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Restriction Map of the 125-Kilobase Plasmid of *Bacillus thuringiensis* subsp. *israelensis* Carrying the Genes That Encode Delta-Endotoxins Active against Mosquito Larvae

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A large plasmid containing all delta-endotoxin genes was isolated from *Bacillus thuringiensis* subsp. *israelensis*; restricted by *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, and *Sal*I; and cloned as appropriate libraries in *Escherichia coli*. The libraries were screened for inserts containing recognition sites for *Bam*HI, *Sac*I, and *Sal*I. Each was labeled with ^{32}P and hybridized to Southern blots of gels with fragments generated by cleaving the plasmid with several restriction endonucleases, to align at least two fragments of the relevant enzymes. All nine *Bam*HI fragments and all eight *Sac*I fragments were mapped in two overlapping linkage groups (with total sizes of about 76 and 56 kb, respectively). The homology observed between some fragments is apparently a consequence of the presence of transposons and repeated insertion sequences. Four delta-endotoxin genes (*cryIVB-D* and *cytA*) and two genes for regulatory polypeptides (of 19 and 20 kDa) were localized on a 21-kb stretch of the plasmid; without *cytA*, they are placed on a single *Bam*HI fragment. This convergence enables subcloning of delta-endotoxin genes (excluding *cryIVA*, localized on the other linkage group) as an intact natural fragment.

Mosquitoes and blackflies are vectors of many human infectious diseases (22). One of the best biocontrol agents against their larvae is the bacterium *Bacillus thuringiensis* subsp. *israelensis* (serovar H14) (9, 20). Its mosquito larvicidal activity is included in five polypeptides of a parasporal crystalline body (delta-endotoxin [17, 20]), CryIVA-D and CytA (134, 128, 78, 72, and 27 kDa in size), encoded by genes which are highly expressed during sporulation (9). These, and all the other genetic elements responsible for toxicity, are located on one of the largest (125-kb) plasmids of *B. thuringiensis* subsp. *israelensis* (9, 16). The gene for a regulatory 20-kDa polypeptide (*p20*) which is required for efficient production of the structural proteins (25–27) is mapped downstream of *cryIVD* (1). A new open reading frame (*p19*) which encodes a putative 19-kDa polypeptide proposed as another chaperone has recently been found upstream of *cryIVD* (12). The DNA sequences indicate that *p19*, *cryIVD*, and *p20* form an operon (12). Several insertion sequences (IS*231*F, V, and W and IS*240*A and B) which seem to allow transposition, duplication, rearrangement, and modification have been found on this 125-kb plasmid (19).

The coding information known to date on the 125-kb plasmid accounts for less than 20% of its total length. The rest (over 80%), still unknown, may regulate expression of the structural genes. It is anticipated that deciphering the roles of other genes involved will enhance development of molecular procedures for improving mosquito biocontrol. A full restriction map will allow location of the toxin genes on the plasmid and improve our understanding of their interactions with other genes.

The plasmid has previously been mapped partially for *Bam*HI (15) and for *Sac*I as well (4). Here, it was completely mapped for *Bam*HI, *Sac*I, and *Sal*I and partially for *Eco*RI, *Hin*dIII, *Kpn*I, and *Pst*I, and all eight currently known genes were localized on the derived map. The apparent convergence of most of them indeed enables subcloning of delta-endotoxin genes as an intact natural fragment.

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MATERIALS AND METHODS

Bacterial strains and plasmid vectors. Strain 4Q5 (original code, 4Q2-72), a derivative of *B. thuringiensis* subsp. *israelensis* cured of all its plasmids except the 125-kb one (8), was kindly supplied by D. H. Dean (Bacillus Genetic Stock Center, Columbus, Ohio) and served as the source for the 125-kb plasmid. Plasmids pUC-9 and pUC-19 were used as cloning vectors, and *Escherichia coli* XL-Blue MRF′ (Stratagene, La Jolla, Calif.), JM83, and JM109 were used as recipient strains.

Recombinant DNA methods. DNA modification and restriction enzymes were used as recommended by the suppliers (mostly New England BioLabs and United States Biochemical), and the procedures were carried out as described by Sambrook et al. (21). Competent cells were prepared and plasmids were isolated by standard procedures. Transformants of *E. coli* were selected on Luria broth plates containing ampicillin (50 to 100 μg ml^{-1}). For screening of recombinants, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or MacConkey plates were employed. Small-volume isolation of recombinant plasmids was performed by alkaline extraction (5). DNA was analyzed by electrophoresis on horizontal agarose slab gels and visualized with ethidium bromide. DNA fragments were purified from the gels by electrophoresis onto DEAE-cellulose membranes (21) or by phenol extraction (23).

Isolation of large plasmid DNA. The 125-kb plasmid DNA was isolated according to the method of Krens and Schilperoort (18) for the Ti plasmids of *Agrobacterium tumefaciens*. *B. thuringiensis* subsp. *israelensis* 4Q5 was grown in Luria-Bertani broth at 30°C. Cells were harvested in the early stationary phase and lysed with sodium dodecyl sulfate (1%) and pronase (0.25 mg ml^{-1}). The pH was raised to 12.2, at which chromosomal DNA but not plasmid DNA denatures, and then slowly lowered to 8.6. The suspension was brought to 1 M NaCl to precipitate denatured DNA and proteins, and the plasmid in the supernatant was precipitated by polyethylene glycol and purified by centrifugation in a CsCl-ethidium bromide gradient.

Southern blot hybridization. DNA of the 125-kb plasmid was digested with several restriction enzymes and subjected to 0.8 and 0.6% agarose gel electrophoresis for short and long runs, respectively. The DNA was denatured and transferred to nitrocellulose filters (Micron Separations, Westboro, Mass.) as described by Davis et al. (7), and conventional hybridization was carried out (21). Autoradiograms were obtained after appropriate exposure to X-ray films at −70°C.

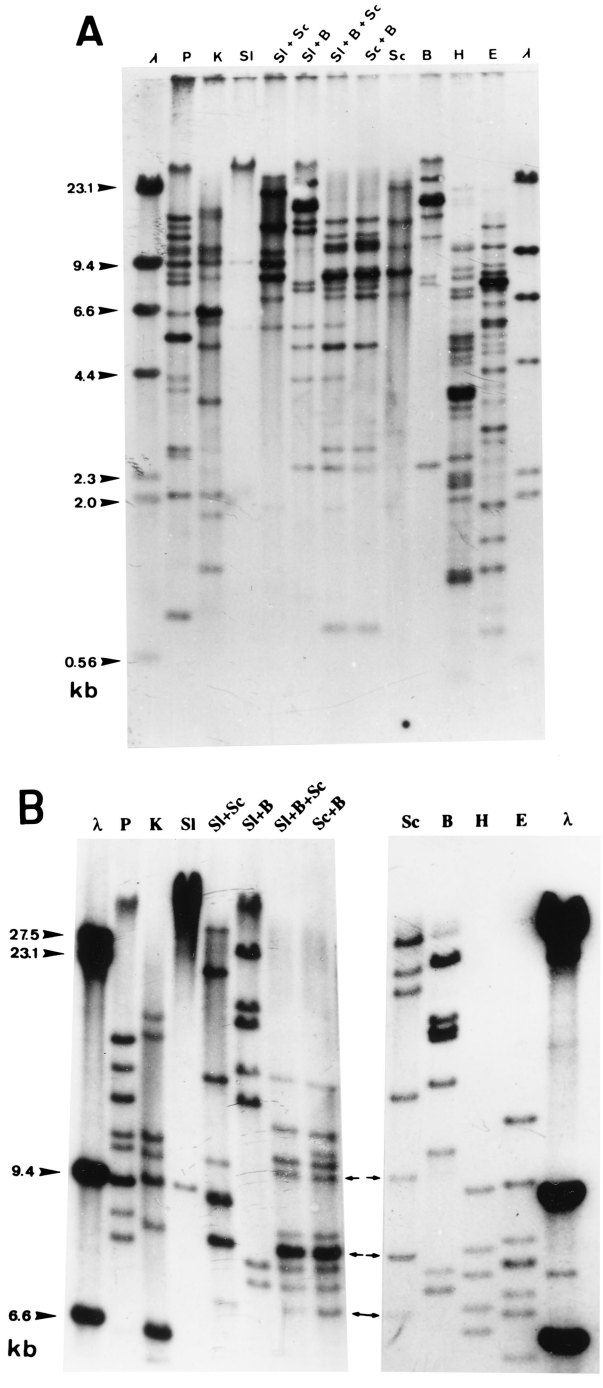

FIG. 1. Autoradiograms of Southern blots of the 125-kb plasmid (hybridized to ^{32}P-labeled plasmid itself as a probe), digested by the following restriction enzymes: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Sc, *Sac*I; Sc+B, *Sac*I-*Bam*HI; Sl+B+Sc, *Sal*I-*Bam*HI-*Sac*I; Sl+B, *Sal*I-*Bam*HI; Sl+Sc, *Sal*I-*Sac*I; Sl, *Sal*I; K, *Kpn*I; and P, *Pst*I. λ, λ_{DNA} digest by *Hin*dIII. The blots are of short (15 h at 50 V) (A) and long (40 h at 40 V) (B) runs on agarose (0.8 and 0.6%, respectively) gels of the restriction digests. Panel B was assembled from two equivalent autoradiograms; internal arrows display identical bands.

RESULTS

Large DNA is more conveniently mapped by rare-cutting restriction enzymes than by frequent cutters. Three enzymes (*Bam*HI, *Sac*I, and *Sal*I) were chosen here because the GC content in their recognition sites is high (4 of 6 bases) while it is relatively low (ca. 35% [2]) in total DNA of *B. thuringiensis* strains.

TABLE 1. Sizes of fragments (derived from Fig. 1) of the 125-kb plasmid by restriction endonuclease digestion

Endonuclease(s)	Sizes (kb) of fragments generated	Total size (kb)
*Sal*I	~110, 9.4, 5.7	~125.1
*Sal*I-*Sac*I	31.0, 22.0, 14.0, 10.5, 9.4, 9.3, 8.5, (8.5?), 7.0, 5.7, 1.8, 0.4	128.1[a]
*Sal*I-*Bam*HI	27.5, 20.0, 18.0, 15.0, 13.5, 8.0, 7.5, 5.7, 5.0, 4.1, 2.5, 0.9	127.7
*Sal*I-*Bam*HI-*Sac*I	15.0, 12.5, 11.0, 10.5, 9.0, 8.5, (8.5?), 8.0, 7.5, 7.0, 5.7, 5.0, 5.0, 4.1, 2.7, 2.5, 1.8, 0.9, 0.7, 0.4	126.3[a]
*Sac*I-*Bam*HI	15.0, 12.5, 11.5, 11.0, 10.5, 9.0, 8.5, (8.5?), 8.0, 7.5, 7.0, 7.0, 5.0, 2.7, 2.5, 0.7, 0.4	127.3[a]
*Sac*I	31.0, 24.0, 22.0, 14.0, 10.5, 8.5, (8.5?), 7.0, 0.4	125.9[a]
*Bam*HI	27.5, 20.0, 18.5, 18.0, 15.0, 11.5, 8.0, 7.5, 2.5	128.5
*Pst*I	16.2, 15.0, 13.0, 11.2, 10.5, 9.3, 8.8, 8.2, 6.4, 5.4, 4.3, 4.2, 4.0, 2.8, 2.7, 2.1, 1.5, 0.82	126.4
*Kpn*I	17.5, 16.5, 11.5, 10.8, 9.4, 8.4, 6.6, 6.4, 5.8, 5.1, 3.8, 3.1, 2.8, 2.4, 2.1, 1.7, 1.2, 0.85	115.9
*Eco*RI	13.0, 10.2, 8.6, 8.0, 8.0, 7.4, 7.0, 6.3, 5.8, 5.7, 5.4, 4.8, 4.3, 3.5, 3.0, 2.8, 2.2, 1.9, 1.6, 1.3, 0.78, 0.13, 0.08	111.8
*Hin*dIII	9.7, 8.4, 7.9, 7.2, 6.8, 5.4, 5.2, 5.0, 4.7, 4.5, 4.1, 4.0, 3.9, 3.7, 3.4, 3.2, 2.6, 2.4, 2.3, 2.1, 1.8, 1.7, 1.5, 1.3, 1.2, 1.0, 0.82, 0.66, 0.48	107

[a] Includes two *Sac*I 8.5-kb fragments, one of which is indicated in the second column of data by a question mark in parentheses.

Preliminary data. The plasmid (125 kb) containing all genetic information required for toxicity was restricted by *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, and *Sal*I, singly or in four

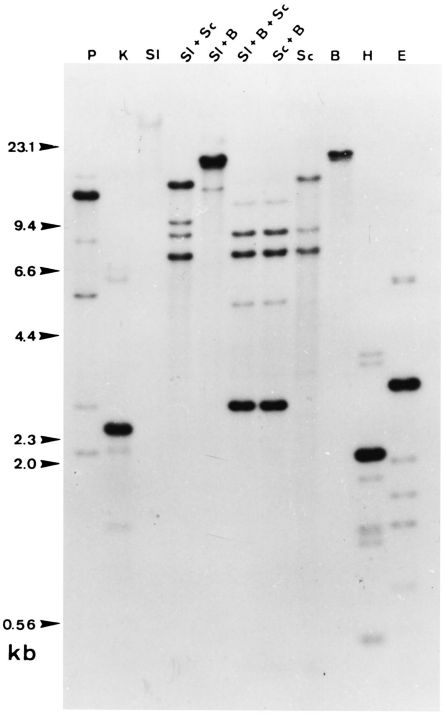

FIG. 2. Autoradiogram of a Southern blot (same as in Fig. 1A) hybridized to the ^{32}P-labeled *Kpn*I 2.4-kb fragment as a probe (Table 2).

TABLE 2. Sizes of plasmid fragments detected by hybridization to eight different probes

Probe[a]	Size(s)[b] of fragments									
	*Sal*I	*Sal*I-*Sac*I	*Sal*I-*Bam*HI	*Sal*I-*Bam*HI-*Sac*I	*Sac*I-*Bam*HI	*Sac*I	*Bam*HI	*Pst*I	*Kpn*I	*Hin*dIII	*Eco*RI
*Kpn*I-2.0 kb (B)	>50	(9.3)	13.5	11, 2.7, (5)	11, 2.7, (5)	14, (8.5)	20, 18.5, 18	13, (5.4)	2.0, (3.8)	2.1, (3.7)	8.0
*Kpn*I-2.4 kb (Sc,Sc)	>50	(9.3)		2.7	2.7	14, 0.4, 7, (8.5)	20	13	2.4	2.1, (0.66)	3.0
*Kpn*I-5.1 kb (Sc)	>50			12.5	12.5	22, 10.5	27.5	10.5, 4.2	5.1	5, 2.3, 1.2, (1.5, 0.48)	4.8, 2.2
*Kpn*I-5.8 kb (B)	>50					30	15, 7.5	8.8, (11.2, 1.5)	5.8	3.7, 2.4, (2.1)	7, 3.5, (13)
*Kpn*I-6.4 kb (B, Sc)	>50			0.7, (5)	0.7, (5)	22, 8.5, (14)	18, 2.5	9.3, 5.4, (0.82)	6.4	4.1, (1.2)	7.4
*Kpn*I-8.4 kb (B, Sl)	9.4	1.8	13.5, 5, 4.1	5, 4.1, 1.8	7	24	18.5, 11.5, (27.5)	16.2, 15, (10.5)	8.4	7.9, 3.7, (9.7)	8.6, 4.3, 3
*Hin*dIII-9.7 kb (B)	(9.4)		5	12.5, 5	12.5	22	27.5, 8, (18.5)	10.5, 9.3	16.5, 8.4	9.7, (7.9)	5.4, 4.3, 3, 0.8
*Pst*I-11.2 kb (B)	>50			9.0	9.0	31	20, 7.5, (15)	11.2, (8.8)	11.5, 2.8, (17.5)	7, 2.1, (4)	13, 7, (8)

[a] Fragment used as the probe, with at least one recognition site for *Bam*HI (B), *Sac*I (Sc), or *Sal*I (Sl) (indicated in parentheses).
[b] Numbers in parentheses designate fragments weakly hybridized, indicating partial homologies to probes.

FIG. 3. Autoradiogram of a Southern blot (same as in Fig. 1A) hybridized to the ^{32}P-labeled *Kpn*I 5.1-kb fragment as a probe (Table 2).

combinations as follows: *Sal*I-*Bam*HI-*Sac*I, *Sac*I-*Bam*HI, *Sal*I-*Bam*HI, and *Sal*I-*Sac*I. The products were separated on agarose gels, and their blots were hybridized to the radiolabeled (nick-translated) plasmid itself (Fig. 1; Table 1). Two relatively small fragments (5.7 and 9.4 kb) were generated by *Sal*I, and the sizes of the rest could not be determined under the conditions employed. Nine fragments (two of which were separable by long runs only on agarose gels), summing up to about 128 kb, were generated by *Bam*HI, and eight fragments, summing up to about 118 kb, were generated by *Sac*I. This apparent discrepancy can be resolved if two fragments of *Sac*I with similar sizes (ca. 8.5 kb) overlap in the gel, as indicated by the higher intensity of the *Sac*I 8.5-kb band than those of the *Sac*I 7- and *Sac*I 10.5-kb bands (Fig. 1). In addition, the sum of the sizes of the fragments generated by digestion with the four combinations (as above) ranged between 125 and 128.5 kb (Table 1). Digestion with the frequent cutters, *Eco*RI, *Hin*dIII, *Kpn*I, and *Pst*I, yielded summed sizes between 107 and 126 kb (Table 1), negatively correlated to the number of fragments generated.

The four largest *Bam*HI fragments (18, 18.5, 20, and 27.5 kb) were cut once each by *Sac*I, and the four largest *Sac*I fragments (14, 22, 24, and 31 kb) were cut once each by *Bam*HI (Fig. 1; Table 1). The double digest yielded seven *Sac*I-*Bam*HI fragments (12.5, 11, 9, 7, 5, 2.7, and 0.7 kb). Two *Bam*HI fragments (11.5 and 18.5 kb) were cut by *Sal*I, and two *Sal*I fragments (9.4 kb and the large one) were cut by *Bam*HI. These double digests yielded four *Sal*I-*Bam*HI fragments (13.5, 5, 4.1, and 0.9 kb). Only one *Sac*I fragment (24 kb) was cut by *Sal*I to generate two *Sac*I-*Sal*I fragments (9.3 and 1.8 kb). The 13.5-kb *Bam*HI-*Sal*I fragment was cut by *Sac*I, and the 9.3-kb *Sac*I-*Sal*I

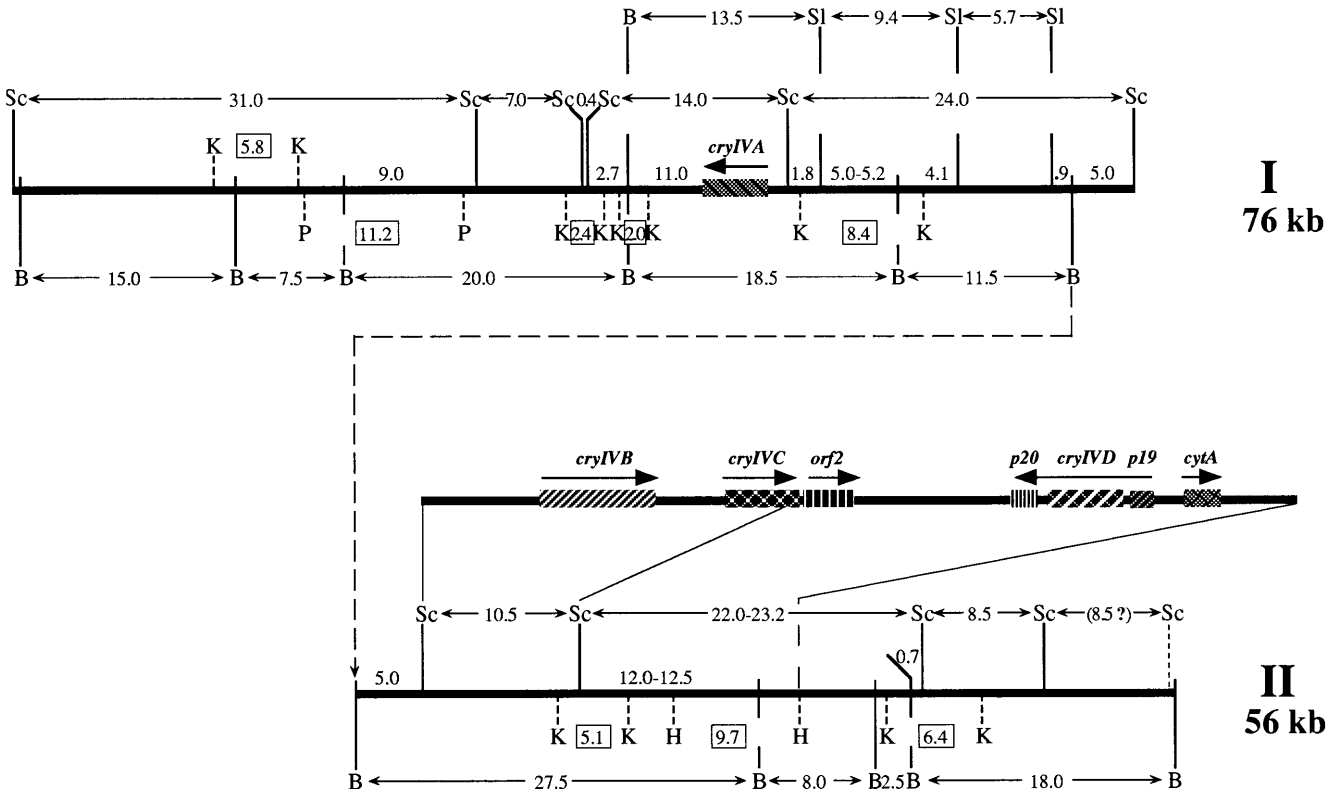

FIG. 4. Partial physical map of the *B. thuringiensis* subsp. *israelensis* H-14 125-kb plasmid. Numbers indicate sizes (in kilobases) of the relevant fragments, some of which (*Bam*HI [B], *Sac*I [Sc], *Sal*I [Sl], and one *Bam*HI-*Sal*I [B-Sl]) are enclosed by double-headed, thin arrows and others of which (those of the probes) are boxed. Other abbreviations are defined in the Fig. 1 legend. The broken arrow displays the overlap site between the two linkage groups (I, of 76 kb; II, of 56 kb). Genes are indicated by hatched boxes, and their transcription directions are indicated by thick arrows. The 26-kb (*Sac*I-*Sac*I-*Hin*dIII) region with most of the known genes is enlarged about 2.3-fold.

fragment was cut by *Bam*HI (triple digest in Fig. 1 and Table 1).

Mapping by hybridization to selected probes. The 125-kb plasmid was restricted by *Hin*dIII, *Kpn*I, and *Pst*I and cloned as appropriate libraries in *E. coli*. The libraries were screened for inserts containing recognition sites for *Bam*HI, *Sac*I, or *Sal*I. Radiolabeled inserts with at least one of these recognition sites each were exploited as probes for hybridization to Southern blots (same as in Fig. 1) to align at least two of the relevant fragments at a time. For example, the labeled *Kpn*I 2.4-kb insert (containing two *Sac*I sites, positioned 0.4 kb apart) detected three *Sac*I bands (0.4, 7, and 14 kb long), a single (20-kb) *Bam*HI band, and a 2.7-kb *Bam*HI-*Sac*I band (Fig. 2; Table 2). This information linked the three *Sac*I fragments to the single *Bam*HI 20-kb fragment (see Fig. 4).

The 5.1-kb *Kpn*I probe (containing one *Sac*I site) similarly linked two *Sac*I fragments (10.5 and 22 kb long), a single (27.5-kb) *Bam*HI fragment, and a 12.5-kb *Bam*HI-*Sac*I fragment (see Fig. 4): it hybridized to the respective bands (Fig. 3; Table 2).

An interesting, crucial probe for the mapping (Fig. 4) is the 8.4-kb *Kpn*I probe: in addition to a *Bam*HI site, it contains one of the only three *Sal*I sites of the plasmid (Fig. 1; Table 1). This probe hybridized to the following bands (Table 2): two *Bam*HI bands (18.5 and 11.5 kb), one *Sac*I band (24 kb), two *Sal*I bands (9.4 and 5.7 kb), three *Bam*HI-*Sal*I bands (13.5, 5, and 4.1 kb), one *Sac*I-*Sal*I band (1.8 kb), and one *Sac*I-*Bam*HI band (7 kb).

Table 2 summarizes the data obtained from these and five additional probes. Together with the preliminary data, they allowed construction of two linkage groups (of 76 and 56 kb) (Fig. 4).

Gene localization and homologies. Hybridization results were not always simple to interpret because of gene duplications and insertion sequences, which had been documented before (6, 19); they indeed hampered map construction. However, once a reasonable map has been assembled (Fig. 4), it became evident that a consistent picture could be obtained. For example, the genes *cryIVA* and *cryIVB*, which are highly homologous, had been mapped on the *Sac*I 14- and 10-kb fragments, respectively (6). When *cryIVA* (on a PCR-derived 3.5-kb DNA [3]) was used as a probe, it indeed detected them both (Fig. 5), as well as the two overlapping *Bam*HI fragments (27.5 and 18.5 kb), confirming the map of Fig. 4.

Four genes which had previously been positioned on the *Hin*dIII 9.7-kb fragment (1, 12–14) were localized on the plasmid relative to the *Bam*HI and *Sac*I maps and to the other genes in this work (Table 2; Fig. 4; see also Discussion).

DISCUSSION

Genes for delta-endotoxin, beta-exotoxin, and thuricin are clustered on either the same or different replicons, usually very large plasmids or the chromosome itself, in various *B. thuringiensis* subspecies (19). *B. thuringiensis* subsp. *israelensis* (serovar H14) is an exclusively delta-endotoxin producer (9). All genes necessary for synthesis of its toxin are located on a single, high-molecular-weight plasmid of about 125 kb (9, 16).

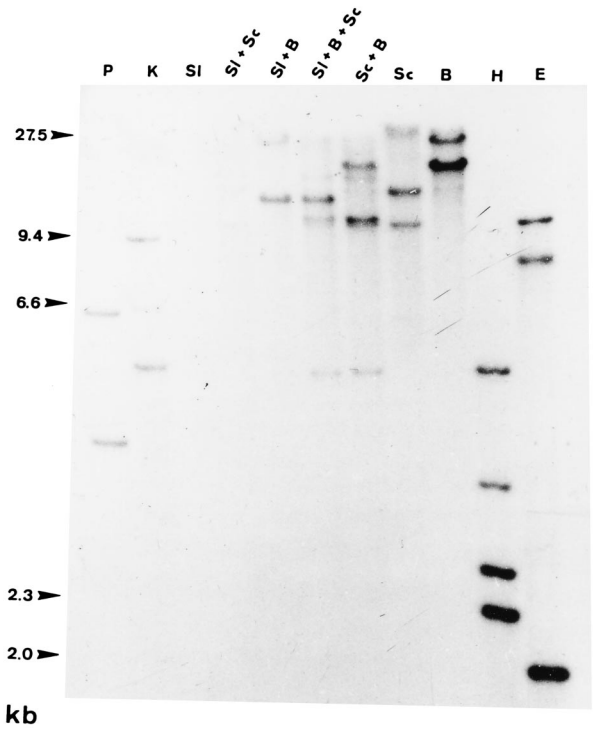

FIG. 5. Autoradiogram of Southern blot (same as in Fig. 1A) hybridized to ^{32}P-labeled PCR-derived *cryIVA* on a 3.5-kb fragment as a probe (Table 2).

The plasmid was partially mapped in this study (Fig. 4) through analysis of products obtained by cleavage with several restriction endonucleases (Fig. 1; Table 1) and hybridization to reference fragments (Fig. 2, 3, and 5; Table 2). The two linkage groups (of 76 and 56 kb) thus constructed (Fig. 4) can logically be joined by the 5-kb *Bam*HI-*Sac*I fragment at the end of each, which seem to overlap. Moreover, it is the only *Sac*I-*Bam*HI fragment that was detected by none of the probes except itself. Construction of a consistent, circular map of the plasmid was obtained by assuming that the second *Sac*I 8.5-kb fragment, proposed in Results, "Preliminary data," is found at the end of the 18-kb *Bam*HI fragment.

Ambiguous results were obtained with several additional probes (data not shown), which could be attributed to the known multiple insertion sequences (IS*231*F, V, and W and IS*240*A and B [1, 11, 19]) and homologous regions (17) on the plasmid. A case in point which could be resolved is the known homology between *cryIVA* and *cryIVB*; they had been mapped on *Sac*I 14- and 10-kb fragments, respectively (6). Labeled *cryIVA* indeed detected both of them, as well as the two overlapping *Bam*HI fragments (18.5 and 27.5 kb) (Fig. 5). In connection, it should be mentioned that the *Sac*I 10-kb fragment contains, in addition to *cryIVB*, most of *cryIVC*. The conclusion (6) that this fragment includes three copies of *cryIVB* becomes very unlikely, considering that most of its length is occupied by these two genes and the stretch between them (10). More data are needed before any conclusions can be drawn about possible interactions between these genes from their proximity on this *Sac*I 10.5-kb fragment.

The *Hin*dIII 9.7-kb fragment (1) was used as a probe (Table 2) to join two *Bam*HI fragments, 8 and 27.5 kb long. Its *Bam*HI site splits *cytA* (1), and three additional genes, *p19*, *cryIVD*, and *p20* (as well as IS*231*W), were located on the larger half (1, 12). These four genes, and the known open reading frame *orf2* (10, 24), are all included on the *Sac*I 22-kb fragment.

It is instructive that seven of the eight known genes (excluding *cryIVA*) were located on a 21-kb section of the plasmid (linkage group II) (Fig. 4). Without *cytA*, they are placed on a single (27.5-kb) *Bam*HI fragment. This convergence enables subcloning of most delta-endotoxin genes as an intact natural fragment.

The gene *cryIVA* is at least 27 kb removed from the cluster of the other seven known genes (Fig. 4). It is flanked by two symmetrical IS*240* sequences (A and B) of 865 bp each on the *Sac*I 14-kb fragment (6, 11). The genes on the stretch of 6 kb downstream of *cryIVA* inside this region are still to be determined.

The plasmid map derived in this study (Fig. 4) will be helpful for discovering new delta-endotoxin genes, localizing them relative to the currently known genes, and understanding interactions between them and with adjacent regulatory elements such as promoters, enhancers, terminators, stabilizers, and mobile elements. It would be indispensable for the ultimate description of the plasmid and for relating nucleotide sequences to the entire genome.

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