Curing of Plasmid pXO1 from *Bacillus anthracis* Using Plasmid Incompatibility

Xiankai Liu*, Dongshu Wang*, Huagui Wang, Erling Feng, Li Zhu, Hengliang Wang*

State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Biotechnology, Beijing, China

**Abstract**

The large plasmid pXO1 encoding the anthrax toxin is important for the virulence of *Bacillus anthracis*. It is essential to cure pXO1 from *B. anthracis* to evaluate its role in the pathogenesis of anthrax infection. Because conventional methods for curing plasmids (e.g., curing agents or growth at elevated temperatures) can induce mutations in the host chromosomal DNA, we developed a specific and reliable method to eliminate pXO1 from *B. anthracis* using plasmid incompatibility. Three putative replication origins of pXO1 were inserted into a temperature-sensitive plasmid to generate three incompatible plasmids. One of the three plasmids successfully eliminated the large plasmid pXO1 from *B. anthracis* vaccine strain A16R and wild type strain A16. These findings provided additional information about the replication/partitioning of pXO1 and demonstrated that introducing a small incompatible plasmid can generate plasmid-cured strains of *B. anthracis* without inducing spontaneous mutations in the host chromosome.

**Introduction**

*Bacillus anthracis*, a gram-positive, rod-shaped, spore-forming bacterium, is the etiologic agent of anthrax, an acute and often fatal mammalian disease [1,2,3]. Virulent forms of *B. anthracis* harbor two large pathogenicity-related plasmids: pXO1 (181.6 kb), which encodes the anthrax toxin genes *pagA*, *lef*, and *cya* [2,4]; the toxin regulatory elements *atxA* and *pagR*, the plasmid-borne germination genes *gerXC*, -A, and -B [2,4,5,6]; and pXO2 (93.5 kb), which carries the genes responsible for capsule synthesis and degradation, *capA*, -B, -C and -D [7,8,9,10,11]. The two large plasmids of *B. anthracis* are essential for full pathogenicity; elimination of either dramatically attenuates the virulence of *B. anthracis*.

Numerous studies have attempted to characterize the role of these plasmids in virulence. Generating a plasmid-free strain is important to elucidate the crosstalk between the plasmids and host chromosome. Conventional curing strategies involve chemical agents (e.g., acridine orange, novobiocin, ethidium bromide), growth at elevated temperature, or treatment with ultraviolet (UV) light [12,13,14,15,16]. These strategies have been used successfully in many bacteria to eliminate resident plasmids. However, these methods are not specific to a particular plasmid but eliminate all resident plasmids. Furthermore, elimination of the resident plasmids is spontaneous; therefore, screening colonies for plasmid-cured strains is a tedious process. Finally, most known plasmid-curing chemical agents are toxic or mutagenic (e.g., acridine orange, ethidium bromide, and sodium dodecyl sulfate) and can therefore induce mutations in the host chromosome [17].

Mutations also occur during harsh physical curing treatments (e.g., high temperature or UV light). The phenotypic changes caused by mutation may interfere with the functional analysis of the plasmid [18]. For these reasons, there is a need to develop a curing method that is safe, reliable, and specific to the plasmid of interest.

Plasmid incompatibility is the inability of two different but related plasmids to coexist stably in the same host cell in the absence of continued selective pressure. Plasmids that share the same mechanisms for replication or partitioning are placed in the same incompatibility group. Plasmid incompatibility may be due to competition for the same replication or segregation sites, or caused by the repression of replication initiation [19]. Thus, introducing a smaller high-copy-number plasmid from the same incompatibility group may specifically eliminate a resident plasmid. This strategy has been demonstrated to be a specific, efficient, and safe technique to generate plasmid-free bacterial strains [20,21].

Tinsley et al. isolated and characterized a minireplicon of plasmid pXO2 [22]. These researchers cloned a 2429-bp region of pXO2 into an *Escherichia coli* vector that is normally unable to replicate in *B. anthracis* and demonstrated that this plasmid replicated when introduced into *B. anthracis*. The pXO2 replicon contains an open reading frame (ORF) encoding the putative replication initiation protein RepS and the putative origin of replication ori. Another ORF (*repB*) was included in this fragment but thought to be dispensable for replication of the pXO2 minireplicon [22]. Based on this information, we successfully cured the large pXO2 plasmid from the *B. anthracis* wild type strain A16 (pXO1pXO2*) using a smaller plasmid in which a 3836-bp
region of pXO2 containing repS, repB, and the putative pXO2 ori was inserted [23]. These results initiated our interest in eliminating the plasmid pXO1 from *B. anthracis* using plasmid incompatibility, which has not yet been reported in the literature. Little is known about the replication and partition properties of pXO1. The origin of replication and genes involved in replication initiation, plasmid partitioning, and plasmid stability have not been identified. Researchers have long sought to characterize the structure of the pXO1 replicon. Robertson et al. attempted to localize the replication origin by cloning pXO1 DNA fragments into an *E. coli* vector and transforming the plasmid into *S. subtilis*. This group identified several clones that mapped to an 11-kb region (coordinates 86249–97209) of the pXO1 sequence (GenBank accession no. AF065404; ORF72-01) (coordinate numbers and ORFs annotated by Okinaka et al. will be used throughout this report) [24]. The products encoded by the 10 ORFs did not show any sequence similarity to rep proteins associated with theta-replicating plasmids. In the past 10 years, data from numerous *B. anthracis* genome-sequencing projects have considerably increased our understanding of the biological roles of plasmids in this species. Plasmid pXO1 of the *B. anthracis* Sterne strain was sequenced by Okinaka et al. using a random whole-genome shotgun strategy, which enabled analysis of its replicon. The annotation predicted 143 ORFs in pXO1, but only 35 have putative functions assigned to them based on similarity to genes in open databases. Unfortunately, no sequences have been found that show significant similarity with known replication initiator proteins encoded by other plasmids [24]. Tinsley and Khan cloned a 5-kb vector and demonstrated that this plasmid could replicate in *E. coli*. Mutational analysis showed that ORF45 was required for replication of the minireplicon, and this ORF was designated repX [25]. In a more recent effort to understand pXO1 replication, Andrei et al. isolated and characterized a new minireplicon of *B. anthracis* plasmid pXO1 using the Cre-loxP recombination system to delete large DNA segments; this region (ORF14–16, nt 19032–24901) differed from those previously described. Deletion analysis showed that only ORF14 and ORF16 were essential for replication of the minireplicon, and ORF16 was thought to be the replication initiator protein with a putative theta-replicating origin of replication [26].

In this report, we cured the virulence-associated plasmid pXO1 from *B. anthracis* vaccine strain A16R and wild type strain A16, using plasmid incompatibility to generate isogenic plasmid-cured and plasmid-containing strains. Our results will lead to a better understanding of the pXO1 plasmid and its interaction with the *B. anthracis* chromosome.

### Materials and Methods

#### Bacterial Strains, Plasmids, and Culture

The bacterial strains and plasmids used in this study are listed in Table 1. *B. anthracis* A16 strain was isolated from the carcass of a mule that had died of anthrax in Langfang city in the Hebei province of China in 1953. The lethal dose of A16 is 100 spores for mice and 150 spores for rabbits administered by subcutaneous injection. *B. anthracis* vaccine strain A16R was derived from A16 by exposure to UV radiation. A culture dish containing the spore suspension of A16 was placed 10 cm from the center of a UV lamp. After a 3-hour UV treatment, the irradiated suspension was spread on agar plates containing 20% bovine serum and incubated in 20% CO$_2$ at 37°C for 24 hours. A rough colony was chosen to streak on an agar plate containing 20% bovine serum, which was incubated in 20% CO$_2$ at 37°C for 24 hours. This procedure was repeated until all descendants produced rough colonies. One of these clones was carried continuously in nutrient medium for 141 passages and in mice for 30 generations. With no descendants producing smooth colonies, this clone was considered stable and designated A16R. Its virulence was greatly attenuated, primarily through loss of the bacterial capsule, and A16R is now used for the human anthrax vaccine in China. For safety reasons and ease of handling in the laboratory, an attenuated *B. anthracis* vaccine strain A16R (pXO1$^+$ pXO2$^-$) was used for the initial experiments in the present study. The experiments were then repeated with the wild type strain A16. The temperature-sensitive shuttle vector pKSV7 (permisive temperature, 30°C; restrictive temperature, 37°C) contains a chloramphenicol resistance gene for selection in *B. anthracis*, an ampicillin resistance gene for selection in *E. coli*, and a multiple cloning site [27]. Strains containing recombinant plasmids were cultured at 30°C, except when curing the plasmid from the host bacteria, when the strains were cultured at 37°C.

| Table 1. Plasmids and bacterial strains used in this study. |
|---|
| **Plasmids and strains** | **Relevant genotype and characteristics** | **Source** |
| pkSV7 | Shuttle vector, temperature-sensitive, Amp$^+$ (gram-negative) in *E. coli* and Cm$^+$ (gram-positive) in *B. anthracis* | [27] |
| pKS4K | Incompatible plasmid, constructed by inserting 4-kb fragment of pXO1 into pkSV7 | This work |
| pKS5K | Incompatible plasmid, constructed by inserting 5-kb fragment of pXO1 into pkSV7 | This work |
| pKS1K | Incompatible plasmid, constructed by inserting 1-kb fragment of pXO1 into pkS7V | This work |
| E. coli DH5$\alpha$ | F', qbld(lacZ+M15, ϕlacZYA-argF)U169, deoR, recA1, endA1, hsdR17(θ+), mcrA, supE44, thi-1, relA1 | This lab |
| E. coli SC5110 | rpsL(StrR), thr, leu, endA, thi-1, lacy, gatK, gat1T, ara, tonA, lxy, dam+, dcm-, supE44(lac-proAB), F- [traD36, proAB, lacQlac2, ϕM15] | This lab |
| *B. anthracis* A16R | Vaccine strain, pXO1$^+$, pXO2$^-$ | This lab |
| *B. anthracis* A16 | Wild type A16R, pXO1$^+$, pXO2$^-$ | This lab |
| *B. anthracis* A16R5K | A16R was cured of pXO1, harbors recombinant plasmid pKS5K, pXO1$^+$, pXO2$^-$, pKSSK | This work |
| *B. anthracis* A16RO | pXO1 plasmid-cured derivative of vaccine strain A16R, pXO1$^+$, pXO2$^-$ | This work |
| *B. anthracis* A16Q1 | pXO1 plasmid-cured derivative of wild type A16, pXO1$^+$, pXO2$^-$ | This work |
Before transformation into *B. anthracis*, all recombinant plasmids were passaged through *E. coli* SCS110 (dom dem mutant) to demethylate the plasmid DNA, thereby facilitating its transformation into bacteria. All strains were cultured in Luria-Bertani (LB) broth (10 g/L tryptone [Oxoid, UK], 5 g/L yeast extract [Oxoid], 10 g/L NaCl) or on LB agar plates. Transformants were selected for ampicillin resistance (100 μg/mL) in *E. coli* or for chloramphenicol resistance (10 μg/mL) in *B. anthracis*.

**Construction of Incompatible Plasmids**

Based on the literature, we evaluated three DNA fragments (11-kb, 4-kb, and 5-kb) containing putative replication origins of pXO1 (Figure 1). The vector pKSV7 was linearized at the *SmaI* restriction site with restriction enzyme (Takara Biotechnology, Dalian, China) and purified after 0.6% agarose gel electrophoresis using an agarose gel DNA purification kit. The 4-kb and 5-kb fragments were amplified with Pyrobest DNA polymerase (Takara Biotechnology, Dalian, China) by polymerase chain reaction (PCR) from total genomic DNA of strain A16R with the three primer pairs 11K_1F/R, 11K_2F/R, and 11K_3F/R. These PCR products were purified with the QIAquick PCR purification kit (Qiagen, Germany) and cloned in tandem into the linearized pKSV7 vector by homologous recombination using the CloneEZ kit (GenScript, Piscataway, NJ, USA), generating the plasmids pKS4K and pKS5K, respectively. The 11-kb fragment was amplified by PCR from total DNA of strain A16R with the three primer pairs 11K_1F/R, 11K_2F/R, and 11K_3F/R. These PCR products were purified with the QIAquick PCR purification kit (Qiagen, Germany) and cloned in tandem into the linearized pKSV7 vector to generate pKS11K. PCR was performed in a 20-μL reaction containing 10 μL of Taq PCR Master Mix, 1 μL template, 7 μL ddH2O, and 1 μL each primer. The primer pair pKSV7-F/R, which flanks the multiple cloning site, was used to determine whether the 4-kb or 5-kb DNA fragment was inserted into pKSV7, and three primer pairs (11K_1F/R, 11K_2F/R and 11K_3F/R) were used to determine whether the 11-kb DNA fragment was inserted (Table S1). The thermal cycling conditions used to analyze transformants were same as those used to amplify fragments from the A16R total genomic DNA. Colonies that were positive for transformation, as assessed by colony PCR analysis, were amplified by restriction digestion and DNA sequencing in an ABI Prism Model 3730XL DNA analyzer (Applied Biosystems). The recombinant plasmids pKS4K, pKS5K, and pKS11K were confirmed by sequencing and introduced into the dom dem E. coli host strain SCS110 by electroporation (200 Ω, 25 μF, 1.8 kV) (Bio-Rad Gene Pulser II electroporator, Hercules, CA, USA) to obtain demethylated plasmid DNA for transformation into the *B. anthracis* A16R strain.

**Curing of Plasmid pXO1 from *B. anthracis* A16R Strain**

The electroporation-competent *B. anthracis* A16R cells were prepared as previously described [28]. The plasmids pKS4K, pKS5K, and pKS11K were isolated (Axygen Scientific, Union City, CA, USA) from *E. coli* SCS110 and introduced into *B. anthracis* A16R by electroporation (200 Ω, 25 μF, 1.8 kV). The cells were grown at 30°C for 3 h in LB broth without antibiotics on a shaker (225 rpm) and then spread onto selective agar plates containing ampicillin. After incubation at 30°C for 12 to 16 h, transformants were picked and analyzed by colony PCR. Briefly, a single colony was suspended in 100 μL ddH2O, heated to 95°C for 2 min, and then cooled to room temperature. Cellular debris was removed by centrifugation at 15,000 x g for 5 min, and 1 μL of the lysate was used as template for PCR amplification. PCR was performed in a 20-μL reaction containing 10 μL 2× Taq PCR Master Mix, 1 μL template, 7 μL ddH2O, and 1 μL each primer. The primer pair pKSV7-F/R, which flanks the multiple cloning site, was used to determine whether the 4-kb or 5-kb DNA fragment was inserted into pKSV7, and three primer pairs (11K_1F/R, 11K_2F/R and 11K_3F/R) were used to determine whether the 11-kb DNA fragment was inserted (Table S1). The thermal cycling conditions used to analyze transformants were same as those used to amplify fragments from the A16R total genomic DNA. Colonies that were positive for transformation, as assessed by colony PCR analysis, were amplified by restriction digestion and DNA sequencing in an ABI Prism Model 3730XL DNA analyzer (Applied Biosystems). The recombinant plasmids pKS4K, pKS5K, and pKS11K were confirmed by sequencing and introduced into the dom dem E. coli host strain SCS110 by electroporation (200 Ω, 25 μF, 1.8 kV) (Bio-Rad Gene Pulser II electroporator, Hercules, CA, USA) to obtain demethylated plasmid DNA for transformation into the *B. anthracis* A16R strain.

---

**Figure 1. Three putative replicons of plasmid pXO1 (GenBank Accession no. AF065404).** Each putative replicon of pXO1 is indicated in a different color, along with primer pairs used for amplification. doi:10.1371/journal.pone.0029875.g001
Elimination of Extraneous Plasmid pKS5K from Strain A16R5K (pXO1− pXO2 − pKS5K)

To obtain a plasmid-cured strain without exogenous DNA, we needed to eliminate the recombinant plasmid from the host A16R strain. The pXO1-cured A16R strain containing the incompatible plasmids was passaged more than six times in 5 mL LB broth without antibiotics at 37 °C for 12 h on a shaker (225 rpm). At each passage, aliquots of the cultures were diluted and spread on agar plates without antibiotics and incubated at 37 °C for 12 h. Single clones were streaked onto two agar plates with or without chloramphenicol. The agar plates were incubated at 30 °C overnight, and chloramphenicol-sensitive clones were considered positive for loss of the incompatible plasmid. The DNA of these clones was extracted and used as template for PCR with two primer pairs specific to plasmid pKSV7 (pKSV7p3 F/R and pKSV7p6 F/R, Table S1) to verify elimination of the incompatible plasmid.

Detection of Anthrax Toxin Protective Antigen by Western Blot Analysis

Western blot analysis was performed to determine the curing of pXO1 from B. anthracis A16R. The A16R strain (pXO1+) and A16R derivative strain (pXO1− pXO2+) were cultured on LB agar supplemented with 5% horse serum and 0.9% sodium bicarbonate at 37°C in 5% CO2 for 12 h. The bacteria were collected, and the cell suspension was separated by 12% bis-polyacrylamide gel electrophoresis. Proteins were transferred with Hoefer TE 77 semi-dry transfer unit (Amer sham Bioscience, USA) from the gel to a polyvinylidine fluoride membrane with a constant current of 35 mA for 1 h 50 min. The transferred membrane was blocked with Tris buffered saline with 0.05% Tween 20 (TBST) containing 5% skim milk and then incubated with the mouse monoclonal antibody against protective antigen (C3; Santa Cruz, CA, USA) diluted in 40 ml TBST for 60 min at room temperature. After washing three times with TBST for 7 min, the membrane was incubated in 40 ml TBST with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:10000) (Santa Cruz) for 60 min, and then washed four times with TBST for 7 min. The membrane was immersed in ECL substrate solution (Thermo Scientific, USA) for 5 min, and then exposed to x-ray film (Kodak RP X-OMAT, USA).

Use of Plasmid Incompatibility to Cure pXO1 from Wild Type Strain A16

The plasmid used to cure pXO1 from B. anthracis vaccine strain A16 was introduced into B. anthracis wild type strain A16 to cure the plasmid pXO1 using the same methods described above. Bacterial capsules were stained with India ink (Gibco, USA) and observed with a phase-contrast microscope (Eclipse TE300, Nikon, Tokyo, Japan).

Results

Identification of Incompatible Plasmids

Colonies were screened to screen for transformants of the incompatible plasmids, and plasmids were isolated from the positive clones. Restriction analysis confirmed insertion of the 4-kb, 5-kb, and 11-kb DNA fragments into the vector pKSV7. The recombinant plasmids were confirmed by DNA sequencing and designated pKS4K, pKS5K, and pKS11K, respectively. These plasmids were introduced into B. anthracis to cure plasmid pXO1 from A16R.

Elimination of pXO1 from A16R by Plasmid Incompatibility

The recombinant plasmids pKS4K, pKS5K, and pKS11K were introduced into B. anthracis vaccine strain A16R and passaged 10 times in chloramphenicol-containing LB. The cultures were plated on selective LB agar plates containing chloramphenicol, and colony PCR was performed with the three pairs of specific primers (pag_F/R, lef_F/R, and cya_F/R) to screen for pXO1-cured colonies (Figure 2). Positive colonies underwent further PCR analysis using 14 pairs of specific primers to confirm the elimination of pXO1 from A16R. None of the primers specific to plasmid pXO1 amplified DNA from the A16R colonies transformed with pKS5K (Figure 3). These results demonstrate the elimination of pXO1 from B. anthracis A16R by the incompatible plasmid pKS5K. The pXO1-cured A16R derivative strain was designated A16R5K (pXO1− pXO2− pKS5K). In contrast, the recombinant plasmids pKS4K and pKS11K did not cure pXO1 from A16R.

Figure 2. Colony PCR screening for pXO1 elimination with three plasmid-specific primer pairs. Results of using recombinant plasmids pKS11K (A), pKS4K (B), and pKS5K (C) to eliminate the large plasmid pXO1 from B. anthracis vaccine strain A16R by plasmid incompatibility. The presence of anthrax toxin genes pagA, lef, and cya was determined by PCR analysis of the vaccine strain A16R (1) and the putative pXO1-cured strain A16R (2). M, DNA marker IV (Real-Times Biotechnology, Beijing, China).

doi:10.1371/journal.pone.0029875.g002
not eliminate pXO1 even after passaging the cells for more than 2 months in chloramphenicol-containing LB broth.

Elimination of Exogenous Plasmid pKS5K from A16R (pXO1 2 pXO2 2 pKS5K+)

After curing pXO1 from A16R, we needed to eliminate the exogenous plasmid pKS5K from the host bacteria. To avoid damage to the chromosomal DNA of A16R, the bacteria containing pKS5K were passaged five times in LB broth without antibiotic at 37°C. The culture was plated on LB agar without antibiotics, and single clones were screened by colony PCR with two primer pairs (pKSV7P3_F/R and pKSV7P6_F/R) specific to vector pKSV7. The pXO1-cured A16R derivative strain (A16R5K) and the candidate clones were analyzed by PCR to confirm elimination of the recombinant plasmid pKS5K. The predicted DNA fragments were amplified from DNA of A16R5K, but not from DNA of the candidate clones. These results indicate that the recombinant plasmid pKS5K was successfully eliminated from A16R5K, and this strain was named A16RO (pXO1 2 pXO2 2) (Figure 4).

Phenotypic Confirmation of A16RO (pXO1 2 pXO2 2)

To confirm elimination of pXO1 from strain A16RO, the presence or absence of the anthrax toxin protective antigen, encoded by pXO1, was determined by western blot analysis. The results revealed the presence of the protective antigen protein in the cell lysate of A16R, but this protein band was not detected in the cell lysate of A16RO, confirming that pXO1 had been eliminated (Figure 5).

Use of Plasmid pKS5K to Cure pXO1 from Wild Type Strain A16 (pXO1+pXO2)

The results of PCR analysis and western blot analysis of the protective antigen demonstrated that pXO1 was successfully eliminated from B. anthracis wild type strain A16; this pXO1-cured strain was named A16Q1 (Figure 6). Although both strains produced capsules (Figure 6 B), the plasmid-cured strain A16Q1 did not produce the anthrax toxin protective antigen (Figure 6 C), which was consistent with the results of PCR analysis (Figure 6 A).

Discussion

In this study, we aimed to eliminate the large plasmid pXO1 from B. anthracis using plasmid incompatibility. Although this strategy has been used in some bacterial species to cure resident plasmids, it has not yet been used in B. anthracis other than our previous work to eliminate plasmid pXO2 from B. anthracis wild type strain A16. This is mainly because the replication and partitioning of the virulence-associated plasmids pXO1 and pXO2 are poorly understood.

Characterizing the replication origin is important to eliminate resident plasmids from the host bacteria. However, only three studies have described the replication and partition properties of pXO1. Furthermore, the replication origins identified by the three reports differed; therefore, we tested the ability of three fragments, each containing one of the putative replication origins, to eliminate pXO1.

Three DNA fragments containing putative origin of replication of B. anthracis were inserted into a temperature-sensitive shuttle vector producing the curing recombinant plasmids. One fragment
sufficient to infer its replication in
However, replication of the shuttle vector in
replication origin of
putative pXO1 replication origins reported by Robertson et al.
A16. Our results suggest that replication is not initiated at the
defined replication in B. anthracis
B. anthracis
using plasmid incompatibility. Our findings indicate that
this fragment is the actual replication origin of plasmid pXO1.
In summary, we cured the plasmid pXO1 from B. anthracis
A16R and A16 using plasmid incompatibility. This result has
provided additional information about the replication and parti-
tioning of pXO1. In addition, we developed a specific and reliable
method to generate plasmid-cured strains of B. anthracis
without inducing spontaneous mutations in the chromosomal DNA.

Supporting Information

Table S1 Oligonucleotides primers used in this study.
(XLS)

Author Contributions
Conceived and designed the experiments: XKL, HJW. Performed the
experiments: DSW HGW ELF LZ. Analyzed the data: XKL LZ.
Contributed reagents/materials/analysis tools: HGW ELF. Wrote the
paper: XKL.

References
1. Hanna PA (1998) Anthrax pathogenesis and host response. Curr Top Microbiol
Immunol 225: 23–35.
2. Koehler TM (2002) Bacillus anthracis genetics and virulence gene regulation.
Curr Top Microbiol Immunol 271: 143–164.
3. Mock M, Fouet A (2001) Anthrax. Annu Rev Microbiol 55: 647–671.
4. Guidi-Rontani C, Pereira Y, Raffin J, Girard JC, Weber-Levy M, et al. (1999)
Identification and characterization of a germination operon on the virulence plasmid
pXO1 of Bacillus anthracis. Mol Microbiol 33: 407–414.
5. Bourgogne A, Drysdale M, Hilterbrand SG, Peterson SN, Koehler TM (2003)
Global effects of virulence gene regulators in a Bacillus anthracis strain with both
virulence plasmids. Infect Immun 71: 2370–2374.
6. Pannucci J, Okinaka RT, Sabin R, Kaske CR (2002) Bacillus anthracis plasmid
sequence conservation among closely related bacterial species. J Bacteriol 184:
134–141.
7. Green BD, Battisti L, Koehler TM, Thorne CB, Iscas BE (1985) Demonstration
of a capsule plasmid in Bacillus anthracis. Infect Immun 49: 291–297.
8. Malino S, Uchida I, Terakado N, Sasakawa C, Yoshikawa M (1989) Molecular
characterization and protein analysis of the cap region, which is essential for
encapsulation in Bacillus anthracis. J Bacteriol 171: 722–730.
9. Uchida I, Sekizaki T, Hashimoto K, Terakado N (1985) Association of the
encapsulation of Bacillus anthracis with a 60 megadalton plasmid. J Gen
Microbiol 131: 363–367.
10. Uchida I, Malino S, Sasakawa C, Yoshikawa M, Sugimoto C, et al. (1993)
Identification of a novel gene, dep, associated with depolymerization of the
capsular polymer in Bacillus anthracis. Mol Microbiol 9: 487–496.
11. Vredeveld PJ, Marzcco R, Hoover TA, Wellko SI (1995) Identification and
classification of a plasmid involved in the regulation of encapsulation by Bacillus
anthracis. Gene 152: 1–9.
12. Brana H, Benada O, Navarat O, Cegka K, Hubacek J (1983) Stability of the
hybrid plasmid pMl130 and its curing by some eliminating agents. Folia
Microbiol (Prague) 28: 441–445.
13. El-Mansi M, Anderson KJ, Inche CA, Knowles KL, Platt DJ (2000) Isolation
and curing of the Klebsiella pneumoniasa large indigenous plasmid using sodium
dodecyl sulphate. Res Microbiol 151: 201–208.
14. Mezzer JM, Rodriguez MC, Mejor MT (2004) Plasmid curing of Oenococcus
oeni. Plasmid 51: 37–40.
15. Keyhani J, Keyhani E, Attar F, Haddadi A (2006) Sensitivity to detergents and
plasmid curing in Enterococcus faecalis. J Ind Microbiol Biotechnol 33:
239–242.
16. Spengler G, Molnar A, Schell Z, Narul L, Sharpes D, et al. (2006) The
mechanism of plasmid curing in bacteria. Curr Drug Targets 7: 823–841.
17. Hovi M, Sukupolvi P, Ekdahl MI, Rhen M (1988) Plasmid-associated
virulence of Salmonella enteritidis. Microb Pathog 4: 383–391.
18. Poppe C, Gyles CL (1988) Tagging and elimination of plasmids in Salmonella
of avian origin. Vet Microbiol 18: 73–87.
19. Novick RP (1987) Plasmid incompatibility. Microbiol Rev 51: 381–395.
20. Ni B, Du Z, Guo Z, Zhang Y, Yang R (2008) Curing of four different plasmids in
Yersinia pestis using plasmid incompatibility. Lett Appl Microbiol 47: 233–240.
21. Urji M, Suzuki K, Yoshida K (2002) A novel plasmid curing method using
incompatibility of plant pathogenic Ti plasmids in Agrobacterium tumefaciens.
Genes Genet Syst 77: 1–9.
22. Tinsley E, Naqvi A, Bourgogne A, Koehler TM, Khan SA (2004) Isolation of a minireplicon of the virulence plasmid pXO2 of Bacillus anthracis and characterization of the plasmid-encoded RepS replication protein. J Bacteriol 186: 2717–2723.

23. Wang H, Liu X, Feng E, Zhu L, Wang D, et al. (2011) Curing the Plasmid pXO2 from Bacillus anthracis A16 Using Plasmid Incompatibility. Curr Microbiol 62: 703–709.

24. Okinaka RT, Cloud K, Hampton OR, Hoffmaster AR, Hill KK, et al. (1999) Sequence and organization of pXO1, the large Bacillus anthracis plasmid harboring the anthrax toxin genes. J Bacteriol 181: 6509–6515.

25. Tinsley E, Khan SA (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.

26. Pomerantsev AP, Camp A, Leppla SH (2009) A new minimal replicon of Bacillus anthracis plasmid pXO1. J Bacteriol 191: 3134–3146.

27. Smith K, Younginan P (1992) Use of a new integrational vector to investigate compartment-specific expression of the Bacillus subtilis spoIIM gene. Biochimie 74: 705–711.

28. Shatalin KY, Neyfakh AA (2005) Efficient gene inactivation in Bacillus anthracis. FEMS Microbiol Lett 245: 315–319.