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Sau1: a Novel Lineage-Specific Type I Restriction-Modification System That Blocks Horizontal Gene Transfer into *Staphylococcus aureus* and between *S. aureus* Isolates of Different Lineages

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The Sau1 type I restriction-modification system is found on the chromosome of all nine sequenced strains of *Staphylococcus aureus* and includes a single *hsdR* (restriction) gene and two copies of *hsdM* (modification) and *hsdS* (sequence specificity) genes. The strain *S. aureus* RN4220 is a vital intermediate for laboratory *S. aureus* manipulation, as it can accept plasmid DNA from *Escherichia coli*. We show that it carries a mutation in the *sau1hsdR* gene and that complementation restored a nontransformable phenotype. Sau1 was also responsible for reduced conjugative transfer from enterococci, a model of vancomycin resistance transfer. This may explain why only four vancomycin-resistant *S. aureus* strains have been identified despite substantial selective pressure in the clinical setting. Using a multistrain *S. aureus* microarray, we show that the two copies of sequence specificity genes (*sau1hsdS1* and *sau1hsdS2*) vary substantially between isolates and that the variation corresponds to the 10 dominant *S. aureus* lineages. Thus, RN4220 complemented with *sau1hsdR* was resistant to bacteriophage lysis but only if the phage was grown on *S. aureus* of a different lineage. Similarly, it could be transduced with DNA from its own lineage but not with the phage grown on different *S. aureus* lineages. Therefore, we propose that Sau1 is the major mechanism for blocking transfer of resistance genes and other mobile genetic elements into *S. aureus* isolates from other species, as well as for controlling the spread of resistance genes between isolates of different *S. aureus* lineages. Blocking Sau1 should also allow genetic manipulation of clinical strains of *S. aureus*.

Staphylococcus aureus is a commensal of the human nose and a common cause of both hospital- and community-acquired infection. It is becoming increasingly virulent and resistant to antibiotics due to the horizontal transfer of mobile genetic elements (MGE) encoding virulence and resistance genes (17). *S. aureus* can be classified into approximately 10 dominant lineages, each with unique surface protein profiles and each capable of causing disease (18). In addition, approximately 15% of any *S. aureus* genome consists of MGE, such as bacteriophage, transposons, plasmids, and pathogenicity islands (17, 18). These elements may be transferred horizontally between isolates at high frequency, both in the laboratory and in vivo (19, 21). However, this is not reflected in the epidemiological spread of resistance and virulence genes among naturally occurring *S. aureus* strains. For example, the transfer of *vanA* to *S. aureus* from vancomycin-resistant enterococci has been exceedingly slow (only four cases) given the high incidence of patients harboring both vancomycin-resistant enterococci and *S. aureus* and treated with vancomycin (30). Another example is that over more than 40 years, only some *S. aureus* lineages have acquired SCC*mec* elements (12, 25) despite the widespread use of methicillin-type antibiotics. Another example is that virulence genes in *S. aureus* carried on MGE include the Panton-Valentine leukocidin genes (*lukS-PV* and *lukF-PV*) found in approximately 2% of all *S. aureus* strains. Only very recently has this toxin been found in isolates that also carry SCC*mec*. These novel methicillin-resistant *S. aureus* (MRSA) isolates are unrelated to hospital-acquired MRSA and are responsible for emerging cases of community-acquired (CA) MRSA causing severe skin and soft tissue infections (5, 6).

In the laboratory, *S. aureus* is notoriously difficult to genetically manipulate and only one available strain, RN4220 (15), can accept *Escherichia coli*-propagated plasmids by electroporation. Consequently, most genetic studies of *S. aureus* are highly dependent on this strain, despite its limited clinical significance. RN4220 is a chemical mutagen of strain 8325-4, which is itself a phage-deficient variant of the clinical isolate 8325 (also known as RN1 or PS47). Neither 8325 nor 8325-4 can accept *E. coli* DNA, suggesting that they carry a specific, active mechanism that blocks uptake. *S. aureus* MGE can be moved between these laboratory strains at a high frequency via transduction, but transfer to clinical isolates is blocked. This prevents the genetic manipulation of representative clinical isolates, such as hospital-acquired MRSA and CA MRSA isolates, and hinders research. We hypothesized that *S. aureus* has specific mechanisms that control the ability of MGE to spread between strains and species and that this has an impact on the evolution of *S. aureus* and our ability to genetically manipulate clinical isolates.

One way in which bacteria may control the uptake of foreign DNA is through the use of restriction-modification (R-M) systems (22). R-M systems are widespread in many types of bacteria. R-M enzymes are complexes that identify specific DNA sequences and modify them, usually by adding a methyl group.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strain		
DH5α	*hsdR17 recA1*	Life Technologies
S. aureus strains		
RN4220	Derivative of 8325-4 that accepts plasmids	15
8325-4	Derived from 8325, missing three prophage	23
879R4RF	Rif^r Fus^r, presumed restriction deficient	29
COL	Laboratory strain, pT181	13
E. faecalis strain		
FA373	JH2-2 carrying pAM378	3
Plasmids		
pSK5632	Replicates in *S. aureus* and *E. coli* (shuttle vector), Amp^r Cm^r	9
p*hsdR*	*Sau1hsdR* from 8325-4 cloned in pSK5632	This study
pMAD	Replicates in *E. coli* (suicide vector), Amp^r *ermC*	1
pT181	Replicates in *S. aureus*, Tet^r	13
pAM378	Pheromone-responsive plasmid, replicates in enterococci, carries Tn*918*, Tet^r	3

If they detect DNA with the same specific sequence that is unmodified, such as that from a foreign bacteriophage, the DNA is digested. The role of R-M is to prevent the uptake of potentially harmful or lethal DNA, such as bacteriophage which lyses and kills bacteria, or prevent the acquisition of superfluous genes that may compromise fitness due to the metabolic demand associated with their expression (7).

There are four types of R-M systems described, including the type II restriction enzymes widely used in genetic laboratories for nucleic acid digestion at specific sequences. Type I R-M systems require three genes, *hsdR* (restriction), *hsdM* (modification), and *hsdS* (specificity). Each gene product forms a subunit that combines as a complex of M_2S or R_2M_2S. M_2S catalyzes the transfer of a methyl group from *S*-adenosylmethionine to adenine residues within a target sequence and protects the DNA from restriction. The R_2M_2S complex is a restriction endonuclease that recognizes DNA unmethylated at the same target sequence and cleaves at a nonspecific distant site to the target sequence. The S subunit determines the specific sequence to be methylated or restricted (22).

Mutagenised *S. aureus* isolates with enhanced susceptibility to bacteriophage lysis were identified many years ago, and it was proposed that R-M systems were responsible for this phenotype (11, 29). The first *S. aureus* R-M system to be characterized in detail was Sau3A, a type II system that specifically digests DNA at GATC sites (28). This system is uncommon in typical *S. aureus* isolates and probably encoded on a MGE. The serotype F bacteriophage-encoded R-M system, Sau42I, was described more recently (4). Sau42I is also relatively rare in *S. aureus* (16) and prevents further phage infection of lysogenized host cells, thereby protecting the interests of the phage rather than the bacterium.

In this study, we aimed to identify the major mechanisms controlling horizontal gene transfer in all *S. aureus* strains. Analysis of the nine available genome sequences of *S. aureus* strains reveals the presence of conserved open reading frames in all strains with high homology to the type 1 R-M systems of other bacteria (10). We have identified a stop mutation in the *sau1hsdR* gene of strain RN4220. Cloning and complementation experiments indicate that this mutation is responsible for the enhanced ability of RN4220 to accept foreign DNA. Furthermore, we show that there is substantial variation in the *sau1hsdS* genes in different strains. These differences correspond to the major lineages of *S. aureus* and suggest that horizontal gene transfer within lineages occurs at a higher frequency than between lineages.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains, plasmids, and bacteriophage used in this study are listed in Table 1. *E. coli* strains were routinely grown in Luria-Bertani (LB) broth or on LB plates. Unless otherwise stated, *S. aureus* isolates were grown in brain heart infusion (BHI) broth or on BHI agar plates. *Enterococcus faecalis* isolates were routinely grown in Todd-Hewitt broth (THB) or on THB agar. Media were supplemented where appropriate with chloramphenicol (10 μg/ml), ampicillin (50 μg/ml), tetracycline (20 μg/ml), fusidic acid (25 μg/ml), or erythromycin (20 μg/ml).

Sequencing. PCR products were amplified using Platinum *Pfx* DNA polymerase (Invitrogen) with conditions of 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 210 s. PCR products were cleaned on QIAquick spin columns (QIAGEN), and both strands of the products were sequenced by Lark Technologies. PCR primers to amplify and sequence the *hsd* genes and their promoters were as follows: *sau1hsdR*, 5′-CGTTTGCGTTGATTGTATTCGG-3′ and 5′-ATGCGGATCCTACACTAATCTAGCGAGG-3′; *sau1hsdM1*, 5′-TCAAAATTAGCTTGAAAGATGG-3′ and 5′-AACCCTGGGAACCTCAATTCTGG-3′; *sau1hsdM*, 25′-CCTGAAATTAAGAAATTCATTGC-3′ and 5′-CCTGGGAATCTCAACTCTGGC-3′; *sau1hsdS1*, 5′-AATGCATACCTGAAAGAACTTGG-3′ and 5′-GACACTGCGCTTTCACAGTGCC-3′; and *sau1hsdS2*, 5′-ATGCATACCTGAAAGAACTTGG-3′ and 5′-CAATTAATAGGTTGTTATCAGG-3′.

Subsequently, all of the *sau1hsdS1* genes from each of the lineages were amplified and sequenced using 5′-CATACCGAGATATGTCGATAC-3′ and 5′-CACTGTGCTATCACAGTGCC-3′.

Complementation of *sau1hsdR*. Plasmid DNA, prepared from bacterial cultures using the QIAGEN plasmid mini kit, was introduced to *E. coli* cells by $CaCl_2$ transformation (26). *S. aureus* transformations were carried out by electroporation, as previously described (27). Primers *hsdR*F (5′-GATCAAGCTTCGTTTGCGTTGATTGTATTCG-3′) and *hsdR*R (5′-ATGCGGATCCTACACTAATCTAGCGAGG-3′) were used to amplify a 3,214-bp fragment encompassing the putative *sau1hsdR* gene and 500 bp of its regulatory region from the strain 8325-4. The resulting PCR product was cloned into the BamHI-HindIII site of pUC18 to give pU*hsdR*. The insert was then subcloned into the KpnI site of pSK5632 (9) to give the plasmid p*hsdR*. Ligations were performed using the rapid DNA ligation kit (Roche) according to the manufacturer's instructions.

Horizontal gene transfer assays and bacteriophage susceptibility. Electroporations were performed according to the method of Schenk and Laddaga (27). The plasmid transferred was pMAD (1), a suicide vector which carries an *ermC*

selectable marker. Plasmid DNA was extracted from *E. coli* DH5α host using QIAGEN plasmid midi kits, and 140 ng total plasmid DNA was added to each electroporation cuvette. Cells were electroporated and recovered for 2 h at 30°C before the entire electroporation mixture was plated and incubated for 48 h at 37°C and erythromycin-resistant colonies were counted.

For conjugation, the method of Clewell et al. (3) was used. The donor bacterial strain was *E. faecalis* FA373 carrying the pheromone-responsive conjugative plasmid pAM378. pAM378 is pAM373 that also carries a copy of Tn*918* encoding a *tet* selectable marker. pAM373 encodes a pheromone receptor expressed on the *E. faecalis* surface that responds to pheromone produced by *S. aureus*. Once triggered, the *E. faecalis* donor produces aggregation substance and binds to *S. aureus*, allowing conjugative transfer of the pAM373 plasmid. The plasmid does not have a functional replication mechanism in *S. aureus* and is lost. However, Tn*918* carried on pAM378 can jump to the *S. aureus* chromosome prior to loss and can be detected by resistance to tetracycline (3). An overnight culture (0.5 ml) of *E. faecalis* donor was mixed thoroughly with 0.05 ml of overnight *S. aureus* recipient strain culture and pipetted onto the surface of a Millipore filter (0.2 mm) placed on THB agar. The matings were incubated at 37°C for 18 h. The cells were then resuspended in 1.0 ml of BHI by vortexing. Aliquots (100 μl) were plated onto mannitol salt agar supplemented with tetracycline to select for *S. aureus* transconjugants. Mannitol salt agar contains salt which inhibits the growth of *E. faecalis*, and the *S. aureus* isolates ferment mannitol, causing their colonies to appear yellow. To allow estimation of transfer frequency per donor, aliquots were also plated on THB agar supplemented with fusidic acid, which is selective for *E. faecalis*. All plates were incubated at 37°C for 48 h, and colonies were counted.

For bacteriophage susceptibility assays, recipient cells were lysed with phage ϕ75 of the international typing set, grown on either *S. aureus* RN4220 or *S. aureus* 879R4RF (3). *S. aureus* 879R4RF is reported to be R-M negative (29), although the reason for this phenotype is unknown and we could find no mutation in its *sau1hsdR* gene. It belongs to lineage CC51, as determined by a microarray analysis (18; data not shown), and has a typical *sau1hsdS* variant profile for this lineage. In comparison, 8325 and its derivatives are from lineage CC8. Recipient bacteria were grown in BHI until mid-log phase and centrifuged, and the pellets were resuspended in 7 ml phage buffer (1 mM $MgSO_4$, 4 mM $CaCl_2$, 0.1 M NaCl, 50 mM Tris-HCl, 0.1% gelatin, pH 7.8) plus 7 ml BHI. Phage stock (100 μl) was added, and the mixture was incubated at room temperature for 10 min. The tubes were transferred to a shaking water bath and incubated at 32°C and 70 rpm for 4 h and/or overnight until the cells had visibly lysed. To prepare a phage stock, lysed cultures were centrifuged to pellet any cellular debris, filter sterilized through a 0.2-μm filter, and stored at 4°C.

For transduction, the marker transferred was the small class I plasmid pT181 carried in the sequenced strain COL and carrying a *tet* selectable marker. The phage stock used was 80α grown on *S. aureus* 879R4RF transduced with the pT181 plasmid. The transduction method developed by Sean Watson in Simon Foster's laboratory was used, derived from Novick (23). Recipient *S. aureus* strains were grown in 50 ml LK broth (1% tryptone, 0.5% yeast extract, 0.7% KCl) overnight and resuspended in 3.5 ml of LK broth. Fifty microliters of 1 M $CaCl_2$ and 1 ml of phage stock were added. The phage stock had a titer of 1.7×10^{11} PFU per ml when grown on RN4220. The mixture was incubated at 37°C statically for 25 min and then for 15 min in a shaking water bath at 37°C. One milliliter of ice-cold 0.02 M sodium citrate was added and centrifuged, and the pellet was resuspended in 1 ml ice-cold 0.02 M sodium citrate and left on ice for 2 h. The entire mixture was spread plated (100 ml per plate) onto LK with 1% agar plates containing 0.2 mg/ml tetracycline and incubated for 1 h at 37°C. An overlay agar (LK with 0.3% agar base and 20 mg/ml tetracycline) was carefully poured onto the plates, which were incubated for 48 h, and transductants were counted.

All bacteriophage susceptibility and horizontal transfer assays were performed in triplicate, and data are expressed as means and standard deviations. Statistical significance was determined using Student's *t* test.

Microarray analyses. Microarray analyses were performed as reported previously (18). Briefly, 161 isolates of *S. aureus* from the noses of healthy donors and from patients with community-acquired *S. aureus* infection were hybridized to a seven-strain *S. aureus* PCR product microarray containing spots for every gene from the seven *S. aureus* sequencing projects (31). When designing the microarray, we specifically included spots corresponding to putative target recognition domain (TRD) regions of *sau1hsdS* that show major sequence variation. They were (with forward and reverse primers) as follows: N315*sau1hsdS2*TRD1 (5′-TGTTTTAACATCGTCAAGACAAG-3′ and 5′-ATCGTCCGACTTTTGAAGATTG-3′), MW2*sau1hsdS1*TRD1 (5′-CAAAGGCATACCATTTTTAAGGA-3′ and 5′-AGACCTTCTCGACTACCTCCA-3′), MW2*sau1hsdS1*TRD2 (5′-TTGAGAATAAGGGTGGCACTG-3′ and 5′-AAAACGCCTTTTTCCTCTCTTT-3′), MRSA252 *sau1hsdS2*TRD2 (5′-CAAGTATATGGAGGCGGAACAC-3′ and 5′-TTTTGAAGTAATCCTTGTTTGAGA-3′), MRSA252*sau1hsdS1*TRD1 (5′-GGAAAAGAATATTTTGGCTCAGG-3′ and 5′-GAAACAGGGTATATGACCTTCA-3′), and MRSA252*sau1hsdS1*TRD2 (5′-AAAAGGCTATATGCAGAAAATC-3′ and 5′-TTAAATTTATGAATCATTCTATGTG-3′). The prefix refers to the sequenced strain used as the template in the PCR. Using GeneSpring version 7.0 (Silicon Genetics) analysis and a gene list containing 728 core variable genes (all genes minus core genes minus MGE genes), the isolates were clustered. The isolates fell into 10 dominant and some minor groups, and these groups correlated with clonal complexes determined by multilocus sequence typing (18). For this study, we focused on the microarray spots corresponding to the *sau1hsdS* gene variants. The presence or absence of an *hsdS* variant region in a test isolate is determined by its hybridization to the relevant spot (detected by Cy3 fluorescence) in comparison to hybridization of the reference strain MRSA252 (detected by Cy5 fluorescence) and is expressed as a ratio. The ratios can be visualized in GeneSpring by their color, with yellow indicating the presence of an *hsdS* variant region in both the test and reference strains, red in the test strain only, and blue in the reference strain only. Fully annotated microarray data have been deposited in BμG@Sbase (accession number E-BUGS-33; http://bugs.sgul.ac.uk/E-BUGS-33) and also ArrayExpress (accession number E-BUGS-33).

RESULTS

Identification of an *hsdR* mutation in RN4220 and complementation. RN4220 was derived from 8325-4 by chemical mutagenesis and is widely used in the laboratory as a recipient for electroporated plasmids from *E. coli* (15). It seemed possible that RN4220's proficiency in accepting foreign DNA was due to some deficiency in its restriction-modification system. The *S. aureus* sequencing projects, including that of 8325 (accession number CP000253), identified a putative type I R-M system common to all nine sequenced *S. aureus* isolates. It consists of one *hsdR* gene found on the chromosome, an *hsdMS* operon on genomic island alpha (GIα), and a second *hsdMS* operon on GIβ. *hsdR* and both *hsdM* genes are highly homologous in the nine whole-genome sequences. They are 75% and 85% homologous to *hsdR* and an *hsdM* gene in *Staphylococcus saprophyticus* ATCC 15205 and 59% and 76% homologous to *hsdR* and an *hsdM* gene in *Staphylococcus epidermidis* RP62A. Otherwise, they do not belong to the type IA to IE families (2) but represent a new family. Following standard nomenclature (24), we propose that the genes be called *sau1hsdR*, *sau1hsdM1*, and *sau1hsdM2* and that their products be called R.Sau1, M1.Sau1, and M2.Sau1, respectively. The two *hsdS* genes varied substantially within each isolate. In addition, there was substantial variation between *hsdS* genes in different sequenced isolates. They have been named according to their lineage, for example, the genes from *S. aureus* N315 of lineage CC5 are named *sau1CC5hsdS1* and *sau1CC5hsdS2* and their subunit products S1.Sau1CC5 and S2.Sau1CC5. While other putative R-M systems are identified in the whole-genome sequencing projects, they are all associated with MGE. Sau1 is the only chromosomal R-M system and the only one with widespread distribution in all *S. aureus* isolates.

All five *hsd* genes from RN4220 were sequenced and compared to those of parent strain 8325-4. The *hsdR* gene from RN4220 contained a G-to-A substitution, which introduces a premature TGA stop codon resulting in a truncated R.Sau1 product of 192 amino acids, which is about 20% of the wild-type length. The sequences of the other *hsd* genes were identical in RN4220 and 8325-4. A lack of functional R.Sau1 is predicted to prevent RN4220 from digesting foreign DNA that is unmodified at sites specified by RN4220 S1.Sau1 and

S2.Sau1; however, normal modification of host DNA should be possible. The mutation would explain the ability of RN4220 to accept foreign DNA from *E. coli*, assuming that *E. coli* DNA is modified differently from RN4220 DNA. It also suggests that RN4220 should be more efficient at accepting foreign DNA from other hosts such as enterococci via conjugation. Furthermore, if there is functional variation in S1.Sau1 and S2.Sau1 in some *S. aureus* isolates, it suggests that RN4220 should be more susceptible to bacteriophage if that phage was grown on an *S. aureus* isolate with a modification or specificity system different from that of RN4220. Similarly, RN4220 should have enhanced ability to accept DNA from *S. aureus* isolates with different modification or specificity systems via transduction compared to 8325-4. To confirm these hypotheses, it was necessary to complement the *sau1hsdR* mutation and determine the ability of this derivative to accept foreign DNA. To facilitate expression of full-length *sau1hsdR* in RN4220, the intact *sau1hsdR* gene from 8325-4 was cloned into the shuttle vector pSK5632 (9) to create p*hsdR*. RN4220 was transformed with p*hsdR* or, as a control, pSK5632, and tested for its susceptibility to bacteriophage lysis and its ability to accept foreign DNA via electroporation, transduction, and conjugation from a variety of donors. Although pSK5632 is a multicopy plasmid, R.Sau1 must combine with M.Sau1 and S.Sau1 to form a functional enzyme complex, limiting the effect of multiple gene copies.

Horizontal gene transfer assays and bacteriophage susceptibility. The electroporation results are presented in Fig. 1A. The two recipients with intact *sau1hsdR*, 8325-4 and RN4220 p*hsdR*, were unable to accept DNA from *E. coli*. In contrast, RN4220 and the control RN4220 pSK5632, both with a mutated *sau1hsdR* gene, accepted plasmid pMAD from *E. coli* at high rates. This result shows that the complemented *sau1hsdR* gene is able to completely block foreign DNA uptake from *E. coli* by RN4220, accounting entirely for this phenotype.

The conjugation transfer results are presented in Fig. 1B. The two recipients with a mutated *sau1hsdR* gene accepted Tn*918* from enterococci at a rate of 80 and 73.3 transconjugants per 10^8 donors. In contrast, RN4220 p*hsdR* accepted Tn*918* at a frequency of 4.6 transconjugants per 10^8 donors, which is 16 times less efficient; this difference is statistically significant ($P < 0.001$). 8325-4 did not accept Tn*918* in this series of experiments at a transfer rate of <2.3 transconjugants per 10^8 donors. These data show that RN4220 can accept foreign DNA via conjugation from enterococcal donors at a significantly higher rate than its parent 8325-4 and that this phenotype is due to *sau1hsdR* mutation.

RN4220 and 8325-4 (lineage CC8) differed in their susceptibilities to lysis by certain phages that have been prepared on other *S. aureus* strains. Cultures of RN4220, but not 8325-4, were readily lysed by particles of ϕ75 that were produced by lytic infection of *S. aureus* 879R4RF (lineage CC51). Cultures of RN4220 pSK5632 were lysed by the same phage, but cultures of RN4220 p*hsdR* were not lysed (Fig. 2). Thus, the mutation in *sau1hsdR* in RN4220 causes the bacterium to be susceptible to lysis, and the complemented *sau1hsdR* gene protects from lysis. Furthermore, our hypothesis predicts that phage grown on *S. aureus* of the same lineage as 8325-4 and RN4220 (CC8) will not be digested in 8325-4 with intact *sau1hsdR*. This is because the phage DNA will be modified and not digested by the Sau1 complex. When infected by ϕ75 grown on RN4220 (using stock phage taken from the earlier experiment; RN4220 is prophage negative), all four recipients were lysed (Fig. 2). This shows that the modification and specificity subunits *sau1hsdM* and *sau1hsdS* are functional and sufficient to modify phage DNA as "self." We could repeat this experiment with a range of different phages grown on 879R4RF, including ϕ80 and ϕ83C from the international phage typing set and the generalized transducing phages 80α and ϕ11.

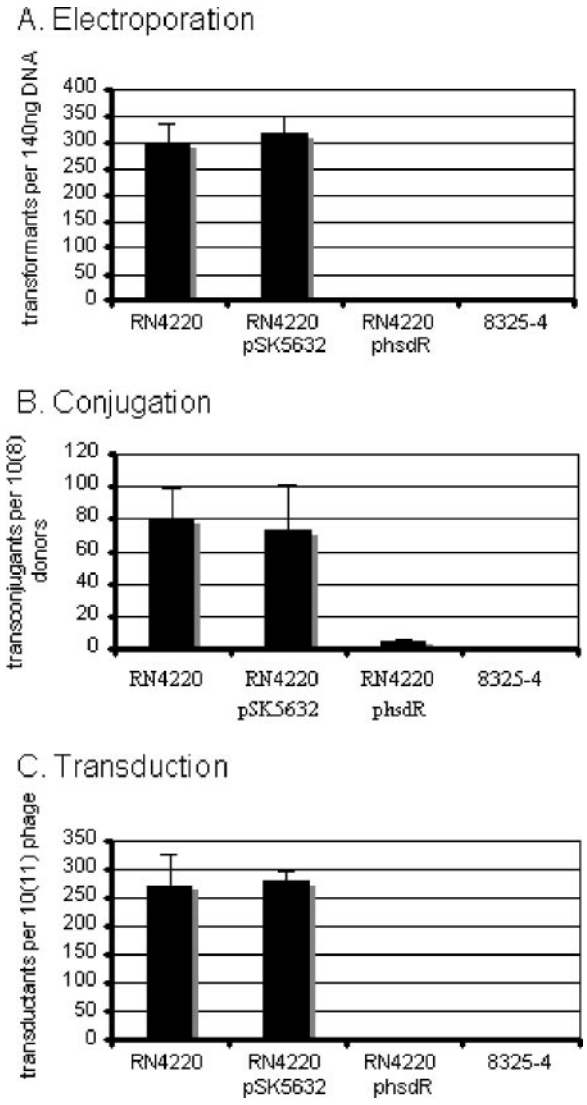

FIG. 1. Horizontal gene transfer assays with *sau1hsdR*-positive and *sau1hsdR* mutant strains. 8325-4 carries *sau1hsdR*, and RN4220 carries the *sau1hsdR* mutation. RN4220 pSK5632 carries the control plasmid, and RN4220 p*hsdR* carries an intact copy of *sau1hsdR*. All experiments were performed in triplicate, and data are presented as means and standard deviations. There was a significant difference between *sau1hsdR* and *sau1hsdR* mutant strains for all three assays ($P < 0.001$). (A) Electroporation. Data are presented as the number of transformants per 140 ng of pMAD plasmid DNA selected using erythromycin. (B) Conjugation. Data are presented as the number of transconjugants carrying Tn*918* per 1×10^8 *E. faecalis* donors selected using tetracycline. (C) Transduction. Data are presented as the number of transductants carrying pT181 per 1×10^{11} donor 80α bacteriophage selected using tetracycline.

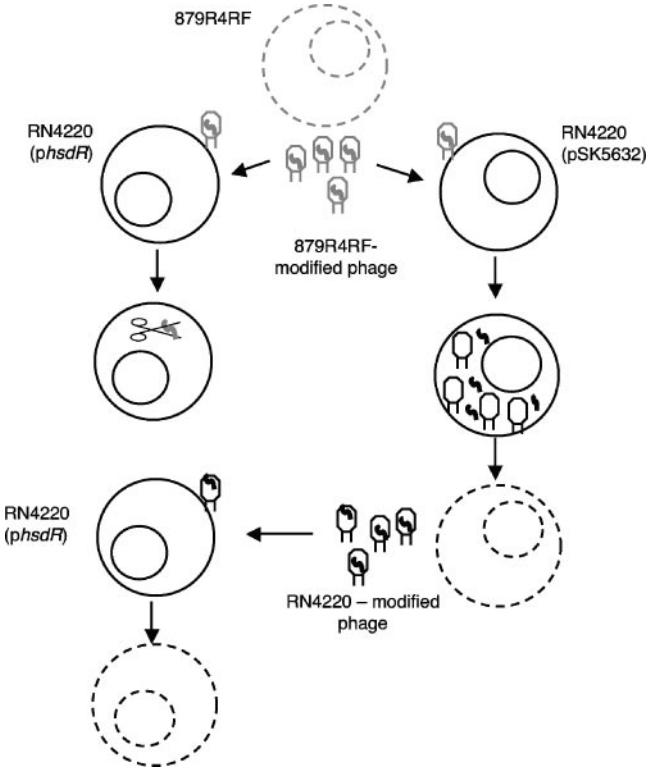

FIG. 2. Illustration of bacteriophage susceptibility experiments. Bacteriophage ϕ75 was grown on *S. aureus* 879R4RF, where we assume its DNA was modified by an R-M system. This phage could infect RN4220 pSK5632 but could not infect RN4220 p*hsdR* as the intact restriction enzyme digested the "foreign" DNA. In the process of lysing RN4220 pSK5632, ϕ75 replicated and its phage genome was modified by the RN4220 R-M system. The progeny phage is now capable of infecting RN4220 p*hsdR* because the phage DNA is modified appropriately and not recognized as "foreign."

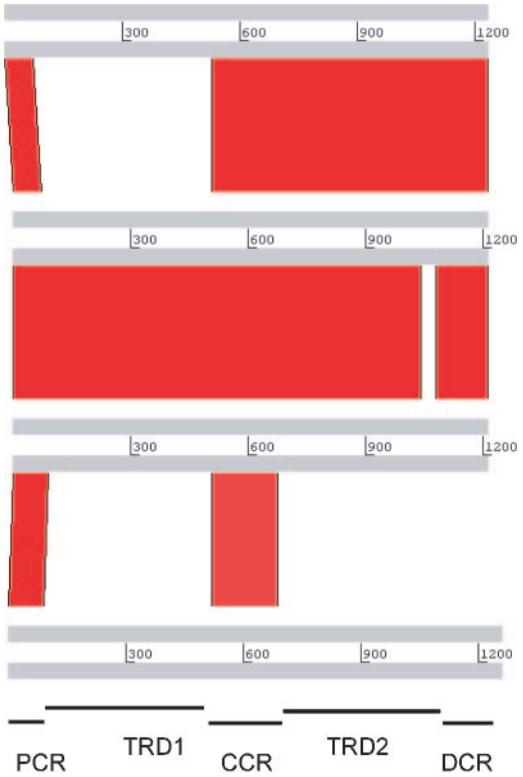

FIG. 3. Variation in *hsdS* genes. Artemis Comparison Tool comparison of *hsdS1* nucleotide sequences of (in descending order) MRSA252, 8325, N315, and MW2. The major regions marked are according to those described by Kim et al. (14) and are TRD1 and TRD2, PrCR (labeled PCR), CCR, and DCR.

Results from the transduction experiments are presented in Fig. 1C. Plasmid pT181 could be transduced from 879R4RF by using generalized transducing phage 80α into RN4220 and RN4220 pSK5632. However, it could not be transferred into 8325-4 or RN4220 p*hsdR*. pT181 could be transferred from RN4220 by using 80α into all four recipient strains (data not shown).

Distribution of *hsdS* types in 161 *S. aureus* isolates. The seven whole-genome sequencing projects revealed that *sau1hsdR* and both copies of *sau1hsdM* are highly conserved in all strains but that significant variation occurs in the two copies of *sau1hsdS* genes (Fig. 3), both between the copies and between strains. For all strains, the two copies of *sau1hsdS* genes were different, and we refer to them as *sau1hsdS1* and *sau1hsdS2* according to the GIs they are carried on, GIα and GIβ, respectively. A short region of conserved sequence is found in the center of the gene in all cases, the central conserved region (CCR). Most *sau1hsdS* genes also have a conserved short 5′ end called the proximal conserved region (PrCR) as well as a 3′ end called the distal conserved region (DCR). The CCR is approximately twice the size of the PrCR or DCR. The majority of the gene variation occurs in two large regions, TRD1 and TRD2. Some strains carry the same TRD1 region in both *sau1hsdS1* and *sau1hsdS2*. We also noted that both *sau1hsdS* genes were highly conserved in the sequenced isolates of the same lineage; 8325, COL, and USA300 of CC8 were the same, MW2 and MSSA479 of CC1 were the same, and N315 and Mu50 of CC5 were the same.

We had previously built a seven-strain *S. aureus* microarray and specifically designed PCR product probes corresponding to TRD1 and TRD2 regions (31). In a study of 161 community isolates of *S. aureus*, the isolates clustered into 10 dominant lineages and several minor lineages based on "core variable" genes, including many surface protein variants (18). Figure 4 shows that each lineage also has a unique pattern of *sau1hsdS* TRD region carriage and that there is a strong similarity between isolates of the same lineage. The *sau1hsdS* genes are carried on genomic islands GIα and GIβ, and these regions are highly variable. However, we have previously reported that they are conserved within lineages and appear stable (18).

Since the microarray carries TRD sequences corresponding to the first seven sequenced strains which come from four lineages (CC1, CC5, CC8, and CC30), it is likely that isolates from nonsequenced lineages carry novel *sau1hsdS* variants and that these regions are not represented on the microarray. We therefore sequenced *sau1hsdS* genes from isolates of other lineages.

Sequencing of *hsdS* from other lineages. Novel *sau1hsdS1* gene sequences from isolates representing lineages CC8, CC12, CC15, CC22, CC25, CC45, CC39, and CC51 were sequenced. We have deposited them in GenBank with accession

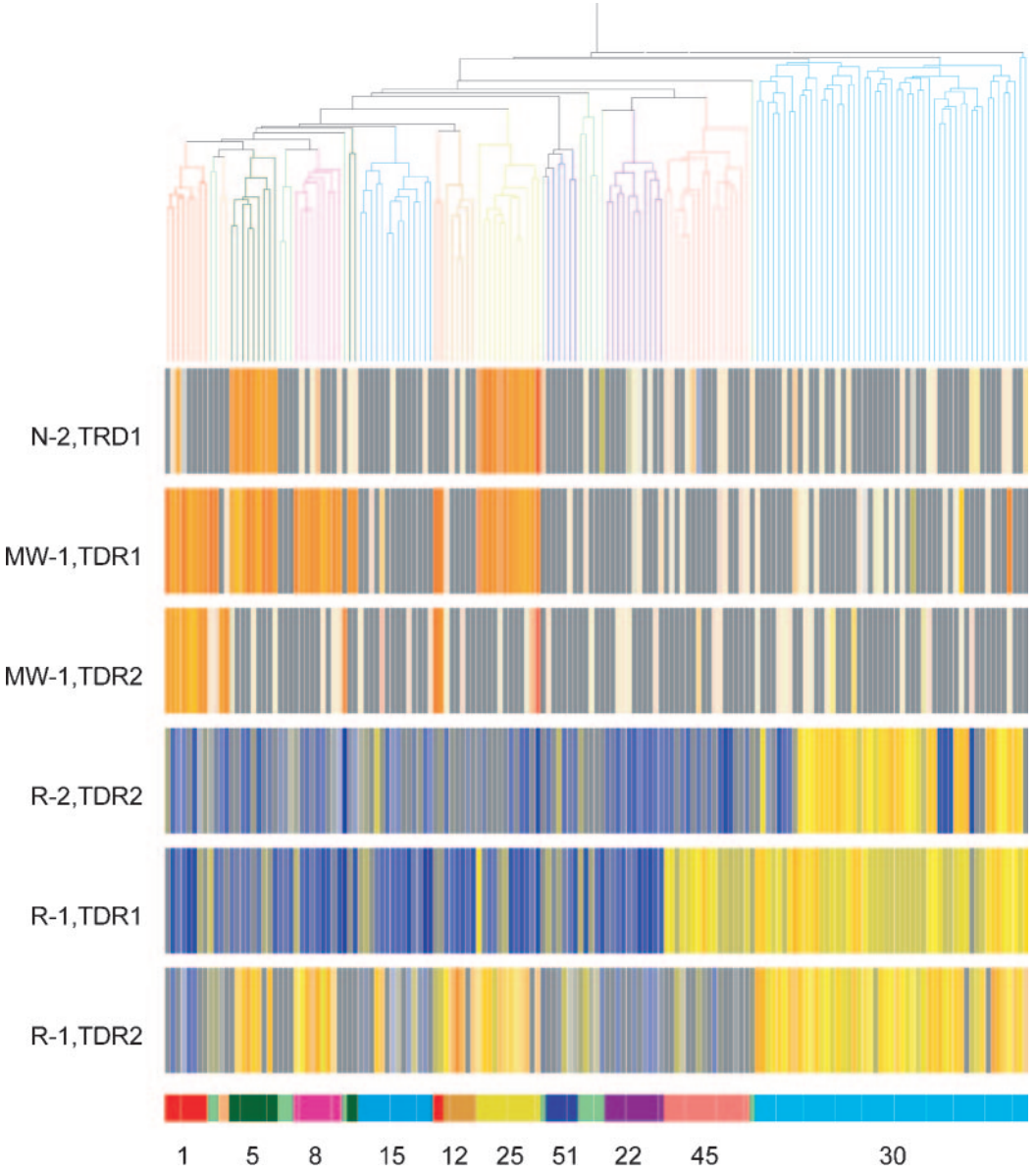

FIG. 4. Distribution of *sau1hsdS* TRD variant regions in 161 community isolates of *S. aureus* by using microarrays. Each vertical line represents an isolate of *S. aureus* and has been clustered into the dominant lineages (marked on the bottom row) by using core variable genes (18). The branches are colored according to the multilocus sequence typing clonal complex, and the two methods show high homology. The horizontal rows correspond to six different TRD-specific PCR product spots on the microarray. In descending order, they are N315*sau1hsdS2*TRD1, MW2*sau1hsdS1*TRD1, MW2*sau1hsdS1*TRD2, MRSA252*sau1hsdS2*TRD2, MRSA252*sau1hsdS1*TRD1, and MRSA252*sau1hsdS1*TRD2. The signal generated on the microarray is a ratio of test strain fluorescence (Cy3) to reference strain fluorescence (Cy5), with the reference strain being MRSA252. A yellow square represents a positive signal in both the test and reference strains. Blue is positive in the reference only. Red is positive in the test only. Negative signals are brown/green or gray. The data show that there is variation in carriage of *sau1hsdS* TRD regions between isolates, and this variation correlates with lineage.

numbers DQ30949 to DQ30955. A Clustal W alignment of these sequences along with those from the sequencing projects is available from the authors on request. As expected, there is enormous variety in *sau1hsdS* gene sequences in the different lineages, and we have identified five types of TRD1 regions and nine types of TRD2 regions which can interchange such that each isolate (representing a lineage) has its own unique combination. Interestingly, *sau1CC12hsdS1* on GIα has a stop mutation leading to a truncated S1.Sau1. To confirm that this was not a mutation in a single strain, a second CC12 isolate was sequenced, and it had exactly the same stop mutation.

DISCUSSION

We have described the major mechanism in *S. aureus* that controls the uptake of foreign DNA in all strains of *S. aureus*. It is a type 1 R-M system encoded by an *sau1hsdR* gene on the chromosome and by two copies of *sau1hsdM* and *sau1hsdS* on

genomic islands GIα and GIβ. GIα and GIβ are stable in *S. aureus* chromosomes, and there is no evidence that they are mobilized (18). The R-M system serves to protect the bacterial cell from phage lysis as well as stringently controls all of the major mechanisms of foreign DNA acquisition, namely, transduction, conjugation, and transformation (via electroporation). Furthermore, we prove that RN4220 has a mutation in *hsdR* that allows it to accept foreign DNA and thus accounts for its usefulness as an intermediary in generating genetically modified *S. aureus* in the laboratory.

The Sau1 type 1 R-M system found in all *S. aureus* strains recognizes and digests "foreign" DNA. But what is foreign? We show here that the 10 dominant lineages of *S. aureus* all carry unique combinations of *sau1hsdS* genes that control the sequence specificity of the system. Thus, DNA from *S. aureus* strains of different lineages is foreign. This has implications for the evolution of *S. aureus*. Firstly, a significant proportion of the *S. aureus* genome consists of MGE carrying various virulence and resistance genes. We therefore predict that MGE present in one strain will transfer horizontally to an *S. aureus* strain of the same lineage at a higher frequency than to *S. aureus* strains of other lineages. This could therefore explain why methicillin resistance (*mecA*) encoded on a staphylococcal cassette chromosome (SCC*mec*) appears to have moved into only six of the dominant *S. aureus* lineages (CC1, CC5, CC8, CC22, CC30, and CC45). Similarly, we might predict that if *vanA* transfers again into *S. aureus* but is not identified and contained, it may not spread immediately to other *S. aureus* lineages. Sau1 likely delays the evolution of new strains carrying multiple virulence and resistance gene combinations.

Secondly, each of the *S. aureus* lineages is very distinct from one another, differing in hundreds of gene and gene variant combinations. Within lineages, there is still some variation, particularly point mutations (10). Genetic exchange of nonmobile DNA between *S. aureus* isolates can occur via generalized transduction and is thought to contribute to the evolution of the different lineages (8, 18). The R-M model suggests that genetic exchange of any kind may be more efficient between isolates with the same *sau1hsdS* profile than between those with different profiles. Therefore, *sau1hsdS* variation could "define" each lineage, limiting the ability of isolates to exchange DNA with other lineages and contributing to the divergence of lineages. As *S. aureus* is known to be unusually clonal compared to other pathogens such as *Neisseria meningitidis*, the Sau1 system may provide a biological explanation for this clonality.

Recently, Kim et al. (14) determined the crystal structure of a type 1 specificity subunit from *Methanococcus jannaschii* and showed that the two TRDs bind independently to specific 3- to 5-base-pair DNA sequences. Both the CCR and DCR form alpha-helices that interact and form a "ruler," separating the TRD regions. This results in a nonspecific gap in the target sequence of 6 to 8 base pairs. This structure agrees with the genomic sequence of the *sau1hsdS* variant genes, although we also notice a PrCR in *sau1hsdS*. Since both the PrCR and DCR are smaller than the CCR in S.Sau1, it is possible that they interact "end to end" to form a functional alpha-helix.

Type I R-M systems are uniquely suited for diversification of sequence specificity because only a single subunit is necessary for specificity (22). The option of two S.Sau1 specificity units in *S. aureus*, which presumably can interchange, adds further flexibility. Others have reported significant chromosomal *hsdS* gene variation between isolates of the same species (20, 22). We believe that this is the first report to link functional *hsdS* variation to stable and well-defined lineages of a bacterial species, suggesting a key role in controlling genetic exchange and evolution of an important bacterial pathogen.

This study also has important implications for the genetic manipulation of *S. aureus* in the laboratory. It is possible to devise a strategy for the manipulation of *S. aureus* from lineages other than CC8, including many hospital-acquired MRSA and CA MRSA isolates. Chemical mutagens of isolates from each of the dominant lineages could be screened for susceptibility to bacteriophage, and the resultant mutants would be novel "intermediate" strains for accepting foreign DNA. Bacteriophage grown on these strains would also serve as useful transducing phage for moving marked DNA into the parental clinical isolates. An alternative approach would be to artificially modify *S. aureus* DNA in *E. coli* or *S. aureus* carrying functional copies of the specific *sau1hsdS* and *sau1hsdM* genes before transfer.

In summary, the Sau1 R-M system is found in all *S. aureus* strains and controls the acquisition of DNA from other bacterial species and from *S. aureus* strains of other lineages. The results of this study suggest that Sau1 has contributed to the evolution of the distinct *S. aureus* lineages and controls the horizontal transfer of MGE that leads to the emergence of increasingly virulent and resistant strains. Manipulation of the R-M system will also enable the generation of molecular tools for the future study of clinical *S. aureus*, leading to greater understanding of pathogenesis and epidemiology and to new strategies for preventing and treating *S. aureus* infection.

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