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Two independent replicons can support replication of the anthrax toxin-encoding plasmid pXO1 of *Bacillus anthracis*

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Abstract

The large pXO1 plasmid (181.6 kb) of *Bacillus anthracis* encodes the anthrax toxin proteins. Previous studies have shown that two separate regions of pXO1 can support replication of pXO1 miniplasmids when introduced into plasmid-less strains of this organism. No information is currently available on the ability of the above two replicons, termed RepX and ORFs 14/16 replicons, to support replication of the full-length pXO1 plasmid. We generated mutants of the full-length pXO1 plasmid in which either the RepX or the ORFs 14/16 replicon was inactivated by TargeTron insertional mutagenesis. Plasmid pXO1 derivatives containing only the RepX or the ORFs 14/16 replicon were able to replicate when introduced into a plasmid-less *B. anthracis* strain. Plasmid copy number analysis showed that the ORFs 14/16 replicon is more efficient than the RepX replicon. Our studies demonstrate that both the RepX and ORFs 14/16 replicons can independently support the replication of the full-length pXO1 plasmid.

Keywords

Plasmid pXO1; Replication; Replicon; TargeTron mutagenesis

1. Introduction

Bacillus anthracis is a Gram-positive, spore forming bacterium that causes anthrax in humans (Hanna, 1998; Mock and Fouet, 2001; Koehler, 2002). Two large plasmids, pXO1 (181.6 kb) and pXO2 (96.2 kb) are required for the full virulence of this organism. Plasmid pXO1 encodes the tripartite anthrax toxin consisting of the protective antigen, lethal factor and the edema factor, while plasmid pXO2 contains genes that are involved in capsule biosynthesis (Koehler, 2002; Pannucci et al., 2002). Plasmid pXO1 is extremely stable (Koehler, 2002; Mock and Fouet, 2001), suggesting that it must have highly efficient replication and partitioning systems. Earlier, we isolated a minireplicon of pXO1 (the RepX

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replicon) and showed that RepX, a 48-kDa protein encoded by pXO1, is essential for the maintenance of this minireplicon in *B. anthracis* (Tinsley and Khan, 2006). The RepX replicon is contained within a 5-kb region (nt positions 54,863-60,166) of pXO1 and RepX harbors limited homology to the bacterial cell division protein FtsZ. Further studies showed that RepX is a GTPase that can form polymers both in vitro and in vivo (Akhtar et al., 2009; Anand et al., 2007). These studies led us to postulate that RepX may play a role in both replication and partitioning of the pXO1 plasmid. Recently, an additional pXO1 minireplicon has been identified (nt positions 19,032-24,981 of pXO1) which contains two open reading frames (ORF 14 and ORF 16) that are essential for miniplasmid replication (Pomerantsev et al., 2009). Sequencing of pXO1-like plasmids isolated from approximately 30 isolates of *B. cereus sensu lato* species showed that all contained the ORFs14/16 replicon and all except a few contained the RepX replicon (Hu et al., 2009a, 2009b) (Auwera, pers. comm.). The conservation of both of these replicons in the pXO1 plasmid family suggests important roles for these two replicons in the maintenance of pXO1-type plasmids in *B. anthracis* and related organisms. The presence of multiple replicons within a single plasmid has been previously observed (Oskam et al., 1991, 1992), although it is uncommon.

Since RepX and ORFs 14/16 replicons independently supported the replication of miniplasmids, we tested whether either or both of these replicons can support the replication of the full-length pXO1 plasmid. Using TargeTron mutagenesis, we generated pXO1 derivatives that lacked either the RepX or the ORFs 14/16 replicon. Our studies show that both the RepX and ORFs 14/16 replicons can independently support replication of the full-length pXO1 plasmid in *B. anthracis*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strains were grown at 37°C in Luria Bertani (LB) broth. *B. anthracis* strains were also grown at 37°C in LB broth and those containing pXO1 derivatives with the spectinomycin resistance (Sp^r) gene were grown in the presence of 100µg/ml of spectinomycin. When necessary, ampicillin (Amp) was used at a concentration of 100µg/ml and erythromycin (Em) at a concentration of 3µg/ml.

2.2. DNA manipulations

DNA manipulations were done using standard protocols (Sambrook et al., 1989). Plasmid DNA was purified using the Promega plasmid isolation kit. Plasmids were introduced into both *E. coli* and *B. anthracis* by electroporation using the Bio-Rad Gene Pulsar.

2.3. Construction of the TargeTron plasmids for gene inactivation in *B. anthracis*

We constructed the TargeTron plasmid, pBSpE194tsPT, for insertional inactivation of genes in *B. anthracis*. First, we generated a co-integrate of the *E. coli* plasmid pBluescript (2,961 bp) and the pE194ts (3,728 bp) plasmid of Gram-positive bacteria by ligating these at their *EcoRI* sites. In the resulting pBSpE194ts plasmid (6,689 bp), the *HindIII* and *BsrGI* sites were destroyed by filling-in with the Klenow fragment of DNA pol I since these sites were to be used later for cloning of the 350 bp mutated PCR product containing *HindIII* and

*Bsr*GI sites into intron-encoding DNA (Yao et al., 2006). Following this, a 526 bp fragment containing the kanamycin resistance (Km^r) gene promoter (Tinsley and Khan, 2006) and *Sa*II linkers was amplified using the forward primer Km^rF and reverse primer Km^rR (Table 1) and plasmid pBSK m (Tinsley and Khan, 2006) as the template. This fragment was cloned into the *Sa*II site of the pBSpE194ts vector generating the pBSpE194tsP plasmid. In the next step, a 3,085 bp fragment (containing the intron and LtrA region) containing *Xho*I linkers was amplified by PCR using the forward primer IntF and the reverse primer IntR (Table 1) using the pJIR750ai plasmid as the template (Chen et al., 2005) and cloned into the *Xho*I site downstream of the Km^r promoter in pBSpE194tsP to generate the TargeTron plasmid termed pBSpE194tsPT (Fig. 1). In this plasmid, the Km^r promoter is expected to drive the expression of the intron RNA and LtrA cassette.

The pBSpE194tsPT plasmid was then used to construct *repX* and ORF16 TargeTron vectors for the insertional inactivation of these genes in the full-length pXO1- Sp^r (hereafter referred to as wt pXO1) plasmid in *B. anthracis*. Target sites in the pXO1 *repX* gene and ORF 16 were identified by the TargeTron design website (<http://www.sigma-genosys.com/targetron/checksequence>), and intron retargeting PCR primers were designed by using a computer algorithm at this web site. Reagents provided in the TargeTron Gene Knockout System were used to construct the retargeted intron according to the manufacturer's instructions. Primers IBS-1070/1071s, EBS1d-1070/1071s, EBS2-1070/1071s and EBS-universal (Table 1) were used for insertion into *repX* (insertion site in the antisense strand at position 237/238 downstream from the initiation ATG codon in the *repX* ORF), and primer IBS-447/448a, EBS1d-447/448a, EBS2-447/448a and EBS-universal (Table 1) were used for ORF 16 (insertion site in the antisense strand at position 447/448 downstream from the initiation ATG codon of ORF 16). These primers were used for one step SOEing (splicing by overlap extension) PCR to amplify a 350 bp intron fragments for targeting *repX* and ORF 16 at nucleotide positions 237 and 447, respectively, downstream from the initiation ATG codons in the antisense strand. The amplified 350 bp fragments were digested with *Hind*III and *Bsr*GI and ligated into the pBSpE194tsPT vector digested with the same restriction enzymes to construct *repX* and ORF 16 TargeTron vectors (pBSpE194tsPT::*repX* and pBSpE194tsPT::ORF16, respectively).

2.4. Isolation and PCR screening of *B. anthracis* strains with insertional inactivation of the pXO1 *repX* and ORF 16 sequences

The pBSpE194tsPT::*repX* and pBSpE194tsPT::ORF 16 plasmids were isolated from the *E. coli* *dam dcm* mutant strain GM2163 and electroporated into *B. anthracis* containing the pXO1- Sp^r (Drysdale et al., 2004) plasmid. Transformants were selected by growing the cells for 48 hr on LB agar plates containing 3 μ g/ml Em and 100 μ g/ml Sp. Doubly-resistant Sp^r and Em^r colonies were isolated, and the insertion of 0.9 kb introns into the *repX* gene and ORF 16 of the pXO1 plasmid confirmed by agarose gel electrophoresis of PCR products using the *repX* forward primer *repXF* and the reverse primer *repXR* (Table 1), and the ORF 16 forward primer ORF 16F and ORF 16 reverse primer ORF 16R (Table 1).

2.5. Curing of the TargeTron plasmids from *B. anthracis* after insertional inactivation of the pXO1 repX and ORF 16 sequences

Once *B. anthracis* strains with insertional mutations in *repX* and ORF 16 were identified, the pBSpE194tsPT::repX and pBSpE194tsPT::ORF 16 plasmids that are temperature-sensitive for replication (*tsr*) were cured by first growing the cells overnight at 30°C in the presence of Em and Sp followed by growing diluted cultures in the brain heart infusion (BHI) medium in the presence of Sp alone at 43°C for 16–18 hr. Serial dilutions of the cultures were then plated on BHI agar plates containing 100 µg/ml Sp and incubated at 43°C overnight. Erythromycin-sensitive colonies were then identified and confirmed for the loss of the TargeTron plasmids by using plasmid specific primers (data not shown).

2.6. Reverse-transcriptase (RT) PCR analysis of repX and ORF 16 expression in *B. anthracis*

Total RNA was isolated from *B. anthracis* strains containing either wt pXO1-Sp^r or its *repX*- and ORF 16-negative derivatives using the Qiagen Total RNA Isolation Kit according to the manufacturer's instructions. Traces of chromosomal DNA in the RNA were removed by treatment with RNase-free DNase I (New England Biolabs) according to the manufacturer's instructions. Complementary (c) DNA synthesis was done in 20 µl reactions containing 250 ng of total RNA and RT and incubation at either 50°C for 1 hr for the *repX* mutant using the reverse primer repXR (Table 1) or at 46°C for 1 hr for the ORF 16 mutant using the reverse primer EBS2-447/448a primer (Table 1). Four microliters of the cDNA reactions were subsequently used for PCR amplification of double-stranded DNA. The primers for *repX* were repXF and repXR and those for ORF 16 were ORF 16F and EBS2-447/448a (Table 1). The reaction products were then analyzed by agarose gel electrophoresis.

2.7. Isolation of full-length plasmid pXO1 and its mutants

Plasmid DNA from *B. anthracis* containing wt pXO1 or its mutants was isolated by a minor modification of a previously published procedure (Green et al., 1985). This procedure utilizes cleared cell lysates that yield chromosomal DNA along with enriched pXO1 plasmid DNA. Cells were grown in 250 ml Erlenmeyer flasks containing 25 ml of the BHI broth with 100 µg/ml of Sp at 37°C for 16 hr. Cell pellets were suspended in 1 ml of E-buffer (40 mM Tris, pH 7.9, 2 mM EDTA and 15% sucrose) and lysed with 2 ml of complete lysis buffer (prepared by adding 0.6 g SDS and 0.6 ml of 5 N NaOH into 20 ml of 50 mM Tris/15% Sucrose). The tubes were rapidly inverted 20 times, incubated at 60°C for 1 hr in a water bath (mixing every 10 min) and chilled in ice for 10 min. The lysates were then centrifuged at 12,000 rpm in a Sorvall SS34 rotor for 20 min at 4°C. Supernatant was collected and 0.5 ml of a solution containing 2 mg/ml of pronase in 2 M Tris (pH 7.0) was then added along with RNase A at a final concentration of 50 µg/ml and incubated for 30 min at 37°C. The supernatant was then extracted with phenol-chloroform (1:1, vol/vol) and plasmids were analyzed by electrophoresis on 0.6% agarose gels.

2.8. Determination of plasmid copy number

To determine the copy number of pXO1 and its derivatives, we first generated a standard curve for both chromosome- and plasmid-specific genes. We PCR-amplified small regions of the chromosomal BA813 ORF of *B. anthracis* (152 bp; genomic control, present in one copy), the *repX* gene (127 bp; to measure the copy number of the RepX minireplicon, the full-length pXO1 plasmid, and the full-length pXO1 plasmid mutated in ORF 16), and the adjacent ORF 15 sequence (147 bp; to measure the copy number of the ORF 14/16 minireplicon and the full-length pXO1 plasmid mutated in the *repX* gene) using primer pairs Ba813-152, repX-127 and ORF 15-147, respectively, and the DNA was cloned into *E. coli* using the TOPO 2.1 vector (Invitrogen). Plasmid DNAs were isolated and diluted to obtain the equivalent of 10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 and 10 copies of the template DNA and QPCR reactions were done in 20 μ l reactions using a BioRad iCycler for 45 cycles (95°C for 15 min, followed by 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec) to generate standard curves (Coker et al., 2003). The absolute copy numbers of the various genes (BA813, *repX* and ORF 15) were calculated from the corresponding standard curves using the C_T values. The copy number of various plasmids was then calculated by dividing the copy number of *repX* or ORF 15 by the copy number of Ba813.

3. Results and discussion

3.1. Generation of an ORF 14/16 miniplasmid

We have previously described the isolation of the RepX replicon of pXO1 (Tinsley et al., 2006). A second replicon of pXO1 containing ORFs 14 and 16 has also been recently described (Pomerantsev et al., 2009). We isolated the ORFs 14/16 replicon of pXO1 by PCR amplification of a ~ 6 kb region of pXO1 (nt positions 18,847-24,882 (27) and cloning it into the *EcoRI* site of the pBS-Cm^r plasmid of *E. coli* (Tinsley et al., 2004). Replication of the ORFs 14/16 miniplasmid in *B. anthracis* was confirmed by introduction of this plasmid into the plasmid-less *B. anthracis* strain UM23C1-1 (Green et al., 1985; Koehler et al., 1994). By frame-shift mutagenesis, we confirmed that both ORFs 14 and 16 were essential for replication of this miniplasmid (data not shown). The conservation of **both** the RepX and ORFs 14/16 replicons in dozens of megaplasmids in the *B. cereus* group of organisms (Hu et al., 2009a, 2009b) suggests important roles for these two replicons in the replication and maintenance of pXO1-type plasmids.

3.2. Inactivation of the RepX and ORFs 14/16 replicons by TargeTron mutagenesis and demonstration that these replicons can independently support replication full-length pXO1

The TargeTron plasmid pBSpE194tsPT (Fig. 1) was used to generate recombinant plasmids in *E. coli* containing specific mutated intron sequences for integration into the *repX* gene or ORF 16 (pBSpE194tsPT::*repX* and pBSpE194tsPT::ORF 16, respectively, as described in materials and methods). These plasmids were then introduced into *B. anthracis* containing the full-length pXO1 Sp^r plasmid and integrants isolated as described in materials and methods. PCR analysis using primers representing *repX* or ORF 16 sequences adjacent to the predicted integration sites in these genes and total DNA isolated from several colonies showed that while the full-length pXO1 plasmid generated a band of 783-bp, integration of the homing intron into *repX* generated a band of 1,683 bp as expected (Fig. 2). Similarly,

while PCR amplification of a portion of the ORF 16 in the full-length pXO1 plasmid generated a band of 570 bp, a 1,470 bp fragment was obtained upon integration of the intron into ORF 16 (Fig. 2). These results showed that the *repX* and ORF 16 sequences were appropriately disrupted by the TargeTron vector. Furthermore, since disruption of either *repX* or ORF 16 still allowed pXO1 replication, these results suggest that both *repX* and ORF 14/16 replicons can independently support replication of the full-length pXO1 plasmid (also see below).

DNA enriched for plasmids was isolated from cleared lysates of *B. anthracis* strains to confirm the presence of full-length pXO1 or its derivatives in the above strains. Agarose gel analysis showed that while the plasmid-less *B. anthracis* strain only contained chromosomal DNA, full-length pXO1 plasmid DNA was present in strains with wt pXO1 DNA as well as in strains containing the pXO1 plasmid with disruption of the *repX* and ORF 16 sequences (Fig. 3). Note that since the pXO1 plasmid is very large (181.6 kb), it migrates slower than the sheared chromosomal DNA band as shown previously (Green et al., 1985).

We further carried out RT-PCR analysis to confirm that *repX* or ORF 16 sequences were disrupted by the TargeTron homing intron in *B. anthracis* strains containing the above pXO1 plasmid derivatives. RT-PCR analysis using total RNA isolated from various *B. anthracis* strains and primers specific for *repX* and ORF 16 (Table 1) showed that the wt pXO1 plasmid with an intact *repX* gene generated a cDNA band of 783 bp while interruption of *repX* resulted in a band of 1,683 bp as expected (Fig. 4). Similarly, the expected 570 bp cDNA band was obtained from a strain with wt pXO1 containing an intact ORF 16 while a 902 bp band was obtained upon disruption of the ORF 16 in pXO1 (Fig. 4). These results confirmed that the *repX* and ORF 16 sequences were disrupted in pXO1 as expected by integration of the introns and their transcripts were altered. Since these insertions occurred in the sequences encoding the amino terminal regions of RepX and ORF, functional proteins are not expected to be made by the above pXO1 mutants. Taken together, our results demonstrate that both the RepX and ORFs 14/16 replicons can independently support replication of the full-length pXO1 plasmid. Although the presence of two replicons within a plasmid has previously been reported (Oskam et al., 1991, 1992), it is uncommon.

3.3. Copy number determination of the full-length pXO1 plasmid and its derivatives

The RepX and ORFs 14/16 minireplicons, as well as the full-length pXO1 plasmid and its derivatives containing only the intact RepX or ORFs 14/16 replicon were analyzed for their copy number by QPCR analysis (Coker et al., 2003). The copy number of the full-length pXO1 plasmid was found to be approximately 3/chromosome (Table 2). The ORFs 14/16 minireplicon had a copy number between 15–20, while the RepX minireplicon had a copy number of approximately one per chromosome (Table 2). Finally, full-length pXO1 containing the intact ORF 16 (*repX*-negative) had a copy number of ~2–3/chromosome while pXO1 with intact *repX* (ORF 16-negative) had a copy number of ~ 1/chromosome (Table 2). These results show that full-length pXO1 is a low-copy plasmid. Furthermore, while the ORFs 14/16 replicon supports replication of the full-length pXO1 to the same levels as wt pXO1 with both the RepX and ORFs 14/16 replicons, the RepX replicon is less

efficient than the ORFs 14/16 replicon. This observation is consistent with the observation that while the RepX minireplicon had a low copy number of one/per chromosome, the ORFs 14/16 minireplicon had a much higher copy number. It is possible that the ORFs 14/16 minireplicon lacks DNA sequences that repress its activity in the wt pXO1 plasmid. Future studies will be directed towards an understanding of the individual contributions of the RepX and ORFs 14/16 replicons in the replication of full-length pXO1 during the lifecycle of *B. anthracis*.

4. Conclusions

Previous studies have shown that two separate replicons can support the replication of mini pXO1 plasmids. In this study, using TargeTron insertional mutagenesis we inactivated the RepX or ORFs 14/16 replicon in the context of the full-length pXO1 plasmid. Plasmid pXO1 derivatives containing only the intact RepX or the ORFs 14/16 replicon were able to replicate in *B. anthracis*, demonstrating that both of these replicons can independently support replication of the full-length pXO1 plasmid. Copy number analysis of various pXO1 derivatives showed that the ORFs 14/16 replicon is more efficient than the RepX replicon. A number of pXO1-like plasmids isolated from *B. cereus sensu lato* species contain both the RepX and ORFs 14/16 replicon, and the presence of two functional replicons in a plasmid in Gram-positive bacteria is rare.

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References

- Akhtar P, Anand SP, Watkins SC, Khan SA. The tubulin-like RepX protein encoded by the pXO1 plasmid forms polymers in vivo in *Bacillus anthracis*. *J Bacteriol*. 2009; 191:2493–2500. [PubMed: 19233922]
- Anand SP, Akhtar P, Tinsley E, Watkins SC, Khan SA. GTP-dependent polymerization of the tubulin-like RepX replication protein encoded by the pXO1 plasmid of *Bacillus anthracis*. *Mol Microbiol*. 2008; 67:881–890. [PubMed: 18179418]
- Chen Y, McClane BA, Fisher DJ, Rood JI, Gupta P. Construction of an alpha toxin gene knockout mutant of *Clostridium perfringens* type A by use of a mobile group II intron. *Appl Environ Microbiol*. 2005; 71:7542–7547. [PubMed: 16269799]
- Coker PR, Smith KL, Fellows PF, Rybachuck G, Kousoulas KG, Hugh-Jones ME. *Bacillus anthracis* virulence in Guinea pigs vaccinated with anthrax vaccine adsorbed is linked to plasmid quantities and clonality. *J Clin Microbiol*. 2003; 41:1212–1218. [PubMed: 12624053]
- Drysdale M, Bourgogne A, Hilsenbeck SG, Koehler TM. *atxA* controls *Bacillus anthracis* capsule synthesis via *acpA* and a newly discovered regulator, *acpB*. *J Bacteriol*. 2004; 186:307–315. [PubMed: 14702298]
- Green BD, Battisti L, Koehler TM, Thorne CB, Ivins BE. Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infect Immun*. 1985; 49:291–297. [PubMed: 3926644]
- Hanna P. Anthrax pathogenesis and host response. *Curr Top Microbiol Immunol*. 1998; 225:13–35. [PubMed: 9386326]
- Hu X, Swiecicka I, Timmerly S, Mahillon J. Sympatric soil communities of *Bacillus cereus sensu lato*: population structure and potential plasmid dynamics of pXO1- and pXO2-like elements. *FEMS Microbiol Ecol*. 2009a; 70:344–355. [PubMed: 19780824]

- Hu X, Van der Auwera G, Timmerly S, Zhu L, Mahillon J. Distribution, diversity, and potential mobility of extrachromosomal elements related to the *Bacillus anthracis* pXO1 and pXO2 virulence plasmids. *Appl Environ Microbiol*. 2009b; 75:3016–3028. [PubMed: 19304837]
- Koehler TM. *Bacillus anthracis* genetics and virulence gene regulation. *Curr Top Microbiol Immunol*. 2002; 271:143–164. [PubMed: 12224521]
- Koehler TM, Dai Z, Kaufman-Yarbray M. Regulation of the *Bacillus anthracis* protective antigen gene: CO2 and a trans-acting element activate transcription from one of two promoters. *J Bacteriol*. 1994; 176:586–595. [PubMed: 8300513]
- Mock M, Fouet A. Anthrax. *Annu Rev Microbiol*. 2001; 55:647–671. [PubMed: 11544370]
- Oskam L, Hillenga DJ, Venema G, Bron S. The large *Bacillus* plasmid pTB19 contains two integrated rolling-circle plasmids carrying mobilization functions. *Plasmid*. 1991; 26:30–39. [PubMed: 1946749]
- Oskam L, Hillenga DJ, Venema G, Bron S. The integrated state of the rolling-circle plasmid pTB913 in the composite *Bacillus* plasmid pTB19. *Mol Gen Genet*. 1992; 233:462–468. [PubMed: 1320190]
- Pannucci J, Okinaka RT, Sabin R, Kuske CR. *Bacillus anthracis* pXO1 plasmid sequence conservation among closely related bacterial species. *J Bacteriol*. 2002; 184:134–141. [PubMed: 11741853]
- Pomerantsev AP, Camp A, Leppla SH. A new minimal replicon of *Bacillus anthracis* plasmid pXO1. *J Bacteriol*. 2009; 191:5134–5146. [PubMed: 19502400]
- Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning: a laboratory manual*. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor; NY: 1989.
- Tinsley E, Khan SA. A novel FtsZ-like protein is involved in replication of the anthrax toxin-encoding pXO1 plasmid in *Bacillus anthracis*. *J Bacteriol*. 2006; 188:2829–2835. [PubMed: 16585744]
- Tinsley E, Naqvi A, Bourgogne A, Koehler TM, Khan SA. Isolation of a minireplicon of the virulence plasmid pXO2 of *Bacillus anthracis* and characterization of the plasmid-encoded RepS replication protein. *J Bacteriol*. 2004; 186:2717–2723. [PubMed: 15090513]
- Yao J, Zhong J, Fang Y, Geisinger E, Novick RP, Lambowitz AM. Use of targetrons to disrupt essential and nonessential genes in *Staphylococcus aureus* reveals temperature sensitivity of L1. *LtrB group II intron splicing RNA*. 2006; 12:1271–1281.

Highlights

1. Two replicons can independently support replication of the full-length pXO1 plasmid.
2. The copy number of the full-length pXO1 plasmid supported by the RepX replicon is approximately one per chromosome.
3. The copy number of the full-length pXO1 plasmid supported by the ORFs 14/16 replicon is approximately three per chromosome.
4. The copy number of the RepX minireplicon is approximately one per chromosome.
5. The copy number of the ORFs 14/16 minireplicon is approximately 15–20 per chromosome.

Fig. 1. Schematic diagram for TargetTron plasmid construction for *B. anthracis*. The plasmids were generated as described under materials and methods. The mutated 350 bp intron PCR product used to retarget the intron into the *repX* and ORF 16 sequences is also indicated.

Fig. 2.

Confirmation of the insertional inactivation of the *repX* gene and ORF 16 in the pXO1 plasmid by PCR and agarose gel electrophoresis. The diagram on the right shows the schematic representation of the insertion of the TargeTron vector into the *repX* or ORF 16 sequences in pXO1 and the sizes of the expected bands generated by PCR (shown by arrows).

Fig. 3.

Agarose gel analysis of DNA isolated from *B. anthracis* strains containing wild-type pXO1 or its *repX*- and ORF 16-negative mutants. DNA bands corresponding to the chromosome and pXO1 plasmid are indicated.

Fig. 4.

RT-PCR analysis of *repX* and ORF 16 expression in *B. anthracis* strains containing either wild-type pXO1, or its *repX*- and ORF 16-negative mutants. The various amplified cDNA products and their sizes are indicated.

Table 1

Oligonucleotide Primers used in this study.

Primer	Nucleotide sequence (5'-3') ^a
repXF	TATCCATATCGTGCATTCTT G
repXR	TCGTAGCGGTGATGTAAC GA
ORF 16F	GTCGCGCAATGTCTACAATTG
ORF 16R	CCGTACATGGTAATGCAG TTG
Ba813-152F	TTAATTCACCTTGCAACTGATGGG
Ba813-152R	AACGATAGCTCCTACATTTGGAG
repX-127F	AGCTCGTAATCCACAGGTTGGTGA
repX-127R	TGTTCCCTCCACCAAGACCACAAGT
ORF 15-147F	ACACAAAAATTCTTTCCCAAGTCGC
ORF 15-147R	GTGTGATGCTACTTATTGAGAAAAAG i
KmF	CTCACTGTGACAAAAGGGAACAAAAGCTGGAG
KmR	ATTTTTGTCGACTCACAAAACGGTTTACAAGCA
IntF	CAGGGGCTCGAGGAAACCAAAGCTTATAATTATCCT
IntR	AGTCGACTCGAGGTGGCTCCAAGTAGCGAAG
IBS-1070 1071s	AAAAAAGCTTATAATTATCCTTACTGCACCC TGCTGTGCGCCAGATAGGGTG
EBS 1d-1070 107 1s	CAGATTGTACAAATGTGGTGATAACAGATAA GTCCCTGCTCATAACTTACCTTTCTTTGT
EBS2-1070 1071s	TGAACGCAAGTTTCTAATTTTCGGTTTGCA GTCGATAGAGGAAAGTGCT
IBS-447 448a	AAAAAAGCTTATAATTATCCTTAATGTACG CTTAAGTGCGCCAGATAGGGTG
EBS1d-447 448a	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGC TTAACTTAACCTTACCTTTCTTTGT
EBS2-447 448a	TGAACGCAAGTTTCTAATTTTCGGTTTACATC CGATAGAGGAAAGTGCT
EBS-universal	CGAAATTAGAACTTGCGTTCAGTAAAC

Table 2

Copy number of pXO1 plasmid and its derivatives.

Plasmid	Copy number ^a
pXO1-Sp ^r	3–3.6
pXO1-Sp ^r - <i>repX</i> mutant	2–3
pXO1-Sp ^r -ORF 16 mutant	0.5–0.9
pXO1 minireplicon	0.8–1
ORF 14/16 minireplicon	15–20

^a Copy numbers of various plasmids were determined by quantitative PCR. Seven independent experiments were done to determine the plasmid copy numbers and the range of values obtained is indicated.