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RESEARCH ARTICLE

Complete Bypass of Restriction Systems for Major *Staphylococcus aureus* Lineages

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ABSTRACT ***Staphylococcus aureus* is a prominent global nosocomial and community-acquired bacterial pathogen. A strong restriction barrier presents a major hurdle for the introduction of recombinant DNA into clinical isolates of *S. aureus*. Here, we describe the construction and characterization of the IMXXB series of *Escherichia coli* strains that mimic the type I adenine methylation profiles of *S. aureus* clonal complexes 1, 8, 30, and ST93. The IMXXB strains enable direct, high-efficiency transformation and streamlined genetic manipulation of major *S. aureus* lineages.**

IMPORTANCE The genetic manipulation of clinical *S. aureus* isolates has been hampered due to the presence of restriction modification barriers that detect and subsequently degrade inappropriately methylated DNA. Current methods allow the introduction of plasmid DNA into a limited subset of *S. aureus* strains at high efficiency after passage of plasmid DNA through the restriction-negative, modification-proficient strain RN4220. Here, we have constructed and validated a suite of *E. coli* strains that mimic the adenine methylation profiles of different clonal complexes and show high-efficiency plasmid DNA transfer. The ability to bypass RN4220 will reduce the cost and time involved for plasmid transfer into *S. aureus*. The IMXXB series of *E. coli* strains should expedite the process of mutant construction in diverse genetic backgrounds and allow the application of new techniques to the genetic manipulation of *S. aureus*.

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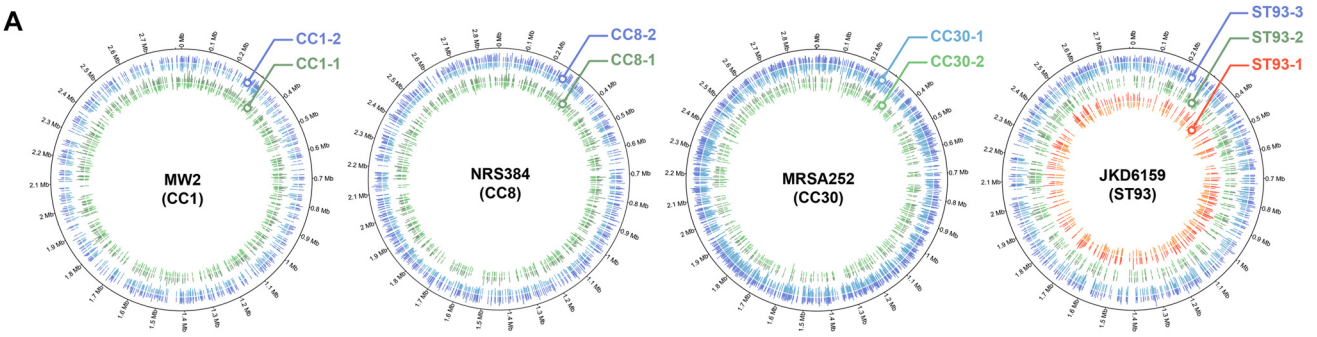
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In order to gain an in-depth understanding of bacterial pathogen biology, genetic studies are required. However, a major impediment to genetic manipulation is the presence of restriction modification (RM) barriers, which hinder the uptake of foreign DNA (1–4). In the absence of host-specific methylation profiles, introduced DNA is recognized by the host as foreign and degraded. The RM barrier can help prevent infection by bacteriophage and transfer of conjugative plasmids and can inhibit the natural uptake of DNA via competence mechanisms (1, 5, 6). It can also prevent the transfer of recombinant plasmid DNA constructed in the laboratory workhorse *Escherichia coli* into organisms such as *Staphylococcus aureus*. The majority of clinical *S. aureus* isolates contain an active type IV system which recognizes systems specific to cytosine methylation (7) and clonal complex (CC) type I (8, 9) that can elaborate complex adenine methylation profiles. The type I RM barrier is comprised of three components, *hsdM* (methylase), *hsdS* (specificity), and *hsdR* (restriction). The $HsdM_2$-$HsdS_1$ protein complex recognizes a target recognition motif (TRM) dictated by the HsdS and detects the methylation status. Appropriately hemimethylated DNA (e.g., replicating chromosomal DNA) is then fully methylated, preventing restriction of the DNA by the $HsdM_2$-$HsdS_1$-$HsdR_2$ complex (8). While most strains contain two active type I RM systems (the *hsdMS* carried on the alpha and beta pathogenicity islands and a single orphan HsdR gene) (10), a third complete HsdRMS can be found in some isolates (11). Additionally, a limited subset of *S. aureus* strains contain type II RM, which includes the enzyme Sau3AI that recognizes GATC motifs (12). Sau3AI sites on plasmids isolated from *E. coli* are blocked by Dam methylation. In *S. aureus* strains of clonal complex 398 (CC398), an active type II RM is encoded that transposes the type IV system. Digestion of CC398 genomic DNA with SmaI is prevented due to cytosine methylation of CCNGG motifs by the cognate type II methylase (13). Plasmids isolated from *E. coli* strain DC10B lacking cytosine methylation (DH10B Δ*dcm*) can bypass the type IV barrier, but maximal transformation efficiency requires the presence of the CC-specific adenine methylation profile (3). Passage of plasmids through the restriction-deficient, modification-proficient *S. aureus* strain RN4220 (14) allows transfer into CC8/sequence type 239 (ST239) hosts at high efficiency. Transfer into other CCs is dependent on the TRM sequences on the particular plasmid. The laboratory *S. aureus* strain RN4220 has been invaluable as

A



B

Staphylococcus aureus	Motif	% Motifs Detected	# Of Motifs Detected	# Of Motifs In Genome	Mean Modification QV [1]	Mean Motif Coverage [2]
MW2						
CC1-1	CCAYNNNNNTTAA	99.6	508	510	98	67.5
(MW0393)	TTAANNNNNRTGG	99	505	510	96.5	68.1
CC1-2	CCAYNNNNNNTGT	97.4	485	498	91.1	67.8
(MW1750)	ACANNNNNNRTGG	99	493	498	94.9	68.5
NRS384						
CC8-1	AGGNNNNNGAT	99.9	690	691	110.7	69.7
(USA300_406)	ATCNNNNNCCT	100	691	691	104.6	68.5
CC8-2	CCAYNNNNNNTGT	97.2	483	497	100.7	70.7
(USA300_1751)	ACANNNNNNRTGG	98.8	491	497	103.9	70.5
MRSA252						
CC30-1	GNAGNNNNNGAT	99.8	1207	1209	104	67.7
(SAR0434)	ATCNNNNNCTWC	99.9	1208	1209	104.4	68.7
CC30-2	GGANNNNNNNTCG	98.9	349	353	100.1	70.5
(SAR1898)	CGANNNNNNNTCC	98.9	349	353	96.6	71.1
JKD6159						
CC93-1	GAAGNNNNNTAC	100	264	264	118.4	71.9
(SAA6159_00387)	GTANNNNNCTTC	100	264	264	110.7	74.2
CC93-2	GGHANNNNNNNTCG	99.7	323	324	105.2	73.8
(SAA6159_01737)	CGANNNNNNNTDCC	97.5	316	324	103.1	74.6
CC93-3	CAGNNNNNNTTC	100	667	667	127.9	74.9
(SAA6159_00055)	GAANNNNNNCTG	99.1	661	667	103.3	74.4

C

Escherichia coli	Motif	% Motifs Detected	# Of Motifs Detected	# Of Motifs In Genome	Mean Modification QV	Mean Motif Coverage
IM01B	GATC[3]	98.7	38074	38594	73.5	42.3
CC1-1	CCAYNNNNNTTAA	98.1	405	413	70.5	41.7
	TTAANNNNNRTGG	98.1	405	413	67.2	42.5
CC1-2	CCAYNNNNNNTGT	96.9	864	892	70.3	42.9
	ACANNNNNNRTGG	97.2	869	892	71.2	42.8
IM08B	GATC	99.2	38291	38594	48.8	47.3
CC8-1	AGGNNNNNGAT	98.9	2015	2038	47	47
	ATCNNNNNCCT	99.3	2024	2038	47.3	46.8
CC8-2	CCAYNNNNNNTGT	98.4	878	892	46.7	46.7
	ACANNNNNNRTGG	98.3	877	892	46.7	46.7
IM30B	GATC	99.2	38289	38594	76.9	40.6
CC30-1	GNAGNNNNNGAT	98.1	1487	1516	77.7	39.2
	ATCNNNNNCTWC	98.6	1495	1516	76.5	40.4
CC30-2	GGANNNNNNNTCG	97.5	1470	1507	73.8	40.4
	CGANNNNNNNTCC	97.1	1463	1507	73.7	40.6
IM93B	GATC	99.4	38370	38594	80.4	47.3
CC93-1	GAAGNNNNNTAC	98.9	454	459	77.2	47
	GTANNNNNCTTC	98.7	454	459	76.9	47.7
CC93-2	GGHANNNNNNNTCG	98.9	2180	2205	76.7	46.7
	CGANNNNNNNTDCC	98.4	2170	2205	76.3	46.9
CC93-3	CAGNNNNNNTTC	53.9	2515	4637	49.9	49.4
	GAANNNNNNCTG	50.8	2362	4637	49.3	49.3

FIG 1 SMRT sequencing for the identification of adenine methylation in *S. aureus* and engineered *E. coli*. (A) The target recognition motifs (TRMs) for the type I HsdMS systems encoded by each *S. aureus* strain were identified, and the positions of methylated adenine residues on the chromosome plotted with Circos (34). Each adenine methylation on the chromosome is represented by a line whose length corresponds with the interpulse duration of the read. (B and C) PacBio reads were analyzed by the SMRT suite pipeline version 2.2.0/motif finder 1.3.1 to identify conserved adenine-methylated residues (in boldface) and the TRMs for the HsdS alleles of each *S. aureus* (B) and IMXXB *E. coli* (C) strain.[1], Mean modification QV is defined as the quality value of the base calls within the motif.[2], Mean motif coverage is defined as the average depth of read coverage within a motif.[3], GATC methylation encoded by *dam* in the *E. coli* strain is not present in *S. aureus*.

an intermediate host for the transfer of plasmid DNA into a restricted set of CCs. However, maintenance of plasmids in *S. aureus* is not ideal, as it requires additional time for growth at 30°C (temperature-sensitive plasmids) and greater cost (enzymatic lysis with lysostaphin) and provides plasmid DNAs of poor quality/yield compared to those isolated from *E. coli*.

A novel method to bypass host-encoded RM, termed plasmid artificial modification (PAM), has been described. Plasmid DNA is premethylated in an *E. coli* strain heterologously expressing methyltransferases of the target host. For *Bifidobacterium breve* (2) and *Bifidobacterium adolescentis* (15), the expression of type II methyltransferases in *E. coli* improved plasmid transfer 1,000 and 10,000-fold, respectively. More recently, a genomic approach was applied to restrictive clones of *Bacillus cereus*, *Bacillus amyloliquefaciens*, and *Nitrobacter hamburgensis* (4). A combination of type II and III RM genes were introduced into *E. coli* with improved plasmid transfer verified. To apply PAM, first the RM systems encoded by the strain in question need to be evaluated and, second, an appropriate method for the cloning and expression of the modification machinery needs to be devised.

To expand the repertoire of clinically relevant *S. aureus* strains that can be manipulated routinely in the laboratory, we have developed CC-specific cloning hosts that stably express CC-specific type I modification genes from the chromosome of *E. coli* DC10B, creating the IMXXB series of *E. coli* strains (XX denotes CC).

RESULTS

Employing the ability of Pacbio single-molecule, real-time (SMRT) sequencing to detect adenine-methylated DNA (16), we sequenced the genomes of the prototypical methicillin-resistant *S. aureus* (MRSA) strains MW2 (CC1), NRS384 (CC8), and MRSA252 (CC30) and identified the adenine methylation of two independent TRMs, with each strain harboring two active HsdS alleles (Fig. 1A and B). These results confirmed the TRMs for CC1 and CC8 strains characterized by Roberts et al. (17). We also identified an adenine methylation pattern for MRSA252 that fits with previously uncharacterized HsdS sequences. The sequencing of a fourth strain, JKD6159 (ST93) (11), which is completely refractory to transformation with RN4220 plasmid DNA, revealed the presence of three active, novel HsdS sequences and TRM sites (Fig. 1A and B).

We then attempted to confer on *E. coli* a CC8 adenine methylation profile. The CC8-1 (nomenclature consistent with Roberts et al. [17]) *hsdMS*, the CC8-2 *hsdMS*, and a CC8-2 *hsdM*/CC8-1 *hsdS* hybrid were expressed using an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible system (pET21) in *E. coli* strain BL21 (naturally *dcm* deficient). The shuttle plasmid pIMK7, which contains two CC8-1 and one CC8-2 TRM, was transformed into the BL21 strains. Methylation of the TRMs was judged to have occurred by the improved transformation of pIMK7 into NRS384 compared to the results for plasmid isolated from the empty pET21 control strain. The frequency of pIMK7 transformation into NRS384 when all CC8-1 and CC8-2 TRM sites were methylated (plasmid isolated from NRS384) was 100-fold greater than for pIMK7 isolated from BL21 containing pET21 (Fig. 2), with the presence of either CC8-1 or CC8-2 methylation alone improving the transformation frequency 30-fold (Fig. 2). Furthermore, we could show that HsdM (CC8-2) could functionally interact with HsdS (CC8-1), because pIMK7 isolated from BL21 containing pCC8-M2S1 improved plasmid transfer ~25-fold, and

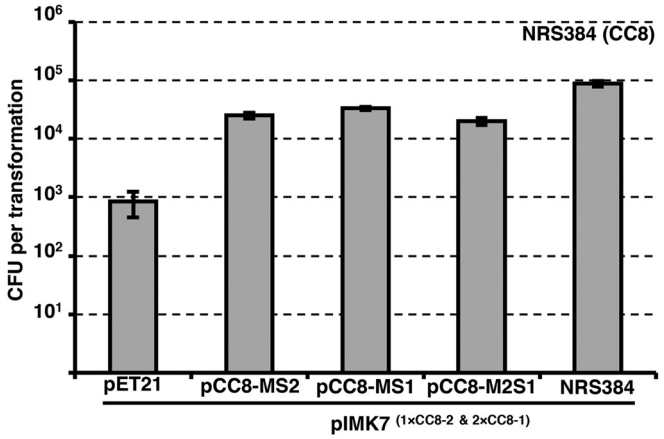


FIG 2 Transformation of NRS384 with HsdMS-methylated plasmid. Three different CC8 HsdMS combinations were expressed from an IPTG-inducible plasmid (pET21) in *E. coli* BL21 (including the hybrid $hsdM_2$-$hsdS_1$). The coextracted shuttle vector pIMK7 (5 μg total plasmid DNA) was transformed into CA-MRSA strain NRS384. pIMK7 isolated from NRS384 was included as the maximal transformation of fully modified plasmid. The number of TRMs on pIMK7 for CC8 strains are indicated next to the plasmid name. The transformation efficiencies are expressed as the mean numbers of all transformants obtained in each experiment ± standard deviations (error bars) from three replicates. The graph shows data representative of the data from three independent experiments.

that maximal transformation efficiency with pIMK7 was dependent on complete methylation of both TRM sites (Fig. 2).

From here, we attempted to express HsdMS CC8-2 from the native or an intermediate or highly expressed promoter on a low-copy-number plasmid (p15A replicon, pIMK series [18]) in the *E. coli* DC10B background. However, we were unable to obtain positive clones from the intermediate or highly expressed constructs and loss of function was observed upon serial passage of the *E. coli* cells containing the CC8-2 *hsdMS* driven by the native promoter (data not shown). These results prompted us to examine the feasibility of integrating the *hsdMS* genes within neutral locations on the DC10B chromosome to improve stability (19) and to produce unmarked *E. coli* strains (Fig. 3A). Initially, we attempted to create a CC8 *E. coli* cloning host to express the equivalent methylation profile of RN4220. By spliced overlap extension (SOE) PCR, we assembled *in vitro* one set of *hsdMS* genes (CC8-2), expressed from a strong constitutive promoter. The fragment was targeted by recombineering to a region of the DC10B chromosome between the *atpI* and *gidB* genes. This yielded *E. coli* SA08B after removal of the *cat* gene. We took advantage of the interaction between HsdM and HsdS to introduce the second *hsdS* (CC8-1) expressed from the strong coliphage promoter P_{N25} (20) between the *essQ* and *cspB* genes of SA08B. This yielded *E. coli* strain IM08B after removal of the kanamycin resistance marker. SMRT sequencing of IM08B genomic DNA revealed the methylation of TRM sites identical to those modified in *S. aureus* strain NRS384 (Fig. 1C) with no detectable off-target modifications by IM08B. Importantly, the efficiency of methylation, as judged by the percentage of motifs methylated in the *E. coli* background, was equivalent to that observed in NRS384 (Fig. 1B and C). To investigate the effect of plasmid methylation by IM08B, either pRAB11 (two CC8-2 TRMs and one CC8-1 TRM) or pIMAY-Z (four CC8-1 TRMs) was isolated from DC10B (lacks type IV restriction) or IM08B and transformed into NRS384, with NRS384 Δ*hsdR* used as a control. The presence of a functional methylation profile was confirmed by the improved transformation efficiencies of pRAB11 (~100-fold) and pIMAY-Z (~500-fold) compared to those of the plasmids isolated from DC10B (Fig. 3B). Using the protocol described for making electrocompetent *S. aureus* cells, we observed a linear increase in the number of transformants recovered with pRAB11 isolated from IM08B for up to 5 μg of plasmid DNA transformed (Fig. 4). Similar results were also observed for pRAB11 isolated from *E. coli* strains IM01B and IM30B, described below.

To extend these results, we sought to introduce the methylation profiles of CC1 (USA400 lineage) and CC30 strains that include common methicillin-susceptible *S. aureus* (MSSA), MRSA, and community-associated (CA)-MRSA strains (21) into DC10B. As *S. aureus* strains from CC8 and CC1 share the *hsdS* allele denoted above as CC8-2 (CC1-2 by Roberts et al. [17]), we introduced the CC1-1 P_{N25}-*hsdS* into SA08B, creating the CC1 *E. coli* strain IM01B. Acquisition of the CC1-1 methylation profile in the CC8-2 *E. coli* background was confirmed by SMRT sequencing (Fig. 1C). We were unable to transform the prototypical CC1 *S. aureus* strain MW2 (USA400) with pRAB11 (two CC8-2 TRMs and one CC1-1 TRM) or pIMAY-Z (three CC1-1 TRMs) isolated from DC10B. However, plasmid DNA methylated with the CC1 profile exhibited at minimum 100- to 1,000-fold improvements in transformation compared to that of plasmids from DC10B (Fig. 3C). A similar approach was used to integrate the CC30-2 *hsdMS* and the CC30-1 *hsdS* genes into the chromosome of DC10B (strain IM30B). The presence of both adenine methylation profiles was confirmed by SMRT sequencing (Fig. 1C). MRSA252 was the most transformable of the *S. aureus* strains tested with plasmids isolated from DC10B (Fig. 3D), yet IM30B improved pRAB11 (two CC30-2 TRMs and one CC30-1 TRM) transfer 12-fold and pIMAY-Z (one CC30-2 TRM and three CC30-1 TRMs) transfer 100-fold. Plasmids isolated from DC10B or IMXXB strains transformed their respective *S. aureus* Δ*hsdR* mutants equivalently, confirming that transformation efficiency is dependent on the plasmid methylation profile and type IV/type I RM barriers.

Alignment of the 11 HsdS amino acid sequences (including CC5-1/CC5-2 described previously [17]) allowed us to decipher the TRMs recognized by each of the CC30 HsdS proteins (Table 1). HsdS contains two target recognition domains, designated TRD1 and TRD2 (Fig. 5). Conserved domains were observed between TRD1 of CC1-1, CC1-2, CC5-2, and CC8-2, correlating with the recognition of a CCAY sequence. HsdS from CC8-2/CC1-2 share identical TRD2s that correlate with the recognition of a TGT motif. TRD2s from CC5-1, CC8-1, and CC30-1 are also conserved, with a GAT motif in common between the three alleles. By exclusion, the motif GW<u>A</u>GN_5GAT was attributed to CC30-1 and GG<u>A</u>N_7TCG to CC30-2 (underlining indicates sites of adenine methylation). Furthermore, the TCG sequence is shared by the CC30-2/CC93-2 HsdS alleles that validated the CC30-2 prediction. In all, recognition sequences for 14/18 of the TRD could be attributed (Table 1) from the SMRT sequencing data.

Transformation of *S. aureus* JKD6159 with plasmid DNA isolated from DC10B, IM93B (Ω*hsdMS-2/S-1*Ω*hsdMS-3*; see Materials and Methods for strain construction), and JKD6159 shed light on the motifs recognized by the unassigned HsdS alleles. The creation of IM93B allowed us to routinely transform plasmid into

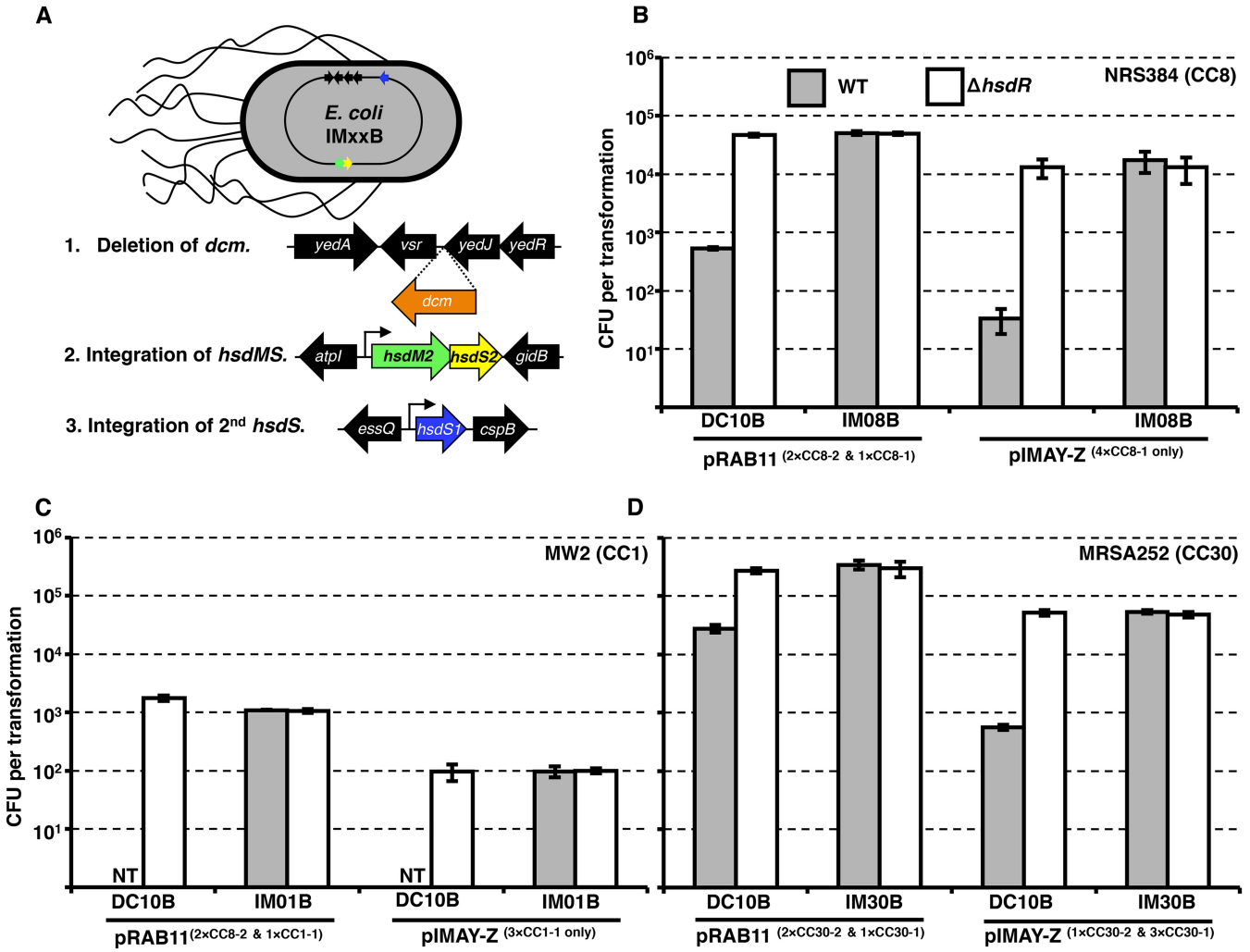


FIG 3 Construction and characterization of IMXXB *E. coli* strains. (A) Schematic of the construction of an *E. coli* strain expressing CC-specific *hsdMS* alleles from strong promoters at neutral locations in the DC10B (DH10B Δ*dcm*) chromosome. (B to D) Transformation profiles of *S. aureus* strains (grey bars) and their isogenic *hsdR* mutants (defective in type I restriction) (white bars) with plasmid pRAB11 (5 μg) or pIMAY-Z (2.5 μg) isolated from DC10B or the respective IMXXB strain of *E. coli*. The number of TRMs on either plasmid for the CC of the strain is denoted next to the plasmid name. NT, no transformants were detected. The transformation efficiencies are expressed as the mean numbers of all transformants obtained in each experiment ± standard deviations (error bars) from three replicates. The graph shows data representative of the data from three independent experiments.

JKD6159. We observed 1,000 times fewer transformants when transforming plasmid from IM93B into JKD6159 compared to the rate of transformation of JKD6159 Δ*hsdR*^{SCCmec} Δ*hsdR*, which is deficient in all type I restriction systems (Fig. 6). The plasmid used for the transformation experiments (pRAB11) contained multiple copies of each JKD6159 *hsdS* TRM. SMRT sequencing of IM93B showed that one of the three alleles was impaired in activity (only ~50% of the motifs were methylated). This was attributed to the HsdS CC93-3 encoded by the gene lying adjacent to the staphylococcal cassette chromosome *mec* element (SCC*mec*), as plasmid DNA isolated from IM93B transformed into JKD6159 Δ*hsdR*^{SCC} 10 times less efficiently than into JKD6159 Δ*hsdR*. This result is consistent with impaired methylation from CC93-3 in IM93B (Fig. 6). Analysis of the genome sequence of IM93B identified a point mutation at the 3′ end of the CC93-3 *hsdS* gene, leading to a predicted amino acid change of E to G at position 402 (E402G), which we speculate might reduce the activity of the enzyme. This allowed us to assign the TRMs recognized by the CC93-1 (GA<u>A</u>GN$_5$TAC) and CC93-2 (GGH<u>A</u>N$_6$TCG) HsdS proteins.

Finally, we tested the transfer of plasmid DNA isolated from IM01B, IM08B, and IM30B into *S. aureus* strain NRS384. We had earlier observed that pIMK7 required both CC8-1 and CC8-2 methylation for maximal transformation efficiency. Plasmid pRAB11 transferred equally efficiently from IM01B and IM08B, with plasmid isolated from IM30B transferred at a level equivalent to that of the unmethylated plasmid from DC10B (Fig. 7). IM01B and IM08B methylate the two CC8-2 TRM sites present on pRAB11, with one additional different TRM methylated by each strain. IM01B still transferred pRAB11 at high efficiency even though a CC1 methylation profile was present on the plasmid. Therefore, in this case, only one of the two TRMs required modification for maximal plasmid transfer and off-target methylation was tolerated, which was different from the results for pIMK7. Only IM08B allowed the transfer of pIMAY-Z at high efficiency.

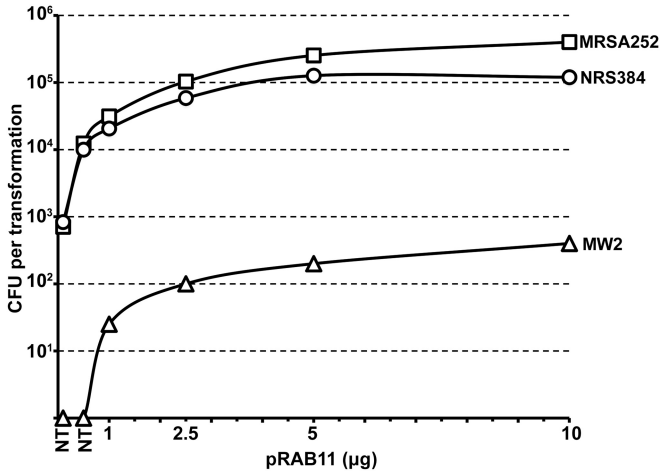


FIG 4 Effect of plasmid concentration for *S. aureus* electroporation. Different concentrations of plasmid DNA (pRAB11 at 0.1, 0.5, 1, 2.5, 5, and 10 μg) were isolated from *E. coli* strains IM01B, IM08B, and IM30B for transformation into *S. aureus* MW2, NRS384, and MRSA252, respectively. A dose-dependent response was observed for up to 5 μg of plasmid with pRAB11. The transformation efficiencies are expressed as the mean numbers of all transformants obtained in each experiment ± standard deviations (error bars) from three replicates. The graph shows representative data from one experiment.

IM01B and IM30B did not methylate the CC8-1 TRMs (4 sites), and additional off-target methylation patterns led to full type I restriction (plasmid isolated from IM01B/IM30B) (Fig. 7). Analysis of the HsdS repertoire of an *S. aureus* recipient strain and sequence analysis of the plasmid to be transformed will allow the use of the *E. coli* strains described here to be applied to *S. aureus* strains from other CCs. For example, we have found that pIMAY-Z isolated from IM08B had improved transformation efficiency into CC5 *S. aureus* strains (data not shown). CC5 strains share a CC8-1 *hsdS* allele (Fig. 5, CC5-1) (17) and encode a novel CC5-2 *hsdS* allele. Plasmid pIMAY-Z contains both TRMs, but CC8-1 methylation alone appears sufficient to significantly improve transformation efficiency into an *S. aureus* CC5 strain. We have tested additional *S. aureus* isolates from CC1, CC8/ST239, and CC30 for their ability to accept plasmid (pRAB11) isolated from either DC10B or the respective IMXXB strain (Fig. 8). Except for PS80 (CC30), we observed improved transformation with plasmid isolated from IMXXB over plasmid isolated from DC10B, showing the general applicability of the IMXXB strains to bypass restriction in these CCs.

DISCUSSION

The method described here is based on engineering *E. coli* to express active type I *hsdMS* genes from *S. aureus*, but this approach can equally be applied to other bacterial species where type I RM is a barrier to genetic manipulation, such as *Streptococcus* spp., *Staphylococcus* spp., and *E. coli* (3, 22–24). Our approach to integrate the *hsdMS* genes onto the chromosome stabilized the DNA, in contrast to the instability/toxicity observed for *hsdMS* genes. However, it should be highlighted that functional *hsdMS* genes have been cloned from multiple bacteria and expressed from plasmids in *E. coli* (16, 25, 26).

Our research builds on previous studies in this area exploring type II and III RM systems. Zhang et al. (4) conducted an in-depth characterization of the restriction barrier in an untransformable strain of *N. hamburgensis* and in two bacilli, *B. cereus* ATCC 10987 and *B. amyloliquefacians* TA208. Through the expression of an assembled operon of active type II or III and/or orphan methylases (determined by Southwestern blotting and liquid chromatography-mass spectrometry [LC-MS]) from each isolate in *E. coli* EC135 (TOP10 Δ*dam* Δ*dcm* Δ*hsd* Δ*mcrBC* Δ*mcrA* Δ*mrr*), recombinant DNA could be transformed into *N. hamburgensis* or the two bacilli at high efficiency. An assumption was made that all naturally occurring methylases in the *E. coli* K-12 TOP10 strain were detrimental to transformation, therefore requiring a "methylase-bald" strain as a starting point for DNA modification (4). No data to support this hypothesis were presented. For all strains of *S. aureus* that we have tested, we have shown that the presence of *dam* methylation was not detrimental to DNA transfer, and in one case, its presence was in fact essential (3). Intact *dam* is important, as deletion of the gene elevates the frequency of transition mutations due to deregulated mismatch repair (27). Furthermore, the deletion of *dam* in a *recA*-deficient background is lethal, so the reinstatement of a wild-type *recA* allele was required prior to *dam* deletion in EC135 (4). It is widely recognized that the presence of functional *recA* can destabilize repetitive DNA (28) and, also, lead to plasmid multimer formation through *recA*-dependent plasmid recombination (29). At least in the context of genetic manipulation of *S. aureus*, we would contend that a methylase-bald, *recA*$^+$ *E. coli* strain is not an ideal cloning host for PAM.

The IMXXB *E. coli* strains we have developed permit the facile construction of unmarked mutations and transposon libraries in *S. aureus* lineages that we could not previously examine (e.g., MW2 and JKD6159) (data not shown). The IMXXB strains will open up new avenues for research, such as the assessment of candidate antigen efficacy in vaccine formulations against *S. aureus* from different lineages that previously could not be assayed (30). The knowledge gained here on the role of type I RM in *S. aureus* and its application to the *E. coli* donor strains tailored to specific *S. aureus* recipients will further improve our ability to conduct sophisticated genetic analysis of this and other important human pathogens.

MATERIALS AND METHODS

Media and reagents. The bacterial strains and plasmids used in this study are described in Table 2. Oligonucleotides were purchased from Integrated DNA Technologies and are listed in Table 3. Genomic DNA was

TABLE 1 Assignment of the TRM arms to each TRD[a]

HsdS	TRD1	N	TRD2
CC1-1	CCAY	5	TTAA
CC1-2	CCAY	6	TGT
CC5-1	AGG	5	GAT
CC5-2	CCAY	6	GTA
CC8-1	AGG	5	GAT
CC8-2	CCAY	6	TGT
CC30-1	GWAG	5	GAT
CC30-2	GGA	7	TCG
ST93-1	GAAG/GTA	5	TAC/TTC
ST93-2	GGHA	7	TCG
ST93-3	CAG/GAA	6	TTC/CTG

[a] Based on the protein alignment (color coded as in Fig. 5) and TRM (obtained from SMRT sequencing), the DNA motif recognized by the TRD was assigned. For ST93-1/-3, it was not possible to assign the TRM for the TRD.

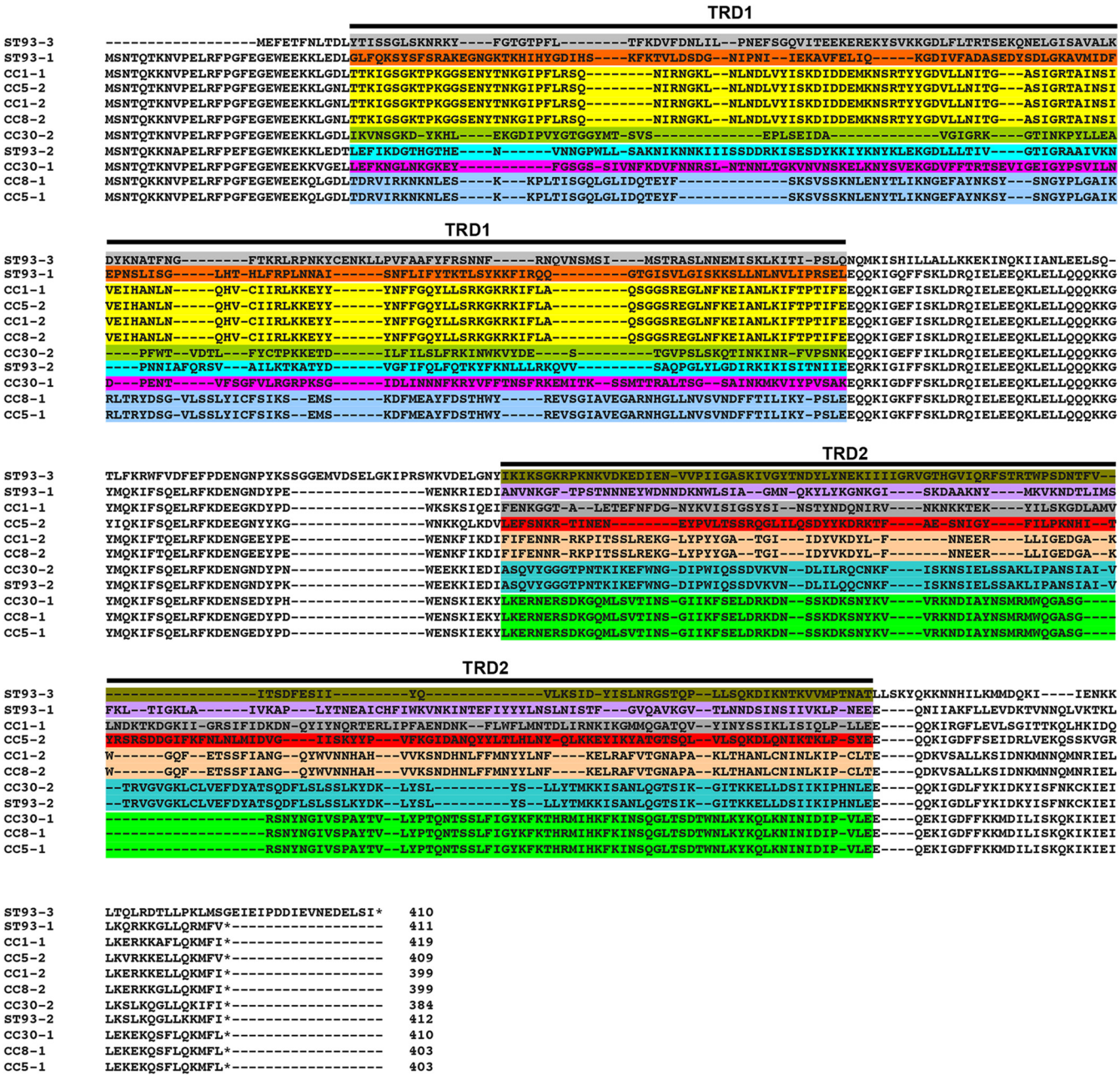


FIG 5 Determination of the TRM for each TRD. Clustal Omega alignment of HsdS proteins from CC1, CC5, CC8, CC30, and ST93. The protein sequences of the HsdS variants were aligned with Clustal Omega, and TRDs that match exactly are color coded in the same color.

isolated using the Qiagen 100/G genomic tip (Qiagen). Weakening of the staphylococcal cell wall required the addition of 100 μg of lysostaphin (Ambi) into the lysis buffer and incubation at 37°C for 30 min. Plasmids and PCR products were isolated using the Wizard plus kits (Promega), with T4 DNA ligase also purchased from Promega. Plasmids were isolated from staphylococci as described previously (3). Restriction enzymes, T4 DNA polymerase, and Phusion DNA polymerase were purchased from New England Biolabs. Phire Hotstart DNA polymerase was purchased from Thermofisher. Sanger sequencing was supplied by Eurofins. Routine manipulation of *S. aureus* and *E. coli* was performed as described by Monk et al. (3). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Melford) was used at 50 μg/ml in *E. coli* and 100 μg/ml in *S. aureus*. Antibiotics were purchased from Sigma Aldrich and used at the following concentrations: carbenicillin (Car), 100 μg/ml; chloramphenicol (Cm), 10 μg/ml; and kanamycin (Kan), 50 μg/ml (*E. coli*) and 100 μg/ml (*S. aureus*).

SMRT sequencing. Genomic DNA samples were submitted to the Duke IGSP Genome Sequencing and Analysis Core Resource for sequencing on a Pacific Biosciences *RS* instrument. Libraries of 8 to 11 kb were constructed and sequenced using P5-C2 chemistry. Data were analyzed using the SMRT Portal version 2.2.0 (Pacific Biosciences) and Motif Finder 1.3.1. Reference *S. aureus* genomes with plasmids omitted were assessed for their type I methylation profiles. DH10B was used as a reference for the IMXXB *E. coli* strains. Additionally, *E. coli* strain DC10B was SMRT sequenced, with only *dam* methylation observed (data not shown). A quality value (QV; predic-

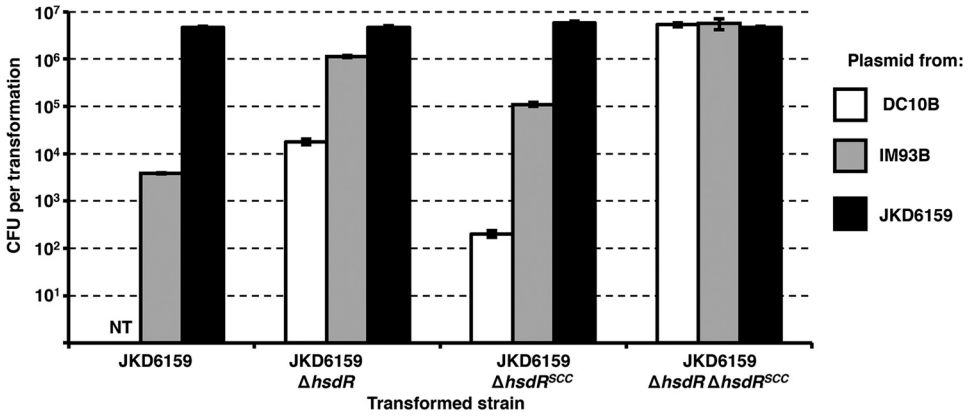


FIG 6 Transformation of JKD6159 and type I restriction mutants with pRAB11. Plasmid pRAB11 was isolated from *E. coli* strains DC10B and IM93B and *S. aureus* strain JKD6159 for transformation into JKD6159 and the respective type I-deficient mutants. pRAB11 contains 4 TRM sites in ST93-1, 2 in ST93-2, and 2 in ST93-3. The transformation efficiencies were expressed as the mean numbers of all transformants obtained in each experiment ± standard deviations (error bars) from three replicates. The graph shows representative data from one experiment. NT, no transformants were isolated.

tion of the error probability of a base call) threshold of 30 was set for adenine modification/motif calling.

Electroporation. *E. coli* and *S. aureus* were transformed as described previously (3), except that staphylococci were grown for 40 min rather than 30 min after dilution back to an optical density at 600 nm of 0.5. MW2 competent cells were created as described by Augustin and Götz (31). Routinely, we used 5 μg of pRAB11 and 2.5 μg of pIMAY-Z with the concentration determined by fluorometric assay (Qubit 2.0; Life Technologies) to transform *S. aureus*. The results presented are from three independent transformation experiments.

SLIC. To construct pIMAY-Z, we applied sequence- and ligase-independent cloning (SLIC) with the modifications described by Bieniossek et al. (32). SLIC allows rapid, flexible, and efficient cloning into plasmid DNA without restriction enzyme sequence site specificity. To insert *lacZ* into pIMAY, the entire 5.7 kb pIMAY plasmid was linearized downstream from the *cat* stop codon and amplified with primers IM452/IM453, and the *E. coli* lacZ gene was amplified from pMUTIN4 using primers IM454/IM455 including a consensus Gram-positive ribosome binding site. The 5′ tails of the *lacZ* primers included 30 nucleotides of homology with the ends of the amplified pIMAY. Both fragments were purified to remove unincorporated deoxynucleoside triphosphates (dNTPs) (Wizard SV Gel and PCR clean up system; Promega). A 1 μg aliquot of each PCR product was treated with 3 U of T4 DNA polymerase (to create single-stranded ends) in the NEB 2 restriction enzyme buffer containing, additionally, 200 mM urea (2 M stock), 10 μg/ml bovine serum albumin (1 μg/ml stock), and 5 mM dithiothreitol (1 M stock), final concentration in 40 μl. The reaction mixture was incubated at 23°C for 20 min, and then the enzyme inactivated by the addition of 2 μl of 500 mM EDTA, pH 8, and further incubation at 75°C for 20 min. Both fragments were mixed (125 ng of each product) and incubated on a thermocycler at 65°C for 10 min, followed by decreases of 1°C per minute to 25°C. The annealed vector and insert were ethanol precipitated and electroporated into *E. coli* strain DC10B, with transformants selected on Luria agar (L agar) plus Cm and X-Gal. To clone into pIMAY-Z, the plasmid was first linearized with KpnI and gel extracted. The vector backbone was amplified (from within the multiple cloning site [MCS] using primers IM427/IM428) with 0.5 U of Phusion and 0.25 U of Phire DNA polymerase (Finnzymes) with annealing at 50°C and extension for 3 min in a 50 μl reaction mixture. Deletion constructs (see below) were amplified by SOE PCR (3) and SLIC cloned into PCR-amplified pIMAY-Z, as described above.

Construction of pIMK7. A kanamycin-resistant shuttle plasmid was created by amplification of (i) the *E. coli* p15a replicon, the pBluescript II

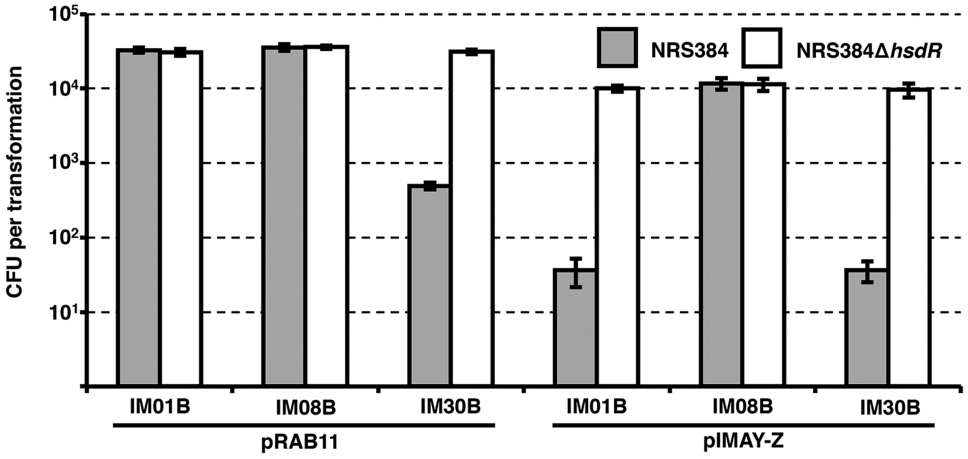


FIG 7 Transformation of *S. aureus* NRS384 and NRS384 Δ*hsdR* with plasmid isolated from IMXXB *E. coli*. Plasmid pRAB11 or pIMAY-Z was isolated from *E. coli* strains IM01B, IM08B, and IM30B for transformation into a CC8 host (NRS384) and the respective *hsdR* mutant. The transformation efficiencies are expressed as the mean numbers of all transformants obtained in each experiment ± standard deviations (error bars) from three replicates. The graph shows representative data from one experiment.

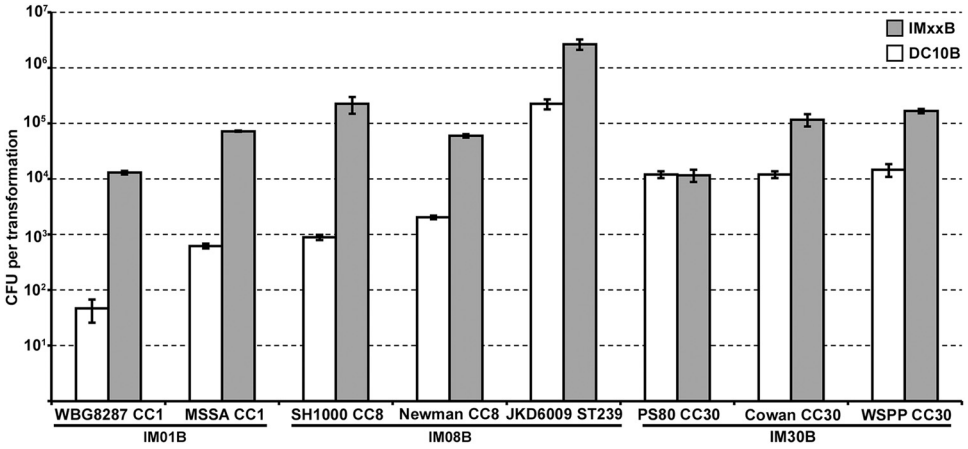


FIG 8 Transformation of additional representative isolates from CC1, CC8/ST239, and CC30 with plasmid isolated from IMXXB strains. Plasmid pRAB11 (5 μg) was isolated from either *E. coli* strain DC10B or the compatible IMXXB strain for transformation into additional representative *S. aureus* isolates of CC1, CC8/ST239, and CC30. The transformation efficiencies are expressed as the mean numbers of all transformants obtained in each experiment ± standard deviations (error bars) from three replicates. The graph shows representative data from one experiment.

KS MCS, and the *aphA3* kanamycin resistance gene (primers IM297/IM49) from pIMK (18) and (ii) the pC194 *S. aureus* replicon (primers IM295/IM296) from pRAB11. The two fragments were joined by SOE PCR (primers IM295/IM49) and transformed into DC10B to generate pIMK7 (4.9 kb).

Cloning *hsdMS* loci into pET21d+. Type I modification genes from *S. aureus* NRS384 were cloned into pET21d+, an IPTG-inducible expression plasmid that is active in the BL21 *E. coli* host (naturally *dcm* deficient). Modification genes were amplified as follows. (i) The internal NcoI site was removed from CC8-1 *hsdMS* by SOE PCR (primers IM517, IM521, IM522, and IM8, incorporating flanking NcoI/SacI sites). (ii) The NcoI$^-$ variant of the CC8-2 *hsdMS* gene was amplified from SA08B (see below) genomic DNA (primers IM516/IM10, incorporating NcoI/XhoI sites). (iii) A hybrid of CC8-2 *hsdM* and CC8-1 *hsdS* was created by SOE PCR (primers IM516, IM519, IM518, and IM8, incorporating NcoI/SacI sites). All PCR products were ligated into complementarily digested pET21d+. Ligations were cotransformed with pIMK7 into BL21 and transformants selected on L agar plus Kan/Car. Plasmids were isolated from BL21 strains after induction overnight with 0.1 mM IPTG.

Construction of *E. coli* IM08B for the transformation of CC8 and ST239. To stably express CC8-2 *hsdMS* genes in *E. coli*, we bypassed the cloning step by *in vitro* PCR assembly of the CC8-2 *hsdMS* joined to an antibiotic resistance marker. The final construct could then be recombineered into the DC10B chromosome at a neutral location. First, we utilized SOE PCR to remove internal NcoI sites from the CC8-2 *hsdMS* of NRS384 (using primers IM373, IM374, IM375, and IM10), and then the PCR product was ligated into NcoI/XhoI-cut pIMK2 (allowing high-level expression from the P_{help} promoter [18]). PCR products comprising P_{help}-*hsdMS* were then amplified from the ligation mixture with primers IM367/IM10. The *cat* gene, flanked by Flp recombinase target (FRT) sites, was amplified with primers IM359/IM360 from plasmid pKD3 and joined to the CC8-2 *hsdMS* amplimer by SOE PCR (IM367/IM360). These primers contained tails with 50 bp of homology for integration into the intergenic region between *atpI* and *gidB* in the DC10B chromosome (33). The PCR products were ethanol precipitated and electroporated into DC10B containing pKD46 made competent as described previously (3). Transformants were selected on L agar plus Cm. Colonies were screened by colony PCR for recombination (primers IM434 and IM435), and positive clones were grown overnight at 43°C to promote loss of pKD46. To excise the FRT site-flanked *cat* gene, the strains were transformed with pCP20 at 28°C, single colony purified at 28°C, and then grown in broth overnight at 43°C to promote loss of the plasmid. Cells were patched onto L agar or L agar plus Cm to confirm the excision of the antibiotic resistance marker, creating strain SA08B. The CC8-1 *hsdS* gene of NRS384 was amplified with primers IM103/IM104 containing the P_{N25} promoter within IM103. The kanamycin resistance marker was amplified with IM105/IM106 from pKD4, and SOE PCR performed to join the two products with IM102/IM106, which target the product to integrate upstream from *essQ* and downstream from *cspB* (33). The amplimer was transformed into SA08B(pKD46) and processed as described above. The Kan^s strain was termed IM08B. Shuttle plasmid (pRAB11 or pIMAY-Z) was isolated from either DC10B or IM08B and used to transform NRS384 or NRS384 Δ*hsdR* to investigate plasmid methylation.

Construction of *E. coli* IM01B for the transformation of CC1. The CC1-1 *hsdS* gene of MW2 was amplified with primers IM103/IM133 containing the P_{N25} promoter within IM103. The kanamycin resistance marker was amplified with IM134/IM106 from pKD4 and SOE PCR performed to join the two products with primers IM102/IM106. The amplimer was transformed into SA08B(pKD46) and processed as described above. The Kan^s strain was termed IM01B. Shuttle plasmid (pRAB11 or pIMAY-Z) was isolated from either DC10B or IM01B and used to transform MW2 or MW2 Δ*hsdR*.

Construction of *E. coli* IM30B for the transformation of CC30. The two native NcoI sites of CC30-2 *hsdMS* of MRSA252 were removed by SOE PCR (using primers IM407, IM408, IM409, IM410, IM411, and IM363, incorporating flanking NcoI/XhoI sites). The MRSA252 CC30-2 *hsdMS* (NcoI$^-$) genes were amplified and ligated into pIMK2 cut with NcoI and XhoI. The P_{help}-*hsdMS* construct was amplified (primers IM367/IM363) from the pIMK2 ligation, and SOE PCR was performed to join the *cat* gene flanked with FRT sites (amplified from pKD3 with primers IM364/IM360). PCR products were electroporated into DC10B (pKD46) and screened as described above. Excision of the *cat* gene yielded *E. coli* strain SA30B. The CC30-1 *hsdS* gene of MRSA252 was amplified with primers IM132/IM104 containing the P_{N25} promoter within IM132. The Kan resistance marker was amplified with IM105/IM106 from pKD4, and SOE PCR with IM102/IM106 performed to join the two products. The amplimer was transformed into SA30B(pKD46) and processed as described above. The Kan^s strain was termed IM30B. Shuttle plasmid (pRAB11 or pIMAY-Z) was isolated from either DC10B or IM30B and used to transform MRSA252 or MRSA252 Δ*hsdR*.

Construction of IM93B for the transformation of ST93. A hybrid *hsdMS-hsdS* operon from JKD6159 was constructed by SOE PCR. The ST93-2 *hsdMS* genes were amplified with IM571/IM573 containing the P_{N25} promoter and joined to the ST93-1 *hsdS* gene amplified with primers IM574/IM575. The products were gel extracted and used as the template in an SOE PCR reaction (primers IM572/IM575). The resultant product

TABLE 2 Strains and plasmids

Strain or plasmid	Description	Reference or source
Escherichia coli strains		
DC10B	DH10B Δ*dcm*; does not methylate DNA on cytosine	3
SA08B	DC10BΩP_{help}-*hsdMS* (CC8-2) (SAUSA300_1751) of NRS384 integrated between the *atpI* and *gidB* genes	This study
SA30B	DC10BΩP_{help}-*hsdMS* (CC30-2) (SAR1898) of MRSA252 integrated between the *atpI* and *gidB* genes	This study
IM01B	SA08BΩP_{N25}-*hsdS* (CC1-1) (MW0393) of MW2 integrated between the *essQ* and *cspB* genes	This study
IM08B	SA08BΩP_{N25}-*hsdS* (CC8-1) (SAUSA300_0406) of NRS384 integrated between the *essQ* and *cspB* genes	This study
IM30B	SA30BΩP_{N25}-*hsdS* (CC30-1) (SAR0434) of MRSA252 integrated between the *essQ* and *cspB* genes	This study
IM93B	DC10BΩP_{N25}-*hsdMS/S* (ST93-2/ST93-1) (SAA6159_01737/00387) of JKD6159 integrated between the *atpI* and *gidB* genes and ΩP_{help}-*hsdMS* (ST93-3) (SAA6159_00055) of JKD6159 integrated between the *essQ* and *cspB* genes	This study
BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3), IPTG-inducible T7 RNA polymerase	Novagen
Staphylococcus aureus strains		
NRS384	CC8, USA300, lineage 14, type strain from NARSA collection	BEI resources
MW2	CC1, USA400 lineage, genome sequenced	35
MRSA252	ST36 CC30, hospital-acquired MRSA, genome sequenced	36
JKD6159	ST93, CA-MRSA, genome sequenced	11
NRS384 Δ*hsdR*	Targetron insertion in the *hsdR* gene of NRS384	3
MW2 Δ*hsdR*	Clean deletion of the *hsdR* gene in MW2	This study
MRSA252 Δ*hsdR*	Clean deletion of the *hsdR* gene in MRSA252	This study
JKD6159 Δ*hsdR*	Clean deletion of the *hsdR* gene in JKD6159	This study
JKD6159 Δ$hsdR^{SCC}$	Clean deletion of the $hsdR^{SCC}$ gene in JKD6159	This study
JKD6159 Δ$hsdR^{SCC}$ Δ*hsdR*	Clean deletion of the *hsdR* gene in JKD6159 Δ$hsdR^{SCC}$	This study
WBG8287	CC1, Western Australian (WA)-MRSA-1, SCC*mec* IV	37
MSSA CC1	CC1, MSSA, pvl^+, Rif^r, fusidic acid resistant	Laboratory strain
SH1000	CC8, MSSA, 8325-4 *rsbU* repaired, genome sequenced	38
Newman	CC8, human clinical MSSA, genome sequenced	39
JKD6009	MRSA, ST239, SCC*mec* III(3A), genome sequenced	40
PS80	CC30, MSSA, phage-propagating strain 80, ATCC 27700	41
Cowan	CC30, ST30, MSSA, high-level protein A producer, ATCC 12598	42
WSSP	CC30, ST30, MRSA, pvl^+, SCC*mec* IV	Laboratory strain
Plasmids		
pRAB11	Anhydrotetracycline-inducible shuttle expression vector, pC194 *S. aureus* replicon; Car^r Cm^r	43
pIMAY	Allelic exchange plasmid for staphylococci; Cm^r (IM452/IM453)	3
pIMAY-Z	Carries Gram-positive ribosome binding site and *lacZ* cloned downstream from the constitutive *cat* gene in pIMAY; 8.8 kb, Cm^r	This study
pIMAY-Z(CC1Δ*hsdR*)	Carries deletion encompassing the entire *hsdR* gene (MW_0169 between the ATG and TAA codons); amplified from MW2 (IM243/IM237/IM238/IM244)	This study
pIMAY-Z(CC30Δ*hsdR*)	Carries deletion encompassing the entire *hsdR* gene (SAR_0196 between the ATG and TAA codons); amplified from MRSA252 (IM236/IM237/IM238/IM239)	This study
pIMAY-Z(ST93Δ*hsdR*)	Carries deletion encompassing the entire *hsdR* gene (SAA6159_00176 between the GTG and TAA codons); amplified from JKD6159 (IM678/IM679/IM680/IM681)	This study
pIMAY-Z(ST93Δ$hsdR^{SCC}$)	Carries deletion encompassing the entire $hsdR^{SCC}$ gene (SAA6159_00056 between the TTG and TAA codons); amplified from JKD6159 (IM684/IM685/IM686/IM687)	This study
pMUTIN4	Template for *E. coli* lacZ; Car^r (IM454/IM455)	44
pIMK	*E. coli*/*L. monocytogenes* phage integrase vector, does not replicate in *S. aureus*; Kan^r	18
pIMK2	Template for P_{help} promoter; Kan^r	18
pKD46	*E. coli* temperature-sensitive plasmid containing λ red recombinase genes under the control of an arabinose-inducible promoter; Car^r	45
pKD3	Plasmid for amplification of FRT-*cat*-FRT for *E. coli* gene deletion; Car^r Cm^r	45
pKD4	Plasmid for amplification of FRT-*kan*-FRT for *E. coli* gene deletion; Car^r Kan^r	45
pCP20	*E. coli* temperature-sensitive plasmid containing *flp* required for antibiotic marker excision; Car^r Cm^r	45
pIMK7	Shuttle vector with staphylococcal pC194 replicon and p15a *E. coli* replicon (IM295/IM296/IM297/IM49)	This study
pET21d+	IPTG-inducible expression vector; Amp^r	Novagen
pCC8-MS1	CC8-1 *hsdMS* genes cloned via NcoI/SacI (IM517/IM521/IM522/IM8) into pET21d+	This study
pCC8-MS2	CC8-2 *hsdMS* genes cloned via NcoI/XhoI (IM373/IM374/IM375/IM10) into pET21d+	This study
pCC8-M2S1	Hybrid CC8-2 *hsdM*–CC8-1 *hsdS* cloned via NcoI/SacI (IM373/IM518/IM519/IM8) into pET21d+	This study

TABLE 3 Oligonucleotides used in the study

Oligonucleotide purpose, name	Sequence (5′–3′)[a]	RE site[b]
Creation of pIMAY-Z		
IM452 cat-lacZ F	**TCAGATAGGCCTAATGACTGGCTTTTATAA**AGGAGGATATCCATGGAAGTTACTGACG	
IM453 cat-lacZ R	**TGTAAAAAGTACAGTCGGCATTATCTCATA**TTATTTTTGACACCAGACCAACTGGTAATGG	
IM454 pIMAY F	TTATAAAAGCCAGTCATTAGGCCTATCTGAC	
IM455 pIMAY R	TATGAGATAATGCCGACTGTACTTTTTACAG	
IM427 pIMAY-Z SLIC F	GGTACCCAGCTTTTGTTCCCTTTAGTGAGG	
IM428 pIMAY-Z SLIC R	GAGCTCCAATTCGCCCTATAGTGAGTCG	
Cloning *hsdMS* genes		
IM9 Pro*MS* (CC8-2) F	ATATGGATCCGATGCAATTATTCAGCCTGGTAGC	BamHII
IM10 *hsdMS* (CC8-2) R	ATATCTCGAGTTAAATAAACATTTTTTGTAATAGTCC	XhoI
IM373 ATG *MS* (CC8-2) F	ATATATGGTTTTGAAAGCATTTGAAAGCTAC	NcoI
IM374 *MS* (CC8-2) NcoI-R	AGTACCTTCATCGTCTAGGTAATGTACC	
IM375 *MS* (CC8-2) NcoI-F	ACATTACCTAGACGATGAAGGTACTATGGCCGTTGTACTCCCACATGG	
IM517 ATG *MS* (CC8-1) F	ATATCCATGGCTATTACTGAAAAACAACGTCAGC	NcoI
IM521 *MS* (CC8-1) NcoI-R	TGTACCTTCATCGTCTAGGTAATG	
IM522 *MS* (CC8-1) NcoI-R	GGTACATTACCTAGACGATGAAGGTACAATGGCCGTTGTACTCCCACATGGTG	
IM8 *MS* (CC8-1) R	ATATGAGCTCTTATAAGAACATTTTTTGTAAAAAGGATTG	SacI
IM518 *S* (CC8-1) F	CCTGAAAGAACTTGGGGTGTTGAAAGATGAGTAATACACAAAAGAAAAATGTGC	
IM519 *M* (CC8-2) R	CTTTCAACACCCCAAGTTCTTTCAGGTATGC	
Recombineering		
IM367 *atpI*-P_{help} F	**CAAAAAGCGGTCAAATTATACGGTGCGCCCCCGTGATTTCAAACAATAAGTA**CGGAGCTCC ATTATGCTTTGGCAG	
IM359 *MS* (CC8-2) −*cat* F	**CAAAAAATGTTTATTTAACTCGAG**ATATGTGTAGGCTGGAGCTGCTTC	
IM360 *gidB* − *cat* R	**ATAACGTGGCTTTTTTTGGTAAGCAGAAAATAAGTCATTAGTGAAAATAT**GTCCATATGAA TATCCTCCTTAG	
IM434 *atpI* OUT F	ACTTTCTTTAAGGCTTAGAGTCAAGC	
IM435 *gidB* OUT R	TTTAACGCCACGTTCACTCTTTTGC	
IM102 *essQ*-P_{N25} F	CCCAAACTGCACCCAAGAGTCAGAACACAGTTTTTCAAGAGTACAAAGGGGTCATAAAAAA TTTATTTGCTTTCAGG	
IM103 P_{N25}-*S*(CC8-1) F	**CATAAAAAATTTATTTGCTTTCAGGAAAATTTTTCTGTATAATAGATTCATAAATTTGAGA** GAGGAGTTATGAGTAATACACAAAAGAAAAATGTGC	
IM104 *S* (CC8-1) R	TTATAAGAACATTTTTTGTAAAAAGGATTG	
IM105 *S* (CC8-1)-*kan* F	CAATCCTTTTTACAAAAAATGTTCTTATAAGTGTAGGCTGGAGCTGCTTC	
IM106 *cspB-kan* R	GCTAACCATTGTGGTGAAGTGCAGGTTTGCTGCATGAATAGTTTTACGGTCCATATGAATA TCCTCCTTAG	
IM113 *essQ* OUT F	CGGCCATTTATACAGGAAAAGCCTA	
IM114 *cspB* OUT R	GTTACCTTCTCTATAGAGAGTGGTG	
IM132 P_{N25}*S* (CC30-1) F	**CATAAAAAATTTATTTGCTTTCAGGAAAATTTTTCTGTATAATAGATTCATAAATTTGAGA** GAGGAGTTATGAGTAATACACAAACGAAAAATGTG	
IM133 *S* (CC1-1) R	TCAAATAAACATTTTCTGTAAAAACG	
IM134 *S* (CC1-1)-*kan* F	CGTTTTTACAGAAAATGTTTATTTGAGTGTAGGCTGGAGCTGCTTC	
pIMK7		
IM295 pC194 replicon F	ATATGCATGCGCTTTTAAAAAGCAAATATGAGCC	SphI
IM296 pC194 replicon R	TTTATCTAAAGTGAATTTAGGAGGC	
IM297 pIMC-pC194 F	GCCTCCTAAATTCACTTTAGATAAAATTCTATAATAGAAGGTATGGAGGATG	
IM49 pIMC R	AGATCTCCTCTCGCCTGTCCCCTCAGTTCAGTAATTTCC	BglII
S. aureus mutants		
IM234 CC30 *hsdR* OUT F	TGTGCGTTCTAATATAAAGTTAGTTGC	
IM235 CC30 *hsdR* OUT R	TCCGACTGTTGTATCTTTGTATCTAGC	
IM236 CC30 *hsdR* FA	**CCTCACTAAAGGGAACAAAAGCTGGGTACC**AGTGTGACAAATTAAAGCAAAGTTCAGG	
IM237 CC1/CC30 *hsdR* RB	CATTCATATCCCCTTCCATACACTTTC	
IM238 CC1/CC30 *hsdR* FC	GAAAGTGTATGGAAGGGGATATGAATGTAATGATTCAGCCCCCTCGCTAG	
IM239 CC30 *hsdR* RD	CGACTCACTATAGGGCGAATTGGAGCTCTAATCTCGTAGACAACGCCTTTACC	
IM240 CC30 *hsdR* OUT R	TCCGACTGTTGTATCTTTGTATCTAGC	
IM241 CC1 *hsdR* OUT F	TCAGTTGCTTGATGAAAAATTGTTGC	
IM242 CC1 *hsdR* OUT R	ACTTTGCAAATATCCGCATTCAACC	
IM243 CC1 *hsdR* FA	**CCTCACTAAAGGGAACAAAAGCTGGGTACC**GACAAATTAAAGCAAAGTTCAGATTTGAGC	
IM244 CC1 *hsdR* RD	CGACTCACTATAGGGCGAATTGGAGCTCAGGACTCTCAGAGACATCATTAGC	
IM678 ST93 *hsdR* FA	**CCTCACTAAAGGGAACAAAAGCTGGGTACC**AGATTGCATGATTTTTGTGACGAATCAAGG	
IM679 ST93 *hsdR* RB	CATTCATATCCCCTTCCGTACACTTTCTATTGC	
IM680 ST93 *hsdR* FC	GAAAGTGTACGGAAGGGGATATGAATGTGTAATGATTCAGCCCCCTCGCTAGATTAG	

(Continued on following page)

TABLE 3 (Continued)

Oligonucleotide purpose, name	Sequence (5′–3′)[a]	RE site[b]
IM681 ST93 *hsdR* RD	**CGACTCACTATAGGGCGAATTGGAGCTC**CCACGCAAATAAGGATAATACATATTAATCC	
IM682 ST93 *hsdR* OUT F	AAACGCATTTACTTGTGTCAACATTTGC	
IM683 ST93 *hsdR* OUT R	TAGGTTGAATACAATCACCAATCAAACC	
IM684 ST93 *hsdR*^{SCC} FA	**CCTCACTAAAGGGAACAAAAGCTGGGTACC**ACCTAGGAGTTGGAAAGTTGATGAATTAGG	
IM685 ST93 *hsdR*^{SCC} RB	TTCACTAAATTGAAAGCTCATCTTCATTCACC	
IM686 ST93 *hsdR*^{SCC} FC	ATGAAGATGAGCTTTCAATTTAGTGAATAAATACTGTTTATATTGTTGACCTGTTAGATAC	
IM687 ST93 *hsdR*^{SCC} RD	**CGACTCACTATAGGGCGAATTGGAGCTC**ATAAAATTATTCCACTTTTATGCTCTTGCC	
IM688 ST93 *hsdR*^{SCC} OUT F	GGTGGAGAGATGGTTGATAGTGAATTGG	
IM689 ST93 *hsdR*^{SCC} OUT R	GTTTCCTTTATCTCTTTTTCACTCTCACG	

[a] Boldface indicates 5′ primer tails complementary to the *E. coli* chromosome or pIMAY-Z.
[b] RE, restriction endonuclease.

was gel extracted and joined to the *cat* PCR product (primers IM576/IM548) amplified from pKD3. The *essQ*-P_{N25}-*hsdMS*-*hsdS*-FRT-*cat*-FRT-*cspB* PCR product was electroporated into DC10B(pKD46) and screened as described above. Excision of the *cat* gene yielded *E. coli* strain SA93B. The *hsdMS* adjacent to the SCC*mec* of JKD6159 was amplified with primers IM466/IM467 and cloned into complementarily digested pIMK2 (NcoI/SalI). The *cat* gene was amplified with primers IM499/IM360 from pKD3, and SOE PCR performed to join the two products with primers IM367/IM360. The amplimer was transformed into SA93B(pKD46) and processed as described above; the Cm^s strain was termed IM93B. Shuttle plasmid pRAB11 isolated from DC10B, IM93B, and JKD6159 was used to transform JKD6159, JKD6159 Δ*hsdR*, JKD6159 Δ*hsdR*^{SCC}, or JKD6159 Δ*hsdR* Δ*hsdR*^{SCC}.

Allelic exchange with pIMAY-Z. The derivatives of pIMAY-Z constructed to delete *hsdR* from MW2 (primers IM243, IM237, IM238, and IM244), MRSA252 (primers IM236, IM237, IM238, and IM239), JKD6159 (primers IM678, IM679, IM680, and IM681), and JKD6159 *hsdR*^{SCC} (primers IM684, IM685, IM686, and IM687) were isolated from 25 ml Luria broth cultures of strains with the respective IMXXB backgrounds. The entire plasmid preparation was ethanol precipitated and transformed into electrocompetent *S. aureus* cells. Transformants were selected on brain heart infusion agar (BHIA) plus Cm at 28°C after 2 days. A single colony containing replicating plasmid was homogenized in 200 μl of tryptic soy broth, and the suspension 10-fold diluted to 10^{-3}. A 100 μl aliquot of each dilution was spread plated on BHIA plus Cm and X-Gal and grown overnight at 37°C. Blue colonies were purified on BHIA plus Cm and X-Gal and grown at 37°C. Potential integrants were screened by colony PCR for loss of the replicating plasmid and the site of integration as described previously (3). Integrants from either side of the gene to be deleted were grown overnight at 28°C without antibiotic selection and then plated on BHIA plus X-Gal at 37°C, screening for white colonies. Plasmid loss was confirmed by the chloramphenicol sensitivity of the white colonies and by colony PCR performed using the chromosomal OUT primers listed in Table 3.

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