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PLASMID BIOLOGY OF BACILLUS THURINGIENSIS

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ABSTRACT

The enlomocidal baclerium, *Bacillus thuringiensis* is presently the subjcct of intense investigation; commercial preparations which utilize the highly-specific entomocidal properties of the organismare currently sold worldwide. Despite the advantage of its specificity, acceplance of this biocontrol agent has been somewhat disappointing because ils effectiveness in the field has not been as promising as results from lests in laboratory experiment. Nonetheless, and because of the selective toxic activity associated with many isolates of *B. thuringiensis,* investigators using the tools of biotechnology foresee a positive potential for developing improved insect biocontrol agents based on *B. thuringien- sis.* It is now well known that subspecies of *B. thuringiensis* harbor a wide array of large transmissible plasmids carrying theinsecticidal parasporal crystal genes which are amenable for geneticengineering of this biocide agent.

The uniqueness of *B. thuringiensis* resides in its ability to produce protein-crystalline inclusions during sporulation (Fig. 1) which are toxic to larvae of many lepidopteran, some dipteran, and a number of coleopteran insects (Dulmage el al., 1981; Goldberg and Margalit, 1977; Krieg et al., 1983). Ingestion of preparations containing the crystalline inclusions by sensitive insect larvae results in release of the protein toxin from the inclusion into the gut; the gut epithelium of the susceptible insect larvae quickly disintegrates due to the toxin's action, and death of the larvae follows (Faust and Bulla, 1982; Heimpel and Angus, 1959; Hofle and Whiteley, 1989). Mounting evidence indicates that the action of the inseciicidal crystal protein is at the cell membrane level (Faust, 1968; Knowles et al., 1984; Knowles and Ellar, 1986; Mulhukumar and Nickerson, 1987). Most investigations on the molecular biology and genetics *of B. thuringiensis* have centered around isolates possessing insecticidal activity for lepidopteran larvae wilh a number of recent studies aimed at isolates toxic to mosquitoes and blackflies (diptera) and to a few coleopteran pests.

Presently, more than 20 subspecies of *B. thuringiensis* have been described based on flagellar antigens (de Barjac and Bonnefoi, 1962; Dean, 1984). Other methods have also been used to separate strains of *B. thuringiensis* that include parasporal crystal shape, size and antigens, biochemical and other phenotypic properties and plasmid profiles (Andrews et al., 1987; Dean, 1984; De Lucca, 1984; Heimpel, 1967; lizuka et al., 1981b; lizuka et al., 1983; Krieg and Langenbruch, 1981; Krieg et al., 1983; Norris, 1964; Smith, 1987).

B. THURINGIENSIS HARBORS A WIDE ARRAY OF PLASMIDS

Plasmid profile studies of many isolates representing most of the subspecies of *B. thuringiensis* have revealed that they harbor plasmids (1 to more than 16) with a wide range in size [1 to more than 150

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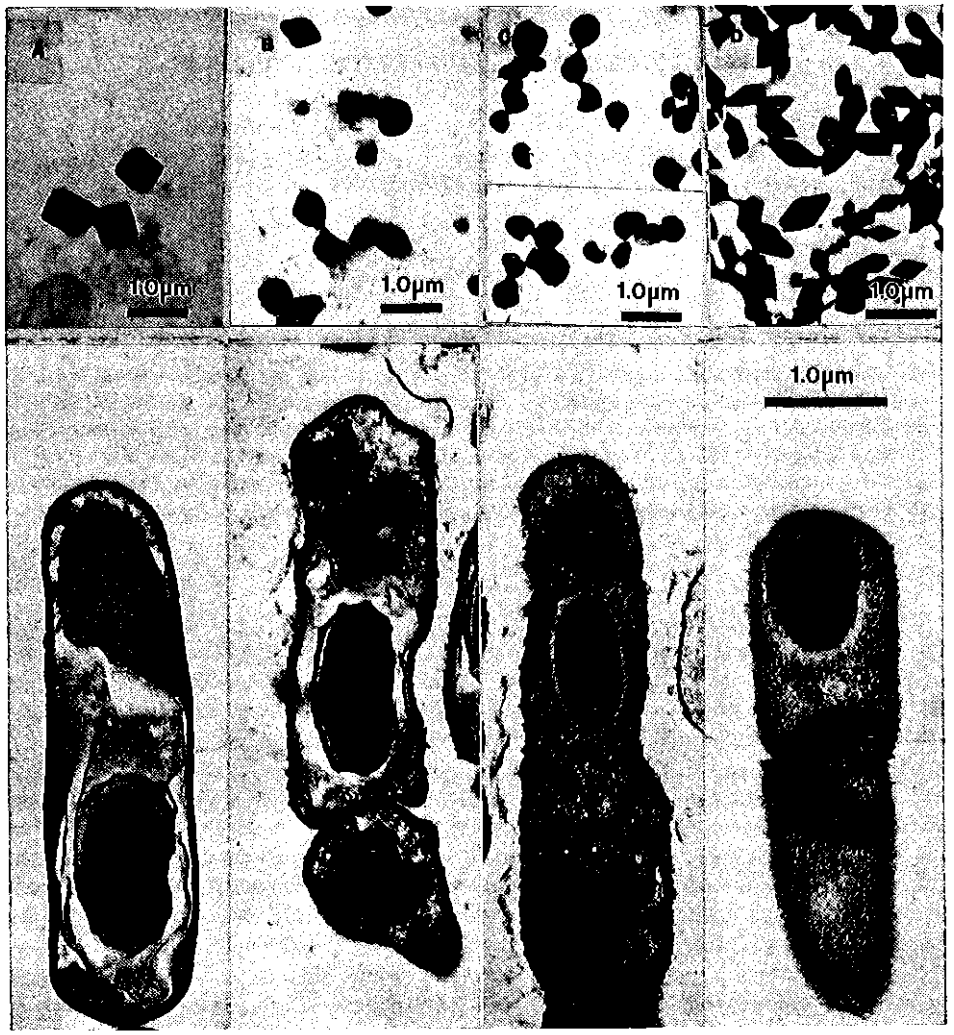

Fig. 1. Representative transmission electron micrographs of *B. thuringiensis* parasporal inclusions (top row) (A) subsp. *tenebrionis*, (B) subsp. *israelensis*, (C) subsp. *darmstadiensis* (73-E-10-2), (D) subsp. *darmstadiensis* (wt) and thin-section electron micrographs of *B. thuringiensis* sporulated cells (A) subsp. *san diego*, (B) subsp. *israelensis* (C) subsp. *darmstadiensis* (73-E-10-2), (D) subsp. *darmstadiensis* (wt) showing the spores and several inclusion types. Subspp. *tenebrionis*, and *san diego* are coleopteran-killing strains, subspp. *israelensis* and *darmstadiensis* (73-E-10-2) are mosquitocidal strains, and subsp. *darmstadiensis* (wt) is a lepidopteran-killing strain.

megadaltons (Mdal)]; the first report of the presence of plasmids was published in 1976 (Zakharyan, 1976) and has subsequently been followed by numerous other reports over the past 10 years (Faust et al., 1979; Gonzalez et al., 1981; Himeno et al., 1985; Iizuka et al., 1981a; Kronstad et al., 1983; Kronstad and Whiteley, 1984; Lereclus et al., 1982). In most cases the plasmid content of a subspecies seems to be rather unique for the subspecies, but exceptions are increasingly being reported. For example, within *B. thuringiensis* subsp. *darmstadiensis* isolates have been described that possess toxicity towards either lepidopteran insects or mosquitoes (Padua et al., 1980) and analysis of the plasmids harbored by these isolates revealed rather differing plasmid patterns (Iizuka et al., 1983). Even among strains of the same subspecies having similar toxicities, there are discrete differences in the profiles, although much similarity still exists. Comparison of *B. thuringiensis* subsp. *kurstaki* HD-1, HD-73 and a multicrystalliferous strain revealed that the *kurstaki* HD-1 strain contained as many as 16 extrachromosomal DNA elements while only 15 were isolated from the *kurstaki* HD-73 and the multicrystalliferous strains (Iizuka et al., 1981b). Only one major difference between the multicrystalliferous and the two single crystalliferous strains that could be noted was the absence of one low molecular weight plasmid in the two single crystalliferous strains, although other differences in the plasmid DNA profiles were also noted among all three strains. In a relatively recent review Carlton and Gonzalez (1985) noted another example — *B. thuringiensis* subsp. *wuhanensis* whose plasmid profile resembles strains of *B. thuringiensis* subsp. *galleriae*. Carlton and Gonzalez (1985) have postulated that perhaps subspecies *wuhanensis* was derived from a strain of subspecies *galleriae* by loss of flagella production.

Other factors which may influence plasmid variation in *B. thuringiensis* include differences in the experimental isolation procedures used by investigators, natural plasmid transfer by cell mating and/or plasmid curing, and deletion or insertional and recombinational events. Some of the larger plasmids (>45Mdal) contain transposon or insertion sequences capable of transferring genetic information (Klier and Rappoport, 1983; Kronstad and Whiteley, 1984; Lereclus et al., 1983; Lereclus et al., 1984; Lereclus et al., 1986; Mahillon et al., 1985). A few unusual extrachromosomal DNA elements along with open circular plasmid DNA and linear forms have also been described (Gonzalez et al., 1981).

LOSS OF SPECIFIC PLASMIDS RESULTS IN LOSS OF PARASPORAL INCLUSION PRODUCTION

Presumptive evidence that parasporal inclusion production was associated with specific plasmids came through plasmid curing studies. Gonzalez et al. (Gonzalez and Carlton, 1980; Gonzalez et al., 1981) demonstrated that the entomocidal protein gene determinants were probably located on plasmids having masses between 30 and 130 Mdal. Five crystal positive strains were used as parents from which acrystalliferous strains were generated in their investigations. Loss of crystal production was associated with loss of a 75 Mdal plasmid in *B. thuringiensis* subsp. *thuringiensis* HD-2 while in *B. thuringiensis* subsp. *kurstaki* HD-73 and subspecies *kurstaki* HD-1 loss of crystal production was associated with a 50 Mdal and 29 Mdal plasmid, respectively. Loss of crystal production in *B. thuringiensis* subsp. *alesti* HD-4 and subspecies *galleriae* HD-8 was found to be associated with the loss of a 105 and 130 Mdal plasmid, respectively. Later, Ward and Ellar (1983) and Himeno et al. (1985) in analyses of a number of isolates of *B. thuringiensis* subsp. *israelensis* cured of one or more plasmids demonstrated that the loss of a 72 Mdal plasmid correlated well with the loss of production of the mosquitocidal protein crystal. In our laboratory we have recently demonstrated such a correlation (Fig. 2) which contrasts to preliminary studies we reported earlier (Faust et al., 1983). Wild types of *B. thuringiensis* subsp. *israelensis* harbor up to 9 extrachromosomal DNA elements (Gonzalez and Carlton, 1984; Tam and Fitz-James, 1986; Ward and Ellar, 1983).

Curiously, Tam and Fitz-James (1986) studying plasmid-cured variants of *B. thuringiensis* subsp. *israelensis* missing the large toxic crystal protein inclusion found that the variants still possessed a

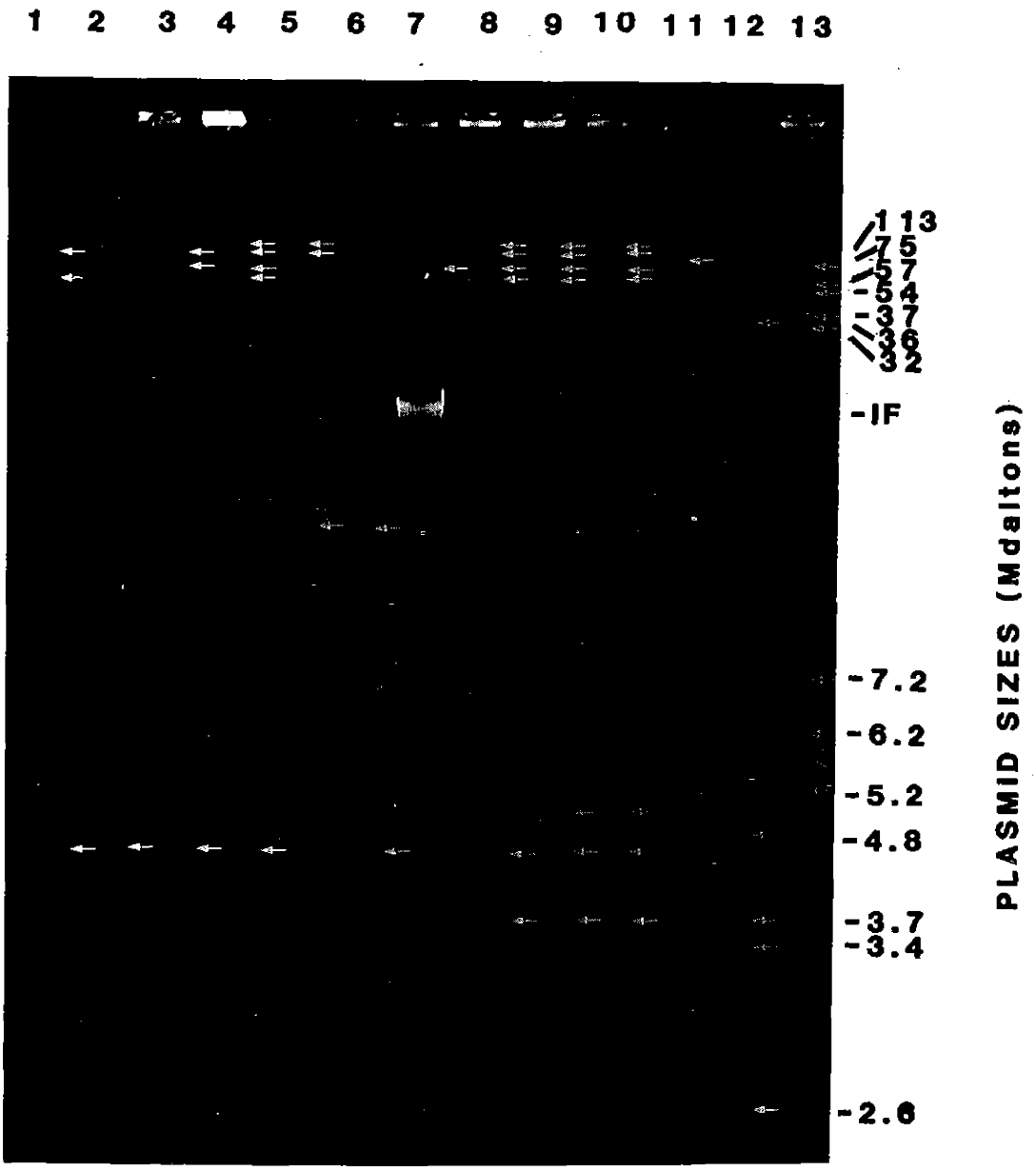

Fig. 2. Representative plasmid patterns of ten isolates of *Bacillus thuringiensis* subsp. *israelensis* as determined by agarose gel electrophoretic analysis. Covalently closed-circular plasmids are indicated with arrows. Other bands visible are open-circular DNA generated occasionally during the isolation procedure. Lane: (1) 922920 cry-, (2) 922918 cry-, (3) 922917 cry+, (4) 922910 cry+. (5) 922906 cry-, (6) 922903 cry-, (7) HD567-61-9 cry +, (8) ONR 60A-IPL wildtype cry+, (9) Goldberg wildtype cry+, (10)4Q1 wildtype cry+. Lanes 11-13 include plasmids of known molecular weights as indicated on the right-hand margin to give an approximate mass scale (IF indicates linear DNA fragments): (11) *Agrobacterium tumefaciens* (pTiA6), (12) *E. coli* V517, (13) *B. thuringiensis* subsp. *thuringiensis* HD-2. Note that in the cry- *israelensis* strains the 72-75 Mdal plasmid is missing (see third pointer from the top in lanes 8–10).

small inclusion similar to the satellite inclusion of the wild type seen in sections of sporulated cells. These "acrystalliferous" variants containing only the satellite inclusions were shown to produce sporulation-dependent phage-like particles, while variants lacking both the insecticidal protein crystal and the satellite inclusion did not harbor the particles. The latter variants were devoided of both the 75- and a 68 Mdal plasmid present in the wild type. The investigators also demonstrated that a crystalliferous, satellite-minus variant possessing the 75-but devoid of the 68 Mdal plasmid harbored the phagelike particles. Analysis of recipient strains that were previously devoid of the insecticidal crystal, the satellite, and plasmids revealed that the satellite and phagelike producing characteristics could have been co-transformed with the 68 Mdal plasmid and that the insecticidal crystal and phagelike particle producing characters could have been co-transformed into a recipient which had acquired the 75 Mdal plasmid. Tam and Fitz-James concluded that the gene coding for the satellite inclusion appears to reside in the 68 Mdal plasmid, while the gene coding for the phagelike particle could reside in either the 68- or 75 Mdal plasmid.

An investigation of Gonzalez and Carlton (1984) with an unusual *B. thuringiensis* subsp. *israelensis* mutant showed that both the 75- and 68 Mdal plasmids could be lost and replaced by two new plasmids of 80-and 63 Mdal. Acrystalliferous variants lost the new 63 Mdal plasmid. After performing Southern blot analysis, they found that the two new plasmids were homologous to the 75 Mdal plasmid which suggested that the 75-and 68 Mdal plasmids recombined in the mutant to yield two new plasmids. The smaller of the two plasmids (i.e., the 63 Mdal plasmid) now carried the functions for insecticidal crystal protein production.

In a study of the crystal protein potency factors of *Bacillus thuringiensis* subsp. *aizawai* Jarrett (1985) has found no consistent correlation between plasmid loss in the mutants examined and any loss or increase of potency indicated by bioassays. Jarrett (1985) concluded that the crystal protein toxins of the isolates studied were composed of at least two toxins and that the genes coding for the production of the toxic materials or for their expression may be carried in some cases on both plasmids and the bacterial chromosome. The majority of work with plasmid curing suggest, however, that single, large plasmids are usually involved in insecticidal crystal protein production.

INSECTICIDAL CRYSTAL PROTEIN GENES ARE CARRIED ON LARGE TRANSMISSIBLE PLASMIDS

More direct evidence that the genes coding for the insecticidal crystal protein are located on the implicated plasmids has come from hybridization, plasmid transfer and gene cloning studies. For example, Kronstad et al. (1983) using an intragenic restriction fragment from a cloned insecticidal protein gene of *B. thuringiensis* subsp. *kurstaki* HD-1 identified the location of the crystal protein genes in 22 *B. thuringiensis* strains representing 14 subspecies by hybridization. In eight of the strains examined, the gene was located on a single large plasmid (subspecies *sotto, darmstadiensis, toumanoffi, alesti, kurstaki* HD-73, *galleriae, thuringiensis*-F, *thuringiensis* HD-2) while in seven other strains (subspecies *tolworthi, kurstaki* HD-244, *kurstaki* HD-1, *thuringiensis* HD-120, *thuringiensis* HD-290, *morrisoni, kurstaki* HD-252) the gene was located on more than one plasmid. Sizes of the implicated plasmids ranged from 33 to more than 150 Mdal. In four other subspecies (*israelensis, dakota, indiana, kyushuensis*) which apparently impart no toxicity to lepidopteran insects, no hybridization occurred. The work also indicated the presence of multiple crystal protein genes in strains of *B. thuringiensis* subsp. *thuringiensis* and subspecies *kurstaki*.

Another hybridization study was more recently reported by Prefontaine et al. (1987) using five oligonucleotide probes to study the relatedness of crystal protein genes among fifteen strains representing 13 serotypes of *B. thuringiensis*. Fourteen of the 15 strains hybridized with at least one probe (subsp. *thompsoni* did not hybridize). Two of the *B. thuringiensis* subsp. *kurstaki* strains (HD-1 and NRD-12, both commercial strains) were indistinguishable using the probes as were *B. thuringiensis* subsp. *dendrolimus* and subspecies *sotto*. Five of the tested strains were determined to contain

only one entomocidal protein gene. A specific probe for the entomocidal protein gene of *B. thuringiensis* subsp. *kurstaki* HD-73 hybridized to only 3 of the 15 strains. The probes also revealed a distinct difference between *B. thuringiensis* subsp. *subtoxicus* and subspecies *entomocidus*. The probe data indicated that *B. thuringiensis* subspecies *aizawai, entomocidus, kenya,* and *thuringiensis* are likely single-gene subspecies, while subspecies *alesti, dendrolimus, galleriae, kurstaki* HD-1, *morrisoni, kurstaki* NRD-12, *sotto, subtoxicus, thompsoni,* and *tolworthi* may contain more than one toxin gene. It was not clear whether *B. thuringiensis* subsp. *kurstaki* HD-73, regarded as a single-gene subspecies, might harbor more than one gene nor could the investigators draw firm conclusions on the exact number of insecticidal crystal protein genes harbored by multiple-gene subspecies.

A conjugal-like plasmid transfer system described by Gonzalez et al., (1982) has been used to further substantiate the contention that the insecticidal crystal protein genes are located on large transmissible plasmids. These investigators demonstrated that both large and small donor plasmids can be transferred equally well between cured strains that are acrystalliferous. The transfer resulted in the ability for the strain to again form insecticidal protein inclusions. This first study demonstrated that within *B. thuringiensis* subsp. *kurstaki*, a 50 Mdal plasmid (HD-73), a 44 Mdal plasmid (HD-263) and a 75 Mdal plasmid of *B. thuringiensis* subsp. *thuringiensis* HD-2 could be transferred into acrystalliferous strains of subspecies *kurstaki* HD-73, subspecies *thuringiensis* HD-2, and into *B. cereus*, thereby converting them to crystalliferous strains. Similar experiments were carried out between acrystalliferous and crystalliferous strains of *B. thuringiensis* subsp. *israelensis*, again implicating a 75 Mdal plasmid involvement in crystal production. In fact a strain of *B. thuringiensis* subsp. *israelensis* harboring only the 75 Mdal plasmid was produced and remained crystalliferous, thus indicating that the other naturally occurring plasmids in subsp. *israelensis* are not necessary for crystal production. The studies also demonstrated that it was possible to produce strains containing and expressing two different insecticidal protein genes (e.g., a subspecies *thuringiensis* HD-2 recipient produced a subspecies *kurstaki* (HD-73 or HD-263) type crystal protein via transconjugation. The transconjugation system requires the growth of mixed cultures of both recipient and donor cells.

ABILITY TO PRODUCE INSECTICIDAL CRYSTALS CAN BE TRANSFERRED TO OTHER BACILLI BY TRANSCONJUGATION

In the previous section we discussed the evidence thus far reported that substantiates the contention that the insecticidal crystal protein genes are carried on large transmissible plasmids. The evidence is convincing and has been obtained through localization of the genes via hybridization, curing, and gene cloning studies as well as by transfer of the implicated plasmids into acrystalliferous strains, thus rendering them with the ability to once again produce crystals. Other evidence has been generated by transconjugation studies involving interspecies transfer.

The transfer of insecticidal crystal-forming abilities to other *bacilli*, such as *B. cereus*, has been reported by Gonzalez et al. (1982) and by McLinden et al. (1985). Additionally, Klier et al. (1983) have transferred cloned crystal genes by mating between *B. subtilis* and *B. thuringiensis*. In this study transconjugation was used to obtain the heterospecific mating between a *B. subtilis* strain harboring the insecticidal crystal protein gene and *B. thuringiensis* subsp. *thuringiensis* (berliner 1715) in addition to other *B. thuringiensis* strains. A recombinant plasmid (pBT42-1) containing the insecticidal crystal protein gene was transferred to an acrystalliferous strain of *B. thuringiensis*; synthesis of an insecticidal crystal protein inclusion was subsequently promoted. The recombinant plasmid was also transferred into the mosquitocidal strain of *B. thuringiensis* subsp. *israelensis* and the transcipient strain produced protein toxins detrimental to lepidoptera and mosquito larvae. Transformation and expression of a cloned delta-endotoxin gene back into *B. thuringiensis* has also been achieved (Lereclus et al., 1989).

Battisti et al. (1985) have reported a mating system for the transfer of plasmids among *B. anthracis*, *B. cereus*, and *B. thuringiensis*. Transfer of plasmid pBC16 carrying tetracycline resistance and other plasmids from *B. thuringiensis* to *B. anthracis* and *B. cereus* recipients resulted from the mating. Two plasmids designated pX011 and pX012, which naturally occur in *B. thuringiensis* appeared to be responsible for the plasmid mobilization. Close cell to cell contact was necessary for plasmid transfer and both *B. anthracis* and *B. cereus*, which subsequently harbored the pX012 plasmid, acquired the ability to produce the insecticidal protein crystals. Later, Aronson and Beckman (1987) demonstrated a low frequency of chromosomal gene transfer from *B. thuringiensis* to *B. cereus* with all of the *B. cereus* transconjugants acquiring at least one plasmid from the *B. thuringiensis* donor encoding the insecticidal crystal protein gene. Interestingly, in transconjugal matings with *B. thuringiensis* subsp. *kurstaki* HD-1, a 29 Mdal plasmid that harbored a 2.5 kilobase (kb) region of homology with the chromosome was always transferred. Since no detectable transfer of chromosomal genes was found in subspecies *kurstaki* HD-1 lacking the plasmid, the investigators suggested that there may be chromosome mobilization.

The mechanism of plasmid transfer in *B. thuringiensis* is only now beginning to be understood. It has been postulated by one group of investigators that it probably does not involve classical mobilization by plasmid encoded transfer genes, nor does it seem that plasmid transfer is due to transformation by DNA released from "leaky" or lysing cells since transfer frequencies were not affected by the presence of DNAs in the mating plates (Fischer et al., 1984). It was proposed, therefore, that the conjugation-like process is dependent on cell-to-cell contact (Fischer et al., 1984). The studies so far reported also demonstrate that the process is highly efficient with up to 75 percent of recipients acquiring one or more plasmids, the transfer is time dependent and DNase insensitive, it occurs bidirectionally in some combinations and the patterns of transfer are variable, and certain plasmids show evidence of incompatibility (Carlton and Gonzalez, 1985; Fischer et al., 1984; Gonzalez and Carlton, 1984; Gonzalez et al., 1982; Klier et al., 1983).

Two additional investigations have broadened, however, our depth of understanding of the phenomenon. Reddy et al. (1987) have recently identified several self-transmissible plasmids in four *B. thuringiensis* subspecies. The investigation was carried out by selecting transcipients which acquired a pBC16 plasmid after mating four *B. thuringiensis* subspecies to *B. anthracis* and *B. cereus*. It was noted that transcipients also inherited a specific large plasmid from each *B. thuringiensis* donor at a high frequency along with an array of smaller plasmids. A number of large plasmids ranging from 50 to 120 Mdal that had originated from *B. thuringiensis* subspecies *morrisoni*, *toumanoffi*, *alesti*, and *israelensis* were shown to be responsible for plasmid mobilization because strains cured of each of the large implicated plasmids were not fertile. These results indicated that these particular plasmids confer conjugation functions. Confirmation that the implicated plasmids were self-transmissible was obtained by the isolation of fertile *B. anthracis* and *B. cereus* transcipients that contained only pBC16 and one of these transmissible plasmids. One particular plasmid, designated pX014 was efficient in mobilizing the insecticidal toxin plasmid, pX01, from *B. anthracis* transcipients to plasmid-cured *B. anthracis* or *B. cereus* recipients. Matings performed between strains harboring the same conjugative plasmid had reduced efficiency of pBC16 transfer. None of the self-transmissible plasmids (pX013, *morrisoni*; PX014, *toumanoffi*; pX015, *alesti*; pX016, *israelensis*) identified in this study encoded the insecticidal crystal protein and no property other than the ability to confer fertility was assigned to them by the investigators. Reddy et al. (1987) concluded that since derivatives of each *B. thuringiensis* strain which lacked the implicated self-transmissible plasmid were nonfertile, these plasmids were responsible for eliciting the cellular contacts required for transfer to occur, and that since tetracycline resistant (encoded on pBC16) *B. cereus* or *B. anthracis* transcipients on inheriting only one of the self transmissible *B. thuringiensis* plasmids were fertile, these plasmids were conjugative.

Unfortunately, neither the exact mechanism for cell-to-cell contact between donor and recipient cells nor the mechanism of the DNA transfer is entirely understood. It has been demonstrated that

some of the large plasmids contain insertion sequences capable of transferring genetic information. The insertion sequences are associated with the insecticidal protein genes (Kronstad and Whiteley, 1984; Lereclus et al., 1983, 1984, 1986; Mahillon et al., 1985). Reddy et al. (1987) have proposed that variant plasmids might result from transposition of sequences form *B. thuringiensis* plasmids to the pX01 plasmid harboring the insecticidal protein gene. Investigators in their laboratory have demonstrated that following mobilization by pX012 (conjugative plasmid encoding crystal formation from *B. thuringiensis* subsp. *thuringiensis*) (Battisti et al., 1985), pX01 was altered and contained some pX012 sequences. Reddy et al. (1987) also reported that there was a variation in the morphology of parasporal inclusions that depended on the presence of a plasmid, pX039 in a strain of *B. thuringiensis* subsp. *israelensis*. We mentioned earlier that Tam and Fitz-James (1986) have associated two different extrachromosomal DNA elements with the formation of the satellite and entomocidal crystal inclusions in *B. thuringiensis* subspecies *israelensis*. The observations of Reddy et al. (1987) are consistent with the work of Tam and Fitz-James (1986). Reddy et al. (1987) have further concluded from plasmid mobilities in agarose gels and DNA restriction enzyme analysis that the self-transmissible plasmids from the various subspecies of *B. thuringiensis* are not related. They have not determined whether or not the plasmids contain conserved sequences encoding conjugative functions as yet.

Finally, the phenomenon of entry exclusion, wherein a resident plasmid interferes with the entry of genetic material by conjugation (Novick, 1969) was observed by Reddy et al. (1987) when strains harbored the same self-transmissible plasmid. However, enhancement of transfer of pBC16 also occurred when the donor and recipient strains contained different conjugative plasmids.

In the second report Koehler and Thorne (1987) demonstrated that the conjugal-like transfer of plasmids in *Bacillus* probably occurs by donation with no physical association of the conjugal and nonconjugal plasmids (i.e., occurs without the benefit of sequences homologous to the donor plasmid and without acquisition of insertion mutations) as opposed to conduction which is mediated by recombination between homologous sequences existing on the two plasmids or by the association of the two plasmids during the translocation of a transposable genetic element (Clark and Warren, 1979; Koehler and Thorne, 1987).

Since the late 1970s much has been learned about the genetics and plasmid biology of *B. thuringiensis*. The insecticidal protein crystal genes have been associated with specific plasmids, mobilized among various bacilli by transconjugation, cloned and in some instances sequenced. The plasmids harboring the toxin genes, along with their transposons and insertion sequences, are of much value for basic and applied research involved with the genetic manipulation of this entomocidal bacterium.

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