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Bacillus anthracis pXO1 Plasmid Sequence Conservation among Closely Related Bacterial Species

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The complete sequencing and annotation of the 181.7-kb *Bacillus anthracis* virulence plasmid pXO1 predicted 143 genes but could only assign putative functions to 45. Hybridization assays, PCR amplification, and DNA sequencing were used to determine whether pXO1 open reading frame (ORF) sequences were present in other bacilli and more distantly related bacterial genera. Eighteen *Bacillus* species isolates and four other bacterial species were tested for the presence of 106 pXO1 ORFs. Three ORFs were conserved in most of the bacteria tested. Many of the pXO1 ORFs were detected in closely related *Bacillus* species, and some were detected only in *B. anthracis* isolates. Three isolates, *Bacillus cereus* D-17, *B. cereus* 43881, and *Bacillus thuringiensis* 33679, contained sequences that were similar to more than one-half of the pXO1 ORF sequences examined. The majority of the DNA fragments that were amplified by PCR from these organisms had DNA sequences between 80 and 98% similar to that of pXO1. Pulsed-field gel electrophoresis revealed large potential plasmids present in both *B. cereus* 43881 (341 kb) and *B. thuringiensis* ATCC 33679 (327 kb) that hybridized with a DNA probe composed of six pXO1 ORFs.

Bacillus anthracis harbors the 181.7-kb plasmid pXO1, which is essential to manifestation of the disease anthrax (25, 35, 36). Recent sequencing and annotation predicted that pXO1 contains as many as 143 open reading frames (ORFs), but only 35 have putative functions assigned based on similarity to genes in open databases (26). A function has been experimentally assigned to only nine pXO1 genes. Eight of the nine characterized pXO1 genes are within a 44.8-kb putative pathogenicity island (PAI) (26). The genes encoding the anthrax toxin proteins (*lef*, *pagA*, and *cya*) (20, 21, 29, 37), the toxin gene transactivator (*atxA*) (9, 34), and the plasmid-borne germination genes (*gerXC*, *-A*, and *-B*) (13, 31) are essential for *B. anthracis* pathogenicity. The functions of the negative regulator of *pagA* (*pagR*) (18) and the topoisomerase gene (*topX*) (11) have also been experimentally determined. Several important functions have not yet been identified within the pXO1 sequence, including an origin of replication and the genes involved in replication initiation, plasmid partitioning, and plasmid stability.

B. anthracis is a member of the *Bacillus cereus*/*Bacillus thuringiensis* phylogenetic group (3, 7, 15). The members of this group are virtually indistinguishable by 16S rRNA sequence analysis, with as much variability present among the multiple rDNA operons of an individual species as among different isolates (4). Multilocus enzyme electrophoresis and comparative DNA sequence analysis suggest that they may represent a single species (15). Many of the functional differences between *B. cereus*, *B. thuringiensis*, and *B. anthracis* isolates are due to the presence of plasmids that can vary in number and size, with some plasmids exceeding 500 kb (7, 24, 27). *B. anthracis* isolates are extremely uniform in chromosome composition and are limited to the presence of the two virulence plasmids, pXO1 and pXO2. This conserved state reflects the relatively recent evolution of *B. anthracis* from a parental *B. cereus* subgroup (15, 19).

The potential for horizontal gene transfer among closely related species is well documented (10, 14, 38), and it is reasonable to expect that plasmid genes are shared among species of the *B. cereus*/*B. thuringiensis*/*B. anthracis* group. For example, the plasmid-borne insecticidal crystal toxin genes of *B. thuringiensis* were identified in certain *B. cereus* strains, making those strains of *B. cereus* appear as crystal toxin- and enterotoxin-producing *B. thuringiensis* (12). Although direct evidence for horizontal gene transfer within the pXO1 sequence has not been found, the presence of 15 plasmid elements with sequence similarity to known insertion elements, transposons, or integration sites suggests that such mechanisms could have played a role in the evolution of pXO1. Movement of pXO1 genetic elements among plasmids from different *B. anthracis* isolates has been observed. Two IS*1627* elements in pXO1 define a 44.8-kb putative pathogenicity island (PAI) that is inverted in two different strains of *B. anthracis* (26). In addition, the presence of three IS*231*-like transposase genes in pXO1 that are present in other *Bacillus* species (22) suggests an exchange between the chromosome or extrachromosomal elements of *B. anthracis* and other related bacteria.

We sought to determine the extent to which other bacterial species contain DNA sequences with high similarity to those of pXO1 ORFs. Eighteen *Bacillus* isolates and species in three other bacterial genera were surveyed to determine if the presence of pXO1 sequences correlated broadly with phylogenetic relatedness. Several recently discovered *B. cereus* and *B. thuringiensis* isolates were also examined for sequence similar to pXO1. The chromosomes of these isolates were analyzed for amplified fragment length polymorphism (AFLP) and were found to be very closely related to that of *B. anthracis* (33; P. J. Jackson, unpublished data).

A combination of DNA-DNA hybridization and PCR am-

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plification was used in a complementary manner for this study. Most PCR results were verified by sequencing the amplified products. Hybridization and PCR both detect exact sequence matches as well as nonidentical but highly similar sequences. DNA-DNA hybridization assays detect sequences with overall similarity among DNA fragments, regardless of occasional base pair mismatches across the interrogated sequence. This approach was used to detect pXO1 sequence similarity across a broad range of bacterial species. In contrast, PCR assays require nearly exact sequence matches between the oligonucleotide primer and the template DNA, but not within the internal sequence. This approach was used to detect sequences similar to pXO1 in closely related *Bacillus* species. A combined approach allowed the identification of more sequences with potential similarity to pXO1 than either approach alone. This study is the first to show substantial conservation between pXO1 sequences and DNA from other *Bacillus* species and represents a starting point for a more thorough investigation of the functions of pXO1 ORFs in *B. anthracis* and other *Bacillus* species.

MATERIALS AND METHODS

Bacterial isolates. Bacterial isolates used in this study are listed in Table 1. These were grown in nutrient broth (NB; Difco Laboratories, Franklin Lakes, N.J.) or on NB agar plates at 28°C. Thirteen bacterial strains from the *B. cereus*/*B. thuringiensis* group were tested for the presence of pXO1 ORF sequences. Eight of these isolates constitute a cluster found by chromosomal AFLP analysis of over 350 *B. cereus*, *B. thuringiensis*, and *B. anthracis* isolates to be the isolates most closely related to *B. anthracis* (P. J. Jackson, unpublished data) and are designated here AFLP group 1. Four *B. cereus*/*B. thuringiensis* group isolates that are members of a separate AFLP group (33) (designated here AFLP group 2) were also examined. In addition, four more-distantly related *Bacillus* species, two *Paenibacillus* species, *Clostridium perfringens*, and *Pseudomonas putida* were included in these experiments. *C. perfringens* was included in the study because there is amino acid sequence homology between the iota toxin of this pathogen and the *B. anthracis* protective antigen (28). Two *B. anthracis* isolates were included as positive controls.

Preparation of bacterial DNA. Total DNA (including chromosomal and extrachromosomal DNA) from *B. anthracis* 91-213C-1 was provided by P. J. Jackson. Total DNA from all other bacterial isolates was extracted as described by Robertson et al. (30) with slight modifications. Cultures grown for 16 h in NB were centrifuged into a pellet, washed in TE (10 mM 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 of lysozyme/ml, 50 mM Tris [pH 7.5], 10 mM EDTA [pH 8.0]), followed by addition of 20% sodium dodecyl sulfate (SDS) containing 0.3% β-mercaptoethanol. A potassium acetate precipitation was performed to further clarify lysed cells (16). DNA was purified by organic extraction and ethanol precipitation. Purified DNA was quantified by UV spectrophotometry. Sodium hydroxide was added to *B. anthracis* Sterne DNA preparations during the SDS incubation step to enrich for pXO1 DNA (32).

Generation of pXO1 ORF primer sets and hybridization probes. Oligonucleotides were identified from 106 pXO1 ORFs and were used as PCR primers to generate probe DNA for hybridization assays. For the remaining 37 ORFs, candidate primers sets with optimal spacing and thermodynamic characteristics were not found due to the A/T richness of the sequence. For this reason, these ORFs were not included in the present survey. PCR primer sets were typically positioned 20 to 50 bases from ORF termini unless the A/T richness of the DNA sequence prohibited the design of primers in that region.

One hundred two individual pXO1 ORF fragments were successfully amplified by PCR in sufficient abundance for use in the hybridization assays. PCR mixtures to generate hybridization probes contained 0.5 to 1.0 ng of pXO1 DNA as the template and 1× PCR buffer containing 1.5 mM $MgCl_2$, 0.8 mM (each) deoxynucleoside triphosphate, 1.25 U of Ampli*Taq* DNA polymerase (Perkin-Elmer, Boston, Mass.), and 45 μM (each) primer per 50-μl reaction mixture. A PTC-200 Peltier thermocycler (MJ Research, Watertown, Mass.) was used for 35-cycle reactions (94°C, 30 s; 48°C, 30 s; 72°C, 30 s; final 5-min 72°C extension). Reaction conditions were optimized to produce one abundant PCR product from *B. anthracis* template DNA. Reaction products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, Calif.). To verify amplification of the correct fragment size, amplicons were resolved on agarose gels, stained with ethidium bromide, and viewed using a UV transilluminator. A hybridization probe was also derived from the *B. anthracis* 16S rRNA gene and an *Hae*III digest of pXO1 DNA. A list of the PCR primers is available at http://biosphere-.lanl.gov/public_html/pxo1conservedseq.html.

TABLE 1. Bacterial isolates used in this study and the number of positive ORFs in hybridization and PCR assays

Species (isolate)	Source or reference[f]	Results[a] of:	
		Hybridization	PCR
B. cereus/*B. thuringiensis* group			
AFLP group 1[b]			
B. anthracis (Sterne)[c]	USAMRIID		106[d]
B. anthracis (91-429C-2)[c]	LSU	102[d]	
B. cereus (S2-8)	FRI		2
B. cereus (DC-17)	FRI	54	16
B. cereus (HRRL HD-571)	USDA		0
B. cereus (3A)	FRI		5
B. cereus (F1-15)	FRI		20
B. cereus (4342)	ATCC		1
B. thuringiensis (A1-Hakam)	UNSCOM		1
B. thuringiensis subsp. *konkukian*	22	2	1
AFLP group 2[e]			
B. cereus (ATCC 43881)	ATCC	41	65
B. cereus (14579)	ATCC	4	4
B. cereus (11778)	ATCC	3	
B. thuringiensis subsp. *kurstaki* (ATCC 33679)	ATCC	29	46
Other bacilli and paenibacilli			
B. mycoides (6462)	ATCC	4	
B. megaterium (15127)	ATCC	1	
B. amyloliquifaciens (23350)	ATCC	2	
B. subtilis (6051)	ATCC	7	9
P. glucanolyticus (49278)	ATCC	3	
P. alvei (6344)	ATCC	3	
More-distantly related bacteria			
C. perfringens (13124)	ATCC	2	
P. putida (12633)	ATCC	0	

[a] Numbers of positive reactions for each assay, except where indicated otherwise.
[b] Group that contains all *B. anthracis* isolates.
[c] Positive control.
[d] Number of ORFs tested.
[e] Group distinct from group 1 (33).
[f] USAMRIID, U.S. Army Medical Research Institute for Infectious Diseases; LSU, M. 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.

PCR assays and amplicon sequencing. PCR assays to detect each of the 106 individual pXO1 ORFs were conducted using DNA from each bacterial species listed in Table 1 as the template. Reaction conditions were identical to those described above for generation of DNA probes for the hybridization assays. Amplified fragments were resolved on agarose gels that were stained with ethidium bromide and viewed using a UV transilluminator. A reaction was considered positive if an abundant fragment that was of the correct size with regard to the primer positions in the pXO1 sequence was amplified.

Most of the PCR products were sequenced using dye terminator chemistry (ABI Prism FS; PE Applied Biosystems, Inc., Boston, Mass.). Sequencing primers were the same as those used in PCR amplifications. Sequencing reactions were resolved on 48-cm-long polyacrylamide gels (4%; 19:1, acrylamide/bisacrylamide; Bio-Rad Laboratories, Hercules, Calif.) using an ABI model 373 fluorescence sequencer (Applied Biosystems, Inc.). DNA sequences were analyzed using Lasergene software (DNASTAR, Inc., Madison, Wis.).

Hybridization assays between pXO1 ORFs and bacterial species. Hybridization experiments to detect pXO1 ORF sequences in DNA from different bacterial species were conducted in two sets. The first set of experiments was designed to determine the extent to which pXO1 ORF sequences were present among bacilli and other bacterial genera with decreasing phylogenetic relatedness to *B.*

anthracis. This set of experiments used a chemiluminescence detection system (ECL direct nucleic acid labeling and detection system; Amersham-Pharmacia Biotech, Piscataway, N.J.) and included the species indicated in Table 1 with the exception of *B. cereus* D-17 and *B. thuringiensis* subsp. *konkukian*. In this experimental format, 1 μg of total DNA from each bacterial strain was denatured by adding 0.1 volume of 1 M NaOH and incubating for 5 min at 37°C. An equal volume of 20× 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, Ill.) presoaked in 10× SSC using a HYBRI-DOT manifold (Life Technologies, Inc., Rockville, Md.). The membrane was exposed to 1,200 mJ of UV light in a UV-Stratalinker 1800 (Stratagene, LaJolla, Calif.) to cross-link DNA to the membrane. Twenty-four replicate membranes were prepared and hybridized with individual pXO1 ORF probes according to the ECL direct nucleic acid labeling and detection system protocol. After hybridization, membranes were washed twice with primary (6 M urea, 0.4% SDS, 0.5× SSC) and secondary (2× SSC) wash solutions for 20 min at 42°C. Membranes were stripped of probe between individual hybridization experiments using 0.4 M NaOH that contained 0.1% SDS for 20 min at 25°C. The stripping solution was neutralized with a solution consisting of 0.1× SSC, 0.1% SDS, and 0.2 M Tris-Cl, pH 7.5. The stripping procedure was tested to insure membranes were cleared of signal before conducting subsequent hybridization experiments.

The discovery of a group of pathogenic *B. cereus* and *B. thuringiensis* isolates that are closely related *B. anthracis* by AFLP analysis (the Food Research Institute, U.S. Department of Agriculture, and United Nations Special Commission isolates comprising AFLP group 1 listed in Table 1) (33; P. J. Jackson, unpublished data) prompted a second set of hybridization experiments. Results from the PCR assays indicated that it would be valuable to conduct hybridization assays on a few of the AFLP group 1 isolates. DNA from individual plasmid ORFs was applied to nylon membranes using the procedure described above. Total DNA extracted from *B. cereus* D-17 and *B. thuringiensis* subsp. *konkukian* was used to synthesize the probe by incorporating [α-^{32}P]dCTP (6,000 Ci/mmol; NEN, Boston, Mass.) into randomly primed DNA synthesis reactions using the Megaprime DNA labeling system (Amersham-Pharmacia Biotech) according to the manufacturer's instructions. With the chemiluminescence assay (described above) the individual ORFs were used as probes to achieve the highest possible detection sensitivity with low background. We found that by using isotopic labeling, the plasmid ORFs could be arrayed on the membrane and probed with a complex DNA probe (total bacterial DNA) with high detection sensitivity. Thus, the entire set of 102 ORFs could be surveyed at one time in a single hybridization experiment. For this format, the membrane was incubated at 50°C in hybridization buffer (0.5 M $NaHPO_4$, 1 mM EDTA [pH 8.0], 7% SDS [8]) for 60 min, followed by hybridization with the probe for 16 h at 50°C. After hybridization, the membrane was washed twice for 10 min at 30°C in 2× SSC containing 0.1% SDS and twice for 10 min at 45°C in 0.2× SSC containing 0.1% SDS. Results were viewed using a Fugi phosphorimager.

Pulsed-field gel electrophoresis (PFGE). Fifteen-milliliter cultures of *B. anthracis* Sterne, *B. cereus* ATCC 43881, and *B. thuringiensis* ATCC 33679 were grown in NB overnight at 37°C with shaking and were prepared for analysis using the method of Barton et al. with a slight modification (5). Briefly, chloramphenicol was added to cultures at a concentration of 180 μg/ml, and cultures were incubated for 60 min. Next, cells were incubated on ice for 10 min and then centrifuged into pellets at 2,500 × *g* for 5 min. Cells were suspended in 1 ml of TE buffer containing 2 mg of lysozyme/ml and incubated at 37°C for 5 min. Lysozyme-treated cells were washed in 1 ml of buffer NT (1 M NaCl, 50 mM Tris, pH 7.5) and suspended in a final volume of 200 μl of buffer NT.

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, Maine) melted in water. Plugs were allowed to solidify at 4°C for 2 h. Thin agarose slices (1 to 3 mm thick) containing embedded bacteria were incubated for 16 h in 500 μl of 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 of proteinase K/ml, and the agarose slices were 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 (Sigma, St. Louis, Mo.) diluted in TE and twice in 0.5× TBE (45 mM Tris-borate [1:1], 1 mM EDTA).

Treatment of agarose slices was sufficient to linearize the large pXO1 plasmid DNA molecules, and that allowed for determination of plasmid size using a concatemerized bacteriophage lambda standard (New England BioLabs, Beverly, Mass.). DNA from agarose slices was resolved on a gel of 1% SeaKem gold agarose melted in 0.5× TBE. Electrophoresis conditions were 175 V in 0.5× TBE at 6°C for 21 h in a CHEF-DR II pulsed-field electrophoresis system (Bio-Rad) with a field switch ramp of 5 to 40 s. Gels were stained with ethidium bromide and viewed using a UV transilluminator.

Transfer and hybridization of pulsed-field gels. The pulsed-field gel was soaked in 0.25 N HCl for 30 min and then transferred to a solution of 3 M NaCl–0.4 M NaOH for 60 min (*Blotting, hybridization, and detection: an S & S laboratory manual*, 6th ed., p. 2–3, Schliecher & Schuell). The gel was prepared for DNA transfer by soaking in 0.5× TBE for 15 min. Electrotransfer of the DNA to a nylon membrane was performed using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) according to the manufacturer instructions. DNA was cross-linked to the membrane by exposure to 1200 mJ of UV light in a UV-Stratalinker 1800 (Stratagene).

The membrane containing *B. anthracis* Sterne, *B. cereus* ATCC 43881, and *B. thuringiensis* ATCC 33679 DNA was hybridized using [α-^{32}P]dCTP. The hybridization probe was prepared from a mixture of six PCR-amplified pXO1 ORF fragments from different regions of pXO1 (pXO1 ORF-18, -32, -51, -85, -101, and -141). Care was taken to avoid the insertion sequence elements present on the plasmid. Probe synthesis, hybridization conditions, and the wash regimen were as described above for *B. cereus* D-17 and *B. thuringiensis* subsp. *konkukian*. Results were viewed using a Fugi phosphorimager.

Nucleotide sequence accession numbers. The sequences determined in this study were deposited in GenBank under accession no. AF442968 to AF443070.

RESULTS

Hybridization results. Replicate hybridization membranes in the chemiluminescent hybridization assay (Table 1) were first probed with a 16S rDNA fragment from *B. anthracis* to determine that adequate and similar amounts of genomic DNA had been bound to each membrane. DNA from each of the bacterial isolates hybridized strongly with the 16S rRNA probe (data not shown), and the *B. anthracis* positive-control spot hybridized strongly with each of the 102 pXO1 ORF probes (Table 1). Each bacterial isolate, with the exception of *P. putida*, hybridized with a probe derived from the total pXO1 sequence (data not shown). As a member of the *Proteobacteria* division, *P. putida* was the species most distantly related to *B. anthracis* that was examined. It was also the only bacterial isolate that did not hybridize with any of the individually tested pXO1 ORF fragments. Sixty-nine of the 102 individual pXO1 ORF probes hybridized with DNA from at least one other species.

Members of the *B. cereus*/*B. thuringiensis* group hybridized with more of the pXO1 ORF sequences than the other *Bacillus* spp., the *Paenibacillus* spp., and more distantly related species (Table 1). DNA from three *Bacillus* isolates in the *B. cereus*/*B. thuringiensis* group, *B. cereus* D-17, *B. cereus* ATCC 43881, and *B. thuringiensis* ATCC 33679, each hybridized with over one-half of the pXO1 ORF probes. The individual ORFs that were detected by hybridization for these isolates are illustrated in Fig. 1.

Bacterial species that hybridized with only a few pXO1 ORF probes are presented in Table 2. DNA fragments from pXO1 ORF-45, -51, and -69 hybridized with DNA from several of the bacterial isolates examined, including all closely related *Bacillus* species and several more distantly related bacteria (Table 2 and Fig. 1). The pXO1 ORF −45 probe (pXO1 nucleotides 58889 to 57960 of the pXO1 complete sequence; GenBank accession no. AF065404) hybridized with DNA from all members of the *B. cereus*/*B. thuringiensis* group and several other *Bacillus* and *Paenibacillus* species. The pXO1 ORF-51 probe (pXO1 nucleotides 65544 to 66457) hybridized with DNA from all but one of the gram-positive bacteria tested (including the

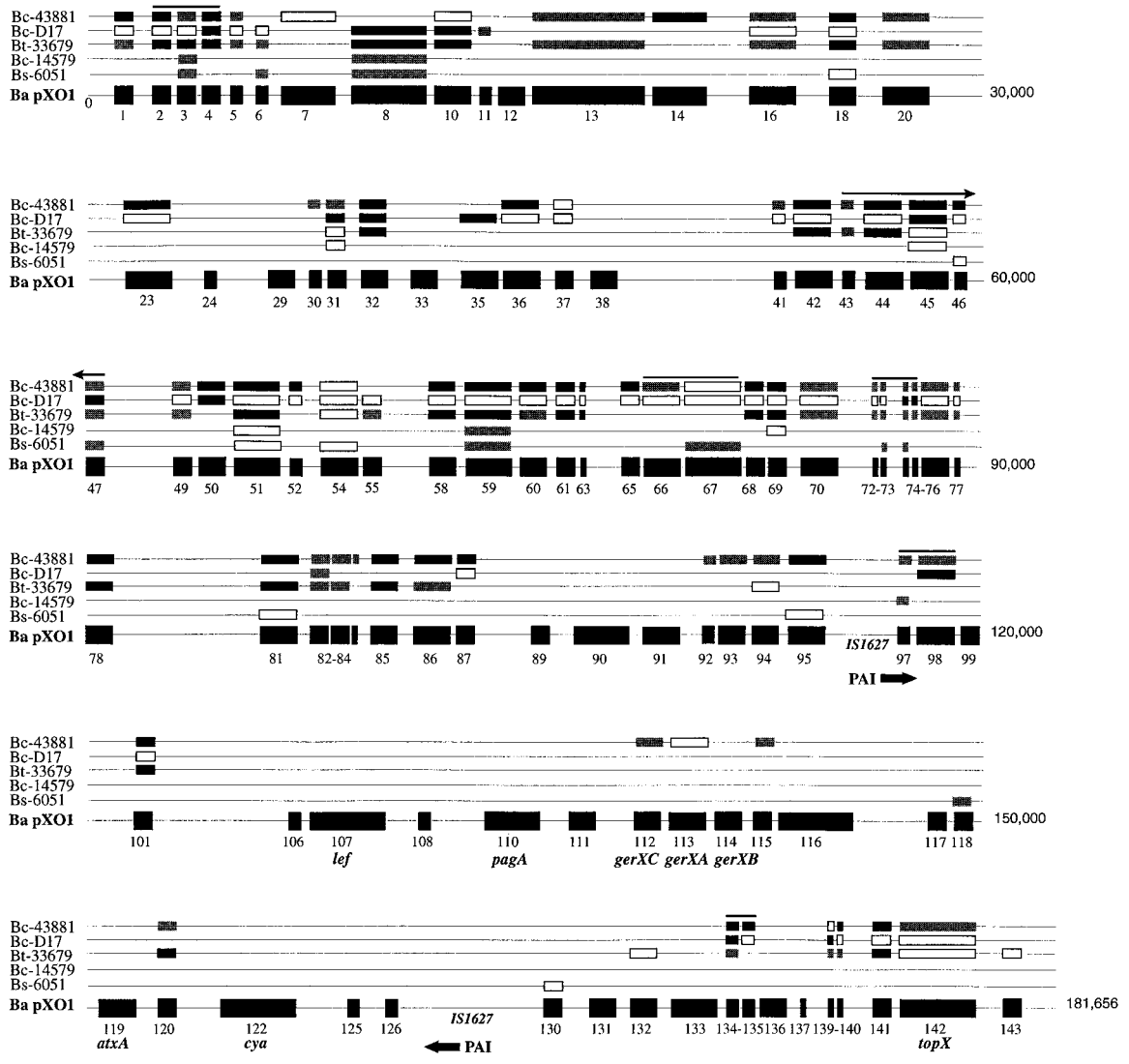

FIG. 1. pXO1 ORF sequences that were detected in the genomes of *B. cereus* ATCC 43881 (Bc-43881), *B. cereus* D-17 (Bc-D17), *B. thuringiensis* ATCC 33679 (Bt-33679), *B. cereus* 14579 (Bc-14579), and *B. subtilis* 6051 (Bs-6051) using a combination of hybridization and PCR assays. The bottom line of each row represents a linear map of the pXO1 ORFs (27), with black boxes representing the ORFs that were examined in this study. Boxes above each pXO1 ORF indicate a positive result that was obtained for each of the five presented isolates (white, grey, and black boxes indicate positive results by hybridization, PCR, or both assays, respectively). This illustration does not imply similar gene order or plasmid arrangement in any of the test species, except as indicated for *B. cereus* ATCC 43881. Lines above the map that span multiple ORFs in five regions indicate conservation of ORF order in those regions for *B. cereus* ATCC 43881.

Bacillus, *Paenibacillus*, and *Clostridium* species), but not with *P. putida*. The probe representing pXO1 ORF-69 (pXO1 nucleotides 82208 to 82604) hybridized with DNA from all isolates from the *B. cereus*/*B. thuringiensis* group that were examined, but not with the more-distantly related species. The *B. thuringiensis* subsp. *konkukian* isolate hybridized with only two pXO1 ORF probes even though it is very closely related to *B. anthracis.* DNA from *B. thuringiensis* subsp. *konkukian* hybridized with probes from ORF-120, encoding a putative transposase (26), and ORF-119, encoding the virulence gene transactivator *atxA*. Both ORFs are in the pXO1 PAI.

PCR results. PCR assays were used to examine the members of the *B. cereus*/*B. thuringiensis* group for similarity to pXO1 ORF sequences to determine whether conserved sequences are more prevalent in AFLP group 1 isolates than in AFLP

TABLE 2. Summary of results for bacterial isolates examined using hybridization assays

Bacterial isolate	Hybridization[a] to pX01 ORF:									
	18	31	45	51	69	93	95	98	119	120
Bt. ko[b]									X	X
B. cereus 11778			X	X	X					
B. mycoides 6462		X	X	X	X					
B. megaterium 15127				X						
B. amyloliquifaciens 23350			X	X						
P. glucanolyticus 49278			X	X				X		
P. alveli 6344	X			X			X			
C. perfringens 13124				X		X				
P. putida 12633										

[a] X, hybridization observed.
[b] Bt-ko, *B. thuringiensis* subsp. *konkukian*.

TABLE 3. Summary of results for bacterial isolates examined by PCR

Isolate[a]	PCR result[b] for pX01 ORF:																							
	2	3	5	6	8	10	11	14	31	32	47	50	61	74	75	89	94	97	98	99	139	141	142	143
S2-8								X											X					
HD-571																								
3A		X		X				X								X			X					
F1-15	X	X	X		X	X	X		X	X	X	X	X	X	X		X	X	X	X	X	X	X	
4342		X																						
Al-Hakam																	X							
Bt-ko																								X

[a] See Table 1. Bt-ko, *B. thuringiensis* subsp. *konkukian*.
[b] X, positive PCR result.

group 2 isolates. *Bacillus subtilis* 6051 was included in the PCR assay as an outlier of the AFLP groups. PCR assays using the 106 individual pXO1 primer sets were all positive with the *B. anthracis* DNA template and detected at least one similar ORF in 11 of the 12 tested species. PCR detection of pXO1 sequences was not correlated with close chromosomal relationship to *B. anthracis* (Table 1). Several of the pXO1 primer sets amplified DNA from four of the bacterial isolates: *B. cereus* D-17, *B. cereus* F1-15, *B. cereus* ATCC 43881, and *B. thuringiensis* ATCC 33679 (Table 1). However, the majority of isolates in this group hybridized with fewer than five of the pXO1 ORFs. The individual pXO1 ORF sequences that were detected in *B. cereus* D-17, *B. cereus* ATCC 43881, and *B. thuringiensis* ATCC 33679 are illustrated in Fig. 1. Table 3 contains results for the remaining isolates that were examined by PCR.

Amplified fragments obtained from *B. cereus* ATCC 43881, *B. cereus* D-17, *B. cereus* 3A, *B. cereus* F1-15, *B. thuringiensis* ATCC 33679, *B. cereus* 14579, and *B. subtilis* 6051 were single pass sequenced from the amplicon ends using the individual ORF primer pairs. DNA sequencing was used to validate the specificity of the PCR assay and to estimate the similarity of conserved DNA fragments, which ranged from 40 to 98% (Fig. 2). The majority of sequences amplified by PCR were greater than 80% similar to that of the corresponding pXO1 ORF.

Combined results. A combination of hybridization and PCR methods was used to detect DNA sequences in the other bacterial species that were similar to pXO1. The two detection methods target different characteristics of interrogated sequences: overall similarity to an entire sequence fragment for hybridization and exact match to small oligonucleotide primers for PCR. Thus, they can detect different sequences. Three isolates with abundant positive results (*B. cereus* D-17, *B. cereus* ATCC 43881, and *B. thuringiensis* ATCC 33679) and two isolates with few positive results (*B. cereus* 14579 and *B. subtilis* 6051) were examined using both hybridization and PCR assays. An ORF map of pXO1 shows the combined results of the hybridization and PCR screening assays for these five isolates (Fig. 1). As expected, the results of the PCR assays did not correspond exactly with results obtained from the hybridization assays. Some of the ORF sequences detected by hybridization were not detected by PCR, and the PCR assay identified the presence of additional conserved ORF fragments that the hybridization assay did not reveal. This was especially true for the chemiluminescence hybridization assay (used to test *B. cereus* ATCC 43881 and *B. thuringiensis* ATCC 33679), which tended to underestimate the extent of sequence conservation.

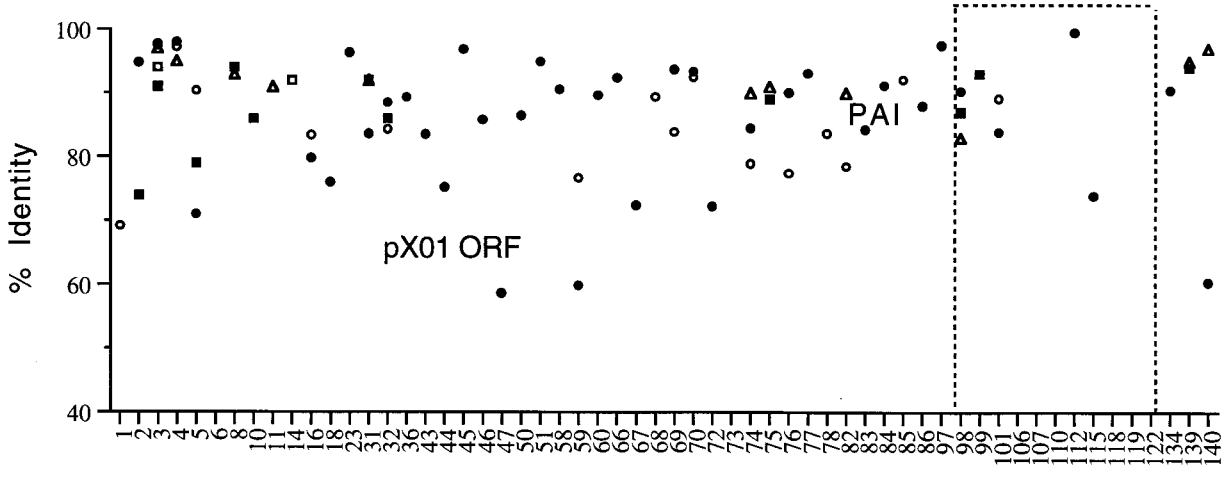

FIG. 2. Sequence similarity of DNA amplified by PCR from *B. cereus* ATCC 43881 (solid circles), *B. thuringiensis* ATCC 33679 (open circles), *B. cereus* F1-15 (solid squares), *B. cereus* 3A (open squares), *B. cereus* D-17 (open triangles), *B. cereus* 14759 (solid triangles), and *B. subtilis* 6051 (open diamonds) to *B. anthracis* pXO1 ORF sequences. The *y* axis is the percent similarity of individual PCR products amplified from five different isolates. PCR amplicons were single pass sequenced from the PCR primer sites as described in Materials and Methods.

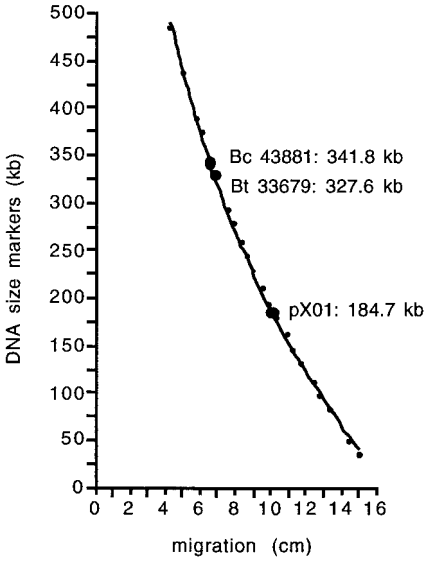

FIG. 3. PFGE of *B. anthracis* Sterne, *B. cereus* ATCC 43881 (Bc 43881), and *B. thuringiensis* ATCC 33679 (Bt 33679) followed by hybridization with a pXO1-derived probe identified large extrachromosomal elements in all three species. A graphic determination of DNA fragment sizes based on a calibrated ethidium bromide-stained gel is shown.

For example, of 65 amplification products obtained by PCR from *B. cereus* ATCC 43881 DNA, only 35 were also detected in the hybridization assay. The use of a combination of methods for detecting similar sequences allowed a greater estimation of sequence conservation between pXO1 and other bacterial species than either method used alone. The total numbers of conserved sequences found by both methods were 72, 54, 53, 8, and 16 for *B. cereus* ATCC 43881, *B. cereus* D-17, *B. thuringiensis* ATCC 33679, *B. cereus* 14579, and *B. subtilis* 6051, respectively.

Conservation of ORF order. PCR assays to test for conservation of ORF order were performed using *B. cereus* ATCC 43881. Primer sets that bridged intergenic spaces and that included multiple ORFs were chosen. Conserved order was found using pXO1 primer sets that spanned ORF-2 to -5, -43 to -47, -72 to -75, -82 and -83, -97 and -98, and -134 and -135 (Fig. 2). DNA sequencing of amplicon ends was used to confirm identity.

Cellular location of pXO1-like sequences in other species. The identification of many conserved pXO1 sequences in other bacterial species, particularly in closely related *Bacillus* species, raised the question of whether the conserved sequences were on a plasmid or chromosome. Total genomic DNA from *B. cereus* ATCC 43881 and *B. thuringiensis* ATCC 33679 DNA was analyzed by PFGE followed by hybridization with a mixed-ORF pXO1 probe. Ethidium bromide staining of pulsed-field gels detected large extrachromosomal DNA molecules in both strains (data not shown). These molecules hybridized with the pXO1 probe mixture and were estimated to be 341.8 kb for *B. cereus* ATCC 43881 and 327.6 kb for *B. thuringiensis* ATCC 33679 (Fig. 3). The pXO1 plasmid was determined to be 184.7 kb in the same experiments. This is within 2% of its size as determined by complete sequencing and within 0.2 kb of the gel-based size determination reported by Robertson et al. (30).

Potential pXO1-specific ORFs. Twenty-one of the pXO1 ORF sequences included in this study were not detected by the hybridization or PCR assays in any of the strains examined. These ORF sequences can be broadly grouped by known function and plasmid location: the known virulence factors (*lef*, *pagA*, and *cya*), other ORFs in the PAI (pXO1 ORF-106, -108, -114, -116, -117, -125, and -126), and ORFs outside the PAI (pXO1 ORF-12, -24, -29, -33, -38, -90, -91, -131, -133, -136, and -137).

DISCUSSION

Among the set of bacterial species examined, the members of the *B. cereus*/*B. thuringiensis* group exhibited more-extensive pXO1 ORF sequence conservation than bacteria from other genera. While the functions of most of these ORFs remain unknown, this result suggests that the pXO1 sequence is associated primarily with certain *Bacillus* species that are closely related to *B. anthracis*. Therefore, these ORFs may be important to the biology of these species and warrant further study. Within the *B. cereus*/*B. thuringiensis* group, the conservation of pXO1 genes did not correlate with phylogeny based on chromosomal analysis using AFLP (33). Two isolates from the relatively distant AFLP group 2, *B. cereus* ATCC 43881 and *B. thuringiensis* ATCC 33679, were found to have extensive sequence similarity to pXO1 ORFs. In contrast, only one of the eight AFLP group 1 isolates, *B. cereus* D-17, was found to contain sequences similar to those of several of the pXO1 ORFs. Seven of the eight *B. cereus* and *B. thuringiensis* isolates that were most closely related to *B. anthracis* by AFLP analysis had relatively little sequence similarity with the pXO1 ORFs examined. Conservation of pXO1 genes in isolates of *B. cereus* and *B. thuringiensis* appears to be independent of evolutionary relatedness based on chromosomal analysis.

The last observation is consistent with the idea that the large number of pXO1-like genes in *B. cereus* ATCC 43881 and *B. thuringiensis* ATCC 33679 is most likely due to the presence of plasmids with features common to pXO1. PFGE results for *B. cereus* ATCC 43881 and *B. thuringiensis* ATCC 33679 suggest that at least some of the sequences similar to pXO1 may be on large plasmids. The DNA bands from *B. cereus* ATCC 43881 and *B. thuringiensis* ATCC 33679 that hybridized with a pXO1 probe are approximately 150 kb larger than the pXO1 plasmid, suggesting megaplasmids of about 330 kb.

None of the bacterial isolates included in this study contained all of the pXO1 ORFs. This indicates conservation of discrete regions rather than the entire pXO1 plasmid among the examined species. The pXO1 sequence contains multiple sites resembling insertion elements, transposons, and integration sites (26). Such sites are associated with DNA rearrangements that result in deletions or insertions (38). The presence of such sites in pXO1 supports the idea that small fragments, rather than the entire plasmid, are transferred and shared among related species. DNA elements introduced into a cell can be unstable due to selection pressures within the host cell. Even if the introduced genes are advantageous to the cell, they may not always be maintained (6). For example, PAIs can be deleted with a high frequency whether on plasmids or chromosomes (38).

Three of the pXO1 ORFs were broadly conserved among all

the bacteria examined. Two of them have no significant DNA similarity to sequences in the current databases, and although they appear widespread in this bacterial collection, their functions remain unknown. There was a limited indication of protein domain similarity (31% similarity over 144 residues; ProDom WU-BLASTP, http://protein.toulouse.inra.fr/prodom) between pXO1 ORF-45 and cell division gene *ftsZ* (23, 26). pXO1 ORF-42, located upstream from ORF-45 in the pXO1 sequence, has limited amino acid similarity (33% similarity over 97 residues; ProDom WU-BLASTP) to the *ftsK* gene and was detected in *B. cereus* D-17, *B. cereus* ATCC 43881, and *B. thuringiensis* ATCC 33679. It is possible that these ORFs may have a role in cell division, but this remains untested. Plasmid-borne cell division genes are not necessary for growth in culture because *B. anthracis* is viable without pXO1, but they could be important under specific environmental conditions such as those in host macrophages.

Twenty-one ORFs from the pXO1 PAI were represented in this study, and 11 of those sequences were detected among the bacteria tested. Three of the pXO1 PAI genes for which a similar sequence was detected in related bacteria have been experimentally characterized as involved in *B. anthracis* pathogenesis: *gerXC* (ORF-112), *gerXA* (ORF-113), and *atxA* (ORF-119). Two other detected PAI ORFs produced strong database matches. pXO1 ORF-101 is nearly identical (blastn E value, 0.0 [2]) to the *B. cereus* enterotoxin gene, *bceT* (1), and pXO1 ORF-115 encodes protein domains resembling those of a transposon-derived resolvase. The sequences of the remaining six ORFs are not similar to current database sequences. In addition to *B. anthracis*, several isolates in the *B. cereus*/*B. thuringiensis* group are known to be pathogens. Many *B. cereus* isolates cause food poisoning and *B. thuringiensis* isolates are well-known insect pathogens. The presence of pXO1 PAI ORF fragments in other related bacteria raises questions of conservation of functions involved in pathogenesis.

Approximately 20% of the tested ORFs were not detected in any of the species examined. While these negative results must be interpreted cautiously, it is possible that some of these sequences are unique to *B. anthracis*. This group of apparently unique ORFs includes the three known anthrax toxin genes (*pagA*, *lef*, and *cya*), six additional ORFs in the PAI, and 10 ORFs from the remainder of the plasmid. In addition to the three virulence factors, only pXO1 ORF-114 has been experimentally defined (*gerXB*) (13, 31) and only pXO1 ORF-116 has a putative function assigned (26). ORF sequences that are potentially unique to *B. anthracis* are candidates for more-focused analysis of their possible roles in the manifestation of anthrax disease.

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