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Minireplicon from pBtoxis of *Bacillus thuringiensis* subsp. *israelensis*▽

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A 2.2-kb fragment containing a replicon from pBtoxis, the large plasmid that encodes the insecticidal endotoxins of *Bacillus thuringiensis* subsp. *israelensis*, was identified, cloned, and sequenced. This fragment contains *cis* elements, including iterons, found in replication origins of other large plasmids and suggests that pBtoxis replicates by a type A theta mechanism. Two genes, pBt156 and pBt157, encoding proteins of 54.4 kDa and 11.8 kDa, respectively, were present in an operon within this minireplicon, and each was shown by deletion analysis to be essential for replication. The deduced amino acid sequences of the 54.4-kDa and 11.8-kDa proteins showed no substantial homology with known replication (Rep) proteins. However, the 54.4-kDa protein contained a conserved FtsZ domain, and the 11.8 kDa protein contained a helix-turn-helix motif. As FtsZ proteins have known functions in bacterial cell division and the helix-turn-helix motif is present in Rep proteins, it is likely that these proteins function in plasmid replication and partitioning. The minireplicon had a copy number of two or three per chromosome equivalent in *B. thuringiensis* subsp. *israelensis* but did not replicate in *B. cereus*, *B. megaterium*, or *B. subtilis*. A plasmid constructed to synthesize large quantities of the Cry11A and Cyt1A endotoxins demonstrated that this minireplicon can be used to engineer vectors for *cry* and *cyt* gene expression.

The various subspecies of *Bacillus thuringiensis* are characterized by the synthesis of parasporal crystals during sporulation. These crystals are typically composed of one or more highly specific insecticidal or nematocidal endotoxin proteins (28). Most isolates of *B. thuringiensis* harbor an array of plasmids with sizes ranging from 2 kb to 600 kb, and the genes coding for endotoxins are typically located on large plasmids (10, 16). The *cis* elements and genes required for replication of small plasmids, such as pTX14-3 (2), have been well characterized. However, little is known about the *cis* elements or mechanisms involved in replicating and partitioning the large endotoxin-encoding plasmids.

One of the most important subspecies of *B. thuringiensis* is *B. thuringiensis* subsp. *israelensis*. This subspecies is highly insecticidal for the larvae of mosquitoes and blackflies and is presently used in many countries to control pest and vector species of these flies. The insecticidal activity of *B. thuringiensis* subsp. *israelensis* results principally from synergistic interactions of major endotoxin proteins, Cry11A (previously CryIVD), Cry4A, Cry4B, and Cyt1A (14, 27, 28). Recently, the nucleotide sequence of the large 128-kb plasmid, pBtoxis, that encodes these proteins was reported (7). Interestingly, comparative analyses of the 158 known and putative proteins encoded by pBtoxis revealed no homology with known plasmid replication proteins. However, comparative nucleotide analysis of pBtoxis and pXO1 of *B. anthracis* (25) suggested that a putative replication origin is present near the assigned first nucleotide position of pBtoxis (7). The region to the right of this position contains an open reading frame (ORF), pBt001, that encodes a putative peptide that shares about 76% amino acid identity with that encoded by pXO1-49, a gene located close to a putative replication origin of pXO1. Based on the relative positions of these genes and the sequence similarity between pBt001 and pXO1-49, it was suggested that they potentially play a role in plasmid replication (7). In addition, ORF pBt156, which occurs to the left of pBt101, encodes a peptide with weak amino acid similarity to the FtsZ/tubulin-like proteins of *Pyrococcus* (BAB17294) and pXO1-45. As the FtsZ protein family is known to function in cell division by forming a ring structure at the dividing septum, it was proposed that pBt156 functions in plasmid partitioning (7).

To determine whether the putative origin of replication, along with pBt001 and pBt156, plays a role in pBtoxis replication, we cloned and performed nested deletions and site-directed mutagenesis of nucleotide sequences within this region. Our results suggest that pBt156 and its flanking ORF (pBt157), but not pBt001, are essential for replication of pBtoxis. In addition, we show that these minimal sequences, referred to here as a minimal replicon (minireplicon), can be used as a replicon to construct plasmids for expression of the *cry* and *cyt* genes of *B. thuringiensis*, thereby providing a new plasmid vector for engineering various combinations of endotoxins into this species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA manipulation. Bacterial strains and plasmids are listed in Table 1. DNA from *B. thuringiensis* and *Escherichia coli* were extracted using QIAGEN plasmid Midi and Maxi kits (QIAGEN), the Wizard plus miniprep DNA purification system (Promega), and the Bactozol kit (Molecular Research Center). DNA fragments were isolated from agarose gels using the QIAquick gel extraction kit (QIAGEN) and ligated using a Fast-Link DNA ligation kit (Epicenter).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
B. thuringiensis		
4Q7	Acrystalliferous strain of *B. thuringiensis* subsp. *israelensis*	*Bacillus* Genetic Stock Center
IPS82	Wild-type strain of *B. thuringiensis* subsp. *israelensis* harboring pBtoxis	*Bacillus* Genetic Stock Center
4Q5	Plasmid-cured mutant of *B. thuringiensis* subsp. *israelensis*; bears only pBtoxis plasmid	*Bacillus* Genetic Stock Center
B. cereus	NRRL B-569	*Bacillus* Genetic Stock Center
B. subtilis	PY79	Global Bioresource Center
B. megaterium	VT1660	Global Bioresource Center
Escherichia coli	DH5α	
Plasmids		
pUC19	High-copy-number *E. coli* vector containing multiple cloning sites; Amp^r	36
pHT3101	Shuttle vector of *E. coli* and *B. thuringiensis*	23
pBtoxis	127-kb endogenous *B. thuringiensis* subsp. *israelensis* plasmid harboring entomocidal crystal toxin genes	7
pBU4	7.3-kb plasmid containing a tetracycline resistance gene	8
pC194	2.9-kb plasmid containing a chloramphenicol resistance gene	18
pUCE	pUC19 containing the erythromycin resistance gene from pHT3101	This work
pBtKp(+)	pUCE containing a 6.3-kb KpnI fragment of pBtoxis; the orientation of *pBt001* is the same as *lacZ* promoter	This work
pBtKp(−)	pUCE containing a 6.3-kb KpnI fragment of pBtoxis; the orientation of pBt001 is opposite to *lacZ* promoter	This work
pBtAg1	Deletion derivative of pBtKp containing a 2.6-kb KpnI/AgeI fragment	This work
pBtAg2	Deletion derivative of pBtKp containing a 3.7-kb KpnI/AgeI fragment	This work
pBtSa1	Deletion derivative of pBtKp containing a 4.5-kb KpnI/SalI fragment	This work
pBtSa2	Deletion derivative of pBtKp containing a 1.8-kb KpnI/SalI fragment	This work
pBtBs	Deletion derivative of pBtKp containing a 2.4-kb KpnI/BstAPI fragment	This work
pBtXm	Deletion derivative of pBtKp containing a 2.9-kb XmnI fragment	This work
pBtSw	Deletion derivative of pBtKp containing a 2.2-kb SwaI fragment	This work
pBtΔ156	pBtSw derivative with ATG of pBt156 replaced by CCC	This work
pBtΔ157	pBtSw derivative with ATG of pBt157 replaced by TTT	This work
pUCEG	pUC19 containing the erythromycin resistance gene from pHT3101 and the *gyrb* gene from the chromosome of *B. thuringiensis* subsp. *israelensis*	This work
pBtH	pBtSw containing ~10-kb HindIII fragment of pBtoxis	This work

PCR. PCR was performed with the Expand Long Template PCR System (Roche Molecular Biochemicals) in a Px2 system thermocycler (Thermo Hybaid) for 30 cycles as follows: 94°C, 30 s; 55°C, 30 s; 68°C, 2 min.

Construction of recombinant plasmids. To construct pUCE, the 1.2-kb fragment containing the erythromycin resistance gene (*erm*) from pHT3101 (23) was amplified using primers Em-S-F and Em-S-R (Table 2), digested with SalI, and cloned into the same site in pUC19. Plasmid pUCEG was constructed by inserting a 1.2-kb *gyrB* gene (AY461780) (21) obtained by PCR with primers gyr-F and gyr-R (Table 2) into the SmaI site of pUCE.

The plasmids pBtKp(+) and pBtKp(−) were obtained by inserting the 6.3-kb KpnI fragment from pBtoxis (AL731825), which spanned nucleotides (nt) 123999 to 2408, into the KpnI site of pUCE. This fragment contains the putative replication origin of pBtoxis (7) and ORFs pBt001, pBt156, pBt157, and pBt158 (Fig. 1). The orientations of pBt001 and the *lacZ* gene in pUC19 are the same in pBtKp(+) but opposite in pBtKp(−). Plasmid pBtKp(+) was double digested with either SmaI and AgeI, SmaI and SacI, or SmaI and XbaI, and the largest fragment from these digests, containing the parental plasmid pUCE and various deletions within the 6.3-kb fragment, were blunted with Klenow enzyme (Biolabs) and self-ligated to generate the pBtKp derivatives pBtAg1, pBtSa1, and pBtXb (Fig. 1). Similarly, pBtKp(−) was double digested with either SmaI and AgeI, SmaI and BstAPI, or SmaI and SacI, and the largest fragments were self-ligated to generate pBtAg2, pBtBs, and pBtSa2 (Fig. 1). Plasmids pBtXm and pBtSw (Fig. 1) were constructed by inserting the 2.9-kb XmnI fragment (nt 124124 to 127052) and the 2.2-kb SwaI fragment (nt 124407 to 126636), from the 6.3-kb KpnI fragment, into the SmaI site of pUCE.

Plasmid pBtH was constructed by inserting the 10-kb HindIII fragment from pBtoxis (nt 14238 to 24460), which contained the 20-kDa gene (34), along with the *cry11Aa* and *cyt1Aa* genes, into the HindIII site of pBtSw (Fig. 1). All constructs were confirmed by restriction enzyme and DNA sequence analyses.

Transformation. Transformation of *B. thuringiensis* subsp. *israelensis* 4Q7, *B. megaterium* VT1660, *B. cereus* NRRL B-569, and *B. subtilis* PY79 with vectors bearing the minireplicon was performed as described previously (29, 31, 34, 35). For controls, *B. cereus* was transformed with pC194 (18) and *B. subtilis* and *B. megaterium* with pBU4 (8).

Site-directed mutagenesis. The putative translational start codons (ATG) for ORFs pBt156 and pBt157 were replaced with CCC and TTT, respectively, by PCR using plasmid pBtSw as the template and primer pairs ORF156-F and ORF156-R, and ORF157-F and ORF157-R (Table 2), which were designed in

TABLE 2. Primers used for PCR amplification

Primer	Sequence (5′→3′)[a]
Em-S-F	.....ACGC**GTCGAC**AGAAGCAAACTTAAGAGTGTG
Em-S-R	.....ACGC**GTCGAC**ATCGATACAAATTCCCCGTAG
ORF156-F	.....TTATCCCTTATTAAACAGTAATGAACTA
ORF156-R	.....AATTCCTCCCCTATACTTATAAATTA
ORF157-F	.....AAACATTTAATAGGGATCACTTTTATAC
ORF157-R	.....AACTCCCATCTGTTTAATTAATTCTTGA
gyr-F	.....AATAATAACTTTATGATAGCGTGC
gyr-R	.....CGGTGGCGGTTACAAAGTTTCTGG

[a] Restriction endonuclease cleavage sites for SalI are in boldface, and the mutated codons are underlined.

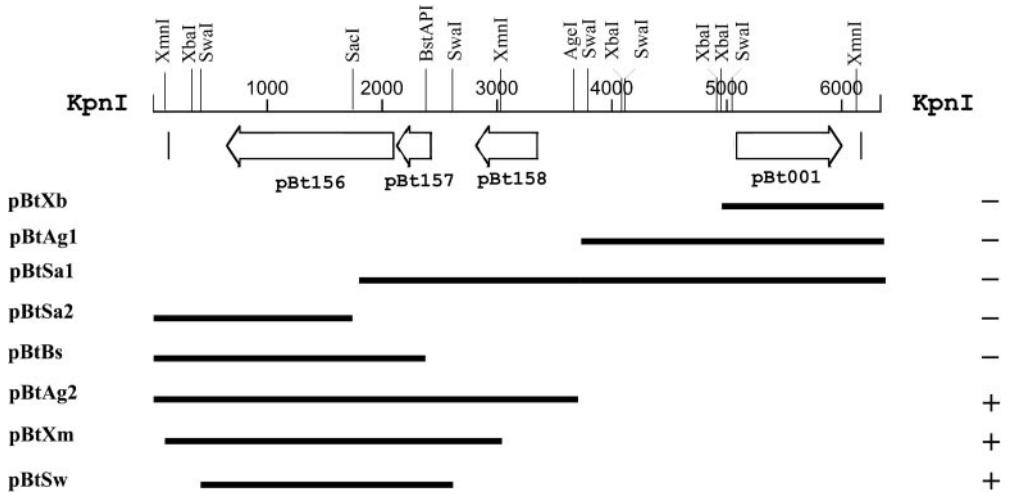

FIG. 1. Deletion mutants of the KpnI fragment used to identify a minireplicon of pBtoxis. The ability of each of the mutants to replicate in *B. thuringiensis* subsp. *israelenses* 4Q7 is indicated (+, capable; −, incapable).

inverted tail-to-tail directions to amplify the entire pBtSw. After PCR, the amplified linear DNAs were self-ligated to generate pBtΔ156 and pBtΔ157.

DNA probes. The 2.4-kb fragment containing the *erm* and *gyrB* genes in pUCEG (see above) was amplified by PCR with primers gyr-F and Em-S-R (Table 2) and labeled using the Oligonucleotide 3′-end labeling kit (second generation; Roche).

Relative copy number determination. The plasmid copy number was determined according to the method of Devine et al. (13), using the chromosome-specific *gyrB* and plasmid-specific *erm* probes. Fragments that hybridized were analyzed with the AlphaEaseFC software, and the ratio of signals from the *erm* gene to the *gyrB* gene was used to estimate of the number of plasmid copies per chromosome equivalent.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Bacterial strains were grown in 20 ml of NBG broth (26) at 30°C for 5 days, by which time the cells had sporulated and lysed. An equal volume (1.2 ml) from each culture was sedimented, and the proteins were seperated in a 10% polyacrylamide gel by SDS-PAGE (27).

Segregation stability assay. The stabilities of plasmids pBtKp(+), pBtKp(−), and pBtSw in *B. thuringiensis* subsp. *israelensis* 4Q7 were determined using methods described previously (22).

RESULTS

Cloning the pBtoxis minireplicon. To determine whether the region in pBtoxis (nucleotide positions 123999 to 2408) identified by Berry et al. (7) contained a replication origin, we cloned the 6.3-kb KpnI fragment containing this region into pUCE, a pUC19-based vector, to generate pBtKp. Following electroporation of *B. thuringiensis* subsp. *israelensis* 4Q7 with pBtKp, the recombinant strain *B. thuringiensis* subsp. *israelensis* 4Q7/pBtKp bearing this plasmid was recovered on LB agar plates containing erythromycin. No *B. thuringiensis* subsp. *israelensis* 4Q7 transformants were recovered after electroporation with pUCE. Plasmid analysis of *B. thuringiensis* subsp. *israelensis* 4Q7/pBtKp showed that pBtKp was present in the strain, demonstrating that the 6.3-kb KpnI fragment contained a replicon.

Minimal sequences (minireplicons) sufficient for replication. In addition to the presence of a replicon, the 6.3-kb KpnI fragment contains four ORFs, pBt001, pBt156, pBt157, and pBt158, the last three of which putatively code for, respectively, a FtsZ/tubulin-related protein, a DNA-binding protein, and a transcriptional regulator. Using a series of overlapping deletion derivatives of pBtKp, the minimal sequences required for replication included those that contained intact ORF156 and ORF157, i.e., plasmids pBtAg2, pBtXm, and pBtSw (Fig. 1). Plasmids containing intact ORF156 but deficient or partially deficient in ORF157 sequences, or vice versa (pBtXb, pBtAg1, pBtSa1, pBtSa2, and pBtBs), did not replicate (Fig. 1). Thus, the smallest autonomously replicating DNA sequence was located on a 2.2-kb SwaI fragment in pBtSw (Fig. 2). This fragment is defined here as a minireplicon of pBtoxis.

Sequence analyses of the pBtoxis minireplicon. Sequence analyses of the minireplicon in the 2.2-kb SwaI fragment (Fig. 2) suggested that it contained an operon composed of ORFs pBt157 and pBt156. Signature ribosome binding sites were found upstream of these ORFs, at positions 181 to 185 (GGAGT) and 514 to 518 (GGAGG), respectively. Our analyses also revealed the presence of a 12-bp direct-repeat DNA motif (TAAAGGTTTAAA; A+T% = 87%) upstream of pBt157 (Fig. 2) that resembled the so-called iteron motif, common in replication origins of theta-replicating plasmids, which serves as a site for interactions of replication proteins (12). In addition, an 8-bp inverted repeat (AAATTTAA) was present upstream of pBt157 at the start of the sequence and within the putative iteron. Four putative DnaA boxes (TTA/TTNCACA) (20), sequences to which the replication initiator protein DnaA binds, were also present (Fig. 2).

The pBt157 gene (nt 192 to 506) coded for a putative peptide (ORF157) of 104 amino acid residues (M_r, 11,800), and pBt156 (nt 527 to 1981) coded for a putative peptide of 484 amino acids (ORF156; M_r, 54,400). Previous analyses suggested that ORF157 is a DNA-binding protein with a predicted helix-turn-helix (HTH) motif at amino acids 41 to 62 (7). In our studies, alignment of ORF157 with MarR, a repressor of multiple antibiotic resistance genes in *E. coli* (1), placed the helix-turn-helix motif at residues 43 to 65 (Fig. 2). Berry et al. (7) reported that ORF156 showed weak similarity to FtsZ/tubulin-like proteins from *Pyrococcus* (BAB17294; 21% identity) and pXO1-45 (21% identity). Our analyses agree with this conclusion. In addition, we identified the signature motif (GG

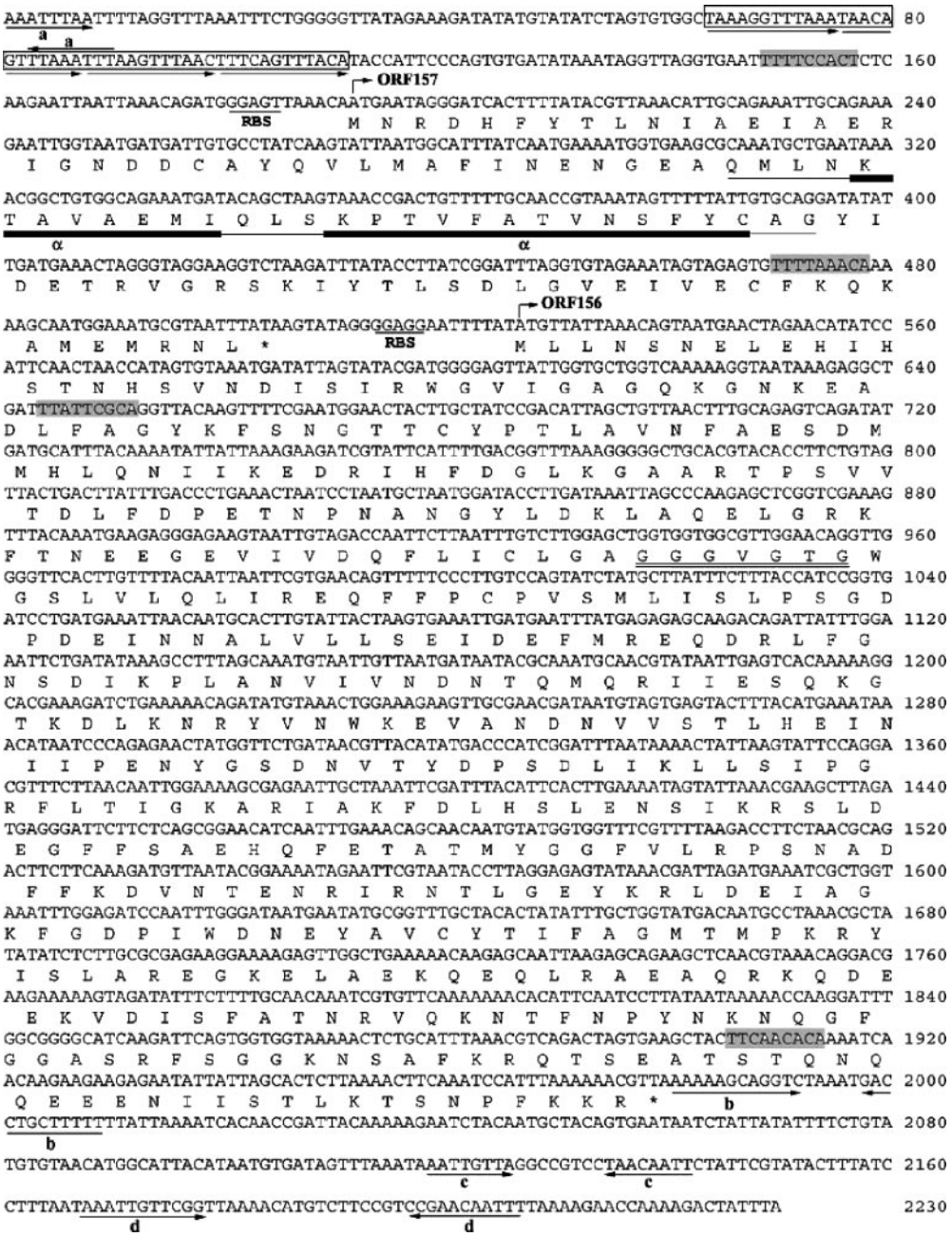

FIG. 2. Nucleotide sequence of the 2.2-kb SwaI region of pBtoxis from *B. thuringiensis* subsp. *israelensis*. The fragment contains a putative operon composed of two ORFs, ORF156 (pBt156) and ORF157 (pBt157). The predicted amino acid sequences are shown beneath the nucleotide sequence. Direct- and inverted-repeat sequences (a, b, c, and d) are indicated by arrows. Signature ribosome binding sequences (RBS) are underlined. Iterons and potential DnaA boxes are boxed and shaded, respectively. The tubulin superfamily signature motif in pBt156 is double underlined, and the helix-turn-helix motifs at positions 43 to 65 with residues that form alpha (α) helices in pBt157 are underlined in boldface.

GTVGTG; residues 138 to 143) (Fig. 2) in ORF156 that is characteristic of the tubulin protein superfamily (11).

pBt156 and pBt157 are required for minireplicon replication. When the translation start codon of either ORF156 or ORF157 was disrupted, *B. thuringiensis* subsp. *israelensis* 4Q7 transformants were not recovered following electroporation with the mutant plasmids (pBtΔ156 and pBtΔ157), indicating that both ORF156 and ORF157 are required for minireplicon replication.

Determination of the copy number for pBtSw. Densitometric analysis of hybridization bands showed that the intensity of the *erm* gene derived from pBtSw was two to three times that of the single chromosomally derived *gyrB* gene (data not shown), indicating a copy number of two or three per chromosome equivalent.

Segregation stability. Studies of the segregation stabilities of pBtKp and pBtSw, which contained the 6-kb KpnI and the 2.2-kb SwaI fragments, respectively, showed that these plas-

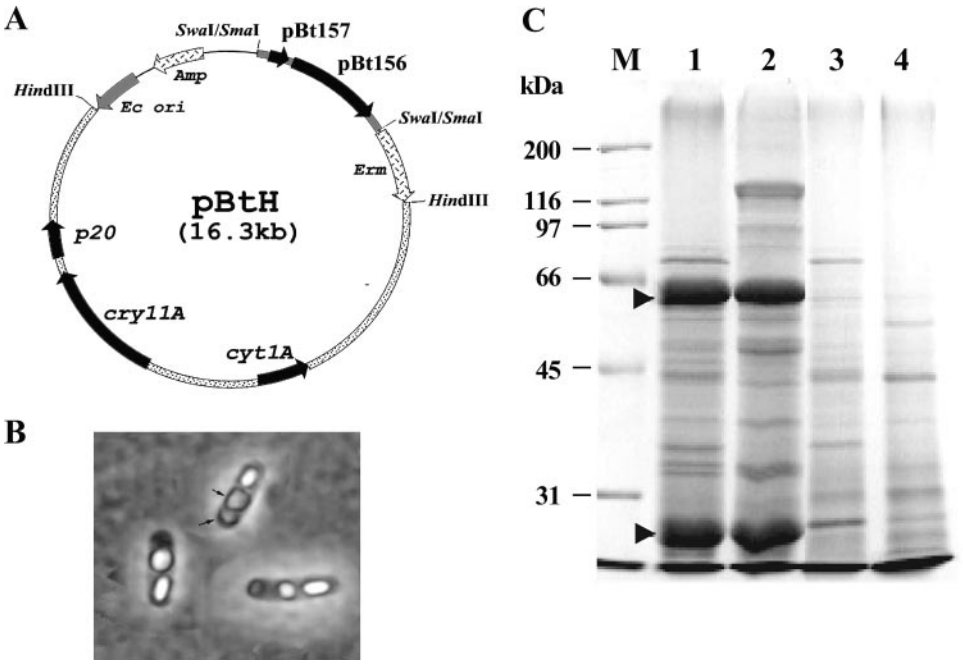

FIG. 3. Synthesis of Cry11A and Cyt1A using the minireplicon sequence derived from pBtoxis. (A) Map of recombinant plasmid pBtH constructed using the minireplicon in pBtSw (Fig. 1) and the ~10-kb HindIII fragment from pBtoxis that contains the *cry11A*, *cyt1A*, and 20-kDa genes. (B) Phase-contrast microscopy showing crystals (arrows) in cells of *B. thuringiensis* subsp. *israelensis* strain 4Q7 transformed with pBtH. (C) SDS-PAGE analyses of recombinant strains and wild-type strains of *B. thuringiensis* subsp. *israelensis*, strain 4Q7 pBtH (lane 1), strain 4Q5 (lane 2), strain pBtSw (lane 3) (Fig. 1), and strain 4Q7 (lane 4). Lane M, protein molecular mass standards.

mids were unstable in the absence of positive selection with erythromycin. Virtually 100% of the cells from colonies grown without selection after 20 generations failed to grow in LB medium with erythromycin.

Replication of the pBtoxis minireplicon in other *Bacillus* species. Plasmid pBtSw, which contained the pBtoxis minireplicon, was tested for its ability to replicate in *B. cereus*, *B. megaterium*, and *B. subtilis*. Whereas the control plasmids replicated after transformation in these species, pBtSw did not. Specifically, pC194 replicated in *B. cereus* (~300 CFU/μg DNA) and pBU4 replicated in both *B. subtilis* and *B. megaterium* (~500 CFU/μg DNA). No colonies were obtained with these species transformed with pBtSw. Furthermore, when wild-type *B. thuringiensis* subsp. *israelensis* IPS82, which contains pBtoxis, was transformed with plasmids containing the minireplicon, very few colonies (~5%) produced crystal toxins, indicating a loss of parental pBtoxis in most transformants. Incompatibility between the *B. thuringiensis* recombinant and resident plasmids from which the replicons in recombinants were derived has been reported previously (6).

Expression of *cry* and *cyt* genes using the minimal replicon. Recombinant 4Q7 cells transformed with pBtH (Fig. 3A), the plasmid containing the minimal replicon, along with the 10-kb HindIII fragment of pBtoxis that contains the *cry11Aa*, *cyt1Aa*, and *p20* genes (7), synthesized large inclusions that were clearly visible by light microscopy (Fig. 3B). The presence of Cry11A and Cyt1A was confirmed by SDS-PAGE analysis, which showed that the recombinant strain containing this plasmid synthesized amounts of these toxins similar to those produced by *B. thuringiensis* subsp. *israelensis* strain 4Q5 (Fig. 3C).

DISCUSSION

In the present study, we demonstrated that a 2.2-kb fragment spanning nucleotide positions 124407 to 126636 in pBtoxis (7) contains an origin of replication, referred to here as a minireplicon, that bears an operon coding for two peptides, ORF156 and ORF157, both of which are required for replication. Our sequence analyses, together with those of Berry et al. (7), show canonical features of plasmid replication origins, including a high A-T ratio, direct-repeat sequences (iterons) and DnaA box motifs for replication initiation, and putative replication (Rep) proteins, characteristic of the group A family of theta replicons (9; http://www.essex.ac.uk/bs/staff/osborn/DPR/DPR_ThetaData.htm).

Modes of plasmid replication from a wide variety of prokaryotes fall within two categories of mechanisms, the so-called rolling-circle mechanism and the theta mechanism. Whereas the rolling-circle mechanism is the preferred mode for replicating small plasmids (less than ~15 kb), large plasmids typically replicate by the theta mechanism (5, 6, 19, 30). At present, five large plasmids with low sequence homology, three (p43, p44, and p60) from *B. thuringiensis* subsp. *kurstaki* HD263 and two (pHT73 and pAW63) from *B. thuringiensis* subsp. *kurstaki* HD73, have been shown to replicate by the theta mechanism (4, 6, 15, 33). Although pBtoxis shares no significant homology with other large plasmids in *B. thuringiensis* (7), the structural features of its minireplicon, which include putative iteron sequences and DnaA boxes, suggest that it also replicates using the theta mechanism.

Although we have shown that ORF156 and ORF157, en-

coded by, respectively, pBt156 and pBt157, are essential for replicating the pBtoxis minireplicon, the precise roles of these proteins remain unknown. The presence of a conserved HTH motif in ORF157 (7), however, suggests a functional role in DNA binding. The HTH motif is present in many prokaryotic and eukaryotic proteins that function in transcription, DNA replication and repair, RNA metabolism, and protein-protein interactions in diverse signaling pathways (3). As plasmid-encoded Rep proteins, which also contain HTH, together with DnaA, accumulate at AT-rich sites at replication origins to initiate plasmid replication (12), we propose a similar role for ORF157. Furthermore, as Rep-DNA-DnaA complexes are also known to play a role in plasmid partitioning (12), ORF157 could play a similar role. In this regard, ORF156, which shows similarity to FtsZ/tubulin-like proteins, could potentially function with ORF157 in plasmid partitioning. The prokaryotic FtsZ protein is a polymer-forming GTPase that shares structural and functional similarities with eukaryotic tubulins (32). FtsZ assembles into a ring structure on the inner surface of the cytoplasmic membrane at the site of cell division. The so-called Z ring is progressively reduced in diameter, a process which leads to invagination of the dividing septum (32). It is possible that ORF156 and ORF157 function together to mediate partitioning of pBtoxis into daughter cells.

The low copy number (two or three copies per chromosome) for the pBtoxis minireplicon was expected, as it is typical for replicons derived from large plasmids (17). The unstable inheritance of the minireplicon when propagated in the absence of antibiotic selection is also not unexpected, because other loci, such as *par* (12), associated with efficient partitioning of this artificial plasmid are absent. The inability of the pBtoxis minireplicon to replicate in *B. cereus*, *B. megaterium*, and *B. subtilis* suggests that these bacteria lack replicative proteins that recognize *cis* elements in the minireplicon or proteins that can interact effectively with ORF156 and ORF157. Alternatively, the recombinant plasmid harboring the minireplicon could have been degraded by host cell restriction modification systems (6, 24).

Finally, for commercial and regulatory purposes, one of our primary interests is to develop expression vectors for the synthesis of insecticidal Cry and Cyt proteins using nucleotide sequences native to *B. thuringiensis* subsp. *israelensis*. Current regulations for the registration of genetically engineered bacterial insecticides favor recombinants that have as little foreign DNA as possible. The high levels of Cry11A and Cyt1A synthesis that we obtained (Fig. 3) demonstrated that the pBtoxis minireplicon can be used for this purpose and thus has utility in genetically manipulating *B. thuringiensis* to produce novel combinations of insecticidal proteins.

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REFERENCES

1. **Alekshun, M. N., S. B. Levy, T. R. Mealy, B. A. Seaton, and J. F. Head.** 2001. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 Å resolution. Nat. Struct. Biol. **8:**710–714.
2. **Andrup, L., J. Damgaard, K. Wassermann, L. Boe, S. M. Madsen, and F. G. Hansen.** 1994. Complete nucleotide sequence of the *Bacillus thuringiensis* subsp. *israelensis* plasmid pTX14-3 and its correlation with biological properties. Plasmid. **31:**72–88.
3. **Aravind, L., V. Anantharaman, S. Balaji, M. M. Babu, and L. M. Iyer.** 2005. The many faces of the helix-turn-helix domain: transcription regulation and beyond. FEMS Microbiol. Rev. **29:**231–262.
4. **Baum, J. A., D. M. Coyle, M. P. Gilbert, C. S. Jany, and C. Gawron-Burke.** 1990. Novel cloning vectors for *Bacillus thuringiensis*. Appl. Environ. Microbiol. **56:**3420–3428.
5. **Baum, J. A., and J. M. Gonzalez, Jr.** 1992. Mode of replication, size and distribution of naturally occurring plasmids in *Bacillus thuringiensis*. FEMS Microbiol. Lett. **96:**143–148.
6. **Baum, J. A., and P. Gilbert.** 1991. Characterization and comparative analysis of replication origins from three large *Bacillus thuringiensis* plasmids. **173:** 5280–5289.
7. **Berry, C., S. O'Neil, E. Ben-Dov, A. F. Jones, L. Murphy, M. A. Quail, M. T. Holden, D. Harris, A. Zaritsky, and J. Parkhill.** 2002. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **68:**5082–5095.
8. **Bourgouin, C., A. Delécluse, F. de la Torre, and J. Szulmajster.** 1990. Transfer of the toxin protein genes of *Bacillus sphaericus* into *Bacillus thuringiensis* subsp. *israelensis* and their expression. Appl. Environ. Microbiol. **56:**340–344.
9. **Bruand, C., E. Le Chartelier, S. D. Ehrlich, and L. Janniere.** 1993. A fourth class of theta-replicating plasmids: The pAMβ1 family from Gram-positive bacteria. Proc. Natl. Acad. Sci. USA **90:**11668–11672.
10. **Carlson, C. R., T. Johansen, M. M. Lecadet, and A. B. Kolsto.** 1996. Genomic organization of the entomopathogenic bacterium *Bacillus thuringiensis* subsp. *berliner* 1715. Microbiology **42:**1625–1634.
11. **de Boer, P., R. Crossley, and L. Rothfield.** 1992. The essential bacterial cell-division protein FtsZ is a GTPase. Nature **59:**254–256.
12. **del Solar, G., R. Giraldo, M. J. Ruiz-Echevarria, M. Espinosa, and R. Diaz-Orejas.** 1998. Replication and control of circular bacterial plasmids. Microbiol. Mol. Biol. Rev. **62:**434–464.
13. **Devine, K. M., S. T. Hogan, D. G. Higgins, and D. J. McConnell.** 1989. Replication and segregational stability of *Bacillus* plasmid pBAA1. J. Bacteriol. **171:**1166–1172.
14. **Federici, B. A., H. W. Park, D. K. Bideshi, M. C. Wirth, and J. J. Johnson.** 2003. Recombinant bacteria for mosquito control. J. Exp. Biol. **206:**3877–3885.
15. **Gamel, P. H., and J. C. Piot.** 1992. Characterization and properties of a novel plasmid vector for *Bacillus thuringiensis* displaying compatibility with host plasmids. Gene **120:**17–26.
16. **González, J. M., Jr., and B. C. Carlton.** 1980. Patterns of plasmid DNA in crystalliferous and acrystalliferous strains of *Bacillus thuringiensis*. Plasmid **3:**92–98.
17. **Gordon, S., J. Rech, D. Lane, and A. Wright.** 2004. Kinetics of plasmid segregation in *Escherichia coli*. Mol. Microbiol. **51:**461–469.
18. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J. Bacteriol. **150:**815–825.
19. **Khan, S. A.** 1997. Rolling-circle replication of bacterial plasmids. Microbiol. Mol. Biol. Rev. **61:**442–455.
20. **Kornberg, A., and T. Baker.** 1992. DNA replication, 2nd ed. Freeman, New York, N.Y.
21. **La Duc, M. T., M. Satomi, N. Agata, and K. Venkateswaran.** 2004. *gyrB* as a phylogenetic discriminator for members of the *Bacillus anthracis-cereus-thuringiensis* group. J. Microbiol. Methods **56:**383–394.
22. **Lereclus, D., and O. Arantes.** 1992. *spbA* locus ensures the segregational stability of pTH1030, a novel type of gram-positive replicon. Mol. Microbiol. **6:**35–46.
23. **Lereclus, D., O. Arantes, J. Chaufaux, and M. Lecadet.** 1989. Transformation and expression of a cloned delta-endotoxin gene in *Bacillus thuringiensis*. FEMS Microbiol. Lett. **51:**211–217.
24. **Murray, N. E.** 2002. Immigration control of DNA in bacteria: self versus non-self. Microbiology **148:**3–20.
25. **Okinaka, R. T., K. Cloud, O. Hampton, A. R. Hoffmaster, K. K. Hill, P. Keim, T. M. Koehler, G. Lamke, S. Kumano, J. Mahillon, D. Manter, Y. Martinez, D. Ricke, R. Svensson, and P. J. Jackson.** 1999. Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. J. Bacteriol. **181:**6509–6515.
26. **Park, H. W., D. K. Bideshi, J. J. Johnson, and B. A. Federici.** 1999. Differential enhancement of Cry2A versus Cry11A yields in *Bacillus thuringiensis* by use of the *cry3A* STAB mRNA sequence. FEMS Microbiol. Lett. **181:** 319–327.
27. **Park, H. W., D. K. Bideshi, M. C. Wirth, J. J. Johnson, W. E. Walton, and B. A. Federici.** 2005. Recombinant larvicidal bacteria with markedly improved efficacy against *Culex* vectors of West Nile Virus. Am. J. Trop. Med. Hyg. **72:**732–738.
28. **Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean.** 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. **62:**775–806.
29. **Schurter, W., M. Geiser, and D. Mathe.** 1989. Efficient transformation of

Bacillus thuringiensis and *B. cereus* via electroporation: Transformation of acrystalliferous strains with a cloned delta-endotoxin gene. Mol. Gen. Genet. **218:**177–181.

30. **Titok, M. A., J. Chapuis, Y. V. Selezneva, A. V. Lagodich, V. A. Prokulevich, S. D. Ehrlich, and L. Janniere.** 2003. *Bacillus subtilis* soil isolates: plasmid replicon analysis and construction of a new theta-replicating vector. Plasmid **49:**53–62.
31. **Turgeon, N., C. Laflamme, J. Ho, and C. Duchaine.** 2006. Elaboration of an electroporation protocol for *Bacillus cereus* ATCC 14579. J. Microbiol. Methods [Online/doi:10.1016/j.mimet.2006.05.005.]
32. **Vaughan, S., B. Wickstead, K. Gull, and S. G. Addinall.** 2004. Molecular evolution of FtsZ protein sequences encoded within the genomes of Archaea, Bacteria, and Eukaryota. J. Mol. Evol. **58:**19–39.
33. **Wilcks, A., L. Smidt, O. A. Okstad, A. B. Kolsto, J. Mahillon, and L. Andrup.** 1999. Replication mechanism and sequence analysis of the replicon of pAW63, a conjugative plasmid from *Bacillus thuringiensis*. J. Bacteriol. **181:** 3193–3200.
34. **Wu, D., and B. A. Federici.** 1993. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. J. Bacteriol. **175:**5276–5280.
35. **Xue, G. P., J. S. Johnson, and B. P. Dalrymple.** 1999. High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J. Microbiol. Methods **34:**183–191.
36. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.