A Complete Physical Map of a *Bacillus thuringiensis* Chromosome

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Received 5 October 1992/Accepted 30 November 1992

Bacillus thuringiensis is the source of the most widely used biological pesticide, through its production of insecticidal toxins. The toxin genes are often localized on plasmids. We have constructed a physical map of a *Bacillus thuringiensis* chromosome by aligning 16 fragments obtained by digestion with the restriction enzyme *NotI*. The fragments ranged from 15 to 1,350 kb. The size of the chromosome was 5.4 Mb. The *NotI* DNA fingerprint patterns of 12 different *B. thuringiensis* strains showed marked variation. The *cryIA*-type toxin gene was present on the chromosome in four strains, was extrachromosomal in four strains, and was both chromosomal and extrachromosomal in two strains. A Tn4430 transposon probe hybridized to 5 of the 10 *cryIA*-positive chromosomal fragments, while *cryIA* and the transposon often hybridized to different extrachromosomal bands. Ten of the strains were hemolytic when grown on agar plates containing human erythrocytes. Nine of the strains were positive when assayed for the presence of *Bacillus cereus* enterotoxin. We conclude that *B. thuringiensis* is very closely related to *B. cereus* and that the distinction between *B. cereus* and *B. thuringiensis* should be reconsidered.

Bacillus thuringiensis is the source of the most widely used biological pesticide in the world today. This gram-positive soil bacterium produces during sporulation crystal inclusion bodies consisting of highly specific insecticidal toxins (for a review, see reference 19). Most toxins are active against larvae of lepidopteran species, but some show toxicity against dipteran or coleopteran species (19). Strains specifically toxic to protozoan pathogens, animal-parasitic liver flukes, or mites have broadened the potential *B. thuringiensis* product spectrum even further (13). Certain strains of *B. thuringiensis* are used in Africa and the United States to control larvae of mosquitos and blackflies, vectors of important human diseases such as malaria (7).

At least 14 distinct crystal-protein genes have been reported, grouped into four gene types, CryI to -IV, and further into subtypes (19). Proteins of the subtypes cryIA to -ID have more than 55% identical residues, while less identity (20 to 50%) was seen between the other types (19).

B. thuringiensis strains are classified into subspecies or varieties, based on flagellar antigens (10). Each subspecies may produce one or several insecticidal toxins. *B. thuringiensis* usually contains one or more large plasmids, on which the *cry* genes are localized (5, 15, 16).

B. thuringiensis is most closely related to *Bacillus cereus*, and plasmids of *B. thuringiensis* coding for insecticidal crystals have been transferred to *B. cereus*, making *B. cereus* look like crystal-producing *B. thuringiensis* (14). *B. cereus* is a common soil bacterium and a frequent contaminant of milk (1, 9). Some *B. cereus* strains produce and secrete an enterotoxigenic protein (39). It is not known yet whether the enterotoxin gene is localized on the chromosome or on a plasmid, and the gene has not been cloned. Since *B. cereus* may produce an enterotoxin(s), it is a potentially harmful bacteria to humans, unlike *B. thuringiensis*, which may be regarded as a beneficial organism because of its insecticidal activity.

It has been claimed that *B. thuringiensis* should be regarded as an insecticide-producing variant of *B. cereus* (17),

and it would therefore seem appropriate to look into the relationship between *B. cereus* and *B. thuringiensis*. We have approached this by analyzing the bacterial chromosomes.

Pulsed-field gel electrophoresis (PFGE) and the construction of a physical map of a genome constitute a very powerful technique for the analysis of genomes when no genetic map is available, as for *B. cereus* and *B. thuringiensis*. Recently, we have constructed physical maps of four *B. cereus* strains by aligning their *NotI* restriction fragments (4, 22). In this report, we present a low-resolution physical map of a *B. thuringiensis* strain. The map is similar to those of the *B. cereus* strains. Our results demonstrate that the *B. thuringiensis* strain is as closely related to the *B. cereus* strains as the *B. cereus* strains are among themselves. Like *B. cereus* strains, *B. thuringiensis* variants show strikingly different *NotI* fingerprint patterns. In addition we have demonstrated that several of the *B. thuringiensis* strains produce enterotoxins. We conclude that the taxonomy of *B. cereus* and *B. thuringiensis* should be reconsidered.

MATERIALS AND METHODS

Bacterial strains. *B. thuringiensis* subsp. *canadensis* HD224 (4H2), *B. thuringiensis* subsp. *darmstadensis* HD146 (4M1), *B. thuringiensis* subsp. *dendrolimus* HD7 (4E2), *B. thuringiensis* subsp. *entomocidus* HD9 (4I4), *B. thuringiensis* subsp. *israelensis* ONR60A (4Q1), *B. thuringiensis* subsp. *israelensis* 4Q2-72 (4Q5), *B. thuringiensis* subsp. *kenya* HD293 (4F3), *B. thuringiensis* subsp. *kurstaki* HD1 (4D1), *B. thuringiensis* subsp. *kurstaki* HD73 (4DH), *B. thuringiensis* subsp. *morrisoni* HD12 (4K1), *B. thuringiensis* subsp. *subtoxicus* HD109 (4I5), and *B. thuringiensis* subsp. *thuringiensis* HD2 (4A3) were obtained from the *Bacillus* Genetic Stock Center, Department of Biochemistry, The Ohio State University, Columbus.

Preparation of DNA. The bacterial cells were grown at 30 or 37°C, and the genomic DNA was prepared in agarose blocks (22, 40). The cells were cast in agarose as previously described (22), and the blocks were incubated in 4 ml of 10 mM EDTA–25 mM Tris pH (8.0)–2% lysozyme–30 µl of

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RNase (10 mg/ml) for 24 h in a shaking water bath at 50°C. The solution was carefully removed, and 2 ml of ESP (0.5 M EDTA [pH 8.0], 1% sodium sarcosinate, 1 mg of proteinase K [Boehringer, Mannheim, Germany] per ml) was added. After 24 h at 50°C the ESP solution was replaced by another 2 ml of ESP and the blocks were kept at 4°C until analysis. Before treatment with restriction enzyme the proteinase K was inactivated by two 30-min treatments at room temperature with 10 ml of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride. The blocks were then washed three times for 2 h each in TE.

Digestion of DNA with restriction enzymes. The restriction enzymes (*AscI* and *NotI*) were obtained from New England Biolabs. Each agarose block was preincubated in the enzyme mixture overnight at 0°C to ensure sufficient diffusion of enzyme and buffer into the agarose block. For complete digests the block was incubated with 20 U of enzyme for 8 h at 37°C, while incubation with 5 U of enzyme for 10 min was used to obtain partial digests. After digestion the block was incubated in ESP overnight at 50°C. The blocks were routinely kept in ESP solution for several days before further analysis.

PFGE. Gene-line 1 from Beckman with transverse electrodes was used. The Chef Mapper from Bio-Rad Laboratories, Richmond, Calif., with hexagonal electrodes was used in some experiments. DNA in one-fifth of an agarose block was electrophoresed through 1% agarose gels (SeaKem GTG, FMC Corp., Rockland, Maine) in 0.25× modified TBE (25 mM Tris-borate buffer, 0.05 mM EDTA). The electrophoresis was regularly run at 15°C with a pulse time of 4 s for the first 10 min at 170 mA, after which the pulse time was varied from 5 to 90 s at 150 mA for 18 to 24 h.

Size markers were *Saccharomyces cerevisiae* chromosomes (size range 260 to 1,300 kb; Pharmacia and New England Biolabs) for fragments larger than 500 kb, phage lambda concatemers, and a mixture of lambda concatemers and *HindIII* fragments produced by New England Biolabs.

Probes. The Bc random probes were obtained from a *B. cereus* ATCC 10987 library in pUC18 prepared in our laboratory. A phosphatidylinositol-specific phospholipase C probe from *B. cereus* (26) was donated by A. Kuppe, University of Oregon, Eugene; phospholipase C from *B. cereus* (21) was donated by T. Johansen, Tromsø, Norway; pyruvate dehydrogenase from *B. subtilis* (18) was donated by H. Hemilä, Helsinki, Finland; β -lacI (32) and β -lacII (2) from *B. cereus* were donated by J. O. Lampen, The State University of New Jersey, Piscataway; pAKI covering *recF*, *gyrA*, and *gyrB* (origin region) (27) as well as genes for the rRNA operon (p14B8) (41) from *B. subtilis* were donated by K. Bott, University of North Carolina, Chapel Hill; *TerC* from *B. subtilis* (6) was donated by G. Wake, University of Sydney, Sydney, Australia; *abrB* from *B. subtilis* (45) was donated by O.-M. Gulliksen, Oslo, Norway; pESI containing a conserved region of the insecticidal *cryI* toxin gene from *B. thuringiensis* (24) was donated by D. J. Ellar, University of Cambridge, Cambridge, England; and pHT44 containing Tn4430 from *B. thuringiensis* (29) was donated by Lereclus, Paris, France. The Bc37 probe from *B. cereus* (21a) contained an open reading frame with 31.4% sequence identity in 86 amino acids to the B chain of a potassium-transporting ATPase from *Escherichia coli*, and an open reading frame of Bc201 from *B. cereus* (35) showed 62% identity to the rod shape-determining protein, MreB, of *E. coli*. The probes were labelled by using random priming and ³²P-labelled dCTP and dATP (11, 12). Whole plasmids with inserts were used as probes, except for the insecticidal toxin

FIG. 1. PFGE of DNA from *B. thuringiensis* subsp. *thuringiensis* HD2. (A) Running conditions: 60-s pulses for 20 h. Lane 1, *S. cerevisiae* chromosomes; lane 2, *B. thuringiensis* subsp. *thuringiensis* HD2 plus *NotI*. (B) Running conditions: 15-s pulses for 10 h and 25-s pulses for 10 h. Lane 1, lambda concatemers; lane 2, *B. thuringiensis* subsp. *thuringiensis* HD2 plus *NotI*; lane 3, *B. thuringiensis* subsp. *thuringiensis* HD2 undigested.

probe, in which two *EcoRI* fragments (F and G) within the toxin gene (24) were used. In some experiments the DNA was labelled directly in a low-melting-point gel, using the Megaprime DNA labelling system of Promega.

Southern blotting and hybridization. The gels were blotted overnight in 20× SSC (1× SSC is 0.3 M NaCl plus 0.03 M sodium citrate) onto supported nitrocellulose filters (Hybond-C Extra; Amersham Corp., Amersham, United Kingdom) and hybridized as described previously (22).

Assay for enterotoxin and hemolysin. For the enterotoxin test the bacteria were grown overnight at 30°C and centrifuged (12,000 × g), the supernatant was filtered (0.22-μm pore size; Millipore), and aliquots were kept at -80°C. The enterotoxin was measured in microtiter plates by a reversed agglutination assay provided by Oxoid (23, 43). The antitoxin-coated latex particles agglutinated in the presence of toxin to give a clear visual reading after 20 h at room temperature.

Hemolytic activity was observed after growth of the bacteria overnight at 37°C on 5% human blood agar plates. A clear zone around the colony indicated secretion of soluble hemolysins.

RESULTS AND DISCUSSION

Size of the *B. thuringiensis* subsp. *thuringiensis* chromosome. Digestion with *NotI* followed by PFGE showed 16 fragments ranging from 15 to 1,350 kb (Fig. 1). Several electrophoretic runs at different pulse times were necessary to obtain good separation of the smaller fragments (Fig. 1B). The intensity of the 60- and 200-kb bands indicated that each of these were doublets; this was confirmed by hybridization experiments of partial digests. The 260-kb band usually appeared broader because of the presence of an extrachro-

FIG. 2. Construction of physical map of *B. thuringiensis* subsp. *thuringiensis* HD2. (A) Fragments after partial *NotI* digests separated at 60-s pulse for 20 h (lanes 1, 2, 3, and 5) and 25-s pulse for 20 h (lane 4) and hybridized to the probes indicated. (B) Assignments of 13 fragments of the strain *B. thuringiensis* subsp. *thuringiensis* HD2 chromosome. A 15-kb fragment, which was flanked by fragments E and I, could not be verified by these experiments.

separating partially digested *NotI* fragments by PFGE, blotting the fragments onto membranes, and hybridizing them by using radioactively labelled probes. Nineteen random (Bc) probes obtained from our *B. cereus* ATCC 10987 library hybridized to *B. thuringiensis* fragments even under stringent conditions. In addition, *B. cereus*, *B. subtilis*, and *B. thuringiensis* gene probes obtained from other laboratories were used.

No hybridization was obtained with the *B. subtilis* plasmid probe pAK1 (covering *recF*, *gyrA*, and *gyrB*) (27) or the *TerC* (6) probe even at low temperature (55°C) and stringency (1× SSC plus 0.1% sodium dodecyl sulfate). The *TerC*

TABLE 1. *NotI* fragments in *B. thuringiensis* subsp. *thuringiensis* HD2 chromosome

Fragment	Size (kb)
A	1,350
B	750
C	680
D	450
E	350
F	340
G	300
H	260
I	200
J	200
K	170
L	130
M	110
N	60
O	60
P	15
Total (without extrachromosomal bands)	5,425

mosomal band (Fig. 1B). The sizes of the fragments were determined from 10 gels at short and long pulse times, and the total size of the chromosome was calculated to be 5,425 kb (Table 1). The sizes of the chromosomes from four *B. cereus* strains determined previously varied between 5.4 and 6.3 Mb (4, 22). The genomes of the *B. thuringiensis* and *B. cereus* strains were 22 to 33% larger than the *B. subtilis* genome (4.2 Mb) (20, 36).

Construction of the physical map of *B. thuringiensis* subsp. *thuringiensis* HD2. The physical map was constructed by

and pAK1 probes gave no or only weak hybridization to the *B. cereus* strains, as earlier reported (4), and it is possible that these DNA sequences are not similar enough to hybridize.

The sizes of the neighboring fragments were determined from partial digests (Fig. 2A). Probes Bc45, -69, -85, and -88 were anonymous probes from a *B. cereus* ATCC 10987 library in pUC18. Partial sequencing of the probes showed that Bc88 is a part of the 23S rRNA gene (4). At least four gels run at different pulse times were analyzed for each probe. Thirteen of 16 fragments were aligned by using six probes (Fig. 2B), and these results were confirmed with 20 additional probes. No signals between the 740-kb partial fragment (F+O+N+K+M) and the compression area (1,350 kb) (Fig. 2A) excluded fragment C (680 kb) as the neighboring fragment to fragment F (340 kb). In addition, when partial digests were hybridized with probes assigned to fragment C, no partial fragments less than 1,400 kb were detected. This indicated that fragment C was linked to fragment B (750 kb) rather than to fragment F (340 kb). The alignment of the largest fragments was A-C-B (Fig. 3). To determine the position of the 15-kb *NotI* fragment, the fragment was separated by PFGE, labelled directly in low-melting-point gel and hybridized against partial digests of the entire chromosome. The hybridization results showed that the 15-kb fragment was flanked by fragments E (350 kb) and I (200 kb) (data not shown). In agreement with this, hybridization experiments with probes assigned to fragment E always hybridized to a wide fragment E band in partial digests, consistent with a very small neighboring fragment. The final arrangement of the fragments is shown in the physical map (Fig. 3).

Physical maps of four different *B. cereus* strains (ATCC 14579, ATCC 10876, ATCC 10987, and ATCC 11778) have earlier been constructed by PFGE and hybridization experiments (4). The *B. cereus* strains had between 10 and 14 *NotI* fragments. The *B. thuringiensis* subsp. *thuringiensis* HD2 genome contained 16 *NotI* sites, and the size was 5.4 Mb, the same as the size calculated for the *B. cereus* type strain ATCC 14579. The maps of the *NotI* fragments of *B. thuringiensis* subsp. *thuringiensis* HD2, *B. cereus* ATCC 10987, and *B. cereus* ATCC 14579 were aligned to obtain maximum apparent similarity (Fig. 4). Probes that reacted with at least two strains were marked by lines between the corresponding

FIG. 3. Physical map of the *B. thuringiensis* subsp. *thuringiensis* HD2 chromosome based on *NotI* fragments.

NotI fragments. The positions of various gene probes and Bc probes were quite similar in the three *Bacillus* strains. In fact, *B. cereus* ATCC 10987 and *B. cereus* ATCC 14579 looked more related to *B. thuringiensis* subsp. *thuringiensis* HD2 than to the two other *B. cereus* strains (4).

Analysis of 12 *B. thuringiensis* strains. (i) *NotI* patterns. Strikingly different *NotI* patterns were seen for most of the strains (Fig. 5A and 5B). *B. thuringiensis* subsp. *kurstaki* HD1 and HD73 appeared almost identical, except that *B. thuringiensis* subsp. *kurstaki* HD1 showed a more complex array of extrachromosomal bands (Fig. 5D). Likewise, the two *B. thuringiensis* subsp. *israelensis* strains were almost identical. *B. thuringiensis* subsp. *dendrolimus* HD7, *B. thuringiensis* subsp. *entomocidus* HD9, and *B. thuringiensis* subsp. *subtoxigenus* HD109 (Fig. 5C) were clearly closely related and different from the other nine strains. *B. thuringiensis* subsp. *kenya* HD293 was extraordinary; the largest *NotI* fragment was 350 kb, the next was 280 kb, and the rest were smaller than 200 kb (Fig. 5A and 5B). When undigested DNA blocks were analyzed by PFGE, one or more extrachromosomal bands with an apparent size of 15 kb or larger were seen for all strains, except for *B. thuringiensis* subsp. *kenya* HD293.

The heterogeneous *NotI* fingerprint patterns were in good agreement with the reported heterogeneity of this group of bacteria (4, 37). This is in contrast to PFGE results obtained with strains of *Lactococcus lactis* (42), *Campylobacter jejuni* (8), and *Neisseria meningitidis* group A (3) or B (21b), for which the fragment patterns were similar. PFGE analysis of *Haemophilus influenzae* showed identical fragment patterns in some strains, while others differed markedly (28).

(ii) **Hybridization with *cryIA* and transposon probes.** Although plasmids usually carry the *cry* toxin genes, the presence of the genes on the chromosome has been reported (44). To decide whether the *cryIA* gene or closely related insecticidal genes were present on the chromosome or in extrachromosomal DNA only in the 12 *B. thuringiensis* strains we analyzed, the hybridization patterns of undigested and *NotI*-digested DNA separated by PFGE were compared. When undigested DNA was run by PFGE, the chromosomal DNA remained in the agarose block in the well.

The *cryIA* gene from *B. thuringiensis* subsp. *kurstaki* HD1 was used as a toxin probe. This probe could cross-react with several, but not all, of the *cryI* to *cryIV* genes (13, 19, 24). Although not proven, our data strongly indicate a chromosomal localization of *cry* genes in six strains, and in two of these strains (*B. thuringiensis* subsp. *kurstaki* HD73 and *B. thuringiensis* subsp. *entomocidus* HD9) the *cry* gene was present in plasmids as well (Table 2; Fig. 6B). The *cry* hybridization signals to the chromosomal fragment were usually weaker than to the plasmids (Fig. 6A and 6B), possibly due to several copies of the plasmid or because the chromosomal *cry* gene may have been less related to the *cryIA* gene probe used in this study. In five strains (the partial fragments in *B. thuringiensis* subsp. *darmstadtensis* HD146 were perhaps too large to detect) hybridization to a partial *NotI* or *AscI* chromosomal fragment was found; an example is shown in Fig. 6C. The *cryIA* probe gave very strong extrachromosomal signals for strains *B. thuringiensis* subsp. *kurstaki* HD1 and *B. thuringiensis* subsp. *kurstaki* HD73 compared with those for the other 10 *B. thuringiensis* strains, implying the presence of multiple closely related crystal toxin genes or gene copies. Since the presence of crystalline inclusion bodies is a typical feature of *B. thuringiensis* strains that produce insecticidal toxin, the bacteria

FIG. 4. Maps of *B. thuringiensis* subsp. *thuringiensis* HD2, *B. cereus* ATCC 10987, and *B. cereus* ATCC 14579 (type strain) were aligned to obtain maximum apparent similarity. Probes that reacted with two or three strains were marked by lines.

were inspected under the microscope. Typical crystal inclusion bodies were observed in the two *B. thuringiensis* subsp. *israelensis* strains as well as in all strains harboring plasmids that hybridized to the *cryIA* probe. In strains with chromosomal location only of the toxin gene, like *B. thuringiensis* subsp. *canadensis* HD224, *B. thuringiensis* subsp. *darmstadtensis* HD146, *B. thuringiensis* subsp. *kenya* HD293, and *B. thuringiensis* subsp. *thuringiensis* HD2, no crystal body was detected. This may be due to either an incomplete toxin gene or the lack of an additional plasmid-borne factor(s) that may be required for crystal formation (16).

The transposon Tn4430 of the Tn3 family (29) and IS231 (33) are usually present in the vicinity of *cryIA* genes. It has been reported by others (25, 30) that the Tn4430 sequence is located on several plasmids of *B. thuringiensis* strains that harbor the crystal gene, and this sequence is conserved without apparent modification among serotypes which differ in the specificity of the *cry* toxin. We have therefore used probe pHT44 (29) containing transposon Tn4430 and IS231.

The transposon probe hybridized to both chromosomal and extrachromosomal bands, and positive chromosomal fragments always hybridized to the *cry* probe also (Table 2). However, the transposon and the *cry* probes hybridized to different extrachromosomal bands in *B. thuringiensis* subsp. *dendrolimus* HD7, *B. thuringiensis* subsp. *morrisoni* HD12, and *B. thuringiensis* subsp. *subtoxius* HD109 (data not shown). Only some of the extrachromosomal bands in *B. thuringiensis* subsp. *kurstaki* HD1 and *B. thuringiensis* subsp. *kurstaki* HD73 hybridized to both the *cry* and transposon probes. The transposon probe hybridized very strongly to both chromosomal and extrachromosomal DNA from *B. thuringiensis* subsp. *darmstadtensis* HD146, indicating the presence of multiple genes copies (Fig. 6D). The *cryIA* toxin probe hybridized only weakly to two chromosomal *NotI* fragments (820 and 900 kb) (Table 2) in this strain.

The two *B. thuringiensis* subsp. *israelensis* strains hybridize neither to the transposon probe nor to the *cryIA* probe.

FIG. 5. *NotI* fragments of DNA from *B. thuringiensis* strains separated by PFGE at 60-s pulses for 20 h (Beckman) (A) and 20-s pulses for 40 h (Bio-Rad) (B). Lanes: A1, *S. cerevisiae* chromosomes; B1, lambda concatemers; 2, *B. thuringiensis* subsp. *kenya* HD293; 3, *B. thuringiensis* subsp. *canadensis* HD224; 4, *B. thuringiensis* subsp. *darmstadtensis* HD146; 5, *B. thuringiensis* subsp. *entomocidus* HD9; 6, *B. thuringiensis* subsp. *kurstaki* HD1; 7, *B. thuringiensis* subsp. *kurstaki* HD73; 8, *B. thuringiensis* subsp. *israelensis* ONR60A; 9, *B. thuringiensis* subsp. *israelensis* 4Q2-72 (C) *NotI* fragments of DNA from three *B. thuringiensis* strains separated at 60-s pulses for 20 h. Lanes: 1, *S. cerevisiae* chromosomes; 2, *B. thuringiensis* subsp. *entomocidus* HD9; 3, *B. thuringiensis* subsp. *dendrolimus* HD7; 4, *B. thuringiensis* subsp. *subtoxius* HD109, (D) DNA from *B. thuringiensis* subsp. *kurstaki* HD1 separated at 60-s pulses for 20 h. Lanes: 1, *S. cerevisiae* chromosomes; 2, *B. thuringiensis* subsp. *kurstaki* HD1 plus *NotI*; 3, *B. thuringiensis* subsp. *kurstaki* HD1 undigested.

TABLE 2. Detection of insecticidal toxin gene, transposon gene, enterotoxin, and hemolytic activity in *B. thuringiensis* strains.

Strain	Hybridization ^a				Enterotoxin ^b	Hemolytic activity ^c
	<i>cryIA</i> probe		pHT44 probe			
	E	C	E	C		
<i>B. thuringiensis</i> subsp. <i>canadensis</i> HD224	–	290 kb	–	–	–	++
<i>B. thuringiensis</i> subsp. <i>darmstadiensis</i> HD146	–	820 kb, 900 kb	+	900 kb	+++	++
<i>B. thuringiensis</i> subsp. <i>dendrolimus</i> HD7	+	–	+	–	+++	+++
<i>B. thuringiensis</i> subsp. <i>entomocidus</i> HD9	+	350 kb, 700 kb	–	700 kb	+++	–/+
<i>B. thuringiensis</i> subsp. <i>israelensis</i> ONR60A	–	–	–	–	++	–/+
<i>B. thuringiensis</i> subsp. <i>israelensis</i> 4Q2-72	–	–	–	–	++	–/+
<i>B. thuringiensis</i> subsp. <i>kenya</i> HD293	–	50 kb, 280 kb, 400 kb	–	50 kb, 280 kb, 400 kb	–	++
<i>B. thuringiensis</i> subsp. <i>kurstaki</i> HD1	+	–	+	–	+++	–
<i>B. thuringiensis</i> subsp. <i>kurstaki</i> HD73	+	290 kb	+	–	+++	–
<i>B. thuringiensis</i> subsp. <i>morrisoni</i> HD12	+	–	+	–	++	–/+
<i>B. thuringiensis</i> subsp. <i>subtoxicus</i> HD109	+	–	+	–	+++	++
<i>B. thuringiensis</i> subsp. <i>thuringiensis</i> HD2	–	340 kb	–	–	–	+

^a E, extrachromosomal; C, chromosomal^b Enterotoxin was measured by immunoreaction.^c Hemolysis of human erythrocytes in agar plates.

When the whole plasmid pES1 (24) was used as a toxin probe, a very weak signal of 400 kb was detected in both strains, indicating that the plasmid contained other sequences similar to chromosomal DNA of *B. thuringiensis* subsp. *israelensis* strains. In *B. thuringiensis* subsp. *israelensis* toxin crystal production has been associated with the presence of a 72-MDa plasmid, but the crystal gene has also been detected on the chromosome (16). Weak or no hybridization probably reflects the presence of relatedness of the different strains with respect to toxin genes. The amino acid identity between the gene probe product of *cryIA* and those

of *cryIVA*, *cryIVB*, *cryIVC*, and *cryIVD* present in *B. thuringiensis* subsp. *israelensis* was below 30% (19). The toxin genes localized on the 72-MDa plasmid are thus most likely too unrelated to hybridize to the *cryIA* probe.

For *B. thuringiensis* subsp. *kenya* it has been reported (34) that a protoxin gene is localized on a high-molecular-mass plasmid estimated to be 50 MDa in size. In *B. thuringiensis* subsp. *kenya* HD293, no plasmid was detected and the *cryIA* probe as well as the transposon probe hybridized to chromosomal *NotI* fragments (Table 2).

The presence of extrachromosomal bands in the 12 *B. thuringiensis* strains was analyzed by regular gel electrophoresis. No bands below 10 kb were detected by ethidium bromide staining or by hybridization using Tn4430 as a probe.

(iii) **Enterotoxin and hemolytic activity.** All *B. thuringiensis* strains except *B. thuringiensis* subsp. *kurstaki* HD1 and HD73 were hemolytic when grown on human blood agar plates, indicating secretion of soluble hemolysins (Table 2). Nine of the *B. thuringiensis* strains produced *B. cereus*-type enterotoxin (Table 2). Both of the nonhemolytic *B. thuringiensis* subsp. *kurstaki* strains were strongly positive for the enterotoxin, as were the apparently closely related strains *B. thuringiensis* subsp. *dendrolimus* HD7, *B. thuringiensis* subsp. *entomocidus* HD9, and *B. thuringiensis* subsp. *subtoxicus* HD109 (Table 2). It is not known yet whether the enterotoxin gene in *B. cereus* is localized on the chromosome or on plasmids, and cloning of the gene has not been reported. When *B. cereus* strains were analyzed for enterotoxin production, 9 of 12 strains were enterotoxin positive (21b), whereas all strains were hemolytic.

Strains derived from *B. thuringiensis* subsp. *kurstaki* HD1 as well as a number of other *B. thuringiensis* strains are today used for the commercial production of *B. thuringiensis* biopesticides (13). The use of biopesticides is expected to increase, and much work is put into the screening of *B. thuringiensis* strains for the presence of Cry toxins with new specificities. Likewise, the *cry* toxin genes are being moved from one *B. thuringiensis* strain to another (14, 31), as well as being expressed in other bacteria (38).

Our results confirm the close relationship between *B. thuringiensis* and *B. cereus* (4). The fact that *B. thuringiensis* strains may produce *B. cereus*-type enterotoxin suggest that these *B. thuringiensis* strains should be regarded as entero-

FIG. 6. DNA from *B. thuringiensis* strains separated at 60-s pulses for 20 h, blotted onto membranes, and hybridized with the *cryIA* toxin probe. (A) Lanes: 1, *B. thuringiensis* subsp. *dendrolimus* HD7 plus *NotI*; 2, *B. thuringiensis* subsp. *dendrolimus* HD7 undigested; 3, *B. thuringiensis* subsp. *subtoxicus* HD109 plus *NotI*; 4, *B. thuringiensis* subsp. *subtoxicus* HD109 undigested. Fainter bands may represent plasmids of lower copy number or perhaps open forms of the more strongly hybridizing plasmids of slightly greater mobilities. (B) Lanes: 1, *B. thuringiensis* subsp. *entomocidus* HD9 plus *NotI*; 2, *B. thuringiensis* subsp. *entomocidus* HD9 undigested. (C) Lane 1, *B. thuringiensis* subsp. *thuringiensis* HD2 plus *NotI*, partial digest. Compr., fragments ≥ 450 kb. (D) Hybridization of the Tn4430 transposon to DNA from *B. thuringiensis* subsp. *darmstadiensis* HD146. Lanes: 1, *B. thuringiensis* subsp. *darmstadiensis* HD146 plus *AscI*; 2 and 3, *B. thuringiensis* subsp. *darmstadiensis* HD146 plus *NotI*; 4, *B. thuringiensis* subsp. *darmstadiensis* HD146 undigested. The sizes of the *S. cerevisiae* chromosomes are indicated.

toxin-producing *B. cereus* strains that have acquired cry-containing plasmids. This should be taken into consideration when screening *B. thuringiensis* strains as potential biopesticides. The taxonomy of *B. cereus* and *B. thuringiensis* should be reevaluated.

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