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Routine Markerless Gene Replacement in *Bacillus anthracis*

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An improved genetic tool suitable for routine markerless allelic exchange in *Bacillus anthracis* has been constructed. Its utility was demonstrated by the introduction of insertions, deletions, and missense mutations on the chromosome and plasmid pXO1 of the Sterne strain of *B. anthracis*.

Bacillus anthracis, a gram-positive, spore-forming bacterium, is the causative agent of anthrax. Full virulence requires the production of a toxin and the formation of a protective capsule. The primary genes involved in the production of these two virulence factors are contained within two large nonessential plasmids, pXO1 and pXO2 (13). Distributed elsewhere on the two plasmids and on the chromosome are genes involved in regulating the expression of anthrax toxin, the capsule, and a number of other genes potentially involved in virulence (2, 6, 11). The entire genomic sequence of the Ames strain of *B. anthracis* has recently been determined, enabling the identification of interesting, potentially virulence-related genes by reverse genetics (19).

The ideal mutation to initially introduce in such "genome-mining" studies is an in-frame deletion of the candidate gene. Such a mutation avoids problems with polarity and other effects on the expression of surrounding genes, which accompany either insertion or less-precise deletion mutations and which can complicate interpretation. In addition, the ability to readily introduce missense mutations, which enables a determination of the effects of single-amino-acid substitutions, is necessary for more-sophisticated genetic analyses of structure-function relationships. Both of these types of desirable mutations are "markerless" in that they are not necessarily associated with a phenotype that can be selected or screened for during genetic manipulation (e.g., antibiotic resistance in the case of an insertion mutation). For *B. anthracis*, markerless gene replacements for some loci have been reported (3), but the methods by which these mutations have been isolated can be time and labor-intensive. This is due to the lack of a counterselection scheme, in which, by selecting for the loss of a plasmid vector, one can select for the second of two successive crossovers between such a vector and the chromosome in order to achieve gene replacement.

An alternative to counterselection schemes involves the use of the intron-encoded homing restriction enzyme I-SceI. The ability of this enzyme, which recognizes an 18-bp sequence, to cleave an introduced site that is essentially unique in a genome has been exploited in the promotion of homologous recombination in organisms as diverse as bacteria, *Drosophila*, and other higher eukaryotes (4, 20, 22). In one case, the use of I-SceI in promoting allelic exchange in *Escherichia coli* has been reported (17). In such a scheme, the integration of a suicide plasmid by a cloned region of homology containing the desired genetic change results in one of the two crossovers required to effect allelic exchange. To promote the second, the synthesis of the I-SceI enzyme results in cleavage at the unique I-SceI site within the vector. This double-stranded break is a potent substrate for host recombination systems that can repair the break by homologous recombination of the regions of sequence homology that flank the ends of the break as a result of the initial plasmid cross-in. As in allelic-exchange schemes driven by counterselectable markers, the loss of the plasmid sequences by homologous recombination leads to a population in which approximately 50% will have undergone the desired gene replacement. We have adapted this procedure for use with *B. anthracis*.

The method, described as follows, uses two plasmids, pBKJ236 and pBKJ223. These are illustrated schematically in Fig. 1, and the steps in the method are depicted in Fig. 2. Gene replacement constructs are first cloned into plasmid pBKJ236 for integration into the *B. anthracis* chromosome by homologous recombination. This vector was constructed by modification of pJRS233, which contains an erythromycin resistance gene, a replication origin for stable maintenance in *Escherichia coli*, and a temperature-sensitive replication origin for conditional maintenance in gram-positive organisms (16). In addition to these features, we added the *oriT* from RP4 to facilitate conjugative transfer from *E. coli* to *B. anthracis* (26) and the 18-bp recognition site for I-SceI. It should be noted that any suicide vector can be modified for use in this method simply by inserting an I-SceI site. We chose to separate the steps of plasmid transfer and recombination with the chromosome in order to overcome the relatively low efficiencies of genetic transfer from *E. coli* to *B. anthracis*. Thus, plasmid integrants are isolated by a shift to the replication-nonpermissive temperature after conjugative transfer and growth at the permissive temperature. The second plasmid, pBKJ233, is then introduced by electroporation and selection for tetracycline

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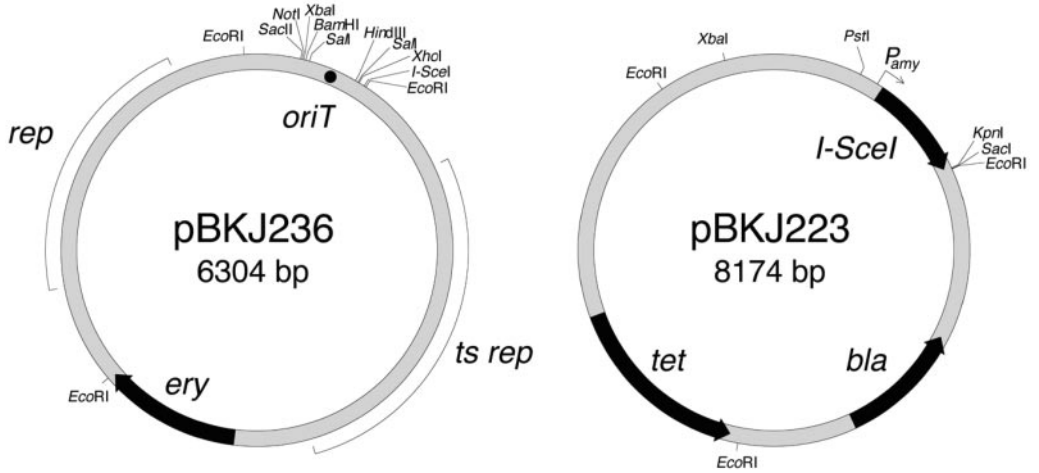

FIG. 1. Plasmids used in allelic exchange. pBKJ236 was created by the addition of an approximately 250-bp Bam-HindIII fragment from pSS1910 (25) containing the *oriT* of RP4 into the same sites of pJRS233 (16) and the subsequent addition at the KpnI site of the complementary oligonucleotides CATAGGGATAACAGGGTAATTGAATTCGGTAC and CGAATTCAATTACCCTGTTATCCCTATGGTAC containing an I-SceI site. To create pBKJ223, two PCR-generated fragments were added between the SalI and KpnI sites of pUTE29 (8). One fragment, from XhoI to NdeI, was created using the primers CGCGAATTCCTCGAGAAGCTTGAAGAAGACCATAAAAATACCTTGTC and CGCTCTAG ACATATGCGTTCTCCTTTCATTTTCTTATACAAATTATATTTT with *Bacillus amyloliquefaciens* chromosomal DNA as a template and was based on that described by Cohen et al. (5). This fragment contains the promoter for amylase and the ribosome-binding site of *B. anthracis pagA* (incorporated into one primer). The promoter sequence differs from the published sequence (5, 15) at several positions: an AT-to-TA transversion at positions −80 and −79 (with respect to the initiating codon) and a T-to-G change at position −63, presumably due to PCR errors. The other fragment, from NdeI to KpnI, was created using the primers CGCTCTAGACATATGCATCAAAAAAACCAGGTAATGAAC and CGCGGT ACCTTATTATTTCAGGAAAGTTTCGGAGGAGAT with pUCRP12 (17) as a template and comprised the ORF for the I-SceI enzyme.

resistance. A derivative of pUTE29 (8), this plasmid contains the gene for the I-SceI enzyme under the control of a hybrid amylase promoter and gram-positive ribosome-binding site. Transformants are streaked twice on solid medium containing tetracycline, and then single colonies are scored for loss of erythromycin resistance. Following screening by PCR for the incorporation of the desired mutation, the pBKJ233 plasmid is lost spontaneously by streaking the cells twice on medium lacking tetracycline and scoring a small number of colonies for tetracycline sensitivity. The replicational instability of the pUTE29 vector has been previously described (21).

In order to demonstrate the utility of this approach, a number of mutations were introduced into *B. anthracis* 7702 (Sterne). We have also used this method with success with a similar strain, 34F2 (data not shown). The structures of the lesions are presented in Table 1, and the efficiency of the pBKJ233-encoded I-SceI nuclease in stimulating the second crossover event and allelic exchange is presented in Table 2. In separate control experiments with the strain harboring the pBKJ240 plasmid integrant, the pUTE29 vector showed no such stimulation (data not shown). Figure 3 shows the results of the PCR analysis demonstrating the incorporation of the altered allele in the resulting mutant strains. The *plcR* locus was used as an initial test of this method because it has previously been documented that the *plcR* gene is nonfunctional in *B. anthracis* due to a frameshift mutation (1). Both a clean, in-frame deletion of the *plcR* gene (Δ*plcR240*) and a deletion marked with a spectinomycin resistance cassette (*plcR241*::*spc*) were successfully introduced. The *scrB* gene was chosen since it presented a target with a potentially scorable colonial phenotype. This gene is predicted to encode sucrose-6-phosphate hydrolase, which is essential for the metabolism of sucrose by the sucrose phosphoenolpyruvate-dependent phosphotransferase system (7, 9). Analysis of the *B. anthracis* (Ames) genome suggested that no other pathway for catabolizing sucrose existed. Indeed, both the insertion mutation (*scrB239*::*spc*) and a missense mutation (*scrB237*) (G214Q) conferred a sucrose uti-

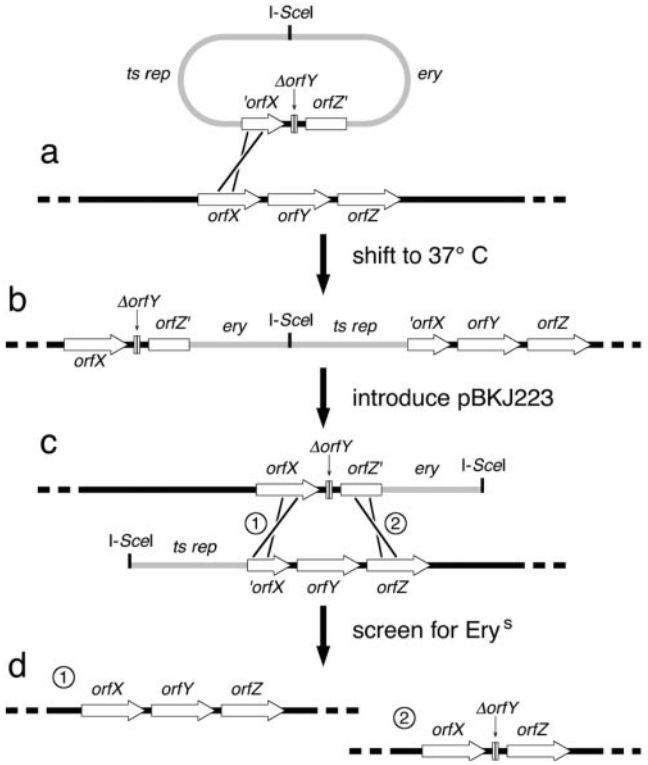

TABLE 1. Description of mutants

Allele	Plasmid[a]	Strain[b]	Upstream homology (bp)	Mutation	Downstream homology (bp)	Size of deletion (bp)[d]
plcR241::*spc*	pBKJ241	BA689	738	Spectinomycin insert[c] in MfeI site of Δ*plcR240*	775	R
Δ*plcR240*	pBKJ240	BA692	738	CAATTG (MfeI site) in place of ORF	775	642
scrB237	pBKJ237	BA693	463	G to A (Gly to Glu at position 214)	539	
scrB239::*spc*	pBKJ239	BA688	419	Spectinomycin insert[c] at native MfeI site	584	I
Δ*spo0A245*	pBKJ245	BA722	422	ATG-GGATTC-TAA	450	825
Δ*pagA242*	pBKJ242	BA690	1,078	ATG-11 codons-GAATTC-3 codons-TAA	1,011	2,247
Δ*lef243*	pBKJ243	BA723	858	ATG-CAATTG-TAA	1,079	2,424
Δ*cya244*	pBKJ244	BA695	447	ATG-CAATTG-TAA	450	2,397

[a] Plasmid containing the allele to be exchanged cloned into pBKJ236.
[b] Resulting *B. anthracis* Sterne strain in which successful allelic exchange has occurred.
[c] The spectinomycin resistance gene cassette was created by PCR with the primers CGCGAATTCGGATCGATCTGTATAATAAAGAATAATTA and CGCGAATTC GCCTAATTGAGAGAAGTTTCTATAGAA with pIC333 (23) as a template.
[d] R, mutation replacing the wild-type *plcR* gene (642 bp) with the ~950-bp spectinomycin resistance gene, so the PCR product is ~300 bp larger than that of the wild type; I, insertion of the spectinomycin resistance gene, so the PCR product is ~950 bp larger than that of the wild type.

lization defect visible as a change of colony color from yellow (wild type) to pink on nutrient agar supplemented with 1% sucrose and 0.0025% phenol red. This result validates the prediction that *scrB* represents the only pathway for sucrose utilization in *B. anthracis*. Since sporulation is also an easily scorable phenotype, we introduced an in-frame deletion (*spo0A245*) into the *spo0A* gene, a transcription factor required in the early steps of sporulation (14). As expected, the resultant mutant strain, BA722, yielded no detectable spores (CFU after treatment at 65°C for 30 min) under conditions (growth in Difco sporulation medium) which, for the *B. anthracis* 7702 parent strain, resulted in nearly 50% of the CFU being spores (data not shown). The identification of a deletion encompassing the *spo0A* gene in *B. anthracis* has been previously reported (27). However, this spontaneous deletion had endpoints in flanking DNA and thus affects other open reading frames (ORF) as well. To ascertain whether the method described here could be used for the replacement of genes on the large virulence plasmids of *B. anthracis*, the genes encoding the three components of anthrax toxin, *pagA*, *lef*, and *cya*, were targeted. Each of the three single mutant strains which resulted demonstrated a lack of production of the corresponding toxin component, but not of the other two toxin components, when tested by Western blotting

FIG. 2. Schematic of allelic-exchange procedure. (a) A modified chromosomal segment containing a deletion of *orfY* with flanking *orfX* and *orfZ* sequences was cloned into pBKJ236 and introduced into *B. anthracis* by conjugation. For conjugation, the desired pBKJ236 construct was first transformed into the *E. coli dam dcm* strain SCS110 (Stratagene, La Jolla, CA). It has previously been reported that propagation of plasmids in *E. coli* strains deficient in adenine and cytosine methylation increases their efficiency of transfer into *B. anthracis* (10). Overnight cultures of this strain grown in LB medium (12) plus 300 μg/ml erythromycin, SS1827 (24) grown in LB medium plus 200 μg/ml ampicillin, and *B. anthracis* Sterne grown in brain heart infusion (BHI) agar (Difco) were washed two times by pelleting them in a microcentrifuge and resuspending them in LB medium. Equal amounts of each suspension were mixed, and 150 μl was spotted onto a BHI agar plate and allowed to dry. The plates were incubated at room temperature for 24 h, at which time the accumulated growth was recovered by scraping and resuspended in 200 μl LB medium, and 150 μl was spotted onto BHI agar containing 5 μg/ml erythromycin and 60 units/ml polymyxin B. After the plates were dried and streaked for single colonies, they were incubated for 48 h at room temperature. (b) *B. anthracis* recombinants harboring the allelic-exchange construct integrated by homologous recombination between cloned and chromosomal sequences were isolated by shifting them to 37°C, a nonpermissive temperature for plasmid replication, while maintaining selection for erythromycin resistance. Exconjugant colonies arising in the previous step were inoculated into BHI broth plus 5 μg erythromycin and grown with shaking at room temperature overnight. The resulting saturated cultures were diluted 1:1,000 in fresh BHI broth plus erythromycin and incubated with shaking at 37°C until saturated (usually 6 to 8 h, although cultures incubated overnight were used successfully as well). A 200-μl sample of these cultures was spotted onto BHI agar plus erythromycin, allowed to dry, streaked for single colonies, and incubated at 37°C overnight. (c) Synthesis of the I-SceI restriction enzyme directed by pBKJ223 resulted in the cleavage of the integrated pBKJ236 vector, creating a potent substrate for bacterial-host recombinational repair systems. Integrant colonies arising in the previous step were inoculated into LB medium plus 0.1% glucose and 5 μg/ml erythromycin, grown with shaking overnight at 37°C, subcultured into fresh medium of the same composition, and used to prepare electrocompetent cells by the method of Quinn and Dancer (18). Plasmid DNA was prepared from SCS110(pBKJ223) using a spin mini-prep kit (QIAGEN, Inc., Valencia, CA). Electrocompetent cells (400 μl) and plasmid DNA (5 μl) were combined in a 0.4-cm-gap electroporation cuvette on ice. Samples were electroporated using a gene pulser (Bio-Rad, Hercules, CA) set at 2.5 V, 200 Ω, and 25 μF, returned to ice, diluted with 0.5 ml of LB medium plus glucose, incubated with shaking for 2 to 4 h, plated on BHI agar plus 10 μg tetracycline, and incubated overnight at 37°C. (d) Repair of the double-stranded break by homologous recombination between flanking repeat sequences results in plasmid excision. Recombination occurring on the same side of *orfY* as the integration event (denoted by circled "1") leads to regeneration of the wild-type sequence. Recombination occurring on the opposite side (denoted by circled "2") leads to the integration of the Δ*orfY* mutation. Tetracycline-resistant colonies arising from electroporation in the previous step were pooled, restreaked on the same medium, and incubated overnight at 37°C. This process was repeated with streaking of colonies from areas of confluent growth. Single colonies from the second streaking were patched onto BHI agar and BHI agar plus erythromycin to screen for erythromycin sensitivity. Chromosomal DNA was prepared from patches of sensitive colonies from the BHI agar (Colony Fast-Screen kit; Epicentre Biotechnologies, Madison, WI) and screened by PCR (FailSafe PCR system; Epicentre Biotechnologies, Madison, WI) with appropriate primers (see legend to Fig. 3) to detect the desired gene replacements. Strains so selected were restreaked on BHI agar and incubated overnight at 37°C, and single colonies were patched onto BHI agar and BHI agar plus tetracycline to screen for spontaneous loss of pBKJ223.

TABLE 2. Frequencies of allelic exchanges generated by the I-SceI system

Allele[a]	% of erythromycin-sensitive colonies[b]	% Allelic exchange[d]
scrB237	25	8
scrB239::*spc*	30	53
Δ*plcR240*	36	50
plcR241::*spc*	2	100
Δ*pagA242*	32	63
Δ*lef243*	36	22
Δ*cya244*	4[c]	40
Δ*spo0A245*	7	85

[a] See Table 1 for a detailed description of each allele.
[b] Percentage of 50 colonies screened after two passages in the presence of pBKJ223. Erythromycin sensitivity indicates a successful second crossover event.
[c] Two additional passages in the presence of pBKJ223 were needed to isolate erythromycin-sensitive candidates in this instance.
[d] Percentage of erythromycin-sensitive colonies incorporating the mutation as detected by PCR.

(data not shown). Finally, in order to demonstrate the use of this tool to perform sequential mutageneses, a double *lef cya* mutant (BA721) was constructed. Synthesis of protective antigen, the product of the *pagA* gene, was normal for this strain, while lethal factor and edema factor were undetectable (data not shown).

In summary, the gene replacement procedure described here has been demonstrated to perform efficiently with *B. anthracis* and has been used to achieve allelic exchange of both marked and unmarked mutations, including deletions, insertions, and gene replacements as large as 1 kb or as small as 1 bp. This procedure can be used to target both chromosomal and plasmid-borne genes and is relatively fast. Once the initial crossover event has occurred, candidates for final screening can be generated in less than a week with little effort. An additional virtue is that genetic modification of the *B. anthracis* strain of interest is not required as it is in counterselection schemes such as that based on *rpsL* mutation and streptomycin sensitivity. Indeed, the natural resistance of *B. anthracis* to the antibiotic polymyxin B renders unnecessary even the introduction of a chromosomal marker for use in selection against the donor *E. coli* in conjugation experiments. As a result, any *B. anthracis* strain can be used, and engineered strains will be isogenic with the parent strain, with the exception of the introduced mutation(s). The ability to routinely introduce engineered alleles which are not marked with antibiotic resistance allows for the serial introduction of an unlimited number of such alterations. It also expands the repertoire of mutations that can be introduced to include single-base-pair changes, thus allowing detailed genetic analyses of the structure-function relationships of proteins and DNA sites in a *B. anthracis* genetic background.

Future work is required for the refinement of this system to accommodate select agents in cases where the use of some of the antibiotic markers implemented here would be prohibited. However, much experimental work is being performed with the non-human-pathogenic strain (Sterne) used in this study. The speed and efficiency of the techniques reported here allow the construction of multiple mutations in parallel and thus will enable types of comprehensive genetic analysis that have not been feasible for *B. anthracis* to date. It is our hope that these methods will facilitate the genetic study and manipulation of this fascinating and currently all-too-important bacterial pathogen.

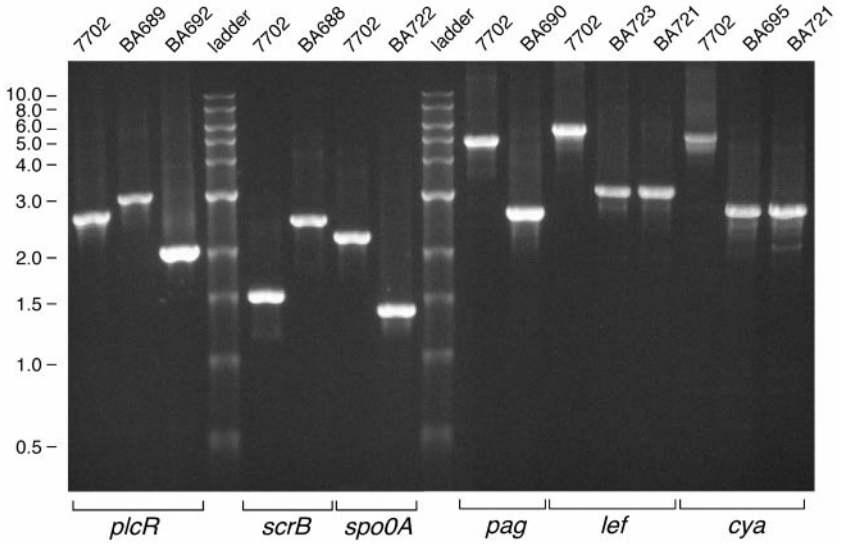

FIG. 3. Diagnostic PCR of the mutant strains made in this study. Refer to Table 1 for more information on each strain and mutant allele, including the expected size differences in the PCR products. PCR products were separated on a 1% agarose gel and visualized by staining with ethidium bromide. Primers that functioned in regions outside and flanking the cloned regions were chosen so that amplification of the plasmid-borne allele would not occur. Primers were as follows: for *plcR*, GGCATAATCAAGGTTTCTCTCACTTAAAAG and CCAAGTGAAGATTTAGCTGCATCG; for *scrB*, ATG TCAAAATATAAAACAATACTGCAATC and TCATACAATCCCTCTTTTCAGCTTATATTG; for *spo0A*, GCATAATCCCCCACAACAGGG and GAAATTAGCGAGGTTCTCACCAGATC; for *pagA*, CGCATATAAGCAAATACTTAATTGGTC and GGATAGGGTTTAACAACTTAA TAATCCC; for *lef*, CACGAGAAGAGTATTTAAAGAAAATC and AACTATAGGACAATATTCATTACCATG; and for *cya*, ATATCAAGTTTA ATTGTTAAGTTTGAAGG and CCCGCGGCCGCAACCAAATGGTTTTCATTTCTTAG.

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