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Note

Creation of deletion and insertion clonal complex 8 *Staphylococcus aureus* mutants using a common cloning vector

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ABSTRACT

Deletion and insertion clonal complex 8 *Staphylococcus aureus* mutants were created without using intermediate host *S. aureus* RN4220 or temperature-sensitive shuttle vectors. These mutants were created using a common cloning vector by passing the constructs through a modification host and recovering the electroporated cells in a large volume of medium.

Staphylococcus aureus is a clinically significant opportunistic pathogen causing a wide range of diseases, such as food poisoning, bacteremia, infective endocarditis, skin and soft tissue infections, and biofilm infections on medical implants (Tong et al., 2015). It is currently listed on the World Health Organization's priority pathogens list for research and development of new antibiotics for many of the emergent drug-resistant bacteria. To understand its physiology and pathogenesis, the Nebraska Center for Staphylococcal Research (CSR) has generated a sequence-defined transposon mutant library consisting of 1952 strains, each containing a single mutation within a nonessential gene of the epidemic community-associated methicillin-resistant *S. aureus* (CA-MRSA) isolate USA300 (Fey et al., 2013). We are interested in creating replacement deletion mutants to study gene function in *S. aureus*, particularly in clinical strains. Unfortunately, *S. aureus* has four restriction modification (RM) systems, and mutant creation in this organism has been severely hampered by these restriction modification systems, particularly the types I and IV systems, which are present in most clinical isolates (Monk et al., 2015).

Type IV RM is represented by the conserved SauUSI which recognizes and cleaves methylated cytosines (Xu et al., 2011). To bypass the type IV barrier and deliver plasmids into *S. aureus*, researchers have been using plasmids isolated from restriction-deficient *S. aureus* strain RN4220 (Kreiswirth et al., 1983) or cytosine methylation lacking *E. coli* strain DC10B (DH10B_*dcm*) (Monk et al., 2012). *S. aureus* strain RN4220 has been invaluable as an intermediate host for the transfer of plasmid DNA into clonal complex (CC) 8/sequence type 239 (ST239) hosts. However, compared to *E. coli*, it is not ideal to maintain plasmids in *S. aureus*- due to the poor quality and low yield of plasmid DNAs and the requirement of enzymatic lysis of the organism with lysostaphin for plasmid isolation. Plasmids isolated from *E. coli* strain DC10B also can bypass the type IV barrier and be transformed into *S. aureus*, but maximal transformation efficiency requires the presence of the CC-specific type I adenine methylation profile of the target strain (Monk et al., 2012).

Type I RM system consists of three components encoded by a specificity gene *hsdS*, a methylation gene *hsdM*, and a restriction gene *hsdR* (Roberts et al., 2003, Waldron and Lindsay, 2006). Genome sequences of prototypical methicillin-resistant *S. aureus* strains (Kuroda et al., 2001) revealed that most *S. aureus* strains contain two CC-specific methylation operons (*hsdMS-1* and *hsdMS-2*) located on the alpha and beta pathogenicity islands respectively for the complete methylation of targets (Monk and Foster, 2012, Monk et al., 2015). To overcome the type I barrier, some research groups have heterologously expressed the methylation operons (*hsdMS-1* and *hsdMS-2*) of the target host in *E. coli* DC10B, either from the recombinant plasmid (Jones et al., 2015) or chromosome (Monk et al., 2015). Although modifying the plasmids using the *hsd* methylation systems in the intermediate host increased the transformation efficiency, but the transformation frequencies varied with different levels of methylation on the plasmids (Jones et al., 2015). Compared to plasmids isolated from *hsdMS-1* containing *E. coli*, plasmids from *E. coli* containing *hsdMS-2* showed higher transformation efficiency (Jones et al., 2015). The transformation efficiency reached the maximum when both target recognition motif sites were methylated by *hsdMS-1* and *hsdMS-2* enzymes (Monk et al., 2015).

The *S. aureus* transposon mutant library from CSR was created in *S. aureus* strain JE2, a CC8 strain that is a derivative of the USA300 *S.*

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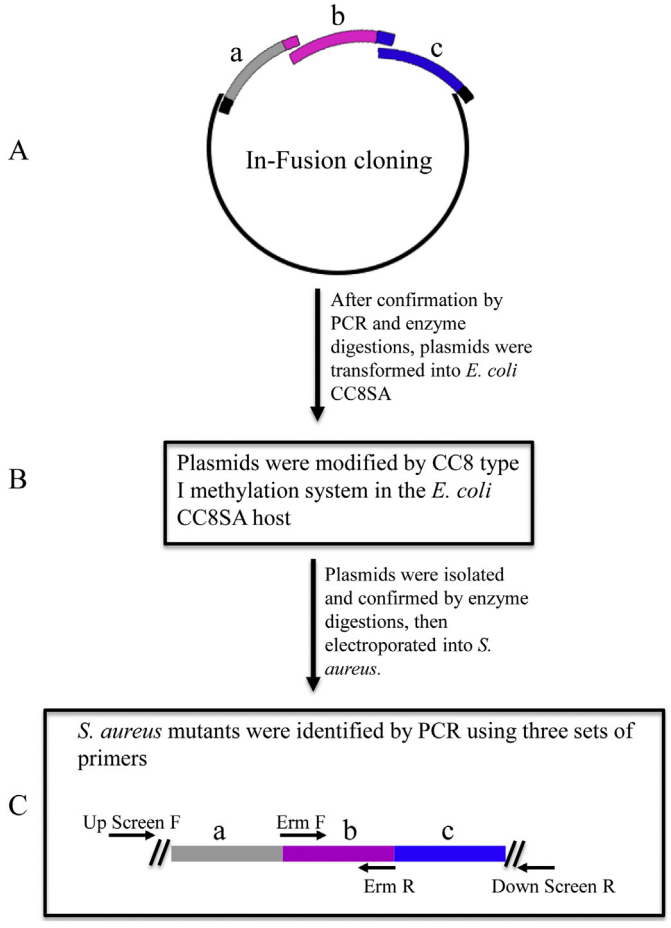

Fig. 1. Schematic of creation of deletion/insertion *S. aureus* mutants with a commonly used cloning vector pCR2.1. (A) The upstream DNA fragment (a) of the target gene, selection marker gene (b), and downstream DNA fragment (c) of the target gene were PCR amplified and cloned into vector pCR2.1. (B) The confirmed plasmids were transformed into *E. coli* CC8SA for CC8 type I methylation modifications. (C) Modified plasmids were electroporated into *S. aureus* strains using the settings described in the text. Transformants were screened for the desired mutation using three sets of primers: Up Screen F and Erm R, Erm F and Down Screen R, Erm F and Erm R. Identified mutants were further studied for changes in function.

aureus strain LAC (Los Angeles County Jail) (Fey et al., 2013). To facilitate the transformation of CC8 strains, several *E. coli* strains expressing CC8 *hsdMS-2* genes for plasmid modifications have been created. Monk et al. created an *E. coli* SA08B strain which has the *hsdMS-2* from *S. aureus* NRS384 (CC8-2) inserted into a region of the DC10B chromosome between the *atpI* and *gidB* genes that are controlled by a strong constitutive promoter (Monk et al., 2015). Jones et al. created an *E. coli* strain ALC7885 which contains the recombinant plasmid expressing *hsdMS-2* from *S. aureus* strain LAC (Jones et al., 2015). By combining the methylation capabilities of these two *E. coli* strains in constructs, we describe here how to create deletion/insertion *S. aureus* JE2 mutants using a common cloning vector pCR2.1as illustrated in Fig. 1.

In our earlier study, we showed that capsule biosynthetic genes (*cap5EF*, *I-P*) were upregulated under *S. aureus* - *Pseudomonas aeruginosa* mixed biofilm conditions, suggesting that *cap* genes in *S. aureus* could be important for interactions with *P. aeruginosa* (Miller et al., 2017). In order to confirm the critical involvement of *cap* genes, we sought to create the *cap5I* deletion mutant. Suicide plasmids were first generated using the In-Fusion® HD Cloning Plus CE (Clontech Laboratories, Inc., Mountain View, CA) as previously described (Miller et al., 2017). Primers used are listed in the supplementary table. Briefly, chromosomal regions corresponding to 1 kb up- and downstream of *cap5I* gene were amplified by PCR from *S. aureus* JE2 chromosome, and a ~1-kb erythromycin antibiotic cassette (*ermC*) was PCR-amplified from pCN57 (Charpentier et al., 2004). The three gel-purified PCR fragments were incubated with the In-Fusion HD enzyme, along with the *Eco*RI linearized pCR2.1 cloning vector and the mixture was transformed into Stellar™ competent cells (Clontech Laboratories, Inc., Mountain View, CA). Plasmids with the correct insert were identified by restriction enzyme digestion.

Before transforming these plasmids into *S. aureus* JE2, the plasmids were modified by the *S. aureus* type I RM system. To enhance the type I methylations on these plasmids, the plasmid pACYC184::*hsdMS*-2^{CC8} that was isolated from *E. coli* strain ALC7885 (kindly provided by Dr. Ambrose L. Cheung) was transformed into *E. coli* strain SA08B (purchased from Lucigen Corp, Middleton, WI). The resultant strain was designated as *E. coli* CC8SA. Although not pursued extensively, integrating transformant was not observed using plasmid isolated from ALC7885 or SA08B for electroporation. All constructs used to transform *S. aureus* strains were isolated from *E. coli* CC8SA. Approximately 30 μg of plasmid DNA was precipitated with Pellet Paint (EMD Millipore, Burlington, MA) and used for each *S. aureus* transformation. The use of 30 μg of plasmid was based on the results from our preliminary trails. No integrating transformant was obtained when less DNA was used.

As shown by previous studies, a number of factors could influence the transformation efficiency of *S. aureus* (Schenk and Laddaga, 1992). These include: (a) growth media; (b) the gap size of the electroporation cuvette; (c) the growth phase of the competent cells when harvested; and (d) the amount of media used during the recovery phase after electroporation. Similar to the earlier studies, in our optimization of transformation procedures, we found that using both B2 broth (10 g/l casamino acids, 25 g/l yeast extract, 1 g/l K2HPO4, 5 g/l D-glucose, and 25 g/l NaCl) as the growth medium for *S. aureus* and the 0.1-cm cuvettes for electroporation gave us the highest transformation efficiency. In addition, it has been reported that bacteria would activate CRISPR-Cas system(s) to target foreign DNA when the cell density was high through the quorum sensing cell-cell communication process (Hoyland-Kroghsbo et al., 2017). To minimize the potential cell density effect, a 10-ml recovery medium was used in the recovery process following electroporation.

To prepare *S. aureus* competent cells for electroporation, 2 ml of overnight culture grown in B2 broth was transferred into 25 ml of fresh B2 broth in a 500 ml flask and incubated at 37 °C with shaking at 150 rpm for 2 h (O.D. 600 ≥ 1.2). The cells were harvested and washed 3 times with an equal volume of water, and once with room temperature 0.3 M sucrose solution. The cells were suspended in 2 ml 0.5 M sucrose + 10% glycerol and incubated at room temperature for 15 min. After the incubation, the cells were pelleted by centrifugation (14,000*g* × 1 min on benchtop microcentrifuge) and re-suspended in 550 μl of 0.5 M sucrose + 10% glycerol. For electroporation, 55 μl of the cell suspension was mixed with 5 μl (approximately 30 μg) Pellet Paint precipitated Δ *cap5I* suicidal plasmid DNA isolated from *E. coli* CC8SA. Electroporation was carried out using an ECM630 Electro Cell Manipulator from BTX (Holliston, MA) in a 1-mm gap width electroporation cuvette with the following settings: 25 μF, 114 Ω, and 2.3 kV based on the optimized settings for *S. aureus* (Schenk and Laddaga, 1992). After electroporation, 10 ml pre-warmed B2 broth was added, cells were transferred to a 50-ml culture tube, and incubated for 3 h at 37 °C with shaking at 150 rpm. At the end of incubation, cells were harvested, re-suspended in 600 μl of medium, and all the cell suspension was plated on four Trypticase Soy Agar (TSA) plates (150 μl/plate) containing 25 μg/ml erythromycin. After the 24 h incubation, colonies were analyzed for proper crossover recombination by PCR. Three Δ *cap5I* suicidal plasmid single integration colonies were obtained (Fig. 2A). We attempted to use kanamycin as the selective marker for double cross over mutant(s) selection, but the kan-gene of pCR2.1 was