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A simple method of markerless gene deletion in *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is a Gram-positive pathogen that causes opportunistic infections and a wide variety of diseases. Methicillin-resistant *S. aureus* (MRSA) is frequently isolated as multidrug-resistant in nosocomial and community infections. Molecular genetic manipulation is an important tool for understanding the molecular mechanism of *S. aureus* infection. However the number of available antibiotic markers is limited due to multidrug resistance. In this study, we constructed two *Escherichia coli–S. aureus* shuttle vectors, pKFT and pKFC, that carry a temperature-sensitive origin of replication in *S. aureus*, *lacZ*(*a*) enabling a simple blue-white screening in *E. coli*, an ampicillin resistant gene, and either a tetracycline resistance gene or a chloramphenicol resistance gene. We report a simple technique using pKFT to construct a markerless gene deletion mutant in *S. aureus* by allelic replacement without the use of a counter-selection marker. Subculture twice at 25 °C was critical to promote an allelic exchange rate in *S. aureus*. This technique is very simple and useful to facilitate genetic research on *S. aureus*.

1. Introduction

Staphylococcus aureus is a Gram positive pathogen that causes suppurative diseases, toxic shock syndrome, pneumonia, food poisoning, staphylococcal scaled-skin syndrome, and so on. It produces a large number of virulence determinants including proteases, enterotoxins, cytolytic toxins, protein A, clumping factor, and others that may play important roles in establishing and maintaining infections. Methicillin-resistant *S. aureus* (MRSA) is frequently isolated in nosocomial and, recently, community infections. The emergence of MRSA, which is resistant to β-lactam antibiotics and quite often to other chemotherapeutic agents, is a serious clinical problem (Fischbach and Walsh, 2009; Grundmann et al., 2006). To study the function of genes in *S. aureus*, classical chromosomal modification could be achieved by inactivation via single-crossover insertion, deletion via double-crossover recombination, or random insertional mutagenesis using transposon. In these steps, a suitable antibiotic resistance gene is introduced into the chromosome for selection and confirmation of the insertion or deletion of the region of interest. In *S. aureus*, several resistance genes such as tetracycline resistant genes (*tetL*, *tetK*), chloramphenicol resistant gene (*cat*), erythromycin resistant genes (*ermB*, *ermC*), ß-lactamase gene (*bla*), kanamycin resistant gene (*aphAIII*), and spectinomycin resistant gene (*aph*) have been used as selection markers (Novick, 1991). Clinically isolated *S. aureus* is frequently resistant to several antibiotics, and MRSA isolated in health care units often manifest multi-drug resistance. Therefore multiple gene deletion in a strain is extremely difficult due to the limited number of available antibiotic markers. In order to overcome this drawback, drug-resistance markerless gene deletion technique has been explored. In *S. aureus*, several genetic tools have been reported including *secY* antisense-dependent repression (Bae and Schneewind, 2006), Cre-loxP system to excise resistance markers (Leibig et al., 2008), and temperature-stable ß-galactosidase for blue-white colony assay (Arnaud et al., 2004).

In this study, we report a very simple technique to achieve markerless gene deletion in *S. aureus* without any means for counter selection using ordinary *Escherichia coli–S. aureus* shuttle vectors with a temperature-sensitive origin of replication in *S. aureus*.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *S. aureus* and *E. coli* were grown with shaking in trypticase soy broth (TSB, Becton Dickinson Microbiology Systems, Cockeysville, MD) or Luria–Bertani broth (5 g yeast extract, 10 g polypeptone, 10 g NaCl per liter; pH 7.2), respectively. When necessary, ampicillin (Ap, 100 μg/ml), chloramphenicol (Cp, 10 μg/ml), or tetracycline (Tc, 3 μg/ml) were added to the medium.

2.2. DNA procedures

Routine DNA procedures such as DNA digestion with restriction enzymes, DNA ligations, and gel electrophoresis were performed

Abbreviations: Methicillin-resistant *Staphylococcus aureus*, MRSA; trypticase soy broth, TSB.

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Table 1
Bacterial strains and plasmids used.

Strains or plasmid	Genotype or characteristics	Source or reference
Strains		
S. aureus		
RN4220	NCTC8325-4, $r^- m^+$	Kreiswirth (1983)
TY34	Clinical isolate (*eta*$^+$, *agrIII*, *mecA*$^+$)	Kato et al. (2011)
FK139	TY34 *sarS*	This work
E. coli		
DH5a	F$^-$, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk$^-$, mk$^+$), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1	TaKaRa
Plasmids		
pUC18	*E. coli* cloning vector	TaKaRa
pHY300PLK	A shuttle vector between *E. coli* and *S. aureus*	TaKaRa
pCL52.1	8.0 kbp temperature-sensitive shuttle vector; Spc^r in *E. coli*, Tet^r in *S. aureus*	Subrata et al. (1997)
pCL15	7.0 kbp IPTG-inducible vector, P_{spac}, Amp^r in *E. coli*, Cm^r in *S. aureus*	Luong and Lee, (2006)
pKFT	5.7 kbp temperature-sensitive shuttle vector; Amp^r Tet^r in *E. coli*, Tet^r in *S aureus*	This work
pKFC	5.1 kbp temperature-sensitive shuttle vector; Amp^r in *E. coli*, Cm^r in *S aureus*	This work
pFK25	pKFT containing regions upstream and downstream of the *sarS* gene	This work

essentially as described previously (Sambrook et al., 1989). The oligonucleotides used in this study are described in Table 2. PCR was performed using the Ex Taq polymerase (TaKaRa Bio, Japan) using the appropriate cycling conditions.

2.3. Construction of temperature-sensitive shuttle plasmids for gene manipulation in S. aureus

The temperature-sensitive *S. aureus* plasmid pKFT was constructed as follows.

The tetracycline resistance gene (*tetL*) was amplified from pHY300PLK (TaKaRa Bio, Japan) using primers TetLF and TetLR, blunt-ended with Mighty Cloning Reagent Set (TaKaRa Bio, Japan), and inserted into the SspI site of pUC18, yielding pTet. A fragment carrying the pE194ts replicon was amplified from pCL52.1 (Subrata et al., 1997) using primers, TsoriF and TsoriR. The resulting PCR product was digested with AatII, and cloned into the same site of the pTet, yielding pKFT (Fig. 1). On the other hand, the plasmid pKFC was constructed as follows. A fragment carrying the pE194ts replicon was amplified from pCL52.1 using primers TsoriF and TsoriR. The resulting PCR product was digested with AatII, and cloned into the AatII site of pUC18, yielding pTS. A chloramphenicol resistance gene (*cat*) was amplified from pCL15 (Luong and Lee, 2006) using primers, CMF and CMR, digested with NdeI and inserted into the same site of the pTS, yielding pKFC (Fig. 1).

Table 2
Oligonucleotide primers used.

Primer	Sequence (5′ to 3′)[a]
TsoriF	TACGAT**GACGTC**TTTTGCGCAGTCGGC
TsoriR	ATA**GACGTC**GTGAGAAACAGCGTACAG
TetLF	TTATTGCAATGTGGAATTCGGAACGG
TetLR	CCGGGAATTCCTGTTATAAAAAAAGG
CMF	TT**CATATG**CCGGCAATAGTTACCCTT
CMR	TT**CATATG**GATCTGGAGCTGTAATAT
sarS1	GCCA**AAGCTT**ATACATGGCTAGTCGG
sarS2	GAAA**GTCGAC**GCTTGTTAACAATAAC
sarS3	CATT**GTCGAC**ATTAAACCTCAGCAC
sarS4	TCAA**GGATCC**ATAGAAGGCGCTTTG
sarS5	CCAAGGATTACTCATTACAACGAAGC
sarS6	ACATTAGTGCTTTGGCTTGGGTCGTC
pUC-UV	CGACGTTGTAAAACGGCCAGT
pUC-RV	CACAGGAAACAGCTATGACCATG

[a] Sequence letters in boldface represent restriction sites.

2.4. Markerless sarS gene deletion using pKFT

A 711-bp DNA fragment containing an upstream region of *sarS* locus was amplified from MW2 genome DNA using primers sarS1 and sarS2. The resultant PCR product was digested with HindIII and SalI, and subcloned into the same site of pKFT to obtain pKFTsarSF. Likewise, a 646-bp DNA fragment containing a downstream region of *sarS* locus was amplified with primers sarS3 and sarS4. The resultant PCR product was digested with SalI and BamHI, and subcloned into the same site of pKFTsarSF, yielding the allelic replacement vector pFK25. The resulting pFK25 was first transformed into DNA restriction system-deficient *S. aureus* RN4220, then a modified plasmid was isolated and electroporated into *S. aureus* TY34 (Kato et al., 2011). Transformants were selected at 30 °C on TSB plates containing tetracycline. Then, transformants were grown at 30 °C with shaking in 3 ml TSB containing tetracycline. Integration of the plasmid into the chromosome by a single crossover event was achieved by incubation at 42 °C, a nonpermissive temperature, on TSB plates containing tetracycline. Correct homologous recombination of the target region was verified by PCR using primers, pUC-UV (plasmid) and sarS5 (chromosome). Then, these integrants were grown at 25 °C or 37 °C overnight with shaking in 10 ml TSB without any antibiotics. 10 μl of the culture was transferred into 10 ml fresh TSB and incubated until full growth at 25 °C or 37 °C. The cells were serially plated on TSB plates at 42 °C. The excision of the plasmid region in the chromosome by a double-crossover event was screened for tetracycline-sensitive colonies by replica-plating candidates on TSB plates versus TSB plates containing tetracycline (3 μg/ml). Culture of the integrant at 25 °C or 37 °C and subsequent replica-plating were independently performed three times, and the data is shown as the total colony count ($n = 100$ in each group). Then, the markerless deletion mutants were screened by PCR using primers sarS5 and sarS6 from tetracycline-sensitive colonies.

2.5. Preparation of cell surface protein fraction and Western blot analysis

Cell surface protein fraction was prepared as described previously (Cheung and Fischetti, 1998). Strains were grown with shaking at 37 °C for 6 h in 3 ml TSB and sub-cultured into 30 ml fresh TSB adjusted to an initial OD_{660nm} of 0.02. The cultures were then incubated with shaking at 37 °C for 4 h. The cell surface proteins were prepared as follows: cells harvested by centrifugation were suspended in 6 ml of digestion buffer (30% raffinose in 0.05 M Tris [pH 7.5] with 0.145 M NaCl) containing 1 mg of lysostaphin (Wako Pure Chemical Industries, Ltd. Japan), 100 mg of DNase (Sigma-Aldrich, Japan), and 1 mM phenylmethylsulfonyl fluoride (NACALAI TESQUE, INC. Kyoto, Japan). The cell mixture was allowed to react for 1 h at 37 °C with gentle shaking. The protoplasts were then removed by centrifugation at 8000 ×*g* for 10 min, and equal aliquots from each supernatant sample were electrophoresed using a 12% polyacrylamide gel and detected by rabbit antibody conjugated HRP (ICN Pharmaceuticals, Inc). Immuno-detection of protein was performed using the ECL Western blot analysis system (GE Healthcare, Japan).

3. Results

3.1. Construction of pKFT and pKFC

Plasmids pKFT and pKFC were constructed carrying a temperature-sensitive origin of replication derived from pE194ts, the *lacZ*(*a*) gene derived from pUC18 that enables a simple blue-white screening of cloning in *E. coli*, and a tetracycline resistance gene or a chloramphenicol