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A GENETIC SYSTEM FOR BACILLUS THURINGIENSIS

The Ohio State University

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A GENETIC SYSTEM FOR

BACILLUS THURINGIENSIS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Phyllis A. W. Martin, B.A.

* * * * *

The Ohio State University
1979

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To Bill
who knows all the reasons
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cloning vehicles for Bacillus subtilis. 1979. Recombinant  

Genetic manipulation in the insect pathogen, Bacillus thur-  
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effects and maintenance mechanisms. C. Studdard (ed.).  

Genetic manipulations in the insect pathogen Bacillus thur-  

FIELD OF STUDY

Microbial Genetics. Professor Donald H. Dean
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INTRODUCTION

Bacillus thuringiensis produces a crystalline protein (\(\delta\)endotoxin) which is toxic to some types of insects. The most significant aspect of this work is determining the location of the gene(s) needed for expression of the \(\delta\)endotoxin. In the strain 3b-1, they appear to be on a large plasmid. This was determined by curing studies and confirmed by transformation of a strain lacking both crystals and plasmids to yield transformants with crystals and one plasmid.

Previous efforts to make B. thuringiensis a more effective microbial insecticide have centered on isolating strains with increased virulence and improving fermentation procedures. Another way to accomplish this is through genetic manipulation. This dissertation is a starting point to establish a workable genetic system for B. thuringiensis. The literature review will describe the state of the art in B. thuringiensis and its genetics when this project was started. Analogies to methods of genetic exchange in other systems have given direction to this work.

Of the two methods attempted, the results of the first, transduction, remain inconclusive. The second method chosen, transformation of protoplasts by plasmids, was successful. Staphlococcus aureus plasmid DNA (pC194) was able to transform B. thuringiensis, to chloramphenical resistance. This plasmid,
a transposon, is not maintained as a plasmid, but rather appears to integrate into the chromosome. This opens the door for transfer of genes from other bacteria into **B. thuringiensis**.
LITERATURE REVIEW

The first isolation of the bacterium now known as *Bacillus thuringiensis* took place in 1901 in Japan. Ishiwata (1901,1902) described the isolation of a spore-forming bacteria from diseased larvae of the silkworm, *Bombyx mori*. This work along with other works describing this disease in the sericulture industry have been overlooked because they were originally published in Japanese and were not translated for many years. In 1915 Aoki and Chigasaki (1915a,b) were able to associate the symptoms of the disease with sporulating cultures of *B. thuringiensis* and not with vegetative ones.

Berliner (1911) is usually given credit for the first description of *B. thuringiensis* which he isolated during an infestation caused by the flour moth, *Ephestia kuhniella*. Berliner (1915) also reisolated this bacterium from the same organism and described an inclusion now known to be a crystalline protein. Mattes (1927) also describes this protein inclusion and calls it "Restkorper".

There were some early attempts to use *B. thuringiensis* in insect control, which were largely unsuccessful, so that the active period of research on *B. thuringiensis* did not begin until the 1950's when Hannay (1953) rediscovered the crystalline inclusion and suggested that this protein may be responsible for the toxic effect. Angus (1954,1956) was able
to show that for the silkworm the parasporal protein inclusion was the agent of pathogenesis.

Taxonomically, *B. thuringiensis* acquired and maintains its species designation because of its toxicity to insects, or more specifically, through the production of the endotoxin which is usually the sole criterion separating *B. thuringiensis* from a related species, *Bacillus cereus*. These species have been shown to share 50-80% DNA sequence homology as determined by hybridization (Seki et al, 1978). Upon loss of the crystal, *B. thuringiensis* can not be distinguished from *B. cereus* by biochemical tests that are usually used to characterize the *Bacillus* species (Gordon, 1975). Nonetheless, because of the economic importance of *B. thuringiensis* and its widespread use by insect pathologists, *B. thuringiensis* will continue to be designated as a separate species. This species can be further subdivided into serologically distinct types as will be discussed later.

*B. thuringiensis* is economically important as a biological insecticide. It is pathogenic to some 180 species of insects yet has no effect on others (including most beneficial insects, Norris, 1970). It can be used as an alternative to chemical insecticides. Since it is related to the ubiquitous soil organism *B. cereus*, its ecological niche would seem to be the soil. However, a recent study by
Delucca et al., (1979) showed it to be present in only 0.05% of some 48,000 soil samples from around the United States. 

B. thuringiensis does not survive in the soil, despite efforts to increase field persistence. This may be due to two causes: first, B. thuringiensis is very sensitive to ultraviolet irradiation and exposure to sunlight decreases its viability (Griego and Spence, 1978); second, upon loss of the crystal B. thuringiensis is indistinguishable from B. cereus so that lack of recovery of B. thuringiensis may be due to this change as well as loss of viability.

Many field studies have been carried out using B. thuringiensis. Certain formulations of δendotoxin have shown considerable promise in control of several major forest pests, including tent caterpillars and webworms, gypsy moth, tussock moths and spruce budworm (USDA, 1978). The δendotoxin is also useful in controlling cotton budworm (USDA, 1978). B. thuringiensis can also control lepidopterous pests in stored grains (USDA, 1978) and may be able to replace some chemical insecticides in this area. The toxin is registered in the United States by the U.S. Department of Agriculture for use on alfalfa, artichokes, bananas, broccoli, cabbage, cauliflower, celery, lettuce, melons, potatoes, tomatoes, tobacco, and various forest trees (Norris, 1970). Despite this widespread use there are just now in progress studies elucidating the
mechanism of toxicity. There is also little knowledge about the ecology and the genetics of this organism.

Up to now improvements in toxicity have followed two paths. The first is to improve formulation. This includes improving media (to produce a greater quantity of crystals or to increase their toxicity - Dulmage, 1971) and increasing persistence in the field by adding UV protectants to spore-crystal preparations (Griego and Spence, 1978). The second is to isolate from diseased insects, examples of *B. thuringiensis* that are more virulent than strains now in collections (Dulmage, 1970).

As an insect pathogen, *B. thuringiensis* is toxic to many different types of insects including the orders: Lepidoptera (caterpillars), Orthoptera (grasshoppers), Coleoptera (beetles and weevils) and Diptera (common flies and mosquitoes) and the family Tenthredinidae (sawflies).

Probably the best characterized activity is against the economically important lepidoptera. There are opposite reasons for studying this activity. *B. thuringiensis* attacks silkworm larvae and therefore much interest is shown in controlling this disease by the sericulture industry. *B. thuringiensis* also attacks crop pests whose control is important in agriculture. Varieties of *B. thuringiensis* have been isolated from the following lepidoptera: *Bombyx mori* (var.
alesi anduze, Delaporte and Bequin, 1955), *Dendrolimus sibericus* (var. *dendrolimus*, Talalaev, 1956), *Galleria mellonella* (var. *galleriae*, Isakova, 1958), *Aphomia gularis* (var. *entomocidus*, Steinhaus, 1951) and have been shown to be toxic to these particular species. However, *B. thuringiensis* var. *finitimus* was isolated from *Malacosoma disstria* (Heimpel and Angus, 1958) and was shown to be not toxic to lepidoptera. The mosquito pathogen, *B. thuringiensis* var. *israelensis* was not isolated from diseased insects, but rather from their breeding grounds in Israel (Goldberg and Margalit, 1977). However, Margalit is of the opinion that a decaying mosquito larvae may have been the actual source (J. Margalit, pers. comm.).

A few lepidopteran insect species have been used to attempt to standardize *B. thuringiensis* crystal toxicity by means of a bioassay (Dulmage et al., 1971). Because of the varying toxicity of a *B. thuringiensis* spore-crystal preparation on different insects, usually more than one species is used. Some of the more common are *Tricuplusia ni*, cabbage looper; *Heliothis zea*, tobacco budworm (Dulmage, pers. comm.) or *Pieris brassicae*, white butterfly; *Mamestra brassicae*, cabbage moth (deBarjac and Bonnefori, 1968).

The *Sendotoxin has also been recently shown to have activity against black flies (*Simulium vittatum*, Lacey and
which serve as vectors for human and animal diseases, including human and bovine onchocerciasis, and leucocytozoan parasites of turkeys and other fowl (Crosskey, 1973). *B. thuringiensis* var. *israelensis* was found to have its crystalline inclusion toxic to mosquito larvae of the species *Aedes aegypti* and *Anopheles stephensi* (deBarjac, 1978b) which are vectors of yellow fever and malaria respectively.

A thermostable toxin (exotoxin) is produced in the supernatants of cultures of H-serotypes 1, 8, 9 and 4b which is active against a wide range of insect orders, including Lepidoptera, Diptera, Hymenoptera, Coleoptera and Orthoptera (McConnell and Richards, 1959; Rogoff, 1966; Norris, 1970; Burgerjon et al, 1974). It affects *Musca domestica* at moulting, or metamorphosis when fed to larvae (Briggs, 1960) resulting in reduced emergence or malformed adults.

Another toxic product produced by serotype 1 is lethal upon feeding for 19 species of sawflies (Smirnoff and Berlinquett, 1966). But it is very labile which will preclude its use in the field.

Even though *B. thuringiensis* is harmful to pest insects it has no harmful effects on useful insects such as bees, other hymenopterous pollinators and insect predators (Norris, 1970). *B. thuringiensis* is not toxic for mammals (Norris, 1970) though the exotoxin does exhibit toxicity.
for mice at oral doses of 400-800mg/kg of body weight and somewhat lower doses upon injection (Norris, 1970). However other studies show that $^{32}\text{P}$-labeled $\beta$-exotoxin is rapidly dephosphorylated to a non-toxic product when administered to mice either orally or by injection (Sebesta and Horska, 1973).

The $\alpha$-endotoxin also has some potential uses in chemotherapy. In 1973 Prasad et al demonstrated antitumoral activity of the $\alpha$-endotoxin against Yoshida ascites sarcoma. And it was later shown to cause non-specific enhancement of the immune response (Prasad and Shethna, 1975).

The mode of action of the crystalline toxin has been best elucidated using lepidoptera. The pathogenicity of this glycoprotein (Bulla et al, 1977) is one of intoxication. An insect consumes crystals, spores or a combination of the two, the alkaline gut contents solubilize the crystal (Faust et al, 1967) and proteases in the gut break down the protein into toxic peptides (Faust et al, 1974a,b). These peptides un- couple oxidative phosphorylation in gut epithelial cells as demonstrated by the effects on isolated mitochondria (Travers et al, 1976). This leads to metabolic imbalance and cell death. This death is histologically observed by the balloon-ing and bursting of the columnar epithelial cells (Angus and Heimpel, 1969; Sutter and Raun, 1967). There is then disruption of the gut wall which allows the contents of the gut
to mix with the hemolymph. It is this leakage of the alkaline contents of the gut which results in the general paralysis (in the silkworm) which is the cause of the death of the insect. An alkaline solution when injected into the hemolymph causes a general paralysis indistinguishable from that caused by the ingestion of the endotoxin (Heimpel and Angus, 1960). It is unclear what exactly the toxic products are in this process. Lysino-alanine has been suggested as the possible toxic product (Dastidar and Nickerson, 1978) due to its presence in alkaline digested crystal preparations and its resemblance to the cyclic peptide, valinomycin, which can uncouple oxidative phosphorolation (Angus, 1968). However the crystals in various serotypes seem to be different, some having several peptides which are toxic, others having only one. It is unclear what proteases break down the crystal protein. In *B. thuringiensis* var. *kurstaki* Bulla et al (1977) have found a serine protease associated with the crystal itself. Toxic products have only been produced using gut juices of insects (Faust et al, 1967; Faust et al, 1974a). The action of the toxin of *B. thuringiensis* var. *israelensis* on mosquito larvae is similar to that on lepidopteran larvae (deBarjac, 1978b), though this has not been extensively studied.

The only other toxin that has been well studied is the betaexotoxin which is produced by only some varieties of
B. thuringiensis: thuringiensis, galleriae, canadensis, aizawai, morrisoni, tolworthi, darmstadiensis, and toumanoffi. This heat stable toxin is produced during exponential growth. It is an ATP analog which competitively inhibits those ATP requiring reactions which convert the triphosphate to the monophosphate (Horska, 1968; Sebesta et al, 1969). The sexotoxin interferes with metamorphosis in the house fly, Musca domestica (Cantwell et al 1974). In this same paper they also reported on the entomogenous effect of the sexotoxin on mosquito larvae. It is also toxic when injected into mice (Heimpel, 1967; Bond et al, 1971). It has been tested for use to control Diptera hatching in the feces of domestic animals (notably chickens and cattle). Gringrich (1975) reported no effect on cattle. No effect on quantity or quality of eggs laid by orally fed hens was observed by Briggs (1960). However internal pathologies in chickens were observed by Barker and Anderson (1975).

The actions of other toxins of B. thuringiensis are less studied. These toxins are: (as per Faust, 1975) αexotoxin or phospholipase C (Toumanoff, 1953); Ψexotoxin (Heimpel, 1967) which clears egg yolk agar; labile exotoxin (Smirnoff and Berlinguet, 1966) active against sawflies; "water soluble toxin" (Fast, 1971) producing symptoms similar to that of the θendotoxin; and "mouse factor toxin" (Krieg, 1971) which is effective against lepidopteran larvae and mice.
The broad spectrum host range leads to important applications. *B. thuringiensis* is presently being used in California to control lepidoptera on cole crops. It is useful against the cabbage looper which is resistant to many chemical insecticides. Those insecticides which are effective leave a toxic residue making the crops less desirable for human consumption (Norris, 1970).

The World Health Organization is now in early stage laboratory trials to test the efficacy of serotype 14 of *B. thuringiensis* verus mosquitoes (WHO, 1977). Mosquitoes are vectors of many diseases of man and animals. The control of mosquitoes, especially in developing countries is of paramount importance for the health of those nations.*B. thuringiensis* is manufactured throughout the world by several companies and can be purchased or is in use under the trade names of Dipel (Abbot Laboratories), Thuricide (Sandoz, Inc.) in the United States; Entobacterin, Dendrobacilline, Insectine and Toxobacterine in the Soviet Union and Bactospeine and Leptox in France (BioChem Products A.G.) (Beegle, 1979).

As a starting point to review the genetics of *B. thuringiensis* it is useful to describe successful methods of gene transfer in other bacteria as well as previous attempts at genetic exchange in *B. thuringiensis*. There are many ways to transfer genes between bacteria that fall into the
general catagories of transduction, transformation and conjugation. These means will be briefly reviewed with emphasis on closely related systems and their possible application to *B. thuringiensis*.

Transduction is the transfer of genetic markers between cells via phage particles. This transfer can be of two types depending on the particular phage selected. Generalized transduction was first discovered in the *Salmonella* phage P22 (Zinder and Lederberg, 1952). PBS-1 of *B. subtilis* has been found to be useful in elucidating the general order of genes in that bacterium. This phage carries about $1-2 \times 10^8$ daltons of DNA and transfers genes in large blocks; it is thus suitable for general ordering of markers, but not for fine structure mapping (Dubnau et al, 1967). PBS-1 attaches to the flagella of *B. subtilis* so that motile bacteria are required for transduction to take place (Joys, 1965). In *Staphlococcus aureus* the transduction system can be controlled so that the phage preferentially transfers chromosomal rather than plasmid DNA by exposing cells with the phage to UV irradiation (Kayser et al, 1972).

By specialized transduction an integrated phage can use illegitimate recombination to pick up genes flanking its integration site. This process is best studied in the coliphage λ which can transduce *bio* or *gal* (Morse et al, 1965). Specialized transduction is also known for *B. subtilis* temperate phages φ105 (Shapiro et al, 1974 and SPβ (Zahler et al,
integrates between leu and phe and transduces leu auxotrophs to prototrophy at low frequencies (Shapiro et al, 1974). Transduction in B. subtilis has been found to be the best means for rough mapping and sometimes the only means for strain construction. For such a system to be useful in B. thuringiensis a suitable phage would have to be found along with the parameters for the propagation of this phage and factors influencing transduction.

Transformation involves the uptake of naked DNA by the bacterial cell. At least three methods are in use at the present time: a competence regime, calcium shock and protoplasting. Competence is defined as the ability of a bacterium to take up DNA. Some bacteria are always competent or are competent over a wide range of their growth cycle (Catlin, 1960; Moseley and Setlow, 1968). B. subtilis, on the other hand is competent only during a short phase of its growth which occurs shortly after stationary phase is reached (Anagnostopoulis and Spizizen, 1961). Competence regimes in B. subtilis utilize two methods. The first involves a media shift from a rich medium to a poor one (Spizizen and Anagnostopoulis, 1959). The second involves exhaustion of the nutrients in a defined medium (Bott and Wilson 1967). It does not seem to matter which method is used and competent cells in the population can reach levels of 0.1 - 0.5% though higher levels have been reported (Wilson and Bott, 1968).
One of the unusual aspects of this process in _B. subtilis_ is that the DNA taken up by the cells is single stranded and the DNA must recombine in order to be expressed (Davidoff-Abelson and Dubnau, 1973). This makes transformation by plasmid DNA particularly ineffective.

Calcium shock (Lederberg and Cohen, 1974) puts "holes" in the cell wall and allows uptake of plasmid DNA in _Salmonella typhimurium_. It has been shown in _Escherichia coli_ that other factors, such as age of the culture, also affect this process (Brown et al, 1979).

Protoplasting (cell fusion) is a technique that has been adapted from tissue culture work (Davidson and Gerald, 1975), for use in bacteria. In _E. coli_ spheroplasts can be used to increase the frequency of transfection over calcium shocked cells (Benzinger et al, 1971). In the _Bacilli_ protoplasting was first used in cell fusion studies. In 1976 Fodor and Alfoldi (in _B. megaterium_) and Schaeffer et al (in _B. subtilis_) fused bacterial auxotrophs of different types using polyethylene glycol and were able to recover prototrophs at a low frequency. These preliminary observations showed that this technique would not be useful in mapping studies because of the confusing and conflicting results obtained (Fodor and Alfoldi, 1979). In 1979 Gabor and Hotchkiss further explored this technique in _B. subtilis_. One of their negative results showed that protoplasts are not transformed by linear DNA.
At the same time, Chang and Cohen (1979) were developing a procedure using this same method to transfer plasmid DNA from *S. aureus* into *B. subtilis*. The efficiency of this procedure is 10-80% which represents a 10- to 100-fold increase over other methods. This high rate would allow for the screening of non-selectable markers such as toxin production.

Conjugation, transfer of genes via plasmids which has been so useful in *E. coli* genetics (Lederberg and Tatum, 1946), is overlooked in the Bacilli. There are many modifications of a basic technique for a variety of organisms. There are some plasmids, such as RP4 which have a wide host range and can be used to introduce DNA into many different species (Datta and Hedges, 1972). This plasmid has been used to transfer genes from *E. coli* to *B. subtilis* where at least some of these genes are temporarily expressed (Buheitel and Klingmuller, 1979). There has been one report of possible conjugation in *Clostridium perfringens* where Rood et al. (1978) have demonstrated the transfer of a tetracycline resistance plasmid. Since *B. thuringiensis* has a variety of plasmids, this system may be of some use.

There are three systems of gene exchange which exist for *B. thuringiensis*, but do not seem to be generally useful or readily adaptable to all serotypes. The earliest report of genetic transfer in *B. thuringiensis* is an unpublished thesis by Reeves (1966) in which he reported the transformation of
serotype 2 (non-toxic variety) with DNA from serotype 1 (toxic variety). Transformation was done using a "modified Spizizen method", but no details are given. He selected transformants based on altered colony morphology and tested their toxicity on house flies ($\beta$ exotoxin) and cabbage looper ($\delta$ endotoxin).

In 1970 deBarjac reported transduction in B. thuringiensis with a phage called Th 1. This phage is able to plate on serotypes 1, 4a, 4b, 7, 9, and 10. deBarjac was able to transduce to prototrophy two mutants of serotype 1; one requiring uracil and the other thyamine. It was necessary to inactivate the phage with UV irradiation for transduction to prototrophy to occur at the rate of $2.8-5.8 \times 10^{-7}$/plaque forming units or $0.67-1.3 \times 10^{-6}$/cell. She was also able to transduce the flagellar antigen from serotype 7 into serotype 1. However this phage is highly virulent, and too has low transducing frequency and too limited host range to be of general use.

The most recent report of transduction was by Thorne (1978). Using CP51 and CP54 phages originally isolated from soil on B. cereus as a host (1968), he was able to transduce to prototrophy several different auxotrophs of different serotypes (12, 2 and 3a). He was able to establish co-transduction frequencies for markers in serotype 3a. CP54 has a wide host range covering serotypes 1, 2, 3a, 3b, 4ab, 4ac, 5ab, 5ac, 6, 7, 8, 9, 10 and 12. However this phage
is too virulent to be useful in transduction of many of the strains it infects. Another problem arises in that transduction of markers between serotypes was largely unsuccessful; this could indicate a lack of homology, although restriction could not be ruled out. This lack of homology could pose a problem in constructing strains via transduction. Thorne was the first to develop and map auxotrophic mutants for genetic studies.

Development of a genetic system requires not only a means of transmission but also genetic variability, natural or induced to be transmitted. There are natural differences in the serotypes and toxins of B. thuringiensis. The serotype differences are based on flagellar antigens or H-antigens (deBarjac and Bonnefori, 1968). The 20 recognized subspecies with their respective H-antigen types are listed in Table 1. The endotoxin differences result from serological, biochemical and toxicity dissimilarity among crystal types. Different serotypes and strains within serotypes produce different toxins (Table 2).

There are three subspecies whose crystal structure has been looked at in some detail: tolworthi, kurstaki and thuringiensis. The molecular weights as well as their antigens seem to differ. Tolworthi was found to contain two components when analyzed by polyacrylamide gel electrophoresis (Herbert et al, 1971), a component with a molecular weight
<table>
<thead>
<tr>
<th>H - serotype</th>
<th>Subspecies</th>
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<tbody>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>finitimus</td>
</tr>
<tr>
<td>3a</td>
<td>alesi</td>
</tr>
<tr>
<td>3ab</td>
<td>kurstaki</td>
</tr>
<tr>
<td>4ab</td>
<td>sotto</td>
</tr>
<tr>
<td>4ab</td>
<td>dendrolimus</td>
</tr>
<tr>
<td>4ac</td>
<td>kenyae</td>
</tr>
<tr>
<td>5ab</td>
<td>galleriae</td>
</tr>
<tr>
<td>5ac</td>
<td>canadensis</td>
</tr>
<tr>
<td>6</td>
<td>subtoxicus</td>
</tr>
<tr>
<td>6</td>
<td>entomocidus</td>
</tr>
<tr>
<td>7</td>
<td>aizawai</td>
</tr>
<tr>
<td>8</td>
<td>anagastae (morrisoni)</td>
</tr>
<tr>
<td>9</td>
<td>tolworthy</td>
</tr>
<tr>
<td>10</td>
<td>darmstadiensis</td>
</tr>
<tr>
<td>11a</td>
<td>toumanoffi</td>
</tr>
<tr>
<td>11ab</td>
<td>kyusuensis</td>
</tr>
<tr>
<td>12</td>
<td>thompsoni</td>
</tr>
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<td>pakistani</td>
</tr>
<tr>
<td>14</td>
<td>israelensis</td>
</tr>
<tr>
<td></td>
<td>dakota</td>
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<tr>
<td></td>
<td>indiana</td>
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after deBarjac (pers. comm.)
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<tr>
<th>Variety</th>
<th>exo</th>
<th>exo</th>
<th>exo</th>
<th>endo</th>
<th>labile</th>
<th>water soluble</th>
<th>mouse factor</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>finitimus</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alesi</td>
<td>+</td>
<td>-</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>sotto</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kenyae</td>
<td>+</td>
<td>-</td>
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<td></td>
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<td>+</td>
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<td></td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
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<td>+</td>
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<td></td>
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<td>entomocidus</td>
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<td>-</td>
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<td>+</td>
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<td></td>
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</tr>
<tr>
<td>toumanoffi</td>
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<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>thompsoni</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* no activity towards lepidoptera

after Faust, 1975
of 55,000 which contained all the toxicity and one of 120,000 which was not toxic. This strain contains two major crystal antigens (Norris, 1970). Kurstaki and thuringiensis however each contain only one polypeptide but the molecular weights differ: 120,000 for kurstaki (Bulla et al, 1977) and 80,000 for thuringiensis (Glatron et al, 1972). These crystals have two (Winkler et al, 1971) and three (Lecadet and Dedoner, 1971) antigenic determinants respectively. Within the kurstaki subspecies two different types of crystal can be found (Kryweinczyk, 1978).

The endotoxins can also be separated by host range and by toxicity to a given insect. While most of the crystals affect some lepidoptera there are two notable exceptions: finitimus and israelensis. Table 3 shows several strains of B. thuringiensis, in which toxicity is compared by use of L.D. 50's and defined by comparison to a standard of 1000 international units/mg (Dulmage, 1971).

These natural occurring differences in insect toxicity are difficult for the average laboratory to work with as they require large amounts of material for analysis. Rearing insects for use in a bioassay requires specialized facilities. A variety of mutants have emerged to overcome these difficulties. These include toxin mutants, auxotrophs and mutants resistant to antibiotics.

The endotoxin mutants are probably the best studied. A spo<sup>+</sup>cry<sup>-</sup> phenotype (sporulates normally, but no longer
TABLE 3

Endotoxin pathogenicity of strains of B. thuringiensis to cabbage looper and tobacco budworm

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Strain No.</th>
<th>I.U./mg*</th>
<th>Tricoplusia ni</th>
<th>Heliothos zea</th>
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<tr>
<td>1</td>
<td>1-2</td>
<td>55%</td>
<td>60%</td>
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</tr>
<tr>
<td></td>
<td>1-3</td>
<td>9,000</td>
<td>1,400</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>13,900</td>
<td>5,900</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2-1</td>
<td>4%</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>3a-1</td>
<td>0</td>
<td>12%</td>
<td></td>
</tr>
<tr>
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<td>2</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>3b-1</td>
<td>39,000</td>
<td>15,400</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>4ab</td>
<td>4ab-1</td>
<td>1,900</td>
<td>490</td>
<td></td>
</tr>
<tr>
<td>4ac</td>
<td>4ac-1</td>
<td>9,400</td>
<td>5,700</td>
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</tr>
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<td>5ab-1</td>
<td>6,700</td>
<td>680</td>
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<td></td>
<td>5ab-2</td>
<td>7,400</td>
<td>3,100</td>
<td></td>
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<td>5ab-3</td>
<td>25,600</td>
<td>22,400</td>
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</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>7-1</td>
<td>8,000</td>
<td>830</td>
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<td></td>
<td>7-2</td>
<td>13,000</td>
<td>1,600</td>
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<tr>
<td>11</td>
<td>11-1</td>
<td>30%</td>
<td>20%</td>
<td></td>
</tr>
</tbody>
</table>

* International units per milligram after Dulmage (in press)
produces a crystal) is the easiest to obtain. It was first described in 1959 (Fitz-James and Young) for serotypes 1 and 4ac as occurring spontaneously. In fact the crystal phenotype is easily lost and care must be taken to prevent the loss of this trait.

Mutants which are cry$^+$spo$^-$ (form crystals, but not spores, Nishiitsutsuji-Uwo et al., 1975) were obtained from serotypes 3ab and 7 using nitrosoguanidine mutagenesis and selection for asporogenous variants which remained toxic to the silkworm. These mutants formed crystals within 48 hrs and most of the cells autolise.

Spo$^-$cry$^-$ (do not sporulate nor form crystals) have been reported with exposure to heat at 42°C (Yousten, 1978) or ethidium bromide (Azizbekyan et al., 1978). These mutants seem to indicate a relationship between sporulation and crystal formation, the nature of which is not yet clear.

Other types of mutants which exist are listed in Table 4. The antibiotic resistance mutants were selected as spontaneous mutations. Auxotrophs were isolated by exposing cells to ultraviolet light, allowing them to sporulate, then germinating the spores in minimal media, killing those that had germinated by boiling and then allowing the survivors to germinate on complete media. These survivors were then tested for auxotrophic markers (Thorne, 1978). There also exist, in individual laboratories; mutants of various serotypes which have not been published (H.deBarjac and H.D. Burges pers.comm.).
### TABLE 4

Auxotrophic and antibiotic resistant mutants in *Bacillus thuringiensis*

<table>
<thead>
<tr>
<th>H-serotype</th>
<th>Mutants</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>thyamine&lt;sup&gt;-&lt;/sup&gt;, uracil&lt;sup&gt;-&lt;/sup&gt;</td>
<td>deBarjac, 1970</td>
</tr>
<tr>
<td>2</td>
<td>tryptophan&lt;sup&gt;-&lt;/sup&gt;, niacin&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Thorne, 1978</td>
</tr>
<tr>
<td>3a</td>
<td>tryptophan&lt;sup&gt;-&lt;/sup&gt;, methionine&lt;sup&gt;-&lt;/sup&gt;, histidine, arginine&lt;sup&gt;-&lt;/sup&gt;, cysteine&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Thorne, 1978</td>
</tr>
<tr>
<td>3a anduze</td>
<td>penicillin&lt;sup&gt;r&lt;/sup&gt;, streptomycin&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Delafield et al, 1968</td>
</tr>
<tr>
<td>3ab</td>
<td>streptomycin&lt;sup&gt;r&lt;/sup&gt;, azide&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Fettig, 1976</td>
</tr>
<tr>
<td>12</td>
<td>tryptophan&lt;sup&gt;-&lt;/sup&gt;, niacin&lt;sup&gt;-&lt;/sup&gt;, methionine&lt;sup&gt;-&lt;/sup&gt;, leucine&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Thorne, 1978</td>
</tr>
</tbody>
</table>
Having established that genetic variety exists at least to a degree in *B. thuringiensis*, the next step in the development of a genetic system is to obtain a means of transmission which involves the isolation of chromosomal DNA or plasmids or phages; and the identification of plasmids or phages.

Isolation of *B. thuringiensis* chromosomal DNA for transformation was demonstrated by Reeves (1966) by the method of Marmur (1961). Plasmids for use in conjugation or transformation have been identified in many serotypes by Faust (1979), Milteva (1979), Stahly et al (1978a,b) and Martin and Dean (1979a,b in press). These studies have consisted mainly of demonstrating the existence of plasmid DNA and attempting to determine molecular weights, though only Stahly uses electron microscopy methods for sizing. There are many plasmids in most strains of *B. thuringiensis*, though until this thesis no one had been able to localize any genes on the plasmids.

Phages could also prove to be a useful tool in studying the genetics of *B. thuringiensis*. Virulent phages of *B. thuringiensis* have been isolated from the soil (C.L. Fort, unpublished data; Thorne, 1968; Thorne, 1978) or associated with bacteria isolated from insects (deBarjac et al, 1974; Colasito and Rogoff, 1969a; Ackerman et al, 1974; Rautenshtein et al, 1972). These phages have been characterized as to their morphology and some according to host range.
in *B. thuringiensis* and serology. Tobek et al (1978) have done restriction analysis on the DNA of one virulent phage. Restriction patterns should prove to be the best means to distinguish different phages (Dean et al, 1978).

Several temperate phages of *B. thuringiensis* have been characterized (deBarjac et al, 1974; Colasito and Rogoff, 1969b; Rautenshtein et al, 1972). They have been induced using mitomycin C, UV light or hydrogen peroxide, but none have been demonstrated to be lysogenic. This variety and abundance of phages may provide a transduction system for *B. thuringiensis*.

One of the reasons *B. thuringiensis* is particularly interesting from a genetic viewpoint is its production of they crystalline toxin during sporulation. The reason for the production of this protein is debatable. It may actually be the excess spore coat(Sommerville et al, 1976) deposited in the sporangium in crystalline form. Or it may only be incidentally related to sporulation and be trapped in the spore coat as it is formed (Stahly et al, 1978a). Some light may be shed on this developmental question by determining the location of gene(s) needed for crystal production. The ability to form crystal is easily lost from some strains of *B. thuringiensis* (Fitz-James and Young,1959). Loss of crystal production can be increased by plasmid curing methods (ethidium bromide, Azizbekyan et al, 1978; heat Stahly et al, 1978a). These studies showed that when crystal
production was lost, all plasmids were lost. Gonzales and Carlton (1977) using similar techniques were unable to demonstrate a correlation between plasmid loss and crystal formation.

Thus while there is evidence to indicate the endotoxin is on a plasmid, it is yet to be conclusively demonstrated that this is the case. An alternative hypothesis which could account for both types of observations is transposition of genes for crystal formation from a plasmid to the bacterial chromosome. Transposition was first observed in the galactose operon (Starlinger, 1977). It was later found that many genes including those for antibiotic resistance and toxin production can move from plasmid to plasmid and from plasmid to chromosome.

The endotoxin makes *B. thuringiensis* a fascinating study from many viewpoints, from its toxicity to insects, to the location of these genes. Progress in making *B. thuringiensis* a better pathogen could be aided by a genetic transfer system. This study is a beginning.
MATERIALS AND METHODS

General Information.

All chemicals and equipment used in this study are listed in the appendix.

The strains of B. thuringiensis that were used are listed in Table 5. Most of the strains which were isolated from nature were from H. Dulmage. Helpful mutants were supplied by P. Fettig (a streptomycin-azide resistant derivative of 3b-2). A. Yousten (several spo⁺cry⁻ derivatives of 3b-1) and A. Aronson (a spo⁺cry⁻ plasmidless, cycloserine resistant derivative of 3b-1).

The other bacterial strains that were used are listed in Table 6 along with their donors. The Bacillus strains were used for host range studies with phages and the Staphlococcus aureus strain, SA231, was the original source of pC194 used in transformation.

The media used are listed in Table 7. Soft agar for overlays was made by adding 6g of agar to the media per liter. Bottom agar plates were made by adding 15g of agar per liter.

Preparation of spore stocks.

Spore for titering and host range experiments in all Bacillus strains used were streaked heavily on DSM, and allowed to sporulate for three days at 30°C; spores were then
TABLE 5

*Bacillus thuringiensis* strains used

<table>
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<th>Serotype</th>
<th>Strain No.</th>
<th>Original Designation</th>
</tr>
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<tr>
<td></td>
<td>14-2</td>
<td>HD-500</td>
</tr>
</tbody>
</table>

HD- from H. Dulmage, <sup>a</sup> from L. Bulla, <sup>b</sup> from P. Fettig, <sup>c</sup> from A. Yousten, <sup>d</sup> from A. Aronson, <sup>e</sup> from L. Goldberg.
TABLE 6

Other bacterial strains used

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain No.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus amyliquefacens</td>
<td>10A1</td>
<td>Y. Ikeda</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>6A1</td>
<td>H. Halvorson</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>7A1</td>
<td>H. Halvorson</td>
</tr>
<tr>
<td>Bacillus pumilis</td>
<td>8A1</td>
<td>F. Young</td>
</tr>
<tr>
<td>Bacillus globiggi</td>
<td>11A1</td>
<td>H. Halvorson</td>
</tr>
<tr>
<td>Bacillus niger</td>
<td>12A1</td>
<td>J. Copeland</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>1A1</td>
<td>J. Shapiro</td>
</tr>
<tr>
<td>Staphlococcus aureus</td>
<td>SA231</td>
<td>S. Ehrlich</td>
</tr>
<tr>
<td>Media</td>
<td>Use</td>
<td>Composition</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Brain Heart Infusion</td>
<td>Bacteriocin testing</td>
<td></td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>E. thuringiensis sporulation</td>
<td></td>
</tr>
<tr>
<td>Trypticase Soy Broth</td>
<td>Bacteriocin testing</td>
<td></td>
</tr>
<tr>
<td>Penassay Broth</td>
<td>Protoplasting</td>
<td></td>
</tr>
<tr>
<td>Difco Sporulation Media</td>
<td>Other Bacilli sporulation</td>
<td>0.8% NB, 0.25M MgSO₄, 0.1% KCl, 10⁻²M FeSO₄, 10⁻⁴M MgCl₂, 10⁻³M CaCl₂.</td>
</tr>
<tr>
<td>L-Broth</td>
<td>Routine growth</td>
<td>1% tryptone, 0.5% yeast extract, 0.5% NaCl.</td>
</tr>
<tr>
<td>P</td>
<td>Phage titering</td>
<td>0.8% NB, 0.5% NaCl, pH 5.9, 1.5ml 1M CaCl₂, 10ml 0.5% MnSO₄, 10ml 2% MgSO₄/ l.</td>
</tr>
<tr>
<td>DM3</td>
<td>Protoplast regeneration</td>
<td>135g Na succinate pH 7.3, 5g casamino acids, 5g yeast extract, 3.5g K₂HPO₄, 1.5g KH₂PO₄, 5g glucose, 20ml 1M MgCl₂, 25ml 2% BSA, 8g agar/l.</td>
</tr>
<tr>
<td>CY</td>
<td>Plasmid isolation from S. aureus</td>
<td>1% casamino acids, 1% yeast extract, 0.1M NaCl, 0.5% glucose 0.05M Na glycerophosphate.</td>
</tr>
</tbody>
</table>
washed off the plates with sterile distilled water. To rid the preparation of vegetative cells, SDS (sodium dodecyl sulfate) was added to 2% and spores were then washed extensively with sterile water to eliminate the SDS. This preparation of spores was then titered in sterile distilled water and about $10^6$ spores were used for indicator lawns.

**Phage Isolation.**

Bacteriophage were isolated by growing strains to an optical density (OD 660) of 0.07 followed in a Spectrophotometer and inducing by the addition of 1 ug/ml mitomycin C and following growth, If cells lysed under these conditions, the lysate was filtered through a 0.45um filter. Dilutions in phage buffer (0.02 MTris PH 7.4; 0.085 M NaCl; 0.02SM Mg Cl2; 0.1% gelatin) were plated on a lawn of 3b-3 on P-media. If plaques were formed, the strain was scored as harboring a phage.

**Lysogeny.**

Of those phage plating on 3b-3, the turbid ones were picked and streaked for isolation on str-azi media (50ug/ml streptomycin, 100ug/ml azide in L-agar, selective for 3b-3) three times. A single colony was then resuspended in L-broth and induction was attempted as described above. Those phage which were capable of lysing 3b-3 under these conditions were called lysogenic and used for further study.
Host Range.

Host range experiments were carried out using the phages of 3b-3 lysogens in a spot test. 10ul aliquots of dilutions' of each phage were spotted on an indicator lawn of different Bacillus species. A phage was said to have a host range which includes a species only if individual plaques were formed on that species. A general clear zone which may be also indicative of bacteriocin production was not scored as plating on that host.

Transduction.

Transduction was attempted using 3b-1 as the host and 3b-3 lysogens as the donors to transfer the str and azi markers. The host was grown to a known cell density in P-broth. Phage were then added at a multiplicity of infection of about 0.1,1 and 10, and incubated for 10 min to allow for adsorption. Cells were pelleted, washed and resuspended in the same volume of P media. After allowing time for expression of the antibiotic resistance, cells were plated on selective media.

Antibiotic Resistance.

Because gram positive organisms are known to have mechanisms of antibiotic resistance that affect colony survival rather than single cell survival (Davies, 1978), several methods were used to test resistance. They were a colony method on L-agar, single cell resistance on agar (Nordstrom et al, 1968) and minimum inhibitory concentration
(MIC in L-broth). Antibiotics which were used are listed in Table 8.

Colony resistance was tested on L-agar. An initial small innoculum was stabbed into an antibiotic-containing plate as well as a control plate without the antibiotic. After three days growth at 30° colonies were measured and scored in the following manner: +, indicated growth comparable to the control; i, indicated growth to less than half the control; and -, indicated no growth.

Single cell resistance was determined by plating approximately 200-800 cells onto various concentrations of antibiotic agar. Single cell resistance was scored as the highest concentration of antibiotic which yielded the same number of cells as the control plate without the drug after 18hrs of growth.

MIC's were determined using microtiter plates in L-broth. The microtiter plates were first filled with a known quantity of growth medium, then the same volume of serial dilutions of antibiotics in L-broth was added and finally approximately 10³ cells were added and the plates were incubated at 30° for 18hrs. MIC's were scored as the lowest concentration of a drug which showed no growth.

**Plasmid Isolation.**

Plasmids of *B. thuringiensis* were isolated according to a modified technique of P. Kretchmer (pers. comm.). pC194
<table>
<thead>
<tr>
<th>Inhibitor or antibiotic</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acriflavine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Wyeth</td>
</tr>
<tr>
<td>Azide (sodium salt)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bryamycin</td>
<td>Bristol</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>Upjohn</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>Upjohn</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Neamine</td>
<td>Upjohn</td>
</tr>
<tr>
<td>Neomycin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Oleandomycin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Polymixin E</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>Upjohn</td>
</tr>
<tr>
<td>Streptolydigin</td>
<td>Upjohn</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Streptovaricin</td>
<td>Upjohn</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tylosin</td>
<td>Lilly</td>
</tr>
</tbody>
</table>
was isolated from *S. aureus* by the method of Novick and Bouanouchaud (1971).

*B. thuringiensis* plasmid isolation was performed as follows. L-broth was inoculated 1:100 with an overnight culture of *B. thuringiensis* also grown in L-broth. This culture was then incubated on a reciprocal shaker at 125 rpms at 30° for about 4 hrs. Cells were harvested, washed in 1/10th volume 0.5M NaCl and resuspended in 1/100th volume of SET (25% sucrose, 0.25M Ethylenediainetetra acetic acid, EDTA, and 0.25M Tris, pH 8). Lysozyme was added to 5mg/ml and the mixture incubated in a 37° water bath with gentle shaking for at least 30 min. RNase (0.1 unit T1, 10ug/ml pancreatic) and pronase (5mg/ml, predigested 30min. at 37°) were added and the mixture lysed while vigorously mixing with an equal volume of 2.4% sarkosyl. This lysed mixture was then incubated at 37° for 20 min. and cleared by centrifugation at 42,000 x g for 35 min. The supernatant (cleared lysate) containing the plasmid is then either run on an agarose gel or saved for further use by storing at 4°.

pC194 was isolated from *S. aureus* in the following manner. Cells were grown in CY media to late log phase, harvested and resuspended in 2.5M NaCl, 0.05M EDTA pH 7. Lysostaphin was added to 15ug/ml. This mixture was maintained at 37° for 15 min. Protoplasts were lysed with 1.5 volumes of 1% Brij 58, 0.04% deoxycholate and 0.3M
EDTA pH 8 while kept on ice. The lysate was cleared by centrifugation at 49,000 x g for 15 min. This lysate was then purified on CsCl as described below.

**Removal of chromosomal DNA from plasmid preparations.**

Chromosomal DNA can be removed from cleared lysates in the following ways: centrifugation through ethidium bromide-cesium chloride (EB-CsCl) gradients, a denaturation step and phenol extraction (Currier and Nester, 1970), or by boiling (van den Hondel et al, 1979).

To isolate plasmid DNA for transformation EB-CsCl gradients were used. CsCl was added to cleared lysates to 1g/ml. EB was added to this solution to yield a light pink color. Gradients were run at 40,000 rps (Ti 75 rotor) in a Beckman L2-65 ultracentrifuge for 40 hrs. Using the short column method of Griffiths (1978), gradients could be run at 55,000 rpm for only 24 hrs. Because of the low concentration of EB, fractions were collected under dim room light as most EB is bound to the DNA. DNA appears as a bright red band against a pale pink background. EB is then removed by extracting as many times as is necessary with equal volumes of isopropanol in CsCl saturated TES (Tris 0.05M pH 8, 0.05M NaCl, 0.05M EDTA). CsCl is then dialyzed out against TES for use in transformation.

To visualize the high molecular weight bands of plasmids obscured by the chromosome in cleared lysates two methods
were employed. The Currier and Nester technique consists of shearing the DNA using a vortex (1 min), denaturing DNA with base (raising the pH to 12 with NaOh), then quickly neutralizing (lowering the pH to 8 with 2M Tris, pH7). This mixture is then extracted with phenol and run on gels. The van den Hondel procedure removes contaminating chromosomal DNA (linear) as well as open circular forms of plasmids. It consists of boiling small amounts of a plasmid DNA preparations for 2 min, cooling quickly on ice and immediately subjecting it to electrophoresis.

Phenol Extraction.

The phenol extraction used is a variation of the standard procedure. Redistilled phenol is equilibrated with TES buffer and the bottom phenol containing layer is added in equal volume to a cleared lysate in an Erlenmyer flask. This mixture is gently shaken on ice for 20 min and then centrifuged at 4°C, 10,000 x g, 15 min. The aqueous (top) layer containing the DNA is pipetted off and reextracted with an equal volume of equilibrated phenol and chloroform in a 1:1 ratio. The final extraction is carried out with an equal volume of chloroform. The aqueous phase is then added to two volumes of freezer cold 95% EtOh and allowed to precipitate overnight in the freezer. The DNA is then centrifuged 20 min at 15,000 rpms. The supernatant is poured off and the white to clear pellet is resuspended in TES.
Electrophoresis.

Plasmids were visualized on 0.5% or 0.7 agarose gels made up in TEA (40 mM Tris, 20 mM Na acetate, 2 mM EDTA pH 8.05 with glacial acetic acid). Electrophoresis was carried out at 20v for 14 hrs. Gels were stained with a solution of 0.5 μg/ml EB in TEA and then destained for 15 min in TEA. Gels were then photographed using a Polaroid MP-4 camera and 667 Polaroid film with a red filter under UV illumination. The F-stop was 4.5 and the exposure was for 2 min. Molecular weights of covalently closed circular (CCC) forms were determined using published molecular weights of 3b-1 plasmids as standards (Stahly et al, 1978a). These data were analyzed graphically or by linear regression.

Plasmid Curing.

All plasmid curing was done on plates using either heat, drugs or a combination of the two. Heat at 37°C and 42°C was used (Yousten, 1978) and colonies of unusual appearance were screened for loss of crystal by phase contrast microscopy and for loss of antibiotic resistance by replica plating on a master plate and various drug containing plates. Acri-flavine curing was also done on plates at 24 μg/ml and scored in a similar manner. Other curing agents such as rifampicin, SDS, trimethoprim and ethidium bromide were also tried.

Bacteriocin Testing.

Bacteriocin testing was done on a few strains using the indirect antagonism method (Frederique, 1949) which calls for
growing bacteriocin producing colonies for 48 hrs, lysing with chloroform vapors and then overlaying with a bacteriocin sensitive lawn (3b-3). Strains were able to be tested in this way because of a media effect pointed out by A. Yousten (pers. comm.). TSA is the media of choice for this type of testing. The direct antagonism method (Barrow, 1963) which entails stabbing bacteriocin producing cells into a lawn of sensitive indicator cells was also employed.

Protoplasting and Transformation.

Protoplasting and transformation was done by a modification of the procedure of Chang and Cohen (1979). Cells for protoplasting were grown to a cell density of \(-1 \times 10^8\) cells per ml. This culture was made by innoculating L-broth 1:100 from an overnight culture in L-broth which was made from a fresh culture on L-agar. Cells were harvested, resuspended in 1/10th volume SMML (2 x 0.5M sucrose, 0.02M maleate, 0.02M MgCl₂ pH 6.5 mixed in equal volumes with 4x L-broth) and lysozyme (20mg/ml in SMM) was added to a final concentration of 7mg/ml. This mixture was shaken at 37° for at least 1 hr and as long as 4 hrs depending on the strain. Protoplast formation was monitored using phase contrast microscopy.

Initially the efficiency of protoplasting was determined by the ratio of spherical forms (protoplasts) to rods. In later experiments efficiency was determined by diluting protoplasts in hypotonic media (0.8% peptone) and plating
on nutrient agar (Gabor and Hotchkiss, 1979). Only those cells which have not formed protoplasts survive. The percentage of non-protoplasted cells (compared to cells before protoplasting) is subtracted from 100 to get the efficiency of protoplasting.

The protoplasts were then harvested at 2600 x g for 15 min, washed in an equal volume of SMML and then resuspended in the same volume. Protoplasts (0.5ml) were added to plasmid DNA in an equal volume of SMM in a sterile 15ml corex centrifuge tube. Plasmid DNA was obtained from CsCl gradients and was extensively dialyzed against TES. A control with no DNA was also done. To both were added 1.5ml PEG (40% w/v polyethylene glycol, MW 6000, in SMM). This mixture was mixed and allowed to incubate at room temperature two min. The mixtures were then diluted with 5 ml of SMML, centrifuged at 2600 x g for 15 min and resuspended in 0.5ml of SMML. Serial dilutions of protoplasts in SMML were then plated on DM3 regeneration media (Table 7).

Cells were allowed to regenerate three days on fresh DM3 at 30°. The frequency of regeneration is calculated as a percentage of input cells. These colonies were then replica plated on selective media, L-agar containing 8ug/ml chloramphenicol for pC194 and nutrient agar for crystal formation. Stability of chloramphenicol resistant colonies was checked by streaking resistant cells for several growth cycles on non-selective media (nutrient agar) and then retesting growth on media containing chloramphenicol.
**CAT Enzyme Assay.**

Chloramphenicol acetyltransferase (CAT) was assayed in chloramphenicol resistant (cam\(^r\)) *B. thuringiensis* transformants by the method of Shaw (1975). Cells were grown in L-broth to stationary phase in 8ug/ml cam. 8ug/ml of cam was added every other cell doubling to insure that CAT was fully induced. Cells were harvested and washed in 50mM Tris pH 7.5 and 0.145M NaCl. Cells were broken by cryo-impacting (Smucker and Pfister, 1975), that is broken by mechanical force at liquid nitrogen temperature. Broken cells were thawed in 50mM Tris pH 7.8 buffer containing 50uM 2-mercaptoethanol. Cell debris was removed by centrifugation at 30,000 x g for 20 min. The following assay was done on the supernatant. 1M Tris-HCl pH 7.8, 50mM acetyl-CoA and 1.6mg/ml cam were made up in advance; 4mg/ml 5,5'dithiobis-2-nitrobenzoic acid (DTNB) in 1M Tris is made up fresh. In a test tube mere mixed 0.2ml DTNB, 0.5ml enzyme, 1.3ml H\(_2\)O and 0.4ml cam which were equilibrated at 37\(^\circ\). 1ml was distributed to two cuvettes, zero set on a recording spectrophotometer then 20ul acetyl Co A was added to the sample cuvette and thereaction followed at 412nm. The amount of enzyme was then calculated to be the net change in extinction per minute divided by 13.6 to yield umoles/min of cam dependent DTNB reacted. Since this is equal to the rate of acetylation, 1 unit of CAT = 1 umole of cam acetylated/ min at 37\(^\circ\).
RESULTS

Isolation and Characterization of Phage.

All available strains of *Bacillus thuringiensis* were induced as described and tested for plating on a single host, 3b-3. The results of this induction and subsequent attempts at lysogeny are given in Table 9. Most strains showed lysis with mitomycin C at the concentration used (1 μg/ml). Only a portion of these lysates were able to plate on 3b-3 with varying efficiencies (1.5 x 10^2 to 5.8 x 10^7 plaque forming units/ml).

Representatives of typical induction attempts are shown in Fig.1. Such a curve for 3b-1 shows the growth of this strain with an initial doubling time of 24 min. An induction attempt that failed with 3b-3 does not show the typical decrease in optical density; the effect of mitomycin C in inhibiting the growth of cells can be seen when compared to growth when no mitomycin C is added.

In order to characterize these phage further and as a possible prelude to transduction studies, lysogens of 3b-3 were made. Out of twelve phage which made turbid plaques on 3b-3, nine were able to lysogenize this strain (Table 9).

These lysogens were further characterized by host range studies in other *Bacillus* species as shown in Table 10. All phage used had titers on 3b-3 of at least 10^6 phage/ml. In
### TABLE 9

**Tau phages of *Bacillus thuringiensis***

<table>
<thead>
<tr>
<th>Strain</th>
<th>Induction with mitomycin C</th>
<th>Titer on 3b-3</th>
<th>3b-3 lysogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>1-2</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>1-3</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>1-4</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>1-5</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>2-1</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>3a-1</td>
<td>+</td>
<td>1.1x10³</td>
<td>+</td>
</tr>
<tr>
<td>3a-2</td>
<td>+</td>
<td>3.3x10²</td>
<td>+</td>
</tr>
<tr>
<td>3b-1</td>
<td>+</td>
<td>1.9x10⁶</td>
<td>+</td>
</tr>
<tr>
<td>3b-2</td>
<td>+</td>
<td>2.0x10⁴</td>
<td>N.D.</td>
</tr>
<tr>
<td>3b-3</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>3b-4</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>3b-5</td>
<td>+</td>
<td>3.2x10⁶</td>
<td>-</td>
</tr>
<tr>
<td>4ab-1</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>4ac-1</td>
<td>+</td>
<td>1.8x10⁵</td>
<td>+</td>
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<td>4ac-2</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>4ac-3</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5ab-1</td>
<td>+</td>
<td>5.8x10⁷</td>
<td>-</td>
</tr>
<tr>
<td>5ab-2</td>
<td>+</td>
<td>2.0x10³</td>
<td>+</td>
</tr>
<tr>
<td>5ab-3</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5ab-4</td>
<td>+</td>
<td>1.2x10⁷</td>
<td>+</td>
</tr>
<tr>
<td>6-1</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>7-1</td>
<td>+</td>
<td>1.5x10²</td>
<td>+</td>
</tr>
<tr>
<td>7-2</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>7-3</td>
<td>+</td>
<td>1.0x10⁷</td>
<td>-</td>
</tr>
<tr>
<td>8-1</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>9-1</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>9-2</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>10-1</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>10-2</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>11-1</td>
<td>+</td>
<td>3.1x10⁵</td>
<td>+</td>
</tr>
<tr>
<td>12-1</td>
<td>+</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>13-1</td>
<td>+</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>14-1</td>
<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>14-2</td>
<td>+</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. not determined
Fig. 1: Induction of *Bacillus thuringiensis* strains 3b-1 and 3b-3 with mitomycin C.

- 3b-1 showing induction with mitomycin C.
- 3b-3 no mitomycin C added.
- 3b-3 showing no induction, but inhibition of cell growth.

→ shows time of addition of mitomycin C.
### TABLE 10

**Host Range of Phages**

<table>
<thead>
<tr>
<th>Host</th>
<th>Phage 1</th>
<th>Phage 2</th>
<th>Phage 3</th>
<th>Phage 4</th>
<th>Phage 5</th>
<th>Phage 6</th>
<th>Phage 7</th>
<th>Phage 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. amyliquifaciens</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. cereus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>-</td>
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<tr>
<td>B. lichenformis</td>
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<tr>
<td>B. megaterium</td>
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<td>B. niger</td>
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<td>-</td>
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<tr>
<td>B. pumilis</td>
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<td>-</td>
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</tr>
<tr>
<td>B. subtilis</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>B. thuringiensis var. kurstaki</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>
summarizing the relayent results of Table 10: three phage, T3a-2, T5ab-2, and T5ab-4 have the same host range (B. cereus, B. subtilis, and B. thuringiensis); T3b-1 and T5ab-1 plate only on B. cereus and B. thuringiensis; all but T4ac-1 plate on B. cereus and no phages plate on B. amyliquifacens, B. licheniformis, B. megaterium, or B. niger.

Transduction.

Transduction, attempted with phages T3a-2 and T5ab-4 was unsuccessful yielding no transductants above the control. Transduction was also done with T5ab-2 and the results of three experiments are given in Table 11. There are few transductants and the efficiency in the best experiment (36 str<sup>r</sup> in 1.2 x 10<sup>7</sup> cells) is only 0.003%. Azir<sup>r</sup> is transduced at an even lower frequency which is not above the control. No linkage can be established between these two markers.

Transduction of the str and azi markers was also attempted using CP-51 (Thorne, 1978). It was too virulent even after UV treatment to leave enough survivors to show transduction.

After these attempts at transduction failed to yield a suitable gene transfer system, a slightly different approach was employed. If genetic differences among strains of B. thuringiensis could be established, then other means of gene transfer might be used.

Antibiotic Resistance.

Strains of B. thuringiensis were screened for antibiotic resistance to determine variations in naturally occurring
### TABLE 11

Transduction of 3b-1 with τ5ab-2

<table>
<thead>
<tr>
<th>Experiment # 1</th>
<th>MOI*</th>
<th>Titer of cells</th>
<th>str&lt;sup&gt;r&lt;/sup&gt;</th>
<th>azi&lt;sup&gt;r&lt;/sup&gt;</th>
<th>str-azi&lt;sup&gt;r&lt;/sup&gt;</th>
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<tr>
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<tr>
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<td>36</td>
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<tr>
<td>0.25</td>
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</table>

* multiplicity of infection
resistance. This was done as described in materials and methods and the results are summarized in Tables 12, 13 and 14. The antibiotics are grouped according to the site of action at the cellular level. Ampicillin affects the cell wall; polymixin, cell membrane; rifampicin and streptolydigin, RNA synthesis; streptomycin through tylosin, protein synthesis; azide, respiration and sulfanilamide and trimethoprim, intermediary metabolism.

In general, using colony resistance or MIC methods, B. thuringiensis strains were resistant to ampicillin, polymixin, acriflavine, spectinomycin, neamine, lincomycin, sulfanilamide and trimethoprim. They are sensitive to nalidixic acid, novobiocin, rifampicin, chloramphenicol, erythromycin, oleandomycin and bryamycin. The single cell resistance in all cases was equivalent to or lower than the results obtained by the other two methods.

Plasmids.

Various serotypes were screened for plasmids. Plasmids could be useful in identifying a particular strain and as a potential means of transferring genes. There was also the possibility that in some strains a plasmid carries the gene for the endotoxin. Attempts were made to locate genes on these plasmids.

Electrophoresis of plasmid DNA from cleared lysates on agarose gels proved to be a suitable way to screen for plasmids. Fig 2 shows a comparison of gel concentrations
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<thead>
<tr>
<th>Antibiotic</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>1-1</td>
</tr>
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<td>20ug/ml ampicillin</td>
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</tr>
<tr>
<td>24ug/ml acriflavine</td>
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</tr>
<tr>
<td>50ug/ml nalidixic acid</td>
<td>-</td>
</tr>
<tr>
<td>50ug/ml rifampicin</td>
<td>-</td>
</tr>
<tr>
<td>20ug/ml streptomycin</td>
<td>-</td>
</tr>
<tr>
<td>50ug/ml spectinomycin</td>
<td>+</td>
</tr>
<tr>
<td>20ug/ml kanamycin</td>
<td>i</td>
</tr>
<tr>
<td>5ug/ml neomycin</td>
<td>+</td>
</tr>
<tr>
<td>10ug/ml chloramphenicol</td>
<td>-</td>
</tr>
<tr>
<td>50ug/ml erythromycin</td>
<td>-</td>
</tr>
<tr>
<td>50ug/ml lincomycin</td>
<td>-</td>
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<tr>
<td>20ug/ml tetracycline</td>
<td>i</td>
</tr>
<tr>
<td>50ug/ml bryamycin</td>
<td>-</td>
</tr>
<tr>
<td>20ug/ml sulfanilamide</td>
<td>+</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Strain</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>ampicillin</td>
<td></td>
</tr>
<tr>
<td>polymixin</td>
<td></td>
</tr>
<tr>
<td>nalidixic acid</td>
<td></td>
</tr>
<tr>
<td>novobiocin</td>
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<tr>
<td>rofampicin</td>
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</tr>
<tr>
<td>streptolydigin</td>
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<td>erythromycin</td>
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<td>clindomycin</td>
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<td>oleandomycin</td>
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<td>tylosin</td>
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</tr>
<tr>
<td>bryamycin</td>
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<td>azide</td>
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</tr>
<tr>
<td>sulfanilamide</td>
<td></td>
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<tr>
<td>trimethoprim</td>
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TABLE 14

Single Cell Resistance of Selected
*Bacillus thuringiensis* Strains

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Strain</th>
<th>1-1</th>
<th>3b-1</th>
<th>4ac-1</th>
<th>6-1</th>
<th>81</th>
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<th>14-2</th>
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<td>ampicillin</td>
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<td>&lt;1</td>
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<td>&lt;1</td>
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<td>1</td>
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<td>1</td>
<td>5</td>
<td>&lt;1</td>
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<td>1</td>
<td>&lt;5</td>
<td>5</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>rifampicin</td>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<td>1</td>
<td>&lt;1</td>
<td>&lt;5</td>
<td>1</td>
<td>1</td>
<td>&lt;1</td>
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<tr>
<td>spectinomycin</td>
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<td>&lt;1</td>
<td>&gt;30</td>
<td>&gt;10</td>
<td>5</td>
<td>&gt;10</td>
<td>ND</td>
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<td>&lt;1</td>
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<td>&lt;1</td>
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<td>1</td>
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<td>&lt;1</td>
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<td>1</td>
<td>&lt;1</td>
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<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>erythromycin</td>
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<td>ND</td>
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<td>&lt;1</td>
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<td>&lt;1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>azide</td>
<td></td>
<td>&gt;10</td>
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<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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</tr>
</tbody>
</table>
Fig. 2: Comparison of 0.5% and 0.7% agarose gels using strain 3b-1.

In the 0.5% gel all six plasmids are visible including both OC and CCC forms of pTX3b-1, pTX3b-2, pTX3b-3 and pTX3b-4.

In the 0.7% gel only four plasmids are visible and only both forms are visible for pTX3b-1, pTX3b-2 and pTX3b-3.
between 0.7% and 0.5% agarose gels. For the same strain, 3b-1, more plasmid forms are visible in the 0.5% gel than in the 0.7% gel. Plasmids were found in all but one serotype (Fig. 3); 2-1 (var. finitimus) lacks all plasmid DNA. It should be noted that one strain, 13-1 consistently had to be grown longer (30 min) than other strains in order for any of the plasmids to be observed in this gel system. None of the different strains have the same plasmid pattern although related strains in the same serotype have similar plasmid patterns (Fig. 4).

The plasmid molecular weights were determined as described and the composite results of several gel measurements are shown in Table 15. The determination of molecular weight was based on comparison of covalently closed circular (CCC) forms of known molecular weight to CCC forms of unknown molecular weight which were isolated in the same manner. CCC forms could be distinguished from open circular (OC) forms by boiling (Fig. 5). During this process the chromosomal DNA as well as the OC forms of the plasmids are lost.

Strain 3b-1 was chosen to continue studies because of its availability, industrial importance, virulence and known plasmid pattern. In order to determine which genes are carried by which plasmids various means of curing were tried. Acriflavine and heat were the most successful methods. Curing experiments with SDA, rifampicin, ethidium bromide and trimethoprim were unsuccessful.
Fig. 3: Plasmid patterns of representative strains of all 17 serotypes.
Fig. 4: Comparison of plasmid patterns within serotype 1.

All strains have plasmid A in common, 1-1 and 1-3 share all but one plasmid, 1-4 and 1-5 share plasmids B, C, and D.
### TABLE 15
Molecular Weights of *Bacillus thuringiensis* Plasmids

<table>
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<th>Strain</th>
<th>Plasmid</th>
<th>Molecular weight $10^6$ daltons +/- standard deviation</th>
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<td>1-1</td>
<td>pTX1-1</td>
<td>5.38 +/- 0.09</td>
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<tr>
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<td>pTX1-2</td>
<td>6.43 +/- 0.2</td>
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<tr>
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<td>pTX1-3</td>
<td>7.69 +/- 0.4</td>
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<tr>
<td>3a-1</td>
<td>pTX3a-1</td>
<td>4.61 +/- 0.08</td>
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<td>pTX3a-2</td>
<td>4.93 +/- 0.09</td>
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<td>pTX3a-3</td>
<td>5.68 +/- 0.16</td>
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<tr>
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<td>pTX3a-4</td>
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<td>pTX3a-5</td>
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<td>pTX7-1</td>
<td>3.4 +/- 0.15</td>
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<td>pTX7-2</td>
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<td>pTX7-3</td>
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<td>pTX7-4</td>
<td>8.45 +/- 0.94</td>
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<td>pTX13-1</td>
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<td>6.17 +/- 1.07</td>
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<tr>
<td>14-2</td>
<td>pTX14-1</td>
<td>3.37 +/- 0.1</td>
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<td>pTX14-2</td>
<td>4.21 +/- 0.25</td>
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<td>pTX14-3</td>
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<tr>
<td></td>
<td>pTX14-4</td>
<td>16.5 +/- 1.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>molecular weights Stahly et al., 1978.

<sup>b</sup>by restriction analysis this strain has more than one plasmid.
Fig. 5: Removal of chromosomal and open circular plasmid forms from cleared lysates.

1 - shows strain 1-1 from a cleared lysate with both open circular (OC) and covalently closed circular (CCC) forms of all its plasmids.

2 - shows same lysate after boiling. Chromosomal DNA is removed as well as the OC forms of the three plasmids.
Using acriflavine or heat curing only two genes could be tentatively assigned to plasmids (Tables 16 and 17). pTX3b-4 determines ampicillin resistance and pTX3b-5 produces the crystal toxin. Fig. 6 shows a gel of some of the cured strains.

Other characteristics could be eliminated from 3b-1 including: sporulation, bacteriocin production and reduction in resistances to tylosin, streptomycin, lincomycin, novobiocin, erythromycin, tetracycline, clindomycin, oleandomycin, streptolydigin, streptovaricin and acriflavine, but these characters show no clear relation to any single plasmid (Table 18). Other characteristics which are not lost are resistances to spectinomycin, azide, neamine, sulfanilamide and trimethoprim.

Transformation.

To more accurately determine the location of certain genes a transformation system is essential. Because protoplasting gives such a high rate of transformation this method was chosen. The method used as described in Materials and Methods was the result of optimizing conditions and a lucky break. Many conditions affect the efficiency of protoplast formation and these are shown in the following tables: Table 19, temperature; Table 20, cell density; Table 21, lysozyme concentration; Table 22, pH; Table 23, salt concentration; Table 24, media effect; and Table 25, strain effect. Of these conditions the most important ones
Relation of pTX3-b-4 to Ampicillin Resistance as Revealed by Curing

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Method of curing</th>
<th>Method of selection</th>
<th>pTX3-b-4</th>
<th>Ampicillin resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>28A</td>
<td>acriflavine</td>
<td>amp$^S$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17A</td>
<td>acriflavine</td>
<td>amp$^S$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M-6</td>
<td>acriflavine</td>
<td>sulf$^S$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>heat</td>
<td>cry$^-$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>28</td>
<td>heat</td>
<td>cry$^-$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3b-3</td>
<td>heat</td>
<td>straz$^{r_1}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30's</td>
<td>acriflavine</td>
<td>tet$^S$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12T</td>
<td>acriflavine</td>
<td>tet$^S$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4T</td>
<td>acriflavine</td>
<td>tet$^S$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>26s</td>
<td>acriflavine</td>
<td>sulf$^S$</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3b-11</td>
<td>heat</td>
<td>cry$^-$</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3b-7</td>
<td>heat</td>
<td>cry$^-$</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3b-8</td>
<td>heat</td>
<td>cry$^-$</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3b-9</td>
<td>heat</td>
<td>cry$^-$</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3b-10</td>
<td>heat</td>
<td>cry$^-$</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*ampicillin resistance only $\frac{1}{2}$ that of parent strain
TABLE 17
Relation of pTX3b-5 to Crystal Production as Revealed by Curing

<table>
<thead>
<tr>
<th>Isolate</th>
<th>pTX3b-5</th>
<th>Spore Production</th>
<th>Crystal Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>3b-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17A</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M9</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3b-7</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3b-11</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>30s</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M6</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18T</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4T</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3b-2</td>
<td>-</td>
<td>+</td>
<td>*</td>
</tr>
</tbody>
</table>

*produces an inclusion, but does not look like a typical crystal.
Fig. 6: Cured strains of 3b-1.

3b-1, retains all plasmids.
3b-2, missing pTX3b-5.
3b-3, accidentally cured strain, plasmids rearranged.
30s, has pTX3b-4 and at least 2 other plasmids of different molecular weights.
17A, missing pTX3b-4 molecular weight of pTX3b-5 slightly reduced.
M-6, retains pTX3b-5 but is spo"cry".
3b-7, seems to be missing pTX3b-3, pTX3b-4, pTX3b-6, pTX3b-5.
3b-11, no plasmids.
Figure 6
TABLE 18

Traits and plasmids lost upon "curing"

<table>
<thead>
<tr>
<th>Trait or plasmid</th>
<th>Isolate</th>
<th>3b-1</th>
<th>17A</th>
<th>3b-7</th>
<th>M-6</th>
<th>30s</th>
<th>3b-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>sporulation</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>crystal</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>bacteriocin</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ampicillin</td>
<td></td>
<td>50</td>
<td>1</td>
<td>13</td>
<td>6</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>novobiocin</td>
<td></td>
<td>50</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>streptomycin</td>
<td></td>
<td>13</td>
<td>3</td>
<td>3</td>
<td>13</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>streptovarin</td>
<td></td>
<td>50</td>
<td>1</td>
<td>13</td>
<td>6</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>streptolydigin</td>
<td></td>
<td>50</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>erythromycin</td>
<td></td>
<td>25</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>clindomycin</td>
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<td>50</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>lincomycin</td>
<td></td>
<td>50</td>
<td>13</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>oleandomycin</td>
<td></td>
<td>50</td>
<td>1</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>tetracycline</td>
<td></td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>tylosin</td>
<td></td>
<td>50</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pTX3b-1</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pTX3b-2</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>-</td>
</tr>
<tr>
<td>pTX3b-3</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>-</td>
</tr>
<tr>
<td>pTX3b-4</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pTX3b-5</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pTX3b-6</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

a + or - indicates whether a characteristic or plasmid is present
b number indicates antibiotic resistance as determined by M.I.C.
c plasmid notation
d has two other plasmids instead


TABLE 19

Effect of temperature on protoplast formation

<table>
<thead>
<tr>
<th>Temperature</th>
<th>% Protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°</td>
<td>1.0</td>
</tr>
<tr>
<td>37°</td>
<td>9.1</td>
</tr>
<tr>
<td>42°</td>
<td>8.3</td>
</tr>
<tr>
<td>65°</td>
<td>21.8</td>
</tr>
</tbody>
</table>

Growth in PAB to O.D.(660) 0.65; 2mg/ml lysozyme
TABLE 20

Effect of cell density on protoplast formation

<table>
<thead>
<tr>
<th>O.D. (660) harvested</th>
<th>% Protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>7.3</td>
</tr>
<tr>
<td>0.4</td>
<td>9.5</td>
</tr>
<tr>
<td>0.5</td>
<td>7.0</td>
</tr>
<tr>
<td>0.6</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Growth in PAB; 2mg/ml lysozyme at 60°
<table>
<thead>
<tr>
<th>Lysozyme concentration mg/ml</th>
<th>% Protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.0</td>
</tr>
<tr>
<td>2</td>
<td>16.1</td>
</tr>
<tr>
<td>3</td>
<td>16.9</td>
</tr>
<tr>
<td>4</td>
<td>17.7</td>
</tr>
<tr>
<td>5</td>
<td>13.7</td>
</tr>
<tr>
<td>6</td>
<td>14.2</td>
</tr>
<tr>
<td>7</td>
<td>21.9</td>
</tr>
<tr>
<td>8</td>
<td>15.3</td>
</tr>
</tbody>
</table>

Growth in PAB to O.D.(660) 0.7; 60°
### TABLE 22

**Effect of pH on protoplast formation**

<table>
<thead>
<tr>
<th>pH</th>
<th>% Protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>17.4</td>
</tr>
<tr>
<td>6.5</td>
<td>15.8</td>
</tr>
<tr>
<td>7.5</td>
<td>13.7</td>
</tr>
<tr>
<td>8.5</td>
<td>12.1</td>
</tr>
<tr>
<td>9.5</td>
<td>9.5</td>
</tr>
<tr>
<td>10.4</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Growth in PAB to O.D.(660) 0.6; 4mg/ml lysozyme at 60°
TABLE 23

Effect of NaCl on protoplast formation

<table>
<thead>
<tr>
<th>NaCl concentration mg/ml</th>
<th>% Protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13.2</td>
</tr>
<tr>
<td>2.9</td>
<td>11.7</td>
</tr>
<tr>
<td>1.0</td>
<td>13.7</td>
</tr>
<tr>
<td>0.5</td>
<td>10.4</td>
</tr>
</tbody>
</table>

Growth in PAB to O.D.(660) 0.6; 7mg/ml at 60°
TABLE 24

Effect of media on protoplast formation

<table>
<thead>
<tr>
<th>Media</th>
<th>% Protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAB</td>
<td>17.8</td>
</tr>
<tr>
<td>PAB (with fructose)</td>
<td>17.0</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>4.5</td>
</tr>
<tr>
<td>Nutrient broth + glucose</td>
<td>10.6</td>
</tr>
<tr>
<td>Brain Heart Infusion</td>
<td>15.2</td>
</tr>
<tr>
<td>Trypticase Soy Broth</td>
<td>13.1</td>
</tr>
<tr>
<td>L-broth</td>
<td>90.0</td>
</tr>
</tbody>
</table>

Growth to O.D.(660) 0.6; 7mg/ml lysozyme at 37°
TABLE 25

Strain effect on protoplast formation

<table>
<thead>
<tr>
<th>Strain</th>
<th>O.D.(660) harvested</th>
<th>% Protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>0.66</td>
<td>0</td>
</tr>
<tr>
<td>3b-1</td>
<td>0.60</td>
<td>99</td>
</tr>
<tr>
<td>4ab-1</td>
<td>0.66</td>
<td>98</td>
</tr>
<tr>
<td>6-1</td>
<td>0.66</td>
<td>10</td>
</tr>
<tr>
<td>10-1</td>
<td>0.62</td>
<td>1</td>
</tr>
<tr>
<td>12-1</td>
<td>0.60</td>
<td></td>
</tr>
</tbody>
</table>

Growth in L-broth; lysozyme 7mg/ml at 37°
affecting the percent of cells protoplasted were media effect
and strain differences. The optimal conditions for 3b-1 were
determined to be growth in L-broth to OD (660) 0.6, then in-
cubation with lysozyme (7mg/ml) at 37°C for 1-2 hrs (protoplast
formation at higher temperatures resulted in lower recovery
due to cell lysis).

These conditions were then used in protoplasting for
transformation. Transformation was successful with, 17A a
derivative of 3b-1, which retains the crystal of the parent
strain, but is ampicillin sensitive and is missing pTX3b-4.
The results of two transformations of this strain with pCl94
are given in Table 26. In the first experiment there was
poor regeneration, but the transformation rate was high, 8.3%.
This initial rate was cut in half upon one subculturing on
selective media (chloramphenicol at 8ug/ml). When tested
for permanent stability by maintaining strains without
selection for at least three subculturings two more clones
were demonstrated to be unstable. Fig. 7 shows plasmid
patterns of four transformants. In stable transformants c2,
c10 or c12 there is no extra plasmid band in the region where
pCl94 should band; however in an unstable transformant, c6,
there is an extra band running slightly larger than pCl94.
The molecular weight of pCl94 is 2 million daltons and this
new plasmid is about 2.2 million. The plasmid pattern of this
clone reverted to the typical pattern (i.e. loss of the plasmid
band) under continued selection on chloramphenicol media and
TABLE 26

Transformation of 17A with pC194

<table>
<thead>
<tr>
<th>Cells regenerated</th>
<th>Transformants</th>
<th>Stable subclones</th>
</tr>
</thead>
<tbody>
<tr>
<td>296</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>3500</td>
<td>115</td>
<td>35</td>
</tr>
</tbody>
</table>
Fig. 7: Chloramphenicol resistant transformants of 17A.
A - controls of parent strain, 17A and plasmid, pC194.
B - transformed strains, c2, c10 and c12 are stable transformants; c6 is unstable.
stabilized (i.e. it can now be maintained without continued selection pressure).

Crude lysates of these transformants were assayed for the presence of chloramphenicol acetyltransferase (CAT) activity and the results of an assay are shown in Fig. 8. Both transformants have activity while the parent strain does not. Using Shaw's method (1975) to calculate the amount of enzyme from the rate of the reaction, both \textit{B. subtilis} (pC194) and c6 have 0.8 units of CAT activity.

In the second experiment the regeneration was increased though the percentage of transformants decreases to 3.3%. Here a greater instability was seen in the first subcloning. Different colony types showed up on retesting, but all stable types showed the parental plasmid pattern including both types of colony morphology when segregation was observed.

A composite rate of about 5% for both experiments made it feasible to look for transformation of a non-selectable marker, endotoxin production. In this experiment 3b-11 (\textit{cry}^-, \textit{plasmid}^-, \textit{cycloserine}^R) a derivative of 3b-1 was used as the recipient and plasmids which were CsCl purified from 3b-1 were the donor DNA. The percent protoplasting in this experiment was 99.8% but the regeneration was only 0.6%. The 39 colonies that were exposed to 3b-1 plasmid DNA which regenerated on DM3 all formed crystals when replica plated on nutrient agar. The 54 colonies of the control (which were not exposed to plasmid DNA) formed no crystals. All colonies
Fig. 8: Chloramphenicol acetyltransferase activity in transformed cells.
grew on cycloserine (50μg/ml). Using the MIC method 31 of 34 of the crystal\(^+\) colonies tested had the cycloserine resistance of the parent 3b-11. Three of these colonies were picked and DNA examined by the cleared lysate method. The plasmid patterns are shown in Fig. 9. All the crystal\(^+\) transformants had one plasmid that corresponds to pTX3b-5 of 3b-1 by molecular weight analysis.
Fig. 9: Transformation of crystal determining plasmid into strain 3b-11.
Figure 9,
DISCUSSION

Phages.

Since both transduction systems known for \textit{B. thurin-giensis} involved the use of virulent phages, perhaps the isolation of temperate phages would be a way to overcome this problem.

The strain 3b-3 was chosen as a host for several reasons. It does not induce with mitomycin C and therefore does not harbor an inducible bacteriophage or produce an inducible bacteriocin. Lack of phage would avoid the problems of immunity. It has two resistance markers (streptomycin and azide) and thus can be picked out from a background of other cells. These markers, assumed to be chromosomal, would be readily available for transduction. A single host strain was needed to rule out restriction-modification problems in transduction.

From Table 9 most strains appear to harbor an inducible bacteriophage, though some fail to plate on 3b-3. Therefore 3b-3 is not in the host range of that particular phage. An alternative explanation is that mitomycin C might be inducing the production of a bacteriocin (Dougan and Sherratt, 1977), which can also cause the lysis of the cell thereby accounting for the decrease in optical density as seen in Fig.1.
The difference in efficiency of plating of the various bacteriophages on 3b-3 may be due to two major reasons. The first is restriction-modification, a well documented phenomenon (Boyer, 1971). In this case failure of a phage to plate at high efficiency may be due to different modification patterns of the host (3b-3) and the incoming phage DNA with the result that the phage is cut by host restriction enzymes. The second is failure of the original host to induce fully, resulting in low numbers of phage released. Other minor considerations may include sub-optimal conditions for induction and/or plating or instability of the phage.

Lysogens of 3b-3 were used to further characterize tau phages. This avoided restriction-modification problems. The use of single donor for transduction would facilitate comparison of transfer frequencies between different phages.

It was immediately clear that an attempt to do host range studies among \textit{B. thuringiensis} serotypes (done by deBarjac et al, 1974) would not distinguish between host range and immunity because most strains appear to be lysogenic. It is also clear that phages of low titer may not plate due to restriction rather than a limited host range.

Phages of \textit{B. subtilis} have been characterized using their host range among other \textit{Bacillus} species (Dean et al, 1978). Because of the close relationship of \textit{B. thuringiensis} and \textit{B. cereus} it was expected that most phage would plate on both
species. The exception of T4ac-1 shows that there has been some divergence in at least phage attachment sites between these two species. A surprising finding was that many of these phages also plated on B. subtilis. B. subtilis is distantly related to B. thuringiensis and possesses a potentially useful gene (UV resistance) for B. thuringiensis if a system of genetic exchange existed. If host range can be used to establish major groups of phages, then by this criterion, there are six of these groups among tau phage.

Some of the phages with host range in B. subtilis were used for transduction. Phage T5ab-2 was able to transduce the str<sup>r</sup> marker of 3b-3 to 3b-1, although this frequency was too low to be useful. The failure of the second marker, azi<sup>r</sup>, to be transduced could be due to too high a level of azide (100ug/ml, which is the maximum for 3b-3) for transductants to be scored.

### Antibiotic Resistance.

In search for more variety, the natural antibiotic resistance of representative strains were determined by three methods. The most sensitive method is the MIC which could screen the widest variety and concentration of drugs. The colony resistance was absolutely essential for later work as replica plating was the only means of scoring transformants. A comparison of these two methods with each other offers some insight into the mode of action of these resistances.
There is a remarkable amount of resistance found to antibiotics among B. thuringiensis strains. This could be because B. thuringiensis shares an ecological niche with soil organisms which produce many of these drugs. Feed additives such as lincomycin and tylosin, which can make their way into the soil through animal feces may be the selection pressure which allows those strains of B. thuringiensis which are resistant to survive.

High levels of resistance need not be explained by a failure of the drug to enter the cell. Acriflavine, for example, must be able to get into cells to explain its curing effect on plasmids. Likewise sulfanilamide and trimethoprim affect the synthesis of nucleic acids from p-aminobenzoic acid and in a rich media such as L-broth; a salvage pathway could be used to circumvent this block. The only exception to this rule is strain 6-1 which may not be able to utilize a salvage pathway and therefore is severely affected by small amounts of both of these drugs.

A possible use of these data is in insect bioassays where antibiotics are used in the insect diet to prevent bacterial infection. These data would enable the selection of an antibiotic which as the least effect on the B. thuringiensis strains being tested and yet control other bacteria.

A clue as to the mode of action of some of these other resistances can be obtained by comparing the single cell
resistance to the MIC. If one recalls that gram positive bacteria, when utilizing enzymes that inactivate drugs, excrete these enzymes directly into the media. Thus a group of cells will survive in a higher concentration of drug than a single cell. This can be demonstrated for ampicillin resistance in most strains of *B. thuringiensis* (suggesting a β-lactamase in strains 3b-1, 4ac-1, 13-1, 14-2) and for some other drug strain combinations. An example of this is strain 1-1 and resistance to spectinomycin. The single cell resistance is less than 1μg/ml, yet using the MIC method, where the effect on groups of cells is seen, the resistance is 50μg/ml. (see Tables 13 and 14 for other examples of spectinomycin, 14-2; tetracycline, 4ac-1; erythromycin, 3b-1). One has to be careful not to extend this analysis to many bacteriocidal drugs, because if cell lysis has taken place this could account for some degree of protection from a drug for a group of cells by forming an environment that decreases the local drug concentration. The discrepancies between colony resistance and MIC are few. These can be explained by the time periods after which the results are read (colony resistance, 3 days; MIC, 18 hrs). In cases where the colony resistance is greater than the MIC this may be evidence for a slowly inducible resistance system. These kinds of enzymes in many systems are known to be carried by plasmids.
Plasmids.

By the simple system of running cleared lysates on gels, molecular weights of plasmids could be determined. For large molecular weight plasmids a 9.5% agarose gel gave better separation of plasmids without concurrent loss or diffusion of low molecular weight plasmids (about 1 million daltons). It was important to know which form (OC or CCC) plasmids were in so that an accurate comparison could be made.

The variety of plasmid patterns is greater in *B. thuringiensis* than in most *Bacilli*, such as *B. subtilis* (Tanaka et al., 1976; LeHegarate and Anagnostopoulis, 1977). *B. pumilis* (Lovett and Bramucci, 1975), and *B. cereus* (Bernhard et al., 1978) but is less than *B. megaterium* (Carlton and Smith, 1974). The small plasmids (less than 9 x 10^6 daltons) conform exactly to molecular weight standards as determined by electron microscopy (Stahly et al., 1978a), however the larger ones only approximate the predicted values. Changes in superhelical density seem to affect larger plasmids to a greater extent. The number of superhelical turns can affect the movement of plasmids in gels. The open circular forms may be present for two reasons. During lysis nicks can be introduced into supercoiled forms, or plasmids may exist in cells as open circles or relaxation complexes (Novick, 1976).

The variety of plasmids found in *B. thuringiensis* forced a choice of a particular strain in which to concentrate
efforts at making a genetic system work. The strain 3b-1 was chosen for the reasons mentioned in the results. "Cured" mutants were relatively easy to obtain. Cry− and spo− were by far the most common mutants obtained. Antibiotic sensitive mutants were picked up at a lower frequency than cry−. This loss of characteristics would be expected if plasmids are being cured. These curing techniques for the most part affected only the larger plasmids of 3b-1 (pTX3b-4, pTX3b-5, pTX3b-6) which was expected from the results of other curing studies. These studies show that larger plasmids are more easily cured and the small ones are refractory to curing by the same techniques (Mitsuhasi et al, 1961). This can be explained by the small size and high copy number of this class of plasmid, thus inactivation of a gene on one plasmid would not necessarily affect the other copies.

Using these methods I never obtained a plasmidless strain. Due to the many strains of B. thuringiensis used in this study it was necessary to be able to identify a particular strain. Because of the easy loss of a variety of traits, some of which may be on plasmids or on the chromosome or may move between them, antibiograms were not particularly useful. Therefore the easiest method found for identifying strains was by plasmid patterns and a strain without plasmids would have been questioned as belonging to serotype 2. The plasmid− sporulation+ mutant from A. Aronson was a great help in this
regard, because before this strain was cured of its plasmids, cycloserine resistance was introduced. The resistance also added to the confusion about which antibiotic resistances are on plasmids. Cycloserine effects the cell membrane and there is a possibility that this particular mutation could alter the permeability of other drugs as well. This may, in part, account for the higher MIC's for 3b11 for certain drugs than other strains only missing a few plasmids (Table 18).

The assignment of ampicillin resistance and crystal production to particular plasmids is at this point tentative. Though the evidence from curing studies for crystal production being on plasmid pTX3b-5 is circumstantial, all spo - cry - cells which have pTX3b-5, also retain toxicity to T. ni larvae (H.T. Duimage pers. comm.). This plasmid is missing in many gels due to poor lysis or is obscured by the chromosome. Plasmid pTX3b-4 is a more interesting case. For the most part a correlation can be established, such that when this plasmid is missing the ampicillin resistance drops from 50ug/ml to less than 5ug/ml. The exceptions to this rule force an alternative hypothesis: transposition. If the ampicillin resistance gene were on a transposon, it might be expected to move to the chromosome. Then the plasmid could be lost but the ampicillin resistance retained. Genes of this type usually produce β-lactamases which act to modify the drug to a harmless derivative. All mutants which retain ampicillin resistance
and no longer have pTX3b-4 showed a lowered resistance to ampicillin that is about 1/4th that of the parent strain, 3b-1. But this is much higher than sensitive strains. This may be due to a gene dosage effect. These strains of intermediate resistance may have lowered resistance because the ampicillin resistance gene which was present in many copies on pTX3b-4 is now present in only one copy on the host chromosome.

Other characteristics seem to be lost in a random manner which may indicate that procedures used to cure plasmids are affecting the cells in other ways. Acriflavine which cures by intercalation is known to cause frameshifts and may nonspecifically cause chromosomal mutations. Heat, on the other hand, is believed to cause curing of plasmids by forcing the faster replication of the chromosome and faster cell division, thus "starving" nonessential plasmids for materials needed for replication. An alternative is that heat can induce lesions in DNA and an inefficient repair system may not repair plasmid lesions which leads to loss of plasmids because of failure to replicate past such lesions. The sensitivity of *B. thuringiensis* to UV light and sunlight may be indicative of an inefficient repair system. Or an increase in temperature may cause mistakes to be made in DNA replication due to some temperature sensitive enzyme(s) so that many types of mutations are actually caused.
Transformation.

With the possibility that many mutations are being introduced into cells during curing, a more direct approach to determine genes on plasmids is to attempt to do transformation. The technique of introducing circular DNA into protoplasts is successfully used in many systems (E. coli, Benzinger et al., 1971; B. subtilis, Chang and Cohen, 1979; Saccaromyces cerevisiae, Hinnen et al., 1978). Starting from the work of Chang and Cohen (1979), all experiments were done determining optimal conditions in penassay broth, until it was pointed out that there may be a media effect due to the sugar in the media which may be directly incorporated into the cell wall as seen in B. megaterium (B. Kolodziej, pers. comm.). The media effect was nothing short of dramatic. Of all the media used only nutrient agar and L-broth do not contain significant amounts of glucose. Even when penassay is made with fructose, protoplasting does not increase significantly. Growth in nutrient broth is much slower than in other media and cells harvested at the optimal cell density (0.6 at 660) seem to be entering stationary phase which could account for their failure to form protoplasts. B. thuringiensis produces pellicle when entering stationary phase which accounts for its higher tolerance to lysozyme (Angus, 1956).

While 3b-1 is particularly useful for studying the endotoxin, it is not an ideal host for transformation. It already has six plasmids of its own and the introduction of
more may lead to incompatibility (Datta. 1975) It is already resistant to many drugs which limits the choice of markers which could be used for such experiments. There are other strains which may be better suited to this type of genetic analysis. Strains 2-1, 4ab-1, 6-1, 10-1 and 12-1 appear to have only one or two plasmids and 6-1 in particular lacks most of the antibiotic resistance of the other strains and makes a non-toxic crystal. However as noted in Table 25 there is a considerable variation occurring in the efficiency of protoplasting in these strains, under the optimal conditions determined for 3b-1. It is evident that conditions for making protoplasts will differ among strains and these conditions will have to be empirically determined. One of the particular problems with 2-1, for example, is that it tends to grow in chains and the first action of the lysozyme is to break up the chains, rather than forming protoplasts.

Having a strain that does protoplast well and a plasmid (pC194) which differs in size from other plasmids of 3b-1 and carries a gene for chloramphenicol resistance (cam⁰), I attempted transformation as described in the Results using 17A. Transformation to cam⁰ was easily obtained and no mutation to cam⁰ of the parent strain was observed even when 10⁸ cells are directly plated on 8ug/ml cam. It was unusual that all stable transformants had no change in plasmid pattern. At this time it was suggested by D. Dubnau that pC194 could act as a transposon (pers. comm.). The resulting stability
of B. thuringiensis transformed with this S. aureus plasmid would then be due to its integration to a B. thuringiensis replicon. The assay for the chloramphenicol acetyltransferase activity proves that this gene has been transferred. The instability of many transformants may possibly mean that the original colony on chloramphenicol is a combination of plasmid$^+$ and plasmid$^-$ cells. Selection of a plasmid$^-$ cell for subcloning would lead to sensitivity. Or perhaps, as seen in c6, pC194 may be able to replicate autonomously for a short time under selection pressure, but will either be lost upon removal of the selection or transpose into the host genome as a preferred state. From these experiments it seems that transposition can and does occur in B. thuringiensis, and if stable clones are indicative of the rate of transposition, this rate of 2% is extremely high. In this system it may be the most energetically favored state.

The results from transformation of 3b-11 with plasmid DNA from 3b-1 may at first glance seem surprising, but on closer inspection there are many reasons for the near 100% transformation. The DNA transformed into 3b-11 was from the parent strain, 3b-1. This eliminates any problems with restriction of newly introduced DNA. Because 3b-11 is a derivative of 3b-1, chromosomal genes needed for maintenance and proper expression of these plasmids are already present. This plasmid, pTX3b-5 is also large ($30.1 \times 10^6$ daltons) as compared to pC194 ($2.0 \times 10^6$ daltons). Novick (in press) has
suggested that during regeneration of protoplasts, aberant cell divisions take place which preferentially cause the loss of small multi-copy plasmids that lack an obligatory segregation mechanism. However, large, single or low copy number plasmids often have such a segregation mechanism linked to that of the chromosome. In these aberant divisions pTX3b-5 would necessarily segregate with a viable cell whereas pC194 may be lost if segregated into a cell without a chromosome.

Since by molecular weight comparison pTX3b-5 is the only plasmid to be transfered in clones of cells, this conclusively shows that the gene(s) needed for the expression of the crystal are found on this plasmid. The most likely possibility is that this plasmid carries the gene(s) that are the structural components of the crystal in \textit{B. thuringiensis} var. kurstaki. This crystal is composed of a 120,000 dalton glycoprotein which is the $\delta$endotoxin (Bulla et al 1977). Associated with this crystal is a serine protease. Thus this particular crystal requires the presence of at least three genes. The first must code for the polypeptidetoxin itself, the second for the enzyme that adds the sugar moiety onto the protein and the third, the serine protease which may have a role in degradation of the crystal in a susceptible insect. Other than a structural role genes on this plasmid could be regulatory in nature capable of turning on specific genes, such as crystal production during sporulation.
While in 3b-1 the crystal gene(s) are plasmid borne, this can not explain all crystal production in \textit{B. thuringiensis}. For example, strain 2-1 of the variety finitimus makes a crystal, but has no plasmids. The single plasmid of 6-1 can also be lost without loss of crystal production. A hypothesis which would encompass both of these observations is transposition. If toxin genes were located on plasmids this might be a means for amplification on the toxin (in the two varieties mentioned, neither crystal is highly toxic: see Table 3). This would be an advantage to the bacteria in an environment where insects are abundant as some 1300 isolates have come from diseased insects (WHO, 1977). In the soil this seems to be a disadvantage as \textit{B. thuringiensis} is not found in the soil (Delucca et al, 1979). Plasmids are easily lost, but a gene could be retained if it transposed to the chromosome in an inactive form or very reduced in activity could explain the lack of recognizable \textit{B. thuringiensis} in soil samples. Transposition can occur in \textit{B. thuringiensis} as demonstrated with pC194 so it is possible with toxin genes, but not as easily demonstrated.

There are many applications of this work to the field of microbial control of insects. This is the first step to use the techniques of genetics to improve existing pathogens of lepidoptera and perhaps mosquitos. There are several directions this research could take. Because \textit{B. thuringiensis}
can express genes from other bacteria, it may be possible to introduce genes from such bacteria, for example, UV resistance from \textit{B. subtilis} to increase field persistence or perhaps toxins from \textit{B. spheracus} or \textit{B. thuringiensis var israelensis} so that a single strain of \textit{B. thuringiensis} is toxic to both moths and mosquitoes. Other possibilities include amplification of the toxin adding additional plasmids to degrade pesticides or even fix nitrogen.

In basic research \textit{B. thuringiensis} can be employed to study transposition, plasmid biology and the mechanism used to control the production of the \textit{endotoxin}, a peculiar specialized gene product.

There are many lifetimes of research in \textit{B. thuringiensis}, whether using classical molecular techniques or recombinant DNA technology or a combination of the two.
APPENDIX

American Optical, Buffalo, N.Y. - phase microscope.

American Sterilizer Co., Erie, Pa. - autoclave.

Ashland Chemical Co., Easton, Pa. - dextrose.

J.T. Baker Chemical Co., Phillipsburg, N.J. - maleic acid, NaCl, glacial acetic acid, HCl.

Baltimore Biological Laboratory, Cockeysville, Md. - typticase soy broth.


Becton-Dickinson Co., Rutherford, N.J. - 1cc tuberculin syringes.


Bristol Laboratories, Syracuse, N.Y. - mitomycin C.

CalBiochem, San Diego, Ca. - pronase B grade.

Chematron Corp., Columbus, Ohio. - liquid nitrogen.

Difco Laboratories, Detroit, Mich. - Bacto agar, Bacto typtone, yeast extract, brain heart infusion, casamino acids, peptone.

Eastman Chemical Co., Rochester, N.Y. - isopropanol.


GSA/Precision Scientific, Chicago, Ill. - Thelco incubators.

ICN K&K Laboratories, Inc., Cleveland, O. - sarkosyl.

Lab-line Instruments, Inc., Chicago, Ill. - water baths.

Laboratory Supplies Co. Inc., Hicksville, N.Y. - hot block.
Lehigh Valley Chemical Co., Easton Pa. - FeCl₃.

Eli Lilly & Co., Indianapolis, Ind. - glassine weighing paper.

Mallinckrodt, Inc., St. Louis Mo. MnCl₂, CaCl₂, MgCl₂, KCl, NaOH, sodium acetate, sucrose, K₂HPO₄, KH₂PO₄, phenol, chloroform.

Marine Colloids CDiv., Rockland Me. - agarose.

Millipore Corporation, Bedford, Mass. - 0.45μm filters.


Reliable Chemical Co., St. Louis Mo. - CsCl.

Sigma Chemical Co., St. Louis, Mo. - SDS, agarose deoxycholate, fructose, glycerophosphate, BSA DNTB, s-adenosyl Co A, Brig 58, lysostaphin T₁ RNase, pancreatic RNase.

Scientific Products, Obetz, O. - vortex, test tubes, petri dishes.

G. Frederick Smith Chemical Co., Columbus, O. - EDTA.

United States Biochemical Corp., Cleveland, O. - Tris.

Ultraviolet Products, Inc., San Gabriel, Ca. - Transilluminator.

Vitar, Japan. - red filter.
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