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Curing Both Virulent Mega-Plasmids from *Bacillus anthracis* Wild-Type Strain A16 Simultaneously Using Plasmid Incompatibility

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Plasmid-cured derivative strains of *Bacillus anthracis* are frequently used in laboratory studies. Plasmid incompatibility, which does not increase the risk of chromosomal mutation, is a useful method for plasmid curing. However, in bacteria containing multiple plasmids, it often requires the sequential introduction of multiple, specific incompatibility plasmids. This lengthy process renders the traditional plasmid incompatibility method inefficient and mutation-prone. In this study, we successfully cured plasmids pXO1 and pXO2 from *B. anthracis* A16 simultaneously using only one recombinant incompatible plasmid, pKORT, to obtain a plasmid-free strain, designated A16DD. This method may also be useful for the simultaneous, one-step curing of multiple plasmids from other bacteria, including *Bacillus thuringiensis* and *Yersinia pestis*.

Keywords: *Bacillus anthracis*, pXO1, pXO2, plasmid curing, plasmid incompatibility

Introduction

Plasmids, which are associated with pathogenicity, virulence, antimicrobial resistance, and other additional metabolic processes, are separate from, and can replicate independently of, chromosomal DNA within bacteria. Many methods have been applied to study the functions of plasmid-encoded genes, among which the construction of plasmid-cured mutant strains is probably the most effective and simple.

Conventional plasmid-curing strategies have been applied to remove plasmids from bacteria. These methods employ acridine dye, ethidium bromide, novobiocin, sodium dodecyl sulfate (SDS), and sublethal temperature or ultraviolet (UV) treatments [1, 5, 9]. However, these steps may damage chromosomal DNA and non-target plasmids in the elimination process. Another disadvantage is that screening colonies for plasmid-cured strains is very time-consuming.

Plasmid incompatibility refers to the fact that two plasmids belonging to the same incompatibility group cannot be stably inherited by the same daughter cell. This may occur *via* replication and/or partition competition [2, 4]. Thus, introducing a second, smaller plasmid of the same incompatibility group to a bacterial cell may cause a native plasmid to be specifically eliminated in the presence of selective pressure. This strategy is generally specific and efficient.

Bacillus anthracis, the causative agent of anthrax, usually harbors two plasmids (pXO1 and pXO2) that are necessary for complete virulence. Conventional strategies using plasmid incompatibility can only eliminate pXO1 and pXO2 sequentially, and they require a long period of continuous passaging, as well as repeated preparations and transformations of competent cells. In the current study, we generated plasmid-free strains in a one-step procedure using a newly constructed, dual-incompatibility plasmid, pKORT. It was derived from shuttle plasmid pKSV7

containing a ColE1 origin for replication in *Escherichia coli* and a pE194ts temperature-sensitive origin for replication in *B. anthracis* [8]. pKORT also contains two inserted DNA fragments: the pXO1 origin of replication (designated OR1 in this report) (nt 19072–23919; GenBank Accession No.: AF065404) [3, 6], and the origin of replication of pXO2 (designated OR2 in this report) (nt 31833–35669; GenBank Accession No.: NC_007323) [10, 11]. pXO1 and pXO2 were simultaneously cured from a wild-type strain of *B. anthracis* by the dual-incompatibility plasmid pKORT.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *B. anthracis* strain A16 (pXO1$^+$, pXO2$^+$; Table 1) was isolated from the carcass of a mule that died from anthrax in Hebei Province, China, in 1953. *B. anthracis* vaccine strain A16R (pXO1$^+$, pXO2$^-$; Table 1) was derived from strain A16 by UV irradiation. *B. anthracis* strain A16Q1 (pXO1$^-$, pXO2$^+$; Table 1) is a pXO1 plasmid-cured derivative of wild-type strain A16 that was generated using the plasmid incompatibility method [3].

Incompatibility plasmid pKORT was derived from the temperature-sensitive shuttle vector pKSV7 [8] (permissive temperature, 30°C; restrictive temperature, 37°C; Table 1), which contains an ampicillin resistance gene for selection in *E. coli*, and a chloramphenicol resistance gene for selection in *B. anthracis*. All strains were cultured in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI, USA) or on LB agar plates (LB broth supplemented with 1.2% agar). *E. coli* and *B. anthracis* strains containing pKORT were cultured at 30°C. When curing the plasmids from *B. anthracis*, bacteria were cultured at 37°C. Transformants were selected on LB agar plates containing ampicillin (100 μg/ml; *E. coli*) or chloramphenicol (10 μg/ml; *B. anthracis*).

Construction of the Dual-Incompatible Plasmid

The primers used in this study are listed in Table 2. Plasmid

Table 1. Plasmids and strains used in this study.

Plasmids and strains	Relevant genotype and characteristics	Source
pKSV7	Shuttle vector, temperature-sensitive, Ampr in *E. coli* and Cm^r in *B. anthracis*	This lab
pKORT	Dual-incompatible plasmid, constructed by inserting OR1 and OR2 fragments into pKSV7	This work
E. coli DH5α	F-,φ80d/*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(r_k^-,m_k^+), *phoA*,*supE*44λ^-, *thi*-1, *gyrA*96, *relA*1	This lab
E. coli SCS110	*rpsL*(StrR), *thr*, *leu*, *endA*, *thi*-1, *lacy*, *galK*, *galT*, *ara*, *tonA*, *tsx*, *dam*-, *dcm*-,*supE*44(lac-proAB), F- [*traD*36, *proAB*, *lacI*^{q}*lacZ*ΔM15]	This lab
B. anthracis A16R	Vaccine strain; pXO1$^+$, pXO2$^-$	This lab
B. anthracis A16	Wild type of A16R; pXO1$^+$, pXO2$^+$	This lab
B. anthracis A16Q1	pXO1 plasmid-cured derivative of A16 strain; pXO1$^-$, pXO2$^+$	This lab
B. anthracis A16ORT	A16 cured of pXO1 and pXO2, harbors recombinant plasmid pKORT; pXO1$^-$, pXO2$^-$, pKORT$^+$	This work
B. anthracis A16DD	pXO1 and pXO2 plasmids-cured derivative of A16 strain; pXO1$^-$, pXO2$^-$	This work

Table 2. Primers used in this study.

Primer name	Sequence	Note
OR1_F	ACGACGGCCAGTGCCAAGCTTTAAATTAACCACTTCTTGTC	Amplification of OR1 fragment
OR1_R	GTAGAGGGCGTTACAGACACTAAAAATAGAGAGCTCATTAC	
OR2_F	GTAATGAGCTCTCTATTTTTAGTGTCTGTAACGCCCTCTAC	Amplification of OR2 fragment
OR2_R	CTATGACATGATTACGAATTCCCAGCATTTCACCAAGCAATTC	
*atxA*_F	CAGGTCGCAGATAAAACAGTC	Screening for pXO1 deletion strains
*atxA*_R	ATGTCTTGGAGTGATTCGTTAG	
*cya*_F	AGGATTGATGTGCTGAAAGGAG	
*cya*_R	TTCGTCTTTGTCGCCACTATC	
*lef*_F	TGAGTGGTCCCGTCTTTATCC	
*lef*_R	AATCGCTCCAGTGTTGATAGTG	
*pag*_F	GTTCCAGACCGTGACAATGATG	
*pag*_R	ACTAGGATTAACCGCCGCTATC	

Table 2. Continued.

Primer name	Sequence	Note
CapA-F	CGATGACGATGGGTGAC	Screening for pXO2 deletion strains
CapA-R	AGATTGAAGTACATGCGGATGG	
CapB-F	GACGAGGAGCAACCGATTAAG	
CapB-R	AAGAACGCAGGCTTAGATTGG	
CapC-F	GTATTAGGAGTTACACTGAGCC	
CapC-R	GGTAACCCTTGTCTTTGAATTG	
pXO1_07F	CGTACTGCTGGAATTGATGG	Identification of pXO1 deletion strain
pXO1_07R	GTCTTGGCTAACACCTGTATG	
pXO1_13F	AGAAATTGAGTTTGAATATGGTGAG	
pXO1_13R	AGGTTGGCTTACTGGAGATAC	
pXO1_16F	AGCACATGACATACGAAGAAC	
pXO1_16R	GAACATAAGAAGTCTGAATGGATAG	
pXO1_23F	AACTAAGACACAACGAATACTACG	
pXO1_23R	CATTATGTGGTCAAGATTATGGTTC	
pXO1_70F	CATACCATTACAGGAGCATCATC	
pXO1_70R	ACCAGGAATCGCAAGAACC	
pXO1_90F	AAGGAAGTAGAGGCAGAAGC	
pXO1_90R	TTAATGTGTTGGCGTTCAGG	
pXO1_95F	GTCTATCAGAAGTAGGTCATAACG	
pXO1_95R	TTCAGTAAGAGCCTCCATAGTAG	
pXO1_98F	GACTGGTATTTCTACTGGGTTTG	
pXO1_98R	GTCCTGCTTCTTGATGATGATG	
pXO1_116F	CCTTCGTTCTGGTGATATGTG	
pXO1_116R	AATAATATGTGGTGCCTCTTCTG	Identification of pXO1 deletion strain
pXO2_007F	GCGATGGTGGAACAGGAATG	
pXO2_007R	TGCGTTGCTGCCGATATTG	
pXO2_039F	GCTTCTCACTGGACACCTAATG	
pXO2_039R	CCACTCGTGCCAATGACTAC	
pXO2_060F	CGAAAGCAACAGGGATACAAAG	
pXO2_060R	AGATACTCTGCCCAACTTTCAC	
pXO2_084F	AGCGTTCAAATACAGTCACATC	
pXO2_084R	TTACCTTTGCGATTTCCTCATC	
pXO2_089F	AACTGACGGTGAATCCATGAAC	
pXO2_089R	ATTGCCTGACTAATCGCTAAGC	
pXO2_094F	CCTGGGCGTAAAGAAGATGG	
pXO2_094R	TCTCGTTGCGTGACATTATCG	
pXO2_097F	AAGCAACCCGTGGAGATTTC	
pXO2_097R	TGGATGTTCCGCACCTTTATAG	
pXO2_107F	TGGACGGAGAACAGGACTATG	
pXO2_107R	GGGCTTGCGGATACTCAGG	
pXO2_111F	ATACAAGCGAAGCATCAGTACC	
pXO2_111R	TCCATCGTTACAACCTCCATTC	
pKSV7P3_F	ATAAGCAGTTACAGAGTGGAATAG	Identification of exogenous plasmid pKORT deletion strain
pKSV7P3_R	CAATGGACAATGGACACCTAG	
pKSV7P6_F	GGTGTCCATTGTCCATTGTC	
pKSV7P6_R	CATCCTATCTTCTGTATCAGTATCC	

pKSV7 was isolated using a plasmid isolation kit (Axygen Scientific, Union City, CA, USA) and then linearized using restriction enzymes *Hin*dIII and *Eco*RI (Takara Biotechnology, Dalian, China). Total DNA was isolated from *B. anthracis* strain A16 using the Biomed Bacteria DNA kit (Biomed, Beijing, China), and used as a template to amplify the OR1 and OR2 fragments using Pyrobest DNA polymerase (Takara Biotechnology) and primer pairs OR1_F/R and OR2_F/R (Table 2). The polymerase chain reaction (PCR) was performed in a 100 µl volume containing 5 U of Pyrobest DNA polymerase (Takara Biotechnology), 10 µl of 10× Pyrobest PCR buffer, 200 mM each of dNTP, 1 µg of A16 total DNA, 0.4 mM of each primer (Table 2), and double-distilled (dd) H_2O up to 100 µl. Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) with an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 56°C for 40 sec, and 72°C for 4–6 min, with a final elongation step of 72°C for 10 min. The PCR products were purified following 0.6% (w/v) agarose gel electrophoresis using a PCR purification kit (Biomed). The purified OR1 and OR2 fragments were cloned in tandem into linearized pKSV7 using the CloneEZ homologous recombination kit (GenScript, Piscataway, NJ, USA). Recombinant plasmids were introduced into chemically competent *E. coli* DH5α and selected using ampicillin. Transformants were picked and verified by colony PCRs containing 10 µl of 2× *Taq* PCR Master Mix, 7 µl of ddH_2O, 1 µl of bacterial suspension, and 0.4 mM of each primer (OR1_F/OR2_R; Table 2). Thermal cycler parameters were as follows: 95°C for 5 min, followed by 25 cycles of 95°C for 40 sec, 52°C for 40 sec, and 72°C for 8 min, with a final elongation at 72°C for 10 min. A single positive clone was selected and confirmed by DNA sequencing (BGI, Beijing, China), and was designated pKORT. pKORT was then purified from *E. coli* DH5α and introduced into *E. coli* SCS110 by electroporation (200 Ω, 25 µF, 1.8 kV).

Curing Native Plasmids from *B. anthracis* Wild-Type Strain A16

pKORT was isolated from *E. coli* SCS110 and introduced into *B. anthracis* A16 electrocompetent cells [7] by electroporation (500 Ω, 25 µF, 0.6 kV). Transformants were screened for chloramphenicol resistance and verified by colony PCR using the primer pair ORTid_F/R (Table 2) and the thermal cycler conditions described above. Positive clones were designated pKORT/A16 ($pXO1^+$, $pXO2^+$, $pKORT^+$). A pKORT/A16 clone was then passaged at least seven times in 5 ml of LB broth containing chloramphenicol (30°C, 225 rpm) for 12 h. Cultures were then diluted and spread onto LB agar plates containing chloramphenicol. Six pairs of specific primers (*pag*-F/R, *lef*-F/R, and *cya*-F/R for pXO1, and *capA*-F/R, *capB*-F/R, and *capC*-F/R for pXO2, Table 2) were used to screen the plasmid-cured strain. Total cellular DNA was then extracted from the putatively positive clone ($pXO1^-$, $pXO2^-$), the A16R ($pXO1^+$, $pXO2^-$) strain, and the A16Q1 ($pXO1^-$, $pXO2^+$) strain and used as template for PCR assays using 18 specific primer pairs (nine each for pXO1 and pXO2; Table 2) to ensure the elimination of the two native plasmids. The plasmid-cured strain was named A16ORT ($pXO1^-$, $pXO2^-$, $pKORT^+$).

Curing the Dual-Incompatible Plasmid from A16ORT

The A16ORT strain was passaged 5–10 times in 5 ml of LB broth incubated at 37°C and 225 rpm for 12 h. The cultures were then diluted and spread onto LB agar plates without antibiotics. A single clone was picked and streaked onto LB agar plates, both with and without chloramphenicol, and the plates were incubated overnight at 30°C. Total cell DNA from the chloramphenicol-sensitive clone was isolated for PCR with two specific primer pairs (pKSV7P3_F/R and pKSV7P6_F/R; Table 2) to verify the elimination of the dual-incompatible plasmid. The plasmid-free strain was designated A16DD ($pXO1^-$, $pXO2^-$).

Verification of Phenotype

The wild-type strain *B. anthracis* A16 and the plasmid-less strains *B. anthracis* A16Q1, A16R, and A16DD were cultured on LB agar plates supplemented with 5% (v/v) horse serum and 0.9% (w/v) sodium bicarbonate at 37°C in 5% CO_2. Capsule formation was confirmed by India ink staining (Remel BactiDrop, Thermo Fisher Scientific, Lenexa, KS, USA) and observed using a phase-contrast microscope (Eclipse TE300; Nikon, Tokyo, Japan).

Western blot analysis was performed to verify the expression of protective antigen, a component of anthrax toxin. A suspension of denatured bacterial cells was loaded on a 12% bis-polyacrylamide gel and separated by electrophoresis. The proteins were then transferred to a PVDF membrane for 1 h and 45 min at 15 V using a Hoefer TE 77 semi-dry transfer unit (Amersham Pharmacia Biotech, Uppsala, Sweden). The transferred membrane was blocked with Tris-buffered saline/0.05% (v/v) Tween-20 (TBST) with 5% (w/v) skim milk powder and incubated with mouse monoclonal antibody against *B. anthracis* protective antigen C3 (Santa Cruz Biotechnology, Dallas, TX, USA), which was diluted to 1:500 in TBST. The membrane was incubated for 60 min and then washed three times, for 7 min each, in TBST. The membrane was then incubated with goat anti-mouse horseradish-peroxidase-conjugated IgG (1:10,000; Santa Cruz Biotechnology) for 60 min. After washing three times with TBST, the membrane was immersed in ECL substrate solution (Thermo Fisher Scientific) for 5 min and then images were collected with a Tanon-5200 Imaging System (Tanon, Shanghai, China).

Results

Identification of the Dual-Incompatible Plasmid

A schematic diagram of the recombinant plasmid is shown in Fig. 1A. PCR analysis using the primer pairs OR1_F/R, OR2_F/R, and OR1_F/OR2_R confirmed the presence of the expected 4 and 5 kb DNA fragments, which were ligated together, in plasmid pKSV7 (Fig. 1B). The recombinant plasmid (pKORT) was confirmed by DNA sequencing (data not shown).

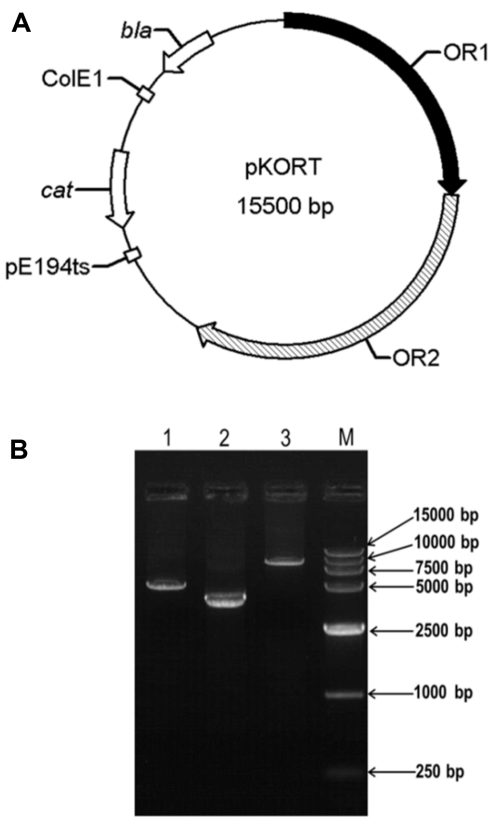

Fig. 1. Analysis of dual-incompatible plasmid pKORT.
(**A**) Schematic representation of the dual-incompatible plasmid pKORT. (**B**) Identification of the recombinant plasmid. Lane 1, amplicon generated using primers OR1_F/R; lane 2, amplicon generated using primers OR2_F/R; lane 3, amplicon generated using primers OR1_F/OR2_R; lane M, DNA Marker D15000 (Tiangen, Beijing, China).

Curing of Native Plasmids from Wild-Type Strain A16

Following passage of the pKORT/A16 (pXO1$^+$, pXO2$^+$, pKORT$^+$) strain under chloramphenicol selection, PCR screening of the plasmid-cured strain revealed that specific bands could not be amplified by the six pairs of primers in the candidate positive clone (Fig. 2). Further screening using 18 specific primer pairs confirmed the elimination of the two native plasmids (Fig. 3), although bands were observed in the positive control lane corresponding to pXO1 and pXO2. These results confirmed that pXO1 and

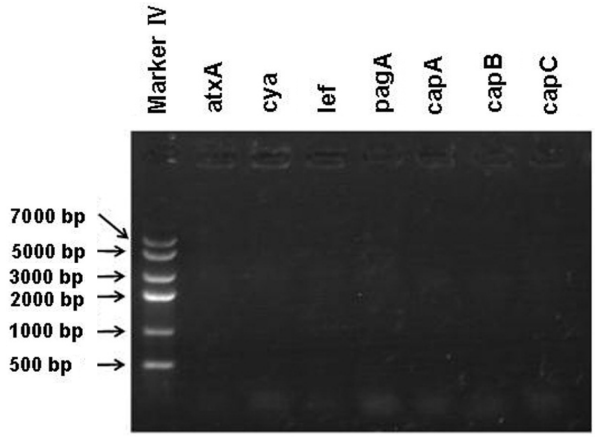

Fig. 2. Results of PCR screening of the plasmid-cured strain using six specific primer sets.
Genes *atxA*, *cya*, *lef*, and *pagA* are located on pXO1, and the *capABC* genes are located on pXO2. Lane M, DNA marker IV (Biomed).

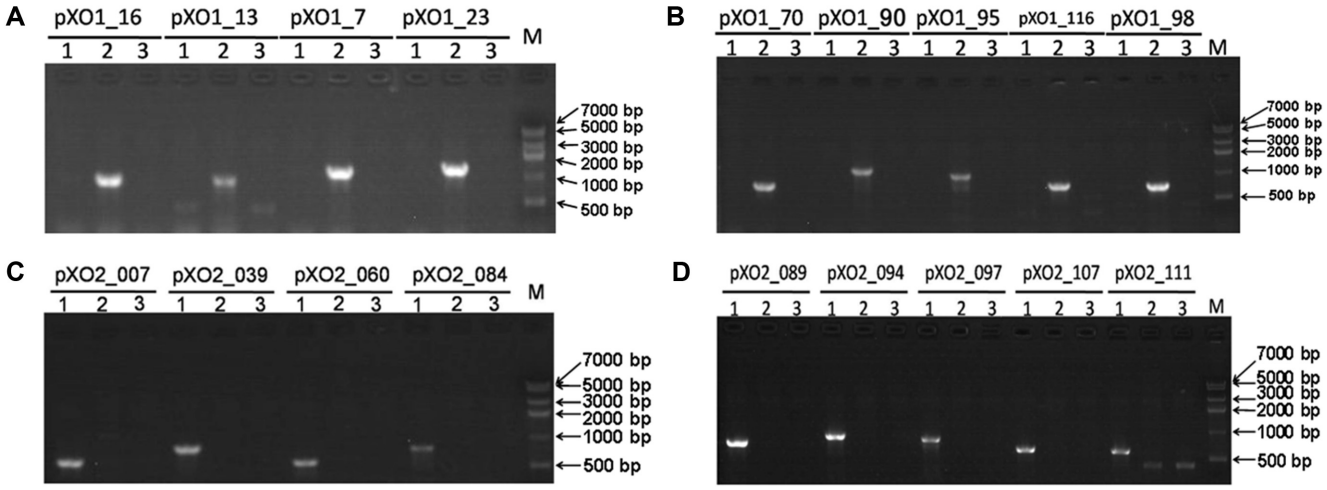

Fig. 3. Identification of the plasmid-cured strain by PCR.
Lanes 1, 2, and 3 show the amplicons resulting from PCR screening of *B. anthracis* strains A16Q1 (pXO1$^-$, pXO2$^+$), A16R (pXO1$^+$, pXO2$^-$), and A16DD (pXO1$^-$, pXO2$^-$), respectively. Lane M, DNA marker IV. Panels **A** and **B** show results generated using primers specific for pXO1 genes. Panels **C** and **D** show the results of pXO2-gene screening.

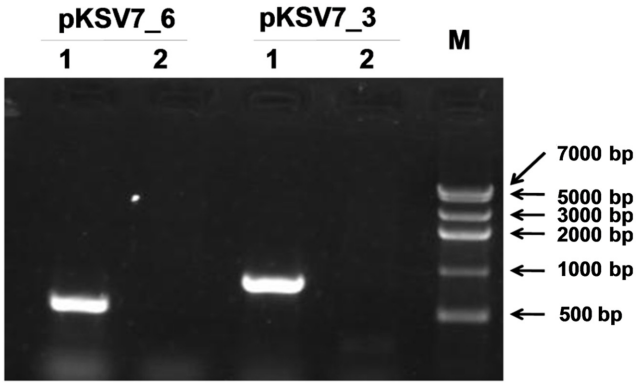

Fig. 4. PCR screening to confirm the elimination of exogenous, incompatible plasmid in *B. anthracis* strain A16ORT.
Lane 1 (*B. anthracis* strain A16ORT) and lane 2 (*B. anthracis* strain A16DD) show amplicons generated using vector-specific primer pairs. Lane M, DNA marker IV.

pXO2 were simultaneously cured by the addition of the pKORT incompatibility plasmid.

Elimination of the Incompatible Plasmid from A16ORT (pXO1$^-$, pXO2$^-$, pKORT$^+$)

After pXO1 and pXO2 had been cured, pKORT was eliminated from the host bacteria by passaging A16ORT (pXO1$^-$, pXO2$^-$, pKORT$^+$) five times in LB broth without antibiotic. PCR screening using two primer pairs specific to vector pKSV7 verified the elimination of pKORT (Fig. 4). This plasmid-free strain was designated A16DD.

Phenotypic Confirmation of A16DD (pXO1$^-$, pXO2$^-$)

Capsule formation was confirmed by India ink staining, and expression of protective antigen was verified by western blotting (Fig. 5). The results revealed that A16DD could not form a capsule and did not express protective antigen, confirming that pXO1 and pXO2 had been cured.

Discussion

Bacterial plasmids are generally not considered necessary for survival; however, they are often very important for resistance, symbiosis, metabolism, and pathogenesis. Some plasmids can be easily eliminated by changing the culture conditions, although this is usually ineffective for large plasmids. Curing plasmids by plasmid incompatibility is an efficient and specific method that does not carry any risk of introducing mutations in the host chromosome.

B. anthracis harbors two plasmids: pXO1 and pXO2. Construction of a *B. anthracis* plasmid-free strain by conventional strategies using plasmid incompatibility involves several time-consuming processes, including preparation of electrocompetent cells, introduction of the first incompatible plasmid, elimination of one resident plasmid, elimination of extraneous plasmid, and then

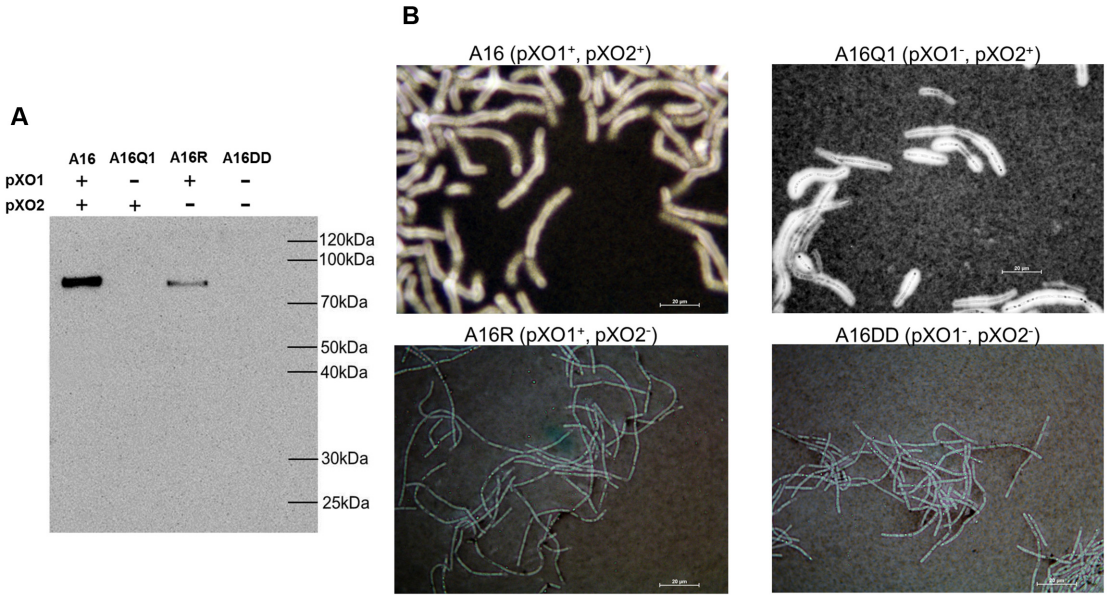

Fig. 5. Biochemical and phenotypic confirmation of *B. anthracis* strain A16DD.
(**A**) Results of western blotting analysis. The pXO1-cured strain A16Q1 and the plasmid-free strain A16DD did not express protective antigen. (**B**) India ink capsule staining. The pXO2-cured strain A16R and plasmid-free strain A16DD did not form capsules.

repeating the above procedure to eliminate the second resident plasmid. This becomes even more difficult when dealing with a bacterium that harbors three or more plasmids.

Previously, we cured pXO2 from *B. anthracis* wild-type strain A16 using the recombinant incompatible plasmid pKSV7-oriIV, in which the OR2 fragment was inserted [11]. pXO1 of the *B. anthracis* vaccine strain A16R was then cured by another recombinant incompatible plasmid, pKS5K, in which the OR1 fragment was inserted [3]. These results not only deepened our understanding of the replication initiation sites in the two large *B. anthracis* plasmids, but also confirmed the effectiveness of OR1 and OR2 in curing large virulence plasmids. These results suggested that it may be possible to eliminate the two *B. anthracis* plasmids using a single incompatible plasmid.

In this study, we constructed plasmid pKORT, which contains two incompatible fragments, and successfully cured pXO1 and pXO2 simultaneously from *B. anthracis* wild-type strain A16. Interestingly, the curing efficiency of pKORT differed for pXO1 and pXO2. pXO2 curing was observed after only 1–2 passages, whereas pXO1 deletion was not detected until passage 5–6 (data not shown). There are two possible explanations for these results. First, OR1 and OR2 use different mechanisms to compete with their target plasmids. Second, replication stability differs between pXO1 and pXO2. There are some naturally pXO2-deficient strains, but a naturally pXO1-deficient strain has not been reported. This seems to support the second explanation, but it is not sufficient to draw any conclusions. Despite the differences in curing efficiency, the dual plasmid-elimination strain can easily be obtained using the method described herein. Therefore, this technique may be useful for the efficient plasmid curing of other bacterial species containing multiple plasmids, such as *Yersinia pestis*, enteropathogenic *E. coli*, *Salmonella*, and *Shigella*.

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