was found to have lost the ~94, 63, 8.8 and 4.9 MDa plasmid (lane 4, Fig. 8). A variant of type II, designated 10-2-3, was found to have lost only the ~94 and 8.8 MDa plasmid while another type II variant, 10-2-4, was found to have lost, in addition, the 4.9 MDa and the ~130 MDa plasmids (lane 5 and 6, Fig. 8). Two randomly chosen heat resistance variants of wild type inclusion morphology were found to have retained all the discrete plasmids of the wild type. The data is summarized in Table III.

The number of plasmids housed by isolate 73-E-10-2 found in this study differs from that reported by Iizuka et al., (1983). Their study showed that isolate 73-E-10-2 carried plasmids with relative MW of 107.15, 9.47, 8.95 and 3.30 MDa. In order to determine if all the distinct bands seen in the gels of this study were CCC DNA, low MW

Fig. 8 Modified Eckhardt's lysate electrophoresis of variants of Bacillus thurigiensis isolate 73-E-10-2 on agarose gel. Lane 1: Plasmids of Bacillus thuringiensis subsp. thuringiensis HD-2 included in the experiment as molecular weight standards. Lane 2 and 3: wild type isolate 73-E-10-2. Lane 4: strain 10-2-2, the type I small inclusion variant. Lane 5: strain 10-2-3, a type II small inclusion variant. Lane 6: strain 10-2-4, a type II small inclusion variant. Lane 7 and 8: two randomly chosen 42°c heat-resistance variants retaining the wild type inclusion morphology. The gels were stained with ethidium bromide. The numbers on the far left indicate the molecular weights (in Megadaltons) of the plasmid standards. The numbers between the two gels indicate the relative molecular weights (in Megadaltons) of the plasmids of wild type isolate 73-E-10-2. The bracket ([) indicates the region in which linear fragments of large plasmids and chromosomal DNA are found.

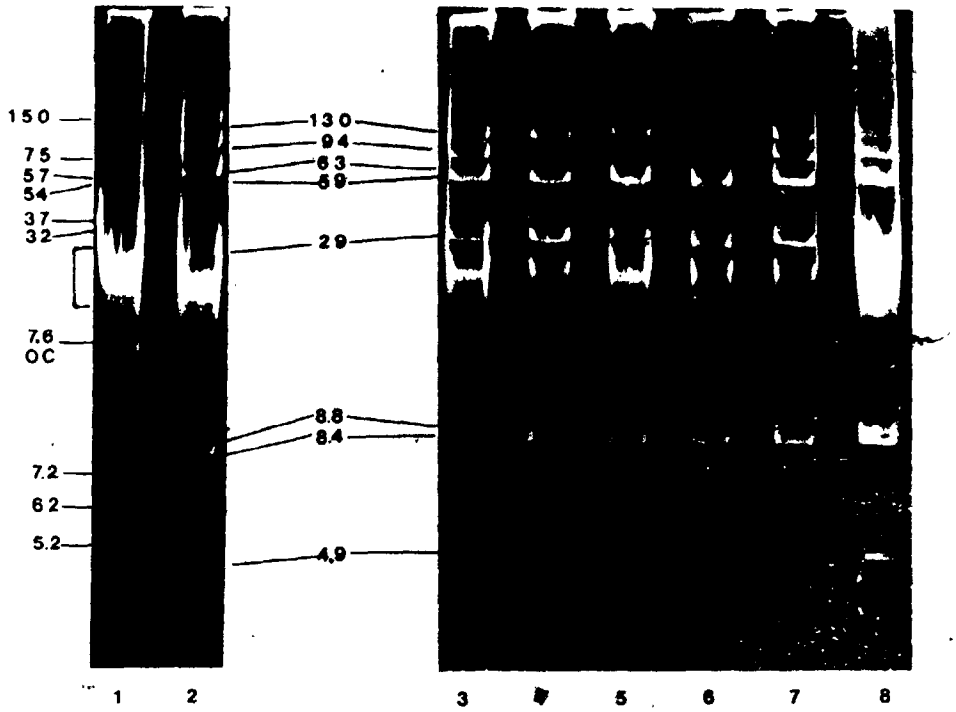

150
75
57
54
37
32
7.6
OC
7.2
6.2
5.2
13.0
9.4
6.3
5.9
2.9
8.8
8.4
4.9
1
2
3
5
6
7
8

TABLE III

Plasmids Present in Bacillus thuringiensis isolate 73-E-10-2 and its Variants

Strain	Plasmids present							
	~130,	~94	63	59	29	8.8	8.4	4.9
Wild type	+	-	-	+	+	-	+	-
Type I variant, 10-2-2	+	-	+	+	+	-	+	+
Type II variant, 10-2-3	+	-	+	+	+	-	+	-
Type II variant, 10-2-4								

Plasmids present in the wild type are listed as their masses. (+) indicates presence of and (-) indicates absence of a plasmid in the variants. Plasmids with relative masses larger than 75 MDa were preceded by (~) to indicate the values are only approximations, since among the plasmid standards used in the gel electrophoresis experiments, only the 75 MDa plasmid and those of smaller mass have been analysed by electron microscopy for mass determinations (González et al., 1981).

plasmids were extracted and analysed. A plasmid preparation derived from CsCl-EtBr gradient centrifugation of the clear lysate of isolate 73-E-10-2 was subjected to gel electrophoresis. The gel revealed plasmid bands with mobilities corresponding to molecular weights of 18 (duplex), 10.5, 8.8, 8.4 and 4.9 MDa (Fig. 9). By the van den Hondel (1979) technique, it was shown that the 18 MDa and 10.5 MDa plasmids were actually open circular forms, probably of the 8.8; 8.4 and 4.9 MDa plasmids.

Low molecular weight plasmid analysis of the type I variant, 10-2-2, showed two plasmid bands with mobilities corresponding to relative MW of 18 and 8.4 MDa while that of the type II variant, 10-2-3, revealed in addition, a 10.5 MDa band and a 4.9 MDa band (Fig. 10). The 18 MDa generated from both preparations appeared to be thicker than the 18 MDa band generated from the plasmid preparation of the wild type. These results further substantiate the probability that the 10.5 MDa band is in fact, the OC forms of the 4.9 MDa plasmid and that the 18 MDa (duplex bands) seen in the preparation of the wild type are actually the OC forms of the 8.4 and 8.8 MDa plasmids. Thus, the high MW plasmids (those above the the linear DNA smear) revealed by the modified Eckhardt's procedure were not OC forms of the low MW plasmids in that they migrated to higher positions than the OC forms of the low MW plasmids. Furthermore, OC forms of plasmids with MW higher than 10 MDa would only form anomalous smears on top of the gel under the conditions in which the modified Eckhardt lysate electrophoresis were performed (González and Carlton, 1980). Thus the bands above the linear chromosomal DNA smear in the gels are likely to be CCC plasmids.

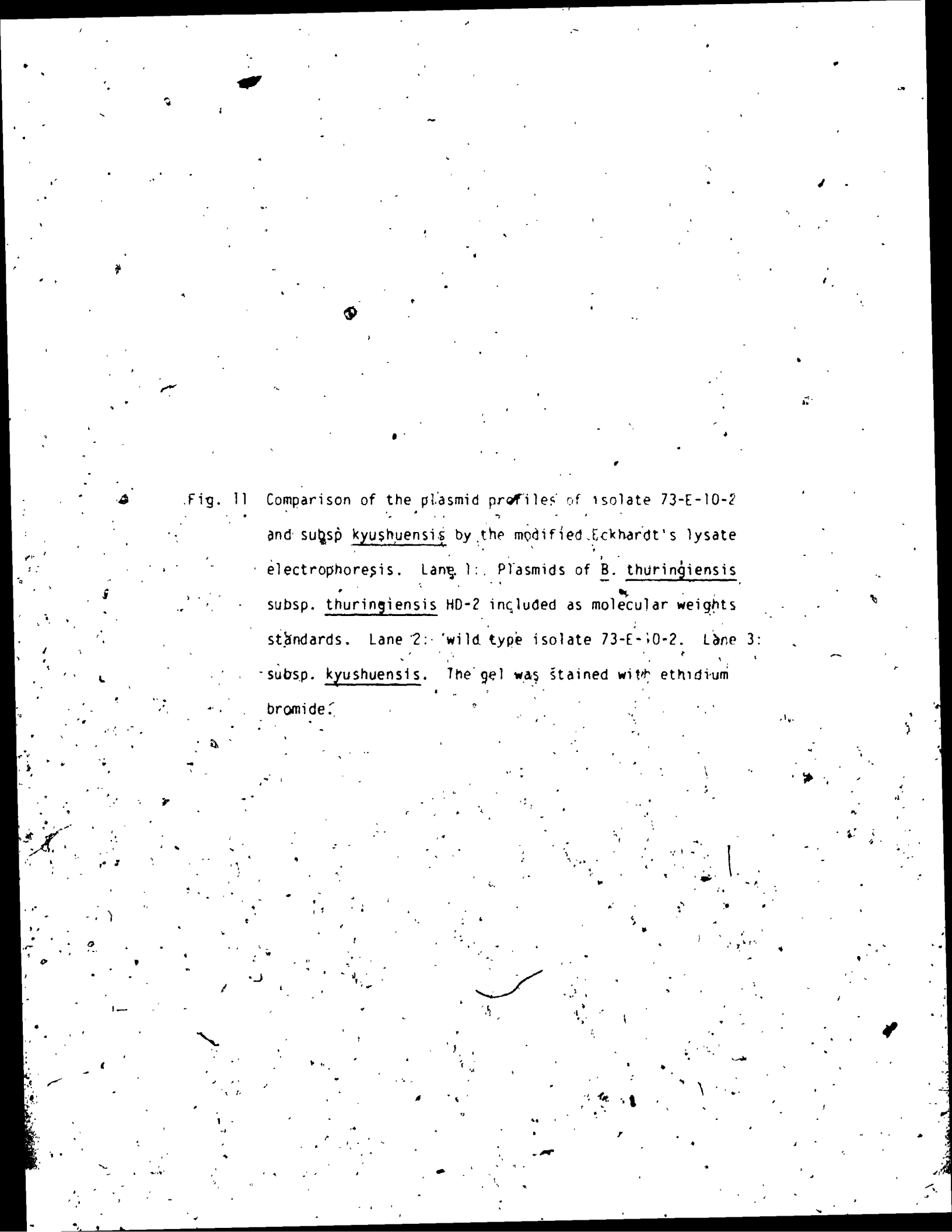
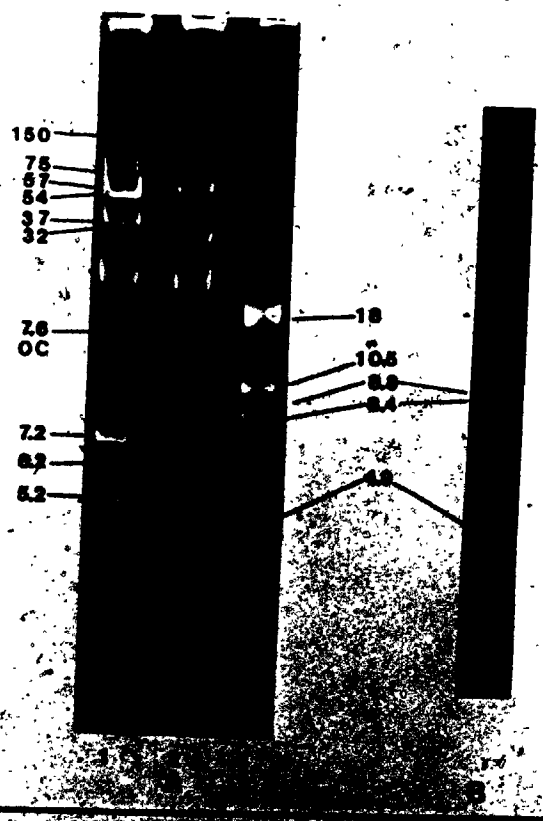


Fig. 11 Comparison of the plasmid profiles of isolate 73-E-10-2 and subsp kyushuensis by the modified Eckhardt's lysate electrophoresis. Lane 1: Plasmids of B. thuringiensis subsp. thuringiensis HD-2 included as molecular weights standards. Lane 2: wild type isolate 73-E-10-2. Lane 3: subsp. kyushuensis. The gel was stained with ethidium bromide.

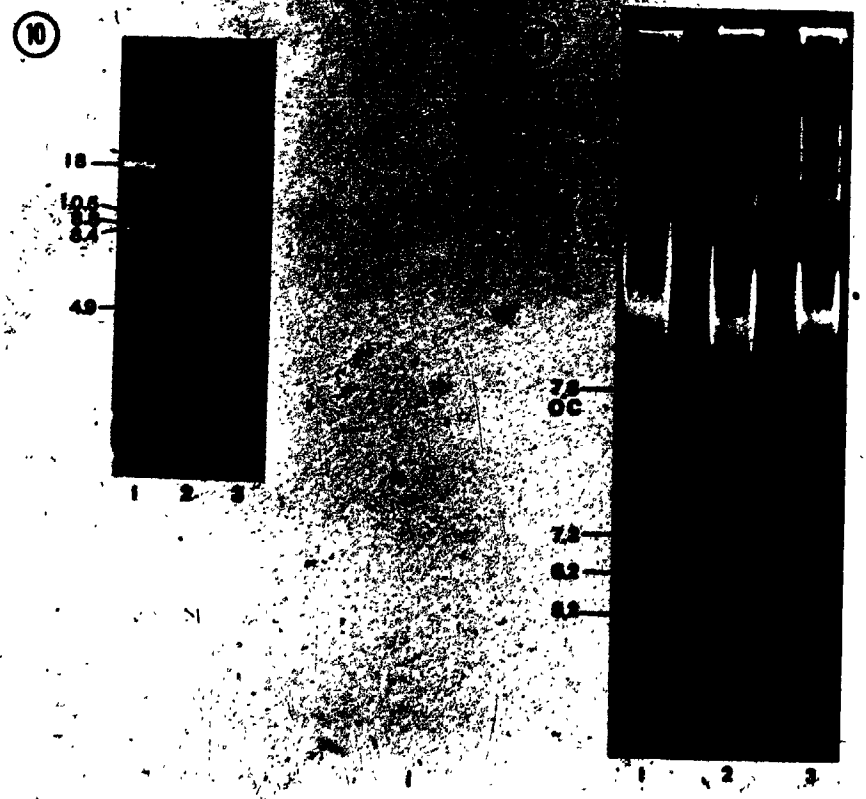
Fig. 9 Analysis of plasmids of Bacillus thuringiensis isolate 73-E-10-2 by agarose gel electrphoresis. Gel A. Modified Eckhardt's lysate electrophoresis in 0.5% agarose gel. Lane 1: plasmids of Bacillus thuringiensis subsp. thuringiensis HD-2 as plasmid standards. Lane 2: isolate 73-E-10-2. Lane 3: purified plasmids of isolate 73-E-10-2 loaded on the gel at the same time the protoplasts were loaded. Gel B. Heat treated purified plasmids of isolate 73-E-10-2 run in a 0.6% agarose gel. The gels were stained with ethidium bromide. The numbers on the far left indicate the molecular weights (in Megadaltons) of the plasmid standards. The numbers between the two gels indicate the apparant molecular weights (in Megadaltons) of the purified plasmids of isolate 73-E-10-2.

Fig. 10 Analysis of low molecular weight plasmids of isolate 73-E-10-2 and its variants. Purified plasmid preparations of strains of isolate 73-E-10-2 were run in a 0.7% horizontal agarose slab gel (10 cm x 15 cm x 0.6 cm) at 60 V for 10 hrs. Lane 1: wild type isolate 73-E-10-2. Lane 2: strain 10-2-2, the type I small inclusion variant. Lane 3: strain 10-2-3, a type II small inclusion variant. The gel was stained with ethidium bromide. The numbers on the right indicate the apparant molecular weights of the plasmids of wild type isolate 73-E-10-2.

(9)


150
75
57
54
37
32
7.6
OC
7.2
6.2
18
10.5
8.6
8.4
4.9

(10)


18
10.5
8.6
8.4
4.9
1 2 3
7.6
OC
7.2
6.2
6.0
1 2 3

Plasmids carried by B. thuringiensis subsp. kyushuensis were also examined by the Eckhardt lysate electrophoresis procedure. It was found that the strain carried 10 plasmids with relative MW of ~150, ~132, ~97, 71.5, 62, 10.3, 9, 5.2, 4.5 and 4.1 MDa (Fig. 11).

4. Discussion

Gel electrophoresis of the inclusion proteins of B. thuringiensis subsp. israelensis, subsp. kyushuensis and isolate 73-E-10-2 carried out in this study show that the molecular weight distribution of inclusion components of the three bacilli are very similar. Since the 36.5 Kd and 34.5 Kd proteins of the inclusion preparation derived from subsp. isralensis are, actually components of the satellite inclusion (Chapter 4), the ovoid crystalline inclusion, by itself, has a protein profile remarkably similar to that of the Japanese strains. However peptide mapping and immunological studies showed the protein components of subsp. israelensis were different from that of the Japanese strains. On the other hand, at least one component, a protein of relative MW of 25.7 Kd is common in the inclusions of isolate 73-E-10-2 and subsp. kyushuensis. It is not known whether the 12.5 and 11.5 Kd proteins of the Japanese strains were unique proteins or degradation products of larger inclusion components. Fitz-James (personal communication) has found that Na_2EDTA at a concentration of 0.01 M in all the buffers used in preparing the inclusions following their release from the cells drastically reduces the intensities of these two fastest running bands of isolate 73-E-10-2. Presumably the Na_2EDTA inhibition of a metal-ion dependent protease prevents the

degradation of larger proteins into the smaller peptides. However, in the following discussion, the two peptides will continue to be regarded as two unique proteins.

Morphologically, isolate 73-E-10-2 stands out from subsp. israelensis and kyushuensis in that it produced inclusions of contrasting sizes influenced by the growth conditions. The data reported here suggests that the size of the inclusions of this strain could be determined by the amount of the 25.5 Kd, 12.5 Kd and 11.5 Kd proteins (in short, 25.5 - 11.5 Kd proteins) present. It is possible that at the high growth temperature (32°C) the production of these proteins was somehow hindered and the inclusion size distribution of the population shifted downward. The result obtained by germinating spores of isolate 73-E-10-2 in nutrient broth before culturing in GBBM could be partly due to nutritional factors. In an experiment (data not shown) in which spores were germinated and cultured at 28°C in 100 ml. of GBBM enriched with 5 ml. of nutrient broth, the proportion of large inclusion producing cells among the whole population was 60%. This value is higher than the 42% obtained when no nutrient broth was added but significantly lower than the 80% when the spores were germinated in nutrient broth (see Table I). Thus the environment in which the spores germinate seems to be a factor determining the proportion of large inclusion producers among the whole population as well as the relative amount of the 25.5 - 11.5 Kd proteins in the inclusions. In any event, this seems to be the first observation of non-coordinate synthesis of protein components of inclusions of B. thuringiensis.

An effective way of quantitating the total PLP proteins in the PLP producing bacilli has not been developed. Yet by polyacrylamide gel electrophoresis of the high speed centrifugation pellets derived from sporulation lysates of isolate 73-E-10-2 grown under different conditions, different amounts of ∅10-2-1 present in the high speed pellets were found. Moreover, the ratio of the amount of the 29.5 Kd ∅10-2-1 protein to total protein of a high speed pellet of any one culture correlated positively with the ratio of the amount of the 25.5 - 11.5 Kd proteins to total proteins of the inclusions derived from a culture grown under identical culture conditions. The results agree with the finding that less purified ∅10-2-1 were obtained from a culture grown at 30-32°C than at 28°C (Chapter 2). Since quantitation of proteins of ∅10-2-1 has not been carried out extensively, conclusive results on the amount of the 29.5 Kd PLP protein in the cells grown under different condition are unknown.

The small inclusions of variants thus far isolated are all devoid of the 25.5 - 11.5 Kd proteins and a 140 Kd protein of the wild type inclusion. Again, this is the first time inclusions of B. thuringiensis missing some components of the wild type have been described. All small inclusion variants were found to be devoid of ∅10-2-1, indicating that the synthesis of the PLP and the protein components missing in such variants could be related. The ease of isolating the variants again indicated that the genes responsible for the synthesis of the proteins involved might not be in the chromosome.

Isolate 73-E-10-2 was shown by this work to house eight plasmids with relative MW ranging from 4.9 MDa to ~130 MDa. This finding is different from that reported by Iizuka et al., (1983) because those

workers did not use the Eckhardt lysate-electrophoresis method. This method is the only reliable procedure for showing large plasmids in B. thuringiensis strains. Analysis of isolate 73-E-10-2 showed that its small inclusion variants are all devoid of both a ~94 MDa and a 8.8 MDa plasmid. Thus either one or both of these plasmids could be responsible for the syntheses of the 25.5-11.5 Kd proteins and the 29.5 Kd protein of ∅10-2-1. Since no genes responsible for the synthesis of the inclusions of B. thuringiensis have ever been found to be carried by plasmids with relative MW less than 44 MDa (González et al., 1981; González et al., 1982; Carlton and González, 1984), and since the gene coding for ∅isr-1 are likely to be on the 75 and 68 MDa plasmids in subsp. israelensis, it is extremely likely that the ~94 MDa plasmid carries the genes responsible for the production of the proteins concerned.

The culturing conditions could have some effects on the multiplication or transcription of the plasmids and these, in turn, altered the rates of synthesis of the proteins. Further investigations are needed to verify. Incidentally, the synthesis of the 83 Kd inclusion protein missing in the type I inclusion but not in the type II might be determined by the 63 MDa plasmid which is missing in the type I variant but not in the type II.

While the aim of this study is to understand the relationship between the ∅10-2-1 and the inclusions of isolate 73-E-10-2, the results obtained tentatively identified the toxic component or components of the inclusions. Inclusions of isolate 73-E-10-2 and of subsp. kyushuensis solubilized in a solvent of 50 mM $NaCO_3$.HCl (pH 10.5) (Thomas and Ellar, 1983a) are found to be non-toxic to mosquito

larvae in this laboratory. Thus identification of the active components cannot be carried out by assaying solubilized protein fractions for toxicity. Nevertheless, the finding that an increase in the relative amount of 25.5 - 11.5 Kd proteins in the inclusions of isolate 73-E-10-2 resulted in an increase in inclusion toxicity indicates that either one or all of the three proteins could be toxic components. Furthermore, the small non-toxic inclusions produced by the heat generated variants lack the 25.5 - 11.5 Kd proteins. Yet the inclusions of subsp. kyushuensis which have higher quantities of the identical 25.5 Kd proteins, as well as the two smaller proteins are less toxic than the inclusions of wild type isolate 73-E-10-2 grown at 28°C. Thus there could be some other factors helping to confer toxicity to these inclusions.

The results of peptide mapping, isoelectric focusing and immunological study showed that the 25.5 Kd inclusion proteins of subsp. kyushuensis and isolate 73-E-10-2 are very similar. Thus this study provided one more example showing that subspecies of B. thuringiensis belonging to different serotypes can produce inclusions containing similar components. Only, in this case, one component out of the ten or eleven produced by the two strains is alike. This further substantiates the general belief that the phylogeny of the B. thuringiensis subspecies does not necessarily play an important role in determining the nature of the inclusions produced. Finally, no plasmids with relative MW of ~94 and 8,8 MDa were found in subsp. kyushuensis, thus the genes coding for the 25.5 - 11.5 Kd proteins and Ökyu-1 must be located on plasmids distinct from the above two plasmids or in the chromosome of this strain.

Chapter 6

General Discussion

This work began as a study of the phage-like-particles of B. medusa but later expanded to cover similar structures found in Bacillus thuringiensis subsp. israelensis, B. thuringiensis subsp. kyushuensis and B. thuringiensis isolate 73-E-10-2. The major findings of this work are the following: the major component of each PLP is a protein of approximately 30 kilodaltons which is similar to but not identical with its counterparts in the other three PLP. The PLP are specifically formed at early stage(s) of sporulation (stage II or III) and at least in subsp. israelensis and B. medusa, the PLP proteins are apparently synthesized at the times the PLP appear in the cells. In subsp. israelensis, the PLP are not produced in variants missing the 75 MDa and the 68 MDa plasmids. Strains possessing either one or both of the plasmids including those strains which previously reacquired the plasmids through transformation are PLP producing. In isolate 73-E-10-2, PLP are not found in variants lacking the ~94 and the 8.8 MDa plasmids. These variants also produce exclusively small inclusions lacking several protein components of the parental inclusions. These observations indicate that the gene determining the synthesis of Øisr-1 in subsp. israelensis is carried by both the 75 MDa plasmid which carries the gene determining the synthesis of the mosquitocidal inclusion (González and Carlton, 1984), and by the 68

MDa plasmid found to be determining the synthesis of a satellite inclusion. In isolate 73-E-10-2, the gene determining the synthesis of Ø10-2-1 could be carried by the ~94 MDa plasmids or the 8.8 MDa plasmids or by both. One of these plasmids possibly also carries the genes determining the synthesis of the missing components in the nontoxic small inclusion. The newly isolated mosquitocidal inclusion producing strains, PG-14 (Padua et al., 1984) was also found to produce similar looking phage-like particles (Fitz-James, personal communication). Hence all mosquitocidal B. thuringiensis producing solely ovoid or round inclusions thus far isolated are PLP producing.

What causes the diverse locations of the genes determining the syntheses of the ~30 K PLP proteins? Why are the PLP initially synthesized at early stage(s) of sporulation? Why are four (including strain PG-14) out of the five bacilli which produce PLP mosquitocidal and why are all mosquitocidal B. thuringiensis, which produce solely ovoid or round inclusions, PLP producing? In the discussion to follow, some attempts will be made to answer the questions.

Since this work is an initial biochemical survey of the PLP, it can only partially speculate on the significance of the PLP. However, in parallel to the ~30 Kd PLP proteins are the much studied lepidocidal inclusion proteins of molecular weight around 135,000 daltons. These P_1 proteins have similar molecular weights, immunologically cross-reacting with each other but are not all identical (Krywienczyk and Angus, 1967; Yamamoto, 1983). The diverse locations of the structural genes of these proteins has been

demonstrated by Kronstrad et al., (1981). González and Carlton, in their studies with some of the lepidocidal strains of B. thuringiensis, have shown that rearrangement of plasmids can occur fairly readily (González et al., 1981). In one study they found that in a cry$^+$ variant of B. thuringiensis subsp. thuringiensis HD-2, the 75 MDa plasmid carrying the structural gene for the P_1 crystal protein was replaced by a 65 MDa plasmid. A loss of the 65 MDa plasmid was accompanied by the disappearance of the crystal. These authors suggested that the 65 MDa plasmid could have derived from the 75 MDa plasmid. This together with the ease whereby plasmids can be transferred in mixed culture leads one to speculate that the diversity in locations of the P_1 structural genes was the result of intraspecific transfer of plasmids carrying the P_1 genes and of plasmid rearrangements. Recently, transposon-like stem and loop structure have been found in electron microscopic studies of plasmids of B. thuringiensis subsp. galleriae, also a P_1 protein producer (Debabov et al., 1980). In another study a 3 MDa DNA sequence likely to have transposition properties was found in plasmids in several strains of B. thuringiensis (Lereclus et al., 1983). Furthermore, inverted repeat DNA sequences were found flanking the crystal gene on a large plasmid of B. thuringiensis subsp. kurstaki HD 73. Copies of these sequences were subsequently found on plasmids from 14 strains of B. thuringiensis (Kronstad and Whiteley, 1984). Thus the diverse locations of the P_1 protein genes could be due to transposable genetic elements.

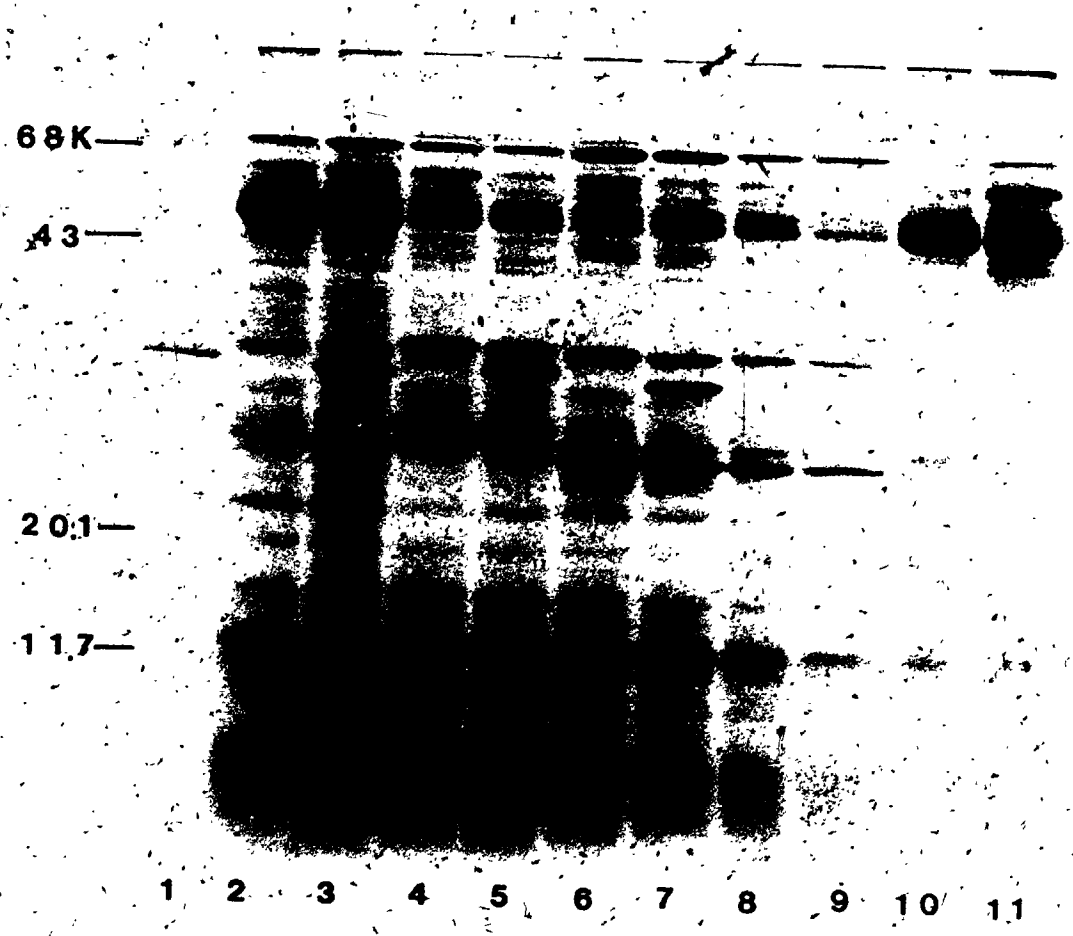
Most bacilli carry temperate phages and many of them are defective (Garro and Marmur, 1970). In cell lysates of sporulated B. medusa and subsp. israelensis, particles with heads and tails, although rare, could readily be seen among the masses of PLP. Possibly the PLP studied in this work are also defective phage. It could be that the viable forms of these now defective phages have become extinct and only the prophages which have incorporated into the DNA of the host are preserved. If this is the case, two hypotheses could be raised to explain the diverse locations of the PLP genes in the PLP producing bacilli. Firstly, at one time, a bacillus could have been infected by the viable form of PLP and lysogeny established. Then through plasmid transfer and rearrangement (not necessarily in that order) involving the prophage, the present state could have been retained attained. With time, mutation might have occurred to individual genes coding for the ~30 K PLP protein, varying the size of the resulting proteins. Secondly, viable forms of the PLP could have been similar to the unique coliphage Mu-1, a prophage which can insert at a wide variety of host sites, probably at any point on the host DNA (Howe and Bade, 1975). Thus when PLP initially infected the bacilli, the prophage integrated itself at different points along the DNA of the bacteria. Ultimately, mutation caused the phages to become defective.

There are two explanations for the production of PLP at early stage(s) of sporulation. Firstly, as the cell enter sporulation, many biochemical changes occur, including the change in sigma factors of

the RNA polymerase. These changes may stop the synthesis of the repressors responsible for maintaining the lysogenic state of the prophage and thus activate the DNA. Secondly, the genes coding for the PLP might be located in a position downstream to some spore specific DNA segments so that the transcription of the PLP proteins is under the control of those segments. In the case of subsp. israelensis and isolate 73-E-10-2, these sporulation specific DNA segments could be the operators of the genes coding for inclusion proteins. It is possible that the DNA of the viable form of PLP also inserted into the host DNA at some other sites, e.g. in or next to a vegetative specific gene, but this situation would have killed the host so that this sort of lysogeny is not seen.

At this time, it is difficult to explain why four out of five of the PLP producing bacilli so far examined are mosquitocidal. The protein components of the inclusions of subsp. israelensis and isolate 73-E-10-2 share few similarities except the molecular size distributions. Thus it is not likely that a PLP prophage and the structural genes for the proteins of one inclusion have been transferred together on one plasmid from one strain to another. Thus far, the genes responsible for the syntheses of both the ~30 Kd protein of β-Kyu-1 and the 25.7 - 11.5 Kd proteins of the inclusion of subsp. kyushuensis have not been shown to be carried by one plasmid. Thus further work will have to be done before any additional speculations can be made.

At this point of departure, this candidate would like to suggest some work to be done in the continuation of this research. At present, it is not absolutely certain that the 75 MDa and the 68 MDa plasmids are indeed carrying structural genes for the 29.7 Kd protein of Øisr-1. To verify this point, these plasmids should be transferred to some other receptive bacteria to see if the transcipients produce the Øisr-1 protein. If so, then one can be sure that the 75 MDa and the 68 MDa plasmids are carrying a structural and not a regulatory gene for the synthesis of the Øisr-1 protein. Secondly, since the 42°C heat treatment did not cure the production of PLP in B. medusa and subsp. kyushuensis, some other plasmid curing methods should be tried. The possibility that the genes determining the syntheses of these PLP are located on the chromosome should also be checked. Thirdly, the increase in production of the 25.5 - 11.5 Kd inclusion proteins and possibly the production of Ø10-2-1 in isolate 73-E-10-2 when grown in particular culturing conditions could be due to an increase in the rates of transcription or translation of the genes or to an increase in the number of the plasmid copies housing the genes. To verify this, quantitation of the number of plasmids involved should first be done. Fourthly, if the PLP determining genes carried by the 75 MDa and the 68 MDa plasmids of subsp. israelensis are the structural genes; then DNA segments carrying the genes should be cloned by DNA recombinant technique. Antiserum against the Øisr-1 proteins could be used in aiding the selection of the clones. DNA sequencing of the genes would possibly reveal the nature of the PLP.


68K
43
20.1
11.7
1 2 3 4 5 6 7 8 9 10 11

The cloned DNA segments could also be used to identify the locations of the PLP genes in B. medusa and in subsp. kyushuensis. Lastly, the investigation of whether there are nucleic acids in Øisr-1 and Ømed-1 has not been exhaustive. These two PLP could have defective capsids such that nucleic acids could easily be lost. Thus, better purifying methods which would not "shock" the PLP should be developed. If nucleic acids were found in these PLP, and if they were phage specific nucleic acids, then investigation could be done directly on the nucleic acids and the DNA cloning step would not be immediately necessary.

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Appendix 1: Reprint of the publication of Hendry et al., 1976.

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Bacteriophage and Bacteriophage-Like Structures Carried by *Bacillus medusa* and Their Effect on Sporulation

G. S. HENDRY,[1] J. B. GILLESPIE,[2] AND P. C. FITZ-JAMES*

Department of Bacteriology and Immunology and Department of Biochemistry, University of Western Ontario, London, Ontario, Canada

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Bacillus medusa was found to carry three phages or phagelike structures named φmed-1, φmed-2, and φmed-3. φmed-1 is a minute, 25-nm-diameter particle without a tail. It was extracted from the sporulation lysate of a φmed-2-minus strain of *B. medusa* and purified by differential centrifugation. The nucleic acid from this structure was shown to be orcinol positive, alkali sensitive, RNase sensitive, and DNase resistant. An RNase-resistant core of nucleic acid was not found, indicating that it was single-stranded RNA. A host strain has not yet been found for φmed-1. Phage φmed-3 was induced with mitomycin C or UV light and consisted of empty heads of 57 nm in diameter, whereas φmed-2 induced with mitomycin was a phage of 60-nm head diameter and 220-nm tail length. The sporulation sequence proceeded faster in those mutants lacking φmed-2, and when the phage was reintroduced to *B. medusa* the extended wild-type sporulation sequence was observed. *B. thuringiensis* var. *schwetzova* was sensitive to φmed-2 and yielded small turbid plaques. *B. medusa* produced small numbers of φmed-2 during growth. The other phage may be produced at the same time but were not detected. φmed-1 was found in sporulating cells by electron microscopy techniques. Its release from these was demonstrated by both electron microscopy techniques and a radioactive assay. It appears to participate in the formation of a surface layer on the parasporal inclusion of *B. medusa*.

Bacillus medusa, a filamentous inclusion-forming bacillus isolated by C. F. Robinow, has a parasporal body with a fuchsin-stainable skin (20) which is more prominent in agar than in shaken cultures (P. C. Fitz-James, unpublished data). A classical description of the organism has been published (6). By its spore morphology it belongs in the *B. cereus* group of sporeformers.

B. medusa strain 9 is a variant selected for its large spores and synchrony of sporulation. A more complete description of this strain and of the structure and composition of its parasporal body, with a comparison to the structure of other parasporal crystals, has been completed (J. B. Gillespie, Ph.D. thesis, University of Western Ontario, London, 1969). *B. medusa* has been used in relatively few studies; it was not mentioned in a recent review of the species *B. thuringiensis* (21), to which *B. medusa* appears to be related.

This study is concerned with the properties and effects on sporulation of three unusual bacteriophage or phagelike particles carried by *B. medusa*, called φmed-1, φmed-2, and φmed-3.

The effect of a temperate phage on the sporulation of *B. megaterium* 899a has been investigated with φT (13), where no effects were noted without induction of the phage. Some characteristics of φT have recently been published (11, 12).

MATERIALS AND METHODS

Bacterial strains. *B. medusa*, its mutants used in this investigation, and their origins are summarized in Table 1. Various *B. thuringiensis* strains, obtained from the culture collection of P. C. F. J., were used as phage indicator strains. Some strains of *B. medusa* are given descriptive letters, and these are described in the footnotes to Table 1.

Medium. The growth and sporulation medium was the same as in previous studies of *B. megaterium* (13), except that $CaCl_2$ was added to give 8.0×10^{-4} M in the final medium.

Buffer and sporulation techniques. The phage buffer and sporulation techniques were also the same as previously reported (13).

Phage assay. The overlay medium used previously (13) was altered for *B. medusa* studies by the addition of 0.2 ml of sterile 1 M $CaCl_2$ solution to 100

[1] Present address: Ontario Department of the Environment, Rexdale, Ont., Canada.
[2] Present address: Department of Industrial Microbiology, University College, Ardmore-Stillargan, Dublin, Ireland.

TABLE 1 *Origin of mutants of B medusa*[a]

Strain	Sporulation blocked at stage	Source of isolation
B medusa 9 B^+Sp^+		*B medusa* (Robinow)
B medusa R B^-Sp^+		*B medusa* 9 B^+Sp^+
B medusa G B^+ Sp	I	*B medusa* 9 B^+Sp^+
B medusa W B^- Sp	III	*B medusa* 9 B^+Sp^+
B medusa Bd^+Sp^+		*B medusa* 9 B^+Sp^+
B medusa Bc^+Sp^+		*B medusa* Bd^+Sp^+
B medusa Bc^+Sp^-	I	*B medusa* Bd^+Sp^+
B medusa L06 B^+Sp^+		*B medusa* 9 B^+Sp^+
B medusa Bd^+Sp^+ (ϕ-med-2)		*B medusa* Bd^+Sp^+

[a] Sp^+, Heat-resistant spores formed; Sp^-, heat-resistant spores not formed, B^+, possesses the parasporal body, Bc^+, abnormal inclusion, core crystal only, Bd^+, possesses a parasporal body with a donut appearance by phase-contrast microscopy, R, rough colony; G, colonies more grey than wild ones; W, colonies more white than wild ones

ml of medium just before use The indicator strains of *B thuringiensis* are listed in Table 3 One milliliter of phage suspension, 0 2 ml of a suspension (optical density at 645 nm [OD_{645}] of 0 5) of bacteria, and 4 ml of overlay agar were mixed before pouring the upper layer The plates were incubated for 12 to 18 h at 29 C

Phage induction. Mitomycin C (Nutritional Biochemicals, 0 35 μg/ml) was added to log-phase cultures having OD_{645} values of 0 3 or less Induction with UV was carried out by exposing a log-phase culture for 25 s in a shallow dish 35 cm from a 15-W GE germicidal lamp to give a dose of 140 ergs/mm^2 per s as measured by a UV intensity meter (Black-Ray J225, Ultraviolet Products, San Gabriel, Calif) This was the minimum UV dose that caused lysis of 95% of the cells upon induction

Phage purification. Bacterial cells were removed from phage or sporulation lysates by centrifugation at 10,000 $\times$ *g* for 10 min The phage, pelleted in the Beckman model L ultracentrifuge in a type 30 rotor at 70,000 $\times$ *g* for 2 h, were resuspended in phage buffer This suspension was purified by two cycles of differential centrifugation to give what is referred to as a "phage pellet", this was resuspended in phage buffer at 1.25 the original volume of phage suspension

The concentrated phage suspension was treated for 30 min with 50 μg of each enzyme, RNase (bovine pancreatic RNase, 5$\times$ crystallized, Calbiochem) DNase (DNase I, Worthington), and lysozyme (Calbiochem) per ml and examined in a CsCl gradient A discontinuous gradient was formed in a 5-ml tube by layering 1 3 ml of CsCl solutions having densities of 1.7, 1 5, and 1 3 g/ml One milliliter of phage suspension was layered on the gradient After centrifugation for 1 h at 100,000 $\times$ *g* in the SW50 rotor, the tube contents were scanned at 254 nm in a gradient analyzer (ISCO model D with UA_2 UV analyzer) and specific fractions were recovered The density of each fraction was determined from the refractive index, and the fractions were dialyzed against phage buffer

Electron microscopy. Phage preparations were examined by potassium phosphotungstate (PTA) or ammonium molybdate negative staining (1) The phage suspension in phage buffer was mixed with 1% PTA at pH 6 9 or with 1% ammonium molybdate at pH 7 2

For freeze-etching, cells and inclusions were harvested from agar cultures, unless specified, and frozen in Freon 22 on scored copper disks These specimens were transferred to the precooled (-150 C) stage of the Balzers Unit (AC Furstentum Liechtenstein) and, after cleavage, etched for 1 5 min at -100 C The cleave-etched surface was shadowed with platinum-carbon and then stabilized with further carbon The replica pieces were flotation cleaned in concentrated sulfuric acid, 6% sodium hypochlorite (Javex), and distilled water before examination in the electron microscope

Samples of the sporulating culture were prepared for thin-section electron microscopy by the method of Kellenberger et al (15), with some modifications described recently (18) A Philips 200 electron microscope was used

Light microscopy. Sporulating cultures were routinely observed by phase-contrast microscopy Spores undergoing whitening when observed by this method are referred to as white spores

Scintillation counting. Samples of cells were prepared for counting by precipitation with cold 10% trichloroacetic acid and were washed on a glass-fiber filter (Whatman GF/A) with cold 10% trichloroacetic acid Similarly, nucleic acid samples for counting were co-precipitated with 100 μg of bovine serum albumin per sample The scintillant used was Econoflour (New England Nuclear) Samples were counted in a Philips liquid scintillation analyzer Carrier-free [^{32}P]phosphoric acid was obtained from Atomic Energy of Canada

^{32}P labeling of the nucleic acid of ϕmed-1. *B medusa* L06 B^+SP^+ (ϕmed-1$^+$, ϕmed-2$^-$, ϕmed-3$^+$) was inoculated on the agar medium containing 0 666 mCi of ^{32}P per Povitsky bottle containing 375 ml of medium After sporulation lysis (2 to 3 days at 29 C), the phage was extracted and purified, and labeled nucleic acid was extracted as described above

Assay of ϕmed-1. A plaque assay method for this phage is not yet available A radioactive assay was applied, similar to that of Huang and Marmur (14) The phage was labeled with ^{32}P and expressed in the

purified phage preparation as DNase- and RNase-resistant, trichloroacetic acid-precipitable ^{32}P counts. The samples were counted in Econoflour in the Philips liquid scintillation analyzer.

Chemical determination of nucleic acid. The purified ϕmed-1 pellet was extracted with hot 5% trichloroacetic acid (22), and the DNA was determined in an aliquot by the method of Burton (3). RNA was determined by the orcinol method (17).

Extraction of phage nucleic acid. Nucleic acid was extracted from the purified ϕmed-1 pellet in standard saline citrate (SSC) buffer, pH 7.0 (16), by extraction with phenol (5) buffered with SSC. The phenol extract was dialyzed against phage buffer.

Hyperchromicity of nucleic acid after heat denaturation. The increase in OD_{260} of phage nucleic acid in SSC in a sealed tube after heat denaturation (121 C for 10 min, followed by cooling in ice) was determined with the Gilford spectrophotometer, model 2400.

Alkali sensitivity of ϕmed-1 nucleic acid. RNA but not DNA, is hydrolyzed to nucleotides by 1.0 M NaOH at 37 C in 18 h (4). A 0.4-ml sample of ^{32}P-labeled phage RNA in SSC was mixed with 1.6 ml of 1 N NaOH and incubated at 37 C for 18 h. A control contained water instead of NaOH. The solution was neutralized by adding 0.27 ml of 6 N HCl. The trichloroacetic acid-precipitable ^{32}P labeled material was then determined.

Sensitivity of ϕmed-1 nucleic acid to specific nucleases. One milliliter of ^{32}P-labeled phage RNA in SSC was mixed with 10 μl of either DNase or RNase (both at 500 μg/ml) or water (control) and incubated at 30 C for 10 min. The reaction was stopped by adding 1 ml of ice-cold 20% trichloroacetic acid and placing the tube on ice. The trichloroacetic acid-precipitable ^{32}P-labeled material was counted.

RESULTS

Detection of *B. medusa* phage. Colonies of old cultures of *B. medusa* 9 were pitted in appearance (see also Fig. 15). This in itself may indicate the presence of temperate bacteriophage. However, when *B. medusa* 9 heat-shocked spores were spot inoculated on a lawn of various *B. thuringiensis* strains (later shown to be sensitive to phage carried by *B. medusa* 9), there was no zone of lysis surrounding the resulting bacterial colony. This fact made it difficult to screen *B. medusa* substrains for the number and type of phage carried. Indicator strains that do react positively this way may yet be found. The phages were encountered initially in electron microscope (EM) preparations of induced or sporulation lysates.

Examination of induced cultures. Induction with mitomycin C of a culture of *B. medusa* 9 caused lysis after 2 h at an OD_{645} of 0.74 (Fig. 1). This OD corresponds to t_0 in this medium, that is, the point in time when the OD rise of the culture starts to decline (dotted line, Fig. 7). Goldberg and Gollakota (9) reported a lytic phage of *B. cereus* which only lysed its host during early sporulation, close to t_0. When *B. medusa* 9 was rejuvenated (i.e., diluted 1 to 4 in fresh medium) and then induced with mitomycin C, lysis occurred after the same eclipse period as before but at an OD_{645} of 0.2 (Fig. 1), so that no special relationship to t_0 exists here. On examination of the phage pellet from induced cultures, masses of empty phage headlike structures with an average diameter of 57 nm were seen (Fig. 2). UV-induced lysis gave similar results. No phage tail structures were seen. This empty particle is named ϕmed-3.

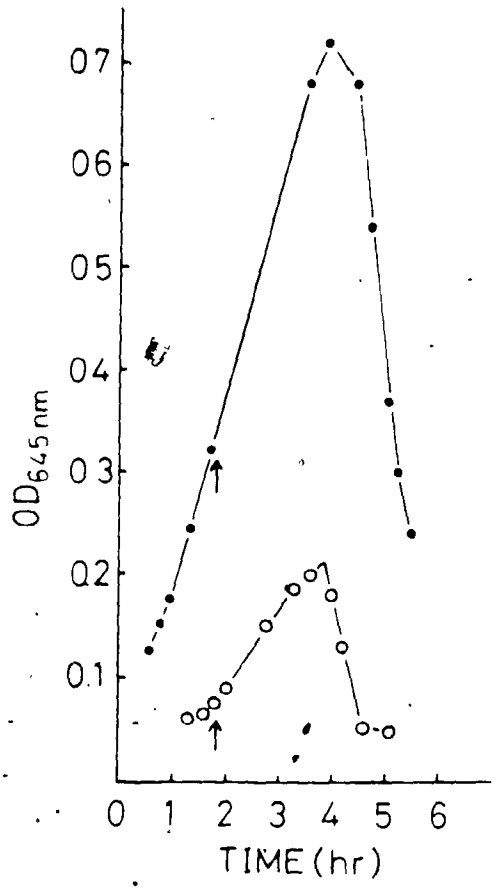


FIG. 1. *Induction of B. medusa 9 cultures with mitomycin C. Symbols: ● unrejuvenated culture, ○ rejuvenated culture (diluted 1 + 4 in fresh medium). The arrow indicates the time of addition of mitomycin C.*

When subsequent studies on sporulation lysates (see below) revealed the presence of the intact phage (ϕmed-2) active against *B. thuringiensis* var. *schwetzova*, vegetative cultures

were tested for the presence of ϕmed-2 using this indicator both before and after lysis induced by mitomycin C. The background phage titer was 7.0×10^{5}/ml and, after lysis, 9.3×10^{6}/ml. Thus ϕmed-2 appeared inducible, but weakly so. The number of cells lysed was 1.4×10^{7}/ml, indicating a burst size of approximately 7×10^{-2}/cell. This is small and may be due to inhibition of virus maturation by the mitomycin C.

The lysate of *B. thuringiensis* var. *schuetzova* cells infected with ϕmed-2 on agar plates was extracted in phage buffer and examined in the EM. Phage with empty heads were seen (Fig. 8). Since ϕmed-2 was usually stable in phage buffer, we assume that these incomplete phage were produced as such by the indicator strain.

Examination of sporulation lysates. Sporulation on agar medium resulted in eventual lysis and release of inclusions and spores from the bacterial cells. This agar growth was washed in phage buffer, and a purified phage suspension was prepared and examined in the EM. Two phage structures were observed (Fig. 3). One was a minute phagelike particle of 20 to 25 nm in diameter (ϕmed-1) and the other was a large phage with a tail. Phage ϕmed-3, prevalent in the mitomycin C- or UV-induced lysates of log-phase cultures, was not found. The minute phage often adhered to the parasporal body where it was usually attached to the fibers of which the parasporal body outer layer is constructed (Fig. 4 and 13). It was also attached to fragments of thin sheets of unknown origin (Fig. 5). The dimensions of the phages are listed in Table 2.

Separation of ϕmed-1 and ϕmed-2. The sporulation lysate of *B. medusa* 9 applied to an overlay agar seeded with *B. thuringiensis* var. *schuetzova* produced small turbid plagues, indicating sensitivity of the bacteria to one or both of the phages.

Although separation was not complete on a CsCl gradient, the phage titer was highest where the large ϕmed-2 was found on the gradient, (ρ = 1.48 g/ml). The small ϕmed-1 adhered largely to membranes and concentrated at a lower density (1.32 g/ml) in the gradient. A ϕmed-2 strain of *B. medusa* was sought and readily obtained. As indicated earlier, a simple scoring technique for the presence of phage was not possible. Therefore single spore isolates were allowed to grow and sporulate on agar, which was then extracted with a small amount of phage buffer, and this extract was spotted on a seeded lawn of *B. thuringiensis* var. *schuetzova*. Only 50 isolates, with no prior mutagenic treatment, were tested before a ϕmed-2 strain was obtained. The ease by which this strain was obtained suggested that this phage was not integrated with the host chromosome and may exist in a carrier state. When this strain, *B. medusa* L06 $B^{+}Sp^{+}$ (ϕmed-1^{-}, ϕmed-2^{+}, ϕmed-3^{+}), sporulated on agar, only ϕmed-1 was obtained (Fig. 5). Moreover, ϕmed-2 was now lytic to this strain as judged by the plaque assay technique (Table 3).

Phage produced without induction. Phage ϕmed-2 was produced during vegetative growth of *B. medusa* in the liquid sporulation medium. The number of phage released showed a marked increase during early log phase of growth of the culture (Fig. 7). The number of cells undergoing lysis in the culture was not small, being about 2%. This amount of lysis became detectable in the phase-contrast microscope during the log phase of growth and coincided with the rise in phage titer. It was assumed that cell lysis was due to phage production, and it is interesting to note that, other than mechanical breakage, phage lysis appeared to be the only way in which the cells in chains of *B. medusa* could be separated. Phage ϕmed-2^{+} strains showed less of this spontaneous lysis during growth but it still occurred, possibly due to the other phages for which we have no indicator system. The sporulation lysis

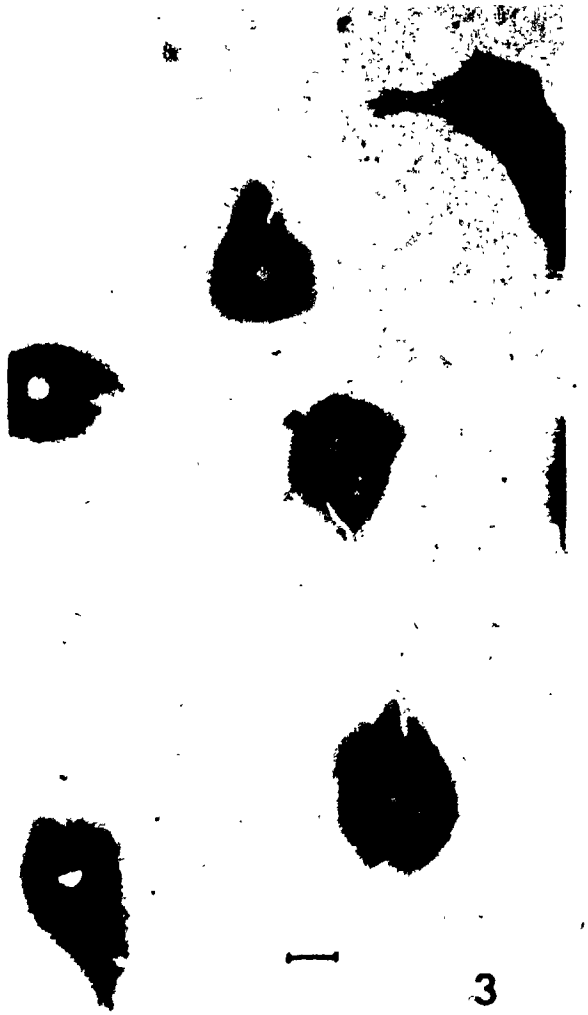
FIG. 2. *PTA-negative stain of phage pellet from B. medusa induced with mitomycin C. Only empty heads (diameter, 57 nm) are seen. This phage is called ϕmed-3. ×67,000. Note: The magnification bar on all micrographs represents 100 nm unless otherwise specified.*

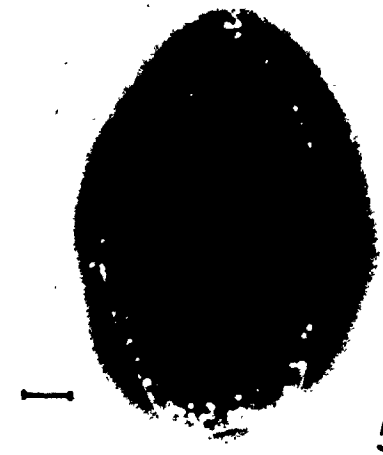
FIG. 3. *PTA-negative stain of a phage pellet from a sporulation lysate of B. medusa 9 (ϕmed-1^{+} ϕmed-2^{-} ϕmed-3^{+}). Two additional phage are seen: a small tail-less phage, ϕmed-1, and a large phage with a tail, ϕmed-2. ×67,000.*

FIG. 4. *Phage pellet from a sporulation lysate of B. medusa L06 (ϕmed-1^{+} ϕmed-2^{-} ϕmed-3^{+}). The parasporal bodies were washed in buffer containing 0.1% Triton X-100, and fibers with ϕmed-1 attached can be seen. ×100,000.*

FIG. 5. *Phage pellet from a sporulation lysate of B. medusa L06 (ϕmed-1^{+} ϕmed-2^{-} ϕmed-3^{+}). Only ϕmed-1 can be seen. It is shown here attached to a membrane of unknown origin. ×67,000.*

FIG. 6. *Thin section of phage pellet from sporulation lysate of B. medusa 9. The sections were stained with magnesium uranyl acetate and lead citrate according to Frasca and Parks (8). The nucleic acid is positively stained, and the arrow indicates what appears to be ϕmed-1 attached to a small piece of membrane lying among more densely stained ϕmed-2. ×100,000.*





FIGS. 2-6

TABLE 2. *Morphology of bacteriophages carried by B. medusa*

Phage	Type	Inducibility	Size (nm)		
			Head diam	Tail length	Tail units[a]
ϕmed-1	Minute, RNA	-	25	None	-
ϕmed-2	Noncontractile tail	+	60	220	4.3
ϕmed-3	Defective, empty heads, no tails	+	57	-	-

[a] Center-to-center spacing

TABLE 3. *Lytic activity of bacteriophages carried by B. medusa to some Bacillus spp.*

Bacillus strain	Lysis by phages[a]		
	ϕmed-1	ϕmed-2	ϕmed-3
B. medusa 9 B⁺Sp⁺	-	+	-
B. medusa W B⁺Sp	-	+	-
B. medusa L06 B⁺Sp⁺	-	+	-
B. medusa R B⁺Sp⁺	-	+	-
B. medusa G B⁻Sp⁺	-	+	-
B. medusa Bd⁺ Sp⁺	+*	+	-
B. medusa Bc⁺ Sp⁺	+*	+	-
B. medusa Bc⁺ Sp⁻	+*	+	-
B. cereus (L)	-	-	-
B. cereus (penase)	-	-	-
B. cereus var. *alesti* A-3	-	-	-
Fowler's bacillus	-	+	-
B. thuringiensis var. *finitimus*	-	+	-
B. thuringiensis var. *schwetzova*	-	+	-
B. thuringiensis var. M-13	-	+	-
B. thuringiensis var. *entomocidus*	-	+	-
B. thuringiensis T_{-5}	-	-	-
B. thuringiensis var. *sotto*	-	-	-

[a] +, Plaques formed; -, no plaques evident; +*, zone of inhibition (plaques were not observed when a more dilute phage preparation was used).

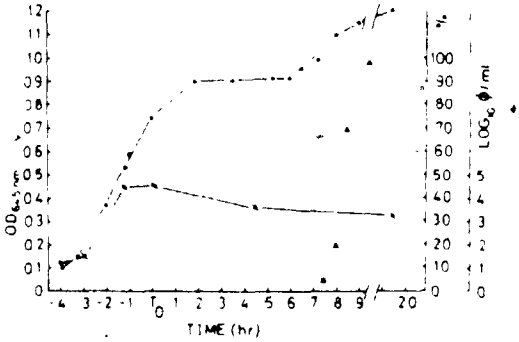


FIG. 7. *Growth and sporulation of B. medusa 9 in a growth and sporulation medium. Symbols: ●, OD_{590}; ▲, spore whitening measured as percentage of sporulating cells containing white spores; ■, phage titer per milliliter using B. thuringiensis var. schwetzova as an indicator strain.*

of medusa (stage VII of sporulation), unlike that of most *B. cereus*-type sporeformers, was extremely slow in liquid medium but more rapid on agar medium.

Characterization of ϕmed-1. Phage ϕmed-1 was found initially in lysates of sporulating cells. It was not found in lysates of induced bacteria. Several methods of induction of vegetative cells, mitomycin C, UV, H_2O_2, and thermal shock, were used without success. Nevertheless, without induction it may have been released from cells during growth in numbers too small to be detected.

Since the development of this peculiar phage seemed to be related to sporulation, we examined a series of mutants blocked at different times during sporulation. The findings suggested that the development of ϕmed-1 occurred during early sporulation. A culture of *B. medusa* G B⁻Sp⁺ (ϕmed-1⁻ ϕmed-2⁻ ϕmed-3⁻) blocked at stage I suddenly ceased its rise in OD_{590} at t_{+2}, and on terminal lysis of its cells produced no ϕmed-1 either free or on its inclusions when examined by PTA smears. However, the stage III mutant *B. medusa* W (B⁺Sp⁻, ϕmed-1⁻ ϕmed-2⁻ ϕmed-3⁺) formed large amounts of ϕmed-1. In spite of the differences in phage production, cultures of both these asporogenous strains were insensitive to ϕmed-1 (Table 3).

The inclusion bodies of strains able to form ϕmed-1 were like those of *B. medusa* 9 covered with a fibrous skin (Fig. 4 and 13), whereas the small inclusions of the stage I-blocked mutant *B. medusa* G, which is unable to form ϕmed-1, were devoid of the fibrous skin. It is interesting that the crystal portion (center) of the parasporal body in *B. medusa*, unlike that of *B. thuringiensis*, first appears at the end of vegetative growth (7), t_{+1} of Fig. 7. Hence the inclusion is not sporulation dependent and continues to enlarge in asporogenous mutants. However the conditions for synthesis of the inclusion skin and the associated ϕmed-1 appear from the comparison of these mutants to be established at, or require, stage II of spore formation.

Other studies were done to eliminate any possibility that the ϕmed-1 particles found at sporulation were carried over from vegetative cell lysis to the sporulation lysate. *B. medusa*

L06 (ϕmed-1⁺ ϕmed-2⁻ ϕmed-3⁺) was allowed to sporulate on agar until the spores and parasporal bodies were complete, before lysis and release of cell contents had begun. The bacterial growth was harvested and washed in phage buffer containing 0.1% Triton X-100 and a "phage pellet" prepared from the washing. The washed bacterial cells were broken in the phage buffer with the MSU ultrasonic disrupter, and another phage pellet was prepared from the cell contents. These phage pellets, which would have contained phage if any were present, were examined as PTA-negative stains in the EM. Phage ϕmed-1 was found mainly in the preparation from the broken cells (Fig. 10 and 11), where it was attached to pieces of parasporal body fibers located within the cells at the time they were sonicated.

A direct localization of ϕmed-1 was next attempted at different times of sporulation. Samples of cells of a fluid culture of *B. medusa* L06 were disrupted in the French press at 5,000 lb/in², and phage pellets were prepared. No ϕmed-1 were seen in PTA smears of these pellets prepared from vegetative cells. Cells at the early stage of inclusion formation (t_{-1}) were likewise apparently free of this particle. At early stage II (t_{-2}) ϕmed-I first became plentiful, thus supporting the finding with the stage I and III mutants that production of this phage-like particle in visible amounts is associated with early sporulation.

When a similar disruption and phage pellet procedure was applied to the stage I-blocked *B. medusa* G aerated in fluid medium, ϕmed-1 could not be found, nor were they seen in phage pellets prepared from a 7-day-old agar culture. Either this mutant is ϕmed-1 free or, because of the block, cannot express the presence of the phage.

In freeze-etch preparations the diameter of the ϕmed-1 structure was 25 nm (Fig. 13), and in PTA preparations it was 20 to 22 nm (Fig. 11). Pentagonal faces forming the surface of the phage suggest it is in the form of dodecahedron.

After trypsin (Worthington) or keratinase (a gift from W. J. Nickerson) digestion (50 μg/ml in 0.01 M triethylamine buffer, pH 8.1) at 4 C for 12 h or at 37 C for 2 h, the ϕmed-1 and its associated membrane were unchanged when examined in PTA smears. At the higher temperature phage in buffer alone were empty; the enzyme-treated samples were protected and intact.

Stained cores of the small ϕmed-1 were occasionally encountered in thin sections in their typical locale or on membrane pieces (Fig. 6).

Some attempts to further characterize the core material of ϕmed-1 were made. Since a plaque assay method has not yet been devised for this phage, particles labeled with ^{32}P were purified by three cycles of differential centrifugation and enzymatic treatment as outlined in Materials and Methods. Electron microscopy showed little or no contamination by membrane vesicles. The phage yield was determined by measurement of ^{32}P counts in the trichloroacetic acid-precipitable material remaining after treatment with nucleases (enclosed nucleic acid). A portion of this preparation was then run in a CsCl gradient, recovered, and dialyzed. The phage structures recovered were now empty. Since further purification could not be achieved by this gradient procedure, the ^{32}P-labeled preparation was analyzed directly.

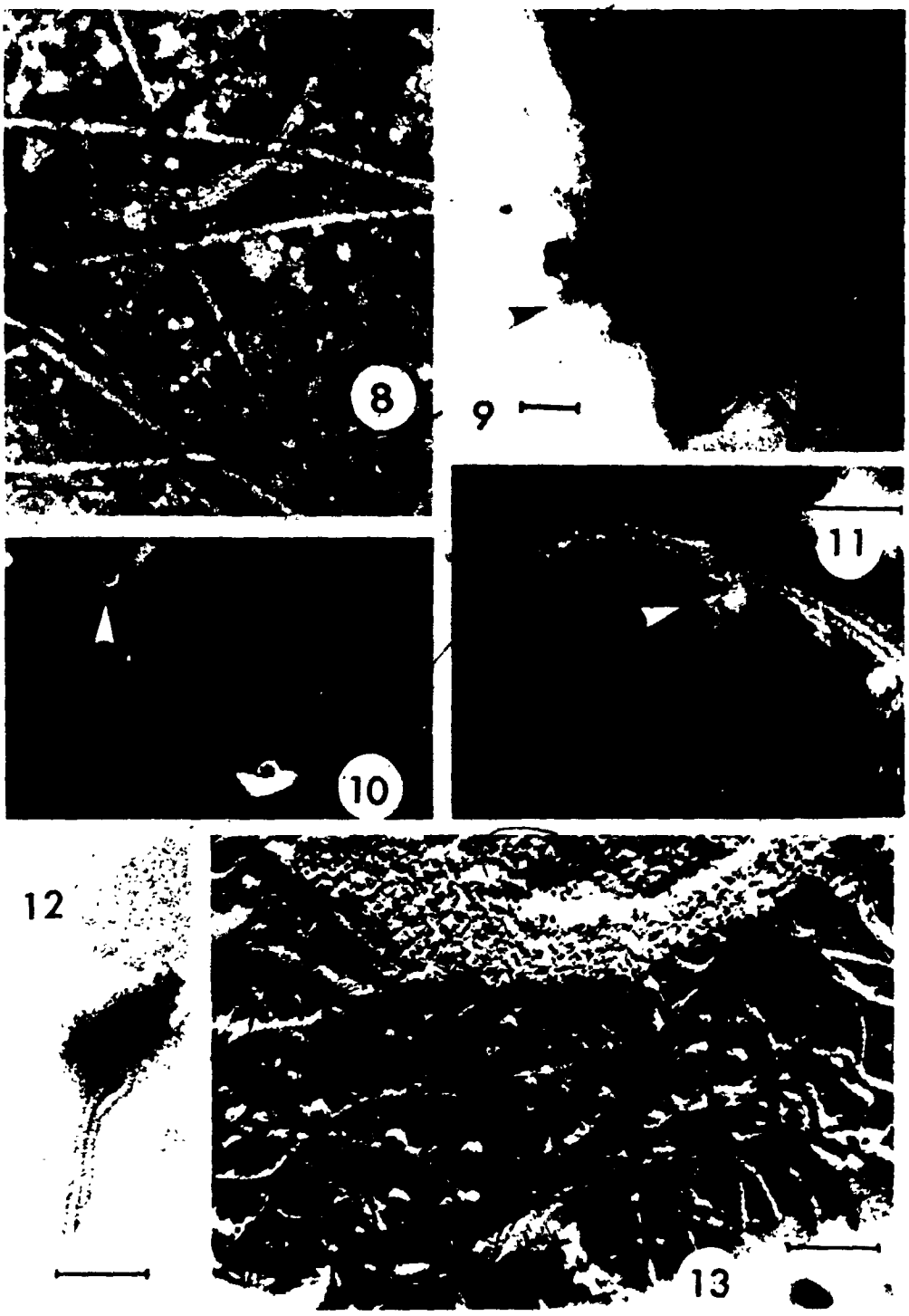
A hot 5% trichloroacetic acid extract of the final phage preparation was tested for DNA and RNA. The latter was positive. A blank, containing that amount of RNA indicated by the orcinol reaction, gave a reading with the Burton method slightly greater than did the phage preparation, and no DNA was detected. The ϕmed-1 nucleic acid was alkali sensitive (Table 4), RNase sensitive, and DNase resistant (Table 5).

The nucleic acid was extracted by phenol from a purified ϕmed-1 preparation and dialyzed against SSC. The UV spectrum of this extract was very similar to that of a purified RNA from yeast.

The ϕmed-1 RNA behaved like single-stranded RNA in that it was extremely sensitive to low concentrations of RNase and left no resistant fraction (Table 5). Heat denaturation of ϕmed-1 nucleic acid in SSC at 121 C for 10 min resulted in an OD_{260} increase of only 7%, which is consistent with the single-stranded form of RNA.

Phage ϕmed-1 was concluded to be a minute, RNA-containing bacteriophage or phagelike particle carried by *B. medusa*. The radioactive assay procedure confirmed the release of this phage after the lysis of sporulating cells (Table 6).

Characterization of ϕmed-2 and ϕmed-3. Initially it was considered that these two phages could be manifestations on only one phage genome and that ϕmed-2 simply represented a few ϕmed-3 that managed to complete synthesis. However, the isolation of ϕmed-2-cured strains of *B. medusa* suggested ϕmed-2 and ϕmed-3 were separate entities; ϕmed-3 was produced by induction of the ϕmed-2⁻ strain, *B. medusa* L06, with mitomycin C or UV. On CsCl density gradient centrifugation these empty heads banded at a density of 1.285 g/ml,



FIGS. 8-13

whereas a DNA-containing *B. thuringiensis* phage of similar size banded at 1.367 g/ml. The ultraviolet absorption spectrum of a suspension of φmed-3 is characteristic of a protein with a flat maximum at 280 nm.

In *B. medusa* 9 only very small quantities of φmed-2 were produced after induction, and a chemical characterization of the phage could not be done. The morphological characteristics of the phages appear in Table 2 and, as illustrated in Fig. 2, 3, 6, and 12, suggest a typical DNA-containing, tailed *Bacillus* phage.

Lytic activity of *B. medusa* bacteriophages to *Bacillus* spp. The sensitivity of various *Bacillus* strains to lysis by phage of *B. medusa* was determined by spotting phage preparations on seeded agar plates as in the phage assay. Phage φmed-1 was prepared from the sporulation lysate of *B. medusa* L06, φmed-2 from induction of *B. medusa* 9, and φmed-3 from induction of *B. medusa* L06. Phage φmed-3 gave no positive reactions (Table 3), and φmed-1 was positive with only a few mutants of *B. medusa*. Moreover, this activity of φmed-1 on *B. medusa* mutants was not observed when the phage preparation was diluted 1/10, suggesting some sort of growth inhibition rather than true phage lytic activity (Table 3). From both their plaque formation with φmed-2 and the absence of this phage by EM search of vegetative cell mitomycin lysates, all *B. medusa* mutants or strains appear to lack φmed-2. The *B. cereus* strains used were insensitive to φmed-2. A few *B. thuringiensis* strains were sensitive. A plating efficiency comparison of φmed-2 on *B. thuringiensis* and *B. medusa* has not been done.

Phage-specific sporulation effects. Old colonies of *B. medusa* 9 showed signs of lysis (Fig. 14) which appeared after secondary growth had formed on the colony. The secondary growth on *B. medusa* Bd was in the form of small discrete colonies that later lysed, forming pits (Fig. 15).

TABLE 4. *Alkali sensitivity of φmed-1 nucleic acid*[a]

Expt. no.	Trichloroacetic acid-precipitable ^{32}P remaining after treatment (counts/min)	
	Control (SSC, 37°C, 18 h)	Treatment (0.08 M NaOH, 37°C, 18 h)
1	9,458	69
2	1,639	1

[a] Material used was ^{32}P-labeled, phenol-extracted nucleic acid.

This type of lysis did not occur in non-spore-forming mutants, which did, however, become glassy with time. The colony type of *B. medusa* L09 was the same as *B. medusa* 9, indicating that this lysis was not affected by loss of φmed-2.

Sporulation of *B. medusa* 9 took many hours more than did that of other sporeformers (Fig. 7). This extended sporulation sequence was originally considered to be due to parasporal body formation. Then it was noted that *B. medusa* L06, a φmed-2-sensitive and presumably cured strain, sporulated rapidly, and spore whitening occurred synchronously at [illegible]. Other φmed-2-sensitive strains (see Table 3) sporulated rapidly, suggesting that φmed-2 was somehow the cause of extended sporulation in the wild type. This was supported by an experiment in which a lawn of a φmed-2-sensitive strain was exposed to a cell-free preparation of φmed-2. Within the zone of lysis, colonies were recovered that now showed the extended sporulation rate and yielded φmed-2 on induction. Thus the extended sporulation sequence of *B. medusa* 9 was reestablished and apparently associated with the carried phage φmed-2.

Phage-specific sporulation products. Since φmed-1 is readily seen in sporulating cells using negative-stain procedures, thin sections of such cells were also examined in the EM. Since

FIG. 8. *PTA-negative stain of phage, extracted from agar containing B. thuringiensis var. schuetzova infected with φmed-2. The large phage heads are unfilled, and some empty phage heads without tails were seen. The arrow indicates one of many small incomplete phage seen. ×140,000.*

FIG. 9. *Ammonium molybdate-negative stain, showing φmed-1 produced by B. medusa W, a sporulation mutant blocked at stage III. The arrow indicates a cluster of φmed-1 attached to a membrane. ×90,000.*

FIG. 10 and 11. *Sporulating, agar-grown cells of B. medusa L06 harvested before lysis began and washed in buffer.*

FIG. 10. *PTA-negative stain of phage found in the phage pellet from the buffer wash. The arrow indicates one of two small broken vesicles which may be from φmed-1. ×90,000.*

FIG. 11. *PTA-negative stain of phage found in the phage pellet from the above cells (Fig. 11, 12) broken in buffer by ultrasonication. The arrow indicates φmed-1 attached to parasporal body skin fibers. Pentagonal faces were seen on two of the phage particles. ×194,000.*

FIG. 12. *Uranyl acetate-negative stain of φmed-2 from the sporulation lysate of B. medusa 9. The tail subunits can be easily seen and were spaced 4.3 nm center to center. ×140,000.*

FIG. 13. *Freeze-etch preparation of agar-grown parasporal bodies of B. medusa 9 showing the fibrous skin with φmed-1 attached. The fiber repeating units were spaced 6.7 nm center to center. Phage φmed-1 diameter was 25 nm. ×140,000.*

TABLE 5. *Nuclease sensitivity of ϕmed-1 nucleic acid*

Material	Expt no	Trichloroacetic acid-precipitable ^{32}P remaining after treatment (counts/min)			
		Control	DNase treatment (5 μg/ml)	RNase treatment	
				5 μg/ml	2.5 μg/ml
^{32}P-labeled, phenol-extracted phage nucleic acid	1	1,621	1,637	81	
	2	1,608	1,613	90	
Native ϕmed-1 nucleic acid		1,588			94
Heat-denatured ϕmed-1 nucleic acid	3	1,588			70

TABLE 6. *^{32}P-labeled ϕmed-1 assay before and after lysis of sporulating cells of B. medusa L06*[a]

Expt no	Trichloroacetic acid-precipitable RNase-DNase-resistant ^{32}P counts/min (total)		% of total radioactivity released by lysis
	Before lysis	After lysis	
1	328	5,358	94.2
2	512	3,850	88.3

[a] Material used was ^{32}P-labeled phage purified as in Materials and Methods.

the cells were still dense, it was difficult to distinguish a possible small phage, which approximates the size of ribosomes and does not stain densely. Some particles thought to be ϕmed-1 were occasionally seen adhering to unusual membrane pieces (Fig. 16).

DISCUSSION

B. medusa carries genetic information for at least three phages or phagelike structures, which have been named ϕmed-1, ϕmed-2, and ϕmed-3. The most unusual was ϕmed-1, an RNA-containing particle with the structural characteristics of a minute phage, which was persistently carried by *B. medusa* and was produced in sporulating cells. Its synthesis appears to depend on the establishment of stage II of sporulation. With the exception of ϕu-4 (23), which seems to have been lost (19), ϕmed-1 is the only minute phage or minute phagelike particle of *Bacillus* spp. reported to date. Moreover, RNA-containing phages have not previously been observed from *Bacillus* spp. (2, 10), and bacterial strains lysogenized by minute phages of any type have not yet been reported. The fact that this one is seen only during spore development makes it especially interesting.

The intracellular localization of ϕmed-1 is also unusual. Often, but not always, it formed part of the fibrous skin of the parasporal body (Fig. 4, 11, 13) or was attached to fine fibers within the sporulating cell (Fig. 16) or to sheets of membrane-like material (Fig. 5, 9, 16). It can be washed off the parasporal body fibers with buffer containing 0.1% Triton X-100. This procedure, however, does not dislodge them from the membrane sheets; from these they are separated by passage through the French press. The origin or fine structure of the membranes is unknown, but they do not appear to be cytoplasmic membrane. The fibrous material making up the inclusion skin and the associated ϕmed-1 were not deposited on nearby spores. This suggests that membrane supporting the clusters of ϕmed-1 was probably not composed of spore-coat protein, although the exosporium is still a possibility.

From cultures free of ϕmed-2, a preparation of ϕmed-1 was prepared from sporulation lysate and used to determine the sensitivity of some bacterial strains to this phage. Some, but not all, strains of *B. medusa* showed a sensitivity to this preparation which did not behave like typical phage lysis (Table 3). We first thought that these strains did not carry ϕmed-1. They were ultimately shown, however, to carry this phage. This plus the loss of sensitivity of the bacterial strains to dilute phage preparations suggests some type of growth inhibition. A bacterial host in which ϕmed-1 clearly replicates has not yet been found. Hence judgement must be withheld on the exact nature of ϕmed-1. It could be a defective minute phage.

ϕmed-1 was very unstable, losing its growth-inhibiting activity after 3 days of storage on ice, after which PTA smears showed many empty phage particles. The phage and associated membranes were resistant to trypsin and keratinase. This instability and lack of inducibility made work with the phage difficult.

The only biologically active phage in the lysate of *B. medusa* 9 induced with mitomycin C was ϕmed-2, which appeared from its morphol-

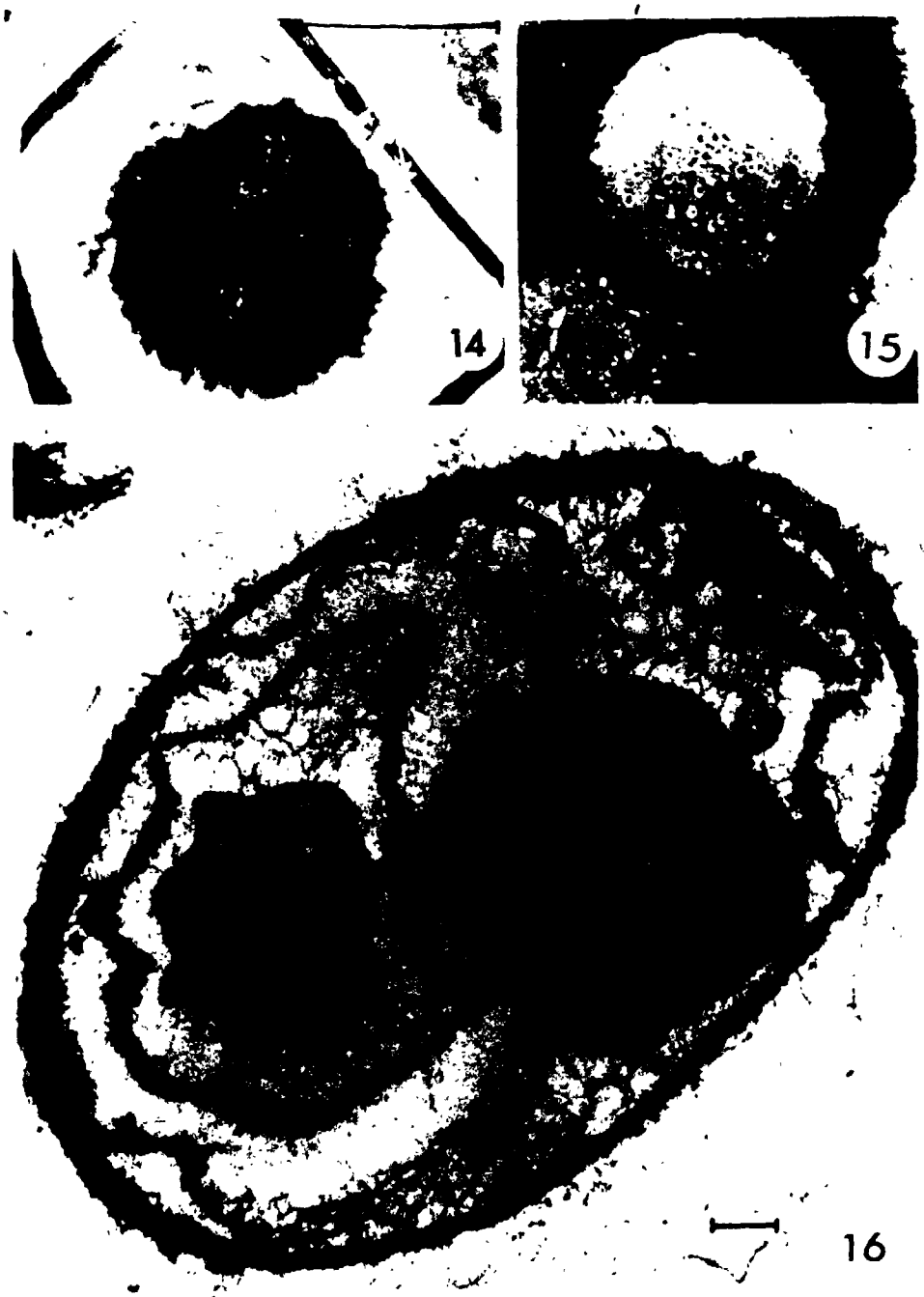


FIG. 14 *Colony of B. medusa L06 on nutrient agar, aged for 10 days to produce the ragged appearance characteristic of B. medusa. ×3. Magnification bar, 0.1 cm.*

FIG. 15 *Colony of B. medusa Bd on nutrient agar, aged for 10 days to produce the pitted appearance characteristic of the donut mutant. ×3.*

FIG. 16 *Thin section of sporulating cell of B. medusa Bd. The cell cytoplasm was clearing prior to sporulation lysis. Small phagelike particles attached to membrane of unknown origin are indicated by arrows. Some of the phagelike particles are attached to a mesh of fibers. The particles are not more than 20 nm in diameter. S indicates the spore surrounded by an exosporium, and the large dark body to the right is the exosporium body. ×102,000.*

ogy and cesium chloride density to be a typical DNA-containing *Bacillus* phage. It is lytic on several strains of *B. thuringiensis* and on Fowler's[3] bacillus (Table 3). The plaques produced on these strains are small, turbid, and easily overgrown. This made it difficult to titer the phage. It was also produced without induction during growth of *B. medusa* 9, although numbers were relatively small. The average burst size of induced cells of *B. medusa* 9 was only 7×10^{-2} ϕmed-2 per cell. The reason for this low burst size cannot be stated with accuracy, but several possibilities are suggested. mitomycin at the concentration used to compare these strains (0.35 μg/ml) is interfering with maturation, the phage is weakly inducible in that only in a few cells is ϕmed-2 induced, ϕmed-3, itself a defective phage, inhibits the production of viable ϕmed-2, ϕmed-2 is defective. A strain of *B. medusa*, cured of all three phages, with which to test these latter possibilities could not be isolated. Comparisons of phage production with UV and lower doses of mitomycin should resolve the first possibility.

The effect of ϕmed-2 on the duration of the sporulation sequence of *B. medusa* 9, which carried this phage, appears to be the first reported case of sporulation effect caused by a persistently carried phage in a *Bacillus*.

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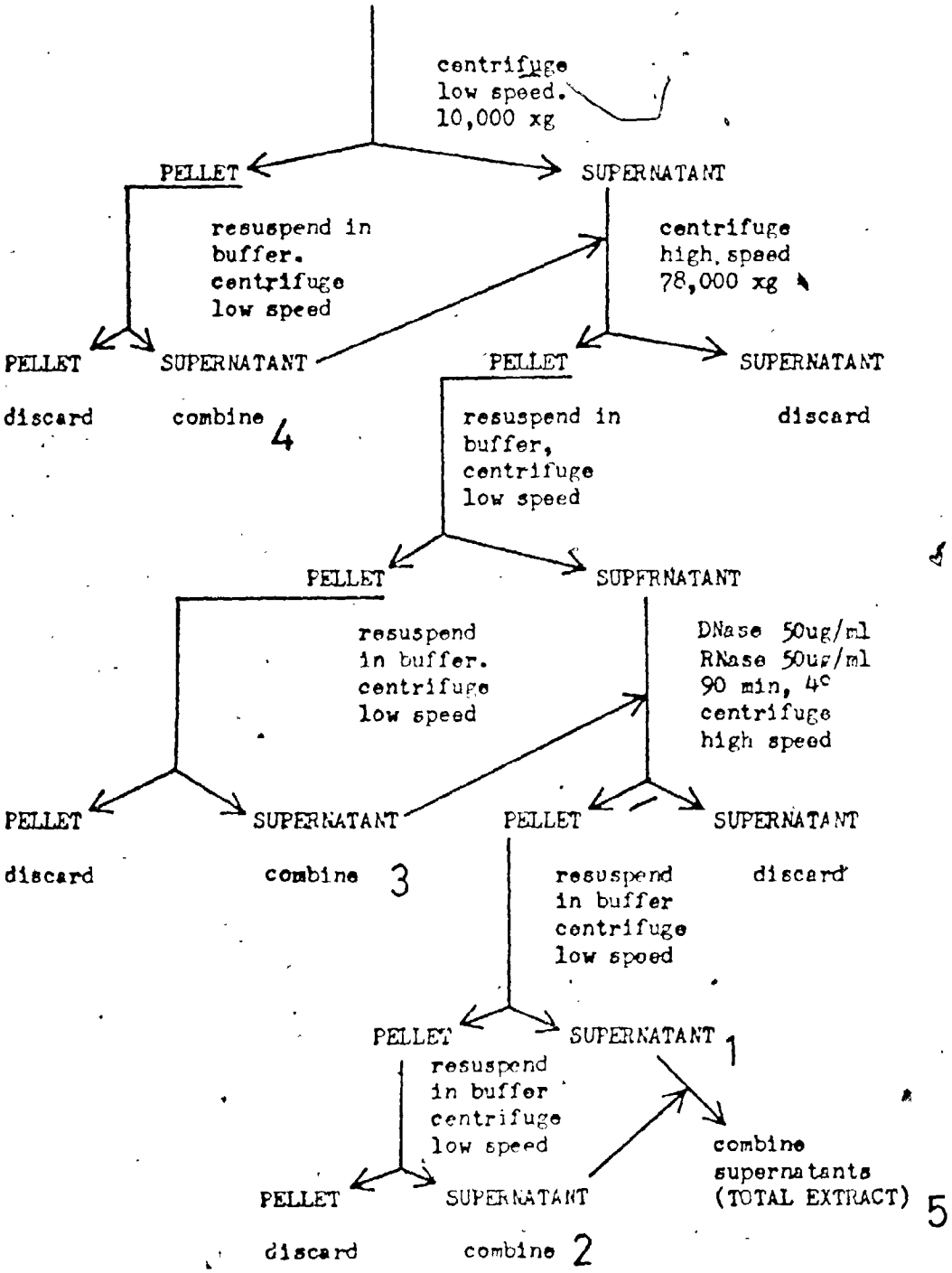
Appendix 2: Reprint of Fig. 28 entitled "Flow diagram of the procedure for the purification of Ømed-1" in the Ph.D. thesis of G. S. Hendry of U.W.O.

Bacillus medusa L06 was grown on sporulation agar until lysis was completed. The spores and BODs were washed off in phage buffer containing 0.1% Triton X100.



Appendix 3. List of Bacillus Strains Related to This Study

Strain	Serotype	Shape of major inclusion	PLP production	Source
B. thuringiensis subsp. *israelensis*				
strain CCEB-950-1	14	ovoid	+	Culture Collection Entomogenous Bacteria
950-2	14	-	+	this work
950-3	14	-	+	this work
950-4	14	-	+	this work
950-5	14	-	-	this work
950-6	14	-	-	this work
950-7	14	-	-	this work
strain HD567-1	14	ovoid	+	B. C. Carlton
HD567-54	14	ovoid	+	B. C. Carlton
HD567-61	14	-	-	B. C. Carlton
HD567-61-6	14	-	-	B. C. Carlton
HD567-61-9	14	ovoid	+	B. C. Carlton

HD567-61-9A	14	-	-	B. C. Carlton
subsp. kyushuensis	11	ovoid	+	M. Ohba
isolate 73-E-10-2	10	round	+	M. Ohba
10-2-2	10	round	-	this work
10-2-3	10	round	-	this work
10-2-4	10	round	-	this work
isolate PG-4	8	ovoid	+	L. E. Padua
subsp. morrisoni	8	bipyramid	-	Insect Path. Research Unit
subsp. toumanoffi	11	bipyramid	-	P.C. Fitz-James
subsp. darmstadiensis	10	bipyramid	-	M. Ohba
subsp. thuringiensis				
HD-2	1	bipyramid	-	B. C. Carlton
subsp. kurstaki				
HD-1	3	bipyramid	-	P. C. Fitz-James
subsp. alesti	3	bipyramid	-	P. C. Fitz-James
subsp. sotto	4	bipyramid	-	P. C. Fitz-James
subsp. galleriae	5	bipyramid	-	P. C. Fitz-James

B. cereus T	NA	NA	-	P. C. Fitz-James
B. medusa	NA	round	+	P. C. Fitz-James

(+) sign in the PLP column indicates PLP producing and (-) indicates non-producing. (-) sign in the Shape of major inclusion column indicates non-producing of the major inlcusion. NA means not applicable

END

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