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DNA sequence conservation between the *Bacillus anthracis* pXO2 plasmid and genomic sequence from closely related bacteria

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Abstract

Background: Complete sequencing and annotation of the 96.2 kb *Bacillus anthracis* plasmid, pXO2, predicted 85 open reading frames (ORFs). *Bacillus cereus* and *Bacillus thuringiensis* isolates that ranged in genomic similarity to *B. anthracis*, as determined by amplified fragment length polymorphism (AFLP) analysis, were examined by PCR for the presence of sequences similar to 47 pXO2 ORFs.

Results: The two most distantly related isolates examined, *B. thuringiensis* 33679 and *B. thuringiensis* AWO6, produced the greatest number of ORF sequences similar to pXO2; 10 detected in 33679 and 16 in AWO6. No more than two of the pXO2 ORFs were detected in any one of the remaining isolates. Dot-blot DNA hybridizations between pXO2 ORF fragments and total genomic DNA from AWO6 were consistent with the PCR assay results for this isolate and also revealed nine additional ORFs shared between these two bacteria. Sequences similar to the *B. anthracis cap* genes or their regulator, *acpA*, were not detected among any of the examined isolates.

Conclusions: The presence of pXO2 sequences in the other *Bacillus* isolates did not correlate with genomic relatedness established by AFLP analysis. The presence of pXO2 ORF sequences in other *Bacillus* species suggests the possibility that certain pXO2 plasmid gene functions may also be present in other closely related bacteria.

Background

Bacillus anthracis contains a 96.2 kb plasmid, pXO2, that is required to cause the disease anthrax [1]. Complete sequencing and annotation (GeneMark.hmm) of pXO2 predicted 85 open reading frames (ORFs) [Genbank accession NC_002146]. Little is known about the identity and function of pXO2 ORFs beyond the virulence genes associated with the *B. anthracis* capsule (*dep, capACB, acpA*) [2–5]. The goal of this study was to determine if many of the novel pXO2 ORFs were unique to *B. anthracis*, or were conserved in other closely related *Bacillus cereus* and *Bacillus thuringiensis* isolates. Conservation of plasmid sequences can provide clues about the origin of the pXO2 plasmid and about potentially conserved gene functions. Identification of ORFs that are specific to *B. anthracis* are potentially useful as markers for detection of the pathogen in clinical and forensic applications.

B. anthracis is a member of the *B. cereus/B. thuringiensis* phylogenetic group [6]. The members of this group are nearly indistinguishable by 16S rDNA analysis [7,8]. Plasmids in the *B. cereus/B. thuringiensis* isolates vary greatly in

number and size, and many of the phenotypic differences among *B. cereus*, *B. thuringiensis*, and *B. anthracis* isolates are conferred by plasmid encoded genes [9–12]. Horizontal plasmid transfer among bacteria, including isolates of the *B. cereus/thuringiensis* group has been documented [12–16].

Amplified fragment length polymorphism (AFLP) analysis of over 350 *B. cereus*, *B. thuringiensis*, and *B. anthracis* isolates, identified several distinct isolate groups [17,18]. Eight of the *B. cereus/B. thuringiensis* isolates were found to be very closely related to all *B. anthracis* isolates and formed a distinct cluster. In the present study, *B. cereus* and *B. thuringiensis* isolates that vary in AFLP-based genomic relatedness to *B. anthracis* were examined for the presence of DNA sequence similar to pXO2, to determine whether portions of this plasmid are conserved in closely related *Bacillus* isolates, and to determine whether the conservation of pXO2 sequences correlated with genomic relatedness established by AFLP comparisons [17,18].

Results and Discussion

PCR was performed using template DNA from 11 *Bacillus* isolates that vary in relatedness to *B. anthracis* with primer sets designed to amplify DNA fragments from 47 different pXO2 ORFs. This method was chosen to detect sequences with potential similarity to pXO2 because it is rapid and the reaction products can be readily sequenced. Table 1 lists the isolates tested, their genomic relatedness to *B. anthracis* as determined by Jaccard distances calculated from AFLP profile comparisons [17,18], and the number of positive PCR reactions obtained for each isolate. DNA sequencing of the amplified PCR products revealed a high degree of sequence similarity to pXO2 ORFs [Genbank accession numbers AF547271-AF547318]. BLAST (blastn) e-values were 6×10^{-13} or less for each ORF fragment detected, which corresponded to sequence similarity of 80% or greater. In a previous study, a similar approach was used to demonstrate that many of the ORFs from pXO1, the toxin-encoding plasmid of *B. anthracis*, were highly conserved in other isolates from the *B. cereus/B. thuringiensis* group [19].

The number of plasmid ORFs detected in a *Bacillus* isolate did not correlate directly with phylogenetic relationship to *B. anthracis* as determined by AFLP. The isolates most closely related to *B. anthracis* as determined by AFLP produced no more than two PCR products each. However, two of the more distantly related isolates, *B. thuringiensis* 33679 and *B. thuringiensis* AWO6, produced 10 and 16 positive PCR reactions, respectively. Neither of these isolates is known to be a human or animal pathogen.

Table 2 lists the 47 pXO2 ORFs that were tested in the PCR assay, their putative functions or similarities to other genes (blastp), and the PCR results obtained in this experiment. Nineteen different pXO2 ORF fragments were detected among the 11 *Bacillus* isolates. Eight of the conserved ORFs were similar to sequences contained in public databases; 11 were unidentified. The only pXO2 ORFs found in common with the isolates most closely related to *B. anthracis* (Jaccard distance of 0.55 or less) were ORFs 47 and 48. These ORFs have sequence similarity to a conserved hypothetical protein found in several bacterial genera and the *tetR* family of transcriptional repressors, respectively.

Table 1: Number of pXO2 ORF fragments detected in *Bacillus* isolates that vary in relatedness to *B. anthracis*.

***Bacillus* species (isolate no.)**	Source[b]	Jaccard Distance[c]	**PCR Products**
B. anthracis (91-429C-2)[a]	LSU	0	47
B. cereus (S2-8)	FRI	0.39	0
B. cereus (3A)	FRI	0.42	1
B. cereus (DC-17)	FRI	0.43	1
B. thuringiensis (Al-Hakam)	UNSCOM	0.46	1
B. thuringiensis konkukian	Hernandez *et al.* [26]	0.46	2
B. cereus (HRRL HD-571)	USDA	0.55	0
B. cereus (F1-15)	FRI	0.55	2
B. cereus (4342)	ATCC	0.67	0
B. cereus (43881)	ATCC	0.69	1
B. thuringiensis (33679)	ATCC	0.69	10
B. thuringiensis (AWO6)	Wilcks *et al.* [12]	0.73	16

[a]Positive control. [b]LSU, M.E. Hugh Jones, Louisiana State University; FRI, A. Wong and D. Beecher, Food Research Institute, University of Wisconsin; USDA, U.S. Department of Agriculture; ATCC, American Type Culture Collection; UNSCOM, United Nations Special Commission. [c]The Jaccard distance is the number of AFLP fragment sizes that occur in only one of the two samples, divided by the number of fragment sizes that occur in both samples plus the number of fragment sizes that occur in only one of the two samples.

Table 2: PCR assay results using primer sets designed for pXO2 ORF sequences.

ORF	Nucleotides	Gene ID or similarity	S2-8	3A	DC-17	ALH	konku	HD571	F1-15	4342	43881	33679	AWO6
5	2240–3088	unidentified											X
7	3449–4057	unidentified											
9	5227–7158	sim. *trsE*, *S. aureus*										X	X
10	7178–7846	unidentified											X
14	8704–11562	c.h.p. *C. perfringens*											X
15	11588–12379	c.h.p. *C. perfringens*											
16	12381–14216	sim. *trsK*, *L. lactis*											X
17	14265–16145	unidentified											
24	18442–18942	unidentified											
25	18975–20306	sim. pX01 ORF-59, *B. anthracis*											X
28	21387–22628	unidentified										X	X
29	22897–24546	unidentified											
30	24561–25625	unidentified											
32	26752–27000	unidentified										X	X
33	27515–28045	unidentified										X	X
35	29882–30571	sim. *rep63A*, AWO6 pAW63											
37	31610–32386	sim. pAW63											X
38	32577–34115	sim. *repS*, AWO6 pAW63										X	X
39	35021–35887	sim. *repB*, AWO6 pAW63											
42	37951–39510	sim. S-layer precursor, *B. anthracis*											
44	40988–41308	unidentified											
45	41900–42211	conserved domain, several bacteria											
46	42260–42925	CAAX amino term. protease family									X	X	X
47	43636–44100	c.h.p. several bacteria					X		X				
48	44477–45067	transcriptional repressor, *tetR* fam.		X	X	X	X		X				
49	45891–46361	*IS*231											
50	46400–46891	*IS*231											
51	47474–47641	sim. bacitracin											
53	49418–50866	sim. to *acpA*, *B. anthracis*											
55	52795–54195	*dep*											
56	54378–55612	*capA*											
57	55625–56074	*capC*											
58	56089–57483	*capB*											
59	60856–61407	signal peptide											
60	61759–62496	unidentified											
61	62841–63251	sim. pX01 *atxA*, *B. anthracis*											
64	68909–70360	*acpA*											
66	73500–75059	*traC*										X	X
68	76097–76690	unidentified										X	X
69	76918–78183	*uvx*										X	
71	79219–80772	unidentified											
73	82311–83936	repressor											
74	85420–85857	unidentified											
76	86664–87491	topoisomerase										X	X
77	87888–88688	unidentified											
80	90752–91735	unidentified											
81	91802–93571	unidentified											X
		Column Totals	**0**	**1**	**1**	**1**	**2**	**0**	**2**	**0**	**1**	**10**	**16**

Bacterial isolates designations are abbreviated as follows: S2-8, *B. cereus* S2-8; 3A, B. cereus 3A; DC-17, *B. cereus* DC-17; ALH, *B. thuringiensis* Al-Hakam; konku, *B. thuringiensis* subsp. *konkukian*; HD571, *B. cereus* (HRRL HD-571; F1-15, *B. cereus* F1-15; 4342, *B. cereus*ATCC 4342; 43881, *B. cereus* ATCC 43881; 33679, *B. thuringiensis* ATCC 33679; AWO6, *B. thuringiensis* AWO6. See Table 1 for source and Jaccard distances of AFLP profiles. 'sim.' = similar to. 'c.h.p.' = conserved hypothetical protein.

A 25.3 kb region that contains the capsule-associated genes has sequence characteristics that are different from the rest of the plasmid. This region of pXO2 spans nucleotides 48242–73500 and includes ORFs 53 through 65 (13 ORFs). In comparison to the rest of pXO2, this region has a larger average gene size (818 bases *vs.* 725 bases), a lower gene density (0.5 gene *vs.* 1.0 gene per kb of sequence), and larger average intergenic spaces (1125 bases *vs.* 260 bases). The region also has a slightly lower percent G+C (~28%) than the rest of the plasmid (~31%). Although the region is not bracketed by *IS* elements or tRNAs that are characteristic of pathogenicity islands (PAIs), it bears features that are similar to the putative PAI identified in the *B. anthracis* plasmid pXO1 [20]. Bacterial sequences with similarity to the *B. anthracis cap* genes are present in sequence databases. However, the capsule-associated genes (*capABC, dep, acpA*) were not detected by PCR in the tested *Bacillus* isolates. The pXO2 ORF sequences detected in *B. thuringiensis* 33679 and *B. thuringiensis* AWO6 were distributed across the entire plasmid sequence, except in the 25.3 kb *cap* gene-containing region, which appeared to be unique to *B. anthracis*.

B. thuringiensis strain AWO6 produced the most products in the PCR assay. A hybridization assay was performed using total genomic DNA from this isolate as a probe against pXO2 DNA targets amplified using the 47 primer sets from the PCR assay (Table 3). The hybridization assay complimented the PCR analysis by identifying nine additional conserved ORF sequences that might not have had exact matches to the PCR primer sequences. Total genomic DNA from *B. thuringiensis* strain AWO6 hybridized with 23 pXO2 ORF fragments, including all ORFs tested in the region between ORF 5 and ORF 38 (Table 3). ORFs in the 25.3 kb pXO2 *cap* gene-containing region did not hybridize with *B. thuringiensis* strain AWO6 DNA.

B. thuringiensis AWO6 is a strain containing a 70 kb plasmid designated pAW63 [12,21]. This strain was derived from *B. thuringiensis* HD73 by curing of its crystal toxin bearing plasmid, pHT73 [12,21]. The pAW63 plasmid contains a replication complex that is classified as a member of the pAMB-1 family of theta replicating plasmids that are present in a broad range of Gram positive species [22]. Plasmid pXO2 also appears to be a pAMB1-like theta replicating plasmid [23] and elements surrounding the replication complex are present in both pXO2 and pAW63 (see pXO2 ORFs 35, 37, 38, 39 in Tables 2 and 3). ORFs 35, 37, and 38 were sufficiently conserved between pXO2 and pAW63 to allow detection by PCR or hybridization (see Tables 2 and 3).

Pulsed field gel electrophoresis was used to separate plasmid and chromosomal DNA in *B. thuringiensis* AWO6, and a Southern hybridization blot using a mixed pool of pXO2-derived probes (ORFs 6, 10, 50, 63, 72, 81) was performed to determine if any of the ORFs were present on the pAW63 plasmid (Figure). A DNA fragment estimated to be 72 kb in size hybridized to the mixed pXO2 probe, which is slightly larger, but within 3% of the reported size of pAW63 (70 kb). This same PFGE protocol produced a similarly accurate measurement of the size of the *B. anthracis* plasmid pXO1 as determined by complete DNA sequencing [19]. The detection of sequences similar to pXO2 ORFs on pAW63 suggests that other pXO2 genes, in addition to those involved with replication, are also located on the pAW63 plasmid.

Conclusions

The presence of pXO2 ORF sequences in 11 *Bacillus* isolates did not correlate with their genomic relatedness to *B. anthracis* as determined by AFLP comparisons. A similar observation was made in previous work that examined the conservation of the *B. anthracis* plasmid pXO1 among closely related bacteria [19].

This study explored the extent of sequence conservation between pXO2 ORFs and total DNA from other *Bacillus* isolates, and detected similar sequences that may be located on the chromosome or any of several plasmids in each isolate. The two isolates with the most sequence conservation with pXO2 ORFs, *B. thuringiensis* isolates 33679 and AWO6, are known to contain large plasmids [12,19]. Four ORFs with high sequence similarity to *B. thuringiensis* AWO6 plasmid pAW63 were detected [22], and a mixed pXO2 ORF probe hybridized with a PFGE fragment similar in size to pAW63. The presence of considerable sequence conservation in more distantly related isolates rather than among close relatives, combined with the observations stated above, is a pattern consistent with the potential plasmid location of these sequences. Comparative sequence analysis of these large plasmids with pXO2 could determine if the observed sequence conservation was located on these plasmids.

Methods

Bacterial isolates and DNA isolation

The genomes of the 11 *Bacillus* isolates selected for study were found by AFLP analysis to vary in relatedness to *B. anthracis*. Isolates with Jaccard distances of less than 0.55 formed a distinct cluster with all of the *B. anthracis* isolates (P.J. Jackson, unpublished data) while the other 4 isolates were present in less closely related clusters (Table 1).

Bacteria were grown in Nutrient Broth (NB; DIFCO Laboratories, Franklin Lakes, NJ) or on NB agar plates at 28°C. Total DNA (including chromosomal and plasmid DNA) was extracted as described by Robertson *et al.* [24] with slight modifications. Cultures grown for 16 h in Nutrient Broth were centrifuged into a pellet, washed in TE (10 mM

Table 3: Comparison of dot-blot hybridization and PCR results for *B. thuringiensis* AWO6.

pXO2 ORF Number	Hybridization	PCR
5	X	X
7	X	
9	X	X
10	X	X
14	X	X
15	X	
16	X	X
17	X	
24	X	
25	X	X
28	X	X
29	X	
30	X	
32	X	X
33	X	X
35	X	
37	X	X
38	X	X
39		
42	X	
44		
45		
46		X
47		
48		
49		
50		
51		
53		
55		
56		
58		
59		
60		
61		
64		
66	X	X
68	X	X
69	X	
71		
73		
74		
76	X	X
77		
80		
81		X
TOTALS	**23**	**16**

Tris pH 7.5/1 mM EDTA pH 8.0), and suspended in 10% sucrose. Cells were incubated at 37°C in lysozyme solution (5 mg/ml lysozyme, 50 mM Tris pH 7.5, 10 mM EDTA pH 8.0), followed by addition of 20% SDS containing 0.3% beta-mercaptoethanol. A potassium acetate precipitation was performed to further clarify lysed cells [25]. DNA was purified by organic extraction and ethanol precipitation. Purified DNA was quantified by UV spectrophotometry. DNA from a *B. anthracis* isolate 91-213C-1 provided by P.J. Jackson was included as a positive control.

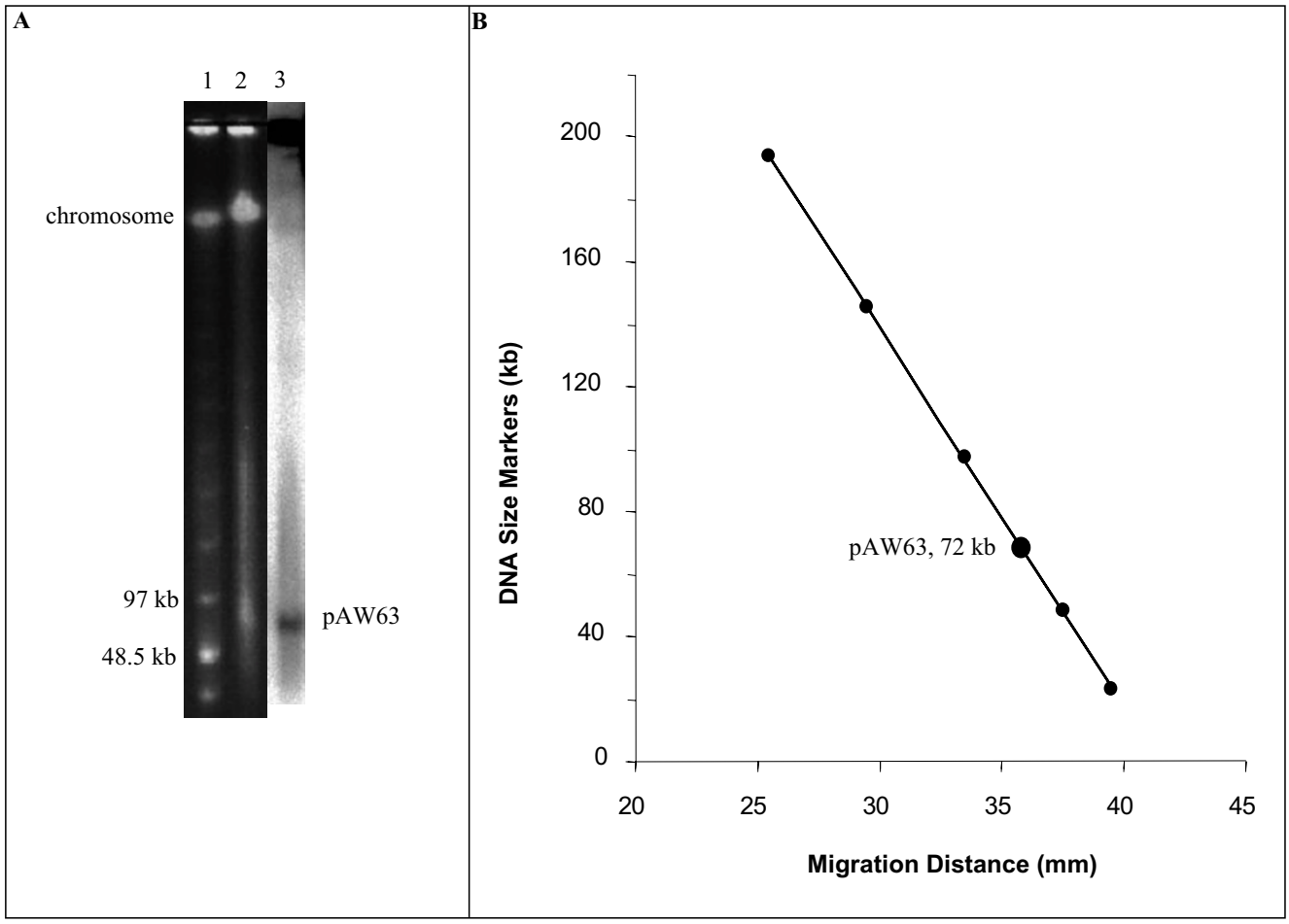

Figure 1
Pulsed field gel electrophoresis of DNA from *B. thuringiensis* AWO6. **Panel A**, Ethidium bromide-stained agarose gel. Lane 1 is the PFGE DNA size marker. Lane 2 is *B. thuringiensis* AWO6 DNA. Lane 3 is a Southern blot of pXO2-derived probes hybridizing to a DNA band the size of the pAW63 plasmid. **Panel B**, size of pAW63 plasmid and hybridizing DNA determined using PFGE.

pXO2 PCR primer sets

Oligonucleotide primer sets were identified for 47 pXO2 ORFs. PCR primer sets were typically positioned 20 to 50 bases from ORF termini unless A/T richness of the DNA sequence prohibited the design of primers in that region. Primer sequences are located at http://bdiv.lanl.gov/databases/databases.html. The remaining 38 pXO2 ORFs were not included in the present survey due to sub-optimal A/T richness, amplicon size, and thermodynamic characteristics of the candidate primer sets.

PCR assays and amplicon sequencing

PCR assays to detect each of the 47 individual pXO2 ORFs were conducted using DNA from each bacterial isolate (Table 1) as template. Fifty μl PCR reactions contained 1X Perkin Elmer PCR buffer with 1.5 mM MgCl2, 0.8 mM each dNTP, 1.25 U AmpliTaq DNA polymerase (Perkin Elmer), and 45 μM of each primer. A PTC-200 Peltier Thermocycler (MJ Research, Watertown, MA) was used for 35-cycle reactions (94°C, 2 min for first cycle only; 94°C, 30 s.; 48°C, 30 s.; 72°C, 30 s). Reactions were resolved on 2% agarose gels that were stained with ethidium bromide and viewed using a UV trans-illuminator. A reaction was considered positive if the amplified fragment was abundant and was the expected size DNA fragment.

The majority of PCR products were sequenced using dye-terminator chemistry (ABI Prism FS, PE Applied Biosystems, Boston, MA). Sequencing primers were the same as those used in PCR amplification reactions. Sequencing re-

actions were resolved on 48 cm polyacrylamide gels (4%, 19:1 acrylamide:bisacrylamide, Bio-Rad Laboratories) using an ABI model 373 fluorescence sequencer (Applied Biosystems, Inc.). DNA sequence was analyzed using Lasergene software (DNASTAR, Inc., Madison, WI). Sequences were deposited in GenBank as accession numbers AF547271 to AF547318.

Hybridization assay

A dot-blot hybridization assay was performed using DNA from *B. thuringiensis* strain AWO6 as probe against PCR-amplified pXO2 ORF DNA applied to a nylon membrane. Ten ng of each pXO2 ORF fragment was denatured by adding 0.1 volume of 1 M NaOH and incubation for 5 min at 37°C. An equal volume of 20X SSC (3 M NaCl, 0.3 M sodium citrate, adjusted to pH 7.0 with 1 M HCl) was added and samples were quickly placed on ice for 2 min. The DNA was then applied to a Hybond-N+ membrane (Amersham, Arlington Heights, IL) pre-soaked in 10X SSC using a HYBRI-DOT Manifold (Life Technologies, Inc., Rockville, MD). The membrane was exposed to 1200 mjoules of ultraviolet light in a UV-STRATALINKER 1800 (STRATAGENE, LaJolla, CA) to crosslink DNA to the membrane. Total DNA extracted from *B. thuringiensis* AWO6 was used to synthesize probe by incorporating [α-^{32}P]dCTP (6000 μCi/mMol) (NEN, Boston, MA) into randomly primed DNA synthesis reactions using the Megaprime DNA Labeling System (Amersham-Pharamacia Biotech, Piscataway, NJ) according to the manufacturer instructions. The membrane was incubated at 50°C in hybridization buffer (0.5 M NaHPO4, 1 mM EDTA pH 8.0, 7% SDS [28]) for 60 min, followed by hybridization with probe for 16 h at 50°C. After hybridization, the membrane was washed twice for 10 min at 30°C in 2X SSC containing 0.1% SDS and twice for 10 min at 45°C in 0.2X SSC containing 0.1% SDS. Results were viewed using a Fugi Phosphorimager.

Pulsed-field gel-electrophoresis (PFGE)

A 15 ml culture of *B. thuringiensis* AWO6 was grown in NB overnight at 37°C with shaking. Chloramphenicol was added at a concentration of 180 μg/ml and the culture was incubated for 60 min. Cells were incubated on ice for 10 min, then centrifuged at 2500 × *g* for 5 min. Cell pellets were suspended in 1 ml TE buffer that contained 2 mg/ml lysozyme and incubated for 5 min at 37°C. Lysozyme-treated cells were washed in 1 ml of Buffer NT (1 M NaCl, 50 mM Tris pH 7.5) and were suspended in Buffer NT to a final volume of 200 μl.

Agarose plugs containing bacterial cells were prepared in a 1 ml syringe by combining cells with an equal volume of 2% SeaKem Gold agarose (FMC BioProducts, Rockland, ME) melted in water. Plugs were allowed to solidify at 4°C for 2 h. Thin agarose slices (1–3 mm) containing embedded bacteria were incubated for 16 h in 500 μl Buffer NTE (100 mM NaCl, 50 mM Tris pH 7.5, 100 mM EDTA pH 8.0) containing 2% lysozyme at 37°C. The lysozyme/Buffer NTE solution was replaced with Buffer NTE that contained 2 mg/ml Proteinase K and incubated for 16 h at 50°C. Slices were then incubated in Buffer NTES (100 mM NaCl, 50 mM Tris pH 7.5, 100 mM EDTA pH 8.0, 1% SDS) for 16 h at 50°C. Before electrophoresis, slices were incubated twice for 30 min in 1.0 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma. St. Louis, MO) diluted in TE and twice in 0.5X TBE (45 mM Tris-borate (1:1), 1 mM EDTA).

Treatment of agarose slices linearized the plasmid DNA and allowed for plasmid size determination using a concatomerized bacteriophage lambda standard (New England BioLabs, Beverly, MA) (5). DNA from agarose slices was resolved on a gel of 1% SeaKem Gold agarose melted in 0.5X TBE. Electrophoresis conditions were 175 V in 0.5X TBE at 6°C for 21 h in a CHEF-DR II Pulsed Field Electrophoresis System (BIORAD, Hercules, CA) with a field switch ramp of 5 to 40 s. Gels were stained with ethidium bromide and viewed using a UV trans-illuminator.

Southern hybridization

The pulsed field gel was sequentially soaked in 0.25 N HCl for 30 min; 3 M NaCl, 0.4 M NaOH for 60 min; and 0.5X TBE for 15 min. Electro-transfer of the DNA to a nylon membrane was performed using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) according to the manufacturer instructions. DNA was crosslinked to the membrane by exposure to 1200 mjoules of ultraviolet light in a UV-STRATALINKER 1800 (STRATAGENE, LaJolla, CA). The membrane containing *B. thuringiensis* AWO6 DNA was hybridized using a [α-^{32}P]dCTP-labeled probe prepared from a mixture of six PCR-amplified pXO2 ORF fragments (pXO2 ORFs 6, 10, 50, 63, 72, 81). Care was taken to avoid the IS elements present on the plasmid. Probe synthesis, hybridization conditions, and wash regimen were performed as described above for hybridization reactions. Results were viewed using a Fugi Phosphorimager.

Authors' Contributions

JP was responsible for experimental design, protocols, and data management. RTO designed the pXO2 oligonucleotides. EW performed PCR reactions and DNA sequencing. RS conducted the hybridization reactions and DNA sequencing. LOT provided AFLP data and statistical analysis. CRK was the principal investigator who began the study and coordinated the work. All authors contributed to preparation of this manuscript.

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