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A New Minimal Replicon of *Bacillus anthracis* Plasmid pXO1▽

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An 8,883-bp mini-pXO1 plasmid containing a replicon from *Bacillus anthracis* pXO1 (181.6 kb) was identified by making large deletions in the original plasmid using a newly developed Cre-*loxP* system. Portions of the truncated mini-pXO1 were cloned into an *Escherichia coli* vector unable to replicate in *B. anthracis*. A 5.95-kb region encompassing three putative genes was identified as the minimal pXO1 fragment required for replication of the resulting recombinant shuttle plasmid (named pMR) in *B. anthracis*. Deletion analysis showed that the only genes essential for replication were the pXO1-14 and pXO1-16 genes, which are transcribed in opposite directions and encode predicted proteins of 66.5 and 67.1 kDa, respectively. The ORF14 protein contains a helix-turn-helix motif, while the ORF16 upstream region contains attributes of a theta-replicating plasmid origin of replication (Ori), namely, an exclusively A+T-containing segment, five 9-bp direct repeats, an inverted repeat, and a σ^A-dependent promoter for the putative replication initiator Rep protein (ORF16). Spontaneous mutations generated in the ORF14, ORF16, and Ori regions of pMR during PCR amplification produced a temperature-sensitive plasmid that is unable to replicate in *B. anthracis* at 37°C. The efficacy of transformation of plasmid-free *B. anthracis* Ames and Sterne strains by the original pMR was ~10^3 CFU/μg, while *Bacillus cereus* strains 569 and ATCC 10987 were transformed with efficiencies of 10^4 and 10^2 CFU/μg, respectively. Around 95% of *B. anthracis* cells retained pMR after one round of sporulation and germination.

Bacillus anthracis, the etiological agent of anthrax, is a gram-positive, rod-shaped, spore-forming bacterium. A key feature distinguishing *B. anthracis* from its nearest neighbors is the presence of two large plasmids that encode key virulence factors. Plasmid pXO2 encodes enzymes that synthesize the poly-γ-D-glutamic acid capsule, whereas pXO1 encodes the anthrax toxins, consisting of protective antigen, lethal factor, and edema factor (10, 15). The complete sequence of pXO1 was first determined by Okinaka et al. (12). However, the sequence was not found to contain regions having homology to any of the *rep* genes associated with origins of replication in large plasmids of gram-positive organisms. Subsequently, two groups sought to characterize the structure of the pXO1 replicon. Robertson et al. (19) cloned pXO1 DNA fragments into an *E. coli* plasmid and obtained shuttle plasmids that could then replicate in *Bacillus* species. This strategy produced several clones containing an 11-kb region between coordinates 86249 and 97209 of the pXO1 sequence (12). This region contains nine open reading frames (ORFs) (ORF72 to ORF80 in the annotation scheme used; GenBank accession no. NC_001496), but the predicted products were again not recognizable as Rep proteins associated with theta-replicating plasmids. (The numbering of nucleotides and ORFs as annotated by Okinaka et al. [12] will be used throughout this report.)

In a more recent effort to explain pXO1 replication, Tinsley and Khan cloned a 5-kb region from *B. anthracis* Sterne plasmid pXO1 into an *E. coli* vector and showed that this plasmid could replicate when introduced into *B. anthracis* (22). This fragment included ORF43 to ORF46 rather than those previously implicated in replication (19). Tinsley and Khan demonstrated that one particular coding sequence, renamed *repX* (ORF45), was required for the initiation of replication. They also identified a 158-bp region (bp 55726 to 55883) that appeared to be required for the initiation of replication. This putative origin is contained within the highly conserved core replication region identified in all pXO1-like plasmids of the *Bacillus cereus* group (17). In this region, *B. anthracis* Sterne pXO1 contains a perfect 24-bp palindrome (22). However, in the several other pXO1 plasmid sequences determined up to now, as well as in the *B. cereus* pXO1-like plasmids, the palindrome is imperfect, having a 2-bp mismatch. The effect, if any, of this difference on plasmid replication is unclear (17). Anand et al. (2) showed that the RepX protein can undergo GTP-dependent dynamic polymerization and found that RepX binds to DNA in a GTP-dependent manner, albeit weakly and nonspecifically. RepX shares homology at its amino terminus with the bacterial cell division protein FtsZ. Both the FtsZ and RepX proteins belong to the tubulin family, members of which act as cytoskeletal proteins involved in cell division and DNA segregation in prokaryotic and eukaryotic cells (2). Later the same group showed that RepX forms polymers in vivo in *B. anthracis* (1). On the basis of these data they speculated that RepX may be a hybrid protein capable of participating in both plasmid replication and segregation. It was also suggested that the megaplasmids found in members of the *B. cereus* group of organisms may have a conserved mechanism which utilizes members of the FtsZ/tubulin family of proteins for plasmid maintenance (1).

We recently described a method for mutating or deleting large DNA segments in *B. anthracis* (15). A plasmid containing two fragments homologous to the target sequence flanking a spectinomycin resistance cassette that is in turn flanked by

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bacteriophage P1 *loxP* sites is inserted by a single crossover event. After selection with spectinomycin and then identification of a subsequent double-crossover event, temporary expression of Cre recombinase excises the spectinomycin marker, leaving a single *loxP* site within the targeted gene or genomic segment. The procedure could then be repeated to inactivate other genes, but in each case a *loxP* sequence remained. An advantage of this method is that the 34-bp *loxP* sequence does not necessarily produce polar effects when introduced into a gene or operon. For example, insertion of the *loxP*-Ω-*sp*-*loxP* cassette into pXO2 between the promoter and the *capB* start codon prevented *capBCAD* operon expression and capsule synthesis because the Ω-*sp* cassette contains transcriptional terminators. However, Cre-mediated excision of the Ω-*sp* cassette restored capsule production even though a single *loxP* site remained between the promoter site and the *capB* start codon (15). In the course of that work, we found that large segments (of at least 30 kb) could be deleted from chromosomal or pXO2 DNA (15). Here we describe a modified Cre-*loxP* system that does not depend on a low-frequency double-crossover event for insertion of the spectinomycin resistance cassette into a selected region of the *B. anthracis* genome. Instead, in the new approach, we used two sequential single-crossover events to introduce *loxP* sites flanking the DNA region targeted for deletion. Cre recombinase action at the *loxP* sites excised the intervening DNA, leaving a single *loxP* site within the targeted gene or selected genomic segment. This strategy allows deletion of DNA fragments from any genome (or plasmid), thereby facilitating functional analysis of specific genes or genome regions. Using this approach, we identified and characterized a new pXO1 minimal replicon different from those reported previously (19, 22).

MATERIALS AND METHODS

Bacterial growth conditions and phenotypic characterization. *E. coli* strains were grown in Luria-Bertani (LB) broth and used as hosts for cloning. LB agar was used for selection of transformants (20). *B. anthracis* as well as *B. cereus* strains were also grown in LB medium. Antibiotics (Sigma-Aldrich) were added to the medium when appropriate to give the following final concentrations: ampicillin (Ap), 100 μg/ml (only for *E. coli*); erythromycin (Em), 400 μg/ml for *E. coli* and 5 μg/ml for *B. anthracis*; and spectinomycin (Sp), 150 μg/ml for both *E. coli* and *B. anthracis*. SOC medium (Quality Biologicals) was used for outgrowth of transformation mixtures prior to plating on selective medium to isolate transformants. *B. anthracis* spores were prepared by growth on NBY-Mn agar (nutrient broth, 8 g/liter; yeast extract, 3 g/liter; $MnSO_4 \cdot H_2O$, 25 mg/liter; agar, 15 g/liter) at 30°C for 5 days (21). Spores and vegetative cells of *B. anthracis* were visualized with a Nikon Eclipse E600W light microscope.

DNA isolation and manipulation. Preparation of plasmid DNA from *E. coli*, transformation of *E. coli*, and recombinant DNA techniques were carried out by standard procedures (20). *E. coli* XL2-Blue and SCS110 competent cells were purchased from Stratagene and *E. coli* TOP10 competent cells from Invitrogen. Recombinant plasmid construction was carried out in *E. coli* XL2-Blue or TOP10. Plasmid DNA from *B. anthracis* was isolated according to the protocol for the purification of plasmid DNA from *Bacillus subtilis* (Qiagen). Chromosomal DNA from *B. anthracis* was isolated with the Wizard genomic purification kit (Promega) in accordance with the protocol for isolation of genomic DNA from gram-positive bacteria. *B. anthracis* was electroporated with unmethylated plasmid DNA isolated from *E. coli* SCS110 (Dam^- Dcm^-). Electroporation-competent *B. anthracis* cells were prepared and transformed as previously described (13) with slight modifications. *B. anthracis* cells grown in 25 ml of LB broth to an optical density at 600 nm of 0.15 to 0.20 were washed three times with 1 ml of ice-cold electroporation buffer (10% sucrose, 15% glycerol, 2 mM phosphate buffer, pH 8.4), resuspended in 200 μl electroporation buffer, mixed with 0.1 to 1.0 μg of plasmid in an ice-chilled electroporation cuvette (0.2 cm), and electroporated using the Bio-Rad Gene Pulser II (voltage, 1.77 kV; capacitance, 25 μF; resistance, 200 Ω). Bacteria were recovered from the cuvette with 1 ml of SOC medium, shaken at 225 rpm and 30 or 37°C for 1 h, and spread on plates with the appropriate antibiotic. Depending on the plasmid and the temperature of incubation used, the plates were kept for 1 to 2 days before further analysis. The same procedure was used for *B. cereus* electroporation except that the voltage used was 1.35 kV.

Restriction enzymes, T4 ligase, T4 DNA polymerase, and alkaline phosphatase were purchased from MBI Fermentas or New England Biolabs. *Taq* polymerase and Platinum PCR SuperMix High Fidelity kits were purchased from Invitrogen. The TOPO TA cloning kit (Invitrogen) and pGEM-T Easy Vector system (Promega) were used for PCR fragment cloning. Ready-To-Go PCR Beads (Amersham Biosciences) were used for DNA rearrangement analysis. For routine PCR analysis, a single colony was suspended in 200 μl of TE buffer (20) (pH 8.0), heated to 95°C for 45 s, and then cooled to room temperature. Cellular debris was removed by centrifugation at 15,000 × *g* for 10 min. Two microliters of the lysate contained sufficient template to support a PCR with the PCR beads. The GeneRuler DNA Ladder Mix (MBI Fermentas) was used for determination of DNA fragment length. All constructs were verified by DNA sequencing and/or restriction enzyme digestion. The oligonucleotides, plasmids, and strains used in this study are listed in Tables 1 to 3, respectively.

Construction of vectors for deletional analysis. The general scheme for producing *B. anthracis* pXO1 deletions using the new Cre-*loxP* system, presented in Fig. 1, employs plasmids we designate generically as pSC, for *s*ingle-*c*rossover plasmid. These were derived from the highly temperature-sensitive plasmid pHY304, which has permissive and restrictive temperatures of 30°C and 37°C, respectively. To produce pSC we cut pHY304 with Ecl136II/BstZ17I and ligated this with a BstZ17I-restricted fragment from a 181-bp DNA sequence (*d*irect *l*oxP repeat [DLR]) synthesized by BlueHeron Biotechnology, WA. The DLR sequence contains a multiple-cloning site between two directly repeated *loxP* sequences. The resulting pDR1 plasmid was cut with BstZ17I and ligated with a pUC18 PvuII fragment, producing the shuttle plasmid pSC (Fig. 1, step I). All the endonuclease restriction sites indicated on the pSC map between two direct *loxP* sequences are unique restriction sites. The plasmid pCrePA, previously used for expression of Cre recombinase, was modified by replacing the Em^r gene with the Sp^r gene from pJRS312, to create pCrePAS. For this purpose, pCrePA was cut with ScaI/FspI and ligated with the FspI/MslI fragment of pJRS312. The resulting pCrePAS plasmid, shown in Fig. 1, step II, has permissive and restrictive temperatures of 30°C and 37°C, respectively. Both pSC and pCrePAS were used in accordance with the schemes presented in Fig. 1 and Fig. 2.

Plasmid construction for the minimal pXO1 replicon search and analysis. Fragments of 5,950 and 5,110 bp were amplified from plasmid Δ_2 using Platinum PCR SuperMix High Fidelity kit (Invitrogen) and the MRF/MRR and MRF1/MRR1 primer pairs, respectively. The PCR products had 3′ adenines added in accordance with the TOPO TA cloning protocol (Invitrogen) and were cloned into pCR2.1-TOPO, producing the TOPO-MR and TOPO-MR1 plasmids. These were cut with EcoRI and ligated into the pΩL plasmid at its EcoRI site, producing plasmids pMR and pMR1. Plasmid pMR was obtained as variants having both orientations of the EcoRI fragment containing pXO1 ORF14 to ORF16. Both variants were used for deletional analysis (see Fig. 4). From the variants having the first orientation, the NruI/PvuI fragment extending into ORF14 was deleted, producing pMRΔ14. The other variant had the Ecl136II/PvuI fragment that included all sequences beyond ORF16 deleted, producing pMR16Δ. Deletion of the C-terminal region of ORF16 from the Ecl136II to the PvuII site produced pMRΔ16. To produce pMRΔ15, we cut pMR with XcmI and treated it with T4 DNA polymerase according to the New England Biolabs protocol for blunting ends by 3′ overhang removal. However, the pMRΔ15 obtained had an unexpected larger deletion of ORF15 and flanking regions (as detailed in Table 2). To produce pMRMut, we repeated the entire cloning scheme used for pMR with the exception that regular *Taq* polymerase was used instead of the Platinum PCR SuperMix High Fidelity polymerase. Point mutations introduced during this PCR amplification to produce pMRMut were subsequently identified by complete sequencing of the pXO1 fragment with the same primers used for Δ_2 plasmid sequencing (see Fig. 4).

Labeling of pXO1 with a spectinomycin resistance gene. To allow convenient assessment of the stability of pXO1, a spectinomycin resistance cassette was inserted downstream of the *pag* gene by a double-crossover event. A 2,265-bp region located immediately downstream of *pag* (protective antigen gene) was amplified with the SPF/SPR primers and cloned into pGEM-T Easy, producing pGEM1. The 2.3-kb BamHI fragment from pΩL containing the Ω-sp cassette was inserted into the unique BamHI site of the amplified *B. anthracis* sequence in pGEM1, producing pGEM1Ω. The EcoRI fragment containing the pXO1 fragment with the internal Ω-sp from pGEM1Ω was then inserted into the EcoRI site of pHY304, producing pHY304S. The Ω-sp from pHY304S was inserted into

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ → 3′)[a]	Relevant properties	Restriction site(s)
DLR	GGTATAC**ATAACTTCGTATAATGTATGCTATACGAAGT TAT**CAGCTGCTCGAGGTCGACGGTATCGATAAGC TTGATATCGAATTCCTGCAGCCCGGGGGATCCAC TAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCT C**ATAACTTCGTATAATGTATGCTATACGAAGTTAT**GT ATACC	Oligonucleotide used to create pSC plasmid; this sequence contains 11 restriction sites as shown in Fig. 1	BstZ17I
D1LF	ACTGCTCGAGGGTGTTTTTCGTTTGAAGAC	Primer pair to amplify fragment defining counterclockwise end of D1 deletion	XhoI
D1LR	ACTGACTAGTTACTTTTCGGCCTCTTCCTG		SpeI
D1RF	ACTGCTCGAGCAGCTTATGGTATAGCAACG	Primer pair to amplify fragment defining clockwise end of D1 deletion	XhoI
D1RR	ACTGACTAGTGTCTTGGATAACCACTATCATA		SpeI
D1FC	TACCATTAATGGCATTGTCTAC	Diagnostic primer pair to amplify internal fragment of D1 deletion	
D1RC	CAGTAGGATTTTCTCGTTGA		
D2F	ACTGCTCGAGATTTGGAGGAACAAACAATGGCTAA	Primer pair to amplify fragment defining counterclockwise end of D2 deletion	XhoI
D2R	ACTGACTAGTAAAACGTCTGGCTTTGTTTCTGGCT		SpeI
D2FC	AATATCAGAAATAGAGACGG	Diagnostic primer pair to amplify internal fragment of D2 deletion	
D2RC	AACCAAGACTAACCACAAAA		
D3F	ACTGCTCGAGTCAGGATCAAAGAAACCAGC	Primer pair to amplify fragment defining clockwise end of D3 deletion	XhoI
D3R	ACTGACTAGTGCAGAGTTCTGAAACAAAGGTATT		SpeI
D3FC	CAGTTCCTTGTGCCGTTTCA	Diagnostic primer pair to amplify internal fragment of D3 deletion	
D3RC	CGAGGGGATTCAATTAAACA		
D3R′	ACTGACTAGTTCAGGATCAAAGAAACCAGC	Primer pair to amplify fragment defining counterclockwise end of Δ_1 region	SpeI
D3F′	ACTGCTCGAGGCAGAGTTCTGAAACAAAGGTATT		XhoI
D4R′	ACTGACTAGTTTATAAAACGTACCATCCAG	Primer pair to amplify fragment defining clockwise end of Δ_1 region	SpeI
D4F′	ACTGCTCGAGGGAGACATTTAAACAACGAT		XhoI
D4RC′	ACTGACTAGTCATTTTAATTGTTCCAGGGG	Primer pair to amplify fragment defining clockwise end of Δ_2 region and diagnostic primer pair to amplify internal fragment of Δ_1 mini-pXO1	SpeI
D4FC′	ACTGCTCGAGATTGCCCGTCACTCCTTTCT		XhoI
DFC	TTTGTCGGTCTTTTGTTTAG	Diagnostic primer pair to amplify internal fragment of TK replicon	
DRC	GGCGTTTTATCATTAGCAAA		
D5R	ACTGACTAGTAACCGTGGCCAATCTAACCT	Primer pair to amplify fragment to divide Δ_2 into $\Delta_{2\text{-}3}$ and Δ_3	SpeI
D5F	ACTGCTCGAGCTGCGTTTGTTGTTTCCCCA		XhoI
D5FC	GAAATTGATACCGATCACAT	Diagnostic primer pair to amplify internal fragment of $\Delta_{2\text{-}3}$ mini-pXO1	
D5RC	GGACTTCATATAAAATGGAGTC		
D6FC	AACTGAACTATGGGTAAAGA	Diagnostic primer pair to amplify internal fragment of Δ_3 mini-pXO1	
D6RC	CCACCAATACTATATGAATGAG		
MRF	ACGACCATGTTTAATCCTCC	Primer pair to amplify MR fragment from Δ_2 mini-pXO1	
MRR	CATTGAAATACCTGCCCCTA		
MRF1	ATGAAGTCCTTTTGATTTGG	Primer pair to amplify MR1 fragment from Δ_2 mini-pXO1	
MRR1	AAATACCTGCCCCTACAAAA		
15DF	TTTCTTGGGAAAATAGGTAC	Primer pair to verify pXO1-15 deletion	
15DR	CCTCCTAATTGCTTCTAATACA		
SPF	CAACGACTTCGTTAGTGTTA	Primer pair to amplify pXO1 fragment for insertion of Ω-sp cassette	
SPR	CCCTTCAACTTTTGGATTAT		
D1CCW	CTTTGACATTAGATTTGAGG	Primer pair to identify *loxP* in junction fragment after D1 deletion	
D1CW	CTGCTTTACTAAGACCCTTA		
D2CCW	GGCGCAAGTTCTTACAAATG	Primer pair to identify *loxP* in junction fragment after D2 deletion	
D2CW	GGTTTAATTTGGGGAACATT		
D3CCW	GGGGAAAGACAGCGATAAGT	Primer pair to identify *loxP* in junction fragment after D3 deletion	
D3CW	GTTAATGGAAAGAGATGGCT		
Δ_1CW	CAAAATAAGCGGTACATATG	Primer pair to identify *loxP* in junction fragment of Δ_1 mini-pXO1	
Δ_1CCW	CCCAGCCATACTATTTCACA		
Δ_2CW	AACGCTATTGTCTCCTATGC	Primer pair to identify *loxP* in junction fragment of Δ_2 mini-pXO1	
Δ_2CCW	GGTGTTAGGGGAAGTGAGTG		

[a] Restriction enzyme recognition sites are underlined; boldface indicates two direct *loxP* sequences.

the homologous region downstream of *pag* by the double-crossover method previously described (15).

Segregational stability assay. The stabilities of plasmids pXO1S, pMR, and pMRMut in *B. anthracis* Ames 35 (pXO1S) and Ames 33 (pMR and pMRMut) were determined using methods described previously (11), with slight modifications. Bacterial strains were inoculated into LB broth with spectinomycin and incubated at 37°C. The cultures then were diluted 10^6-fold into LB broth without antibiotic and incubated overnight at 37°C. Sixteen passages in nonselective LB medium were performed at daily intervals. At each passage, aliquots were plated on agar without spectinomycin and about 100 colonies were transferred to agar containing spectinomycin to determine the percentage of cells retaining the plasmid. To determine plasmid stability during sporulation, overnight cultures from LB medium with spectinomycin were spread on NBY-Mn agar without the antibiotic and incubated for 5 days at 30°C. Cultures were suspended in water and heated at 70°C for 30 min to kill the nonsporulated cells, and the percentage of plasmid-containing spores was then determined as described above.

DNA sequencing and analysis. Plasmid DNAs were sequenced using primers listed in Table 1. All primers for sequencing were synthesized by Operon Biotechnologies, Inc., AL, or the FDA core facility, Bethesda, MD. Sequences were determined using a primer walking strategy (Macrogen). Sequence data were

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristic(s)	Source or reference
pXO1	*B. anthracis* Ames 35 plasmid	17
pGEM-T Easy	Cloning vector for PCR products; Ap^r in *E. coli*	Promega
pBS246	Contains two directly repeated *loxP* sites flanking a-multiple cloning site; Ap^r in *E. coli*; pUC18 replicon	GIBCO-BRL
pJRS312	pUC18 carrying an Ω element with spectinomycin resistance marker *aad9* (Ω-*sp*) from *Enterococcus faecalis*; Sp^r in *E. coli* and *B. anthracis*	15
pΩL	2.3-kb BamHI fragment with Ω-sp inserted between two directly repeated *loxP* sequences of pBS246; Ap^r Sp^r in *E. coli*; pUC18 replicon	15
pGEM1	2.3-kb PCR fragment (SPF/SPR primers) of *B. anthracis* cloned into pGEM-T Easy	This work
pGEM1Ω	2.3-kb BamHI fragment with Ω-sp inserted into BamHI site of *B. anthracis* fragment of pGEM1 plasmid; Ap^r Sp^r in *E. coli*	This work
pHY304	Contains Em^r gene and strongly temp-sensitive replicon for both *E. coli* and gram-positive bacteria; Em^r both in *E. coli* and *B. anthracis*	15
pHY304S	pHY304 with pXO1-fragment containing Ω-sp from pGEM1Ω inserted into EcoRI; Em^r Sp^r both in *E. coli* and *B. anthracis*	This work
pXO1S	pXO1 with Ω-sp inserted into BamHI site located 136 bp downstream of *pag* ORF	This work
pCrePA	pHY304 containing entire Cre recombinase gene under the control of *pagA* promoter	15
pCrePAS	pCrePA with Em^r replaced by Ω-sp; Sp^r both in *E. coli* and *B. anthracis*	This work
pDR1	BstZ17I fragment of DLR oligonucleotide is inserted into large BstZ17I/Ecl136II fragment of pHY304	This work
pSC	PvuII fragment of pUC18 is inserted into BstZ17I site of pDR1; Ap^r in *E. coli*; Em^r both in *E. coli* and *B. anthracis*	This work
Δ_2	Mini-pXO1 plasmid encompassing pXO1-14 to pXO1-19 ORFs	This work
pCR 2.1-TOPO	Cloning vector for PCR products; Ap^r Km^r in *E. coli*	Invitrogen
TOPO-MR	MRF/MRR PCR fragment of Δ_2 inserted into pCR 2.1-TOPO	This work
TOPO-MR1	MRF1/MRR1 PCR fragment of Δ_2 inserted into pCR 2.1-TOPO	This work
pMR	MRF/MRR EcoRI-fragment from TOPO-MR1 inserted into pΩL	This work
pMR1	MRF1/MRR1 EcoRI-fragment from TOPO-MR inserted into pΩL	This work
pMRΔ14	pMR with NruI/PvuI fragment deleted	This work
pMRΔ15	pMR with pXO1-15 ORF deleted; deletion includes ORF15 along with 185 downstream and 56 upstream bp	This work
pMRΔ16	pMR with Ecl136II/PvuII fragment deleted	This work
pMR16Δ	pMR with Ecl136II/PvuI fragment deleted	This work
pMRMut	pMR with four spontaneous point mutations indicated in Fig. 4	This work

assembled using either the Clustal module of the GCG-Lite suite of sequence analysis program (http://molbio.info.nih.gov/gcglite) or Vector NTI software (Invitrogen). The National Center for Biotechnology Information (NCBI) BLAST and FASTA programs (http://www.ncbi.nlm.nih.gov) were used for homology searches in GenBank and nonredundant protein sequence databases. Repeat Finder software (http://www.proweb.org/proweb/Tools/selfblast.html) was used to locate direct and inverted repeat (IRs). Protein motifs were identified using SignalP software version 3.0 (http://www.cbs.dtu.dk/services/SignalP) for signal sequences, TMpred software for prediction of transmembrane regions and orientation, and NPS@ software (http://npsa-pbil.ibcp.fr/cgi-bin/primanal_hth.pl) for helix-turn-helix prediction.

TABLE 3. Bacterial strains used in this study

Species and strain	Relevant characteristics	Reference
B. anthracis		
Ames 35	Ames $pXO1^+$ $pXO2^-$ strain	15
Ames 35S	Ames 35 with pXO1S plasmid	This work
Ames 35D1	Ames 35 with pXO1D1 plasmid (D1 region is deleted)	This work
Ames 35D2	Ames 35 with pXO1D2 plasmid (D2 region is deleted)	This work
Ames 35D3	Ames 35 with pXO1D3 plasmid (D3 region is deleted)	This work
Ames 35Δ_1	Ames 35 with Δ_1 mini-pXO1 plasmid	This work
Ames 35Δ_2	Ames 35 with Δ_2 mini-pXO1 plasmid	This work
Ames 33	Ames $pXO1^-$ $pXO2^-$ strain	15
Sterne 34F2	Sterne $pXO1^+$ $pXO2^-$ strain	14
SdT	Sterne $pXO1^-$ $pXO2^-$ strain	14
B. cereus		
569	Wild-type strain	14
ATCC 10987	Wild-type strain	16

RESULTS

Cre-*loxP*-generated deletions identify a mini-pXO1 replicon. Although two regions of pXO1 have been implicated in its replication, the mechanism(s) of their action is not understood. To identify regions that are either necessary or sufficient for pXO1 maintenance, we modified a Cre recombinase-based system (15) that is able to generate large deletions in pXO1 (Fig. 1). Unlike the prior method, this one does not require or involve low-frequency double-crossover events. The scheme is illustrated in Fig. 1, where, for illustration of the method, the Δ_1 region is defined as containing an independent replicon. The scheme begins by insertion of a single *loxP* site into the target region using two consequent steps: step I, insertion as a single crossover of a plasmid ($pSCL_1$) having the (left) L_1 region of homology bracketed by *loxP* sites, followed by, step II, excision of the pSC vector backbone from the target by means of Cre recombinase treatment along with elimination of the plasmid by growth at a restrictive temperature (Fig. 1). This leaves a single *loxP* site flanked by duplicate copies of the L_1 sequence. The process continues with a second single-crossover event (step III) that inserts two additional *loxP* sites flanking pSC in a manner similar to step I. Cre recombinase treatment of that transformant excises sequences between the three

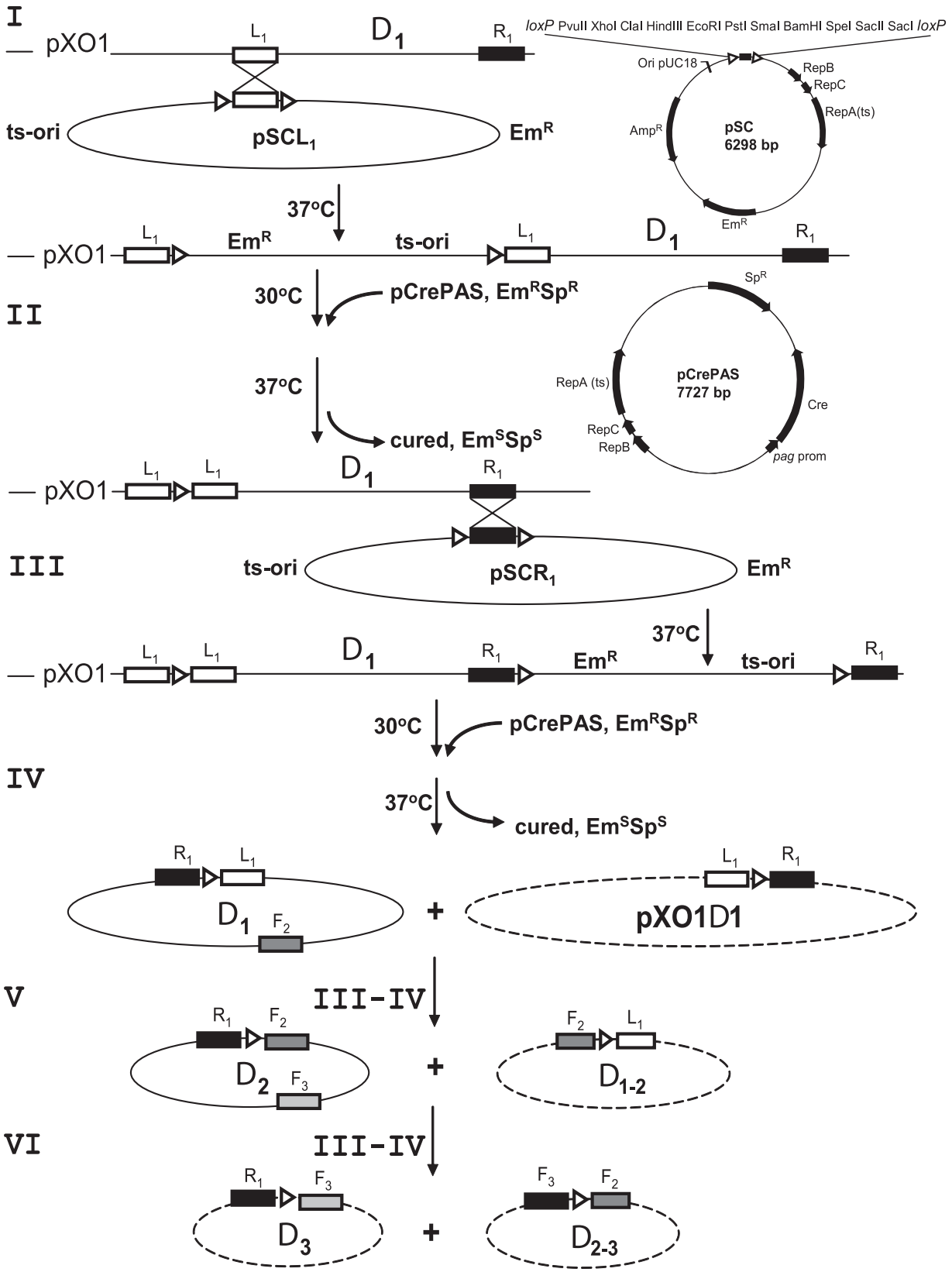

FIG. 1. Cre-*loxP* system used for locating pXO1 minireplicons. (I) Region of plasmid pXO1 containing a putative plasmid replicon $\Delta_1$flanked by the sequences L_1 and R_1 on the left and right, respectively. The L_1 and R_1 fragments are separately cloned into a temperature-sensitive (ts) plasmid, pSC, between *loxP* sites. The plasmid containing L_1 ($pSCL_1$) is transformed into *B. anthracis*, which is then grown at the restrictive temperature. The single-crossover insertion event is selected by the Em^r phenotype. (II) Removal of the Em^r along with the pSC backbone from

loxP sites to produce several possible circular DNAs, most simply those designated Δ_1 and pXO1Δ1 (Fig. 1). In the present case, where Δ_1 is suggested to be the only replicon present in pXO1, the minireplicon plasmid Δ_1 survives and the larger pXO1Δ1does not. Repetition of steps III and IV performed on the Δ_1 plasmid using pSC containing fragments F_2 andF_3 could further reduce its size, until a Cre recombinase treatment removed an essential part of the replicon, in which case no plasmids would survive; this is the case illustrated in step VI (Fig. 1).

Results from application of this strategy to pXO1 are shown in Fig. 2. At first, to validate the new Cre-*loxP* strategy, we eliminated the region described as a pathogenicity island (PAI). The PAI region is a 44.8-kb region defined by terminal inverted IS*1627* elements and containing ORF97 to ORF126 (12). In addition to the toxin genes, the PAI contains a set of regulatory elements that control the expression of the toxin genes, a spore germination response operon, and 19 other ORFs, including several transposition-related elements (23). This PAI is inverted in some strains (21). To eliminate the PAI, we inserted a single *loxP* site into the PAI's counterclockwise (or left) end IS*1627* element using an L_1 fragment corresponding to bp 117241 to 117872. The insertion of the *loxP* sites at the clockwise end of the PAI using a fragment homologous to bp 160861 to 161664 was followed by Cre recombinase treatment to generate the D1 deletion, which was confirmed by PCR analysis (Fig. 2A and B, left panel). The independent (i.e., not sequential) D2 deletion used again the *loxP* site introduced at the counterclockwise end of the PAI and a fragment amplified with primers D2F/D2R, corresponding to bp 67668 to 68049. This deletion was intended in part to test the requirement for the replicon reported to be within bp 86249 to 97209 (R replicon in Fig. 2A) (19). The resulting pXO1 deletant lacking (only) the D2 region (as confirmed by PCR analysis) was retained (Fig. 2A and B, left panel). Deletions were then continued extending clockwise from the PAI. The pSC plasmid having a fragment of DNA homologous to the clockwise end of the PAI that was used above to delete the PAI was now used to insert a single *loxP* site. PCR with primers D3F/D3R generated a fragment homologous to bp 18001 to 18672 that was used to insert *loxP* sites which, after Cre treatment, defined the clockwise end of the D3 deletion (Fig. 2A and B, left panel). Again, deletion of (only) D3 did not prevent pXO1 replication.

None of the three large deletions described above prevented retention of the resulting pXO1 variant plasmids by *B. anthracis* cells, indicating that other regions contained one or more functional replicons. Taking into account the location of the putative TK replicon at bp 54863 to 60166 (22), we designed a fourth independent deletion (provisionally designated D4) that began with insertion of a single *loxP* site using a fragment homologous to bp 18001 to 18672 that was produced with primers D3F′/D3R′. This was followed by insertion of two *loxP* sites using a fragment homologous to bp 54639 to 55390 that was produced with primers D4F′/D4R′. Expression of Cre recombinase in bacteria having the resulting three *loxP* sites did not cause deletion and loss of the targeted (D4) region but instead led to its independent replication. This DNA fragment (bp 18001 to 55390) replicated independently, and the remaining larger portion of pXO1 encompassing bp 54639 to 181677 and continuing through bp 1 to 18672 bp was lost. This result defines a new minireplicon of pXO1 and also calls into question the essentiality of the putative TK replicon. In accordance with the scheme in Fig. 1, we designated the replicative pXO1-fragment Δ_1 (Fig. 2A and B, left panel).

To further define the replicon within Δ_1, we inserted additional *loxP* sites at bp 25803 and 26801 bp (primers D4FC′/D4RC′ were used for the fragment amplification). The PCR analysis performed following Cre treatment revealed a shortened self-replicating part of pXO1 that we designated Δ_2 (Fig. 2). The Δ_2 plasmid contains ORF13 to ORF19 of pXO1 (Fig. 3, middle image). To confirm all of the deletions described above, we synthesized clockwise- and counterclockwise-facing primers (Table 1) to amplify fragments spanning the junction sites of the deletions. The fragments obtained were of the expected size, as seen in Fig. 2B, right panel. All these junction fragments were sequenced and found to have the predicted sequences flanking a single *loxP* site. The *loxP* site, primer locations, and sequence of the junction in the Δ_2 plasmid are shown in Fig. 2C.

A further attempt to truncate the 8883-bp Δ_2 plasmid using the Cre-*loxP* system did not yield smaller replicative plasmids. Genetic analysis of Δ_2 plasmid ORFs indicated that the final recombination event (Fig. 3, top two images, corresponding to step VI in Fig. 1) separated ORF14 and ORF15 (in plasmid Δ_3) from ORF16 to ORF19 (plasmid $\Delta_{2\text{-}3}$). This separation was fatal for both Δ_3 and $\Delta_{2\text{-}3}$. This information and the manageable size of the replicon defined to this point allowed the use of alternative strategies to identify the regions essential for replication of the Δ_2 plasmid.

Cloning of a 5,950-bp region containing the pXO1 minireplicon. Based on indications that ORF14 to ORF16 might constitute the replicon, the region defined by the MRF/MRR primer pair was amplified and cloned into pΩL via pCR 2.1-TOPO cloning according to the scheme presented in Fig. 3, bottom. The resulting plasmid, pMR, equally transformed both *B. anthracis* Ames 33 and SdT strains. The efficacy of transformation was around 10^3 CFU per μg of the plasmid DNA. No

the chromosome is achieved by Cre-mediated recombination (excision) after transforming the strain with pCrePAS at 30°C. Growth at 37°C eliminates both the Cre recombinase-producing plasmid pCrePAS and pSC. The result is insertion of a single *loxP* site between two L_1 fragments. (III) The plasmid containing R_1 (pSCR_1) is transformed into *B. anthracis*, which is then grown at the restrictive temperature. The second single-crossover event is selected by the Em^r phenotype. (IV) Cre recombinase action at the three *loxP* sites then creates three (or potentially more, if Cre action is incomplete) circular DNAs: (i) the Δ_1 fragment, (ii) the large deleted pXO1Δ1, and (iii) the pSC backbone (not pictured). Growth at 37°C eliminates ts plasmids pSC and pCrePAS as well as pXO1Δ1, which cannot replicate owing to lose of the replicon. (Dashed lines denote nonreplicative DNAs.) Only the Δ_1 plasmid carrying the pXO1 replicon is maintained in *B. anthracis*. (V) To reduce the size of the mini-pXO1 plasmid Δ_1, the same operations (III and IV) are repeated sequentially with internal sequences F_2, F_3, etc. The result can be a shortened mini-pXO1 plasmid, Δ_2. (VI) At some point, the iterative process results in the separation of essential elements and failure to replicate.

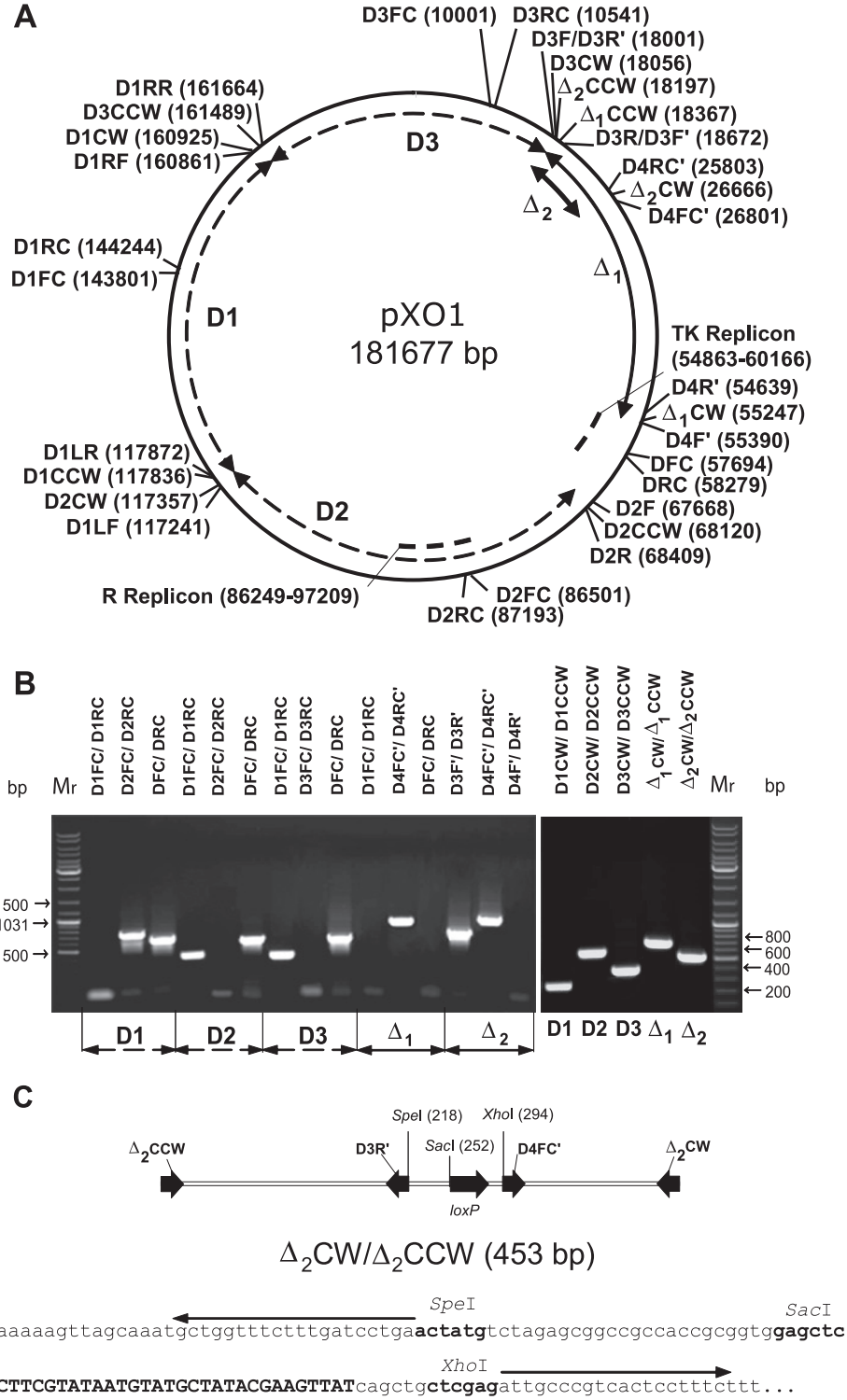

FIG. 2. Creation and analysis of deletions generated in pXO1. (A) Locations of oligonucleotide primers and of large deletions created by the scheme described in Fig. 1. As an example, the D1LF/D1LR primer pair was used for PCR amplification of the left (counterclockwise)-end homologous fragment used to introduce a *loxP* site at the counterclockwise end of deletion D1 (F and R denote forward and reverse), and the D1RF/D1RR primer pair was used for PCR amplification of the D1 right (clockwise)-end fragment. Similarly, the D3F′/D3R′ primer pair along with the D4F′/D4R′ pair amplified fragments that were used to generate the first mini-pXO1 plasmid, Δ_1 (the symbol ′ denotes that the *loxP* sequences introduced were facing in the counterclockwise direction, as opposed to the clockwise *loxP* sites used for all other deletions in this figure). The TK and R replicons as well as D1, D2, and D3 pXO1 fragments were unable to replicate as circular DNAs or maintain deleted pXO1 variants in the cells of *B. anthracis* (dashed lines). Both Δ_1 and Δ_2 mini-pXO1 plasmids were replicative as circular DNAs (solid lines). (B) The left panel shows PCR verification of the deleted pXO1 plasmids. Primers used to verify retention of specific segments are indicated at the top of the gel. The Mr lane is a GeneRuler DNA Ladder Mix for comparison (sizes of bands, from the top, are 10,000, 8,000, 6,000, 5,000, 4,000, 3,500,

transformants were expected or obtained when the parental pΩL plasmid was similarly electroporated into Ames 33 or SdT. These results showed that the 5,950-bp region at bp 19032 to 24981 contains a functional minireplicon of the pXO1 plasmid. A parallel cloning of the shorter region defined by primer pair MRF1/MRR1 was done, and no replicative plasmid was obtained, suggesting that the region between ORF16 and ORF17 is also essential (see also results for pMR16Δ, below). The pMR plasmid was isolated from *B. anthracis*, and the region corresponding to the MRF/MRR fragment was sequenced. The sequence was identical to that of the original pXO1 plasmid (GenBank accession no. NC_001496). The sequence was seen to contain two stem-loop structures (Fig. 4A) that are potential transcription terminators for the pXO1-15 or pXO1-16 genes (see below).

Analysis of the pXO1 minireplicon. To determine which of the ORFs and adjacent regions of the pMR plasmid are required, various truncations and deletions of the ORFs were constructed (Fig. 4A). The ORF15 deletion (in pMRΔ15) also encompassed the left stem-loop structure (bp 21717 to 21741). The right stem-loop structure (bp 24889 to 24935) was deleted along with the region downstream of ORF16 in pMR16Δ (Fig. 4A). Electroporation of the resulting plasmids into *B. anthracis* showed that both pMR and pMRΔ15 gave transformants at efficacies of $\sim 10^3$ CFU/μg, while pMR16Δ, pMRΔ14, and pMRΔ16 gave no transformants at either 30 or 37°C. We also found that the pMRMut plasmid had a drastically reduced ability to transform *B. anthracis* at 37°C, yielding small colonies at a 100-fold-lower frequency than for pMR. However, the ability of this plasmid to transform *B. anthracis* was restored at 30°C, although the colonies remained smaller than for pMR transformants (Fig. 5A). Both the pMR and pMRMut plasmids were tested for their ability to replicate in *B. cereus* strains 569 and ATCC 10987. The plasmids transformed these strains in the same temperature-sensitive manner as found for *B. anthracis* but with efficiencies that were higher for *B. cereus* 569 ($\sim 10^4$ CFU/μg) and lower for *B. cereus* ATCC 10987 ($\sim 10^2$ CFU/μg). The presence of the plasmids in the *B. cereus* transformants was confirmed by PCR analysis and sequencing (data not shown).

Identification of the putative origin of replication of plasmid pXO1. Like a majority of large plasmids, pXO1 probably replicates by the theta-type mechanism (6). In many cases, replicons of plasmids using the theta-type mechanism of replication contain an origin of replication (Ori) and an adjacent gene that codes for a replication initiator protein (Rep). Replication origins often consist of multiple *cis*-acting elements, including direct repeats (iterons), AT-rich regions, and IRs (5). Analysis of the regions adjacent to ORF14 and ORF16 showed that only the latter has features characteristic of an origin of replication: a 24-bp element with 100% AT content preceding one slightly divergent and four perfect repeats of a core 9-bp iteron sequence (TTTCCCAAG) not found elsewhere in pXO1 and an IR located between the iterons and the putative promoter of the adjacent pXO1-16 gene (Fig. 4B). It is notable that one of the mutations that singly or in combination make pMRMut temperature sensitive for replication is a single-nucleotide deletion in this region (Fig. 4A).

Comparison of the pXO1 minireplicon to known genes. BLAST analyses revealed that both the ORF14 and ORF16 amino acid sequences are well conserved among *B. cereus* group plasmids (Table 4). The ORF14 protein has 39 to 99% identity with conserved hypothetical proteins in a number of these plasmids, while the ORF16 protein is more strongly conserved among the same plasmids, having at least 64% identity. It is interesting that the ORF14 protein contains a helix-turn-helix motif at amino acids 516 to 537, in the C-terminal region of the protein.

The ORF15 protein is much less conserved. It is 100% identical to a hypothetical protein of pBCXO1 (8) but less than 40% identical to proteins of pBc239 (24) and pG9842 from *B. cereus* G9842 (NC_011775). This protein is predicted to have a signal sequence of 32 amino acids, suggesting that it may be secreted or surface associated.

Segregation stability. The segregational stabilities of the pXO1 derivative plasmids pXO1S, pMR, and pMRMut differed greatly when the host strains were grown in the absence of positive selection by spectinomycin (Fig. 5B). The pXO1S plasmid, which is simply a spectinomycin-marked, full-size pXO1, was very stable, with less than 5% plasmid loss from the *B. anthracis* Ames 35S population after 16 passages at 37°C. The pMR plasmid, which contains only the pXO1 minireplicon, was found in 80% of the *B. anthracis* Ames 33(pMR) cells after the 5th passage but disappeared by the 16th passage at 37°C. Only two passages at 37°C were required to eliminate pMRMut from the population of *B. anthracis* Ames 33(pMRMut). We also found that the pMR plasmid was retained during sporulation, since 95% ± 5% of both *B. anthracis* Ames 33(pMR) and SdT(pMR) bacteria produced by germination of spores had retained the plasmid.

DISCUSSION

In the present study, we demonstrated that a 5.95-kb fragment spanning bp 19032 to 24981 in pXO1 (12) is able to replicate independently and therefore constitutes a minireplicon. This region contains genes potentially encoding three proteins, ORF14 to ORF16, two of which, ORF14 and ORF16, are required for replication. Our sequence analyses, together with those of Okinaka et al. (12) and Rasko et al. (17), iden-

3,000, 2,500, 2,000, 1,500, 1,200, 1,031, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp). Internal primers pairs D1FC/D1RC, D2FC/D2RC, D3FC/D3RC, and D4FC′/D4RC′ were designed for the D1, D2, D3, and Δ_1 PCR verification. The D4FC′/D4RC′ PCR fragment was also used to produce shortened mini-pXO1 plasmid Δ_2. The DFC/DRC primer pair was designed to assess presence of the TK replicon region (22), while the D2FC/D2RC primer pair was used to assess the R Replicon (19). The right panel shows PCR verification of the fragments of the expected size that span the junction sites of the deletions. For example, the D1CW/D1CCW primer pair was designed to produce a junction fragment in the plasmid deleted of D1. (C) Graphic representation of the junction site for the Δ_2 plasmid (upper panel) along with the sequence indicating the *loxP* site within the Δ_2 junction site (arrows indicate D3R′ and D4F′] primers; endonuclease restriction sites are shown in bold and *loxP* in bold uppercase).

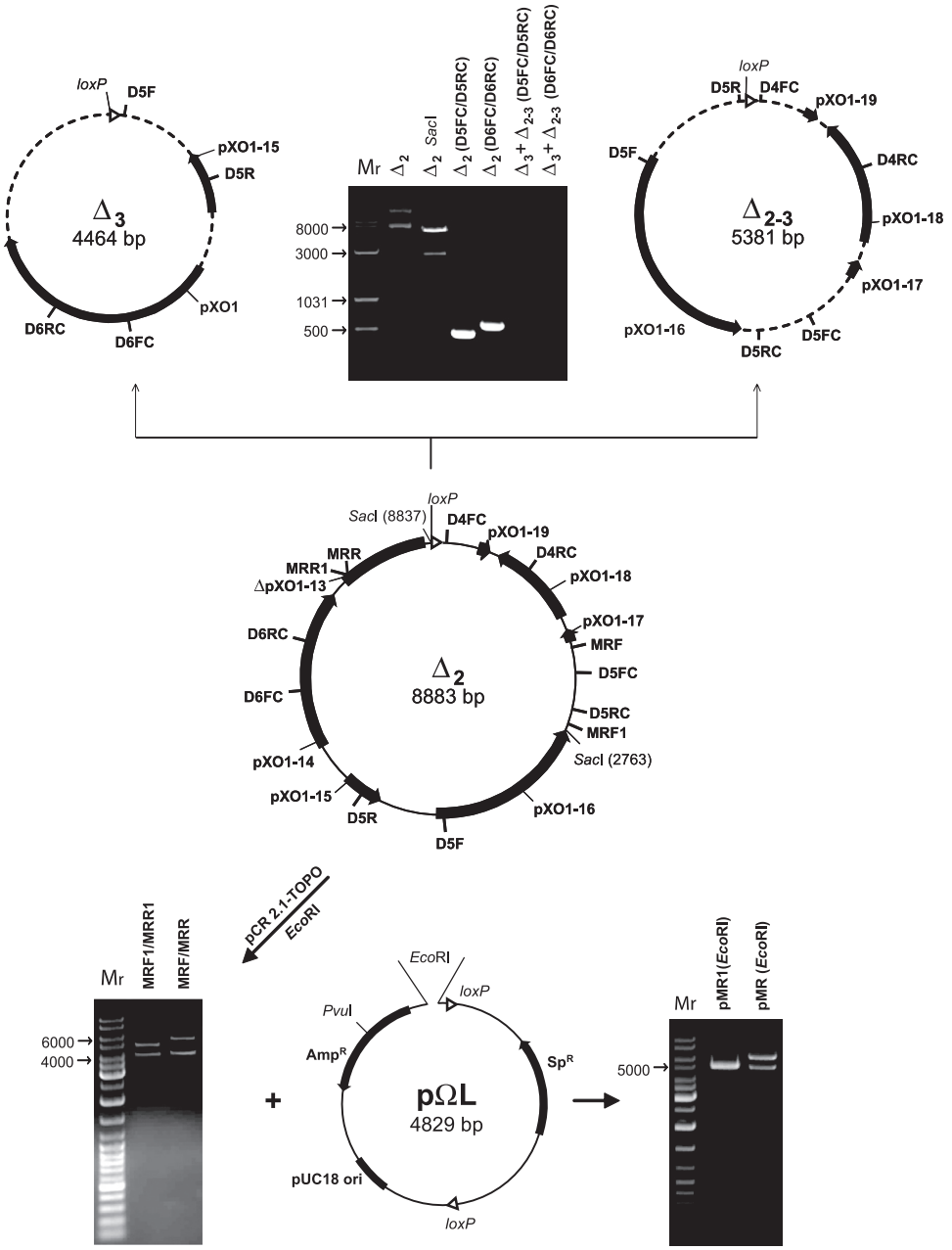

FIG. 3. Identification of the pXO1 minimal replicon. The middle panel shows the map of the mini-pXO1 plasmid Δ_2 with primer locations, diagnostic SacI restriction sites, and predicted ORFs (12). ORF13 is truncated. The D5F/D5R primer pair amplifies a fragment (corresponding to F_3 in Fig. 1, step VI) that was used with the single existing *loxP* site to divide plasmid Δ_2 into candidate plasmids Δ_3 and Δ_{2-3} (upper panel). Internal primer pairs D5FC/D5RC and D6FC/D6RC were designed to characterize the shortened plasmids. The MRF1/MRR1 and MRF/MRR primer pairs (MR is abbreviation for minireplicon; F and R indicate forward and reverse) were designed to isolate potential shorter minimal replicons from Δ_2 (shown in the lower panel). The upper panel shows electrophoretic analysis of DNAs produced by division of Δ_2. Mr, molecular mass markers. The mini-pXO1 Δ_2 plasmid was isolated from *B. anthracis* (Δ_2) and cut with SacI (Δ_2 SacI). The sizes of the SacI fragments as well as the presence of the PCR fragments Δ_2(D5FC/D5RC) and Δ_2(D6FC/D6RC) confirm the Δ_2 minireplicon map. The absence of $\Delta_3+\Delta_{2\text{-}3}$(D5FC/D5RC) and $\Delta_3+\Delta_{2\text{-}3}$(D6FC/D6RC) PCR fragments indicates that neither Δ_3 nor $\Delta_{2\text{-}3}$ could maintain itself in *B. anthracis*. Accordingly, both Δ_3 and $\Delta_{2\text{-}3}$ are shown as dashed lines. The lower panel illustrates the cloning of MRF1/MRR1- and MRF/MRR-amplified fragments into pCR 2.1-TOPO. The gel at the left shows TOPO-MR1 and TOPO-MR digested with EcoRI. The EcoRI-digested fragments were then recloned into the single EcoRI site of pΩL. The gel at the right shows pMR1 and pMR plasmids isolated from *E. coli* SCS110 and digested with EcoRI. The EcoRI digest of pMR1 gives two fragments of similar size that migrate together as a dense band: pΩL at 4,829 bp and MRF1/MRR1 fragment at 5,110 bp. Mr, molecular mass markers. Similar to the case for Fig. 2, we used the same GeneRuler DNA Ladder Mix for comparison in both the upper and lower panels.

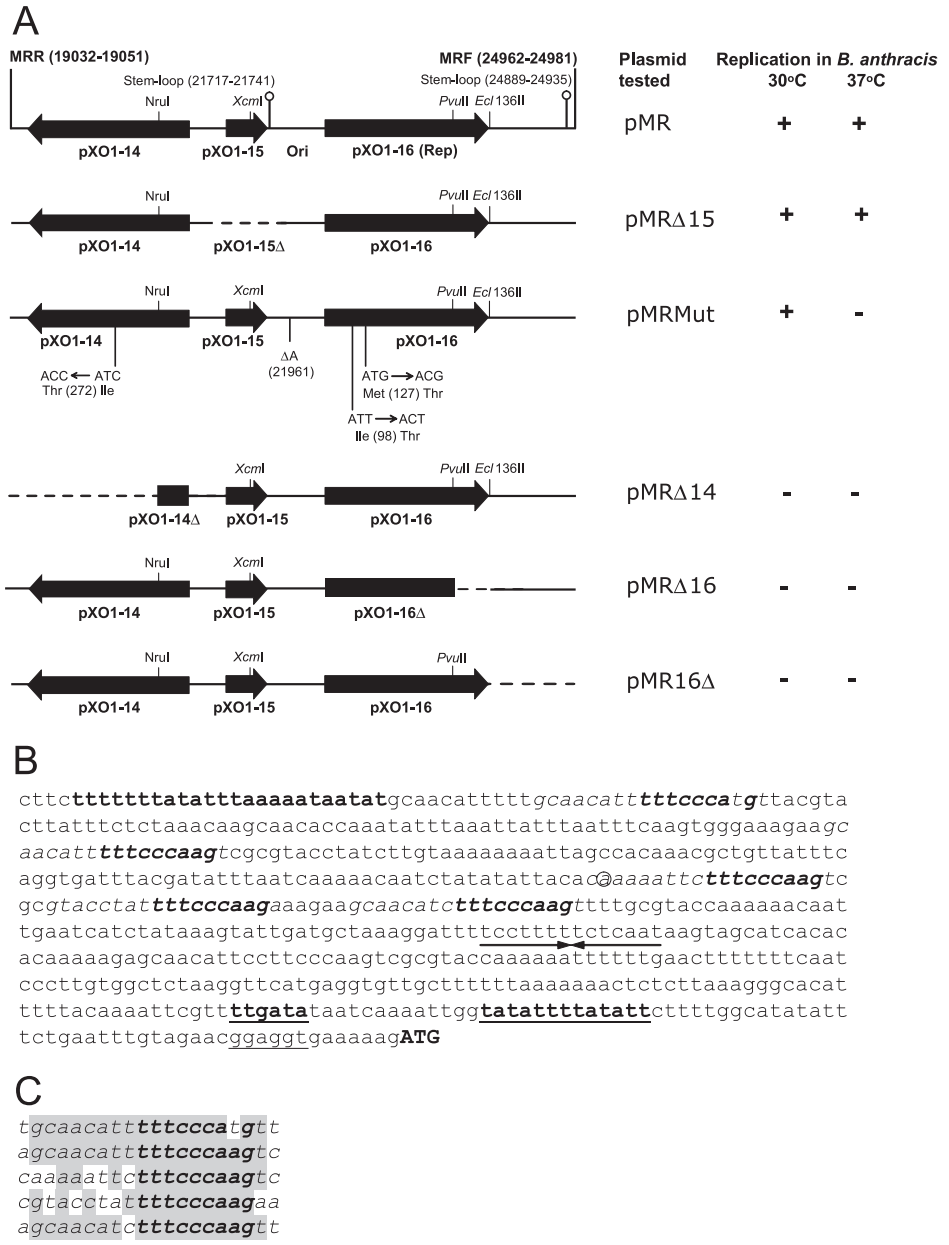

FIG. 4. The pXO1 minimal replicon and its putative origin of replication. (A) Regions of pXO1 plasmid DNA cloned into plasmid pΩL to produce plasmid pMR. The minimal replicon contains three pXO1 ORFs. The numbers correspond to the nucleotide coordinates of pXO1 (12). Derivative plasmids have regions deleted or truncated (dashed lines). The plasmid pMRMut contains three amino acid substitutions in the essential ORFs and a deletion of one nucleotide in the putative origin of replication (Ori). The ability of the plasmids to replicate in *B. anthracis* is indicated at the right. (B) Nucleotide sequence of the putative origin of replication of pXO1. The sequence shown is located immediately downstream of the stem-loop that follows pXO1-15. Features of the Ori are (i) an AT-rich segment (bold), (ii) five direct repeats (italic, with the most conserved sequence in bold italic), and (iii) an IR (converging arrows are above the repeat). The −35 and −10 regions of a σ^A-like promoter are denoted in underlined bold, while the putative Shine-Dalgarno sequence is underlined. The ATG start codon of the putative Rep protein is indicated in uppercase bold. The "@" represents the adenine at position 21961 that is deleted in pMRMut (A). (C) Alignment of the five direct repeats (from the sequence in panel B), with the most conserved residues in bold.

tified canonical features of plasmid replication origins, including an AT-rich region, direct repeat and IR sequences, and an adjacent putative replication (Rep) protein, ORF16. This protein is very similar to a hypothetical protein encoded by a number of other plasmids from the *B. cereus* group. The level of amino acid identity for ORF16 is >64% for 10 different plasmids and >90% for 8 of them. One of these proteins, pBt007 (from pBtoxis) has 96% identity with pXO1-16 and was recognized as widespread in *B. thuringiensis* isolates containing different virulence plasmids (3). Although the pXO1-16 protein shares no homology with Rep proteins of other large plasmids, the structural features of its putative Ori, which in-

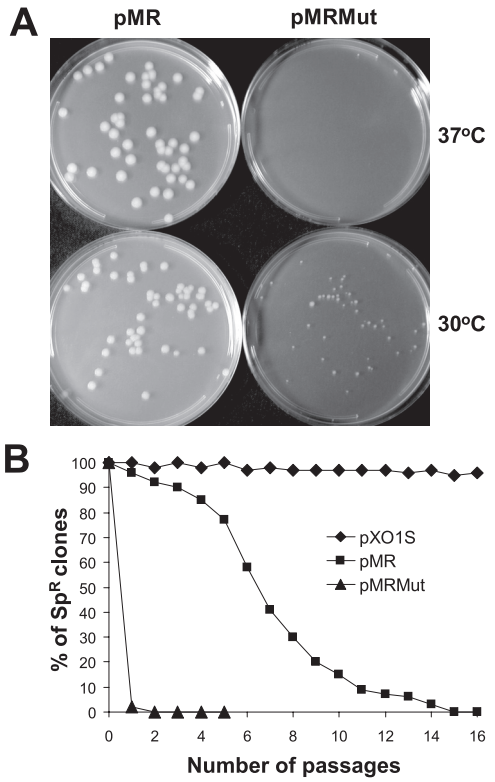

FIG. 5. Plasmid stability and temperature-sensitive replication of plasmid pMRMut. (A) The plasmid-free *B. anthracis* strain Ames 33 was electroporated with pMR and pMRMut, spread on plates containing 150 μg/ml spectinomycin, and incubated overnight at 30 or 37°C. (B) Percentage of spectinomycin-resistant (i.e., plasmid-containing) bacteria in cultures of *B. anthracis* Ames 35S (Ames 35 containing pXO1S), Ames 33(pMR), and Ames 33(pMRMut). Cultures were subcultured daily in LB medium without spectinomycin and grown at 37°C. Bacteria from each culture were diluted and plated on LB agar without spectinomycin (150 μg/ml) and colonies streaked to agar with spectinomycin to see the fraction retaining the plasmids. Maximum root mean square deviations in the percentage of spectinomycin-resistant colonies did not exceed 5%.

cludes iteron sequences, an AT-rich region, and an IR, suggest that this protein could induce replication of pXO1 using the theta mode of replication.

However, the inability of ORF16 (alone) to maintain the $\Delta_{2\text{-}3}$ plasmid in *B. anthracis* cells (Fig. 3, upper panel) indicates that ORF14 is also essential for maintenance of the pMR minireplicon. Moreover, both proteins are functional only when the corresponding genes are located within the same plasmid, as in plasmid Δ_2. Separation of these two genes to different plasmids, Δ_3 and $\Delta_{2\text{-}3}$, resulted in the loss of both the plasmids. Although we showed that both ORF14 and ORF16 are absolutely necessary for pXO1 minireplicon maintenance, the precise roles of the proteins remain unknown. Further studies will be needed to fully understand the roles of both ORF14 and ORF16 in initiation of pXO1 replication and its maintenance in *B. anthracis* cells.

The ORF15 protein was not found to be essential for replication of pMR and therefore is probably dispensable for pXO1 replication. Proteins having some homology to ORF15 were found only in pBCXO1 (8) and (with lower homology) in pBc239 (24) and pG9842 (NC_011775). It is not evident from an evolutionary point of view how and for what purpose a gene encoding a putative secreted protein would be inserted between two genes encoding intracellular proteins essential for pXO1 replication.

Decreased segregational stability of pMR in comparison with pXO1S was observed in *B. anthracis* during repeated passage at 37°C. This could result from the absence of a functional partition system within pMR. Partition is an active process needed by low-copy-number plasmids to ensure accurate distribution of plasmids to both daughter cells. Partition modules typically contain three components: a *cis*-acting site consisting of a set of sequence repeats (*parS*) and two genes, one coding for an ATPase (ParA) and the other for a *parS*-binding protein (ParB) (4). No components of a partition system have been identified on the pXO1 genome, and we could not identify motifs characteristic of *parA* genes in pMR. This suggests that RepX (ORF45) and its DNA target may participate in pXO1 partitioning, as an alternative partition module (1, 2, 22).

We found that the pXO1 minireplicon can replicate in *B. cereus* strains 569 and ATCC 10987. Both strains contain other plasmids: three in *B. cereus* 569 (14, 18) and one in *B. cereus* ATCC 10987 (16). The ability of pMR to transform these *B. cereus* strains regardless of their endogenous plasmid content suggests that plasmids derived from pMR may have value in genetic manipulation of many members of the *B. cereus* cluster. The differences in transformation efficiencies observed between the two *B. cereus* strains could be due to differences in

TABLE 4. Similarity of the ORF14 to -16 proteins to predicted proteins of plasmids from the *B. cereus* group

Plasmid	Host strain	% Identity to pXO1 protein:			GenBank accession no.	Reference
		ORF14	ORF15	ORF16		
pBCXO1	*B. cereus* G9241	99	100	64	NC_010934	8
pCER270	*B. cereus* AH187	96	NA[a]	96	NC_010924	17
pPER272	*B. cereus* AH820	95	NA	96	NC_010921	17
pBC10987	*B. cereus* ATCC 10987	95	NA	95	NC_005707	16
p03BB108_282	*B. cereus* 03BB108	95	NA	96	NZ_ABDM02000062	7
pBtoxis	*B. thuringiensis* subsp. *israelensis*	89	NA	96	NC_010076	3
pG9842	*B. cereus* G9842	61	34	94	NC_011775	
pBc239	*B. cereus* Q1	42	39	97	NC_011973	24
pBWB401	*B. weihenstephanensis* KBAB4	41	NA	94	NC_010180	
pE33L466	*B. cereus* E33L	39	NA	64	CP000040	17

[a] NA, no identity was found in the BLAST search.

restriction/modification systems or possibly could result from plasmid incompatibilities. These issues merit further study.

The inability of pMRMut to replicate efficiently at 37°C indicates that this mutant plasmid contains a temperature-sensitive replicon. The four mutations in this plasmid include a single-nucleotide deletion in the putative Ori region and three relatively conservative amino acid substitutions in the essential ORFs, in each case introducing a Thr residue (Fig. 4A). Further analysis would be needed to determine which of these mutations, individually or in combination, accounts for the temperature-sensitive phenotype. In any case, the very low stability of this plasmid at 37°C in the absence of positive selection with spectinomycin suggests that pMRMut-based cloning vectors will be useful in situations where it is advantageous to conditionally eliminate a vector (9, 15).

The minireplicon identified here differs from those previously described. The first report on a pXO1 origin of replication (19) did not actually demonstrate replication in *B. anthracis* but instead involving cloning of the region containing ORF72 to ORF80 as a shuttle vector into *B. subtilis*. The work was reported in a publication of a type that did not include details about the plasmid used for the cloning or the methods used for *B. subtilis* transformation or mini-pXO1 isolation. For these reasons and because the result has not been confirmed by others, the conclusion that this region contains a functional replicon must be viewed with skepticism. In our limited analysis of the region containing ORF72 to ORF80, we did not obtain a self-maintained replicon.

Surprisingly, the other reported pXO1 minireplicon containing the *repX* gene and adjacent *ori* region along with surrounding ORFs (22) also did not emerge from the Cre-*loxP* deletional analysis. This minireplicon did not maintain the remainder of pXO1 after deletion of the pXO1 region encompassing ORF14 to ORF16. Although the authors of the publication clearly demonstrated the presence of the *repX*-containing DNA in *B. anthracis* cells by Southern hybridization, no data on the stability of the putative minireplicon in *B. anthracis* were presented. The very low efficacy of *B. anthracis* transformation (1 to 2 CFU/μg) also raises concerns. The data presented do not appear to rigorously exclude the possibility that the plasmid DNA was spontaneously integrated into the *B. anthracis* genome in a *recE*-independent manner. Subsequent publications on RepX and the adjacent region have studied its biochemical properties in detail but have not directly shown that it plays a role in initiating pXO1 replication. Instead, evidence suggesting that this protein may play a role in plasmid segregation and partitioning has been presented (1, 2).

Although the two prior analyses of pXO1 discussed above identified regions that could replicate independently (19, 22), we did not observe deleted variants of pXO1 that contained those regions and lacked the ORF14 to -16 region (which we show can replicate independently). However, we did not use other approaches to rigorously examine or directly test the ability of the other two putative replicons to support independent replication. Thus, our analysis does not preclude the existence of other independent replicons within pXO1.

The success of the Cre-*loxP* strategy used here to identify the minireplicon results in part from its lack of bias. Deletions of large portions of pXO1 were achieved in an "in vivo" environment that does not require or involve cloning, transformation, or passage through alternative host organisms (e.g., *E. coli*). Furthermore, no assumptions need to be made about the structure of the replicon. All regions of the targeted plasmid are retained following Cre recombinase action, which creates two (or more) circular DNAs, each of which has the potential to replicate provided that it contains the necessary genes for replication and partition between dividing progeny bacteria. The method is therefore applicable to analysis of both simple and compound replicons. In the case of simple replicons, the essential genes will be isolated as a small plasmid, as occurred here for pXO1. For a compound replicon, any distantly located, separated, essential genes will be revealed when deletions of each required gene are made.

ACKNOWLEDGMENT

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REFERENCES

1. **Akhtar, P., S. P. Anand, S. C. Watkins, and S. A. Khan.** 2009. The tubulin-like RepX protein encoded by the pXO1 plasmid forms polymers in vivo in *Bacillus anthracis*. J. Bacteriol. **191:**2493–2500.
2. **Anand, S. P., P. Akhtar, E. Tinsley, S. C. Watkins, and S. A. Khan.** 2008. GTP-dependent polymerization of the tubulin-like RepX replication protein encoded by the pXO1 plasmid of *Bacillus anthracis*. Mol. Microbiol. **67:**881–890.
3. **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.
4. **Bouet, J. Y., K. Nordstrom, and D. Lane.** 2007. Plasmid partition and incompatibility—the focus shifts. Mol. Microbiol. **65:**1405–1414.
5. **Chattoraj, D. K.** 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. Mol. Microbiol. **37:**467–476.
6. **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.
7. **Hoffmaster, A. R., K. K. Hill, J. E. Gee, C. K. Marston, B. K. De, T. Popovic, D. Sue, P. P. Wilkins, S. B. Avashia, R. Drumgoole, C. H. Helma, L. O. Ticknor, R. T. Okinaka, and P. J. Jackson.** 2006. Characterization of *Bacillus cereus* isolates associated with fatal pneumonias: strains are closely related to *Bacillus anthracis* and harbor *B. anthracis* virulence genes. J. Clin. Microbiol. **44:**3352–3360.
8. **Hoffmaster, A. R., J. Ravel, D. A. Rasko, G. D. Chapman, M. D. Chute, C. K. Marston, B. K. De, C. T. Sacchi, C. Fitzgerald, L. W. Mayer, M. C. Maiden, F. G. Priest, M. Barker, L. Jiang, R. Z. Cer, J. Rilstone, S. N. Peterson, R. S. Weyant, D. R. Galloway, T. D. Read, T. Popovic, and C. M. Fraser.** 2004. Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proc. Natl. Acad. Sci. USA **101:**8449–8454.
9. **Janes, B. K., and S. Stibitz.** 2006. Routine markerless gene replacement in *Bacillus anthracis*. Infect. Immun. **74:**1949–1953.
10. **Leppla, S. H.** 2006. *Bacillus anthracis* toxins, p. 323–347. *In* J. E. Alouf and M. R. Popoff (ed.), The comprehensive sourcebook of bacterial protein toxins. Academic Press, Burlington, MA.
11. **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.
12. **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.
13. **Park, S., and S. H. Leppla.** 2000. Optimized production and purification of *Bacillus anthracis* lethal factor. Protein Expr. Purif. **18:**293–302.
14. **Pomerantsev, A. P., K. V. Kalnin, M. Osorio, and S. H. Leppla.** 2003. Phosphatidylcholine-specific phospholipase C and sphingomyelinase activities in bacteria of the *Bacillus cereus* group. Infect. Immun. **71:**6591–6606.
15. **Pomerantsev, A. P., R. Sitaraman, C. R. Galloway, V. Kivovich, and S. H. Leppla.** 2006. Genome engineering in *Bacillus anthracis* using Cre recombinase. Infect. Immun. **74:**682–693.
16. **Rasko, D. A., J. Ravel, O. A. Okstad, E. Helgason, R. Z. Cer, L. Jiang, K. A. Shores, D. E. Fouts, N. J. Tourasse, S. V. Angiuoli, J. Kolonay, W. C. Nelson, A. B. Kolsto, C. M. Fraser, and T. D. Read.** 2004. The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. Nucleic Acids Res. **32:**977–988.

17. **Rasko, D. A., M. J. Rosovitz, O. A. Okstad, D. E. Fouts, L. Jiang, R. Z. Cer, A. B. Kolsto, S. R. Gill, and J. Ravel.** 2007. Complete sequence analysis of novel plasmids from emetic and periodontal *Bacillus cereus* isolates reveals a common evolutionary history among the *B. cereus*-group plasmids, including *Bacillus anthracis* pXO1. J. Bacteriol. **189:**52–64.
18. **Reddy, A., L. Battisti, and C. B. Thorne.** 1987. Identification of self-transmissible plasmids in four *Bacillus thuringiensis* subspecies. J. Bacteriol. **169:** 5263–5270.
19. **Robertson, D. L., T. S. Bragg, S. Simpson, R. Kaspar, W. Xie, and M. T. Tippetts.** 1990. Mapping and characterization of *Bacillus anthracis* plasmids pXO1 and pXO2. Salisbury Med. Bull. **68**(Spec. Suppl.)**:**55–58.
20. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. **Thorne, C. B.** 1993. *Bacillus anthracis*, p. 113–124. *In* A. B. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, DC.
22. **Tinsley, E., and S. A. Khan.** 2006. A novel FtsZ-like protein is involved in replication of the anthrax toxin-encoding pXO1 plasmid in *Bacillus anthracis*. J. Bacteriol. **188:**2829–2835.
23. **Van der Auwera, G., and J. Mahillon.** 2005. TnXO1, a germination-associated class II transposon from *Bacillus anthracis*. Plasmid **53:**251–257.
24. **Xiong, Z., Y. Jiang, D. Qi, H. Lu, F. Yang, J. Yang, L. Chen, L. Sun, X. Xu, Y. Xue, Y. Zhu, and Q. Jin.** 2009. Complete genome sequence of the extremophilic *Bacillus cereus* strain Q1 with industrial applications. J. Bacteriol. **191:**1120–1121.