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Introduction and expression of the *Streptococcus faecalis* transposon Tn*916* into *Bacillus thuringiensis* subsp. *israelensis* and its use in comobilizing the *Staphylococcus aureus* resistance plasmid pC194

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Introduction and expression of the *Streptococcus faecalis* transposon Tn*916* into *Bacillus thuringiensis* subsp. *israelensis* and its use in comobilizing the *Staphylococcus aureus* resistance plasmid pC194

by

Joseph Gregory Naglich

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GENERAL INTRODUCTION

Objective

The regulation of the B. thuringiensis sporulation and protein toxin synthesis has been studied, but many aspects still remain incompletely understood. One way to investigate the regulation of spore and parasporal crystal formation in B. thuringiensis is to study mutants that are blocked in sporulation. Recently, Youngman et al. (1983) obtained such sporulation mutations in B. subtilis by utilizing transposon-mediated insertional mutagenesis. These investigators introduced a S. faecalis transposon, Tn917, into B. subtilis by transforming competent cells with the plasmid pAM-alpha-1::Tn917, and recovering insertions of Tn917 into many chromosomal sites. These insertional mutations then provided a means for studying sporulation in B. subtilis.

However, very little is known about the basic molecular biology of B. thuringiensis; especially when it is compared to other microorganisms such as E. coli or B. subtilis. By using the concept of transposon-mediated insertional mutagenesis in B. thuringiensis, information could be obtained concerning: (A) the process of transposition in B. thuringiensis; (B), the regulation of sporulation and paracrystal formation in B. thuringiensis; and (C), identification of the location of the genes

responsible for sporulation and parasporal crystal formation in the B. thuringiensis genome.

There are several problems to overcome before transposon-mediated insertional mutagenesis can be used in B. thuringiensis. Presently, there is no information available concerning the introduction of any transposon, Gram negative or Gram positive, into B. thuringiensis. Second, protoplast transformation has been reported in B. thuringiensis (primarily the lepidopteran subspecies), but the efficiency of transformation has been low and inconsistent. Therefore, before transposon-mediated insertional mutagenesis can be used in B. thuringiensis, there must be a means to consistently introduce the transposon into the cell and still have it function efficiently. The major objective of this study was to develop a procedure that would consistently introduce foreign DNA into B. thuringiensis. This methodology could then provide a means to study the basic molecular biology of B. thuringiensis.

Bacillus thuringiensis: An Introduction

Microorganisms pathogenic for insects, or entomopathogens, are varied and diverse. They include viruses, fungi, and bacteria. Each sub-group is composed of a spectrum of microorganisms that vary in their mode of infection, site of replication, and mechanism of

pathogenicity (Miller et al., 1983). While some of these groups exhibit a broad host range, others are more specific and are selectively pathogenic for either larval or adult life stages.

Interest in microbial insecticides is largely a result of the many problems associated with the extensive use of chemical pesticides. Not only do chemical pesticides affect beneficial insects as well as pest species, but insects tend to acquire resistance to the chemicals. Chemical residues also raise environmental and health concerns. Moreover, with the high cost of the new chemical insecticides, it becomes more apparent that pest control can no longer be solely dependent on the use of chemicals. Thus, microbial insecticides are seen as an alternate means of pest control that could play an important role in pest management.

Approximately 1500 naturally occurring microorganisms or microbial by-products have been identified as potentially useful insecticidal agents (Miller et al., 1983). Some of these microbial insecticides are commercially available today. Despite the number entomopathogens that have been proposed as insecticides, insecticides formulated with B. thuringiensis have achieved the most extensive use.

B. thuringiensis is a Gram-positive, rod-shaped, sporeforming, aerobic bacterium that produces a proteinaceous, insecticidal toxin (Andrews et al., 1987; Aronson et al., 1986; Bulla et al., 1980). B. thuringiensis was first described by Ishiwata, in 1901, as the causative agent for "sotto disease", a disease of the Japanese silkworm larvae (Bombyx mori). Since his discovery, a large number of subspecies of B. thuringiensis have been described. Most of these subspecies synthesize insecticidal toxins which vary in their insect specificity (Andrews et al., 1987; Aronson et al., 1986; Bulla et al., 1980), and some of these toxins are used commercially in many countries including the United States, Czechoslovakia, Yugoslavia, Germany and the Soviet Union (Andrews et al., 1987; Bulla et al., 1980).

Initially, B. thuringiensis insecticidal toxins were thought to be limited to lepidopteran insects (i.e., moths and butterflies), but more recently other strains of B. thuringiensis have been isolated. B. thuringiensis subspecies israelensis and kysusuensis have been shown to produce toxins specific for the dipteran insects (i.e., mosquitoes and blackflies) (Andrews et al., 1987; Aronson et al., 1986; Margalit and Dean, 1985; Undeen and Nagel, 1978). Moreover, a recently described isolate, B. thuringiensis subsp. tenebrionis, produces a toxin

specific for coleopteran larvae (i.e., beetles) (Aronson et al., 1986; Herrnstadt et al., 1986).

Taxonomy

It has been demonstrated, by using sequence homology, that B. thuringiensis is closely related to two other Bacillus species, B. cereus and B. anthracis (Andrews et al., 1987; Kaneko et al., 1978; Seki et al., 1978). Some B. cereus strains have been shown to share some flagella antigens with B. thuringiensis (Baumann et al., 1984). B. thuringiensis differs from B. cereus and B. anthracis because it synthesizes, in addition to the endospore, a characteristic parasporal inclusion body within the exosporangium (Bechtel and Bulla, 1976; Bulla et al., 1980). Therefore, the principal criterion used to classify an organism as B. thuringiensis rather than B. cereus is the presence of the entomocidal parasporal crystal (Andrews et al., 1987; Aronson et al., 1986; Baumann et al., 1984). The species B. thuringiensis can be divided into smaller groups based on plasmid profiles, crystal toxin type, phenotypic properties and flagellar antigen serotype. However, taxonomic classification of B. thuringiensis has been complicated because the various methods used to make these determinations seem to provide conflicting information (Andrews et al., 1987; Dean, 1984). Many factors may be responsible for these

conflicts, but the major factor is the ability of these organisms to exchange their genetic information with either members of B. thuringiensis, B. cereus or B. anthracis (Battisti et al., 1985; Dean, 1984; Gonzalez et al., 1982; Ruhfel et al., 1984). Although there are problems associated with the classification of B. thuringiensis based on flagellar serotypes, grouping subspecies based on flagellar antigen content is presently the primary classification method until a better method(s) is made available. These smaller groupings have been called either varieties or subspecies in the literature. Therefore, in order to be consistent with the International Code of Nomenclature of Bacteria (Lapage et al., 1975), the term subspecies (subsp.) will be used to denote subgroupings within the species B. thuringiensis.

Bacillus thuringiensis: Sporulation and Germination

The life cycle of B. thuringiensis, like that of other sporulating bacilli, is characterized by two distinct stages: vegetative cell division and sporulation (Andrews et al., 1987; Bulla et al., 1980). Vegetative cell division is characterized by the formation of division septa which are initiated along the plasma membrane at sites where membrane-like vesicles termed mesosomes, occur (Bechtel and Bulla, 1981; Bechtel and Bulla, 1976; Bulla et al., 1980). These division septa appear to be

extensions of the cell wall and cell membrane, and upon completion of cell division, the vesicles can be observed in the two identical daughter cells.

Sporulation Sporulation is considered to be a primitive form of differentiation because one daughter cell, the spore, is unique from the other, the mother cell, in both its biochemical constitution and morphological structure. Bechtel and Bulla (1976) performed a comprehensive, ultrastructural analysis of sporulation and parasporal crystal development in B. thuringiensis. Using the conventional sporulation nomenclature (Piggot and Coote, 1976), the investigators defined the sequence of B. thuringiensis subsp. kurstaki spore development as follows: Stage 0, was characterized by vegetative bacilli. The transition from vegetative growth to the first stage of sporulation (Stage I, 7 h) was characterized by the nucleoid becoming condensed into a compact and elongated axial filament. Immediately following Stage I, forespore septa began to develop and were recognized as invaginations of the plasma membrane at sub-polar regions of the cell (Stage II, 7-8 h). Once the forespore septum was completed, engulfment of the forespore began (Stage III, 8-9 h); this engulfment stage was distinguished by the junction of the forespore septum and plasma membrane that resulted in the detachment of the

septum from the cell membrane. Immediately following the detachment of the septum, several changes occurred (Stages IV to VI, 8-13 h); these included the development of the exosporium, primordial cell wall, cortex, and spore coat. Sporulation was then completed when the mature spore was released from the mother cell (Stage VIII).

Parasporal Crystal Formation One of the most striking features of sporulation in B. thuringiensis is the synthesis of the insecticidal parasporal crystal. The temporal relationship of its appearance to sporulation is fairly consistent among the different strains and isolates of B. thuringiensis as is its site of synthesis within the cell (Andrews et al., 1987; Tyrell et al., 1981). For example, B. thuringiensis subsp. kurstaki synthesizes parasporal crystalline inclusion bodies only from phases III to VI of sporulation (Tyrell et al., 1981). In some strains, more than one crystal can develop within a cell (Bechtel and Bulla, 1976). As the crystal develops, an ovoid inclusion appears, but more than one such inclusion has not been observed in a sporangium (Bechtel and Bulla, 1976). The ovoid inclusion has never been observed to exhibit crystal lattice fringes as does the parasporal crystal, but whether this ovoid inclusion is toxic to insect larvae remains to be known (Andrews et al., 1987; Bechtel and Bulla, 1976).

Bacterial Germination During spore germination, simultaneous structural and physiologic changes are observed (Bechtel and Bulla, 1976; Bulla et al., 1980). The process of spore germination consists of three sequential phases: Phase 1, is an activation phase in which the spore is conditioned to germinate in a suitable environment; Phase 2, is the germination phase in which the characteristic properties of the dormant spore are lost, and Phase 3, is the outgrowth phase in which the spore is converted into a new vegetative cell. Activation can be achieved by exposure of a dormant spore to sublethal heat treatment in an appropriate medium, or to low pH, thiol compounds, or strong oxidizing agents. The simplest method to accomplish a state of activation in B. thuringiensis seems to be heat shock (Bechtel and Bulla, 1976; Bulla et al., 1980).

Germination is an irreversible sequence of events triggered by the exposure of activated spores to specific stimulants such as amino acids, nucleosides, or glucose (Bechtel and Bulla, 1976; Bulla et al., 1980). There are also lytic enzymes or physical initiators that can facilitate the release of a protoplast as a new vegetative cell. During the germination process, a major part of the spore's dry weight is lost and this material includes the exosporangium, cortex and cortical substances like

proteins, peptides, or calcium dipicolinate (Bechtel and Bulla, 1976; Bulla et al., 1980).

Bechtel and Bulla (1976) and Bulla et al. (1980) performed extensive studies on spore germination. During the early stages of germination (Stages I and II), there was a loss of refractility because more than 95% of the spores appeared dark under phase-contrast microscopy. Stage II, was characterized by the appearance of fine nuclear fibrils, cytoplasm, and swelling of the cortex. Stages III to V were considered to be the outgrowth stages because the germinated spore began to swell and develop into a vegetative cell. During these outgrowth stages, the protoplast's surface area increased about three and a half times over that of a dormant spore. The protoplast began to elongate, and mesosomes, typical of vegetative cells, began to appear. Finally, vegetative bacilli emerged shedding their outer fibrous coat, exosporium, and lamellar spore coats. Stage VI was considered to be the first cell division characterized by the formation of division septa.

Biochemistry of Sporulation

The biochemical events during sporulation of a bacterial cell take place in a highly ordered well-defined sequence. The morphological sequence of events leading to spore formation is well understood, but the biochemical

and genetic events are not (Hoch, 1976; Mandelstam, 1976; Piggot and Coote, 1976). At some point in the development of a cell, the metabolism is irreversibly channeled in the direction of sporulation. There is no single point of commitment for the sporulation process to occur, but rather, a series of commitments. The biochemical activities that appear during sporulation may not be necessary or specific for the process. These activities include: (i) the appearance of specific components that are only observed in sporulating cells and not in vegetatively growing cells; examples include the presence of an unique peptidoglycan layer in the cortex, or the proteins in the spore coat. (ii) changes in vegetative cellular functions that are directed towards spore formation; i.e., an increase in the enzymatic activity of the tricarboxylic acid cycle. (iii) the occurrence of secondary metabolic products, e.g., antibiotics and proteases. These secondary metabolic products, in themselves, do not play a direct role in sporulation, but they do provide biochemical markers which can be used to analyze sporulation mutants (Hoch, 1976; Mandelstam, 1976; Piggot and Coote, 1976).

Bacillus thuringiensis: Biochemistry of the Parasporal Crystal

There are a number of subspecies of B. thuringiensis, most of which synthesize insecticidal toxins with some variation in insect specificity. When lepidopteran toxic crystal proteins are compared to dipteran toxic crystal proteins, several differences can be noted: (i) The most obvious difference is their insect specificity. The insecticidal toxins produced by B. thuringiensis subsp. kurstaki, tolworthi, alesti, or berliner are toxic towards lepidopteran insects (tobacco hornworm, tobacco budworm, cabbage looper, or gypsy moths) whereas, those crystals produced by B. thuringiensis subsp. israelensis and kysusuensis are toxic towards dipteran insects (mosquitoes and black flies) (Andrews et al., 1987; Aronson et al., 1986). Moreover, Tyrell et al. (1981) have shown that the B. thuringiensis subsp. israelensis crystals were lethally toxic to mosquito larvae and nontoxic towards tobacco hornworm larvae; the converse was also true, i.e., crystals from the lepidopteran subspecies killed tobacco hornworm larvae but were ineffective against mosquitoes larvae. (ii) The crystal morphology between the toxins differs because the majority of lepidopteran toxic crystals are bipyramidal-shaped, whereas the crystals synthesized by B. thuringiensis subsp. israelensis and B.

thuringiensis subsp. kysusuensis are ovoid (Bulla et al., 1980; Lee et al., 1985; Tyrell et al., 1981). (iii) Upon solubilization, the B. thuringiensis subsp. israelensis toxin lyses erythrocytes and cultured mammalian cells as well as cultured insect cells (Gill et al., 1987; Thomas and Ellar, 1983), whereas solubilized lepidoptera-active toxin display no corresponding cytotoxicity. (iv) The disulfide bonds of B. thuringiensis subsp. israelensis may be reduced and blocked without loss of toxicity (Pfannenstiel et al., 1985), whereas similar modification of the B. thuringiensis subsp. kurstaki HD-1 crystals resulted in a 160-fold reduction in toxicity (Schesser et al., 1977).

There has been conflicting information about the immunological cross-reactivity between the antigens found on the dipteran and lepidopteran toxic polypeptides. Wie et al. (1984) were able to show, by using polyclonal antisera, and enzyme-linked immunosorbent assays, that the dipteran crystal toxin produced by B. thuringiensis subsp. israelensis shared a few antigens with the lepidopteran toxin from B. thuringiensis subsp. kurstaki, tolworthi, berliner, and alesti. However, monoclonal antiserum specific for certain B. thuringiensis subsp. israelensis toxic antigens did not recognize the B. thuringiensis subsp. kurstaki HD-1 toxin (Armstrong et al., 1985;

Krywienczyk and Fast, 1980). This tends to suggest that the toxins contained distinct and similar antigenic components, but it is important to realize that different purification protocols were used. Hence, it is possible for the crystal toxin preparations to contain antigens other than the toxins, and this should be kept in mind when evaluating the data.

The major component of the native parasporal crystals is a glycoprotein which represents 20 to 30% of the cell's dry weight (Bulla et al., 1981; Dean, 1984). The crystal is primarily composed of protein with only five percent being of carbohydrate nature (about 1.2% mannose and 3.8% glucose). The structure of these individual carbohydrate side chains and their specific attachment site(s) is not known (Tyrell et al., 1981). In vitro, these crystals can be solubilized in mild alkaline solutions, which preserves the toxic activity, or in denaturing solvents, which destroys the insecticidal activity (Bulla et al., 1981; Lee et al., 1985; Tyrell et al., 1981). When a susceptible insect ingests the crystals, they are in a protoxic form, and the protoxin is solubilized by the combined action the gut proteases and gut alkaline pH to generate a toxic peptide (Bulla et al., 1981; Tyrell et al., 1981).

Lepidopteran Toxin The mechanism(s) of action of the toxic lepidopteran peptide is not understood. Electron microscopy of midgut epithelium and studies of the effect(s) of the the toxin on the permeability of cultured insect cell lines indicate a general cytolytic effect (Andrews et al., 1987). Scanning electron micrographs of tobacco hornworm larvae show that within one hour after per os administration of crystal toxin, the microvilli in the midgut become shorter, and by four hours, extensive damage to the midgut has already occurred.

Accompanying the histological changes in the insect's midgut are changes in the physiology of the larvae. There is a severe restriction of potassium transport and this transport is thought to be responsible for the maintenance of the high midgut pH (Andrews et al., 1987). However, Fast and Donaghue (1971) have shown that within one minute of ingestion of the B. thuringiensis toxin, there is a dramatic decrease in the cellular ATP levels in midgut epithelial cells with a corresponding increase in the uptake of glucose. Travers et al. (1976) have shown in isolated midgut cells, that solubilized crystal proteins apparently uncouple mitochondrial oxidative phosphorylation. However, English and Cantley (1985) have demonstrated that the B. thuringiensis subsp. kurstaki

toxin inhibited the potassium dependent vanadate sensitive ATPase from Manduca sexta. This inhibition, they suggested, reduced the potassium concentration in the midgut epithelial cells, and increased the potassium ion concentration in the insect hemolymph.

Dipteran Toxin The tissue most affected by the insecticidal diptern toxin is the midgut epithelium (Lacey and Federici, 1979; Tyrell et al., 1979). The toxin of B. thuringiensis subsp. israelensis has been shown to affect sensitive larvae in a manner very similar to that observed in Lepidoptera. This toxin induces a cessation of feeding, followed by paralysis of the larval midgut. Due to the technical problems associated with working with small mosquito larvae, the histological characterization of the effects of B. thuringiensis have been limited.

The crystal toxin of B. thuringiensis subsp. israelensis is made up of several protein components (Lee et al., 1985; Tyrell et al., 1981). The crystals formed by most lepidopteran toxic strains contain a single, bipyramidal crystalline body whereas subsp. israelensis contains a number of crystals which vary in size. Hurley et al. (1985; 1987) have reported that the cytolytic activity and the mosquitocidal activity could be separated by gel filtration chromatography; an almost pure protein migrating at M_R = 68,000 was highly mosquitocidal but not

cytolytic, whereas a smaller protein M_R = 28,000 was cytolytic but relatively nontoxic towards mosquitoes. Hurley et al. (1987) have further shown that mixtures of the two purified proteins were significantly more toxic to mosquito larvae than either protein alone. Therefore, these investigators suggested that it was likely that both the mosquitocidal and the cytolytic protein play a role in the overall insecticidal action of this B. thuringiensis parasporal crystal. Finally, the toxic activity of B. thuringiensis is restricted to a very limited group of insects, whereas the cytolytic activity has been observed with several different insect and mammalian cells. Therefore, it is difficult to ascribe a specific toxic activity to such a protein.

Genetics of Bacillus Endospore Formation

Most of the details of the genetics of endospore formation have been obtained from studies on B. subtilis (Hoch, 1976; Losick et al., 1986; Piggot and Coote, 1976; Piggot and Hoch, 1985). By utilizing transformation and transduction methodology, a linkage map of this organism has been constructed. It has been demonstrated by these studies, that the sporulation loci are not confined to one segment of the genome, but rather they are distinctly clustered and widely dispersed around the B. subtilis chromosome. Expression of these gene products require a

highly regulated, complex sequence of events (Hoch, 1976; Losick et al., 1986; Piggot and Coote, 1976; Piggot and Hoch, 1985). While a few of the gene products encoded by these loci have been identified, it has been shown that some of these gene products function as either regulatory components of the process, structural constituents of the spore, or spore-specific products.

Genes whose products are specifically required for endospore formation in B. subtilis are called spo genes (Hoch, 1976; Losick et al., 1986; Piggot and Coote, 1976; Piggot and Hoch, 1985). These genes have been identified by mutations which impair spore development, but have little or no effect on vegetative growth. Hundreds of these spo mutations have been isolated and grouped into more than 500 genetic loci based on their position on the B. subtilis chromosome and the stage at which they interfere with spore development (Hoch, 1976; Losick et al., 1986; Piggot and Coote, 1976; Piggot and Hoch, 1985). For example, mutations spo0 loci prevent the formation of the polar septum that partitions the sporangium into mother cell and forespore compartments. There have also been mutations isolated that block the later stages of sporulation (from stages II to VI), germination (ger), and outgrowth (out).

In the first stages of sporulation, nutrient-deprived microorganisms cease normal cell division and form polar septa which partition the sporulating cells into mother-cell and forespore compartments (Losick et al., 1986; Piggot and Coote, 1976). At least ten loci are involved in the initation of this developmental process (Losick et al., 1986). A mutation in any one of these spo genes blocks spore formation prior to completion of any of the morphological changes characteristic of early spore development. Although several spo cistrons have been isolated by gene cloning methods little is known about the nature and function of their products.

Genetics of Sporulation and Parasporal Crystal Development in Bacillus thuringiensis

The synthesis of the crystalline toxins in B. thuringiensis is a sporulation specific event (Andrews et al., 1987; Tyrell et al., 1981). It has been demonstrated that the crystals and spores appear almost simultaneously. Not only do the times of the appearance of the spore and crystal overlap, but they are formed in close proximity. Moreover, the crystal toxin is a spore coat protein because large quantities of the protoxin are found in the spore coat (Aronson et al., 1982). Further, in other sporeforming bacteria, coat synthesis is a sporulation-specific event (Aronson and Fitz-James, 1976).

Tyrell et al. (1981) demonstrated in B. thuringiensis subsp. kurstaki that maximum synthesis of the crystal toxin occurred between stages III and VI of sporulation. Moreover, cells in exponential growth phase or in early stages of sporulation did not contain the crystal toxin antigen and were nontoxic. B. thuringiensis parasporal crystals have been shown in other sporeforming bacteria to be synthesized during sporulation (to be discussed in a later section).

Functions of Bacillus thuringiensis Plasmids

Since B. thuringiensis initial discovery many important aspects of the expression, synthesis, and mechanism of action of the protein toxin have been studied, but these still remain incompletely understood (Gonzalez et al., 1981; Gonzalez and Carlton, 1980, 1984). Strains that normally produce the crystal toxin can readily lose the ability to form the parasporal crystals and hence, the insecticidal activity is lost. Reversion to a crystal-producing, insecticidal phenotype occurs rarely, if at all (Gonzalez et al., 1981; Gonzalez and Carlton, 1980). This irreversible loss of crystal toxin synthesis has suggested a possible plasmid-mediated inheritance for this property, and several reports on the presence of extrachromosomal DNA in B. thuringiensis have been published (Faust et al., 1983; Gonzalez et al., 1981;

Gonzalez and Carlton, 1980, 1984; Stahly et al., 1978). Subspecies of B. thuringiensis typically have unique complex arrays of extrachromosomal DNA. Many subspecies have up to 11 or 12 plasmids per cell which range in size from 1.4 to approximately 150 megadaltons (Gonzalez et al., 1981; Whiteley and Schnepf, 1986). More importantly, it has been demonstrated that special care is required to extract the very large plasmids (Gonzalez et al., 1981). Further, most B. thuringiensis plasmids can be cured spontaneously, by growth at elevated temperatures, or by growth in the presence of sodium dodecyl sulfate (Chapman et al., 1985).

Stahly et al. (1978) were the first to attempt to correlate the presence of plasmids with crystal production. They isolated spore producing, crystal non-producing B. thuringiensis mutants and made the important observation that these mutants lost all six plasmids that were detected in the wild type subsp. kurstaki HD-1. This observation implied that one or more of the plasmids were involved in crystal production. Later, Gonzalez et al. (1981) determined the relationship between crystal toxin production and plasmid content in several different B. thuringiensis subspecies: e.g., kurstaki, thuringiensis, alesti, and galleriae. They demonstrated a correlation between the presence of a specific plasmid and crystal

production. Further, the specific plasmids responsible for crystal toxin synthesis were of different sizes in each strain and the other plasmids seen in these strains could be lost without any effect on crystal production (Gonzalez et al., 1981; Gonzalez and Carlton, 1980). These analysis though, could not determine whether the specific plasmid encoded for the structural genes of the parasporal toxin or for a trans-regulatory component.

Faust et al. (1983) attempted to correlate toxin production with the presence of a specific plasmid in B. thuringiensis subsp. israelensis. They utilized crystal producing (Cry+) and crystal-nonproducing (Cry-) mutants, either induced or spontaneously derived from a Cry+ parent; these were assayed for the presence of covalently closed circular DNA. All of the Cry+ strains were shown to contain plasmid DNAs in the molecular weight range of 4.0 to 13.0 megadaltons. All of the Cry- mutants derived from the Cry+ parent were shown to lose a 4.0 - 4.4 megadalton plasmid, and bioassay data confirmed the loss of toxin production in the Cry- variants. Moreover, the plasmid patterns for several of the Cry- derivatives were shown to be different than those seen in parental strains. The investigators concluded that the loss of a specific plasmid was correlated with the simultaneous loss of crystal toxin formation, but in order to obtain strict

proof, the missing plasmid would have to be reintroduced into the Cry- strain and the Cry+ phenotype be restored. However, these studies would not show whether the plasmids carried the actual toxin gene(s) or, regulatory genes controlling the expression of chromosomal toxin genes.

Gonzalez et al. (1982) described a natural plasmid transfer system which allowed them to analyze more extensively the relationship between crystal production and plasmid content. They noticed high frequency transfer of plasmids during logarithmic growth between Cry+ strains and Cry- strains. For instance, when B. thuringiensis subsp. kurstaki Cry- recipients were mated with Cry+ B. thuringiensis subsp. thuringiensis donors, the B. thuringiensis subsp. kurstaki strains were converted to Cry+. The size of the transmissible crystal-coding plasmid varied with the donor strain and immunological analysis showed the B. thuringiensis subsp. kurstaki Cry+ transcipients to be hybrid strains, i.e., having flagella sereotype of the recipient and crystals of the donor serotype. These results demonstrated that the structural genes for the crystal toxin were plasmid related. In another series of experiments from the same study, the investigators were able to demonstrate the transfer of the crystal-coding plasmids into two related strains of Bacillus cereus. The resulting transcipients were again

of hybrid nature.

Gonzalez and Carlton (1984), utilizing their high-frequency plasmid transfer system of B. thuringiensis, genetically analyzed crystal toxin production in B. thuringiensis subsp. israelensis. They showed that B. thuringiensis subsp. israelensis contained a complex array of plasmids, eight in all, ranging in size from 3.3 to 135 megadaltons. Moreover, when these plasmids were cured from B. thuringiensis subsp. israelensis, they were able to show that a 75 megadalton plasmid was responsible for toxin production because fifteen B. thuringiensis subsp. israelensis Cry- isolates were shown to lack this 75 megadalton plasmid. After utilizing their high-frequency plasmid transfer system between Cry+ and Cry- B. thuringiensis, these investigators demonstrated that only the transfer of the 75 megadalton plasmid was required to convert the Cry- recipient to crystal toxin production.

Cloning of the B. thuringiensis lepidopteran toxins

The above data indirectly suggests that the structural and regulatory genes for the crystal toxin were plasmid related, but these data do not directly distinguish if the loss of the crystal producing ability in B. thuringiensis was due to the loss of a plasmid structural gene or to the loss of a plasmid-encoded regulatory gene. Therefore, another approach was to locate the crystal gene, clone it,

express the cloned gene in another species, and characterize the gene produces according to their physical and immunological properties.

Klier et al. (1983) took advantage of the high frequency transfer of plasmids between strains of B. thuringiensis to perform interspecies matings between B. thuringiensis and B. subtilis. A plasmid pBT 42-1 was constructed by ligating a BamHl fragment derived from the 42 megadalton plasmid of B. thuringiensis subsp. berliner 1715 to the bifunctional cloning vector pHV33. The pBT 42-1 plasmid was transformed into either B. subtilis or E. coli. The resulting transformants synthesized the B. thuringiensis subsp. berliner 1715 crystal toxin. This demonstrated that the plasmid pBT 42-1 carried the information necessary for the production of parasporal crystals. The pBT 42-1 plasmid was then transferred from B. subtilis to either B. thuringiensis subsp. kurstaki or B. thuringiensis subsp. israelensis utilizing the methodology of Gonzalez et al (1982). The investigators observed a lower frequency of plasmid transfer, and they attributed it to B. thuringiensis and B. subtilis not being closely related. When the pBT 42-1 plasmid was transferred into a B. thuringiensis subsp. kurstaki Cry- recipient, it was converted to a Cry+ phenotype where it produced a lepidopteran protein toxin similar,

immunologically and biologically, to purified berliner 1715 crystals. Moreover, when the pBT 42-1 plasmid was introduced into B. thuringiensis subsp. israelensis, the transcipients produced both the dipteran and lepidopteran crystal toxins which were biologically active.

Schnepf and Whiteley (1981) have successfully cloned and expressed a B. thuringiensis subsp. kurstaki crystal protein gene in E. coli. B. thuringiensis subsp. kurstaki HD-1 plasmid DNA was partially digested by the action of Sau3Al. The DNA was ligated into the BamHl site of the E. coli cloning vector pBR322, and used to transform E. coli cells. The resultant transformants were screened for the production of the crystal toxin by utilizing immune sera directed against the intact parasporal crystals. One transformant ES 12, was isolated using this methodology, and it contained a 16.5 Kb plasmid. DNA sequence homology existed between the plasmid isolated from ES 12 and pBR322 as well as two plasmids contained within the B. thuringiensis subsp. kurstaki genome, each of which had a molecular weight of 45 and 71 Kb, respectively. ES 12 synthesized only small amounts of the crystal protein compared to that synthesized by B. thuringiensis subsp. kurstaki, and these investigators suggested that the B. thuringiensis subsp. kurstaki may regulate the production of the crystal toxin differently than E. coli. The

protein produced by ES 12 had the same electrophoretic mobility as purified B. thuringiensis subsp. kurstaki crystal protein, and protein extracts of ES 12 were biologically active against the tobacco hornworm larvae, Manduca sexta.

A crystal protein gene from subsp. kurstaki strain HD-1 was cloned by Held et al. (1982). Total B. thuringiensis subsp. kurstaki DNA was partially digested with EcoRI, and the fragments were cloned into the bacteriophage vector Charon 4A. E. coli cells were then infected with Charon 4A recombinants and the resulting recombinants were assayed for the presence of the protoxin by radioimmunoassay which utilized antibody directed against the B. thuringiensis subsp. kurstaki protoxin. One of these recombinants, C4K6c, produced a protein antigen that was the same molecular weight as the purfied B. thuringiensis subsp. kurstaki crystal toxin, and it was shown to be lethal to M. sexta. Using the recombinant phage as a probe, homologous sequences were found on a 4.6-kb EcoRI fragment derived from a 45 Kb plasmid. Since the C4K6c DNA hybridized with 45 Kb plasmid, these investigators suggest that the protoxin gene was contained within this plasmid. The C4K6c DNA was then cut with EcoRI, ligated to cloning vectors pHV33 and pBR328, and transformed into either B. subtilis or E. coli. The

resultant transformants were screened, and their DNA assayed for hybridization towards B. thuringiensis subsp. kurstaki plasmids. Several transformants were found to contain a 4.6 Kb pair insert and several produced the protein toxin. When the 4.6 Kb EcoRI fragment was used as a probe, hybridization was found to both plasmid DNA and chromosomal DNA. Interestingly, plasmidless mutants of the parent strain did not produce crystals, suggesting that the chromosomal gene was "silent", although it could be expressed when cloned on a phage or plasmid vector. Held et al. (1982) suggested that a plasmid-encoded gene may regulate the synthesis of the toxin protein.

The protoxin genes from several lepidopteran specific B. thuringiensis subspecies have been isolated using different gene identification techniques and vector cloning systems. In most cases, the gene products from these clones were confirmed by their reactivity with antibodies raised against B. thuringiensis crystal proteins and by insecticidal activity to lepidopteran hosts. Further, it has been revealed that there are multiple heterogenous copies of the protoxin gene in some of the subspecies, but the meaning of this gene diversity is not yet fully understood.

Cloning of the B. thuringiensis dipteran toxins

The work discussed so far has concerned B. thuringiensis subspecies toxic towards lepidopteran insects. The majority of parasporal crystals produced by lepidopteran subspecies are primarily composed of a single polypeptide (130,000 to 160,000) (Tyrell et al., 1981). In contrast, the B. thuringiensis subsp. israelensis produces crystals that are composed of several proteins ranging in size from 26,000 to 135,000 (Tyrell et al., 1981). Hurley et al. (1985) reported that the cytolytic activity and the mosquitocidal activity could be separated by using gel filtration chromatography. An almost pure protein migrating at M_R = 68,000 was highly mosquitocidal, but not cytolytic whereas, a smaller protein M_R = 28,000 was cytolytic, but relatively nontoxic. Armstrong et al. (1985) described a M_R = 25,000 protein which was thought to be a proteolytic cleavage product of the M_R = 28,000 protein, and to be primarily responsible for the hemolytic activity of B. thuringiensis subsp. israelensis. Other crystal proteins, M_R = 130,000 and M_R = 230,000, are noncytolytic and have been shown to contribute to the insecticidal action of B. thuringiensis subsp. israelensis. It is thought that these noncytolytic toxins act synergistically with the M_R = 25,000 protein (Gill et al., 1987).

In order to determine which protein was the protoxin, Ward et al. (1984) constructed recombinant plasmids containing the subsp. israelensis crystal toxin gene by inserting the HindIII fragments of the B. thuringiensis subsp. israelensis 75 Mdal plasmid into the E. coli vector pUC12. Two clones (pIP173 and pIP174) were recovered using an E. coli in vitro transcription-translation system, and these clones produced a 26,000 protein which was precipitable by immune sera directed against the native subsp. israelensis 26,000 protein. Moreover, E. coli cells harboring pIP174 were lethal to mosquito larvae and cytotoxic to mosquito cells in vitro. The biological activity of this protein was further confirmed by its neutralization with immune sera. These investigators did not assay their clone for biological activity against other insect and mammalian cells. Because several workers have observed a potent nonspecific activity associated with the B. thuringiensis M_R = 26,000 protein, these data suggest that this cloned gene was the cytolysin gene and not the protoxin gene.

Using a similar approach to that of Ward et al. (1984), Waalwijk et al. (1985) cloned a subsp. israelensis HindIII crystal gene fragment into pBR322 to generate p425. This clone produced in vitro, a M_R = 28,000 protein which was immunoprecipitable with sera directed against

the native B. thuringiensis protein. By hybridizing p425 DNA with B. thuringiensis subsp. israelensis RNA isolated at different stages of growth, these investigators were able to demonstrate that transcription of this cloned gene was restricted to early stages of sporulation. However, there was limited expression of p425 in E. coli, and it was thought that B. thuringiensis subsp. israelensis promoters were poorly recognized by E. coli RNA polymerases. The biological activity of this clone was not reported, and it is not known whether it is active against mosquito larvae.

To determine whether the expression of the B. thuringiensis subsp. israelensis protoxin was sporulation specific, Sekar and Carlton (1985) developed a vector shuttle system using protoplast transformation and a B. cereus tetracycline resistance plasmid, pBC16. After transforming B. megaterium, one clone, VB131, was isolated and it produced crystals during sporulation. Solubilized crystals obtained from this clone were toxic to mosquito larvae and were immunoprecipitable with sera directed against the native, solubilized B. thuringiensis crystals. The molecular weight(s) of the cloned protein(s) was not determined so it was not known which protein(s) was toxic. However, in a subsequent study, Sekar (1986) using VB131 determined immunologically that the crystal toxin of the

recombinant strain contained only the 130,000 protein and not the 68,000 or 28,000 proteins of the crystal toxin. Moreover, nucleotide sequencing analysis of this cloned B. thuringiensis gene identified only one open reading frame corresponding to the 130,000 peptide. It was concluded that the 130,000 protein was primarily responsible for the mosquitocidal activity, but neither the stability of this protein, or its immunological activity after protease digestion were assayed.

McLean and Whiteley (1987) cloned a ca. 10 Kb HindIII fragment of plasmid DNA from B. thuringiensis subsp. israelensis into the E. coli vector pUC9. Extracts of the recombinant strain were toxic to mosquito larvae, and contained a M_r = 27,000 hemolytically active protein that reacted with antibodies directed against a 27,000 protein isolated from B. thuringiensis subsp. israelensis crystals. Analysis of maxicells demonstrated that the 10 Kb insert also coded for 67,000, 20,000 and 16,000 proteins. Therefore, these investigators were careful to point out that it was still possible that intact crystals may contain one or more additional toxic peptides, or that several peptides may act synergistically. More importantly, full expression of this 27,000 protein required the presence of a ca. 0.8 Kb region of DNA located 4 Kb upstream from the structural gene. This 0.8

Kb region could be present in *cis* or *trans* relative to the gene and apparently acted post-transcriptionally. It was also suggested that the 20,000 protein was encoded by the 0.8 Kb DNA region.

The *B. thuringiensis* subsp. *israelensis* protoxin has not been identified. It is clear that the *B. thuringiensis* subsp. *israelensis* toxin contains several different proteins. It is possible that the cloned 130,000 protein (Sekar and Carlton, 1985) from *B. megaterium* is the protoxin, but the protein needs to be assayed for its immunological and biological activity after being treated with various proteases. The M_R = 25,000 to 28,000 hemolytic proteins appear to enhance the insecticidal activity of the 65,000 to 68,000 toxic proteins. Overall, many important aspects of the synthesis, expression, and mode of action of the crystal toxin need to be determined.

Cross-Homologies and IS-like Sequences

Southern hybridization has revealed some homology among the plasmids isolated from different subspecies (Lereclus *et al.*, 1982). For instance, a small plasmid from *B. thuringiensis* subsp. *berliner* 1715 contained no DNA sequence homology with the chromosome, but exclusively hybridized to the smaller plasmids of other subspecies (Lereclus *et al.*, 1982). Alternatively, the larger

plasmids from B. thuringiensis subsp. dendrolimus (50 Kb) and B. thuringiensis subsp. subtoxicus (78 and 84 Kb) did not hybridize with any small plasmids, but rather to the chromosome and other large plasmids (Lereclus et al., 1982).

Lereclus et al. (1983) described an unique 4.2 Kb DNA sequence that was found on several large B. thuringiensis toxin-coding plasmids. This particular sequence (Th-sequence) was found as an insertion in the S. faecalis plasmid, pAM-beta-1, which was introduced into B. thuringiensis subsp. berliner 1715. It resembled an IS-like element, and was found in close proximity to the protoxin genes from several B. thuringiensis subspecies. A 1.3 Mdal segment of DNA separates the Th-sequence from the berliner 1715 protoxin gene and a similar DNA segment is located on the other extremity of the gene but in the opposite orientation. These DNA segments contain inverted repeated sequences and are referred to as IRls. When a plasmid carrying the gene, IRls and the Th-sequence was observed with an electron microscope, it appeared as a transposable element (Lereclus et al., 1984). Therefore, the Th-sequence is now referred to as Tn4430, and it is the first transposon isolated from the genus Bacillus (Lereclus et al., 1986). This DNA segment is similar to the Tn3 family in structure and transpositional activity

(Lereclus et al., 1986). In addition to its insertion properties, Tn4430 has short terminal inverted repeats and promotes deletions adjacent to its insertion site similar to that observed with Class II elements (Lereclus et al., 1984, 1986). Nonetheless, the function of this transposon has not been identified.

Similar inverted sequences, IR1750 and IR2150, flanking the 68 Kb plasmid-encoded protoxin gene of subsp. kurstaki HD-73 have been identified (Kronstad and Whiteley, 1984). Southern hybridization confirmed that these sequences were located in close proximity to the protoxin genes from fourteen other strains of B. thuringiensis, and it was thought that IR1750 and IR2150 may be insertion sequences that mediate transposition.

Nucleotide sequencing of one IR1 element revealed all the characteristic features of an insertion sequence and it was referred to as IS231 (Mahillon et al., 1985). IS231 [1656 base pair (bp)] was isolated from subsp. berliner 1715 and found to be closely linked to the protoxin gene. It is delineated by two 20 bp inverted repeats that are flanked by two 11 bp direct repeats. IS231 contains an open reading frame that spans almost the entire sequence, and DNA sequence homology has been observed between IS231 and the E. coli IS4 element.

Mahillon et al. (1987) sequenced two other insertion

sequences flanking the *berliner* 1715 crystal toxin gene and showed them to be variants of IS*231*. These sequences were referred to as iso-231 elements. When the nucleotide sequences surrounding the iso-231 elements were compared to IS*231*, it was shown that a structural association existed between these elements and the transposon Tn*4430*. It appeared that two IS*231* elements transposed into Tn*4430* where both the IS*231*s and the transposon Tn*4430* remained structurally intact. This structural association between IS*231* and Tn*4430* is similar to the organization of class I mobile genetic elements (i.e., Tn*5* or Tn*10*), and although the exact function of the IS*231* elements is unknown, it is thought that they control Tn*4430* transposition (Mahillon *et al.*, 1987).

In summary, the presence of repeated elements around crystal protein genes may provide two possible mechanisms for the dispersal of the toxin gene on different plasmids, and for the integration of this gene into chromosomal DNA. The evidence for transposition of the Th-sequence into other plasmids, and the arrangement of the repeated elements suggest that the toxin gene could undergo transposition. Alternatively, the presence of repeated elements on many different plasmids and on chromosomal DNA would also provide a mechanism for rearrangements of DNA. Therefore, additional genes must be analyzed for the

organization of repeated elements in order to understand the function(s) of these sequences.

Gram Positive Transposons

Streptococcus faecalis transposon Tn916 The conjugative plasmids of the genus Streptococcus can be placed into two general categories. Members of one group, transfer at a relatively high frequency (10^{-1} to 10^{-3} per donor) in broth, but transfer has only been demonstrated to occur between S. faecalis cells (Dunny et al., 1978, 1979). This high-efficiency transfer in broth is due to the presence of sex-pheromones which generate cell-to-cell contact (Dunny et al., 1978, 1979). The other group is represented by certain MLS plasmids (conferring resistance to macrolides, lincosamides and streptogramin B antibiotics) which transfer poorly in broth (usually less than 10^{-6} per donor), but fairly efficiently (10^{-4} to 10^{-2} per donor) when the matings are only carried out on filter membranes; transfer of this group of plasmids occurs across a wide range of streptococcal and other species (Clewell, 1981).

When multiple drug resistance appeared in clinical isolates of S. pneumoniae a number of investigators revealed that related R plasmids were not present (Buu-Hoi and Horodniceanu, 1980; Shoemaker et al., 1979; Smith and Guild, 1979). However, they observed that these

resistance determinants could be transferred to recipient strains on membrane filters by a conjugation-like process resistant to DNase (Buu-Hoi and Horodniceanu, 1980; Shoemaker et al., 1980). Moreover, similar observations were made involving other streptococci; S. faecalis, S. pyogenes, S. agalactiae, and streptococcal Lancefield groups F and G (Franke and Clewell, 1981; Horodniceanu et al., 1981).

S. faecalis strain DS16 a multiple antibiotic-resistant, hemolytic clinical isolate that harbors two plasmids, pAD1 and pAD2 (Tomich et al., 1980). The pAD1 plasmid (58 Kb) encodes hemolysin-bacteriocin activity, resistance to ultraviolet light, and confers a sex pheromone-inducible, conjugative mating response (Clewell et al., 1982; Clewell, 1981). The pAD2 resistance plasmid (25 Kb) is nonconjugative and confers resistance to streptomycin, kanamycin and erythromycin (Tomich et al., 1980; Clewell et al., 1982). The erythromycin resistance determinant resides on a transposon referred to as Tn917 (Tomich et al., 1980). When DS16 was cured of both plasmids, the tetracycline resistance determinant was shown to be located on the chromosome (Tomich et al., 1979).

Franke and Clewell (1981) reported that the chromosome-borne tetracycline resistance element in S.

faecalis DS16 had the characteristics of a transposon. This 15-17 Kb transposon, Tn916, was capable of transposition to several different conjugative hemolysin plasmids (pAD1 and pOB1) at a frequency of approximately 10^{-6} (Franke and Clewell, 1981). Moreover, Tn916 was also capable of transposition at low frequency (10^{-8}) from plasmid-free derivatives of DS16 to plasmid-free recipients (Franke and Clewell, 1981). This transfer of tetracycline resistance was resistant to the action of DNase, did not require homologous host-mediated recombination functions, and occurred by a conjugation-like event that required direct contact between the donor and recipient (Franke and Clewell, 1981).

Tn916 (16.4 Kb) is classified as a Class IV transposon (Kleckner, 1981). Transposable elements are classified on the basis of mechanistic differences, their DNA sequence homology, and genetic organization (Kleckner, 1981). Class I transposons are IS-like modules and composite elements formed from IS elements (Kleckner, 1981). Class II transposons are a family of Tn3 related elements (Kleckner, 1981). Class III elements are transposing bacteriophages (for example, Mu and D108) (Kleckner, 1981). The Class IV transposons are a miscellaneous group of transposons in which their behavior is not fully understood and their nucleotide sequences not completely

known.

Tn916 has been transferred to a number of streptococcal species by the filter mating technique (Franke and Clewell, 1981; Nida and Cleary, 1983; Wanger and Dunny, 1985; Weiser and Rubens, 1987). Furthermore, it has been transferred by filter matings to S. aureus, L. monocytogenes, and Mycoplasma hominis (Kathariou et al., 1987; Jones et al., 1987; Roberts and Kenny, 1987). The conjugative transfer of Tn916 in the absence of plasmid DNA occurs at a frequency of 10^{-5} to 10^{-8} per donor, and DNA hybridization data has demonstrated that Tn916 inserts itself into different sites on the recipient chromosome (Gawron-Burke and Clewell, 1982).

Insertional mutagenesis using Tn916 has been reported (Kathariou et al., 1987; Nida and Cleary, 1983). Kathariou et al. have reported that a L. monocytogenes genetic determinant essential for hemolysin production was inactivated by insertion of Tn916 into L. monocytogenes DNA. Weiser and Rubens (1987) reported that a virulent, beta-hemolytic group B streptococcus strain was mutagenized with Tn916 to derive isogenic strains with mutations only in the genes responsible for beta-hemolysin biosynthesis. Nida and Cleary (1983) demonstrated that Tn916 was capable of insertion into several chromosomal sites including inactivation of streptolysin S. Tn916

excises precisely (Gawron-Burke and Clewell, 1982). Nida and Cleary (1983) and Kathariou et al. (1987) observed a common event of precise excision to restore hemolytic activity. The hemolytic revertants were void of Tn916 or only carried it at a new chromosomal site.

The current hypothesis for the nature of Tn916 movement maintains that Tn916 transposition may follow excision with circularization and reinsertion of Tn916 similar to phage lambda integration (Gawron-Burke and Clewell, 1982, 1984). Excision of Tn916 from the donor replicon represents the rate-limiting step in its transposition, regardless of whether the insertion occurs in a target located in the same or different cell. There is no direct evidence of what form the transposon will take when it excises from the chromosome, but it is thought that it might form a circular intermediate which is responsible for the activation of the transposon-encoded functions necessary for its transfer and/or insertion.

Tn916 has been cloned as an intact element on a pBR322-derived vector in Escherichia coli by selecting for tetracycline resistance (Gawron-Burke and Clewell, 1984). This transposon has a single HindIII site and lacks recognition sites for EcoRI (Gawron-Burke and Clewell, 1982). Utilizing this information, a restriction fragment

containing the entire transposon was cloned onto pGL101 (a pBR322 derivative) to generate the chimeric plasmid pAM120 (Gawron-Burke and Clewell, 1984). Interestingly, in the absence of selection for tetracycline resistance, Tn916 excised from pAM120 at a high frequency, and only inserted into the E. coli CG120 chromosome (Gawron-Burke and Clewell, 1984).

The ability to clone Tn916 in E. coli facilitated the generation of a restriction map for the transposon (Gawron-Burke and Clewell, 1984). By comparing restriction fragments derived from pAM120 with those obtained from another plasmid, pAM120LT (pGL101 with the regenerated pAD1 EcoRI fragment from which Tn916 excised), it was possible to identify Tn916 specific fragments. Restriction enzymes that cleave this transposon at multiple sites are HincII, HpaI, and Sau3A, and enzymes which only cleave once are HindIII, BstXI, SstI, and KpnI. This transposon does not have any restriction sites for EcoRI, HaeIII, BamHI, BglII, SalI, XhoI, NcoI, SphI, XbaI, and ClaI.

Yamamoto et al. (1987) have utilized Tn5 mutagenesis to study the genetics of Tn916 behavior and the results are providing structural information on this transposon. The data from this study indicate that most of the region right of the tet^r determinant is required for conjugation.

Moreover, similar results have been observed for a Tn916 derivative containing Tn917 in a site in the right half of the transposon (Clewell and Gawron-Burke, 1986).

The complete nucleotide sequence of Tn916 has not been reported. It is known that Tn916 does not contain significant sequence repetition at its ends, and that zygotic induction occurs when it enters a new host (Clewell and Gawron-Burke, 1986). These properties resemble those of the S. aureus transposon Tn554. Tn554 (6.7 Kb) encodes resistance to erythromycin and spectinomycin, does not contain significant repeated sequences at its ends, and is not conjugative (Phillips and Novick, 1979). Even though Tn554 is nonconjugative it would be interesting to determine whether certain basic aspects of transposition prove to be similar in the two different transposons.

Other Conjugative Transposons

Streptococcus faecalis transposons Tn918 and Tn919

Tn918 was discovered in a hemolytic, tetracycline resistant, clinical isolate of S. faecalis RC73 and there are several similarities between Tn918 and Tn916 (Clewell et al., 1985). Both appear to be identical in size, have similar conjugative properties, and contain homologous sequences. Moreover, Tn918 has been shown to insert into pAD1 and give rise to hyper-hemolytic derivatives similar

to those observed with Tn916 (Clewell and Gawron-Burke, 1986).

Tn919 was originally discovered and isolated from S. sanguis FC1 (Fitzgerald and Clewell, 1985). This conjugative transposon encodes tetracycline resistance, and has been shown to insert into pAD1 to give rise to hyper-hemolytic derivatives similar to those observed with Tn916 (Clewell and Gawron-Burke, 1986). Hybridization analysis has also shown strong sequence homology between Tn919 and Tn916 (Fitzgerald and Clewell, 1985). Tn919 has been introduced into lactic acid bacteria by filter matings with S. faecalis (Hill et al., 1987), and it is being used to study the basic molecular biology of the industrially important lactic acid bacteria.

Both Tn918 and Tn919 have been cloned in E. coli (Clewell et al., 1985; Fitzgerald and Clewell, 1985). When these transposons were on the multicopy plasmid vector (pGL101) in E. coli they exhibited a high degree of excision and segregation similar to Tn916.

Streptococcus faecalis transposon Tn917 Tn917 was discovered as a MLS^r determinant (i.e., resistance to macrolides, lincosamides, and streptogramin type B antibiotics) associated with the nonconjugative S. faecalis plasmid pAD2 (Clewell et al., 1982; Tomich et al., 1980). It was observed that this MLS^r determinant

could move from pAD2 to several different conjugative plasmids in S. faecalis at a frequency of approximately 10^{-6}, and it is now known that Tn917 is able to transpose to at least four different sites in the conjugative plasmid, pAD1 (Clewell et al., 1982; Tomich et al., 1980). This transposon is 5.4 Kb in length, confers inducible erythromycin resistance, and has an interesting property in that its translocation can be induced by growing the cells in subinhibitory concentrations of erythromycin (Clewell et al., 1982; Tomich et al., 1980).

Restriction mapping and heteroduplex analysis have revealed extensive homology throughout virtually their entire lengths between Tn917 and the S. aureus transposon Tn551 (Youngman et al., 1984a). DNA sequencing studies have revealed a remarkably high degree of sequence homology within the terminal inverted repeats of Tn917, Tn551 and the gram-negative transposon Tn3 (Khan and Novick, 1980). Finally, Tn917 has also been shown to generate a 5 basepair duplication upon insertion, as do Tn3 and Tn551 which strengthens the conclusion that these three transposons are members of a highly dispersed family of related insertion elements which populate both gram-positive and gram-negative bacteria (Khan and Novick, 1980; Perkins and Youngman, 1984).

Use of Transposon 917 to Identify and Clone B. subtilis Sporulation Genes

One way of identifying developmentally regulated genes of B. subtilis whose products are required for sporulation would be to identify mutations that block the sporulation process (Hoch, 1976; Piggot and Coote, 1976; Youngman et al., 1985). Such mutations could be obtained by using the concept of transposon-mediated insertional mutagenesis which was described by Kleckner (1981). The concept is based on the ability of a transposon to insert itself as a discrete, nonpermutated DNA segment into many random sites in the prokaryotic genome. Once the transposon is in the genome it is possible that it will interrupt certain host gene functions. The inserted transposon could then act as an antibiotic resistance selective marker, therefore permitting selection for inheritance of the insertion and any mutation caused by it. Moreover, because there is an identifiable segment of transposon DNA inserted into the region of the gene, it is possible to locate the gene and clone it for in vitro structure/function analysis. This approach has been successfully used to study the basic molecular biology of various bacteria which include Caulobacter, Myxococcus, Rhizobium, and Bacillus (Youngman et al., 1985).

A system of transposon-mediated insertional mutagenesis was developed in B. subtilis using the S. faecalis transposon Tn917 (Youngman et al., 1983). Most Staphylococcus and Streptococcus plasmids are able to function as stable, autonomous replicons after being introduced into B. subtilis; therefore Tn917 was introduced into B. subtilis by transformation of competent cells with the S. faecalis plasmid pAM-alpha-1::Tn917 (Youngman et al., 1983). pAM-alpha-1 is a 9.6 Kb nonconjugative plasmid of S. faecalis which confers amplifiable tetracycline resistance (Perkins and Youngman, 1983). Restriction mapping analysis of pAM-alpha-1 has shown it to be virtually identical to the 4.6 Kb tetracycline resistance plasmid of B. cereus, pBC16, that is known to show extensive homology to plasmid isolates from Staphylococcus species as well as other Bacillus species (Perkins and Youngman, 1983). Once the cells were transformed with the pAM-alpha-1::Tn917 plasmid, the transpositional activity of Tn917 was assayed by selecting for insertions of Tn917 into the temperate B. subtilis bacteriophage, SP-Beta. This 130 Kb phage was chosen because it was known that it contained relatively extensive regions of "nonessential" DNA, and could accommodate extra DNA without causing headful packaging problems (Youngman et al., 1983). The pAM-alpha-1::Tn917

SP-Beta lysogen was subsequently isolated and subjected to restriction and Southern hybridization analysis. From the data obtained using this bacteriophage, Youngman et al. (1983) were able to conclude: (i) that Tn917 was transpositionally active in B. subtilis; (ii) Tn917 was a highly effective agent for insertional mutagenesis of B. subtilis chromosomal DNA; and (iii) that Tn917 could act as a random chromosomal mutagen.

Youngman et al. (1983) constructed a transposition selection vector by placing Tn917 on a plasmid vector which was temperature sensitive for replication. The plasmid vector used, pBD95, was a derivative of the S. aureus plasmid, pE194. A region of pE194 was deleted which normally encoded for macrolide-lincosamide-streptogramin resistance (MLS^r), and was replaced with a S. aureus chloramphenicol resistant determinant (Cm^r) to yield pBD95. The pBD95 plasmid was utilized because it was able to replicate in B. subtilis, and it was temperature-sensitive for replication (Gryczan et al., 1982). A restriction fragment from pAM-alpha-1::Tn917, containing Tn917 was ligated into pBD95 to produce a plasmid approximately 12 Kb in size. This plasmid was referred to as pTV1. B. subtilis was then transformed with pTV1 and Tn917 chromosomal insertions were selected by shifting to the nonpermissive temperature while

maintaining selection for erythromycin resistance.

Using pTV1, Youngman et al. (1983) were able to demonstrate that Tn917 could produce different kinds of insertional auxotrophs and sporulation-defective (spo) mutants in B. subtilis. Because insertional mutations were found, Youngman et al. (1984b) wanted to clone these genes for in vitro structure/function analysis. The most common method used to facilitate the molecular cloning of neighboring chromosomal sequences is simply to select for the transposon-borne drug-resistance gene in a "shot-gun" cloning of insert-containing chromosomal DNA into an appropriate plasmid vector (Kleckner et al., 1977). However, such a strategy can not be used for the cloning of Tn917-linked B. subtilis chromosomal DNA into E. coli because the MLS resistance gene encoded by Tn917 is not expressed at selectable levels in E. coli (Youngman et al., 1984b). Moreover, if the cloning were to be performed in a Bacillus host, it most likely required the use of recombination-deficient protoplasts (Youngman et al., 1984b).

Youngman et al. (1984b) devised an alternate cloning strategy by using the beta-lactamase (amp) gene of the E. coli plasmid vector, pBR322, and the chloramphenicol acetyltransferase (cat) gene of the S. aureus plasmid, pC194. The strategy involved constructing chimeric

plasmids which could be expressed and maintained in either E. coli or B. subtilis. These chimeric plasmids were constructed by ligating a SalI restriction fragment containing the amp gene (obtained from a pBR322-derived replicon, pHW9), into the temperature-sensitive plasmid, pTV5, (that contained the pC194 cat gene and Tn917). The resulting chimeric replicons were referred to as pTV20 and pTV21. When these chimeric plasmids were linearized with restriction enzymes and used to transform B. subtilis strains already containing Tn917 inserts, the Tn917-pBR322 sequences efficiently became integrated into the chromosomal copy of the transposon by homologous recombination because Tn917 insertions provided portable sites of homology. This strategy then made it possible to clone B. subtilis chromosomal sequences adjacent to either transposon insertion junction in E. coli using ampicillin resistance selection. Youngman et al. (1984b) using this strategy were able to isolate cloned sequences from the region of the spoIIH locus, but these investigators did not elaborate about the significance of these genes.

Transposon-Mediated Gene Fusions

One way to quantitatively assess the transcriptional activity of a sporulation gene is to use a cloned copy of the gene itself as a hybridization probe to measure the presence of the messenger RNA in the bacterium at

different times during its development (Youngman et al., 1985). Perhaps the single most powerful application of transposons for in vivo analysis of gene expression and regulation is their use to generate transcriptional gene fusions (Youngman et al., 1985). This application was pioneered by Casadaban and Cohen using the transposon-phage Mu, referred to as Mud (ampicillin resistant (Ap^r), lac) (Youngman et al., 1985). These Mud (Ap^r, lac) elements were defective mutants of Mu engineered to carry promoterless copies of the E. coli lactose operon, near one end of the Mu genome. When Mu inserted into a chromosomal transcription unit, the lactose operon was oriented such that it was under control of the promoter and other regulatory elements of the interrupted transcription unit.

This work pioneered by Casadaban and Cohen suggest that any transposon could be made to function like Mud (Ap^r, lac), but the key requirement was to identify a site near one end of the transposon where a promoterless lacZ gene could be inserted without disrupting sequences or genes important for transposition activity. Youngman et al. (1984a) performed a thorough physical analysis of Tn917 and identified such a site, a HpaI site located approximately 270 bp from its erm-proximal end. The investigators modified this HpaI site and a promoterless

lacZ was inserted into it such that its orientation made its expression dependent on transcription coming into the transposon from the outside (Youngman *et al.*, 1985; Perkins and Youngman, 1986). This fusion-generating transposon, Tn*917 lac* was ligated into the plasmid pTV30, and in its construction, the *lacZ* gene was furnished with a ribosome-binding site appropriate for the efficient initiation of translation in *B. subtilis* (Youngman *et al.*, 1985; Perkins and Youngman, 1986). The pTV30 plasmid was then transformed into a *B. subtilis* strain containing pTV1, and through *in vivo* homologous recombination a temperature sensitive, Tn*917-lacZ* (pTV32) replicon was obtained (Youngman *et al.*, 1985; Perkins and Youngman, 1986). Because Tn*917-lacZ* was unimpaired in its transpositional activity, and it created transcriptional *lacZ* fusions when it inserted into a chromosomal gene, Perkins and Youngman, (1986) were able to obtain two independent insertions of Tn*917-lacZ* into the *B. subtilis gltA* gene and one insertion into the *trpE* gene. These Tn*917-lacZ* insertions were studied in great detail, and the results confirmed that these Tn*917-lacZ*-mediated transcriptional fusions produced enough beta-galactosidase to accurately reflect the regulated expression of interrupted genes.

Identifying Developmentally Regulated Genes by Expression

Another important application of transposons that generate gene fusions is the identification of genes on the basis of expression or change of expression under specific conditions (Youngman *et al.*, 1985). Youngman *et al.* (1985) developed two strategies for the identification of genes that became active during sporulation in *B. subtilis*. The first strategy involved a transposon that simultaneously generated an operon fusion to both the *lacZ* and *cat*. This transposon, Tn*917 cat-lac*, contained an *E. coli* promoterless *lacZ* gene and promoterless *B. pumulis cat* gene arranged as a tandem pair, with no transcription terminator between them. By using this Tn*917 cat-lac* replicon, and simple phenotypic assays (i.e., blueness on X-gal plates and chloramphenicol sensitivity), the investigators were able to evaluate whether the expression of the Tn*917 cat-lac*-mediated transcriptional fusion was restricted to vegetative or sporulating *B. subtilis* cells. For example, if chloramphenicol sensitive blue colonies were observed, this indicated that the Tn*917 cat-lac* operon was activated during sporulation.

The other approach utilized a beta-galactosidase indicator which provided the investigators with the actual time at which the gene was activated (Youngman *et al.*, 1985). In this system, when the indicator substrate,

4-methylumbelliferyl-beta-D-galactoside (MUG), was hydrolized by beta-galactosidase, the MUG was converted to 4-methylumbelliferone. This hydrolyzed substrate was highly fluorescent under long-wavelength ultraviolet light, and could easily be detected even when present in small amounts. In this approach, a population of B. subtilis cells, containing Tn917 lac-generated inserts, were plated under conditions which favored sporulation. The cells were allowed to grow until they entered sporulation, and then they were sprayed with a solution of MUG. The colonies of B. subtilis that contained fusions of Tn917-lacZ to active spo genes were immediately apparent from their fluorescence under long-wavelength ultraviolet light.

The major advantage of utilizing these fusion-generating transposons is that it is no longer necessary to go through the laborious process of cloning; instead it is simply a matter of obtaining the desired transposon-mediated insertional mutations with the fusion-generating transposon. Moreover, it is possible to identify sporulation genes that are active regardless of whether their products are required for spore-formation. Finally, Tn917 is known to function efficiently in several Gram-positive genera (Streptococcus, Staphylococcus and Bacillus), and it is known to display a high degree of

randomness in its insertions into bacterial genomes which makes it an attractive tool in studying the basic molecular biology of other Gram positive species.

SECTION I. INTRODUCTION OF THE *STREPTOCOCCUS FAECALIS* TRANSPOSON TN*916* INTO *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS*

Introduction of the *Streptococcus faecalis* transposon Tn*916* into *Bacillus thuringiensis* subsp. *israelensis*

by

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ABSTRACT

The conjugative Streptococcus faecalis transposon Tn916 was introduced into Bacillus thuringiensis subsp. israelensis by filter matings with S. faecalis. B. thuringiensis transconjugants resistant to tetracycline were detected at a frequency of approximately 7.0×10^{-7} per recipient cell when filter matings were performed, but tetracycline-resistant transconjugants were not observed when a broth-mating protocol was used. The tetracycline resistance phenotype was not lost during serial passage of B. thuringiensis subsp. israelensis without antibiotic selection. Southern hybridization analysis revealed that Tn916 had inserted into several different sites on the B. thuringiensis subsp. israelensis chromosome. Conjugation-like movement of Tn916 was demonstrated when tetracycline-resistant B. thuringiensis transconjugants were mated with isogenic recipients.

INTRODUCTION

Tn916 is one of several Streptococcus transposons that have been identified and characterized (Gawron-Burke and Clewell, 1982). This transposon is 16.4 kilobases (Kb) in length and encodes tetracycline resistance. Tn916 is capable of conjugative transposition in the absence of plasmid DNA at frequencies ranging between 10^{-5} to 10^{-8} per donor and does so in the absence of host-mediated homologous recombination (Franke and Clewell, 1981). Tn916 has been transferred to a number of streptococcal species (Franke and Clewell, 1981; Nida and Cleary, 1983; Wanger and Dunny, 1985), Staphylococcus aureus (Jones et al., 1987), and Listeria monocytogenes (Kathariou et al., 1987). DNA hybridization data have demonstrated that Tn916 inserts into different sites on the recipient chromosome. The conjugative properties of Tn916 require cell-to-cell contact but are independent of a pheromone response because transfer occurs only during filter matings and not broth matings (Clewell and Gawron-Burke, 1986). Tn916 has also been cloned in Escherichia coli where the tetracycline resistance phenotype was expressed (Gawron-Burke and Clewell, 1984).

This laboratory is interested in studying the basic molecular biology of the industrially important bacterium B. thuringiensis. One subspecies, B. thuringiensis subsp.

israelensis, produces a toxin active against certain diptera larvae including mosquitoes, black flies, and horn flies, but is nontoxic towards other insects, plants or animals (Undeen and Nagel, 1978; Tyrell et al., 1981; Temeyer, 1984; Margalit and Dean, 1985). The insecticidal properties of B. thuringiensis subsp. israelensis reside in a plasmid-encoded parasporal crystal protein that is formed only during sporulation of the organism (Aronson et al., 1986). The toxin is composed of at least two separable activities, a broadly cytolytic component (M_R = 28,000) and a highly specific entomocidal component (M_R = 68,000) (Hurley et al., 1985; 1987).

Basic genetic studies in B. thuringiensis have been hampered by a lack of efficient and versatile techniques for genetic manipulation of this organism. Selectable markers in B. thuringiensis have been limited primarily to plasmid- and chromosome-determined antibiotic resistance, which has placed severe constraints on genetic studies in this organism (Aronson et al., 1986). This paper demonstrates that Tn916 can be transferred by a conjugative process from the chromosome of S. faecalis to B. thuringiensis subsp. israelensis. It can also be transferred from the chromosomes of transposon-containing transconjugants to isogenic recipients at frequencies

adequate to facilitate the use of Tn916 as a tool in genetic studies of B. thuringiensis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media

B. thuringiensis subsp. israelensis, strain LB-2, was provided by Lee A. Bulla Jr.; Department of Biotechnology, University of Wyoming, Laramie, WY 82071. S. faecalis CG110 (Gawron-Burke and Clewell, 1982) and Escherichia coli CG120 (Gawron-Burke and Clewell, 1984) were obtained from Dr. Peter A. Pattee, Iowa State University, Ames, Iowa. E. coli CG120 contains a chimeric plasmid, pAM120, composed of Tn916, and E. coli plasmid pGL101 (Gawron-Burke and Clewell, 1984).

B. thuringiensis and S. faecalis were grown in brain heart infusion (BHI; Difco) at 30°C. For the isolation of DNA, cultures were grown in Luria-Bertani (LB) broth (Maniatis et al., 1982). Antibiotics used were kanamycin (200 ug/ml), tetracycline (10 ug/ml), and rifampcin (30 ug/ml).

Broth-mating protocol

Transfer conditions described by Gonzalez et al. (1982) were used for broth mating. After mating, the cell suspensions were plated onto the appropriate selective media and incubated at 30°C overnight. Donor and recipient cell controls were separately treated in the same fashion as the mating mixtures. Transfer frequency was determined by dividing the number of transconjugants

by the number of recipient cells.

Filter-mating protocol

Conjugation experiments on nitrocellulose filters (0.45 um, Millipore Corp.; Bedford, MA) were done according to the method of Lereclus et al. (1983) with the following modifications: 0.5 ml of midexponential-phase donor and recipient cultures were placed onto nitrocellulose membrane filters, which were laid on absorbent pads. After 10 min., the filters were placed upright on the surface of BHI agar plates and incubated (30°C) for different periods of time. The growth was then suspended in 5 ml of BHI, diluted, spread onto the appropriate selective agar media, and incubated (30°C) overnight. Donor and recipient cell controls were separately treated in parallel with the mating mixtures. Transfer frequency was determined by dividing the number of transconjugants by the number of recipient cells.

Assessment of stability

To determine the stability of the antibiotic-resistance markers in B. thuringiensis subsp. israelensis, cultures were first isolated on BHI agar containing the appropriate antibiotic. After overnight incubation at 30°C, each of several colonies was inoculated into BHI (100 ml) and grown at 30°C with vigorous shaking. After overnight incubations, serial dilutions of these cultures

were spread onto BHI agar with and without selection. The numbers of colonies on selective and nonselective media were then compared.

DNA purification

Total B. thuringiensis DNA was obtained by using the cell lysate procedure of Kronstad et al. (1983) with the following modifications: Cells in logarithmic growth (A_{600}=0.6) were harvested by centrifugation, and the pellets were resuspended in TEG [Tris-HCl (10 mM, pH 7.5)-EDTA (50mM)-glucose 1% (w/v)] buffer containing 15 mg/ml lysozyme. The cell suspension was then lysed in a 1% (w/v) sodium dodecyl sulfate-TEG solution, followed by two chloroform:isoamyl alcohol extractions. After the final extraction, cold ethanol was added, and the DNA was concentrated by centrifugation. To further purify the DNA, RNA was removed by precipitation with ammonium acetate (2.5 M), and covalently closed circular plasmid and linear DNA forms were separated using CsCl-ethidium bromide gradient centrifugation (Maniatis et al., 1982).

Plasmid DNA was extracted from E. coli CG120 by using the alkaline denaturation procedure of Birnboim and Doly (1979). The plasmid preparations were further purified on CsCl-ethidium bromide gradients (Maniatis et al., 1982).

Preparation and analysis of DNA restriction fragments

Digestions with HindIII and EcoRI were performed by using the conditions described by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). Restriction enzyme digests and plasmid samples were analyzed by agarose gel electrophoresis [0.7% (w/v)] (Maniatis et al., 1982). After electrophoresis, the gels were stained with ethidium bromide, and the DNA was visualized on a UV transilluminator.

Southern hybridization analysis

DNA was transferred from the agarose gel to Hybond N (TM) (0.45 um, Amersham Inc., Boston, MA) according to the method of Southern (1975). Plasmid DNA was labelled with ^{32}P-dATP by using a nick-translation kit from Bethesda Research Laboratories, Gaithersburg, MD. Hybridization with the labelled probe was carried out in 50% formamide in 0.1% (v/v) SSC (1X SSC is 0.15 M NaCl-0.015 M sodium citrate, pH 7.0) for 24 h at 42°C. The posthybridization washes and detection of the ^{32}P-labelled probe were performed as recommended by Bethesda Research Laboratories, Gaithersburg, MD.

RESULTS

The frequency of spontaneous mutation to kanamycin resistance (Kan^r) or rifampcin resistance (Rif^r) in B. thuringiensis subsp. israelensis ranged from 5.0×10^{-8} to 1.0×10^{-9}. Apart from antibiotic resistance, the Kan^r or Rif^r mutants were phenotypically indistinguishable from the LB-2 wild-type. Two of these mutants, AN-142 (Kan^r) and AN-146 (Rif^r), were used for further study. When AN-142 was used as the recipient in overnight membrane-filter matings with S. faecalis CG110, AN-142 acquired Tn916 at an average frequency of 10^{-7} per recipient cell.

To further characterize the transfer of Tn916, CG110 and AN-142 were mated on a filter or in broth for 4, 8, 12, and 18 h. As shown in Table 1, optimal transfer of Tn916 occurred when a 8 to 12 h mating time was used. Moreover, Table 1 shows that tetracycline resistant AN-142 transconjugants were not obtained under these conditions with use of broth matings. Tetracycline resistance in AN-142 parents was not observed under these conditions. Transfer of Tn916 was resistant to DNase (150 ug/ml) incorporated in the mating agar plates. All B. thuringiensis Tet^r transconjugants were resistant to tetracycline at concentrations of at least 50 ug/ml.

To determine the stability of the Kan^r and Tet^r phenotypes, two randomly selected Kan^r and Tet^r

Table 1. Frequency of transfer of Tn916 from *Streptococcus faecalis* to *Bacillus thuringiensis* subsp. *israelensis*

Time (hours)	Mating	Frequency/recipient[a]
4	Filter	$< 5.0 \times 10^{-8}$ [b]
8	Filter	9.0×10^{-6}
12	Filter	1.4×10^{-5}
18	Filter	6.2×10^{-7}
4	Broth	$< 5.0 \times 10^{-8}$ [b]
18	Broth	$< 5.0 \times 10^{-8}$ [b]

[a] Represents the average of two mating experiments.

[b] Transfer not detected.

B. thuringiensis subsp. israelensis transconjugants (AN-950 and AN-953) were grown in a nonselective medium, and the frequency of Tet^r was determined in the resulting cultures. The experiments were performed in duplicate on three different occasions. Because comparable numbers of colony-forming units appeared on a medium containing tetracycline and on the same medium without tetracycline, it seemed that the Tet^r phenotype was stably maintained and expressed in both AN-950 and AN-953 for three serial passages (Table 2). However, when the number of colony-forming units on a kanamycin medium are compared with those obtained on the same medium without kanamycin, it is evident that the Kan^r phenotype is not as stable as the Tet^r phenotype; there was approximately a 2.5-fold reduction in the number of colony-forming units observed on the kanamycin medium as compared with the number of colony forming units observed on the nonselective medium. To confirm this observation about the reduction of kanamycin resistant colonies, a more accurate method was used. The transconjugants (AN-950 and AN-953) were grown in a nonselective medium and after the second passage, the cells were plated onto BHI agar medium and incubated overnight (30° C). One hundred colonies from each transconjugant were replicate plated onto media containing antibiotics and the number of colonies compared

Table 2. Stability of the Tn916 phenotype of two representative Bacillus thuringiensis subsp. israelensis transconjugants during serial passage in nonselective medium

Transconjugant	Selection[a]	Number of colony-forming units x 10^7 per milliliter		
		Pass number[b]		
		1	2	3
AN-950	-	12.1	12.7	13.0
	Kan	7.5	9.1	10.7
	Tet	10.6	17.3	16.3
AN-953	-	6.1	13.5	11.1
	Kan	4.8	13.5	5.4
	Tet	5.5	16.0	19.9

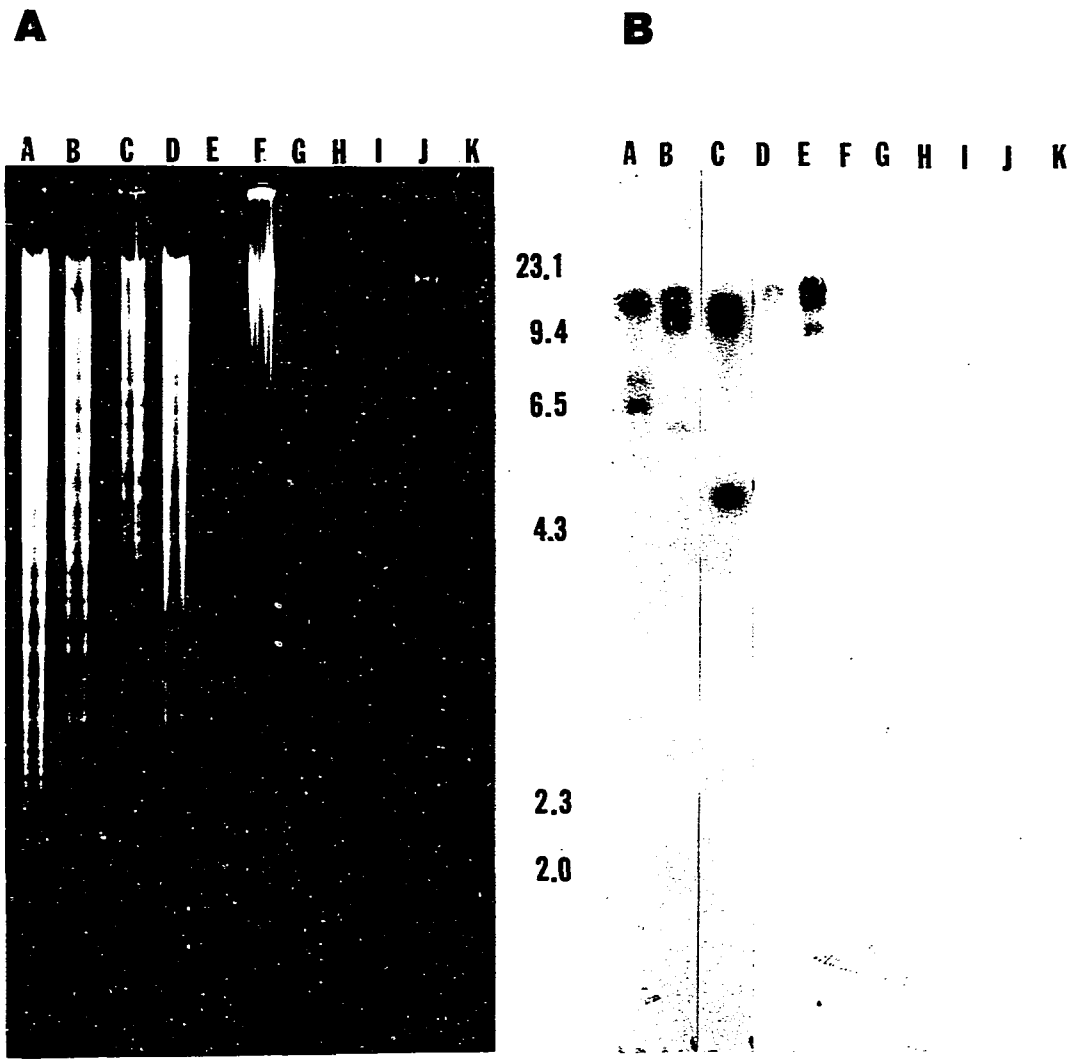
[a]Kan = kanamycin; Tet = tetracycline. Two overnight broth cultures were each serially diluted and plated on media with and without selection after incubation in brain heart infusion broth.

[b]Each experiment represents two complete sets of experimental data for each transconjugant. The pass number represents a serial transfer of each transconjugant.

between selective and nonselective media. It is evident that the Tet^r marker in AN-950 and AN-953 is stably maintained because all of the colonies (100) assayed were resistant to tetracycline. All of the AN-950 colonies were resistant to kanamycin. However, one AN-953 colony became sensitive to kanamycin.

To confirm the introduction of Tn916 in AN-142 Tet^r transconjugants, total DNA was extracted from randomly selected transconjugants and analyzed by Southern hybridization. Figure 1 shows that Tn916 is present in all five of the Tet^r AN-142 transconjugants assayed. Total AN-142 DNA (Figure 1, lane F) did not hybridize with the probe, indicating that there was no homology between the pAM120 probe and the parental AN-142 DNA. Tn916 lacks EcoRI sites and contains a single HindIII site (Gawron-Burke and Clewell, 1982). The single hybridizing band observed in EcoRI-digested AN-142 transconjugant DNAs were large (17 to 20 Kb), and presumably consisted of the entire transposon (16.4 Kb) and flanking B. thuringiensis subsp. israelensis DNA sequences. Such fragments were poorly separated electrophoretically because of their size (data not shown). Therefore, hybridizations with HindIII-digested AN-142 transconjugant DNA were used to determine the number of inserts of Tn916 in AN-142. Each insert of Tn916 should produce two hybridizing fragments,

Figure 1. Hybridization analysis of Tn*916* insertions using ^{32}P-labelled pAM120 DNA to probe *Hind*III-digested DNA in various *B. thuringiensis* subsp. *israelensis* transconjugants. (A) Ethidium bromide-stained 0.7% agarose gel of *Hind*III-digested DNA. (B) Hybridization analysis of the DNA shown in panel A after transfer to a nylon membrane and probed with ^{32}P-labelled pAM120::Tn*916*. Lanes (both panels) A-E, AN-142 Tet^r cesium chloride purified transconjugant chromosomal DNA; F, AN-142 total parental cesium chloride purified DNA; G-K, AN-142 Tet^r cesium chloride purified transconjugant plasmid DNA. Sizes (in kilobase pairs) of DNA *Hind*III-generated restriction fragment markers are noted between panels A and B


A
A B C D E F G H I J K
23.1
9.4
6.5
4.3
2.3
2.0
B
A B C D E F G H I J K

each consisting of part of Tn916 and flanking B. thuringiensis chromosomal DNA. Hybridizations of the Tn916 probe with HindIII-digested DNA from five Tet^r AN-142 transconjugants showed that, in one transconjugant, there was a single copy present because only two hybridizing bands were observed (Figure 1, lane D). Multiple bands were observed in the other four Tet^r AN-142 transconjugants, indicating multiple inserts of the transposon. In two transconjugants (Figure 1, lanes A and C) only three bands are evident. This observable disparity may reflect the fact that two of the transposon-chromosome junction fragments are the same size or that these transconjugants contain a tandem insertion of Tn916. The reason(s) for these bands is unclear. Southern hybridization analysis confirmed that Tn916 had inserted into several different sites in the B. thuringiensis subsp. israelensis chromosome because no hybridizing plasmid bands were detected when cesium-chloride purified plasmid DNA was probed with pAM120 (Figure 1, lanes G-M).

To determine intra-subspecies transfer of Tn916, two different Kan^r Tet^r B. thuringiensis subsp. israelensis transconjugants (AN-950 and AN-953) were chosen as donors of the Tn916 element. The Rif^r (AN-146) B. thuringiensis mutant was used as the recipient. Transfer of Tn916 from one B. thuringiensis subsp. israelensis to another was

demonstrated by mating AN-950 or AN-953 with AN-146 and selecting for the Kan^s Tet^r Rif^r phenotype (Table 3). However, when the parental donor controls were grown on a medium containing rifampcin and tetracycline, the cells became spontaneously resistant to rifampcin at an approximate frequency of 4.0×10^{-7} (Table 3). Transfer of Tn916 was not observed when the broth mating protocol was utilized. In filter matings, transfer of Tn916 was resistant to 150 ug/ml of DNase in the mating medium. All AN-146 Tet^r transconjugants were resistant to 50 ug/ml of tetracycline and were sensitive to kanamycin.

When plasmid pAM120 was used as a probe, hybridization was detected with the total DNA from 25 Kan^s Tet^r Rif^r AN-146 transconjugants. Examples of such experiments are shown in Figs. 2 and 3. AN-146 DNA (Figure 2, lane A; Figure 3, lane F) did not hybridize with the probe, indicating a lack of DNA homology between pAM120 and B. thuringiensis. Lane B (Figure 2) contains total DNA from one Kan^r Tet^r-resistant donor (AN-950), whereas lane A (Figure 3) contains total DNA from the other donor (AN-953). The single fragments observed in hybridizations with EcoRI-digested B. thuringiensis subsp. israelensis DNA were large (17 to 20 Kb), but such fragments were poorly separated electrophoretically because of their size (data not shown). When the hybridization patterns of

Table 3. Intra-subspecies transfer of Tn916 from two different Bacillus thuringiensis subsp. israelensis kanamycin- and tetracycline-resistant donors to an isogenic rifampcin resistant recipient

Donor x Recipient	Selection[a]	Frequency/ recipient[b]
AN-950 x AN-146	Kan^s, Tet^r, Rif^r	1.1×10^{-5}
AN-953 x AN-146	Kan^s, Tet^r, Rif^r	6.7×10^{-6}
AN-950	Tet^r, Rif^r	3.3×10^{-7}
AN-953	Tet^r, Rif^r	4.5×10^{-7}
AN-146	Kan^r, Tet^r, Rif^r	$<5.0 \times 10^{-8}$[c]

[a] Kan^r = kanamycin resistant; Kan^s = kanamycin sensitive; Tet^r = tetracycline resistant; Rif^r = rifampicin resistant.

[b] The entry represents the average of two mating experiments.

[c] Not detected.

Figure 2. Hybridization analysis of Tn916 insertions using ^{32}P-labelled pAM120 DNA to probe HindIII-digested DNA in various B. thuringiensis subsp. israelensis transconjugants. (A) Ethidium bromide-stained 0.7% agarose gel of HindIII-digested DNA. (B) Hybridization analysis of the DNA shown in panel A after transfer to a nylon membrane and probed with ^{32}P-labelled pAM120::Tn916. Lanes (both panels) A, AN-146 recipient DNA; B, AN-950 Tet^r donor DNA; C-F, AN-146 Tet^r transconjugant DNA. Sizes (in kilobase pairs) of DNA HindIII-generated restriction fragment markers are noted between panels A and B

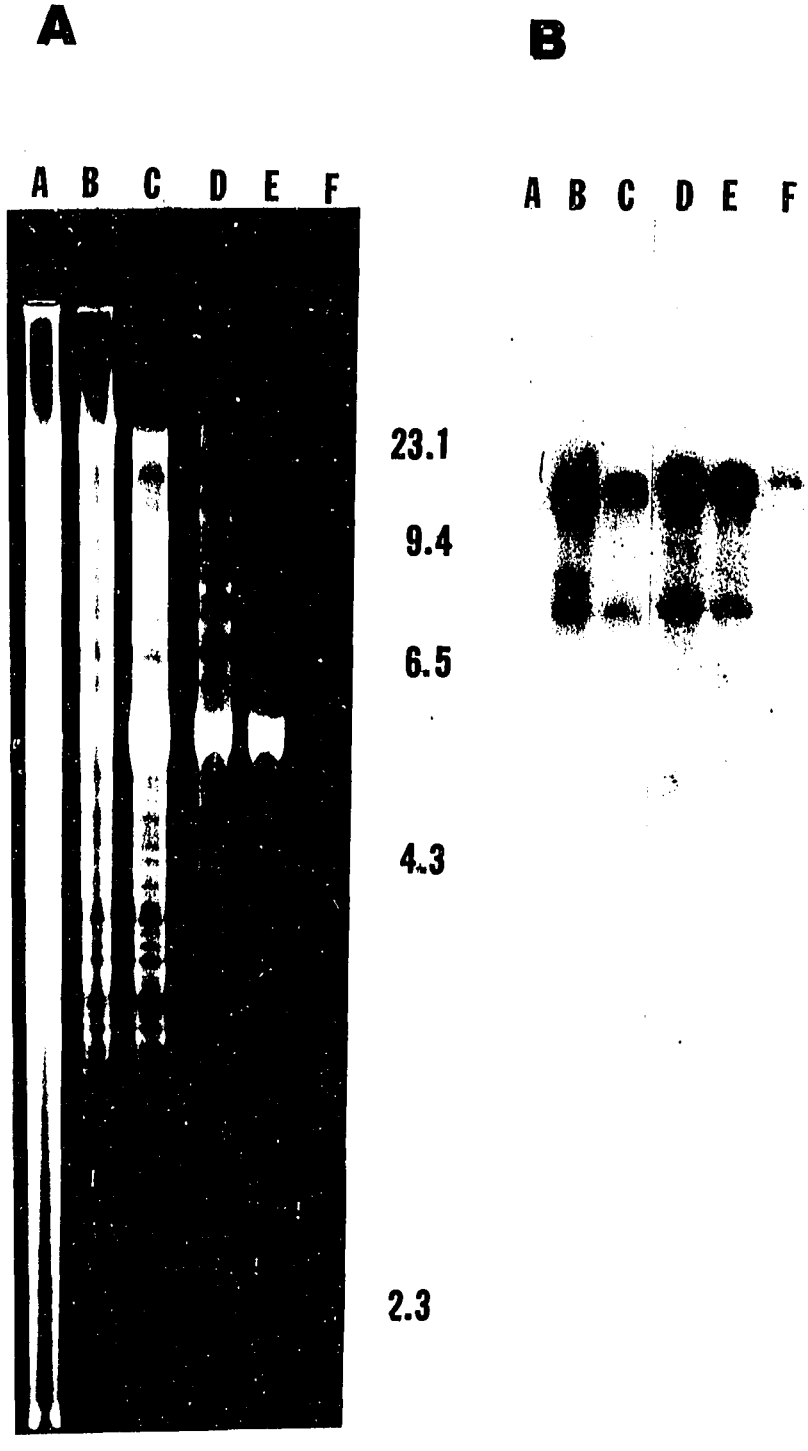
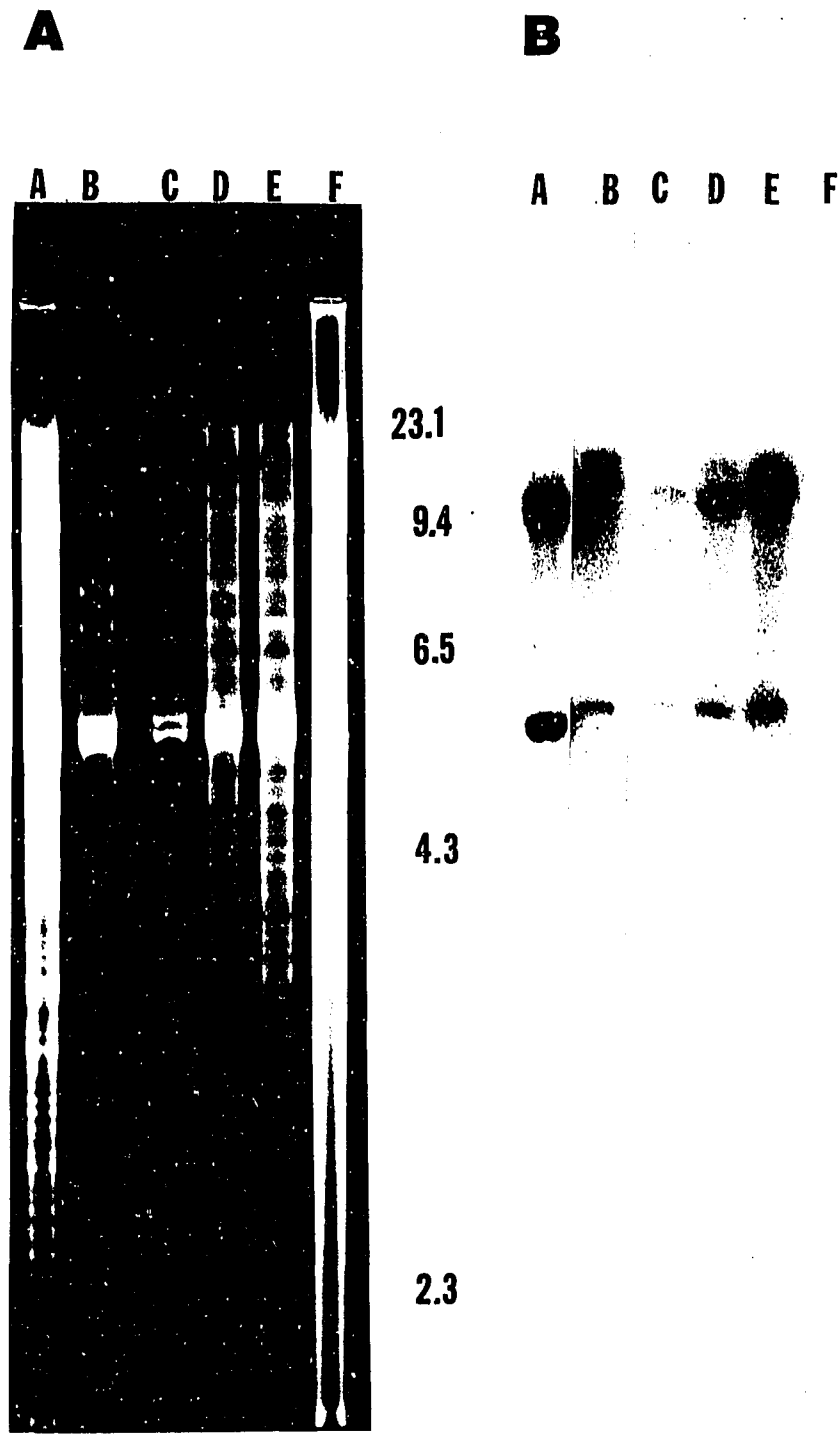

A
A B C D E F
23.1
9.4
6.5
4.3
2.3
B
A B C D E F

Figure 3. Hybridization analysis of Tn916 insertions using ^{32}P-labelled pAM120 DNA to probe HindIII-digested DNA in various B. thuringiensis subsp. israelensis transconjugants. (A) Ethidium bromide-stained 0.7% agarose gel of HindIII-digested DNA. (B) Hybridization analysis of the DNA shown in panel A after transfer to a nylon membrane and probed with ^{32}P-labelled pAM120::Tn916. Lanes (both panels) A, AN-953 Tet^r donor DNA; B-E, AN-146 Tet^r transconjugant DNA; F, AN-146 recipient DNA. Sizes (in kilobase pairs) of DNA HindIII-generated restriction fragment markers are noted between panels A and B


A
B
A B C D E F
23.1
9.4
6.5
4.3
2.3
A B C D E F

HindIII-digested DNA were compared (Figure 2), all the Kan^s Tet^r Rif^r transconjugant DNAs analyzed contained a common 7.8-Kb fragment, whereas, in Figure 3, each of the Kan^s Tet^r Rif^r transconjugant DNAs contained a common 6.3-Kb fragment. Moreover, when the different intra-subspecies transconjugant HindIII-digested DNA profiles are compared with their respective donors, striking similarities are observed between them (Figures 2 and 3). For instance, the transconjugant 7.8-Kb hybridizing fragment is also observed in the AN-950 donor HindIII-digested DNA (Figure 2, lane B), whereas the transconjugant 6.3-Kb fragment is present in the AN-953 donor HindIII-digested DNA (Figure 3, lane A). Regardless of the Tn916 B. thuringiensis subsp. israelensis donor, there was some small variation in the hybridization profiles among the different intraspecies transconjugant HindIII-digested DNAs (Figures 2 and 3).

DISCUSSION

The conjugative S. faecalis transposon Tn916 was introduced into B. thuringiensis subsp. israelensis by using overnight, interspecies, filter matings. The transfer of this transposon required cell-to-cell contact and was resistant to DNase. Therefore, it is likely that the transfer of Tn916 from S. faecalis to B. thuringiensis occurs by a conjugative mechanism (Franke and Clewell, 1981; Clewell and Gawron-Burke, 1986). These observations are similar to those of other interspecies matings; for instance, in Staphylococcus aureus (Jones et al., 1987) and in Listeria monocytogenes (Kathariou et al., 1987).

Several comparisons between the transfer of Tn916 to B. thuringiensis and transfer of this transposon to other genera can be drawn. First, the frequencies of Tn916 transfer into B. thuringiensis subsp. israelensis are similar to those obtained in intraspecies streptococcal matings (Hartley et al., 1984). Second, Southern hybridization analysis has confirmed that Tn916 has inserted into several different sites in the B. thuringiensis subsp. israelensis genome; this diversity has been observed in several other genera (Nida and Cleary, 1983; Jones et al., 1987). Third, molecular DNA homology between Tn916 and the B. thuringiensis genome was not detected; this observation is similar to that seen

with other interspecies transconjugants (Nida and Cleary, 1983; Jones et al., 1987; Kathariou et al., 1987). Finally, the transposon is expressed and stably maintained without selection in B. thuringiensis subsp. israelensis, as is true with other genera (Nida and Cleary, 1983; Jones et al., 1987; Kathariou et al., 1987).

The intra-subspecies transfer of Tn916 occurs in B. thuringiensis, at frequencies approximately 10-fold greater than those observed with its transfer from S. faecalis to B. thuringiensis. Moreover, hybridization analysis demonstrated that, with intra-subspecies matings, there is a limited number of Tn916 insertions in different regions of the B. thuringiensis subsp. israelensis genome compared with the number of insertions observed in the donor. The data in Table 2 suggest that the kanamycin phenotype is not as stably maintained as the tetracycline phenotype. There is approximately a 2.5-fold difference between the total number of cells versus the number of kanamycin-resistant cells. However, when a more accurate assay was used to measure the stability of the Tet^r and Kan^r markers, it was shown that these markers are stably maintained; only one out of 100 became sensitive to kanamycin and all were resistant to tetracycline. Therefore, it is not possible that the AN-146 transconjugants were either AN-950 or AN-953 donors that

became resistant to rifampcin and sensitive to kanamycin.

Twenty-five randomly chosen B. thuringiensis Tet^r transconjugants obtained from intraspecies matings were analyzed by using Southern hybridization, and examples of such experiments are shown in Figures 2 and 3. When the hybridization profiles of these transconjugants and their respective donor were compared, striking similarities were observed. If Tn916 were to insert into the same site of the B. thuringiensis genome, one would expect to see hybridization profiles that are independent of the donor, but this was not the case; the locations of some Tn916 inserts were in the same locations as those of the donor. However, there was some small variation in the hybridization profiles among the different intraspecies transconjugant HindIII-digested DNAs (Figures 2 and 3), which suggest that Tn916 had indeed undergone some movement.

Alternatively, it is possible that the transposon had inserted into a B. thuringiensis cryptic plasmid and that this chimeric plasmid was being transferred from one cell to the other. If Tn916 was on a subsp. israelensis cryptic plasmid, the plasmid-transposon junction fragments would have been of identical size in all transconjugants, and they would have resembled the profile of the donor cells. This explanation is unlikely because: (i) The

pAM120 probe did not hybridize with any of the AN-142 transconjugant plasmid DNA analyzed (Figure 1, lanes G-M). (ii) The Southern hybridization profiles were not exactly identical to the donor profile. Other investigators have analyzed B. thuringiensis plasmid profiles by using a modified Eckhardt procedure (Gonzalez et al., 1981). Therefore, it is still possible that the transposon resides on a cryptic plasmid, but it was not detected with the technique utilized here to obtain DNA.

Another possibility is that homologous recombination could have taken place between the donor and the recipient. Aronson and Beckman (1987) observed low-frequency chromosomal gene transfer of a tryptophan marker from B. thuringiensis subsp. kurstaki HD-1 to B. cereus. The process was resistant to DNase and was not mediated by cell fitrates. Moreover, no detectable transfer of chromosomal genes was found in B. thuringiensis subsp. kurstaki HD-1 strains lacking a 43.5-Kb plasmid that contained a ca. 2.5-Kb region of homology with the chromosome, suggesting that there may be chromosomal mobilization. It is possible that a similar phenomenon could be occurring in B. thuringiensis subsp. israelensis. If chromosomal mobilization was occurring, it would also explain some of the variation observed between the different AN-146 transconjugant hybridization profiles.

Although the data herein strongly suggest self-mobilization of Tn916 among subsp. israelensis strains, such transfer has not been proved. What is needed for the understanding of intraspecies transfer are more stable, easily selectable chromosomal markers. Until they are available, it will be difficult to study the behavior of this transposon in B. thuringiensis subsp. israelensis.

Since the initial discovery of the crystal toxin, many important aspects of its synthesis, expression, and mechanism of action have been studied, but the basic molecular biology of B. thuringiensis is not well understood. The most widely used methods for studying the basic molecular biology among the spore-forming bacilli are transduction and transformation. Generalized transducing phage isolated from various subspecies have been used for preliminary mapping studies (Thorne, 1978; Lecadet et al., 1980; Landen et al., 1981; Heierson et al., 1983; Ruhfel et al. 1984). By using these transducing phage and different subspecies of B. thuringiensis, some data have been obtained, but there are limitations in studying the basic molecular biology of B. thuringiensis by using this form of genetic exchange.

Although transformation of B. thuringiensis protoplasts has been reported (Alikhanian et al., 1981; Martin et al., 1981; Miteva et al., 1981), the efficiency

of transformation was low when compared with those achieved in B. subtilis (Chang and Cohen, 1979). Moreover, when the Staphylococcus aureus plasmid pC194 was used in transformation of B. thuringiensis protoplasts, covalently closed-circle forms could not be detected in the transformants (Martin et al., 1981). The investigators suggested that pC194 integrated into the chromosome (Martin et al., 1981).

The complete nucleotide sequence of Tn916 has not been reported. Yamamoto et al. (1987) utilized Tn5 mutagenesis to study the genetics of Tn916 behavior, and their results are providing structural information of this unique 16.4-Kb transposon. Their studies indicate that most of the region right of the tet^R determinant is required for conjugation. Therefore, it should be possible to remove certain nonessential regions of Tn916, replace them with cloned genes of interest, and introduce the modified form of Tn916 into B. thuringiensis subsp. israelensis by using the methodology described herein.

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SECTION II. INTRODUCTION OF THE *STREPTOCOCCUS FAECALIS* TRANSPOSON TN*916* INTO *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS* AND ITS USE IN COMOBILIZING THE *STAPHYLOCOCCUS AUREUS* RESISTANCE PLASMID pC194

Introduction of the *Streptococcus faecalis* transposon Tn*916* into *Bacillus thuringiensis* subsp. *israelensis* and its use in comobilizing the *Staphylococcus aureus* resistance plasmid pC194

by

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ABSTRACT

The Staphylococcus aureus plasmid, pC194, was introduced into Bacillus thuringiensis subsp. israelensis using the Streptococcus faecalis transposon Tn916 as a mobilizing agent. Transfer of pC194 only occurred when B. thuringiensis subsp. israelensis was mated with a B. subtilis donor containing pC194 and Tn916. B. thuringiensis transconjugants resistant to chloramphenicol and tetracycline were detected at a frequency of 1.96 x 10^{-6} per recipient cell after filter matings. Transconjugants resistant to chloramphenicol were not observed in broth matings. The tetracycline resistance phenotype was maintained during serial passage of B. thuringiensis without antibiotic selection, whereas the chloramphenicol resistance phenotype was not. Southern hybridization analysis demonstrated that Tn916 had inserted into several different sites on the B. thuringiensis chromosome, and pC194 was maintained as an autonomous plasmid in B. thuringiensis genome.

INTRODUCTION

The Gram-positive sporulating bacterium, Bacillus thuringiensis subsp. israelensis, produces insecticidal, proteinaceous crystals that are toxic toward certain diptera larvae (including mosquitoes, black flies, and horn flies), but are relatively nontoxic toward other insects, plants or animals (Undeen and Nagel, 1978; Tyrell et al., 1981; Temeyer, 1984; Margalit and Dean, 1985). The insecticidal properties of B. thuringiensis subsp. israelensis reside in a plasmid-encoded parasporal crystalline protein that is formed only during sporulation of the organism (Aronson and Beckman, 1986). The toxin is composed of at least two separable activities, a broadly cytolytic component (M_R = 28,000), and a highly specific entomocidal component (M_R = 68,000) (Hurley et al., 1985; 1987).

B. thuringiensis has been of interest because of its ability to produce insecticidal toxins during sporulation, but basic genetic studies in B. thuringiensis have been hampered by a lack of efficient and versatile techniques for genetic manipulation in this organism. Transformation of B. thuringiensis protoplasts have been reported (Alikhanian et al., 1981; Martin et al., 1981; Miteva et al., 1981, Fischer et al., 1984) by using methods similar to those developed for B. subtilis (Chang and Cohen,

1979). The Staphylococcus aureus plasmid, pC194 (Iordanescu, 1975), has been transferred to protoplasts of B. thuringiensis subsp. kurstaki, as well as to other subspecies (Martin et al., 1981; Fischer et al., 1984). Further, Alikhanian et al. (1981), using the B. cereus plasmid pBC16, and Miteva et al. (1981), using the S. aureus plasmid pUB110, have transformed B. thuringiensis subsp. galleriae protoplasts. However, in all cases, (i) the efficiency of transformation has been low when compared with those frequencies achieved in B. subtilis (Chang and Cohen, 1979); and (ii), it has been difficult to prepare and regenerate B. thuringiensis protoplasts (Heierson et al., 1987).

The Streptococcus faecalis transposon, Tn916, is probably the best characterized of what have come to be known as "conjugative transposons" (Clewell and Gawron-Burke, 1986). This transposon (16.4 Kb) encodes tetracycline resistance (Tet^r), and is capable of self-transfer, in the absence of plasmid DNA, at frequencies ranging between 10^{-5} to 10^{-8} per donor. Tn916 has been transferred to a number of streptococcal species (Franke and Clewell, 1981; Nida and Cleary, 1983; Wanger and Dunny, 1985; Weiser and Rubens, 1987), B. thuringiensis (Naglich and Andrews, submitted for publication, ca. 1987), S. aureus (Jones et al., 1987), Listeria

monocytogenes (Kathariou et al., 1987), and Mycoplasma hominis (Roberts and Kenny, 1987). DNA hybridization data have demonstrated that Tn916 inserts itself into different sites on the recipient chromosome. The conjugative properties of Tn916 are thought to require cell-to-cell contact, but not to involve a pheromone response because transfer occurs only during filter matings, and not broth matings (Clewell and Gawron-Burke, 1986; Gawron-Burke and Clewell, 1982). Furthermore, the conjugation-like properties of Tn916 do not require intact recombination functions in either parental cell (Franke and Clewell, 1981).

Recently, Naglich and Andrews (submitted for publication, ca. 1987) have demonstrated that Tn916 can be transferred by a conjugation-like process from the chromosome of S. faecalis to B. thuringiensis subsp. israelensis and from the chromosome of transposon-containing B. thuringiensis subsp. israelensis transconjugants to isogenic recipients. Lereclus et al. (1983) transferred a S. faecalis conjugative plasmid, pAM-beta-1, from S. faecalis to several strains of B. thuringiensis. Once in B. thuringiensis, pAM-beta-1 was capable of comobilization of B. thuringiensis plasmids. Therefore, it was of interest to determine if Tn916 was able to induce the movement of a nonconjugative plasmid

from B. subtilis to B. thuringiensis. This paper demonstrates the moblization of pC194 from B. subtilis to B. thuringiensis subsp. israelensis using the S. faecalis transposon Tn916 as the mobilizing agent.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth media

The bacterial strains and plasmids used in this study are described in Table 1. *B. thuringiensis*, *B. subtilis*, and *S. faecalis* were grown on a brain heart infusion medium (BHI; Difco, Detroit, MI) at 30°C. For the isolation of DNA, cultures were grown in Luria-Bertani (LB) broth (Maniatis *et al.*, 1982). Antibiotics used were kanamycin (200 ug/ml), tetracycline (10 ug/ml) and chloramphenicol (10 ug/ml).

Mating protocols

Broth-mating transfer conditions were as those described by Gonzalez *et al.* (1982). To introduce Tn*916* and pC194 into *B. thuringiensis*, filter-mating experiments on nitrocellulose filters (0.45 um, Millipore; Bedford, MA) were done according to the method of Naglich and Andrews (submitted for publication, ca. 1987). Tn*916* was introduced into *B. subtilis* by filter-matings with *S. faecalis* exactly as described for *S. faecalis* to *B. thuringiensis* matings (Naglich and Andrews, submitted for publication, ca. 1987). In all matings, donor and recipient cell controls were separately treated in parallel with the mating mixtures. Transfer frequency was determined by dividing the number of transconjugants by the number of recipient colonies.

Table 1. Designation, characteristics, and origins of bacteria and plasmids used

Strain or Plasmid (Source)	Genotype or Description	Origin or Reference
B. thuringiensis		
AN-142 (LB-2)[a]	*kan*	Naglich and Andrews, ca. 1987
B. subtilis		
AN-503	*trp*, (pC194)	This Study
AN-883	*trp*, (pC194), [Chr::Tn*916*]	This Study
S. faecalis		
CG110 (ISP 871)[b]	[Chr::Tn*916*]	Gawron-Burke and Clewell, 1982
E. coli		
CG120 (ISP 1399)	(pAM120)	Gawron-Burke and Clewell, 1984
S. aureus 8325		
RN2425 (ISP 856)	8325-4 *pig-131*, (pC194)	Goering[c]
Plasmids		
pC194	Cm^r	See ISP 856 above
pAM120	pGL101::Tn*916*	See ISP 1399 above

[a]LB-2 = was provided by Lee A. Bulla Jr., Department of Biotechnology, University of Wyoming, Laramie, Wyoming.

[b]ISP = Peter A. Pattee culture collection, Iowa State University, Department of Microbiology, Ames, Iowa.

[c]Richard V. Goering, Department of Medical Microbiology, Creighton University, Omaha, Nebraska.

Protoplast Transformation

B. subtilis 168 was transformed with pC194 DNA by using the lysozyme-PEG-procedure described by Chang and Cohen (1979). The pC194 DNA was extracted from S. aureus 8325 by using the alkaline denaturation procedure of Birnboim and Doly (1979).

Assessment of stability

The stability of either the tetracycline or chloramphenicol resistance markers in B. thuringiensis subsp. israelensis transconjugants was determined by serial passage in nonselective media. The transconjugants were first isolated on BHI agar containing either tetracycline, chloramphenicol, or both antibiotics. After overnight incubation at 30°C, several colonies were randomly selected; each was inoculated into BHI (100 ml), and grown overnight at 30°C with vigorous shaking. Serial dilutions of these cultures were spread onto BHI agar, with and without selection, and the total number of colonies that grew on a selective medium were compared to the number that grew on a nonselective medium.

DNA purification

Total B. subtilis and B. thuringiensis DNA was obtained by using the cell lysate procedure described by Naglich and Andrews (submitted for publication, ca. 1987).

Plasmid DNA was extracted from E. coli CG120 by using

the alkaline denaturation procedure of Birnboim and Doly (1979), whereas plasmid DNA from S. aureus or B. subtilis was obtained by using the alkaline denaturation procedure of Birnboim and Doly (1979) with the following modification: Cells in logarithmic growth (A_{600}=0.6) were harvested by centrifugation and the pellets were resuspended in TEG [10mM Tris-HCl, pH 7.5 - 50mM EDTA - 1% (w/v) glucose)] buffer containing the appropriate enzyme [lysozyme (2 mg/ml) for B. subtilis; lysostaphin (0.4 mg/ml) for S. aureus]. Cell suspensions were lysed in a 1% (w/v) sodium dodecyl sulfate-0.2 N NaOH-TEG solution, and deproteinized with two chloroform:isoamyl alcohol extractions. After the final extraction, the DNA mixture was treated according to the method of Birnboim and Doly. All plasmid preparations were further purified on CsCl-ethidium bromide gradients (Maniatis et al., 1982).

Preparation and analysis of DNA restriction fragments

Digestion with the restriction enzymes, HaeIII or HindIII, were performed using the conditions described by the supplier (Bethesda Research Laboratories, Gaithersburg, MD). Restriction enzyme digests and plasmid samples were analyzed by agarose gel electrophoresis [0.7% (w/v)] (Maniatis et al., 1982). After electrophoresis, gels were stained with ethidium bromide, and the DNA was visualized on a UV transilluminator.

Southern hybridization analysis

DNA was transferred from the agarose gel to Hybond N (TM) (0.45 um, Amersham Inc., Boston, MA) according to the method of Southern (1975). Plasmid DNA was labelled with ^{32}P-dATP by using a nick translation kit from Bethesda Research Laboratories, Gaithersburg, MD. Hybridization with the labelled probe was carried out in 50% (v/v) formamide in 0.1% (v/v) SSC (1X SSC is 0.15 M NaCl-0.015 M sodium citrate, pH 7.0) for 24 hours at 42°C. The posthybridization washes and detection of the ^{32}P-labelled probe were performed as recommended by Bethesda Research Laboratories (Gaithersburg, MD).

^{32}P-labelled DNA probes were stripped off nylon membranes using 0.1 X SSC-0.1% SDS (SB) buffer. Boiling (100°C) SB buffer was poured over the membrane and the membrane was incubated at 65°C for fifteen minutes. This procedure was repeated three times. To insure that the ^{32}P-labelled probe had been removed from the membrane, the membrane was exposed to a piece of XAR-5 x-ray film (Kodak, Rochester, NY) overnight. The membrane was rehybridized using a different ^{32}P-labelled probe.

RESULTS

B. subtilis protoplasts were transformed with pC194 to construct *B. subtilis* strain AN-503 (data not shown). When AN-503 was used as the recipient in overnight membrane filter matings with *S. faecalis* CG110, Tn*916* was introduced into AN-503 with an average frequency of 10^{-6} per recipient cell. AN-503 transconjugants were resistant to tetracycline at concentrations of at least 50 ug/ml, and chloramphenicol at concentrations of at least 20 ug/ml. One AN-503 transconjugant, AN-883, was randomly chosen to be used as the donor of Tn*916* and pC194. Southern hybridization analysis demonstrated that Tn*916* was introduced into the chromosome of AN-883 (Figure 1B, lane B), and pC194 was present (Figure 1C, lane B).

To determine whether Tn*916* could mobilize the nonconjugative pC194 plasmid into *B. thuringiensis* subsp. *israelensis*, AN-142 (a *B. thuringiensis* subsp. *israelensis* mutant resistant to kanamycin, Table 1) was mated with *B. subtilis* AN-883. An example of such a mating is shown in Table 2. The concomitant transfer of the Tn*916* and pC194 phenotypes into AN-142 occurred at a frequency of 1.96×10^{-6} under simultaneous selection with tetracycline and chloramphenicol (Table 2). However, with tetracycline selection, the transfer of the Tn*916* phenotype into AN-142 occurred at a frequency of 1.09×10^{-4} per recipient cell

Figure 1. Hybridization analysis of B. thuringiensis subsp. israelensis transconjugant HaeIII-digested DNA. (A) Ethidium bromide-stained 0.7% agarose gel of HaeIII-digested DNA. (B) Hybridization analysis of the DNA shown in panel A after transfer to a nylon membrane and probed with ^{32}P-labelled pAM120::Tn916. (C) Hybridization analysis of the same DNA contained on the nylon membrane used in panel B. The ^{32}P-labelled pAM120::Tn916 probe was removed from the membrane, and the transconjugant DNA was hybridized with a ^{32}P-labelled pC194 probe. Lanes (all panels) A, AN-142 total parental cesium chloride purified DNA; B, total AN-883 Cm^r/Tet^r cesium chloride purified DNA; C-D, AN-142 total Cm^r/Tet^s cesium chloride purified transconjugant DNA; E-F, AN-142 Cm^s/Tet^r cesium chloride purified transconjugant DNA; G-J, AN-142 Cm^r/Tet^r cesium chloride purified transconjugant DNA. Sizes (in kilobase pairs) of DNA HaeIII-generated restriction fragment markers are noted between panels A - B and B - C

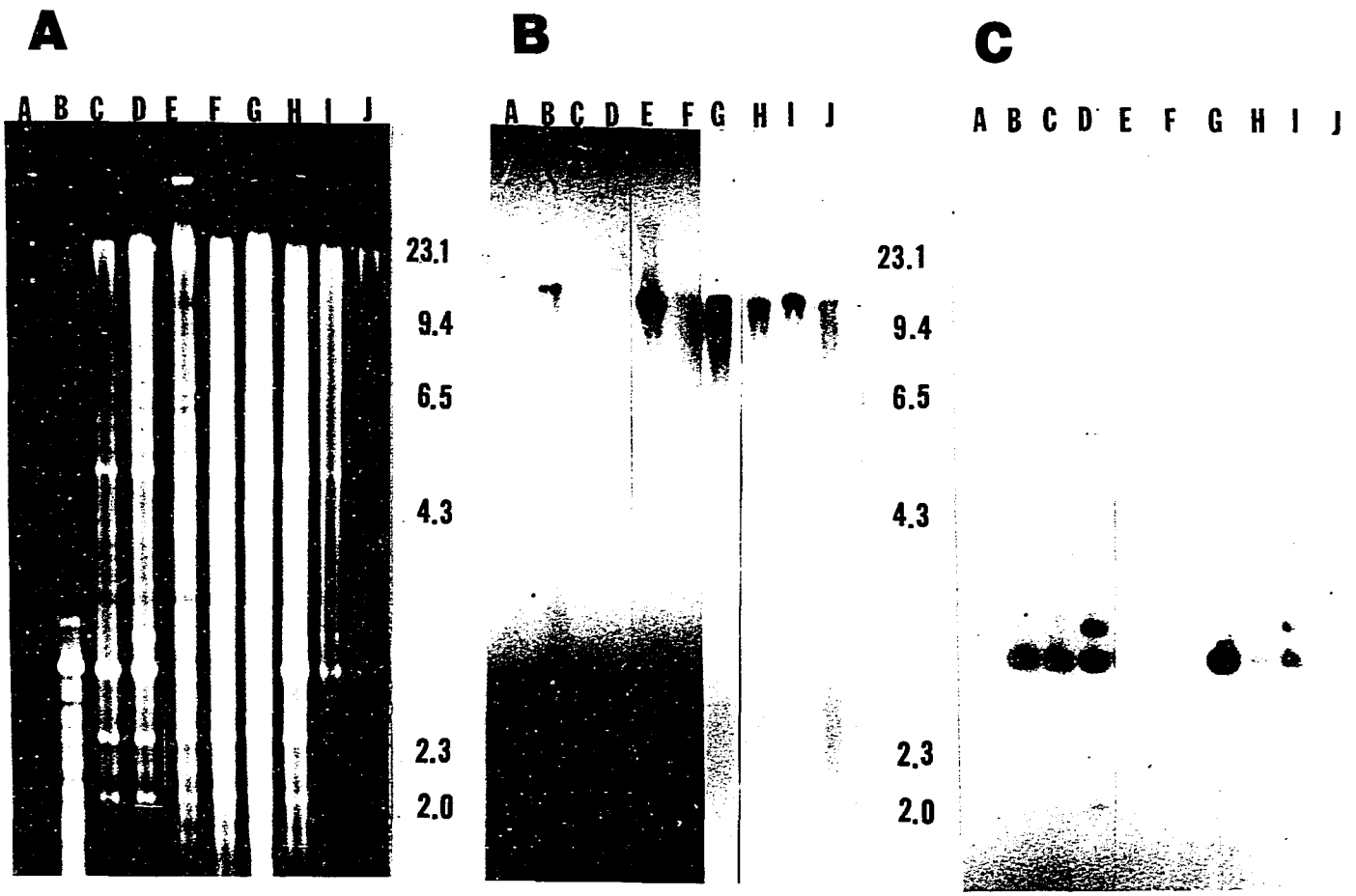

A
A B C D E F G H I J
23.1
9.4
6.5
4.3
2.3
2.0
B
A B C D E F G H I J
23.1
9.4
6.5
4.3
2.3
2.0
C
A B C D E F G H I J

Table 2. Transfer of Tn916 and pC194 from Bacillus subtilis to Bacillus thuringiensis subsp. israelensis

Filter Mating[a]		Number of transconjugants/ml	Frequency/ recipient[b]
Donor[c]	Selection[d]		
Tet/Cm	Tc	8.85×10^3	1.09×10^{-4}
	Cm	2.37×10^3	2.94×10^{-5}
	Tc,Cm	1.58×10^2	1.96×10^{-6}
Cm	Tc	-	$< 5.00 \times 10^{-8}$[e]
	Cm	-	$< 5.00 \times 10^{-8}$[e]

Broth Mating[a]		Number of transconjugants/ml	Frequency/ recipient[b]
Donor[c]	Selection[d]		
Cm	Tc	-	$< 5.00 \times 10^{-8}$[e]
	Cm	-	$< 5.00 \times 10^{-8}$[e]

[a] See text.

[b] Kanamycin resistant Bacillus thuringiensis subsp. israelensis (AN-142) recipient.

[c] Tet/Cm = Bacillus subtilis AN-883 donor; Cm = Bacillus subtilis AN-503 donor.

[d] Tc = tetracycline Cm = chloramphenicol.

[e] Transfer not detected.

(Table 2). When these tetracycline resistant (Tet^r) AN-142 transconjugants were replicate-plated onto a chloramphenicol containing medium, approximately 99% of the Tet^r putative transconjugants were sensitive to chloramphenicol. When selection was with only chloramphenicol, the transfer of the pC194 phenotype into AN-142 occurred at a frequency of 2.94×10^{-5} (Table 2). Approximately 90% of these chloramphenicol resistant (Cm^r) AN-142 transconjugants were sensitive to tetracycline when replicate-plated onto a tetracycline containing medium. The AN-142 transconjugants were resistant to tetracycline at concentrations of at least 50 ug/ml, or chloramphenicol up to 20 ug/ml.

The transfer of pC194 evidently requires the presence of the Tn916 transposon because transfer of the pC194 phenotype was only observed when B. thuringiensis subsp. israelensis was mated on membrane filters with a B. subtilis donor containing pC194 and Tn916 (Table 2). Transfer of the pC194 phenotype was not observed with broth matings or with a B. subtilis donor containing pC194 (Table 2). Tetracycline or chloramphenicol resistance in AN-142 parents was not observed under these conditions.

To confirm the transfer of Tn916 from B. subtilis to B. thuringiensis subsp. israelensis, total DNA was extracted from six randomly selected Tet^r transconjugants,

and analyzed by Southern hybridization. Tn916 does not contain a HaeIII site (Clewell and Gawron-Burke, 1986). When HaeIII-digested AN-142 Tet^r transconjugant DNA was probed with Tn916, only a single band appeared in each Tet^r transconjugant (Figure 1B, lanes E-J). The single hybridizing band observed in HaeIII-digested AN-142 transconjugant DNAs were large (17 to 20 Kb), and presumably consisted of the entire transposon (16.4 Kb) and flanking B. thuringiensis subsp. israelensis DNA sequences. A single band was also observed in the HaeIII-digested B. subtilis donor DNA (Figure 1B, lane B). These data confirm that the Tet^r phenotype corresponds with the presence of Tn916 in the genome because the Tet^s/Cm^r transconjugant DNA (Figure 1B, lanes C-D) did not hybridize with the probe. Moreover, total AN-142 DNA (Figure 1B, lane A) did not hybridize with the probe indicating that there is no homology between AN-142 DNA and the Tn916 probe.

Hybridizations with HindIII-digested AN-142 Tet^r transconjugant DNA were used to determine the number of Tn916 insertions in the B. thuringiensis subsp. israelensis genome. Because Tn916 contains only one internal HindIII site (Gawron-Burke and Clewell, 1982), two hybridizing fragments would be expected for each Tn916 insert. Therefore, if Tn916 was site specific, digestion

of the chromosome with HindIII would always yield two fragments which would be identical in size in all of the examined transconjugants. However, Figure 2B (lanes C-H) shows that Tn916 inserted into several different sites in the B. thuringiensis subsp. israelensis genome because two unique hybridizing fragments were observed in all of the Tet^r transconjugants. Tet^s/Cm^r transconjugant DNA (Figure 2B, lanes A-B) did not hybridize with the probe which confirms the data in the previous experiment.

Total DNA was extracted from six Cm^r AN-142 transconjugants. When the DNA profiles of these Cm^r transconjugants were compared with the AN-142 parent, a new band of similar size to pC194 was present (Figure 3A, lanes C-D, G-J). This band was not seen in the Tet^r/Cm^s AN-142 transconjugants (Figure 3A, lanes E-F). To prove that pC194 was in these Cm^r transconjugants, Southern hybridization analysis was performed. DNA bands in the Cm^r transconjugants (Figure 3B, lanes C-D, G-J) hybridized to the pC194 probe. Because pC194 contains a single HindIII and HaeIII site (Horinouchi and Weisblum, 1982), the same nylon membranes used in the previous experiments (Figures 1B and 2B) were stripped of the Tn916 probe, and hybridized with nick-translated pC194. Figures 1C (lanes C-D, G-J) and 2C (lanes A-B, E-H) show that the probe hybridized with pC194 in the B. thuringiensis genome. The

Figure 2. Hybridization analysis HindIII-digested DNA from various B. thuringiensis subsp. israelensis transconjugants. (A) Ethidium bromide-stained 0.7% agarose gel of HindIII-digested DNA. (B) Hybridization analysis of the DNA shown in panel A after transfer to a nylon membrane and probed with ^{32}P-labelled pAM120::Tn916. (C) Hybridization analysis of the same DNA contained on the nylon membrane used in panel B. The ^{32}P-labelled pAM120::Tn916 probe was removed from the membrane (see text), and then the transconjugant DNA was hybridized with a ^{32}P-labelled pC194 probe. Lanes (all panels) A-B, AN-142 total Cm^r/Tet^s cesium chloride purified transconjugant DNA; C-D, AN-142 Cm^s/Tet^r cesium chloride purified transconjugant DNA; E-H, AN-142 Cm^r/Tet^r cesium chloride purified transconjugant DNA. Sizes (in kilobase pairs) of DNA HindIII-generated restriction fragment markers are noted between panels A - B and B - C

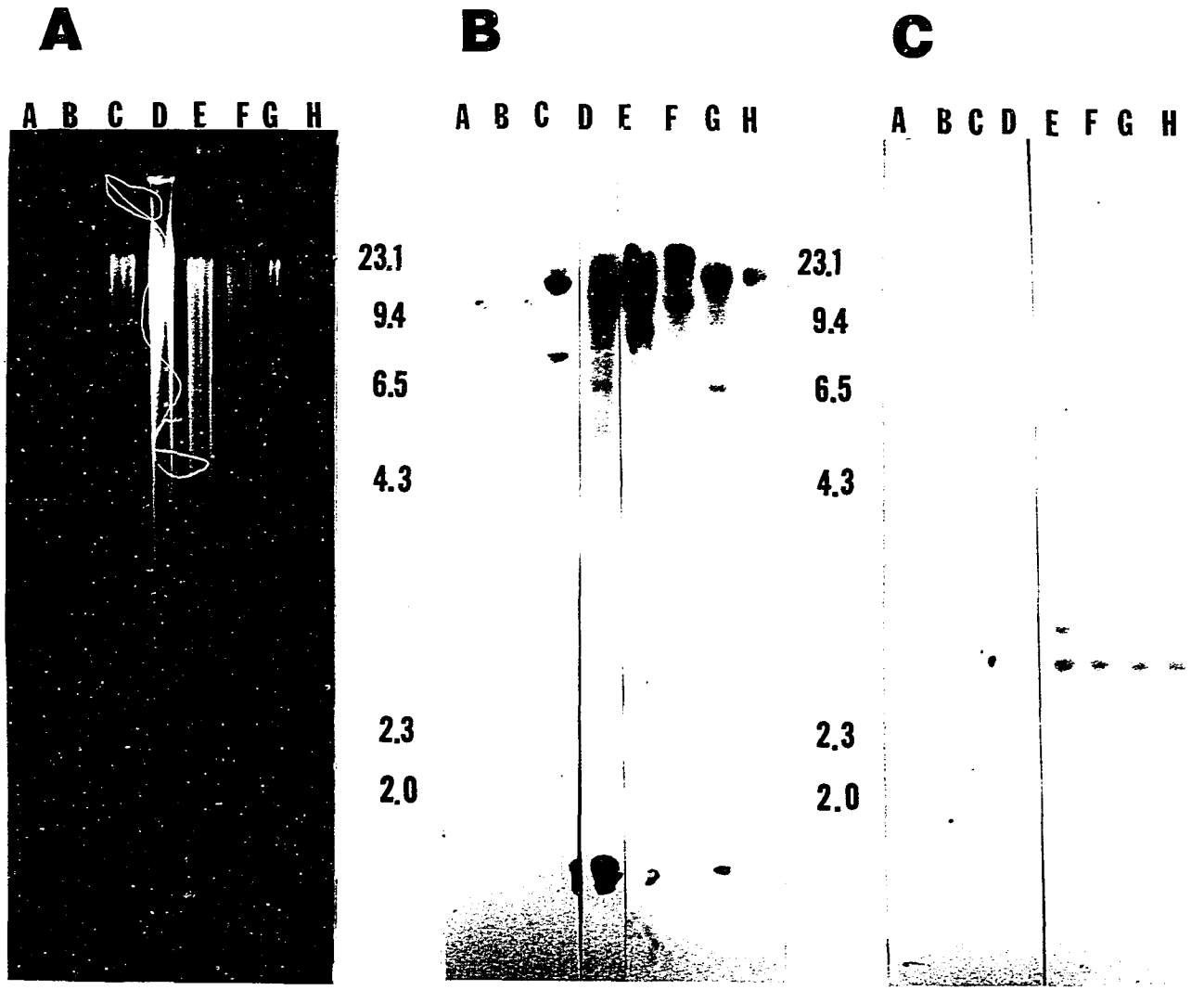
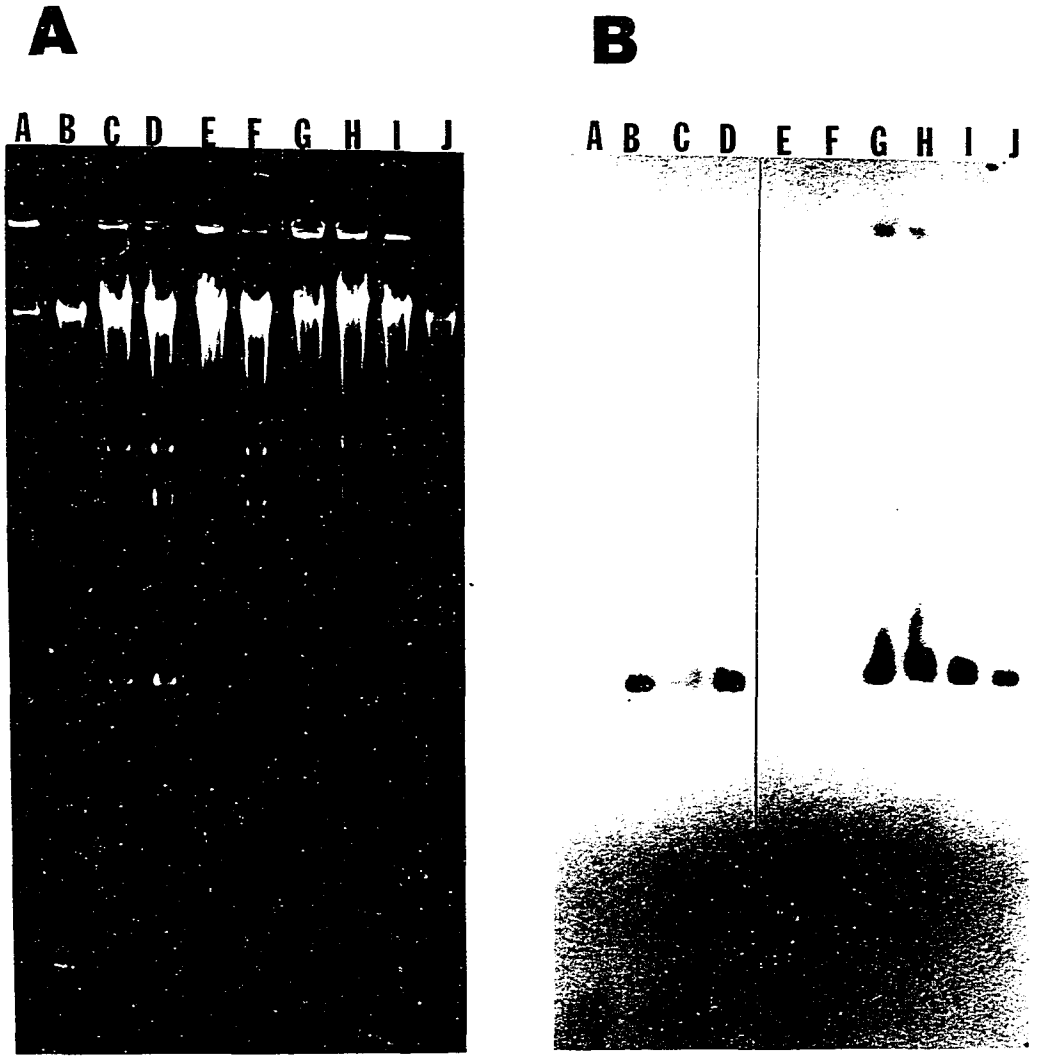

A
A B C D E F G H
B
A B C D E F G H
23.1
9.4
6.5
4.3
2.3
2.0
C
A B C D E F G H
23.1
9.4
6.5
4.3
2.3
2.0

Figure 3. Hybridization analysis of B. thuringiensis subsp. israelensis transconjugant DNA using a ^{32}P-labelled pC194 probe. (A) Ethidium bromide-stained 0.7% agarose gel of B. thuringiensis subsp. israelensis DNA. (B) Hybridization analysis of the DNA shown in panel A after transfer to a nylon membrane and probed with ^{32}P-labelled pC194. Lanes (both panels) A, AN-142 total parental cesium chloride purified DNA; B, total AN-883 Cm^r/Tet^r cesium chloride purified DNA; C-D, AN-142 total Cm^r/Tet^s cesium chloride purified transconjugant DNA; E-F, AN-142 Cm^s/Tet^r cesium chloride purified transconjugant DNA; G-J, AN-142 Cm^r/Tet^r cesium chloride purified transconjugant DNA


A
A B C D E F G H I J
B
A B C D E F G H I J

location of the hybridizing bands corresponded to the reported molecular weight of pC194. Further, Figure 1C shows that the bands which hybridized to the pC194 probe were in the same location as the pC194 band in the B. subtilis donor (lane B). Total Cm^S AN-142 HaeIII-digested DNA (Figure 1C, lanes A, E-F) or HindIII-digested DNA (Figure 2C, lanes C-D) did not hybridize with the probe indicating that: (i) there is a lack of homology between the pC194 probe and B. thuringiensis, (ii) pC194 was not present in these transconjugants, and (iii) that the Cm^S phenotype is not due to partial transfer of pC194, or to a rearrangement of the pC194 DNA once it entered B. thuringiensis.

Tn916 and pC194 were not on the same DNA hybridizing fragment in either B. subtilis or B. thuringiensis (Figures 1 and 2). In no case, when the same nylon membrane was probed with two different probes, were bands of identical mobilities observed that hybridized with both probes.

To determine the stability of the tetracycline and chloramphenicol phenotypes, two randomly selected Tet^r and Cm^r subsp. israelensis transconjugants were grown in a nonselective liquid medium. After growth, the cells were plated onto media that contained chloramphenicol, tetracycline, or both antibiotics. The fraction of cells

resistant to either or both antibiotics were determined in the resulting cultures. The experiments were performed in duplicate on three different occasions. Because comparable numbers of colony forming units appeared on a medium containing tetracycline and on the same medium without tetracycline, the Tet^r phenotype was relatively stable (Table 3). However, after the first passage in nonselective media, the number of transconjugants resistant to chloramphenicol (AN-1240 and AN-1245) decreased 500-fold when compared with those obtained on the same medium without chloramphenicol. When cells from this nonselective culture were subjected to a second pass through a nonselective medium, without an intermediate selection step, these ratios did not change. Further, the number of transconjugants resistant to both tetracycline and chloramphenicol (AN-1270 and AN-1275) decreased 100-fold. Therefore, it is apparent that the Cm^r phenotype is not stable, but in spite of this initial decrease, the number of transconjugants resistant to chloramphenicol (AN-1240 and AN-1245) or chloramphenicol and tetracycline (AN-1270 and AN-1275), were maintained throughout the subsequent serial passages.

Two randomly selected Tet^r and Cm^r AN-1270 and AN-1275 isolates were obtained from a selective medium after their first passage in a nonselective medium. These

Table 3. Stability of the Tn916 or pC194 phenotype in representative Bacillus thuringiensis subsp. israelensis transconjugants during serial passage in nonselective medium

Transconjugant (Phenotype[b])	Selection[c]	Number of colony forming units x 10^7 per milliliter		
		Pass number[a]		
		1	2	3
AN-1240 (Cm^r,Tet^s)	-	24.6	12.4	15.3
	Tc	0.00	0.00	0.00
	Cm	0.15	0.54	0.17
AN-1245 (Cm^r,Tet^s)	-	12.9	13.9	15.7
	Tc	0.00	0.00	0.00
	Cm	0.19	0.03	0.07
AN-1250 (Cm^s,Tet^r)	-	11.2	9.25	24.7
	Tc	16.8	10.7	22.6
	Cm	0.00	0.00	0.00
AN-1255 (Cm^s,Tet^r)	-	15.2	15.2	12.5
	Tc	18.0	12.3	10.9
	Cm	0.00	0.00	0.00
AN-1270 (Cm^r,Tet^r)	-	10.5	7.75	11.6
	Tc	13.1	8.70	13.3
	Cm	0.05	0.03	0.07
	Cm, Tc	0.68	0.22	0.49
AN-1275 (Cm^r,Tet^r)	-	11.1	15.0	14.6
	Tc	12.1	11.5	10.2
	Cm	0.10	0.05	0.09
	Cm, Tc	0.39	0.15	0.64

[a]Each experiment represents two complete sets of experimental data for each transconjugant. The pass number represents a serial transfer of each transconjugant.

[b]Cm = chloramphenicol; Tet = tetracycline. Overnight broth cultures were each serially diluted and plated on media with and without selection after incubation in brain heart infusion broth.

[c]Cm = chloramphenicol; Tc = tetracycline.

transconjugants were again passed in a nonselective medium and plated onto selective media to determine the stability of the Tet^r and Cm^r phenotypes (Table 4). Because the same number of transconjugants appeared on media with and without tetracycline, the Tet^r phenotype was stably maintained. Interestingly, the Cm^r phenotype was not as stable because there was a 500-fold decrease in the number of transconjugants resistant to chloramphenicol compared to those grown on a medium without chloramphenicol (Table 4).

To determine if the pC194 plasmid was still intact after serial passage in a nonselective medium, total DNA was obtained from AN-1270 and AN-1275 after each serial passage. Total DNA obtained from AN-1270 and AN-1275, grown in a selective medium, is shown in lanes A and F (Figure 4). After one serial passage in a nonselective medium, the pC194 replicon is indeed intact in AN-1270 (Figure 4, lanes B-C) and AN-1275 (Figure 4, lanes G-H). Moreover, when AN-1270 and AN-1275 were passed one time in a nonselective medium, and two randomly selected transconjugants were obtained on a selective medium, and again passed in a nonselective medium, the pC194 replicon was still intact (Figure 4, lanes D-E, I-J).

Table 4. Stability of the Tn916 and pC194 phenotype in representative chloramphenicol- and tetracycline-resistant Bacillus thuringiensis subsp. israelensis transconjugants during a single passage in non-selective medium

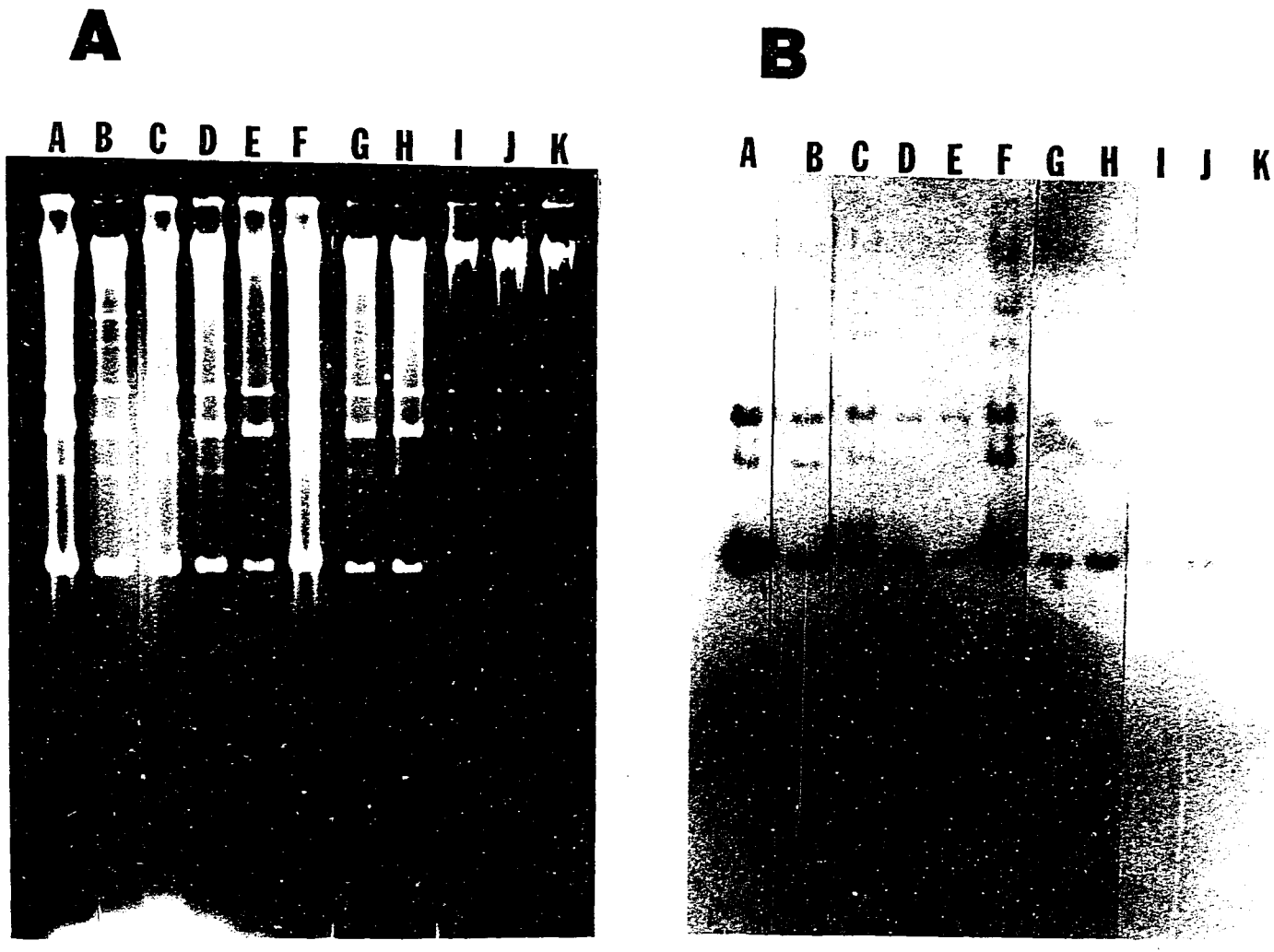
Transconjugant	Number of colony forming units x 10^7 per milliliter[a]			
	Selection[b]			
	-	Tc	Cm	Cm, Tc
AN-1270 (Parent)[c]	13.5	8.75	0.11	0.14
AN-1270-1	5.27	3.80	0.05	0.15
AN-1270-2	10.8	12.9	0.10	0.13
AN-1270-3	4.10	5.55	0.03	0.07
AN-1275 (Parent)[c]	12.9	5.56	0.14	0.06
AN-1275-1	6.53	4.65	0.05	0.05
AN-1275-2	9.98	7.08	0.09	0.04
AN-1275-3	6.80	8.01	0.06	0.14

[a]Each experiment represents two complete sets of experimental data for each transconjugant.

[b]Cm = chloramphenicol; Tc = tetracycline.

[c]AN-1270 and AN-1275 were grown overnight in brain heart infusion broth without selection. The cultures were each serially diluted and plated on media with and without selection. Three representative colonies from each parent were obtained from the chloramphenicol/tetracycline brain heart infusion agar medium and were grown in nonselective brain heart infusion broth. Overnight broth cultures were each serially diluted and plated on media with and without selection.

Figure 4. Hybridization analysis of Cm^r/Tet^r B. thuringiensis subsp. israelensis transconjugant DNA after serial passage in a nonselective medium. (A) Ethidium bromide-stained 0.7% agarose gel of B. thuringiensis subsp. israelensis DNA. (B) Hybridization analysis of the DNA shown in panel A after transfer to a nylon membrane and probed with ^{32}P-labelled pC194. Lanes (both panels) A, AN-1270 total DNA; B-C, AN-1270 total DNA obtained after one passage in a nonselective medium; D-E, AN-1270 total DNA obtained from two randomly selected transconjugants that were passed one time in a nonselective medium, then isolated on selective media and again passed in a nonselective medium; F, AN-1275 total DNA; G-H, AN-1275 total DNA obtained after one passage in a nonselective medium; I-J, AN-1275 total DNA obtained from two randomly selected transconjugants that were passed one time in a nonselective medium, then isolated on selective media and again passed in a nonselective medium; K, AN-142 total parental DNA.


A
A B C D E F G H I J K
B
A B C D E F G H I J K

DISCUSSION

The conjugative S. faecalis transposon Tn916 and S. aureus plasmid pC194 were introduced into B. thuringiensis subsp. israelensis by using overnight filter matings with B. subtilis. The B. subtilis donor was constructed by using the Chang and Cohen (1979) protoplast transformation system to first introduce the S. aureus plasmid pC194. Tn916 was then introduced into the B. subtilis, containing pC194, by a membrane mating with S. faecalis. Reciprocal experiments where Tn916 was first introduced followed by protoplast transformation have not been performed, so it is not known whether the same results obtained herein would occur.

The frequency of Tn916 transfer into B. subtilis is what would be expected; it is approximately equal to that obtained in intraspecies streptococcal matings (Hartley et al., 1984; Weiser and Rubens, 1987), and in other genera (Naglich and Andrews, submitted for publication, ca. 1987; Roberts and Kenny, 1987). Southern hybridization analysis suggest that there is a single insert of Tn916 in the Tet^r B. subtilis transconjugant, AN-883 (Figure 1B, lane B), but it is unknown whether this insertion affects any AN-883 phenotype.

When B. thuringiensis subsp. israelensis (AN-142) was mated with AN-883, transfer of the Tn916 and pC194

phenotypes occurred at a frequency of 1.96×10^{-6} when simultaneously selecting for resistance to tetracycline and chloramphenicol (Table 2). Under chloramphenicol selection, the transfer of the pC194 phenotype occurred at a frequency of 2.94×10^{-5} (Table 2). Interestingly, when the AN-142 transconjugants were only selected for tetracycline resistance, the frequency of Tn916 transfer was 100-fold greater than that observed with tetracycline and chloramphenicol selection (Table 2), and 1000-fold greater than that observed when B. thuringiensis is mated with S. faecalis (Naglich and Andrews, submitted for publication, ca. 1987). There could be several explanations for the higher frequency of Tn916 transfer. First, once Tn916 is maintained and expressed in B. subtilis, it could be modified such that it is not recognized as being totally foreign in B. thuringiensis. Second, all of the matings took place on a BHI medium versus a nutrient agar medium (Naglich and Andrews, submitted for publication, ca. 1987); there could be some component in BHI which enhances/promotes the transfer of Tn916. Third, it has been suggested that Tn916 could be transferred from B. thuringiensis subsp. israelensis to isogenic recipients (Naglich and Andrews, submitted for publication, ca. 1987); because all of the matings took place overnight on a nonselective medium, it is not known

whether Tn916, once in B. thuringiensis, was capable of transfer from one B. thuringiensis to another before the cells were plated onto selective media. Fourth, Franke and Clewell (1981) found in S. faecalis that Tn916 was transferred at a low frequency in the absence of plasmids and that its transfer increased in the presence of the conjugative hemolysin plasmid pAD1. Moreover, similar results were observed using pAD1 and the S. agalactiae transposon Tn3951 (Smith and Guild, 1982; Vijayakumar et al., 1986). Because B. thuringiensis has been shown to transfer plasmids by conjugation it may be possible that a similar phenomenon is occurring in B. thuringiensis. Finally, it is possible that the transfer functions encoded by Tn916 cause a cell-to-cell bridge to form. Once the bridge is formed, the DNA contained within the cell is free to independently move across the bridge at a rate controlled by either its origin, size or conformation. Therefore, it is possible that Tn916 is capable of transferring across the bridge at a greater rate than does pC194.

The transfer of the pC194 phenotype only occurred when: (i) AN-142 was mated with AN-883, and not AN-503 (Table 2) and, (ii) membrane filter matings were used (Table 2). The mechanism is not understood, but the data indicate that the movement of pC194 requires the presence

of the transposon, and cell-to-cell contact. However, the data also show independent transfer of either pC194 or Tn916 (Table 2). Not all of the Tet^r AN-142 transconjugants (99%) were resistant to chloramphenicol, and not all of the Cm^r AN-142 transconjugants (90%) were resistant to tetracycline. More importantly, no Cm^s/Tet^r B. thuringiensis transconjugant DNA hybridized to the pC194 probe which shows that the Cm^s phenotype is not due to partial transfer of the pC194 replicon, or to a rearrangement of the DNA after it enters B. thuringiensis. Further, no molecular DNA homology between Tn916 and the Cm^r/Tet^s B. thuringiensis transconjugants was detected; this confirms that Tn916-like DNA sequences were not present at all in these transconjugants.

These data suggest two possible mechanisms by which pC194 could be transferred into B. thuringiensis. First, the presence of a gene(s) carried on Tn916 could promote the transfer of pC194. However, this possibility would not explain why the Cm^r/Tet^s transconjugants were isolated; even if there was partial transfer of Tn916 which became either deleted or rearranged once in B. thuringiensis, these sequences were not detected in the Cm^r/Tet^s transconjugants (Figures 1 and 2). Alternatively, a cointegrate could have formed between Tn916 and pC194 because there have been reports describing

certain recombination sites (RS) found on several S. aureus plasmids (Gennaro et al., 1987; Novick et al., 1984). If the cointegrates were stably maintained in the AN-142 transconjugants it would have been expected to find: (i) the same transconjugant DNA band hybridizing to both probes, but these bands were not observed (Figures 1 and 2), and (ii) all of the transconjugants to be resistant to chloramphenicol and tetracycline, but Cm^r/Tet^s and Cm^s/Tet^r transconjugants were isolated. It is still possible that a cointegrate could form between Tn916 and pC194 before its transfer into B. thuringiensis, but once this cointegrate was transferred into B. thuringiensis, it would dissociate into two separate entities. This could explain why there were Cm^r/Tet^s and Cm^s/Tet^r AN-142 transconjugants, but these cointegrates were not detected in the AN-883 donor (Figure 1, lane B).

Table 3 shows that the tetracycline resistant transconjugants (AN-1250, AN-1255, AN-1270 and AN-1275) expressed and stably maintained the Tn916 phenotype because comparable numbers of colonies appeared on a medium containing tetracycline and on the same medium without tetracycline. This observation is very similar to that seen with other interspecies transconjugants (Nida and Cleary, 1983; Jones et al., 1987; Kathariou et al., 1987; Naglich and Andrews, submitted for publication, ca.

1987). However, the chloramphenicol resistant transconjugants (Table 3, AN-1240 and AN-1245) did not stably maintain the pC194 phenotype because after the first passage; there was approximately a 500-fold reduction in the number of colonies on the chloramphenicol medium compared to the number of colonies on the a nonselective medium. Moreover, there was approximately a 100-fold reduction in the number of transconjugants that were resistant to both antibiotics (Table 3, AN-1270 and AN-1275). In spite of the initial decrease in the number of transconjugants resistant to chloramphenicol, the fraction of cells resistant to chloramphenicol, after this decrease, were maintained throughout subsequent serial passages.

One possible explaination for loss of a plasmid borne phenotype would be that pC194 was incompatible with the native B. thuringiensis plasmids. Plasmid incompatibility, however, would not explain why 1% of the cells stably maintained the plasmid phenotype. To investigate the nature of this stability, chloramphenicol resistant B. thuringiensis subsp. israelensis colonies were selected immediately after passage in a nonselective medium. These colonies again exhibited the same instabilities as did the original transconjugants (Table 4). The DNA profiles of these transconjugants were

compared with each other, and Figure 4 shows that the stable fraction of transconjugants did not result from a change in pC194 to yield a stable phenotype, nor did these transconjugants result from integration of the *cat* gene into the *B. thuringiensis* genome.

Alternatively, *B. thuringiensis* has been shown to exchange plasmids by conjugation (Gonzalez *et al.*, 1982). Therefore, it is possible that in a nonselective medium pC194 is being lost at a constant rate. At the same time it is possible that pC194 is being transferred by a conjugation-like mechanism to a new *B. thuringiensis* host, i.e., the reverse of the plasmid-loss process. These two phenomena may balance each other out and an equilibrium is established.

When *Hind*III-digested, or *Hae*III-digested Cm^r AN-142 DNA was probed with a nick-translated pC194, the plasmid was detected in an intact form in the *B. thuringiensis* genome because pC194 contains a single restriction site for these enzymes. Moreover, hybridization analysis shows that Tn*916* has inserted into several different sites in the *B. thuringiensis* subsp. *israelensis* genome when it was mated with *B. subtilis*. Although the data herein reveals mobilization of pC194 into *B. thuringiensis*, the mechanism(s) need to be clarified. However, it is apparent that the pC194 phenotype is expressed in *B.*

thuringiensis providing an antibiotic resistance marker to study the basic molecular biology of B. thuringiensis.

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CONCLUSION

This laboratory is interested in advancing the understanding of genetic systems in the industrially important bacterium, B. thuringiensis. These microorganisms have been the subject of intensive research with most of the emphasis on the plasmid encoded insecticidal toxin. However, the B. thuringiensis chromosome remains relatively unexplored. The data presented here show the transfer of the conjugative S. faecalis transposon Tn916 into B. thuringiensis subsp. israelensis using overnight, interspecies, filter matings. Insertions of Tn916 were obtained at a frequency indicating efficient movement of the element from the chromosome of either S. faecalis or B. subtilis into a B. thuringiensis subsp. israelensis recipient. Moreover, Tn916 is stably maintained in B. thuringiensis subsp. israelensis. This is the first report of a foreign transposon being introduced and expressed in B. thuringiensis, and Southern hybridization analysis has shown that Tn916 inserted into several different sites on the B. thuringiensis subsp. israelensis chromosome.

This ability of Tn916 to insert into different chromosomal sites may provide a means for the targeting and cloning of B. thuringiensis genes. By using the cloning strategy developed by Gawron-Burke and Clewell

(1984), it may be possible to clone the B. thuringiensis genes in E. coli. However, Tn916 insertional mutations have not been obtained and until they are, it is not known whether this strategy can be utilized.

There is now a consistent means to introduce a foreign transposon into B. thuringiensis; therefore, it should be possible to study the basic molecular biology of B. thuringiensis subsp. israelensis using Tn916 and certain cloned genes. For example, Youngman et al. (1984a; 1985) used the S. faecalis transposon Tn917, to introduce promoterless copies of the E. coli lacZ gene and the B. pumulis cat gene into B. subtilis; this provided the investigators with a direct means of measuring the sporulation events which occur in B. subtilis. By using a similar strategy, it could be possible to remove certain nonessential regions of Tn916, replace them with other cloned genes of interest, e.g., lacZ or cat, and introduce the modified form of Tn916 into B. thuringiensis subsp. israelensis.

Several reports have shown that other S. faecalis plasmids were capable of self-transfer as well as mobilization of nonconjugative plasmids and chromosomal genes, but this is the first report of a conjugative transposon being able to mobilize another plasmid. More importantly, it is now possible to construct more stable,

effective, and selective B. thuringiensis subsp. israelensis strains because Tn916 could be used as a means to consistently introduce foreign plasmid DNA into B. thuringiensis. The strategy would simply involve cloning the gene(s) of interest (e.g., engineered protoxins) into a pC194 replicon, transforming it into a Tn916-containing B. subtilis strain, and mating the recombinant B. subtilis strain with a B. thuringiensis recipient. Alternatively, it may be possible to introduce the Youngman et al. (1985) transposition-selection-vectors into B. thuringiensis because they are constructed on a similar S. aureus replicon, pE194. These transposition-selection-vectors may provide a means to study the regulation of B. thuringiensis sporulation and crystal toxin production.

However, several questions still remain to be answered. First, it is not known whether the transfer of Tn916 or pC194 will occur between nonisogenic B. thuringiensis strains. This is a very important question because most of the work has concerned B. thuringiensis subsp. israelensis and it is not known whether Tn916 or pC194 can be introduced and expressed in the other lepidopteran-specific subspecies. Second, the mechanism(s) of Tn916 transfer from S. faecalis to B. thuringiensis or from B. thuringiensis to an isogenic recipient is not understood. It appears that the Tn916

element does not reside on a B. thuringiensis conjugal element, but it is not known whether this transposon can use such an element for its transfer between isogenic strains. Third, because Southern hybridization analysis of intraspecies transconjugant DNA showed small variation in the hybridization profiles, it is not known whether the insertion of Tn916, after an intraspecific mating, is site-specific. This question can now be better answered because there is an additional antibiotic resistance marker available, the pC194 Cat^R gene. Fourth, it is not known whether the transfer of pC194 will occur when a tetracycline/chloramphenicol resistant B. thuringiensis subsp. israelensis is mated with either a isogenic or nonisogenic recipient. Moreover, it is not known if pC194 will be transferred when just a chloramphenicol resistant B. thuringiensis subsp. israelensis is mated with either a isogenic or nonisogenic recipient. Finally, it is not known if other S. aureus cloning vectors can be transferred into Bacillus thuringiensis subsp. israelensis utilizing Tn916 as a mobilizing agent.

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