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Refined, Circular Restriction Map of the *Bacillus thuringiensis* subsp. *israelensis* Plasmid Carrying the Mosquito Larvicidal Genes[1]

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All the genetic elements responsible for the mosquito larval toxicity of *Bacillus thuringiensis* subsp. *israelensis* are located on one of its largest plasmids, nicknamed pBtoxis. Two linkage groups (with sizes of about 75 and 55 kb) have previously been mapped partially with respect to *Sac*I and *Bam*HI restriction sites (Ben-Dov *et al.,* 1996), but linking them to a single circular plasmid unambiguously was impossible with the available data. To finalize the plasmid map, another rare cutting restriction endonuclease, *Alw*NI, was used in addition. The two linkage groups and the fragments generated by *Alw*NI were aligned on the circular plasmid, and known insertion sequences were localized on the refined map. Pulsed-field electrophoresis revealed that the total size of pBtoxis (137 kb) was larger than thought before.

The mosquito larvicidal activity of *Bacillus thuringiensis* subsp. *israelensis* is included in at least five polypeptides of a parasporal crystalline body (δ-endotoxin), encoded by the respective genes which are highly expressed during sporulation (Federici *et al.,* 1990; Porter *et al.,* 1993). These, and all the other genetic elements responsible for toxicity, are located on one of the largest (125 kb) transmissible plasmids of *B. t. israelensis* (Andrup *et al.,* 1998; Ben-Dov *et al.,* 1996; Gonzalez and Carlton, 1984), nicknamed pBtoxis. This plasmid also harbors several insertion sequences (ISs):[3] IS*231,* F, V, and W, and IS*240,* A and B, which seem to allow transposition, duplication, rearrangement, and modification of the genes for the crystal polypeptides (Lereclus *et al.,* 1993; Mahillon *et al.,* 1994). IS*240,* for example, is present in all *B. thuringiensis* strains exhibiting toxicity against larvae of Dipteran species, suggesting that this element facilitates mobilization of toxin genes between them (Rosso and Delecluse, 1997).

The meaningful (coding and otherwise) information known to date on pBtoxis accounts for less than 20% of its total length. The rest may contain additional coding sequences and regions of regulatory importance. A full physical map of this plasmid will be helpful in discovering new genes and regulatory elements adjacent to the currently known genes and in deciphering the interactions between them. It will aid in the procedure to complete the nucleotide sequence of this biotechnologically important plasmid.

Two linkage groups (with sizes of about 75 and 55 kb) have previously been mapped partially with respect to *Sac*I and *Bam*HI restriction sites, but closure to a single circular plasmid relied on an assumption that two identical *Sac*I fragments (of 8.5 kb) existed (Ben-Dov *et al.,* 1996). Our efforts to demonstrate such twin fragments have failed, implying that fragment size determination was not sufficiently accurate. Mismatch between the ends was derived from differences in the total sum of fragment sizes

[1] This article is dedicated to our colleague, Yoel Margalith, the discoverer of *Bacillus thuringiensis* subsp. *israelensis,* on his 65th birthday.

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[3] Abbreviations used: IR, inverted repeats; IS, insertion sequences; PFE, pulsed field electrophoresis.

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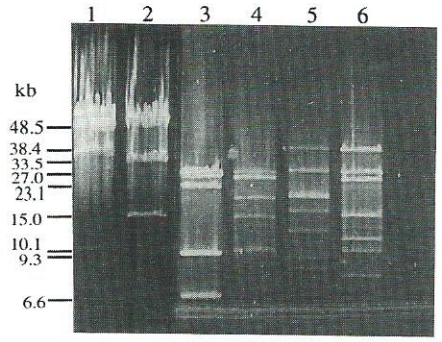

FIG. 1. Pulsed field electrophoresis on an agarose gel (see text) of fragments of pBtoxis digested by the following restriction enzymes: lane 4, *Alw*NI; lane 5, *Bam*HI; lane 6, *Sac*I; lanes 1–3, molecular weight markers with sizes (in kb) indicated on left. λ_{DNA} cleaved by: lane 1, *Apa*I; lane 2, *Xmn*I; lane 3, *Hin*dIII. Fragment sizes are listed in Table 1.

obtained with different enzymes measured from agarose gel electrophoresis (Table 1 in Ben-Dov *et al.,* 1996); this method is not sufficiently sensitive to determine sizes at the larger scale. The present investigation was aimed at resolving both problems simultaneously, real size and linking the two groups, using an additional restriction enzyme and a more sensitive resolving method, pulsed field electrophoresis (PFE).

REEVALUATION OF PLASMID SIZE

Two rare cutting restriction endonucleases, *Bam*HI and *Sac*I, have previously been used to map pBtoxis because the G + C contents in their recognition sites is high, while it is relatively low in total DNA of *B. thuringiensis* strains (Andrup *et al.,* 1993). *Alw*NI was chosen here as an additional enzyme to discover how the two linkage groups are connected and hence to refine the plasmid map.

DNA modification and restriction enzymes were used as recommended by the suppliers (mostly New England BioLabs) and carried out as described by Sambrook *et al.* (1989).

The "125-kb plasmid" was isolated from strain 4Q5 (original code 4Q2-72) of *B. t. israelensis* according to the method of Krens *et al.* (1984) for the Ti plasmids of *Agrobacterium tumefaciens,* as previously described (Ben-Dov *et al.,* 1996). This strain, a derivative of the wild-type cured of all its plasmids except pBtoxis (Zeigler, 1999), was kindly supplied by Dr. D. R. Zeigler (Bacillus Genetic Stock Center, Columbus, OH). It was restricted by *Bam*HI, *Sac*I, and *Alw*NI and fractionated by PFE according to Bio-Rad's directions under the following conditions: 1% agarose, 0.5× TBE, 14°C, 1- to 6-s switch time, 6 V/cm, 16 h. Fragment sizes (Fig. 1; Table 1) were indeed slightly different than those previously derived (Ben-Dov *et al.,* 1996) and helped to construct a self-consistent circular map (see below). The large fragments have previously been substantially underestimated. For example, the largest *Sac*I fragment, of 40 kb, has been estimated to be 31 kb long by regular agarose gel electrophoresis. The plasmid, which was originally estimated to be about 110 kb (Gonzalez and Carlton, 1984; Ward and Ellar, 1983) and later refined to 125 kb (Douek *et al.,* 1991; Ben-Dov *et al.,* 1996) is now more realistically estimated at 137 kb, but the final resolution will stem from its full sequencing.

Two pairs of similar *Alw*NI fragments (two of 9.5 kb, one of 26 kb, and one of 26.5 kb) were not separated in PFE (Fig. 1). Their presence was deduced because they were detected by five different *Alw*NI-containing probes, as detailed

TABLE 1

Sizes of Plasmid Fragments Obtained with Pulsed-Field Electrophoresis

Endonuclease	Sizes (kb) of fragments generated[a]	Total size (kb)[a]
*Alw*NI	27.0, 26.5, 26.0, 21.0, 15.0, 9.5, 9.5, 5.0, 0.4	139.9
*Bam*HI	27.5, 22.0, 21.0, 20.0, 16.0, 12.5, 8.0, 7.5, 2.5	137.0
*Sac*I	40.0, 27.0, 25.0, 15.0, 11.5, 9.5, 7.0, 0.4	135.4

[a] Fragments smaller than 6 kb were out of the PFE gel and inferred from regular gels.

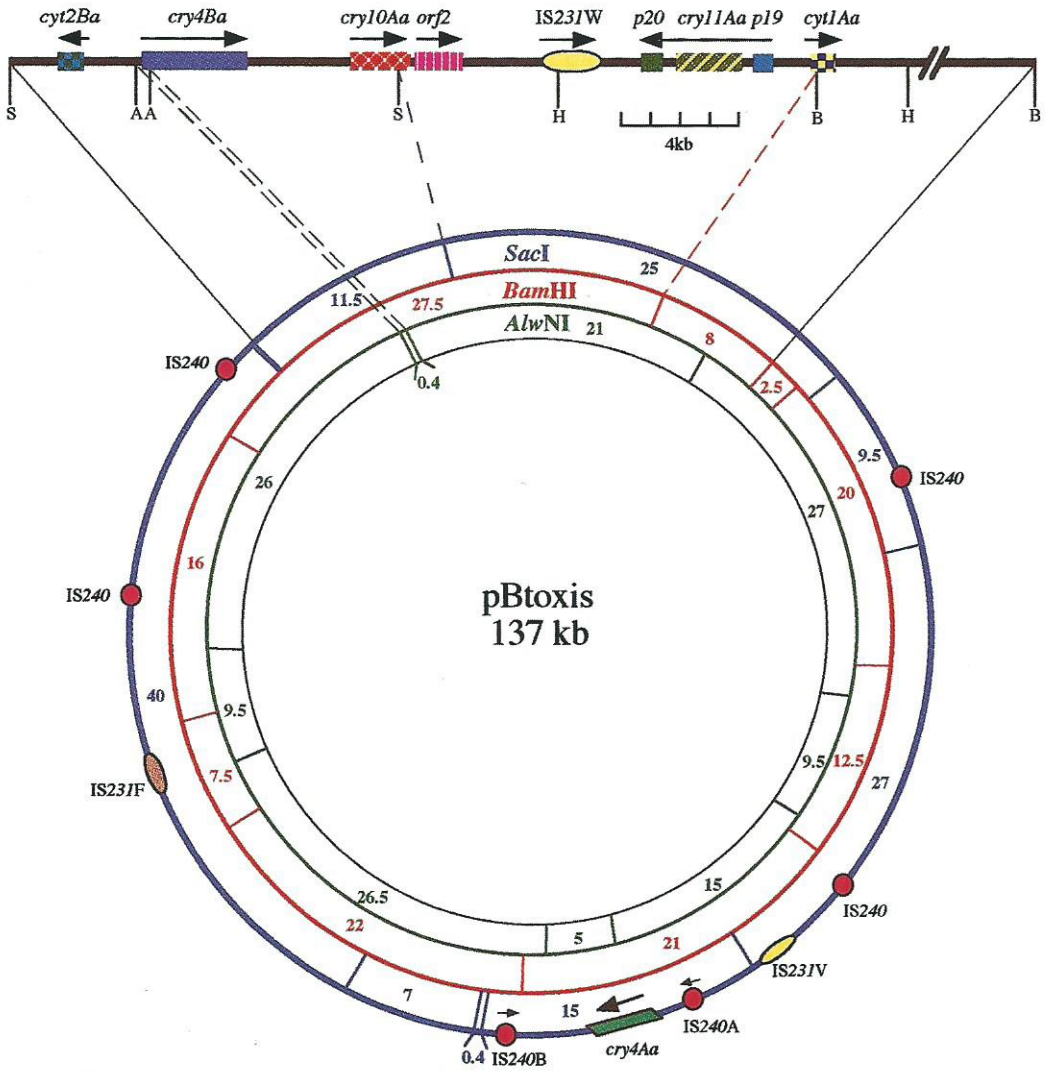

FIG. 3. Restriction map of the 137-kb *B. thuringiensis* subsp. *israelensis* pBtoxis plasmid. Numbers represent sizes of the relevant fragments of *Bam*HI (B), *Sac*I (S), and *Alw*NI (A). The region containing most of the known genes, of about 26 kb (*Sac*I–*Sac*I–*Bam*HI–*Hin*dIII), is enlarged about 1.5-fold (see 4 kb bar). Genes are indicated by colored boxes and direction of transcription—by arrows. Insertion sequences are marked as circles (the IS*240*s) or oval bodies (IS*231*s).

(b) The other end of the 26-kb fragment was linked to the 21-kb *Alw*NI fragment with an 878-bp PCR probe, through the 0.4 *Alw*NI fragment included in the probe. The PCR product was obtained with the following primers, derived from a known region of *cry4Ba* and upstream (Sen *et al.*, 1988): 5′-CGATTTGAATTTTCTGAATATCGAAC-3′ (direct) and 5′-TTATTAGGCTCTCTTTTCCAAGTG-3′ (reverse). (c) The other end of the 26.5-kb fragment was linked to the 5-kb *Alw*NI fragment with the *Eco*RI 8.0-kb probe. (d) The second *Alw*NI 9.5-kb fragment was detected by the *Eco*RI 5.8-kb probe, linking it to another large, 27-kb *Alw*NI fragment.

Table 2 summarizes the data obtained with all nine probes used here, together allowing construction of a consistent circular map containing

TABLE 3

Sizes of Plasmid Fragments Hybridized to Two Different IS Probes[a]

Probe[a]	Size(s) of fragments		
	*Alw*NI	*Bam*HI	*Sac*I
IS*240*	27.0	27.5	40
	26.5	22	27
	26	21	15
	15	20	9.5
		16	
IS*231*	21	27.5	27
	15	21	25

[a] Amplified by PCR (see text).

all sites of the three enzymes (Fig. 3). The two 0.4-kb fragments, of *Alw*NI and *Sac*I, were found fortuitously: the former by having the sequence of *cry4Ba* and flanking region (Sen *et al.,* 1988) and the latter by finding a probe (*Kpn*I 2.4) with two *Sac*I sites (Ben-Dov *et al.,* 1996). Our data do not preclude existence of additional nearby sites for each of these enzymes similarly generating small fragments upon cleavage.

THE INSERTION SEQUENCES

PCR products were used as probes to locate the known ISs on the fragments of this map, IS*231,* V and W, and IS*240,* A and B. The probes were obtained using the inverted repeats (IR) of the ISs as primers from their known sequences (Delecluse *et al.,* 1989; Rezsöhazy *et al.,* 1993), radiolabeled and hybridized to the blots. The probes were prepared with the following primers: 5′-GGGGTACGGCAACA-TCGCCATCAAGC-3′ for IS*231* (V and W) and 5′-AAGGTTCTGGTGCAAA-3′ for IS*240* (A and B). Table 3 summarizes the data obtained by such analyses with reference to the known information and results incorporated into the complete map (Fig. 3).

IS*231,* V and W, of 1964 bp, each encoding two slightly overlapping ORFs (275 and 229 amino acids for IS*231*V and 259 and 250 for IS*231*W), labeled two fragments from each restriction enzyme (Rezsöhazy *et al.,* 1993). Their locations were easily identified by hybridizing to the fragments harboring them since they have no recognition sites for the enzymes defining the map (*Bam*HI, *Sac*I, and *Alw*NI). The sizes of the *Bam*HI fragments to which they were assigned differ from those previously found (Rezsöhazy *et al.,* 1993): 21 kb (rather than 17 kb) for IS*231*V and 27.5 kb (rather than 19 kb) for IS*240*W. The sequence of another IS*231,* F, of 1655 bp (encoding a 477-amino-acid transposase), is carried on the 7.6-kb *Bam*HI restriction fragment of pBtoxis (Rezsöhazy *et al.,* 1992); it is thus included in Fig. 3.

The two IS*240,* A and B (865 bp long, encoding a putative transposase of 235 amino acids), lie in opposite orientations and form a composite transposon-like structure (Bourgouin *et al.,* 1988; Delecluse *et al.,* 1989). At least six copies of IS*240* were found on pBtoxis (Bourgouin *et al.,* 1988; Rosso and Delecluse, 1997). *cry4Aa,* carried on the 15-kb *Sac*I fragment, is flanked by two copies (IS*240*A and IS*240*B (Bourgouin *et al.,* 1988), while IS*231*W is adjacent to *cry11Aa* (Mahillon *et al.,* 1994). It is reasonable to suppose that the other ISs flank additional genes which are yet to be discovered.

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