RESEARCH ARTICLE

Transforming the Untransformable: Application of Direct Transformation To Manipulate Genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*

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ABSTRACT **The strong restriction barrier present in *Staphylococcus aureus* and *Staphylococcus epidermidis* has limited functional genomic analysis to a small subset of strains that are amenable to genetic manipulation. Recently, a conserved type IV restriction system termed SauUSI (which specifically recognizes cytosine methylated DNA) was identified as the major barrier to transformation with foreign DNA. Here we have independently corroborated these findings in a widely used laboratory strain of *S. aureus*. Additionally, we have constructed a DNA cytosine methyltransferase mutant in the high-efficiency *Escherichia coli* cloning strain DH10B (called DC10B). Plasmids isolated from DC10B can be directly transformed into clinical isolates of *S. aureus* and *S. epidermidis*. We also show that the loss of restriction (both type I and IV) in an *S. aureus* USA300 strain does not have an impact on virulence. Circumventing the SauUSI restriction barrier, combined with an improved deletion and transformation protocol, has allowed the genetic manipulation of previously untransformable strains of these important opportunistic pathogens.**

IMPORTANCE **Staphylococcal infections place a huge burden on the health care sector due both to their severity and also to the economic impact of treating the infections because of prolonged hospitalization. To improve the understanding of *Staphylococcus aureus* and *Staphylococcus epidermidis* infections, we have developed a series of improved techniques that allow the genetic manipulation of strains that were previously refractory to transformation. These developments will speed up the process of mutant construction and increase our understanding of these species as a whole, rather than just a small subset of strains that could previously be manipulated.**

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Functional genomic analyses of the Gram-positive opportunistic pathogens *Staphylococcus aureus* and *Staphylococcus epidermidis* have been limited by the inability to manipulate genetically the majority of clinical isolates. In general, wild-type strains of *S. aureus* and *S. epidermidis* have an impenetrable restriction barrier preventing the uptake of "foreign" DNA (1–3). A plethora of knowledge has been generated through the mutagenesis of "laboratory" *S. aureus* isolates such as Newman (4), 8325-4 (5), and more recently some strains of the community-acquired methicillin-resistant *S. aureus* (CA-MRSA) USA300 (6). For *S. epidermidis*, the transformable strains 1457 (7) and O-47 (8) have been the mainstay of genetic studies, but the genome sequences for these isolates are not currently available. The emergence of CA-MRSA and the high frequency of methicillin resistance found in hospital isolates of *S. epidermidis* have highlighted the need to understand better the evolution and biology of these medically important staphylococci at a genetic level (9, 10). The majority of MRSA isolates currently cluster into five clonal complexes (clonal complex 5 [CC5], CC8, CC22, CC30, and CC45) of which only CC5 and CC8 have been successfully manipulated genetically (9). Pathogenomic studies on CC5 and CC8 strains stem from the isolation of *S. aureus* RN4220 almost 30 years ago. This is a restriction-defective mutant that can still modify DNA, which was obtained by extensive chemical mutagenesis of strain 8325-4 (11). Strain RN4220 accepts DNA derived from wild-type *Escherichia coli*, unlike the parental strain 8325-4. Passage of DNA through RN4220 has allowed the subsequent transfer of DNA by electroporation or generalized phage transduction to a small subset of closely related strains, but not to more distantly related *S. aureus* or *S. epidermidis* isolates.

Experimental evidence presented by Waldron and Lindsay (3) suggested that the conserved type I restriction-modification (RM) system was solely responsible for the inability to transform *S. aureus* isolates with *E. coli*-derived plasmid DNA. They identified a premature stop codon in the type I restriction gene (*hsdR*) in *S. aureus* RN4220. Complementation with full-length *hsdR* expressed from a low-copy-number plasmid rendered strain RN4220 incapable of accepting plasmids electroporated from *E. coli*, reduced the rate of conjugation with *Enterococcus faecalis*, and prevented transduction with phage isolated from a distantly

related strain of *S. aureus*. However, inactivation of *hsdR* (1, 2) in wild-type *S. aureus* isolates did not yield a transformable strain, suggesting at least one other pathway is involved. The gene responsible for preventing the transformation of *S. aureus* with *E. coli*-derived plasmid DNA was recently identified and characterized by Corvaglia et al. (1) in two clinical isolates of *S. aureus* (UAMS-1 and SA564). The biochemical properties of the conserved type IV modification-dependent restriction endonuclease (termed SauUSI) were subsequently characterized in detail (12). Cytosine methylated DNA was identified as the motif recognized by SauUSI, with plasmid isolated from a B strain of *E. coli* (which is naturally a *dcm* mutant) able to bypass the type IV barrier. Inactivation of both *hsdR* and *sauUSI* yielded a strain that was transformable at equivalent efficiency with plasmid DNA isolated either from *E. coli* or from the parental *S. aureus* strain. These data demonstrate that only these two pathways contribute to the restriction barrier in the strains analyzed (12).

Here we have characterized the RM systems of the widely used laboratory *S. aureus* strain Newman and corroborated the previous findings (1, 12). We have created a *dcm* mutation in the high-efficiency *E. coli* cloning strain DH10B (called DC10B). We show that direct transformation can be performed with plasmid DNA isolated from DC10B, but not the progenitor DH10B, into *S. aureus* strains from the majority of clonal complexes tested. Furthermore, we show that *S. epidermidis* contains an ortholog of SauUSI (termed McrR) which also recognizes cytosine methylation. McrR can also be bypassed with plasmid DNA isolated from DC10B. To take advantage of these findings, we have developed a robust method for creating mutations by allelic exchange in both laboratory strains and previously untransformable strains of *S. aureus* and *S. epidermidis* and have developed an improved electroporation protocol. The virulence in an intravenous mouse infection model of *S. aureus* USA300 strain NRS384 was not affected by the mutation of *hsdR* or *sauUSI* singly or in combination. These developments open up previously recalcitrant strains of *S. aureus* and *S. epidermidis* to genetic analysis, which will ultimately improve our understanding of these important pathogens.

RESULTS AND DISCUSSION

Bypassing the restriction barrier. Transformation of *S. aureus* strain Newman by the protocol of Augustin and Götz (13) with 5 μg of shuttle plasmid pRMC2 DNA (14) isolated from the wild-type *E. coli* K-12 strain BW25113 (15) consistently failed to yield transformants. Similar results were observed with plasmid isolated from the high-efficiency cloning strain of *E. coli* K-12, DH10B. However, if the plasmid was isolated from the restriction-defective *S. aureus* strain RN4220 or from strain Newman itself, we would routinely obtain 10^4 CFU with the same concentration of pRMC2. These results show that a strong restriction barrier is present in the Newman strain, which impedes the uptake of *E. coli* K-12-derived plasmid DNA. When we applied the electroporation protocol for *Staphylococcus carnosus* developed by Löfblom et al. (16), we observed a 50-fold improvement in the transformation efficiency, and for the first time, a low number of transformants were obtained with plasmid DNA isolated from strain BW25113 (Fig. 1). A second advantage of the *S. carnosus* protocol is the reduced time required for production of competent cells (2 h instead of 4 h). The protocol is also applicable to *S. epidermidis*, albeit with a marked reduction in efficiency (a maximum of 10^3 CFU was obtained in *S. epidermidis* RP62a with pRMC2 DNA isolated from the same strain).

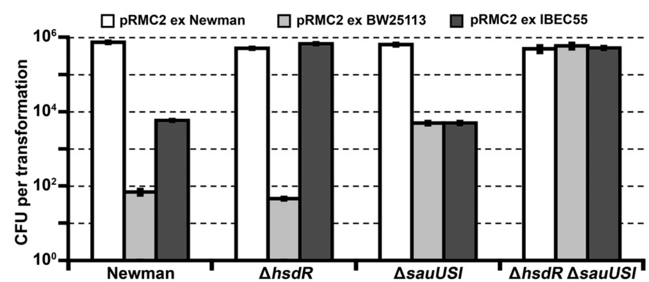

FIG 1 Transformation of *S. aureus* Newman and isogenic mutants defective in restriction. Concentrated pRMC2 DNA (5 μg) isolated from *S. aureus* Newman, *E. coli* BW25113 (dam^+ dcm^+), or *E. coli* IBEC55 (dam^+) were electroporated into Newman or Newman restriction mutants. The transformation efficiency was expressed as the mean number of all transformants obtained in each experiment ± standard deviation (error bar) of three replicates. The graph shows data representative of the data from three independent experiments.

Construction of pIMAY. The role of RM was assessed by Corvaglia et al. (1) in two clinical strains using the targetron system (17) for gene disruption rather than the more traditional allelic exchange approach for the creation of marked or unmarked mutations. The targetron system is useful for the rapid disruption of genes and for testing gene essentiality. However, allelic exchange allows precise control over the mutation allowing the deletion of entire genes, introduction of point mutations, insertion of hybrid genes, and gene restoration to eliminate the possibility of polar effects (18). The plasmid pE194ts replicon is commonly used for allelic exchange in staphylococci, as exemplified by pKOR1 (19). Chromosomal integration of the plasmid requires growth at the high temperature of 43°C (19). It was reported recently that mutations in the *sae* genes encoding a two-component system can be selected during growth at high temperature in the presence of the antibiotic erythromycin or chloramphenicol (20). To avoid these concerns, we have developed a new vector for allelic exchange in staphylococci called pIMAY (Fig. 2 and 3A). The vector utilizes the plasmid pWV01ts replicon (21), which is highly temperature sensitive in staphylococci and thus allows plasmid integrants to be selected at 37°C. PCR can easily be applied to (i) demonstrate that extrachromosomal plasmid is no longer present with primers external to the multiple cloning site (MCS) on pIMAY (IM151 and IM152 [IM151/IM152] [Fig. 3B]) and (ii) determine whether plasmid integration has occurred via the upstream or downstream region of homology cloned into pIMAY (Fig. 3C). Growth at a temperature permissive for pIMAY replication (below 30°C) and the induction of *secY* antisense RNA (derived from pKOR1) prevents growth of cells that retain the integrated plasmid and selects for cells that have lost the plasmid (19). Additionally, a high level of chloramphenicol (Cm) resistance is obtained by expression of *cat* from a strong promoter (22). This reduces the pressure for selection of variants with increased Cm resistance, which, combined with the low copy number of the plasmid, lessens the chance of tandem duplication occurring during chromosomal integration.

Deletion of *hsdR* and *sauUSI* in *S. aureus* Newman. To assess the roles of *hsdR* (encodes the restriction component of a type I RM system) and *sauUSI* (encodes a type IV restriction gene) in *S.*

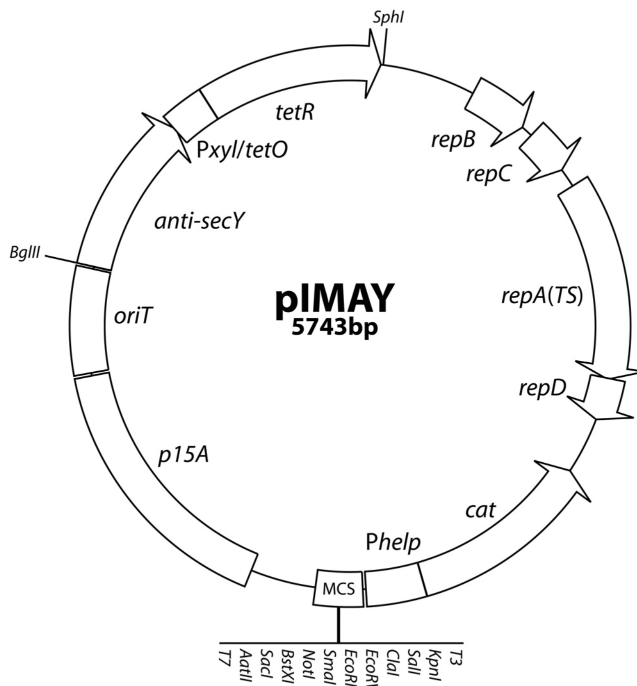

FIG 2 Genetic map of pIMAY. The *E. coli*/staphylococcal temperature-sensitive plasmid pIMAY comprises the low-copy-number *E. coli* origin of replication (p15A), an origin of transfer for conjugation (*oriT*), the pBluescript multiple cloning site (MCS), and the highly expressed *cat* gene (P*help-cat*) derived from pIMC (34). The temperature-sensitive replicon for Gram-positive bacteria (*repBCAD*) and the tetracycline-inducible antisense *secY* region (*anti-secY*) were amplified from pVE6007 (21) and pKOR1 (19), respectively. The restriction sites listed are unique. Primers (IM151/152) bind external to the MCS of pIMAY and are used to screen clones in *E. coli* (amplify 283 bp without a cloned insert) and to determine the presence of a replicating plasmid in staphylococci.

aureus Newman, we created unmarked deletion mutations in each of the genes and a double mutant with pIMAY. No differences in delta toxin production, hemolysis, growth rate, or final growth yield were observed for the mutants compared to Newman (data not shown) indicating the integrity of Agr and Sae. We then transformed wild-type Newman and Newman Δ*hsdR*, Newman Δ*sauUSI*, and Newman Δ*hsdR* Δ*sauUSI* mutants with pRMC2 DNA isolated from either Newman, *E. coli* BW25113 (*dam*$^+$ *dcm*$^+$ *hsd*$^+$) or *E. coli* IBEC55 (*dam*$^+$ only) (15). In line with the experimental evidence presented for the clinical isolate UAMS-1 (1), the Newman Δ*hsdR* Δ*sauUSI* mutant exhibited the highest transformation efficiency, which was equivalent to wild-type Newman transformed with plasmid DNA isolated directly from Newman (Fig. 1). In contrast, a ca. 130-fold reduction in the transformation efficiency of strain Newman was observed with plasmid DNA isolated from *E. coli* IBEC55. Transformation of the Δ*hsdR* mutant with plasmid DNA isolated from strain IBEC55 yielded a high transformation efficiency equivalent to that of DNA isolated from Newman or transformation into Newman Δ*hsdR* Δ*mcrR* mutant. Our data have also shown that the role of the type I RM system in Newman is to prevent the uptake of DNA from other staphylococci as well as from foreign sources. Plasmid DNA isolated from either Dcm$^+$ or Dcm$^-$ *E. coli* was transformed into the *sauUSI* mutant at a ca. 100-fold reduced level compared to plasmid DNA isolated from wild-type Newman (Fig. 1). The gene upstream of *sauUSI* is predicted to encode a nudix hydrolase which is potentially involved in the degradation of mutagenic nucleotide triphosphates (23). Deletion of this gene did not yield the same transformable phenotype observed in the Δ*sauUSI* strain (data not shown), which suggests that the putative nudix hydrolase is not required for SauUSI activity.

In *S. aureus* RN4220, premature stop codons are present in *hsdR* and *sauUSI*, producing a strain that can modify, but not restrict, foreign DNA. We restored the *sauUSI* mutation to the wild type, creating the RN4220 *sauUSI*$^+$ strain, which was poorly transformable with *E. coli*-derived pRMC2 DNA with 10^2 transformants compared to 10^6 CFU for RN4220. This confirms a dominant role for *sauUSI* over *hsdR* as was also observed in Newman.

C-terminally His-tagged SauUSI is an endonuclease. To characterize the barrier to transformation encoded by *sauUSI*, we assessed the function of the purified SauUSI protein. The full-length SauUSI protein of Newman was expressed in *E. coli* BL21(DE3) from pET21d+ (C-terminal hexahistidine tag vector) and purified by nickel affinity chromatography, yielding SauUSI-his. We then tested the effect of incubating SauUSI-his with pRMC2 DNA isolated from a panel of isogenic methylation-defective mutants of *E. coli* BW2511. The pRMC2 DNA profiles (Fig. 4A) demonstrate that SauUSI-his can digest plasmid DNA isolated from both *E. coli* BW25113 (*dam*$^+$ *dcm*$^+$ *hsd*$^+$) and IBEC56 (*dcm*$^+$ only), while pRMC2 isolated from *E. coli* IBEC55 (*dam*$^+$ only), IBEC57 (*hsd*$^+$ only), and IBEC58 (unmethylated) was unaffected. This indicates that cytosine methylation is the signal that is recognized by SauUSI. This occurs in all strains derived from *E. coli* K-12 (e.g., DH5α, TOP10, or XL1-Blue) but not in those derived from *E. coli* B (e.g., BL21). For this reason, we could express SauUSI-his in strain BL21 without toxicity. There was also a strict requirement for ATP, as DNA digestion was not observed when ATP was omitted from the buffer. The frequencies of transformation of *S. aureus* Newman and Newman Δ*sauUSI* with pRMC2 isolated from *E. coli* BW25113 and the methylation mutants were consistent with the digestion profiles, with wild-type Newman being transformed efficiently only if cytosine methylation was absent from the DNA (Fig. 4B).

Isolating mutations in *S. epidermidis* RP62a and *S. aureus* Cowan. We sought to apply these findings and to attempt allelic exchange in the genome-sequenced *S. epidermidis* strain RP62a (24) and *S. aureus* Cowan (25), two strains that we had not previously been able to manipulate genetically. We were able to transform both strains with pIMAY isolated from *E. coli* IBEC55 (at a very low efficiency for RP62a) and subsequently to construct mutations in the *sauUSI*-like genes in each. We called the gene of *S. epidermidis* RP62a *mcrR* for methylated cytosine recognition and restriction. Similar transformation profiles were obtained as observed for *S. aureus* Newman with regard to the involvement of cytosine methylation (Fig. 5). Additionally, the CA-MRSA strain NRS384 and the isogenic *sauUSI* targetron mutant also had the same transformation profile as Newman and Newman Δ*sauUSI* mutant (Fig. 5). However, Cowan exhibited a strict requirement for the presence of adenine methylation on the transforming plasmid DNA, as transformants could not be obtained in its absence in either the wild type or *sauUSI* mutant. Out of 15 *S. aureus* strains used in this study, only Cowan genomic DNA was sensitive to digestion with the restriction enzyme DpnI, which recognizes adenine methylation at a 5′ GATC 3′ motif. This suggests the

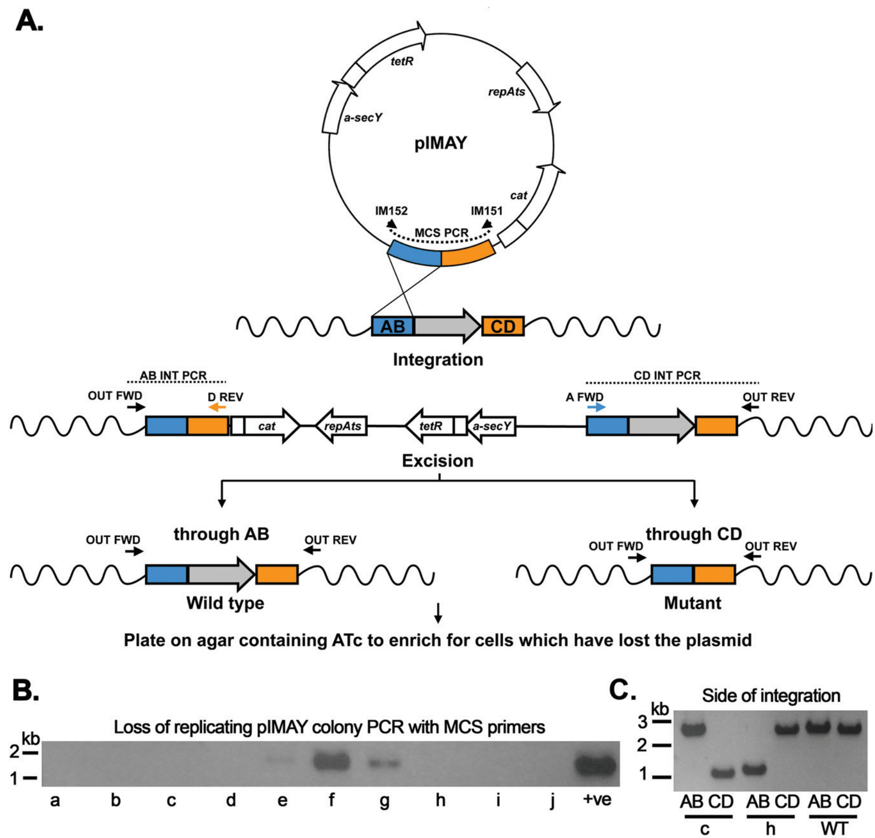

FIG 3 Schematic of allelic exchange with pIMAY. (**A**) A plasmid isolated from *E. coli* DC10B is transformed into staphylococci at 28°C, and single-crossover (SCO) integration was stimulated by growth at 37°C in the presence of chloramphenicol. The loss of replicating plasmid is assayed by colony PCR with MCS primers (IM151/IM152). Clones negative for replicating plasmid are then screened for the side of integration with a combination of chromosomal and cloning primers (e.g., OUT FWD/D REV [AB integration {AB INT}] or OUT REV/A FWD [CD integration {CD INT}]). The diagram details an integration event through the AB side (equivalent to clone h in panels B and C). A clone from either AB or CD integration event is grown at 28°C in broth without antibiotic selection to stimulate rolling circle replication and then plated on TSA with 1 μg/ml ATc. Expression of the *secY* antisense RNA (*a-secY*) inhibits growth of cells maintaining the plasmid. Plasmid excision through the AB side recreates the wild-type locus, while CD excision yields a mutated gene. (B) Colony PCR from 10 randomly chosen clones (clones a to j) after growth at 37°C for the presence of replicating plasmid. The absence of product indicates that the plasmid has integrated. Colony PCR from cells grown at 28°C is included as a positive control (+ve). (C) Two clones without replicating plasmid (clones c and h) were shown by colony PCR to have integrated either on the AB (upstream [clone h]) or CD (downstream [clone c]) side of the gene to be deleted. Wild-type (WT) genomic DNA was included as a control.

presence of a novel adenine methylase expressed by Cowan and indicates the possibility of an additional restriction system in this strain.

Creation of *E. coli* DC10B—a universal staphylococcal cloning host. *E. coli* IBEC55 is not an ideal cloning host, as it does not contain mutations associated with the production of good-quality plasmid DNA (*recA endA*) and is not highly transformable. Through recombineering (recombination-mediated genetic engineering) (26) in a high-efficiency cloning strain of *E. coli* (DH10B), we created DC10B, a mutant with the *dcm* gene deleted. Plasmid DNA isolated from DC10B was capable of transforming Newman at the same efficiency as that of IBEC55 (data not shown). Eleven representative *S. aureus* strains from a diverse selection of multilocus sequence types (STs) (Fig. 6A) and *S. epidermidis* RP62a were tested as recipients for transformation with pRMC2 DNA isolated from either DH10B or DC10B (Fig. 6B). Strains from sequence type 1 (ST1), ST8, ST10, ST22, ST30, ST36, and ST45 could be transformed only with DNA from *E. coli* DC10B, while ST5, ST25, and ST121 yielded colonies irrespective of the plasmid source. This was expected for the ST5 strain N315, because a premature stop codon is present in the *sauUSI* gene. We have not investigated the restriction status of the transformable ST25 and ST121 clones. The isolate chosen from ST97 did not transform with plasmid DNA from either DH10B or DC10B. It is possible that this strain requires different growth conditions or treatments prior to electroporation to become competent (1) or an additional RM system may be present (27). When pRMC2 was reisolated from the different hosts, no evidence of deletion or rearrangement was observed (see Fig. S1 in the supplemental material). *E. coli dam* mutants have a higher frequency of spontaneous mutation compared to wild type or *dcm* mutants (41). Thus, the *dcm* mutant of DH10B is an ideal host for the construction of recombinant plasmids for subsequent direct transformation into *S. aureus* or *S. epidermidis*. We are currently investigating the possibility of bypassing the type I restriction barriers in *S. aureus* by *E. coli* to further improve the transformation efficiency. The knowledge gained here could be applied to other bacteria where DNA uptake is impeded by RM.

Impact on virulence of restriction mutants of *S. aureus* USA300 strain NRS384. We sought to assess the role that RM systems might play in influencing the ability of bacteria to grow *in vivo*. Instead of using the laboratory strain Newman, we constructed restriction-deficient mutations in the CA-MRSA strain NRS384 (NARSA USA300-014 clone) using the targetron system. The double mutant ($hsdR^{INT}$ $sauUSI^{INT}$) was reverted by allelic exchange to restore wild-type *sauUSI* (Fig. 7A). The strains were phenotypically identical with no morphological or growth rate differences. The transformation profiles were similar to those observed for Newman. Restoration of *sauUSI* in the double mutant dramatically reduced the transformation frequency (Fig. 7B). In the AJ mouse intravenous infection model, no significant differences in the bacterial numbers recovered from the kidneys at day 7 were observed (Fig. 7C). Under the conditions tested, the conserved type I RM and type IV restriction system do not affect the virulence of *S. aureus*.

Cytosine methylation—a barrier for DNA transfer? Previously, it was shown that plasmid DNA isolated from *Enterococcus faecalis* (a major potential reservoir of vancomycin resistance)

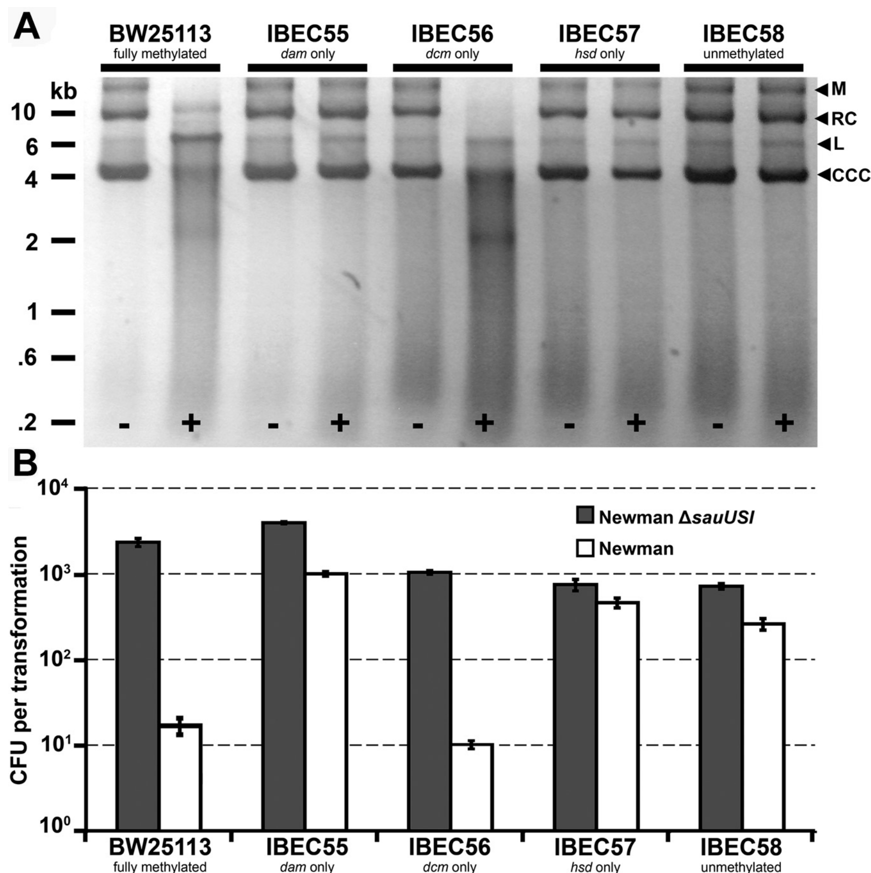

FIG 4 Assays for the activity of SauUSI. (A) The shuttle plasmid pRMC2 was isolated from isogenic E. coli methylation mutants and incubated in NEB ligation buffer with (+) or without (−) purified SauUSI-his for 1 h at 37°C. The DNA was then purified and run on a 1% agarose gel. The positions of linearized (6.4 kb) (L), relaxed circular (RC), covalently closed circular (CCC), and multimers (M) of pRMC2 are indicated by the black arrowheads to the right of the gel. (B) Concentrated pRMC2 DNA (5 μg) isolated from isogenic *E. coli* methylation mutants was electroporated into either *S. aureus* Newman or Newman Δ*sauUSI* mutant. The transformation efficiency was expressed as the total number of transformants obtained in each experiment ± standard deviation (error bar) of three replicates. The graph shows data representative of the data from three independent experiments.

could not be electroporated into wild-type *S. aureus* unless a deletion in *sauUSI* was first constructed (1). This suggests that a barrier to plasmid transfer between *E. faecalis* and *S. aureus* might be cytosine methylation. From the genome sequences of *E. faecalis* strains submitted to public databases, we could identify a putative cytosine methyltransferase in all strains examined. However, when genomic DNA was isolated from *E. faecalis* strains OG1RF, V583, and JH2-2 (grown in brain heart infusion broth [BHI] at 37°C), only DNA from OG1RF could be digested with purified SauUSI (data not shown), suggesting that some *E. faecalis* strains but not others could potentially act as reservoirs for the transfer to DNA to *S. aureus*. Naturally occurring vancomycin-resistant *S. aureus* clones are all in ST5 (CC5) (28). Both genome-sequenced isolates from CC5 (29) contain a truncated version of the *sauUSI* gene, leading to the possibility that acquisition of *vanA* mediated by conjugation from enterococci could occur in strains that lack functional *sauUSI*. Further investigation into the *sauUSI* status of vancomycin-resistant strains of *S. aureus* is required.

Summary. The availability of the *E. coli dcm* mutant (DC10B) along with an improved transformation protocol and the construction of the temperature-sensitive plasmid pIMAY has dramatically accelerated our ability to generate mutations in staphylococci. We have been successful in creating mutations in *S. aureus* strains Newman, NRS384 (USA300), and Cowan and *S. epidermidis* RP62a as described above but also in the hospital-acquired MRSA (HA-MRSA) strain MRSA252 (CC30) and two additional CA-MRSA strains (TCH1516 and LAC, both CC8). From the start of cloning to the confirmation of the mutation takes 2 weeks, under conditions that are less stressful to the bacterium to be altered than previously published protocols. Breaking the restriction barrier opens up clinical isolates such as MRSA lineages CC22, CC30, and CC45 to genetic manipulation (e.g., allelic exchange and transposon mutagenesis) and will ultimately improve our understanding of staphylococcal genetics, potentially leading to novel methods to combat these deadly opportunistic pathogens.

MATERIALS AND METHODS

Media and reagents. Bacterial strains, plasmids, and oligonucleotides used in this study are described in Table 1. *E. coli*, *S. aureus*, and *S. epidermidis* were routinely cultured at 37°C in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), Trypticase soy broth (TSB) (Difco) or brain heart infusion broth (BHI) (Difco). For growth on agar, L broth or brain heart infusion broth was solidified with 1.5% agar, yielding LBA and BHIA, respectively. The following antibiotics and concentrations were used: chloramphenicol (Cm), 10 μg/ml; kanamycin, 50 μg/ml; erythromycin (Em), 25 μg/ml; and carbenicillin, 100 μg/ml (Sigma).

Oligonucleotides and DNA sequencing were purchased from IDT. Restriction enzymes and LigaFAST T4 DNA ligase were purchased from NEB and Promega, respectively. High-fidelity PCR was performed with KOD Hotstart DNA polymerase (Novagen) or Phusion DNA polymerase (Finnzymes) on genomic DNA isolated with the Genelute bacterial genomic DNA kit (Sigma). Plasmids and PCR products were purified using Wizard*Plus* kits (Promega). To isolate plasmid DNA from *S. aureus*, a 10-ml overnight culture was treated with 100 μg lysostaphin (Ambi Products, New York) in P1 buffer for 30 min at room temperature and then processed as recommended by the manufacturer (GeneJET plamsid miniprep kit; Fermentas).

For colony PCR, a small amount of colonial growth was touched to the side of a PCR tube and microwaved for 5 min at 800 W. The tube was placed on ice, Phire Hotstart II master mix (Finnzymes) was added to the PCR tube, and thermocycling conditions were conducted as recommended by the manufacturer.

Construction of temperature-sensitive plasmids pIMC5 and pIMAY. A new temperature-sensitive vector was constructed for allelic replacement mutagenesis in *S. aureus*. The vector is based on the replicon of the lactococcal plasmid pWV01ts (from pVE6007 [21]) rather than the commonly used staphylococcal pE194ts replicon (30). A hybrid vector

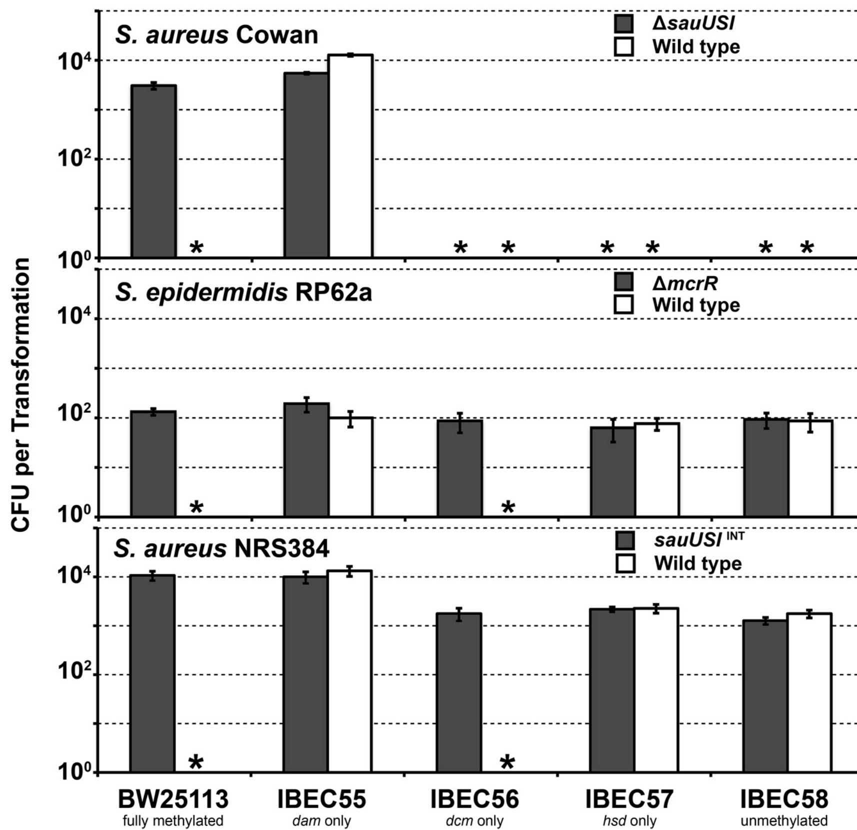

FIG 5 Transformation of *S. aureus* Cowan, *S. epidermidis* RP62a, and *S. aureus* NRS384. pRMC2 DNA (5 μg) isolated from isogenic *E. coli* methylation mutants was electroporated into either the wild type or the corresponding Δ*sauUSI* or Δ*mcrR* mutant of the strain specified. The transformation efficiency is expressed as the total number of transformants obtained in each experiment with standard deviation of three replicates. An asterisk denotes that no transformants were detected.

(pIMC5) was created by spliced overlap extension (SOE) PCR. It comprises (i) the *repBCAD*(Ts) genes from pVE6007 and (ii) the *E. coli* backbone p15A *rep* and pBluescript KS multiple cloning site and the highly expressed chloramphenicol acyltransferase marker from pIMC (31). The counterselection marker encoding tetracycline-inducible antisense *secY* RNA was amplified from pKOR1 and introduced between the novel BglII and SphI sites to form pIMAY.

Electroporation. Electroporation was conducted essentially as described by Löfblom et al. (16). Overnight cultures of *S. aureus* or *S. epidermidis* were grown in 10 ml of either TSB or BHI (in 50-ml tubes) and then diluted to an optical density at 578 nm (OD_{578}) of 0.5 in fresh prewarmed media. The cultures were reincubated for 30 min and then chilled in an ice slurry for 10 min, with all subsequent steps performed at 4°C on ice. The cells were harvested at 4,000 × *g* for 10 min, and the pellets were resuspended in an equal volume of autoclaved ice-cold water. The centrifugation and resuspension steps were repeated. The cells were then repeatedly centrifuged and resuspended first in 1/10, then in 1/25, and finally in 1/200 the volume of autoclaved ice-cold 10% (wt/vol) glycerol. Aliquots of 50 μl were frozen at −70°C. For electroporation, cells were thawed on ice for 5 min and then left at room temperature for 5 min before being centrifuged (5,000 × *g* for 1 min) and resuspended in 50 μl of 10% glycerol and 500 mM sucrose (filter sterilized). Plasmid DNA (5 μg) was precipitated with pellet paint (Novagen) and added to the cells, transferred to a 1-mm electroporation cuvette (Bio-Rad) at room temperature, and pulsed at 21 kV/cm, 100 Ω, and 25 μF. The cells were incubated in 1 ml of TSB supplemented with 500 mM sucrose (filter sterilized), incubated at 28°C or 37°C for 1 h before plating on BHIA plus 10 μg of Cm per ml (Cm10), and incubated at 28°C or 37°C.

Allelic exchange with pIMAY. Deletion constructs for the *hsdR*, *sauUSI*, $sauUSI^{ECORV}$, and *mcrR* genes and a putative nudix hydrolase-encoding gene (Table 1) were PCR amplified as follows. A sequence upstream of the gene to be deleted was amplified with oligonucleotides A and B (A/B) (up to the start codon) and the downstream sequence with oligonucleotides C/D (down from the stop codon) separately. The upstream and downstream PCR products were

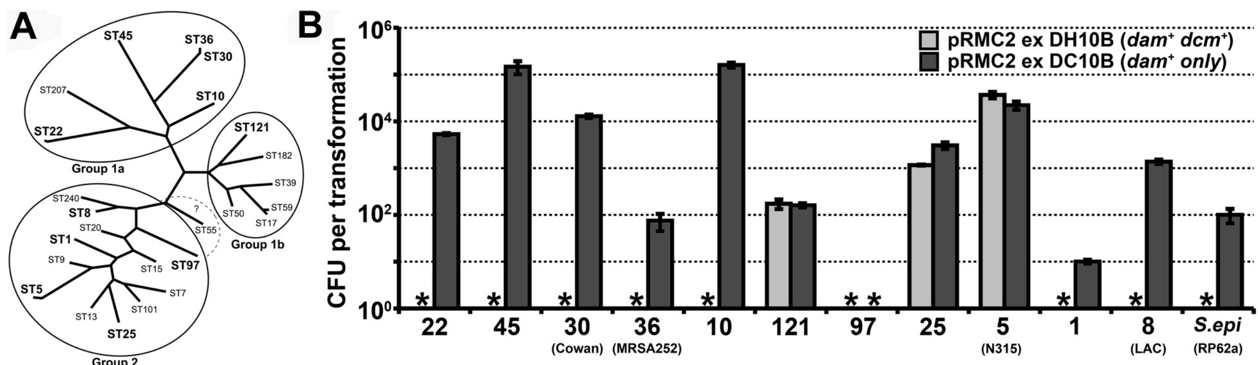

FIG 6 Transformation of strains from a diverse selection of *S. aureus* sequence types and an *S. epidermidis* isolate. (A) Phylogenetic relatedness of the *S. aureus* strains selected for transformation, adapted from reference 35 with permission of the publisher. Representative sequence types used for transformation are highlighted in bold type. (B) Concentrated pRMC2 DNA (5 μg) isolated from *E. coli* DH10B (dam^+ dcm^+) or DC10B (dam^+) was electroporated into strains from different *S. aureus* STs (denoted by the number on the *x* axis) including strains Cowan, MRSA252, N315, LAC, and *S. epidermidis* RP62a. The transformation efficiency was expressed as the total number of transformants obtained in each experiment ± standard deviation of three replicates. An asterisk denotes that no transformants were detected. The graph shows data from one experiment.

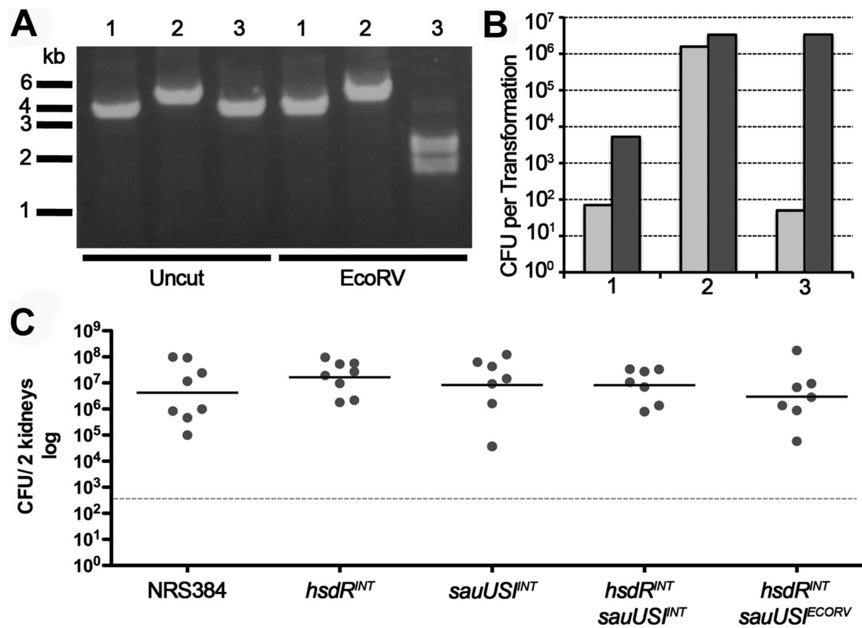

FIG 7 Transformation and virulence of restriction mutants of *S. aureus* USA300 strain NRS384. (A) PCR profiles (primers IM110/IM111) of the *sauUSI* region amplified from NRS384 (lanes 1), NRS384 *hsdR*^{INT} *sauUSI*^{INT} (lanes 2), and NRS384 *hsdR*^{INT} *sauUSI*^{ECORV} (lanes 3) without or with EcoRV digestion. (B) Concentrated pRMC2 DNA (5 μg) isolated from *E. coli* DH10B (*dam*$^+$ *dcm*$^+$) (light grey bars) or DC10B (*dam*$^+$) (dark grey bars) was electroporated into the strains described above, and transformants were enumerated. (C) Intravenous injection of 2 $\times$ 10^6 CFU into 6- to 7-week-old female A/J mice. On day 7 of infection, the mice were euthanized, both kidneys were aseptically removed, and the bacterial CFU were enumerated as described in Materials and Methods. Each symbol represents the value for an individual mouse, and the short black line represents the mean for the group of mice. The broken line denotes the limit of detection at 333 CFU for the two kidneys.

diluted 1:20, and 1 μl of each was used as the template in a second SOE PCR with the A/D primers. Deletion constructs were cleaved at endonuclease sites introduced into A and D primers during PCR and ligated into pIMAY cut with the same enzymes and then transformed into *E. coli* DC10B. The plasmid DNA was sequenced. The DNA was then electroporated into the target strain and plated onto BHIA plus Cm10 at 28°C.

To correct the premature stop codon in the *sauUSI* gene of *S. aureus* RN4220, the wild-type sequence from *S. aureus* 8325-4 was amplified as a 1-kb fragment centered on the RN4220 premature stop codon and then processed as described above.

To complement the *sauUSI* mutation in *S. aureus* NRS384 *hsdR*^{INT} *sauUSI*^{INT}, the *sauUSI* deletion mutant was reverted to the wild type by allelic exchange. To differentiate NRS384 *hsdR*^{INT} from the NRS384 *hsdR*^{INT} *sauUSI*^{ECORV} complemented strain, a new EcoRV restriction site (http://emboss.bioinformatics.nl/cgi-bin/emboss/silent) was introduced into the complementation construct without altering the coding sequence. Phenotypically, no differences were observed between NRS384 *hsdR*^{INT} and NRS384 *hsdR*^{INT} *sauUSI*^{ECORV} mutants.

To integrate pIMAY into the chromosome, a single colony from the transformation plate was homogenized in 200 μl of TSB. The suspension was diluted 10-fold to 10^{-3}, and 100 μl of each dilution was spread on BHIA plus Cm10 and incubated overnight at 37°C. For *S. epidermidis*, a colony from the transformation plate was inoculated into BHI plus Cm10 and grown overnight at 37°C, and diluted suspensions of the bacterial growth were plated for single colonies. For both *S. aureus* and *S. epidermidis*, large colonies were streaked on BHIA plus Cm10 and incubated overnight at 37°C, and colony PCR analysis was performed to determine (i) the absence of extrachromosomal plasmid DNA (with MCS oligonucleotides IM151/152) (Fig. 3B) and (ii) whether plasmid integration had occurred upstream or downstream of the gene (OUT F/D Rev oligonucleotides or OUT R/A Fwd oligonucleotides, e.g., *hsdR* IM5/94 or IM6/93) (Fig. 3C). Overnight cultures of both the upstream or downstream crossover that were free of replicating plasmid were grown at 28°C without chloramphenicol and then plated onto BHIA containing 1 μg/ml anhydrotetracycline (Vetranal; Sigma) (BHIA plus ATc). The plates were incubated at 28°C for 2 days. Large colonies were patched on BHIA plus ATc and BHIA plus Cm10 and grown at 37°C overnight. Chloramphenicol-sensitive colonies were screened by colony PCR with oligonucleotides to identify clones containing the desired mutation (OUT F/OUT R [e.g., Δ*hsdR*-IM5/6]). Putative mutants were validated by PCR amplification of genomic DNA flanking the deletion and DNA sequencing.

Purification of SauUSI. The entire *sauUSI* gene was amplified from *S. aureus* Newman genomic DNA with primers IM196 and IM197, digested with NcoI and XhoI, and cloned into the C-terminal hexahistidine tag vector pET21d$^+$. Plasmid DNA was transferred from *E. coli* DH10B to BL21(DE3) for protein expression. An overnight culture was diluted 1:100 in fresh L broth (500 ml) and grown at 37°C to an OD_{600} of 1.0. The temperature of the culture was reduced to 28°C, and expression of SauUSI was induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG). After 3 h, the induced cells were harvested by centrifugation (4,000 $\times$ *g* for 20 min at 4°C). The cell pellet was suspended in 10 ml of native *E. coli* lysis buffer (50 mM HEPES [pH 8], 500 mM NaCl, 10 mM imidazole, 5% glycerol, 1 complete protease inhibitor tablet [Roche] containing 125 U Benzonase [Novagen] and 10 μg of lysozyme). The cells were frozen at $-$70°C and then freeze-thawed 3 times before centrifugation (20,000 $\times$ *g* for 30 min at 4°C). The supernatant was filter sterilized (0.45-μm filter) before it was passed twice through Histrap FF column (GE Healthcare) equilibrated with native lysis buffer at 1 ml/min. The column was then washed with 25 ml of wash buffer (native *E. coli* lysis buffer containing 30 mM imidazole). The protein was eluted from the column in ten 500-μl aliquots of native lysis buffer containing 250 mM imidazole. The SauUSI-his eluates were visualized on a 10% SDS-polyacrylamide gel, and the protein-containing aliquots were combined. The imidazole in the buffer was diluted (112,500 times) by centrifugation three times through a 50-kDa-molecular-size-cutoff filter (Millipore) with the volume made up to 15 ml with 50 mM HEPES [pH 8], 500 mM NaCl, 5 mM $MgCl_2$, and 5% glycerol (32). Aliquots were stored at 4°C.

Activity of SauUSI. pRMC2 plasmid DNA (1 μg) isolated from isogenic *E. coli* strains derived from strain BW25113 containing different methylation enzymes were mixed with SauUSI-his (ca. 425 ng) in ligation buffer (50 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$, 10 mM dithiothreitol [DTT], 1 mM ATP [final concentration]) and incubated at 37°C for 30 min. To separate the DNA from SauUSI, an equal volume of membrane binding solution (Promega) was added, and the DNA was ethanol precipitated with pellet paint. The samples were then run on a 1% agarose gel and compared to an untreated plasmid DNA control.

Recombineering in *E. coli* DH10B. Strain DH10B is a high-efficiency cloning strain of *E. coli* K-12 (Invitrogen). Even though it has the *recA1* mutation, in our hands, it is still amenable to genetic manipulation by recombineering. The plasmid pKD46 (26) was transformed into strain DH10B using the electroporation protocol of Sheng et al. (33) and se-

TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Bacterial strain, plasmid, or oligonucleotide[a]	Description (relevant genotype or phenotype) or sequence (5′ to 3′)[b]	Source, reference, or RE site[c]
Escherichia coli strains		
DH10B (K-12 strain)	*dam*$^+$*dcm*$^+$ Δ*hsdRMS endA1 recA1*	Invitrogen
BW25113 (K-12 strain)	*dam*$^+$*dcm*$^+$ *hsdMS*$^+$ *hsdR514*	15
IBEC55	Δ*dcm* Δ*hsdMS* in the BW25113 background; Dam methylation only	15
IBEC56	Δ*dam* Δ*hsdMS* in the BW25113 background; Dcm methylation only	15
IBEC57	Δ*dam* Δ*dcm* in the BW25113 background; Hsd methylation only	15
IBEC58	Δ*dam* Δ*dcm* Δ*hsdMS* in the BW25113 background; no methylation	15
DC10B	Δ*dcm* in the DH10B background; Dam methylation only	This study
BL21(DE3) (B strain)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3); IPTG-inducible T7 RNA polymerase	Novagen
Staphylococcus strains		
Newman	ST8; CC8 isolated in 1952 human clinical MSSA; genome sequenced	4
Newman Δ*hsdR*	Newman with a deletion of Sae0139	This study
Newman Δ*sauUSI*	Newman with a deletion of Sae2386	This study
Newman Δ*hsdR* Δ*sauUSI*	Newman with a deletion of both Sae0139 and Sae2386	This study
Newman Δ*hsdR sauUSI*^{ECORV}	Restoration of Sae2386 in the Δ*hsdR* Δ*sauUSI* background	This study
Newman Δ*nudix*	Newman with a deletion of Sae2385	This study
NRS384	USA300-14 clone obtained from NARSA	NARSA collection
NRS384 *hsdR*^{INT}	Targetron insertion at nucleotide 735 of *hsdR*	This study
NRS384 *sauUSI*^{INT}	Targetron insertion at nucleotide 739 of *sauUSI*	This study
NRS384 *sauUSI*^{INT} *hsdR*^{INT}	Targetron insertion in *hsdR* made in the NRS384*sauUSI*^{INT} background	This study
NRS384 *sauUSI*^{ECORV} *hsdR*^{INT}	Restoration of the *sauUSI* mutation with a silent EcoRV site in the double insertion mutant background	This study
RN4220	ST8; CC8; chemically mutagenized derivative of 8325-4, transformable with *E. coli* DNA; premature stop codon in both *hsdR* and *sauUSI*	11
RN4220 *sauUSI*$^+$	Nonsense mutation in *sauUSI* corrected to wild type with 8325-4 sequence	This study
Cowan	ST30; CC30 MSSA;high-level protein A producer; ATCC 12598	25
Cowan Δ*sauUSI*	Deletion of *sauUSI*	This study
N315	ST5 CC5 MSSA; genome sequenced	29
LAC	ST8 CC8 CA-MRSA; USA300	6
MRSA252	ST36 CC30 MRSA; genome sequenced	36
Oxford 13	ST22 CC22	37
Oxford 19	ST10 CC16	37
Oxford 71	ST1 CC1	37
Oxford 159	ST25 CC25	37
Oxford 207	ST15 CC15	37
Oxford 233	ST45 CC45	37
Oxford 560	ST121 CC51	37
Oxford 3177	ST97 CC16	37
RP62a	Methicillin-resistant, biofilm-forming *Staphylococcus epidermidis* isolate; genome sequenced	24
RP62a Δ*mcrR*	Deletion of Serp2052; able to accept DNA at a low frequency from wild-type *E. coli*	This study
Enterococcus faecalis strains		
OG1RF	Rifampin- and fusic acid-resistant *E. faecalis* clone derived for OG1	38
JH2-2	Rifampin- and fusic acid-resistant *E. faecalis* clone derived for JH2	39
V583	Vancomycin-resistant clinical isolate of *E. faecalis*	40
Plasmids		
pNL9164	Temperature-sensitive targetron plasmid for *S. aureus* pT181 replicon; Amp^r Ery^r	Sigma
pNL9164(*hsdR*)	pNL9164 retargeted for *hsdR* of NRS384	This study
pNL9164(*sauUSI*)	pNL9164 retargeted for *sauUSI* of NRS384	This study
pKD4	Plasmid for amplification of *frt-kan-frt* for *E. coli* gene deletion; Amp^r Kan^r	26
pKD46	*E. coli* temperature-sensitive plasmid containing λ red recombinase genes under the control of an arabinose-inducible promoter; Amp^r	26
pCP20	*E. coli* temperature-sensitive plasmid containing *flp* required for antibiotic marker excision; Amp^r Cm^r	10
pIMC	Site-specific integrating vector; p15A low-copy-number origin of replication; RP4 conjugative origin of transfer and Phelp-driven chloramphenicol resistance marker; pBluescript MCS; Cm^r	34
pKOR1	Temperature-sensitive shuttle vector for allelic exchange in *S. aureus*; Amp^r Cm^r	19
pVE6007	pWV01ts-derived plasmid that cannot replicate in *E. coli*; Cm^r	21
pIMC5	Temperature-sensitive Gram-positive replicon from pVE6007 with an *E. coli* replicon; MCS and antibiotic resistance from pIMC; Cm^r (IM46/IM47/IM48/IM49)	This study
pIMAY	pIMC5 with tetracycline; inducible *secY* antisense from pKOR1; Cm^r (IM72/IM73)	This study
pIMAYΔ*hsdR*	A deletion encompassing the entire *hsdR* gene (between the ATG and TAA codons); amplified from Newman (IM93/IM3/IM4/IM94)	This study

(Continued on following page)

TABLE 1 (Continued)

Bacterial strain, plasmid, or oligonucleotide[a]	Description (relevant genotype or phenotype) or sequence (5′ to 3′)[b]	Source, reference, or RE site[c]
pIMAYΔ*sauUSI*(CC8)	A deletion encompassing the entire *sauUSI* gene (between the ATG and TAA codons); amplified from Newman (IM89/IM90/IM91/IM92)	This study
pIMAYΔ*sauUSI*(CC30)	A deletion encompassing the entire *sauUSI* gene (between the ATG and TAA codons); amplified from Cowan (IM89/IM90/IM91/IM150)	This study
pIMAYΔ*mcrR*(*S.epi*)	A deletion encompassing the entire *mcrR* gene (between the ATG and TAA codons); amplified from RP62a (IM216/IM217/IM218/IM219)	This study
pIMAY *sauUSI*^{EcoRV}	A silent EcoRV site was introduced into the middle of the *sauUSI* gene (with DNA flanking for gene restoration in the Δ*sauUSI* mutant) (IM89/IM350/IM351/IM92)	This study
pIMAY(RN4220*sauUSI*$^+$)	A 1-kb fragment amplified from Newman surrounding the premature stop codon in RN4220 *sauUSI* (IM108/IM109)	This study
pIMAYΔ*nudix*	A deletion encompassing the entire putative nudix gene (between the ATG and TAA codons); amplified from Newman (IM222/IM223/IM224/IM225)	This study
pET21d$^+$	C-terminal hexahistadine tagging vector; Amp^r	Novagen
pET21d$^+$*sauUSI*	The entire *sauUSI* gene amplified from Newman and fused to a C-terminal His tag (IM196/IM197)	This study
Oligonucleotides		
IM46 (pVE6007 F)	ATATGCATGCGTTTTAGCGTTTATTTCGTTTAGTTATCGG	SphI
IM47 (pVE6007 R)	GTATTGCTATTAATCGCAACATCAAACC	
IM48 (pIMC F)	GATGTTGCGATTAATAGCAATACATTCTATAATAGAAGGTATGGAGGATG	
IM49 (pIMC R)	AGATCTCCTCTCGCCTGTCCCCTCAGTTCAGTAATTTCC	BglII
IM72 (anti *secY* F)	ATATAGATCTTGATCTAATGATTCAAACCCTTGTG	BglII
IM73 (anti *secY* R)	ATATGCATGCTGAAGTTACCATCACGGAAAAAGG	SphI
IM93 (Δ*hsdR*-AFwd)	ATATGGTACCGTGGCCACACATTACAGTATTCCC	KpnI
IM2 (Δ*hsdR*-B)	CATTCATATCCCCTTCCATACACTTTCTATTGC	
IM3 (Δ*hsdR*-C)	*TATGGAAGGGGATATGAATG*TAATGATTCAGCCCCCTCGCTAGATTAGTG	
IM94 (Δ*hsdR*-DRev)	ATATGAGCTCATTCATCTTTGTATTCTTTCATGTTTCC	SacI
IM5 (*hsdR*-outF)	AGTCATAGTGAATTGCAGTCAATTGC	
IM6 (*hsdR*-outR)	ATATAACAAGAACTTAATTTCAGCCG	
IM89 (Δ*sauUSI*-AFwd)	ATATGGTACCGTGTATGAAAATGCATGGAGTAGAGC	KpnI
IM90 (Δ*sauUSI*-B)	CATATTATCCCTCAGTCATAATTTTATTAACG	
IM91 (Δ*sauUSI*-C)	*CGTTAATAAAATTATGACTGAGGGATAATATG*TAATGTAAACCGAAAAATG AATGTTAGTAAAG	
IM92 (Δ*sauUSI*-DRev)	ATATGAGCTCCCAATCCTCTGGATTCCATATTCTTTCC	SacI
IM150 (CC30 *sauUSI*-DRev)	ATATGAGCTCAAACTCTTCGTCACGAAATCCTTCC	SacI
IM110 (*sauUSI*-OUT F)	ACAGCCCCAAGACAATACTTTTCAC	
IM111 (*sauUSI*-OUTR)	ATACAGGACCAATCCTCTGGATTCC	
IM108 (RN*sauUSIcomp*)F	ATATGGTACCGTGCATTAGATGTTAGAGAAGTAAACC	KpnI
IM109 (RN*sauUSIcomp*)R	ATATGAGCTCATTTAATGATACTGCATCCAATGAATTG	SacI
IM350 (384*sauUSIcomp*)B	GATATCACTTTCTAATGCTGCTTGTAACC	EcoRV
IM351 (384*sauUSIcomp*)C	ACAAGCAGCATTAGAAAGTGATATCTTATGTCCATTTCATTATTTTGGTGTG	EcoRV
IM196 (His-SauUSI F)	ATATCCATGGGTAGATTACTAAATGATTTCAATC	NcoI
IM197 (His-SauUSI R)	ATATCTCGAGATTTGTTAGATAACGATATATATCATCTC	XhoI
IM216 (Se Δ*mcrR*-AFwd)	ATATGTCGACTCTAATATATTAAGTATGTAAACCACG	SalI
IM217 (Se Δ*mcrR*-B)	CAATCTAATTCTCCTCTATTATACG	
IM218 (Se Δ*mcrR*-C)	*GTATAATAGAGGAGAATTAGATTG*TAATTACTTATACTAAATTATTATTTATTG	
IM219 (Se Δ*mcrR*-DRev)	ATATGAATTCTGAATCACAGATCAAAAATGAAGACC	EcoRI
IM220 (Se *mcrR*-OUTF)	GAATTGAAAATTTTAGGTATTCAGATGG	
IM221 (Se *mcrR*-OUTR)	AAACCTTTAATAATTATCAAGACAGC	
IM222 (Δ*nudix*-AFwd)	ATATGGTACCACCTTCACCAAGACCGAATTTTCC	KpnI
IM223 (Δ*nudix*-B)	CATAAGACTCACCCTTCAATTTAAAATC	
IM224 (Δ*nudix*-C)	*TTAAATTGAAGGGTGAGTCTTATG*TAATATGAGTAGATTACTAAATGATTTC	
IM225 (Δ*nudix*-DRev)	ATATGAGCTCATAGTAGACAGTAAAACATTATGC	SacI
IM226 (*nudix* OUT F)	TTTAAATAACGCGCTAAACCTAATGC	
IM227 (*nudix* OUT R)	CACTATCAACTAAATCGCCATTTTTC	
IM261 (Ec Δ*dcm* F)	**TGTAATTATGTTAACCTGTCGGCCATCTCAGATGGCCGGTGAAATCTATG**GTG TAGGCTGGAGCTGCTTC	
IM262 (Ec Δ*dcm* R)	**TTGTGCCTCTTGCTGACGCAACGCCACCGCCTGTTTGATTTTTGGCTCAAG**GTCCAT ATGAATATCCTCCTTAG	
IM251 (Ec Δ*dcm* OUTF)	AGAAGAGACGCGTCGCCTGCTCC	
IM252 (Ec Δ*dcm* OUTR)	TACTGGTCACGTTGGGAAAATATCTC	
IMS80 (*hsdR* IBS)	AAAAAAGCTTATAATTATCCTTACTTCTCCCGCATGTGCGCCCAGATAGGGTG	HindIII
IMS81 (*hsdR* EBS1d)	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCCGCAT ACTAACTTACCTTTCTTTGT	BsrGI

(Continued on following page)

TABLE 1 (Continued)

Bacterial strain, plasmid, or oligonucleotide[a]	Description (relevant genotype or phenotype) or sequence (5′ to 3′)[b]	Source, reference, or RE site[c]
IMS82 (*hsdR* EBS2)	TGAACGCAAGTTTCTAATTTCGGTTAGAAGTCGATAGAGGAAAGTGTCT	
IMS83 (*hsdR* OUT F)	AGTATACGACTTACCTCAA	
IMS84 (*hsdR* OUT R)	TCAGTTGTTTCTGCCACG	
IMS85 (*sauUSI* IBS)	AAAAAAGCTTATAATTATCCTTAAAAGACAAGGCGGTGCGCCCAGATAGGGTG	HindIII
IMS86 (*sauUSI* EBS1d)	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAAGGCG TTTAACTTACCTTTCTTTGT	BsrGI
IMS87 (*sauUSI* EBS2)	TGAACGCAAGTTTCTAATTTCGGGTTTCTTTCCGATAGAGGAAAGTGTCT	
IMS90 (*sauUSI* OUT F)	ATGAGTAGATTACTAAATG	
IMS91 (*sauUSI* OUT R)	CGTTACTACGTTTGAACC	
IMSuni	CGAAATTAGAAACTTGCGTTCAGTAAAC	
IM151 (pIMAY MCS F)	TACATGTCAAGAATAAACTGCCAAAGC	
IM152 (pIMAY MCS R)	AATACCTGTGACGGAAGATCACTTCG	

[a] *S. aureus* and *S. epidermidis* gene designations are taken from http://kegg.jp. For oligonucleotides, anti *secY* stands for antisense *secY* RNA.
[b] The description (relevant genotype, phenotype, or other characteristic) is shown for bacterial strains and plasmids. MSSA, methicillin-sensitive *S. aureus*.
The primers used in the construction of recombinant plasmids are shown in parentheses at the end of the entry. The sequences for primers are shown. Restriction sites are indicated by underlining. Regions of homology for SOE PCR with the B primer are shown in italic type, and regions of homology for recombineering in *E. coli* are shown in bold type.
[c] The source or reference is shown for bacterial strains and plasmids. The restriction enzyme (RE) site is shown for oligonucleotides.

lected on 100 μg/ml carbenicillin at 30°C. Strain DH10B carrying pKD46 was made electrocompetent as described above, except that the cells were grown at 30°C (instead of 37°C), and once the culture reached an OD_{600} of 0.4, filter-sterilized arabinose (Sigma) was added to a final concentration of 0.2%. The culture was incubated for 1 h to induce *exo*, *beta*, and *gam* expression, and the cells were washed and stored at −70°C. The plasmid pKD4 (26) was used as a template to PCR amplify the kanamycin resistance marker flanked by two flippase recognition target (FRT) sites. The forward and reverse primers (IM261/IM262) were tailed with 50 nucleotides complementary up to the *dcm* gene start codon and 23 codons down from the stop codon to generate an in-frame deletion within the *dcm* gene. The linear amplimer was electroporated into the electrocompetent *E. coli* DH10B(pKD46), and transformants were selected on LBA containing 50 μg/ml of kanamycin at 37°C. One kanamycin-resistant clone was selected and plated on LBA at 43°C to eliminate pKD46. An ampicillin-sensitive derivative was made competent and transformed at 30°C with pCP20 (26) (selected on LBA with 10 μg/ml Cm) to excise the kanamycin resistance marker. Finally, pCP20 was eliminated by plating at 43°C to yield *E. coli* DH10BΔ*dcm* (called DC10B). The loss of cytosine methylation was confirmed phenotypically through the inability of SauUSI to digest DC10B plasmid or genomic DNA and transformation of wild-type *S. aureus* and *S. epidermidis* strains with a shuttle plasmid isolated from DC10B.

Creation of targetron insertion mutants in *S. aureus* NRS384. Targetron insertion mutants were created in *S. aureus* NRS384 following the protocol of the manufacturer (Sigma) (17). Primers were designed to retarget the intron for *hsdR* (IMS80/IMS81/IMS82) or *sauUSI* (IMS85/IMS86/IMS87). To amplify the 350-bp retargeted amplicon for *hsdR* or *sauUSI*, the above primers were combined with IMSuni. The amplicon was digested with HindIII/BrsGI and cloned into similarly cut pNL9164. pNL9164*hsdR* or pNL9164*sauUSI* was then passaged through *S. aureus* RN4220 at 30°C before being electroporated into NRS384 at 30°C. A single colony was streaked on Trypticase soy agar (TSA) plus 25 μg of Em per ml (Em25) containing 10 μM $CdCl_2$ and incubated overnight to induce intron mobilization. Single colonies were screened for insertion of the intron into *hsdR* (IMS83/IMS84) or *sauUSI* (IMS90/IMS91) by colony PCR. The plasmid was cured by overnight growth in broth at 43°C followed by plating onto TSA at 30°C. Colonies were patched onto TSA and TSA plus Em to identify plasmid-free isolates. The double mutant was created in the *sauUSI*^{INT} background.

Intravenous infection of A/J mice. Overnight cultures of *S. aureus* NRS384, *hsdR*^{INT}, *sauUSI*^{INT}, *hsdR*^{INT} *sauUSI*^{INT}, and *hsdR*^{INT} *sauUSI*^{ECORV} strains were diluted 1:100 in TSB and grown to an OD_{600} of 0.5 to 0.6. The cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS) and resuspended to an OD_{600} of 1 (5×10^8 CFU/ml). The inoculum was diluted to 2×10^7 CFU/ml, and 100 μl was injected into the tail vein of a 6- to 7-week-old female A/J mouse. Eight mice were used for each strain. At day 7 postinfection, the mice were euthanized, and the total bacterial loads in both kidneys were determined by serial dilution and plating.

Nucleotide sequence accession number. The nucleotide sequence of pIMAY was deposited in GenBank under accession number JQ621981.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00277-11/-/DCSupplemental.

Figure S1, TIF file, 0.4 MB.

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