Mining the Methylome Reveals Extensive Diversity in Staphylococcus epidermidis Restriction Modification

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ABSTRACT Staphylococcus epidermidis is a significant opportunistic pathogen of humans. Molecular studies in this species have been hampered by the presence of restriction-modification (RM) systems that limit introduction of foreign DNA. Here, we establish the complete genomes and methylomes for seven clinically significant, genetically diverse S. epidermidis isolates and perform the first systematic genomic analyses of the type I RM systems within both S. epidermidis and Staphylococcus aureus. Our analyses revealed marked differences in the gene arrangement, chromosomal location, and movement of type I RM systems between the two species. Unlike S. aureus, S. epidermidis type I RM systems demonstrate extensive diversity even within a single genetic lineage. This is contrary to current assumptions and has important implications for approaching the genetic manipulation of S. epidermidis. Using Escherichia coli plasmid artificial modification (PAM) to express S. epidermidis hsdMS, we readily overcame restriction barriers in S. epidermidis and achieved electroporation efficiencies equivalent to those of modification-deficient mutants. With these functional experiments, we demonstrated how genomic data can be used to predict both the functionality of type I RM systems and the potential for a strain to be electroporation proficient. We outline an efficient approach for the genetic manipulation of S. epidermidis strains from diverse genetic backgrounds, including those that have hitherto been intractable. Additionally, we identified S. epidermidis BPH0736, a naturally restriction-defective, clinically significant, multidrug-resistant ST2 isolate, as an ideal candidate for molecular studies.

IMPORTANCE Staphylococcus epidermidis is a major cause of hospital-acquired infections, especially those related to implanted medical devices. Understanding how S. epidermidis causes disease and devising ways to combat these infections have been hindered by an inability to genetically manipulate clinically significant hospital-adapted strains. Here, we provide the first comprehensive analyses of the barriers to the uptake of foreign DNA in S. epidermidis and demonstrate that these are distinct from those described for S. aureus. Using these insights, we demonstrate an efficient approach for the genetic manipulation of S. epidermidis to enable the study of clinical isolates for the first time.

KEYWORDS DNA methylation, Staphylococcus aureus, coagulase-negative staphylococci, generalized transduction, genetic manipulation, genome analysis, type I restriction modification

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**Staphylococcus epidermidis** is a ubiquitous colonizer of human skin (1). Invasive medical procedures, specifically, insertion of prosthetic devices on which the bacteria can form a biofilm, enable evasion of both antibiotics and the host immune system, which has contributed to its increasing importance as a significant nosocomial pathogen. A leading cause of surgical-site- and central-line-associated bloodstream infections (2), *S. epidermidis* poses a major economic burden (3). In the hospital environment, two multilocus sequence types (MLSTs), ST2 and ST23, account for most clinical disease (4, 5). Three hospital-adapted clones (two ST2 and one ST23) were recently demonstrated to be globally disseminated and to have evolved to become untreatable with first-line agents though the acquisition of multiple antibiotic resistance determinants and resistance-conferring mutations (5). Efforts to increase knowledge concerning the molecular genetics, pathogenesis, and treatment of *S. epidermidis* have been limited by barriers preventing the genetic manipulation of clinically relevant isolates and the assumption that the characteristics of *S. epidermidis* are similar to those of *S. aureus*.

Restriction-modification (RM) systems have evolved as a form of bacterial immunity that degrades incoming DNA from foreign donors such as bacteriophage (6). Type I and IV RM systems represent a significant barrier to genetic manipulation of staphylococci. Type I RM systems are comprised of three host specificity for DNA (hsd) genes that encode (i) a specificity protein (HsdS), (ii) a modification protein (HsdM), and (iii) a restriction endonuclease (HsdR). Together, these function as a single protein complex in which HsdS determines the DNA target recognition motif (TRM) in which adenine residues are methylated by HsdM, while HsdR cleaves unmodified and non-self-modified DNA (7, 8). Type IV RM systems consist of a single restriction endonuclease that cleaves DNA with inappropriate modification (8).

Plasmid artificial modification (PAM) is a method to overcome the barrier imposed by RM systems where plasmid DNA is passaged through a cytosine methylation-deficient *Escherichia coli* host (DC10B) that has been engineered to heterologously express the *hsdMS* system of the staphylococcal strain to be transformed. Plasmid DNA extracted from this *E. coli* host mimics the DNA methylation profile of the target strain, thus enabling introduction of plasmid DNA and subsequent genetic manipulation (9).

Type I RM systems of staphylococci are best understood in *S. aureus*. The distribution of *hsdS* alleles corresponds to clonal complex (CC) for the 10 dominant *S. aureus* lineages (10). Far less is known about the type I RM systems in *S. epidermidis*. A recent study suggested that *S. epidermidis* type I RM systems adhered to lineage-specific groupings like *S. aureus*. However, this inference was based on analysis of only four new *S. epidermidis* methylomes (11) plus the one methylome that had already been characterized; namely, the ST2 reference genome of strain BPH0662 (12).

Here, we present the first systematic genomic analyses of the type I RM systems in *S. aureus* and *S. epidermidis* and demonstrate how these data can be used to predict functionality of type I RM systems and associated competence of strains. We show that PAM is a highly efficient method to enable genetic manipulation of *S. epidermidis*, particularly hospital-adapted isolates that possess multiple functional type I RM systems.

**RESULTS AND DISCUSSION**

**S. aureus type I RM systems are lineage specific.** We began this study by testing the notion that *S. aureus* type I RM systems are lineage specific. We compared 128 publicly available finished *S. aureus* genome sequences (see Table S1A in the supplemental material) and confirmed that the chromosomal location and structure of type I RM systems in *S. aureus* are highly conserved. A total of 110 genomes had a single *hsdR* gene and two copies of *hsdMS* with the first in forward orientation located in the alpha pathogenicity island and the second in the opposite orientation and located within the beta pathogenicity island (10, 13) (Fig. 1A). The remaining 18 strains possessed *hsdR* and a single copy of forward oriented *hsdMS* in the alpha pathogenicity island. Five of the 128 strains possessed a third type I RM system at a nonmobile chromosomal...
location downstream of lacA. Twenty-three strains carried an additional type I RM system on a mobile genetic element, 22 were on staphylococcal cassette chromosome (SCC) elements (Fig. 2; see also Fig. S1 in the supplemental material), and 1 carried a type I RM system on a plasmid (strain HUV05). Single variants of both HsdR (NCBI protein accession no. WP_000331347.1; n = 127) and HsdM (WP_000028628.1; n = 222) were demonstrated for the type I RM systems situated in nonmobile chromosomal locations, indicating stable vertical inheritance. Interruptions in hsdR and hsdMS due to horizontal gene transfer were rarely seen in S. aureus. A solitary example of hsdS truncation due to insertion of a bacteriophage was noted in strain Sa17_S6. Most changes were due to single nucleotide polymorphisms (SNPs) leading to amino acid substitutions (n = 35) or nonsense mutations (n = 31) in hsdS (Fig. 2). See Table 1 for a comparison of S. aureus and S. epidermidis type I RM systems.

We next established a high-resolution phylogeny using 144,727 core genome SNPs for the 128 S. aureus genomes covering 40 STs (Fig. 2). Occurrences of hsdMS genes were mapped across the phylogeny for each genome. A total of 48 HsdS subunits were identified with associated TRMs (Table 2; see also Table S2A [https://melbourne.figshare.com/articles/Sa_HsdS_48_fasta/7986956]). Although the same hsdMS genes were present in genetically distinct lineages, the combinations of hsdMS genes were conserved within each lineage (Fig. 2). For example, the same two HsdMS products were present in ST250 and ST254, which are single-locus variants of ST8. A notable exception to the lineage specificity was represented by the type I RM systems carried on SCC elements (Fig. S1B), which may have been acquired from coagulase-negative staphylococci (CoNS). Complete S. epidermidis BPH0736 hsdMSR genes (nondisrupted, identical sequences) were observed in four S. aureus strains from three different STs (ST5, ST59, and ST338), suggesting gene transfer between the species (Fig. S1).

**S. epidermidis type I RM systems are carried on mobile genetic elements.** Seven complete S. epidermidis reference genomes were publicly available at the beginning of this study (Table S1). Of these, only BPH0662 (12) and RP62a had characterized type I RM system motifs. However, the RP62a methylome was determined independently (11) of the finished genome (14). The methylomes of S. epidermidis isolates 1457 (15) and 14.1.R1 (16) confirmed that, consistent with the absence of hsdM genes, neither possessed a functional type I RM system. To improve understanding of the type I RM systems in S. epidermidis, we conducted PacBio SMRT sequencing and established complete genomes and adenine methylomes for six additional S. epidermidis strains.
FIG 2  *S. aureus* native type I restriction-modification systems are lineage specific. The figure shows a maximum likelihood core SNP-based phylogeny of 128 closed *S. aureus* genomes originating from 40 STs, using Newman_UoM as the reference genome. Overlaid are the results (Continued on next page)
from ST2, ST5, ST59, and ST358 and we resequenced RP62a (ST10) (See Table S3 for metadata).

The typical chromosomal arrangement of the type I RM system in S. epidermidis is shown for BPH0736 (ST2) (Fig. 1B). Unlike S. aureus (Fig. 1A), type I RM systems in S. epidermidis are arranged as a complete three-gene operon in either an hsdRMS or hsdMSR organization, unless interrupted (Fig. S1). Analyses of the 11 closed S. epidermidis genomes containing type I RM systems demonstrated their co-occurrence with cassette chromosome recombinase (ccr) genes (with or without the presence of mecA).

The 16 type I RM systems present within these 11 genomes were located a mean distance of 11.5 kb (minimum, 2.3 kb; maximum, 51.0 kb) from the nearest ccr (Fig. S1A). Similarly, in the 22 S. aureus genomes with a SCC-associated type I RM system, the mean distance between hsdRMS and hsdMSR and the nearest ccr was 6.9 kb (minimum, 1.6 kb; maximum, 20.9 kb) (Fig. S1B).

Cassette chromosome recombinases typically integrate at orfX (corresponding to the last 15 nucleotides of the rRNA large subunit methyltransferase [17]), which is located at 31.6 kb in the S. epidermidis chromosome (33.3 kb in S. aureus). This represents the start of a highly plastic region of the chromosome, in which multiple antibiotic resistance genes and genes corresponding to drug transporters and insertion sequence (IS) elements have accumulated (12) (Fig. S1). All 11 S. epidermidis and 22 S. aureus genomes with ccr-associated type I RM systems were integrated at orfX (Fig. S1).

For type I RM system variants present in multiple isolates, conservation of genes surrounding the system and ccr was observed, consistent with the mobilization of an entire element (Fig. S1). Preserved cassette structure between isolates and in both species (Fig. S1B) led us to hypothesize that the movement of type I RM systems in S. epidermidis is mediated by ccr, enabling mobilization on SCC elements between strains and to other staphylococcal species. Localization in this region of the genome also predisposes S. epidermidis type I RM systems to disruption, potentially rendering variants restriction deficient. This was seen with interruption of hsdR by IS elements in BPH0736 (Fig. 1B).

**S. epidermidis type I RM systems are not strictly conserved within lineages.** To expand the S. epidermidis data set, we added short-read data from 234 publicly acquired genomes. S. epidermidis is a species with highly dispersed populations, many of which present unique type I RM systems, as seen in Table 1. Four different ccr genes were observed (Fig. 1B), each with a distinct chromosomal location.

### Table 1 Comparison of S. aureus and S. epidermidis type I restriction-modification systems

| S. aureus type I RM system characteristics | S. epidermidis type I RM system characteristics |
|-------------------------------------------|-----------------------------------------------|
| RM system organized as a single hsdR gene separated from one, two, or three distant hsdMS gene pairs | RM system organized as complete three-gene operon (hsdRMS or hsdMSR) |
| Conserved, stable chromosomal location for each gene | Close proximity to ccr genes integrated at orfX, located in a highly plastic region of the genome |
| Most strains have two type I RM systems | Most strains have a single type I RM system |
| All strains have at least one type I RM system | Many (38.1%) strains have no type I RM system |
| Up to three functional type I RM systems per isolate | Up to three functional type I RM systems per isolate |
| 99.7% amino acid pairwise identity for all native HsdRs | At least five identified variants of HsdR |
| 99.3% amino acid pairwise identity for all native HsdMs | At least six identified variants of HsdM |
| At least 48 different variants of HsdS (8 likely imported from coagulase-negative staphylococci) | At least 31 different variants of HsdS |
| Relative conservation of HsdS present within ST groups | No clear conservation of HsdS according to ST group |
| Conservation of HsdM provides redundancy, enabling interaction with multiple different HsdS | Each HsdS is capable of interacting only with the corresponding paired HsdM; therefore, not all orphan hsdS genes are functional |
| Complete three-gene hsdRMS/MSR type I systems carried on SCC elements do not adhere to lineage specificity and are likely imported from coagulase-negative staphylococci | |

**FIG 2** Legend (Continued)

Of in silico multilocus sequence type (MLST), clonal cluster (CC), Bayesian analysis of population structure (BAPS), presence of CRISPR-Cas systems, and type I restriction-modification system HsdS variants. Bold red font indicates isolates with PacBio-characterized methylomes. Bold blue font indicates isolates with methylomes determined by DNA cleavage with purified enzyme. Boxes around strain names are colored according ST type.

Open circles represent amino acid substitutions present in HsdS. An asterisk (*) indicates a truncated HsdS subunit. The scale bar indicates the number of nucleotide substitutions per site (bold) with an approximation of SNP rate (in parentheses).
available *S. epidermidis* genomes to the 13 finished genomes (Table S1). In contrast to the *S. aureus* data, variability was noted in both the HsdR and HsdM subunits for *S. epidermidis*. Across the 247 genomes, 183 intact HsdR genes were identified, including five major HsdR variants (Table S2). The two variants of HsdR in strain BPH0662 shared only 22% amino acid identity. Similarly, 178 complete HsdM genes were identified with six major variants (Table 3; see also Table S2).

### TABLE 2

| Representative HsdS | Motif | N* isolates | MLST | HsdS NCBI Protein Accession |
|---------------------|-------|-------------|------|-----------------------------|
| **Newman_Uom_MS1** | AGG(N),GAT | 60 | CC5 [5 (225, 105)], CC8 [239, 8 (250, 254, 923)] | WP_00072584.1 |
| **Newman_Uom_MS2** | CCA(Y),GTT | 42 | 151, 1, CC8 [8 (250, 254, 923)] | WP_00072566.1 |
| **MW2_MS1** | CCA(Y),TTA | 3 | 1 | WP_00072566.1 |
| **N315_MS2** | CCA(Y),GTA | 30 | CC5 [5 (225, 105), 228] | WP_00072627.1 |
| **FDAARGOS_150_MS1** | CCA(Y),GAT | 10 | CC5 [5, 228], 25 | WP_00072620.1 |
| **NCTC13435_MS3** | TCTA(N),RTTC | 4 | 25, 72 | WP_00072557.1 |
| **NCTC13435_MS4** | GAC(N),TTYG | 3 | 72 | WP_00072638.1 |
| **DAR4145_MS1** | CT(A),TAG | 6 | 772, 72 | WP_00072692.1 |
| **DAR4145_MS2** | GAA(A),TTTG | 4 | 772, 27 | WP_00072555.1 |
| **ST2013039_MS1** | predict ?-RTGA | 3 | 582, 15 | WP_00066753.1 |
| **ST2013039_MS2** | predict GGA?-TTYG | 3 | 582, 15 | WP_00072556.1 |
| **CNI1_MS1** | GARA(N),RTG | 5 | unclassified, 72 | WP_00072573.1 |
| **CNI1_MS2** | GGA(N),TGC | 5 | unclassified, 72 | WP_00072558.1 |
| **AUS0325_MS1** | GAC(N),TGC | 2 | 1093, 88 | WP_00069200.1 |
| **HO 5096 0412_MS1** | AGG(N),TGA | 3 | 22 | WP_00072655.1 |
| **HO 5096 0412_MS2** | not functional | 3 | 22 | WP_00032390.1 |
| **MRSA252_MS1** | GWAG(N),GAT | 8 | unclassified, 30 (36, 243), 433 | WP_00072632.1 |
| **MRSA252_MS2** | GGA(N),TGC | 10 | unclassified, 30 (36, 243), 433 | WP_00072622.1 |
| **IB565_MS1** | predict AGG?- | 2 | 151 | WP_00072611.1 |
| **Tager 104_MS2** | predict ?-RTTC | 3 | 152, 50, 49 | WP_00072571.1 |
| **S0385(ST398_MS1)** | ACC(N),RTGA | 8 | 398 | WP_00072668.1 |
| **CA-347_MS1** | GWAG(N),TAAA | 2 | 45 | WP_00072579.1 |
| **XO_MS1** | GGA(N),CCT | 2 | 121 | WP_05080335.1 |
| **XO_MS2** | GAC(N),TAYG | 2 | 121 | WP_05080121.1 |
| **SA40_S** | GGA(N),RTG | 6 | 59 (338) | WP_00072659.1 |
| **JKD6159_MS1** | CAG(N),TCC | 1 | 93 | WP_00072616.1 |
| **JKD6159_MS2** | GGHA(N),TCC | 1 | 93 | WP_00072554.1 |
| **RF122_MS1** | unknown | 1 | 151 | WP_00072697.1 |
| **RF122_MS2** | not functional | 1 | 151 | WP_00072697.1 |
| **ED133_MS1** | CAG(N),RTGA | 1 | 133 | WP_00072617.1 |
| **ED133_MS2** | GGA(N),TTRG | 1 | 133 | WP_00072626.1 |
| **LGA251_MS1** | GWAG(N),RTGA | 1 | 425 | WP_00072635.1 |
| **LGA251_MS2** | not functional | 1 | 425 | WP_04412248.1 |
| **Tager 104_MS1** | predict CA?-RTGA | 49 | WP_00072580.1 |
| **JS365_MS1** | predict CAG?- | 1 | 1093 | WP_00072352.1 |
| **FDA206P_MS1** | CCAY(N),RTC | 1 | 464 | WP_00072686.1 |
| **FDA206P_MS2** | predict CCAY?-TTYG | 1 | 464 | WP_04721036.1 |
| **RK4_MS1** | TCTA(N),TGA | 1 | 27 | WP_00072580.1 |
| **RK4_MS2** | predict ?-RTTC | 1 | 7 | WP_00072588.1 |
| **AUS0325_MS1** | ACC(N),RTG | 1 | 88 | WP_00072611.1 |

**Imported systems**

| **J KD6159_MS3R** | GAA(A),TAC | 7 | 93, 425, 1093, 22, 464 | WP_00039404.1 |
| **BPH0736_MS3R** | GAT(N),CCTA | 4 | 59 (338), 5 | WP_00046234.1 |
| **I2228_Rum_MS3** | GAA(N),CCTA | 3 | 5, 8 | WP_00163102.1 |
| **DAR4145_MS3R** | TTAC(N),TAC | 3 | 772 | WP_00038605.1 |
| **CA-347_MS2** | unknown | 2 | 45 | WP_00080914.0 |
| **RVM3897_MS2R** | unknown | 1 | 398 | WP_06058513.0 |
| **MSSA476_MS3R** | unknown | 2 | 1 | WP_00085806.1 |
| **HUV05_RMS3** | unknown | 1 | 8 | WP_04520050.1 |
The amino acid sequences of these six variants were markedly divergent. The two variants of HsdM present in BPH0662 shared only 31% amino acid identity.

A maximum likelihood phylogeny for the 247 S. epidermidis genomes, derived from 83,210 core SNPs and sampled from 72 STs, was established, and the 31 different S. epidermidis HsdS subunits identified were overlaid (Fig. 3). Where known, their associated TRMs are shown in Table 3 with NCBI protein accession numbers (see also Table S2). Amino acid sequences of all 31 HsdS are available from Figshare (https://melbourne.figshare.com/articles/Se_HsdS_31_fasta/7986911). The distribution of S. epidermidis HsdS proteins within the population differed markedly from that observed within S. aureus, with no strict concordance to lineage specificity. For example, HsdS from BPH0723 (BPH0723-S; Table 3) was present in 13 isolates from five STs (ST5, ST21, ST46, ST210, and one unclassified ST), while BPH0662-S2 was identified in 52 isolates from three STs (ST2, ST23, and ST35). Although a high proportion of ST2 isolates shared the same predicted methylome (Fig. 3), the majority of these were known to be clones of internationally disseminated, multidrug-resistant strain BPH0662 (5). However, even within this highly clonal group (n = 36), some predicted

| Representative HsdS | Motif | N isolates | MLST | HsdS NCBI Protein Accession | Interacting HsdM |
|---------------------|-------|------------|------|----------------------------|------------------|
| 1. 12228-S<sup>a</sup> | GAA(N)_CTTA | 64 | 22, 5, 6, 8, 59, 22, 2 (185) | WP_001631029.1 | BPH0662-M1 |
| 2. BPH0662-S2 | CAG(N)_ATC | 52 | 23, 35, 2 | WP_002504701.1 | BPH0662-M2 |
| 3. BPH0662-S1 | ATT(N)_TTC | 43 | 2 | WP_002504637.1 | BPH0662-M1 |
| 4. BPH0723-S | TACG(N)_ATC | 18 | 210, 16, 2 | WP_000459234.1 | BPH0662-M2 |
| 5. BPH0723-S | GAA(N)_TGC | 13 | 32, 46, 83, 5, 210, 21 | WP_002469391.1 | BPH0662-M2 |
| 6. RP62a-S | GAG(N)_TAC | 9 | 230, 71, 23, 10, 40 | WP_002469618.1 | BPH0662-M1 |
| 7. BPH0697-S | CA(N)_TGT | 4 | 89, 4, 22 | WP_002505983.1 | BPH0697-M1 |
| 8. BPH0711-S | GGA(N)_TAG | 2 | 59 | WP_002457322.1 | BPH0662-M2 |
| 9. BPH0704-S | CYX(N)_GTT | 1 | 358 | WP_061544233.1 | BPH0662-M1 |
| 10. BPH0747-S | CNA(N)_RTTA | 1 | 2 | WP_100481761.1 | BPH0662-M2 |

<sup>a</sup>The ATCC 12228 type I RM system is nonfunctional, with a truncated hsdR gene, a complete hsdS gene, and no hsdM gene. All 64 isolates possessed the same incomplete type I RM system. The motif was identified based on the methylome determined for NIH4008 due to the presence of an HsdM protein capable of interacting with 12228 HsdS.

<sup>b</sup>14.1.R1 type I RM system is nonfunctional, with truncated hsdR, complete hsdS, and no hsdM.

<sup>c</sup>1L1M substitution.

<sup>d</sup>First 81 amino acids truncated.

<sup>e</sup>S295P substitution.

<sup>f</sup>11 amino acid substitutions.

<sup>g</sup>Isolate HsdS motifs were collated from methylomes newly characterized in this study and from publications by Lee et al. (12) and Costa et al. (11). HsdS names in bold black font have motifs determined by PacBio sequencing of the isolate after which the representative HsdS was named. The multifocus sequence types (MLSTs) in which each HsdS was found are listed according to the order in which they appear in the Fig. 3 phylogeny (clockwise). ST185 is a single-locus variant of ST2. trunc, truncated; A (red), methylated adenine residue; T, complementary partner to methylated adenine residue.

(https://melbourne.figshare.com/articles/Se_HsdS_31_fasta/7986911). The amino acid sequences of these six variants were markedly divergent. The two variants of HsdM present in BPH0662 shared only 31% amino acid identity.

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S. epidermidis type I restriction-modification systems are not conserved within lineages. The figure shows a maximum likelihood, core SNP-based phylogeny for 247 S. epidermidis genomes, including 7 newly closed reference genomes, 6 existing reference genomes, 156 genomes curated from the NCBI sequence read archive (SRA), 75 isolates from a study by Lee et al. (5); and the 3 draft genomes with methylation data (11). BPH0736 was used as the reference genome for analyses. Overlaid are the results of in silico multilocus sequence type (MLST), Bayesian analysis of population structure (BAPS), presence of CRISPR-Cas systems, and type I restriction-modification system HsdS variants. Bold red font indicates isolates with characterized methylomes. Isolates were from 70 recognized and two unclassified MLST groups. Boxes around strain names are colored according ST type; where background color is same as that of the BAPS group, the result indicates an ST represented by a single isolate. An asterisk (*) represents a truncated HsdS subunit. The scale bar indicates the number of nucleotide substitutions per site (bold) with an approximation of SNP rate (in parentheses).
methylation variation existed. For example, BPH0662-S2 was absent from two isolates, six isolates (including BPH0662) had a truncation in the 12228-S orphan system, and two isolates were missing the 12228-S orphan system completely. Furthermore, within ST2, seven different variants of HsdS were identified in 11 arrangements, including the absence of any type I RM system (Fig. 3). Of the 247 S. epidermidis genomes analyzed, 38% did not contain any hsdS alleles and were predicted to be restriction deficient.

Methylation analysis performed using our Pacbio-sequenced, Illumina read-corrected RP62a_UoM genome as a reference indicated the presence of a single type I RM system with a G\(^A\)GN7TAC TRM (Table 3). Although this motif was consistent with that previously reported by Costa et al. (11), the three additional motifs previously described (lacking apparent associated genes) were not detected by our methylome analysis; the low complexity of the motifs (e.g., GGBNNH) and the low frequency of detected methylation (12% to 29%) (11) suggest that these might have represented artifacts rather than true motifs. Similarly, the three additional low-complexity and low-frequency motifs reported for VCU036 (11) probably represented artifacts. Although the ST type was not specified, VCU036, which shared the same methylome with ST89 isolate NIH051475, was reported as CC89 by Costa et al., leading to the conclusion that S. epidermidis type I RM systems follow S. aureus-like lineage specificity (11). We performed in silico MLST by two independent methods and determined that VCU036 belongs to ST4. Furthermore, our analysis of the 247 S. epidermidis genomes demonstrated VCU036 to be phylogenetically distinct from ST89 (Fig. 3).

Overall, our analyses demonstrated that, in contrast to current assumptions (11), the type I RM systems of S. epidermidis do not adhere to the lineage-specific distribution observed in S. aureus. These differences are attributable to the arrangement of S. epidermidis type I RM systems as complete three-gene operons that reside within a highly mobile region of the chromosome, the movement of which we hypothesize to be mediated by ccr.

**Recombinant target recognition domains generate HsdS variants with low conservation of amino acid identity.** The structure of a typical type I RM system HsdS allele is shown in Fig. S2A and is composed of two highly variable target recognition domains (TRDs) flanked and separated by conserved regions (CRs) that collectively determine the methylation of the TRM by HsdM. Recombinant pairings of TRDs result in different variants of HsdS (13, 18). Alignments of the range of S. aureus and S. epidermidis HsdS proteins identified in this study are shown in Fig. S2B and S3, respectively. Within our S. aureus and S. epidermidis collections, 77 variants of HsdS that shared only 24% pairwise identity were identified. This low level of conservation poses a potential challenge to the high-throughput bioinformatic screening for HsdS variants within genomic data sets. However, using HsdS from ATCC 12228 as the reference translation with our described method, we were able to detect the partial if not complete presence of all HsdS variants in both species. Of note, 12228-S was the only HsdS variant found within both species that clustered with the majority of S. aureus variants. In comparison, RP62a-S captured only 18 of the 31 S. epidermidis HsdS variants and fragments of fewer than half of the S. aureus HsdS variants.

**S. epidermidis HsdS variants interact only as part of a specific complex.** The arrangement of some S. epidermidis type I RM systems, with the presence of a truncated hsdS gene between complete hsdR and hsdMS genes, suggested the occurrence of recombination of component genes (e.g., S. epidermidis BPH0662I [SepiBPH0662I], SepiRP62al, and SepiBPH0704I; Fig. S1). Analyses of the 247 genomes indicated that each variant of hsdS in S. epidermidis was always associated with a specific hsdR gene and hsdM gene, with a gene arrangement that was conserved (unless interrupted), frequently with the same surrounding genes found in association with ccr (Fig. S1A). These observations support our hypothesis of a role for SCC elements in the mobilization of S. epidermidis type I RM systems.
The presence of an orphan hsdS gene without a partner hsdM gene in S. epidermidis introduces additional complexity to the prediction of type I RM system functionality. This was demonstrated by the presence of 12228-S, the most prevalent HsdS within the data set, in 64 S. epidermidis isolates (Table 3) (Fig. 3) and three S. aureus isolates (Table 2 and Fig. 2). All examples of this hsdS variant followed a truncated hsdR gene, without an hsdM gene. We determined that 12228-S was expressed only when the corresponding specific interacting variant of hsdM (BPH0662-M1; WP_002504638.1) was also present (see Table S4 for full explanation). In contrast, conservation of a single variant of hsdM present twice within the same S. aureus genome provides redundancy for the expression of type I RM methylation. This is consistent with previous findings where the product of a single copy of the conserved S. aureus hsdM allele could functionally interact with both CC8 HsdS products when heterologously expressed in E. coli (9).

**Plasmid artificial modification to overcome the type I RM systems in S. epidermidis provides electroporation efficiency equivalent to deletion of functional type I systems.** To determine the restriction barrier posed by type I RM systems in S. epidermidis and assess the efficiency of PAM as a means of bypassing restriction barriers (Fig. 4), ΔhsdS mutants and E. coli hosts for PAM were constructed for S. epidermidis isolates BPH0662, RP62a, and BPH0736. Two different plasmids (pRAB11 [19] and pIMAY [8]) were used in electroporation experiments, as each carried a different number of TRMs recognized by the type I RM systems present in each isolate (Fig. 4D). A clinical ST2 isolate, BPH0662-WT (BPH0662 wild type), was found to have an intractable restriction barrier unless both functional type I RM systems were overcome by complete bypass with PAM in an E. coli host (Ec_Se662I-II) or by deletion of both complete hsdS genes (BPH0662 ΔhsdSI ΔhsdSII) or by a combination of the two approaches (plasmid from Ec_Se662I transferred into BPH0662 ΔhsdSII or plasmid from Ec_Se662II transferred into BPH0662 ΔhsdSI) (Fig. 4A).

Using our protocol, the type I restriction barrier in RP62a-WT was found to be incomplete. Low numbers of transformants (10^1 CFU/ml) were obtained with plasmid DNA isolated from DC10B, indicating that bypassing the type IV restriction barrier alone was sufficient to allow genetic manipulation of this strain (Fig. 4B) as previously demonstrated (8). Complete bypass of the single type I RM system in this isolate with E. coli host Ec_SeRP62al significantly improved electroporation efficiency to 10^4 CFU/ml, which was equivalent to the complete absence of a functional type I RM system as determined with the RP62a ΔhsdS mutant (Fig. 4B). In contrast, when expressing the RP62a hsdMS genes from a plasmid in DC10B, Costa et al. were unable to completely bypass the type I RM barrier. This discrepancy was attributed to the presence of additional RM systems with low-frequency methylation (11). However, our results showed that only one type I RM system is present in RP62a, suggesting that the heterologous expression of type I RM systems on a plasmid in DC10B rather than from a single copy of the genes integrated into the chromosome may be suboptimal. Previously, we found that plasmid-based expression of hsdMS was unstable and that cells were unable to tolerate the high level of expression required for complete methylation of the target DNA (9).

Clustered regularly interspaced short palindromic repeat (CRISPR) loci confer sequence-directed immunity against phages and other foreign DNA and represent another recognized barrier to horizontal gene transfer in S. epidermidis (20). Our analysis of the CRISPR spacers for RP62a (Table S3D) did not demonstrate the presence of any targets on pSK236 (5.6 kb) as used by Costa et al. or on pRAB11 (6.4 kb) or on pIMAY (5.7 kb) as used in this study that would account for the fact that their electroporation efficiency (10^2 CFU/ml per 5 μg plasmid DNA) (11) was lower than that determined by our protocol (10^4 CFU/ml per 5 μg plasmid DNA for both pRAB11 and pIMAY).

Isolate BPH0736-WT was predicted to be naturally restriction deficient due to the interruption of hsdR by IS elements (Fig. 1B), but PacBio sequencing demonstrated that it retained functional methylation conferred by an intact hsdMS system. Due to
the complex and infrequently occurring TRM dictated by the single hsdS (Table 3), neither pIMAY nor pRAB11 had any BPH0736-S TRMs present. Therefore, pIMAY bearing the Δ736 hsdS insertion (pIMAY Δ736 hsdS) was used as this contained three TRMs (Fig. 4D). BPH0736-WT was functionally confirmed to be restriction deficient, with the same electroporation efficiency (10^4 CFU/ml) demonstrated for both BPH0736-WT and BPH0736 Δ hsdS using plasmid isolated from nonspecific E. coli host DC10B and PAM-tailored mutant Ec_Se736I (Fig. 4C). Further supporting our bioinformatic predictions, like BPH0736, ATCC 12228 (truncated hsdR and no hsdM; ST8), 1457 (truncated HsdS containing only one TRD, a truncated hsdR, and no hsdM; ST86), and BPH0710 (truncation at amino acid 81 of HsdS; ST2) were all
predicted to have no functional restriction barrier. Similarly to BPH0736, these three strains were transformable at levels on the order of $10^4$ CFU/ml with plasmid isolated from DC10B, suggesting that this was the maximum electroporation efficiency expected for our protocol. A clinical ST2 strain, BPH0676, was also predicted to have no restriction barrier and the complete absence of a type I RM system; however, similarly to BPH0662, the maximum electroporation efficiency achieved was only $10^3$ CFU/ml, suggesting that inherent strain-dependent factors other than type I RM systems, e.g., cell wall thickness (21), impacted the electroporation of these isolates.

Although the data presented above demonstrate that PAM is an efficient method to overcome the type I restriction barrier of *S. epidermidis*, we observed potential instability with the integration of multiple *S. epidermidis* hsdMS genes of particular TRMs in a DC10B *E. coli* background. With serial passage of Ec_Se662I-II, the electroporation efficiency of plasmid isolated from this *E. coli* host into BPH0662 declined from $10^3$ to $10^1$ CFU/ml despite all other experimental parameters remaining the same. This was not observed for any of the *E. coli* PAM mutants expressing a single hsdMS gene, including Ec_Se662I and Ec_Se662II, which maintained high-level methylation (89.65% to 99.90%) of motifs within the genome (12) (Table S3). Illumina sequencing of the Ec_Se662I-II genome confirmed integration of both hsdMS genes at the expected chromosomal sites but loss of approximately half of the coding sequence of both hsdS genes for the majority of the population sequenced. This instability was hypothesized to be due to the burden of excessive DNA methylation (10,930 sites of heterologous expression of two BPH0662 *S. epidermidis* type I RM systems in addition to 38,592 sites of endogenous *E. coli* dam methylation) that may interfere with normal cellular function, rendering expression toxic in *E. coli*. The same likely accounts for the poor electroporation efficiency seen in experiments using PAM for NIH4008 (100-fold lower than that observed for isolates with only a single type I RM system) as reported previously by Costa et al. (11). NIH4008 possesses the same type I RM systems as BPH0662, without the truncation of the orphan hsdS gene (Fig. 3). Furthermore, although stable chromosomal integration of three *S. aureus* hsdMS systems in *E. coli* DC10B (JM93B) was described previously by Monk et al., decreased efficiency of methylation was observed, with only 10,135 of a total of 14,602 TRM sites demonstrating detectable methylation (9).

Collectively, our current and previous (9, 12) data suggest that DC10B *E. coli* is unlikely to consistently maintain heterologous expression of staphylococcal type I RM systems in the setting of high-frequency methylation ($\geq$10,000 sites). This limitation should not impact plasmid electroporation for mutant creation by allelic exchange, which theoretically requires only a single transformant. However, should high-efficiency electroporation be sought (e.g., for direct transposon mutant library selection), then suitable strains can be predicted using genomic data to identify restriction-deficient isolates, such as our newly described reference isolate BPH0736, representing a clinically significant ST2 isolate. Clinical metadata, genome characteristics, CRISPR spacers (when present), *in silico* resistome, an Vitek 2 antibiogram representing clinically relevant antibiotics, and common plasmid selection markers for the seven new reference isolates and BPH0662 are shown in Table S3. Metadata and sequencing accession numbers for mutant isolates are listed in Table S5.

**Phage transduction of plasmid is subject to type I restriction.** Phage transduction is an alternative method for the genetic manipulation of *S. epidermidis*. In particular, *S. aureus* ST395 lineage-specific φ187 shares wall teichoic acid (WTA) receptors with *S. epidermidis* (22, 23). Depending on the incidental packaging of plasmid introduced into a restriction-deficient intermediary host, *S. aureus* PS187 ΔhsdR ΔsauPSI, with φ187 phage machinery (24), the method can be used to transduce a number of CoNS strains but is not universally applicable to all *S. epidermidis* isolates (23). The observed ability of ST395 *S. aureus* to exchange DNA with some CoNS strains led
Winstel et al. to conclude that overlap of the DNA methylation of ST395 S. aureus and that of CoNS strains that share the same WTA receptors may exist (22, 23). Results of phage /H9021 transduction experiments performed using our WT isolates and ΔhsdS mutants for BPH0662, RP62a, and BPH0736 representing the transfer of pRAB11 are shown in Fig. 5. These experiments demonstrated that even if successfully transduced into a S. epidermidis isolate, plasmids are still subject to degradation by type I RM systems if they bear a recognized TRM. However, in BPH0736 (absent type I restriction) or in mutant strains in which systems have been rendered inactive, transduced plasmid remains viable. The methylome for ST395 S. aureus has not been characterized; however, the draft genome sequence for PS187 (GCA_000452885.1) indicates that the two type I RM systems in this isolate are identical to those in S. aureus isolate JS395 (ST1093, belonging to CC395 [25]). We predicted the methylome of the isolate to include GAGN,TCG (same as AUS0325-MS2) and another unknown TRM (Fig. 2) (Table 2). The results of our experiments and analyses of the diversity of S. epidermidis type I RM systems suggest that successful phage transduction of some S. epidermidis isolates with Φ187 is more likely related to the absence of a functional system than to the presence of a methylome shared with ST395 S. aureus. This is further supported by data from experiments performed by Winstel et al. (23) in which Φ187 was found to be able to transduce pTX15 (26) only into RP62a and not into pKOR1 (27). On the basis of our characterized RP62a TRM, we determined that pTX15 possesses no RP62a motifs whereas pRAB11 and pKOR1 each bear four motifs, explaining why neither plasmid is transducible into RP62a-WT.

A temperature-sensitive plasmid, pIMAY, is frequently used for allelic exchange in staphylococci due to the presence of inducible secY antisense counterselection and the lower likelihood of unintended mutations (that occurs with pKOR1) as integrants are selected at 37°C instead of 43°C (28). However, we found that Φ187 was not capable of transducing pIMAY into any of the tested strains, including the ΔhsdS mutants and naturally restriction-deficient BPH0736. We hypothesized this was due to the low copy number of pIMAY in staphylococci, resulting in low levels of incidental packaging of the plasmid within Φ187, compared to high-copy-number plasmid pRAB11. Other limitations of Φ187 transduction include a recommendation to use plasmids of <10 kb (24); however, that should not have impacted pIMAY (5.7 kb), which is smaller than pRAB11.
Although a simplified harvesting and infection protocol was used compared to that described by Winstel et al. (24), we achieved an efficiency of $10^4$ transductants per ml with PRAB11, equivalent to their anticipated results of $10^3$ to $10^4$ (24), in restriction-deficient *S. epidermidis* strain BPH0736. Of note, the levels of efficiency of both ΔhsdS mutants, BPH0662 ΔhsdSI ΔhsdII and RP62a ΔhsdS, were 2 logs lower than those measured for BPH0736 (Fig. 5), further supporting the theory that strain-dependent factors beyond the barriers posed by type I RM systems and WTA are present in these backgrounds.

**Conclusions.** Our results demonstrate marked differences between the type I RM systems in *S. aureus* and *S. epidermidis*, which had hitherto been assumed to share the same characteristics (11). These differences are predominantly attributable to the arrangement and genome location of the *S. epidermidis* type I system as a complete three-gene operon, which we hypothesize to be mobilized by ccr. Localization of the operon in a highly plastic region of the chromosome increases the likelihood of horizontal transfer of these complete systems between *S. epidermidis* strains as well as to other staphylococci. This results in a lack of lineage specificity and a higher probability of spontaneous interruption of component genes. This is in contrast to *S. aureus*, where the type I systems are typically arranged as one hsdR and two hsdMS genes located apart from one another in stable regions of the chromosome. The evolutionary impact of these differences in the type I RM systems of *S. epidermidis* and *S. aureus* are unknown and warrant future research. The diversity of *S. epidermidis* type I RM systems that do not strictly adhere to ST/CC groupings indicates that genetic manipulation of *S. epidermidis* requires tailoring for each isolate of interest. Attempting electroporation without genomic analysis of the methylome could be successful, as our analyses found that 38% of *S. epidermidis* strains did not possess a type I RM system, and not all systems pose an intractable barrier (e.g., RP62a). However, some isolates such as internationally disseminated, almost pan-drug-resistant clone BPH0662 have complex and absolute type I restriction barriers.

We have demonstrated that PAM using a DC10B *E. coli* host is a simple and effective means to bypass the type I RM barrier in *S. epidermidis*, with plasmid transfer efficiency equivalent to that seen in the complete absence of type I RM systems. The decreasing cost and ready availability of whole-genome sequencing has made the sequencing of isolates planned for mutagenesis and their mutant derivatives commonplace and a practice that is recommended to ensure the absence of acquired secondary mutations (29). If the genome sequence of an isolate is known, then its methylome and ability to be transformed can be predicted as follows. (i) Does the isolate possess an intact type I RM system? If not, type I methylation would not be expressed and the isolate should be inherently transformable. (ii) Each complete type I RM system within a genome should be functional. For an HsdS protein with known TRMs, the presence of the TRMs on a vector would likely prevent electroporation. (iii) Orphaned, complete hsdS genes may be expressed in the absence of an adjacent hsdM if the associated hsdM allele is present elsewhere in the genome. In view of the guidelines presented above, when designing an *E. coli* PAM host, to ensure complete recapitulation of the endogenous type I methylome, we recommend including all complete hsdMS genes and any complete orphan hsdS genes from the *S. epidermidis* strain to be manipulated.

The 247 genomes that we analyzed are by no means an exhaustive representation of all *S. epidermidis*, and additional examples of type I RM systems will undoubtedly be catalogued as further sequencing of this organism is performed. However, this genomic sampling and our functional data were sufficient to draw the conclusions presented above. In view of the identified complexities associated with the genetic manipulation of *S. epidermidis*, the BPH0736 reference isolate should prove particularly useful. A clinical ST2 isolate that is representative of international circulating clones (5), BPH0736 is naturally type I restriction deficient due to the spontaneous interruption of hsdR, rendering it highly amenable to both electroporation and phage transduction and making it an ideal strain for future molecular studies.
MATERIALS AND METHODS

Media and reagents. Bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table S6 in the supplemental material. S. epidermidis were routinely cultured at 37°C in brain heart infusion (BHI) broth (Difco). See Text S1 in the supplemental material for detailed descriptions of culture media, antibiotics, and enzymes.

Genome sequencing and analysis. The genome sequencing and analysis procedures are described in Text S1.

Electroporation. Early (8-h)-stationary-phase cultures of S. epidermidis grown in 10 ml of B media (BM) were added to 90 ml of fresh, prewarmed BM. Cultures were reincubated to an optical density at 600 nm (OD600) of between 0.8 and 0.9 and chilled in an ice slurry for 10 min. Cells were harvested at 3,900 × g for 5 min at 4°C in a swinging bucket rotor, and the cell pellet was resuspended in 100 ml of autoclaved, ice-cold water. Centrifugation was repeated, and the pellet was resuspended in 50 ml of autoclaved ice-cold water. Cells were centrifuged and successively resuspended in 20 ml, 10 ml, and 250 μl of autoclaved ice-cold 10% (wt/vol) glycerol. Equal aliquots (50 μl) were frozen at –80°C. Prior to electroporation, cells were thawed on ice for 5 min and then at room temperature for 5 min. Following centrifugation at 5,000 × g for 1 min, cells were resuspended in 50 μl of 10% glycerol–500 mM sucrose (filter sterilized). Pellet paint (Novagen) precipitated plasmid DNA was added to the cells, and then the cells were transferred into a 1-mm-path length electroporation cuvette (Bio-Rad) and pulsed at 21 kV/cm, 100 Ω, and 25 μF at room temperature. Routinely, 5 μg of plasmid DNA was used, with concentrations determined by fluorometric assay (Qubit 2.0; Life Technologies). Cells were incubated in 1 ml of BHI broth supplemented with 500 mM sucrose (filter sterilized) at 28°C for 2 h prior to plating on BHI agar (BHI agar) containing chloramphenicol (10 μg/ml).

Construction of Ec Se736I and Ec SeRP62aI E. coli hosts. E. coli mutants expressing the relevant S. epidermidis type I RM systems in a DC108 background were created as previously described (8, 9, 12) using the primers listed in Table S6. Details of the methodology are provided in Text S1.

Construction of S. epidermidis ΔhsdS mutants. The pIMAY(ΔhsdS) vectors were constructed using amplification by overlap extension PCR (30) with the A/B/C/D primer sets specified for each strain in Table S6, cloning into the pIMAY vector backbone, and subsequent cloning of the insertion into the vector. Mutant selection and screening were conducted as previously described (5). Details of the methodology are provided in Text S1.

Harvesting Φ187 plus pRAB11/pIMAY lysate from S. aureus PS187 ΔhsdR ΔsauPSI. Φ187 containing pRAB11/pIMAY was harvested from S. aureus PS187 ΔhsdR ΔsauPSI using a protocol adapted from Winstel (24). See Text S1 for detailed methodology.

Φ187 plus pRAB11/pIMAY transduction of S. epidermidis. A phage transduction protocol was adapted a method described previously by Foster et al. (31). Details of the methodology are provided in Text S1.

Data accessibility. Isolate BPH0662 has been deposited with the NCTC (NCTC accession no. 14219). The data sets supporting the results of this article are available from NCBI (BioProject PRJNA32483) and ENA (BioProject PRJEB135032) (sequencing and closed genome assemblies) and Figshare (https://melbourne.figshare.com/articles/Se_HsdR_5.fasta/798693 [S. epidermidis HsdR]; https://melbourne.figshare.com/articles/Se_HsdM_6 fasta/7986956 [S. epidermidis HsdM]; https://melbourne.figshare.com/articles/Se_HsdS_31 fasta/7986911 [S. epidermidis HsdS]; https://melbourne.figshare.com/articles/Se_HsdS_6 fasta/7986827 [S. epidermidis HsdS]; https://melbourne.figshare.com/articles/Se_HsdR_S fasta/7986893 [S. epidermidis HsdR]).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02451-19.

TEXT S1, DOCX file, 0.04 MB.
FIG S1, PDF file, 1.6 MB.
FIG S2, PDF file, 0.9 MB.
FIG S3, PDF file, 0.6 MB.
TABLE S1, XLSX file, 0.1 MB.
TABLE S2, XLSX file, 0.04 MB.
TABLE S3, XLSX file, 0.03 MB.
TABLE S4, DOCX file, 0.02 MB.
TABLE S5, XLSX file, 0.02 MB.
TABLE S6, DOCX file, 0.04 MB.

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