Genomic organization of the entomopathogenic bacterium *Bacillus thuringiensis* subsp. *berliner* 1715

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A physical and genetic map of the *Bacillus thuringiensis* subsp. *berliner* 1715 (serotype 1) chromosome was constructed by pulsed field gel electrophoresis using three restriction enzymes, *Ascl*, *NotI* and *SfiI*. A total of 24 restriction enzyme sites and 28 probes were located on the map. The chromosome size was 5.7 Mb. Consistent with previous mapping of *Bacillus cereus* chromosomes, the genes were nonrandomly distributed. Genes which are often plasmid-encoded were located on one half of the chromosome, whereas the other half contained rRNA genes and the origin region. Hybridization with macro-restriction fragments showed that the region containing rRNA genes and the origin was similar to that of the *B. cereus* type strain, ATCC 14579, and confirmed that the region was conserved between the *B. cereus* and *B. thuringiensis* chromosomes. The insecticidal toxin probe *cryIA* or the transposon probe Tn4430 hybridized to six extrachromosomal elements of 60, 60, 100, 130, 270 and 600 kb, indicating that the genome size was at least 6.9 Mb.

Keywords: *Bacillus thuringiensis*, physical mapping, transposon, insecticidal genes, pulsed field gel electrophoresis

INTRODUCTION

Bacillus thuringiensis is a spore-forming Gram-positive soil bacterium. During sporulation, a crystal containing an insecticidal protoxin is formed inside the cell. Because of its production of insecticidal toxins, *B. thuringiensis* is the most widely used source of biological pesticides (Aronson, 1993; Höfte & Whiteley, 1989). At least 50 protoxins have been characterized by gene sequencing and toxicity profiles (Aronson, 1993). Four major classes of insecticidal protoxin genes have been proposed (*cryI–IV*) (Höfte & Whiteley, 1989) and each class has been further divided into several subclasses (*cryIA–cryID*). Most toxins are active against larvae of lepidopteran species, but some show toxicity against dipteran or coleopteran species (Höfte & Whiteley, 1989). The *cry* genes are usually located on large plasmids (50 kb or larger) (Carlton & Gonzales, 1985; Kronstad *et al.*, 1983), although chromosomal *cry* genes have been reported in some *B. thuringiensis* strains (Carlson & Kolstø, 1993; Klier *et al.*, 1982; Kronstad *et al.*, 1983). A transposon of the Tn3 family (Tn4430) flanked by IS elements is often, but not always, present in the vicinity of the *cry* genes

(Lereclus *et al.*, 1986; Mahillon *et al.*, 1985; Menou *et al.*, 1990).

The technique of pulsed field gel electrophoresis (PFGE) allows separation of megabase-sized linear DNA fragments (Schwartz & Cantor, 1984; Smith *et al.*, 1988) and is useful for the construction of physical maps of bacterial chromosomes (for a review, see Smith & Condemine, 1990). Cloned genes may easily be located on these physical maps and thus a genetic map can be constructed. Circular DNA moves aberrantly by PFGE, or not at all, depending on size and topology (Beverley, 1988; Hightower & Santi, 1989). We have previously used PFGE to construct a map of one *B. thuringiensis* chromosome of 5.4 Mb (subsp. *thuringiensis* HD2; Carlson & Kolstø, 1993). *Bacillus cereus* does not produce insecticidal protoxin crystals, but otherwise these bacteria cannot be distinguished from *B. thuringiensis* (Carlson *et al.*, 1994; Gordon *et al.*, 1973; Smith *et al.*, 1952). The chromosome size of ten *B. cereus* strains have been determined, and chromosome maps constructed for six of them (Carlson *et al.*, 1992; Carlson & Kolstø, 1994; Kolstø *et al.*, 1990). The chromosomes ranged in size from 2.4 Mb to 6.3 Mb. A

Fig. 1. PFGE of DNA from two *B. thuringiensis* strains. (a) Lanes: 1, lambda concatemers (48.5–1018.5 kb, New England Biolabs); 2, undigested *B. thuringiensis* subsp. *berliner* 1715. Running conditions: 4 s for 10 min at 170 mA, then 30 s pulses for 20 h at 150 mA, 15 °C (Beckman). (b) Lanes: 1, MegaBase IV (size range 1.03–3.30 Mb, Gibco, BRL); 2, *Saccharomyces cerevisiae* (225–1900 kb, New England Biolabs); 3–5, *B. thuringiensis* subsp. *berliner* 1715 digested with *AscI* (lane 3), *NotI* (lane 4), *SfiI* (lane 5). Running conditions: 100 s pulses for 20 h at 150 mA, 15 °C (CHEF Mapper, Bio-Rad Laboratories). The *AscI* and *SfiI* digestions were digested for only 1 h, resulting in two partial fragments of 230 kb (A6 and A7) and 770 kb (A5, A6 and A7) in the *AscI* digests, and a weak fragment of 910 kb (S4 and S6) in the *SfiI* digest. The DNA in the compression region (com.) corresponds to partial fragments larger than 1850 kb. A weak plasmid band of 270 kb was seen in all three digests (co-migrating with the 280 kb *SfiI* fragment in lane 5). (c) Lanes: 1, lambda concatemers; 2, *S. cerevisiae*; 3, *B. thuringiensis* subsp. *thuringiensis* HD2 digested with *NotI*; 4, *B. thuringiensis* subsp. *berliner* 1715 digested with *NotI*. Running conditions: as (a). (d) Lanes: 1, lambda concatemers and *HindIII* fragments of lambda (0.13–194 kb, New England Biolabs); 2, *B. thuringiensis* subsp. *berliner* 1715 digested with *NotI*. Running conditions: 4 s for 10 min at 170 mA, then 6 s pulses for 20 h at 150 mA, 15 °C (Beckman).

comparison of the maps showed a similar organization in one half of the chromosome harbouring the origin of replication, while the other half appeared to have greater variation and probes in this area may be present extra-chromosomally in strains with a small chromosome (Carlson & Kolstø, 1994). We report here a physical map of the *B. thuringiensis* subsp. *berliner* 1715 chromosome for three restriction enzymes and have assigned probes to the map. This strain has been widely used for research purposes, and its *cryIA* genes and IS elements have been cloned and sequenced.

METHODS

Bacterial strains. *B. thuringiensis* subsp. *berliner* 1715 was obtained from the International Entomopathogenic *Bacillus* Centre (IEBC Collection), Institut Pasteur, Paris. *B. thuringiensis* subsp. *thuringiensis* HD2 was obtained from the *Bacillus* Genetic Stock Center, Department of Biochemistry, The Ohio State University, Columbus. *B. cereus* type strain ATCC 14579 and *B. cereus* ATCC 10987 were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA.

Preparation, digestion and separation of DNA. Cells were grown overnight at 37 °C. They were then harvested and total

DNA was prepared in agarose blocks as previously described (Carlson & Kolstø, 1993; Kolstø *et al.*, 1990; Smith *et al.*, 1988). The DNA was digested with *AscI*, *NotI* and *SfiI* (New England Biolabs) and separated by Gene-line 1 (Beckman Instruments) or the CHEF Mapper or the CHEF Mapper XA System (Bio-Rad Laboratories) (Carlson & Kolstø, 1993, 1994).

Southern blotting and hybridization. DNA was blotted overnight onto nylon membranes (MSI) (Kolstø *et al.*, 1990). Genomic DNA was hybridized with gene probes from *B. cereus*, *B. thuringiensis* and *Bacillus subtilis*. The references of these probes are given in Table 2. *B. cereus* (Bc) random probes were obtained from a *B. cereus* ATCC 10987 library in pUC18 prepared in our laboratory. When chromosomal fragments were used as probes, the fragments were separated in low-melting-point agarose (LMA) by PFGE: 25 µl of a fivefold dilution of LMA containing the DNA was boiled, then the DNA labelled by using random priming and the Klenow enzyme (New England BioLabs) and ³²P-labelled dCTP and dATP (Feinberg & Vogelstein, 1983, 1984). The probe was incubated for 1 h at 37 °C to complete the polymerase reaction. After incubation, the denatured probe was added directly to the (pre)hybridization solution (Kolstø *et al.*, 1990), containing denatured and fragmented salmon sperm DNA, without further cleaning. A temperature of 65 °C was used for all hybridization and washing procedures, except for the *B. subtilis* probe pAK1 where a temperature of 55 °C was used.

RESULTS AND DISCUSSION

Digestion of *B. thuringiensis* subsp. *berliner* 1715 genomic DNA with three restriction enzymes and chromosome size estimation

Before digestion of the genomic DNA with restriction enzymes, the uncut DNA was analysed by PFGE. Several weak bands were observed (Fig. 1a). These extra-chromosomal bands may originate from large linear plasmids, or circular plasmids that have been partially linearized during the preparation of the DNA as previously reported for *Agrobacterium tumefaciens* (Allardet-Servent *et al.*, 1993) and *Rhizobium meliloti* (Sobral *et al.*, 1991), or they might be smaller circular plasmids that migrate in a pulse-time-independent manner (Beverley, 1988; Hightower & Santi, 1989).

The size of each restriction fragment generated by *AscI*, *NotI* and *SfiI* restriction enzymes was determined by using CHEF and Beckman separation systems. The restriction fragments obtained by digestion with the three enzymes were numbered as shown in Table 1. The sizes of the fragments were determined from four gels at long pulse times separating fragments up to about 2 Mb (Fig. 1b), and three gels at short pulse times separating

fragments smaller than 500 kb (Fig. 1c). Sizes of the double-digested fragments (Table 2) were determined from two gels at long pulse times and one gel at short pulse times (not shown). *AscI* cleaved the *B. thuringiensis* subsp. *berliner* 1715 chromosome into seven fragments ranging in size from 70 to 1790 kb (Fig. 1b, lane 3), whereas *SfiI* generated six fragments ranging from 280 to 1770 kb (Fig. 1b, lane 5). *NotI* generated 16 genomic fragments ranging from 50 to 1460 kb (Fig. 1b-d). At least four of the *NotI* fragments arose from linearized plasmids (see below) and were excluded in the estimation of chromosome size. The *NotI* digest of *B. thuringiensis* subsp. *thuringiensis* HD2 (Fig. 1c, lane 3) is shown for comparison and is clearly different from that of *B. thuringiensis* subsp. *berliner* 1715 (lane 4). The *B. thuringiensis* subsp. *berliner* 1715 chromosome size is 5.7 Mb based on the average sum of the sizes of the restriction fragments using both standards (Table 1). Any fragments smaller than 23 kb may not have been detected by PFGE, but their contribution to the chromosome size would be small.

Physical map of the *B. thuringiensis* subsp. *berliner* 1715 chromosome

To determine the relative order of the restriction fragments generated by *AscI*, *NotI* and *SfiI*, the fragments were blotted and hybridized to various probes. Enzyme digestion involved double, partial and complete digests using the three enzymes. In some experiments, a chromosomal fragment from a digest with one enzyme of the *B. thuringiensis* subsp. *berliner* 1715 chromosome or a *B. cereus* chromosome (ATCC 14579) was used as a linking probe to analyse the fragments generated by a second enzyme.

mreB, the gene coding for a shape-determining protein (Narahara *et al.*, 1992), was mapped to the *AscI* fragment A5, the *NotI* fragment N1 and the *SfiI* fragment S1 (Table 2). Hybridization to partial *AscI* digests revealed that A4 and A7 were the neighbouring fragments of A5, and that A6 neighboured A7. Hybridization to partial *NotI* digests showed that a small *NotI* fragment of about 50 kb (N10) was the neighbouring fragment of N1. rRNA genes were mapped to A5, and to the neighbouring fragment A4, and hybridization with an rRNA probe confirmed the alignments of the fragments obtained by the *mreB* probe. The rRNA probe also hybridized weakly to N4 and it is possible that this *NotI* fragment contains one copy of the gene operon. This gene operon was mapped to the *SfiI* fragment S5. Of five *B. cereus* probes also mapped to N4, one (Bc209) hybridized to S4 and four (Bc52, Bc54, *trr* and *syn*) hybridized to S6. Using these results, the three smallest *SfiI* fragments (S4, S6 and S5) were aligned. S1 was linked to S5 by using a linking fragment probe of 290 kb from the *B. cereus* ATCC 14579 chromosome, whereas S2 was linked to S4 when N6 was used as a linking fragment probe (Table 2). The *AscI* site between A1 and A3 was located on the map when β -*lacII* and Bc45 (both mapped to N2 and S2) were hybridized against *AscI*

Table 1. Sizes of *AscI*, *NotI* and *SfiI* restriction fragments and estimations of the size of the *B. thuringiensis* subsp. *berliner* 1715 chromosome

Sizes of fragments larger than 500 kb were determined using *Saccharomyces cerevisiae* chromosomes and MegaBase IV as standards. Phage lambda concatemers were used for fragments below 500 kb.

<i>AscI</i>		<i>NotI</i> *		<i>SfiI</i>	
Fragment	Size (kb)	Fragment	Size (kb)	Fragment	Size (kb)
A1	1790	N1	1460	S1	1770
A2	1420	N2	860	S2	1410
A3	920	N3	780	S3	1280
A4	850	N4	750	S4	630
A5	550	N5	650	S5	380
A6	160	N6	450	S6	280
A7	70	N7	330		
			(300)		
			(270)		
		N8	250		
			(100)		
			(60)		
		N9	50		
		N10	50		
Total					
chromosome:	5760		5630		5750

* At least four *NotI* fragments (in parentheses) originated from extrachromosomal DNA and are not included in the chromosome size estimation. The 100 kb and 60 kb *NotI* bands were doublets.

Table 2. Probes which hybridized to *AscI*, *NotI*, *SfiI* and double-digested DNA from *B. thuringiensis* subsp. *berliner* 1715

Probe	Origin	Size of hybridizing restriction fragments (kb)*						Reference	
		<i>AscI</i>	<i>NotI</i>	<i>SfiI</i>	<i>AscI</i> + <i>NotI</i>	<i>SfiI</i> + <i>NotI</i>	<i>AscI</i> + <i>SfiI</i>		
Gene probes									
<i>pAK1</i>	<i>B. subtilis</i>	(850) 550	1460	1770 (380)	(800) 550	1460 (200)	(850) 550 (320)	Lampe & Bott (1985)	
<i>rRNA</i>	<i>B. subtilis</i>	850, 550, ND	1460, 750	1770, 380	800, 750, 550	1460, 200	850, 550, 320	Stewart <i>et al.</i> (1982)	
<i>inA</i>	<i>B. thuringiensis</i>	1420	780	1280	780	700	1220	Lövgren <i>et al.</i> (1990)	
<i>flaBI</i>	<i>B. thuringiensis</i>	ND	780	ND	780	ND	ND	Lövgren <i>et al.</i> (1993)	
<i>bblA</i>	<i>B. cereus</i>	920	650	1410	460	400	860	Heinrichs <i>et al.</i> (1993)	
<i>β-lacI</i>	<i>B. cereus</i>	920	650	1410	460	400	860	Madonna <i>et al.</i> (1987)	
<i>β-lacII</i>	<i>B. cereus</i>	ND	860	1410	400	860	550	Ambler <i>et al.</i> (1985)	
<i>PLC</i>	<i>B. cereus</i>	850	ND	ND	800	ND	850	Johansen <i>et al.</i> (1988)	
<i>PI-PLC</i>	<i>B. cereus</i>	ND	50	1410	50	50	550	Kuppe <i>et al.</i> (1989)	
<i>PDH</i>	<i>B. subtilis</i>	ND	450	630	450	360	360	Hemilä <i>et al.</i> (1990)	
<i>SASP</i>	<i>B. cereus</i>	850	1460	1770	800	ND	850	Loshon <i>et al.</i> (1986)	
<i>Tn4330†</i>	<i>B. thuringiensis</i>	—	60, 100	—	60, 100	60, 100	—	Lereclus <i>et al.</i> (1986)	
<i>cryIA†</i>	<i>B. thuringiensis</i>	—	270	—	270	270	—	Kronstad <i>et al.</i> (1983)	
<i>mreB</i>		550	1460	ND	550	1460	550	Narahara <i>et al.</i> (1992)	
<i>chrA</i>		850, 70	ND	ND	800, 70, 50	ND	850, 70	I. K. Hegna and others (unpublished)	
<i>bgIII</i>		70	ND	ND	70	ND	ND	I. K. Hegna and others (unpublished)	
<i>tdb</i>		1420	780	1280	780	700	ND	I. K. Hegna and others (unpublished)	
<i>dnaA</i>		550	ND	ND	550	ND	ND	K. Kvaløy and others (unpublished)	
<i>alkA</i>		850	1460	1770	800	1460	850	L. Lillehagen and others (unpublished)	
<i>ter</i>		<i>B. cereus</i> ATCC 10987	ND	750	280	750	280	280	A. Grønstad and others (unpublished)
<i>pur9</i>		850	ND	ND	800	1460	850	A.-B. Kolstø and others (unpublished)	
<i>syn</i>		ND	750	280	750	280	280		
Fragment probes									
Bc45		920	860	1410	460	860	860		
Bc54		ND	750	280	750	280	280		
Bc68		850	ND	ND	800	ND	850		
Bc72		ND	780	ND	780	700	ND		
Bc85		1420 (850, 550)	780	ND	780	80	200		
Bc86		ND	750	280	750	280	280		
Bc209		ND	750	630	750	270	630		
Bc540		ND	330	1280	330	330	1220		
Large anonymous probes									
1300 kb	<i>B. cereus</i> ATCC 14579	850, 550, 70	1460, (750)	1770, (380)	850, 150, 70, 50	1460, (200)	850, 550, (330)	A.-B. Kolstø and others (unpublished)	
820 kb		1790, (850), (550)	(1460), 750	(1770), 630, 380 280	(850), 750, (550)	(1460), 630, 280, 200	(850), (550), 330, 280		
400 kb		ND	ND	630	450	360	630		
370 kb		ND	780	630	450	360, 80	200		
290 kb		160	250	1770, 380	120	180, 70	320, 60		
40 + 40 + 50 kb		1420, 850	50	1770	50	50	200		
15 kb‡		—	—	—	—	—	—		

ND, Not determined; —, negative.

* Fragments in parentheses contained rRNA genes and gave weak cross-hybridization.

† On extrachromosomal DNA (see text).

‡ The 15 kb fragment was extrachromosomal.

Fig. 2. (a) Physical map of *B. thuringiensis* subsp. *berliner* 1715 showing *AscI* (inner circle), *NotI* (outer circle) and *SfiI* (middle circle) sites. The hatched circle shows restriction fragments that hybridized to various probes. The arrow indicates a region of the chromosome which contains at least two more *NotI* restriction sites, the positions of which have not been determined. Some of the *NotI* fragments of the *B. cereus* ATCC 14579 chromosome are drawn outside the corresponding part of the chromosome of *B. thuringiensis* subsp. *berliner* 1715. (b) Proposed extrachromosomal DNA elements larger than 50 kb in *B. thuringiensis* subsp. *berliner* 1715 detected by using a *cryIA* probe and a Tn4430 probe. Tn4430 has also previously been shown to be located on the *cryIA*-positive plasmid of 60 kb (see text).

and *SfiI* double-digested DNA. Alignment of the *NotI* fragments N3, N7 and N5, and linking of S2 to S3 and A2 to A3, were achieved by hybridization of *hblA* (mapped to N5), Bc540 (mapped to N7) and *inA* (mapped to N3) to single and double digests using the three enzymes (Table 2) and to partial *NotI* digests. A2 was linked to fragment A4 by using a fragment probe from *B. cereus* ATCC 14579 consisting of three neighbouring *NotI* fragments of 40, 40 and 50 kb (Table 2) (Carlson *et al.*, 1992). Hybridization with this linking probe showed that this part of the *B. thuringiensis* subsp. *berliner* 1715 chromosome also contained several smaller *NotI* fragments, including N10, of 50 kb. The position of N10 relative to the *AscI* site

between A2 and A4 has not been determined. This linking probe hybridized to the same *AscI* and *SfiI* double-digested fragment of 200 kb as Bc85 (Table 2). Bc85 also hybridized to A2, N3 and the *NotI* and *SfiI* double-digested fragment of 50 kb, and by combining these results with the results obtained by the linking probe above, S1 could be aligned next to S3. The final arrangement of the *AscI*, *NotI* and *SfiI* restriction sites in the physical map of *B. thuringiensis* subsp. *berliner* 1715 is shown in Fig. 2(a). Any fragments smaller than 23 kb after double digestion have not been determined.

Assignment of probes to the restriction fragments

We used Southern hybridization to place 28 gene probes and anonymous probes on the restriction map (Fig. 2a) (Table 2). Most of the probes were obtained from *B. cereus* ATCC 10987 and *B. thuringiensis*, but three were from *B. subtilis* (Table 2). Hybridization with all these probes to *AscI*, *NotI*, *SfiI* fragments and combination thereof confirmed the order of the restriction fragments in the chromosome map (Fig. 2a).

The fragments from *B. cereus* ATCC 10987 have been partly or fully sequenced and contain putative genes such as *ter* (A. Grønstad and others, unpublished), *chrA*, *bglII*, *tdh* (I. K. Hegna and others, unpublished), *pur9*, *syn*, *gltP*, *aspA* (A.-B. Kolstø and others, unpublished), *dnaA* (K. Kvaløy and others, unpublished), *alkA* (L. Lillehagen and others, unpublished) and *mreB* (Narahara *et al.*, 1992). Bc85 hybridized most strongly to a 780 kb *NotI* fragment, but also weakly to other parts of the chromosome (to two *AscI* fragments of 550 and 850 kb). This anonymous probe also hybridized strongly to yeast chromosomes. *chrA* hybridized most strongly to A7 of 70 kb, but also weakly to A4 of 850 kb (Table 2). Most of these probes were located on one half of the chromosome map. The *dnaA* probe hybridized to a 550 kb *AscI* fragment (A5), indicating that the origin of replication is most likely located on this part of the chromosome. The rRNA genes were mapped to three restriction fragments (A4, A5 and S5 in Fig. 3) including the fragment containing the proposed origin region. A probe from *B. subtilis* (pAK1) covering *recF*, *gyrA* and *gyrB* (Lampe & Bott, 1985) hybridized most strongly to the fragment containing the origin region (A5), but also weakly to A4 and S5. The other half of the chromosome contained sequences which are often plasmid-encoded such as β -lacI, β -lacII, *inA* and *hblA*. The *hblA* gene (mapped to N5) codes for the B component of haemolysin BL which may be responsible for the diarrhoeal form of *B. cereus* food poisoning (Beecher & Macmillan, 1990, 1991). We have previously shown that several *B. thuringiensis* strains produce enterotoxin (Carlson & Kolstø, 1993).

A probe for an insecticidal toxin gene, *cryIA*, hybridized to a *NotI* fragment of 270 kb, and a probe for Tn4430 hybridized to two *NotI* fragments of 60 kb and 100 kb. As discussed later, these *NotI* fragments were derived from extrachromosomal DNA.

Fig. 3. For legend see facing page.

Fig. 4. Comparison of the linearized chromosome maps of *B. thuringiensis* subsp. *berliner* 1715, *B. cereus* ATCC 10987 and *B. thuringiensis* subsp. *thuringiensis* HD2. Each line represents the position of a probe, which within the fragment, is arbitrary. A, *Ascl*; N, *NotI*; S, *SfiI*.

Cross-hybridization between restriction fragments from *B. thuringiensis* subsp. *berliner* 1715 and *B. cereus* type strain ATCC 14579

Chromosomal *NotI* fragments from the *B. cereus* type strain ATCC 14579 were also used as probes in the mapping of the *B. thuringiensis* subsp. *berliner* 1715 chromosome (Table 2). The fragment probes used were mainly from the conserved part of the *B. cereus* chromosome surrounding the putative origin (Carlson & Kolstø, 1994). Hybridization with these fragments gave unambiguous results, indicating conserved regions between the chromosomes. One exception was the 1300 kb *NotI* and 800 kb *NotI* fragments from *B. cereus*, both containing rRNA genes, which hybridized weakly to all fragments in *B. thuringiensis* containing rRNA genes (Table 2). The corresponding chromosomal regions of the two strains are shown in Fig. 2a. When a *NotI* fragment of 370 kb from the proposed variable region of *B. cereus* ATCC 14579 was used as a probe, hybridization to two different locations in the *B. thuringiensis* subsp. *berliner* 1715 chromosome (fragments N3 and S4) was obtained, indicating that these regions are less conserved than the rest of the chromosome. An extrachromosomal fragment of 15 kb from *B. cereus* ATCC 14579 did not hybridize to the *B. thuringiensis* subsp. *berliner* 1715 genome.

Presence of extrachromosomal DNA

The Tn4430 probe and a *cryIA* probe were used in hybridization experiments against blots containing undigested DNA (Fig. 3a, b, lane 7), single digests (Fig. 3a, b, lanes 1, 2 and 3), and double digests (Fig. 3a, b,

lanes 4, 5 and 6) using *AscI*, *NotI* and *SfiI*, and partial *NotI* digests (Fig. 3a, b, lane 2). A similar hybridization pattern in all lanes indicated that the probes were located on only extrachromosomal DNA. Tn4430 hybridized to at least six extrachromosomal bands, three of them were separated in a pulse-time-dependent manner as linear fragments of 60, 100 and 130 kb (Fig. 3a). These three bands probably originate from plasmids that were linearized during preparation of the DNA. The 60 and 100 kb bands hybridized more strongly to the Tn4430 probe in all *NotI* digests (Fig. 3a, lanes 2, 4 and 5; 15 min exposure), indicating that these two plasmids contained a *NotI* site. The 130 kb extrachromosomal band hybridized weakly in all digests. The other three bands migrated in a manner independent of the pulse time as shown in Fig. 3(a) where DNA is separated at both 6 s pulse time and 50 s pulse time for 20 h. These results suggest that these bands correspond to smaller supercoiled plasmids (Hightower & Santi, 1989) which is consistent with the presence of several smaller plasmids in *B. thuringiensis* subsp. *berliner* 1715 (Klier *et al.*, 1982; Lereclus *et al.*, 1988).

cryIA hybridized strongly to three extrachromosomal bands of about 60, 270 and 600 kb (Fig. 3b). All three bands were separated in a pulse-time-dependent manner indicating that they were linear. However, in a complete *NotI* digest (Fig. 3b, lanes 4 and 5) the 600 kb band disappeared and the *cryIA* probe hybridized strongly to a 270 kb *NotI* fragment. When genomic DNA was partially digested with *NotI* (Fig. 3b, lane 2) the 600 kb was still present, indicating that the 270 kb *NotI* fragment originated from the 600 kb plasmid. The rest of the

Fig. 3. DNA from *B. thuringiensis* subsp. *berliner* 1715 digested with *Ascl*, *NotI* or *SfiI*, or combinations thereof, and separated by three different pulse regimes [4 s for the first 10 min at 170 mA, and then 6 s pulses for 20 h (a, e), 30 s pulses for 18 h (b, f) or 50 s pulses for 20 h (c, d, g, h), 150 mA, 15 °C (Beckman)]. DNA was then blotted onto membranes and hybridized with the Tn4430 probe (a–d) or the *cryIA* probe (e–h). The autoradiograms were exposed for the times indicated. Lanes: 1, *Ascl* digest; 2, *NotI* partial digest; 3, *SfiI* digest; 4, *Ascl* and *NotI* digest; 5, *NotI* and *SfiI* digest; 6, *Ascl* and *SfiI* digest; 7, undigested (less DNA was loaded). The sizes of some of the linear fragments that hybridized and yeast chromosomes are indicated on the left. Com. indicates the compression region (see legend to Fig. 1). The arrows indicate extrachromosomal DNA that hybridized to the Tn4430 probe and might be supercoiled molecules.

plasmid may correspond to the *NotI* fragment of about 300 kb and perhaps one or two of the smaller *NotI* fragments that have not yet been placed (Table 1, Fig. 1d). Thus, *B. thuringiensis* subsp. *berliner* 1715 contained several large plasmids that were linearized by *NotI*. These *NotI* fragments were not derived from the chromosome since the *cryIA* and Tn4430 probe did not hybridize to any *AscI* and *SfiI* chromosomal fragments, and these *NotI* fragments were not connected with the chromosomal fragments in any partial *NotI* digests. It has been reported that the crystal protein gene is located on the chromosome as well as on plasmids in *B. thuringiensis* subsp. *berliner* 1715 (Klier *et al.*, 1982), while other investigators have only identified extrachromosomal *cry* genes in this strain (Kronstad *et al.*, 1983). It is possible that the *cryIA* gene previously identified as chromosomal in location may have originated from a very large plasmid. In our previous mapping study of a *B. cereus* strain (Carlson & Kolstø, 1994), we found that the large extrachromosomal DNA elements were isolated together with the chromosomal DNA by a caesium chloride gradient (A.-B. Kolstø, unpublished). A plasmid of 64 kb containing both *cryIA* and Tn4430 as well as IS elements is present in the *B. thuringiensis* subsp. *berliner* 1715 strain (Menou *et al.*, 1990). It is likely that this plasmid corresponds to the *cryIA*-positive plasmid without any *NotI* site with the apparent size of 60 kb. However, the presence of Tn4430 on this plasmid was not easily verified in our experiments because Tn4430 also hybridized strongly to another 60 kb *NotI* fragment. Fig. 2b illustrates the large extrachromosomal DNA elements that were detected by PFGE analysis and hybridization with the *cryIA* and the Tn4430 probe. The sum of the six plasmids or the extrachromosomal DNA was 1.2 Mb indicating that the genome size was at least 6.9 Mb.

In the hybridization experiments we also used probes containing *B. thuringiensis* plasmids of 9.2 and 10.6 kb, and about half of a third plasmid of 8.2 kb (Mahillon *et al.*, 1988). Except for the 9.2 kb plasmid, which also contained the Tn4430 element, these probes hybridized to yet additional extrachromosomal DNA elements that had not been identified using the Tn4430 probe or the *cryIA* probe (data not shown). We also used a probe from the replication origin of a *B. thuringiensis* plasmid (*ori44*) (Baum & Gilbert, 1991). The *ori44* probe hybridized to one of the 60 kb elements that was positive for the Tn4430 probe and the *cryIA* probe (Fig. 2b). These results confirm that a large amount of extrachromosomal DNA is present in the *B. thuringiensis* subsp. *berliner* 1715 strain.

The 5.7 Mb chromosome of *B. thuringiensis* subsp. *berliner* 1715 is about the same size as the *B. thuringiensis* subsp. *thuringiensis* HD2 chromosome (Carlson & Kolstø, 1993) and the largest *B. cereus* chromosomes (5.3 Mb–6.3 Mb; Carlson *et al.*, 1992; Carlson & Kolstø, 1994; Kolstø *et al.*, 1990). Both *B. thuringiensis* strains belong to serotype 1 and have previously been analysed by multilocus enzyme electrophoresis. *B. thuringiensis* subsp. *thuringiensis* HD2 clustered together with *B. cereus* strains, including *B. cereus* ATCC 10987 (Carlson *et al.*, 1994), whereas *B. thuringiensis*

subsp. *berliner* 1715 clustered with several *B. thuringiensis* strains (D. Caugant, unpublished). Fig. 4 shows the positions of the probes within the linearized *AscI*, *NotI* and *SfiI* map of *B. thuringiensis* subsp. *berliner* 1715 compared to that of the *NotI* maps of *B. thuringiensis* subsp. *thuringiensis* HD2 and *B. cereus* ATCC 10987. Although the genomic fingerprint patterns of the two *B. thuringiensis* strains are different (Fig. 1c, lanes 3 and 4), the genetic organization appears similar in one half of the chromosomes (to the left in the figure). This corresponds to the conserved region of the *B. thuringiensis* subsp. *berliner* 1715 and *B. cereus* type strain ATCC 14579 chromosomes (Fig. 2a). The chromosomal regions to the right in Fig. 4 are more variable. The two arrows indicate the borders between the conserved and variable parts previously identified in the *B. cereus* ATCC 10987 chromosome (Carlson & Kolstø, 1994). These borders were clearly less well defined when comparing the probe positions within the three maps. However, the genetic organization within the chromosome map of *B. thuringiensis* subsp. *berliner* 1715 was as similar to *B. thuringiensis* subsp. *thuringiensis* HD2 (also serotype 1) as the latter was to the larger *B. cereus* strains previously mapped (Carlson & Kolstø, 1993). The results are consistent with our proposition that the large chromosomes of *B. cereus*/*B. thuringiensis* consist of a conserved region containing the origin and rRNA genes, and a more variable region which contains genes which are often plasmid-encoded (Carlson & Kolstø, 1994).

When probes for the genes previously mapped by transduction (Heiersen *et al.*, 1983; Lecadet *et al.*, 1980) are available, the physical map could be combined with the genetic map (Vary, 1993).

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