Transfer of Chromosomal Genes and Plasmids in 
*Bacillus thuringiensis*

ARTHUR I. ARONSON\* AND WILLIAM BECKMAN

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Received 22 December 1986/Accepted 31 March 1987

A low frequency of chromosomal gene transfer from *Bacillus thuringiensis* to *Bacillus cereus* was detected by cell mating, with a tryptophan marker being the most frequently transferred gene among four that were tested. The process was resistant to DNase and was not mediated by cell filtrates. Among several *B. thuringiensis* subspecies tested, transfer was best with a derivative of *B. thuringiensis* subsp. *kurstaki* HD1, which lost several plasmids. All of the *B. cereus* recombinants contained at least one plasmid from the donor *B. thuringiensis*; frequently, it was a plasmid that encoded a protoxin gene. In matings with *B. thuringiensis* subsp. *kurstaki* HD1, a 29-megadalton plasmid that contained a ca. 2.5-kilobase region of homology with the chromosome was always transferred. No detectable transfer of chromosomal genes was found in *B. thuringiensis* subsp. *kurstaki* HD1 strains lacking this plasmid, suggesting that there may be chromosome mobilization.

The most prevalent methods for gene transfer among the spore-forming bacilli are transformation and transduction. Only the latter process has been described for *Bacillus thuringiensis* subspecies, and several generalized transducing phage have been isolated (4, 13, 18, 19, 25). In addition, a system that appears to involve cell to cell contact has been documented for the transfer of plasmids from certain *B. thuringiensis* subspecies to *Bacillus cereus* (11), *Bacillus anthracis* (5), and plasmid-cured variants of *B. thuringiensis* (11). Among the plasmids transferred frequently were those encoding protoxin genes (5, 9, 11, 22) or those involved in the regulation of protoxin synthesis (22).

Very few *B. thuringiensis* subspecies transfer plasmids at a sufficiently high frequency to be detectable without selectable markers, although in some cases colonies of derivatives that do or do not produce inclusions can be distinguished on certain media (11). Unfortunately, there are few if any such markers encoded by indigenous plasmids, so low-frequency transfer events are difficult to detect. In such cases, plasmid transfer can be found by selecting for the transfer of chromosomal markers and then screening the recombinants for plasmids from the donor strain. In this way, it has been possible to demonstrate the transfer to *B. cereus* of several different protoxin-encoding plasmids (9, 22).

The interrelationship between chromosome and plasmid transfer has been most extensively studied in *B. thuringiensis* subsp. *kurstaki* HD1, and as discussed in this report, it may involve chromosome mobilization. In addition, further documentation of the transfer of chromosomal markers and plasmids by several *B. thuringiensis* subspecies is included.

**MATERIALS AND METHODS**

The strains of *B. thuringiensis* and *B. cereus* used in this study are listed in Table 1. Auxotrophs were obtained after ethyl methanesulfonate treatment of spores (15) and replica plating from an enriched medium, G-Tris (1), to a minimal medium (22). All auxotrophs reverted at frequencies of between 1/5 × 10⁷ and 1/1 × 10⁸. Streptomycin- or cycloserine-resistant *B. cereus* 569 isolates were selected by plating ca. 5 × 10⁸ cells on G-Tris agar containing 70 μg of the antibiotic per ml.

For matings, cells grown overnight on LB agar (8) at 30°C were used as inocula for LB broth cultures grown at 30°C in an incubator shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) for about 4 to 5 h (early exponential phase of growth). Matings were then done in liquid as described previously (11, 22), or an approximately equal number of colonies (ca. 3 × 10⁸ each) were mixed and collected on filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The filters were placed on LB agar and incubated for 18 to 21 h at 30°C. Filters were then suspended in 10 ml of 0.05 M Tris hydrochloride (pH 7.6), and portions were plated on G-Tris for total cell counts and on G-Tris plus 70 μg of streptomycin per ml for *B. cereus* recipients. Revertants, resistant mutants, and recombinants were scored by plating 1 × 10⁶ to 5 × 10⁷ cells on the minimal agar medium supplemented with the appropriate nutrient and antibiotic. Supplements used were the L-forms of amino acids at 50 μg/ml, guanosine at 30 μg/ml, streptomycin at 70 μg/ml, and D-cycloserine at 70 μg/ml. Recombinants were scored after 4 days of incubation at 30°C and replica plated on selective media to confirm the phenotypes.

For the examination of plasmid profiles, colonies were streaked on SCGY agar (11), and the plates were incubated at 30°C for 12 to 15 h. Portions were lysed and analyzed on agarose gels by the procedure described by Eckhardt (10) as modified by Gonzalez and co-workers (11, 12).

Parasporal inclusion formation in recombinants was initially determined by growth on G-Tris-streptomycin agar at 25°C for 3 to 4 days and the screening of wet mounts in a phase microscope. Confirmation was obtained by fractionation of extracts of spores plus inclusions on 5 to 20% gradient acrylamide gels and by immunoblotting (22, 26). Toxicity to *Manduca sexta* larvae was measured by spreading 10⁵ to 10⁷ spores (plus crystals) on the surface of an artificial diet. Lethality and the concentration that killed 50% of larvae were determined after 7 days.

The cloning of a region of the 29-megadalton (MDa) plasmid from *B. thuringiensis* subsp. *kurstaki* HD1 has been described elsewhere (14). This clone hybridized to chromosomal DNA from a plasmid-free derivative of *B. thuringi-
ensis subsp. kurstaki HD1 (strain S1) (24) and was used initially to isolate a clone of a 13-kilobase (kb) BamHI fragment from strain S1 DNA (cloned in plasmid pLP1201 [23] in Escherichia coli HB101). Subsequent subcloning and cross-hybridization were done to define the regions of homology (see Fig. 2) for sequencing. The EcoRI-BamHI fragment from the 29-MDa plasmid and the SstI-MstI fragment from the chromosomal DNA were sequenced. These inserts were also nick translated and used for hybridization to Southern transfers of plasmids as described elsewhere (9, 22).

Cross-hybridizing portions of the plasmid and chromosomal clones were initially sequenced by the procedure described by Maxam and Gilbert (21) and subsequently via subcloning into M13 vectors for kilo-sequencing (3). The details of the sequencing strategy and the sequences are not included because they are not essential to the results presented here.

RESULTS

Plasmid transfer may be independent or coupled. *B. thuringiensis* subsp. kurstaki HD73 transferred plasmids to *B. cereus* 569 (Str') at a high frequency (Table 2) (11) such that plasmid-containing transciptives could be detected by selecting only for *B. cereus* recipients. In most cases plasmids of 5 to 6 MDA were transferred, but there was also a relatively high frequency of transfer of a 50-MDA plasmid that contained the protoxin gene (Fig. 1, K19) (16). Two different *B. cereus* 569 auxotrophs (Trp, Met) were used as recipients in these matings. No prototrophs were found among 100 randomly picked transciptives that contained HD73 plasmids or by replica plating of over 2,000 *B. cereus* colonies (following filter mating).

In a similar mating employing *B. thuringiensis* subsp. kurstaki HD1 (or the derivatives HD1-7 and HD1-9) and *B. cereus* 569K, none of 100 *B. cereus* colonies picked at random from G-Tris-streptomycin plates were prototrophs, nor did they contain *B. thuringiensis* subsp. kurstaki HD1 plasmids. *B. cereus* prototrophs were found in such matings after they were plated on minimal media containing streptomycin. The frequency varied, depending on the selectable marker (Table 2). Among the four markers tested, trp was the most frequently transferred, while only one of two gaa markers was barely detectable. All of the *B. cereus* prototrophs resulting from the matings contained one or more HD1 plasmids (Fig. 1 and Table 2). In every case a plasmid of 29 MDA was present; some also contained a 44-MDA plasmid that encodes a major protoxin of *B. thuringiensis* subsp. kurstaki HD1 (16; unpublished results). In addition, a plasmid of 4.9 MDA and, occasionally, one of 5.2 MDA were also transferred. These plasmids have a role in regulating protoxin synthesis, at least in *B. cereus* recombinants (22). Very few if any of the other HD1 plasmids were detected among *B. cereus* prototrophs.

The frequency of chromosome transfer (at least for the four markers studied) was enhanced considerably if HD1 derivatives lacking several plasmids present in the parental strain were used in the matings (Table 2). These derivatives still produced toxic inclusions. Strain HD1-7 contained

<table>
<thead>
<tr>
<th>TABLE 1. Bacterial strains used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td><em>B. cereus</em> 569</td>
</tr>
<tr>
<td><em>B. cereus</em> 569K</td>
</tr>
<tr>
<td><em>B. cereus</em> 569KC</td>
</tr>
<tr>
<td><em>B. cereus</em> 569A</td>
</tr>
<tr>
<td><em>B. cereus</em> 569B</td>
</tr>
<tr>
<td><em>B. cereus</em> 569A</td>
</tr>
<tr>
<td><em>B. cereus</em> 569B</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. kurstaki HD1</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. kurstaki HD1-7</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. kurstaki HD1-9</td>
</tr>
<tr>
<td>CryB</td>
</tr>
<tr>
<td>S1</td>
</tr>
<tr>
<td>Mit6</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. kurstaki HD73</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. alesi</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. finitimus</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. berliner</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. galleria HD834</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. aizawai HD133</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> subsp. kurstaki HD1-7(pBC16)</td>
</tr>
</tbody>
</table>

a Presence or absence of parasporal inclusion (Cry+ or Cry-). Confirmed in transciptives and recombinants by immunoblotting and toxicity assays with *M. sexta* larvae.

b Smaller, less stable inclusions.

c Cured by heating spores twice at 80°C for 20 min.

d Curing as described above in footnote c; spores were essentially devoid of coat and were very sensitive to lysozyme in contrast to CryB.

e See reference 6; codes for Te'.
plasmids of 44 and ca. 110 MDa, both of which contain prototoxin genes (16), whereas HD1-9 lost the larger plasmid and produced smaller, less stable inclusions if grown at 25°C but no inclusions when grown at 30°C (22). Strain HD1-9 was at least 10 times more efficient in the transfer of chromosomal markers than the original B. thuringiensis subsp. kurstaki HD1 strain.

This chromosome-plasmid transfer was resistant to DNase (40% decrease in Trp+ prototrophs with 50 μg/mL in either a liquid or filter mating; Table 2), as was found for the plasmid transfer system (11). No chromosomal marker transfer occurred in liquid matings of these strains if a 0.45-μm-pore-size filter was placed between the two cultures in a U-tube or if supernatants of B. thuringiensis subsp. kurstaki HD1 or HD1-9 were added to B. cereus auxotrophs. While there are several B. thuringiensis generalized transducing phage (4, 13, 18, 19, 25), none could package sufficient DNA to account for the simultaneous transfer of both chromosomal genes and a large contingent of plasmids. It is likely, therefore, that the process requires cell contact.

Possible chromosome mobilization by the 29-MDa plasmid.

The presence of the B. thuringiensis subsp. kurstaki HD1 29-MDa plasmid in all B. cereus prototrophic transconjugants implied a role for this plasmid in chromosome mobilization. Several derivatives of strain HD1 that lost all plasmids or all plasmids larger than 5.2 MDa were obtained after spores were heated or cells were treated with a sublethal concentration (0.2 μg/mL) of mitomycin C (Table 1). None of these strains (a total of six were tested) was capable of transferring markers to B. cereus 569 auxotrophs (Table 2 shows results with B. cereus 569K, but B. cereus 569KC and 5695 were also tested).

There was a region of homology of ca. 2.5 kb between the 29-MDa plasmid and the B. thuringiensis subsp. kurstaki HD1 chromosome (Fig. 2). The 0.6-kb region of 91% homology was highly conserved, with only 3 amino acid differences and 45 third-position differences. There appeared to be two open reading frames (ORFs), and one of them (ORF2) had limited homology (7 of 21 amino acids) to a part of the conserved region of neomycin phosphotransferase genes, especially one from plasmid pHL1 (27). In fact, E. coli HB101, which contained an 8.3-kb HindIII clone from the chromosome region (Fig. 2, p62-9), was resistant to 5 μg but not to 20 μg of kanamycin or neomycin per ml nor to gentamicin. An EcoRI subclone (pSM36) also conferred a low level of resistance, whereas others (p62-9-3 and p62-9-5), which did not contain all of ORF2, failed to confer resistance (including a clone of the PstI-BamHI fragment from the 29-MDa plasmid). There was no detectable neomycin phosphotransferase activity in extracts of E. coli clones containing plasmid p62-9, however, so another type of activity may be present. Interestingly, B. thuringiensis subsp. kurstaki HD1 was less resistant to kanamycin than plasmid-cured derivatives (i.e., CryB, Mit6, or S1), suggesting the cryptic nature of the gene in plasmid-containing strains or, perhaps, a lowered permeability to kanamycin in these cured strains. The ORF1 sequences were not identified with any known gene.

The cloned regions of homology from the 29-MDa plasmid (EcoRI-BamHI fragment) and the B. thuringiensis subsp.
from the plasmid appeared to be indicative of map positions relative to the point of mobilization, but there is not yet sufficient mapping data to test this possibility (2).

B. thuringiensis subsp. kurstaki HD73 transferred plasmids at a much greater frequency than the chromosomal markers tested, and in many cases the 50-MDa protoxin-encoding plasmid was transferred. The location of protoxin genes may be dependent on transposons (17, 20), so their presence in particular plasmids could be fortuitous. In all of the other subspecies examined, plasmids were not as readily transferred. Even the presence of a transmissible plasmid such as pBC16 (5) in B. thuringiensis subsp. kurstaki HD1-7 did not enhance plasmid or chromosome transfer (Table 2).

B. cereus transciipients containing the 50-MDa protoxin-encoding plasmid from B. thuringiensis subsp. kurstaki HD73 did not transfer this plasmid to other B. cereus 569

**DISCUSSION**

Transfer of several chromosomal markers from B. thuringiensis to B. cereus appeared to require cell contact and was invariably accompanied by the transfer of one or more plasmids. This process was resistant to DNase and was unlikely to be mediated by a transducing phage because there were no prototrophs when cell filtrates were added to B. cereus auxotrophs or when a 0.45-μm-pore-size filter was placed between donor and recipient cultures. Markers were transferred from B. thuringiensis to these B. cereus auxotrophs via the transducing phage CP51 (25), but the relative frequencies were not the same as the apparent gradient found in matings (Table 2). In particular, the guaA markers were transduced about as efficiently as the his+ markers; met+ and trp+ were also transduced about equally well (unpublished results). The gradient of marker transfer may be indicative of map positions relative to the point of mobilization, but there is not yet sufficient mapping data to test this possibility (2).

**FIG. 1.** Agarose gel pattern of plasmids present in parental strains (HD1-9 and B. cereus 569K) and B. cereus prototrophic recombinants K4 and 8 (4.9, 29, and 44 MDa) and 4 (29 MDa). K19 (4.9, 5, 2, and 50 MDa) is a recombinant from the mating of B. thuringiensis subsp. kurstaki HD73 and B. cereus 569K. Arrow indicates 9-MDa plasmid indigenous to B. cereus 569K; CHR, chromosomal DNA.

**FIG. 2.** Cloned fragments of the 29-MDa plasmid and chromosomal DNA (CHR) from B. thuringiensis subsp. kurstaki HD1 containing the region of homology as determined by Southern hybridization. An EcoRI-BamHI subclone of the plasmid clone and an SstI-MstI subclone of the chromosomal clone were sequenced. Regions designated ORF1 were similar to each other, as were the ORF2 regions, although only ORF1 from the plasmid appeared to be completely sequenced. Other subclones (in plasmid pLP1201) of the chromosomal clone were tested for their capacity to confer resistance to 5 μg of kanamycin (KmR or KmS) per ml to E. coli HB101.

B. thuringiensis subsp. kurstaki HD73 plasmid DNA was transfected into B. thuringiensis subsp. finitimus clones and was found to be an efficient donor of DNA from a plasmid-cured derivative (9). A low frequency of transfer of the trp marker to B. cereus was found for B. thuringiensis subsp. kurstaki HD73, B. thuringiensis subsp. finitimus, and B. thuringiensis subsp. aizawai but not for B. thuringiensis subsp. alsestis, B. thuringiensis subsp. berliner, or B. thuringiensis subsp. galleriae (Table 2); so there was no correlation of either the presence of strain HD1 sequences in the chromosome or of a region of plasmid and chromosome homology with the low-frequency transfer of the trp marker.
strains (cycloserine resistant) at a very high frequency (<3% of randomly screened recipients). Properties of strain HD73 cells may be important for the efficient transfer of plasmids. In addition, there were no detectable transfer of plasmids from *B. thuringiensis* subsp. *kurstaki* HD73 to a plasmid-free derivative of *B. thuringiensis* subsp. *kurstaki* HD1 (strain S1) or of chromosomal markers from *B. thuringiensis* subsp. *kurstaki* HD1-9 to auxotrophs of strain S1. Because plasmid transfer between *B. thuringiensis* strains has been reported (11), it may be possible to find suitable strains for chromosome transfer.

Transfer of plasmids from *B. thuringiensis* subsp. *aizawai*, *B. thuringiensis* subsp. *kurstaki* HD1, and *B. thuringiensis* subsp. *finitimus* (9) was too low to be detected by random screening of recipient *B. cereus* cells. In these cases plasmid transfer was found only among Trp+ prototrophic recombinants, and all such recombinants contained one or more plasmids from the donor. In a large fraction a protoxin-encoding plasmid was transferred. Other smaller plasmids were also transferred, including one in *B. thuringiensis* subsp. *kurstaki* HD1 that appeared to function in the regulation of protoxin synthesis (22); but there were several plasmids, especially for *B. thuringiensis* subsp. *kurstaki* HD1, that were never found in *B. cereus* recombinants.

In the case of *B. thuringiensis* subsp. *kurstaki* HD1 (or its derivatives), a 29-MDa plasmid with some homology to the chromosome was always transferred. It is tempting to speculate that chromosome mobilization occurred, perhaps at a region encoding a kanamycin resistance gene. The lack of mating by *B. thuringiensis* subsp. *kurstaki* HD1 derivatives cured of the 29-MDa plasmid is consistent with mobilization, but the efficiency of transfer by strains that contained this plasmid still varied 10-fold (i.e., HD1 versus HD1-9). Unfortunately, it has not yet been possible to reintroduce the 29-MDa plasmid or the cloned region of homology into these cured strains. In addition, some *B. thuringiensis* subsppecies with no evidence for regions of homology between chromosomal and plasmid sequences transferred chromosomal markers with about the same frequency as that of *B. thuringiensis* subsp. *kurstaki* HD1. In these cases, transfer functions on plasmids (usually those encoding protoxin genes) may aid chromosome marker transfer or the transfer functions may already be present in the chromosome.

The reason for differences in chromosome transfer, especially the apparent relative inefficiency of wild-type strains, is not understood. It may be due in part to the presence of plasmids that encode bacteriocins that are lethal for *B. cereus*. Indeed, *B. thuringiensis* subsp. *kurstaki* HD1-9 no longer produced bacteriocin, in contrast to strain HD1 or HD1-7 (unpublished results). A similar phenomenon was noted in matings of *B. thuringiensis* subsp. *finitimus* in which loss of a 77-MDa bacteriocin-encoding plasmid resulted in an increased efficiency of transfer of the trp+ marker and of a 98-MDa protoxin-encoding plasmid (9). A second possibility is a change in surface properties of donor strains (7) that may enhance mating with *B. cereus*. Many strains with altered plasmid profiles appeared to have surface properties different from that of the parental strain, i.e., colony morphology and the extent of clumping in suspension.

The chromosome transfer system described here appears to be rather inefficient, at least for the markers tested, and the strains most proficient in coupled transfer were rather unexpected. Transfer was not detected for several subspecies and was very low for others. The wild-type strain *B. thuringiensis* subsp. *kurstaki* HD1 was 10-fold less efficient than a derivative, HD1-9, which had lost several plasmids (22), including one of ca. 110 MDa that probably contains genes for two or three protoxins (16; unpublished results). The strain HD1-9 derivative thus produced only a single species of protoxin in contrast to the original strain *B.
thuringiensis subsp. kurstaki HD1 which forms two types of inclusions, including one with two to three species of protoxins (16, 22, 28). In addition, protoxin in the inclusion of HD1-9 appeared to be less stable than that in HD1 because toxicity was more rapidly lost on storage and less (135 kDa) protein was found on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (unpublished results). Perhaps the increased mating efficiency of strain HD1-9 is related to this less stable and less abundant protoxin. Chromosome transfer may not function primarily for recombination but, rather, to enhance the transfer of plasmid-encoded protoxin genes to other cells in which the interaction of two or more protoxins may result in more stable and toxic inclusions.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant GM 34035 from the General Medical Institute. W.B. was supported by a postdoctoral fellowship from Eli Lilly & Co., Indianapolis, Ind. Kathy Morgan, Michelle Sims, and Cynthia Elliott, summer Minority Access Research Careers students, also participated in the research. The technical assistance of Gail Sudlow is greatly appreciated.

LITERATURE CITED