

# Genetic Variation among Hospital Isolates of Methicillin-Sensitive *Staphylococcus aureus*: Evidence for Horizontal Transfer of Virulence Genes

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*Staphylococcus aureus* strains often carry in their genomes virulence genes that are not found in all strains and that may be carried on discrete genetic elements. Strains also differ in that they carry one of four classes of an accessory gene regulator (*agr*) locus, an operon that regulates virulence factor expression and that has been proposed to be a therapeutic target. To look at their distribution among hospital strains, we investigated 38 methicillin-sensitive *S. aureus* isolates, classifying the isolates by *agr* class and screening them for the presence and restriction fragment length polymorphisms (RFLPs) of 12 core and 14 accessory virulence genes. Twenty-three (61%) were *agr* class I, 10 (26%) were *agr* class II, and 5 (13%) were *agr* class III. None were *agr* class IV. The *S. aureus* strains had distinguishable RFLP profiles, although clusters of isolates with clearly related core gene profiles were found among our strains, including all five *agr* class III strains, two sets of six strains within *agr* class I, and six strains within *agr* class II. Within these clusters there was evidence of horizontal acquisition and/or loss of multiple accessory virulence genes. Furthermore, two isolates from the same patient were identical except for the presence of the *sea* gene, indicating that movement of mobile elements may occur in vivo. Several strong correlations with the carriage of virulence genes between strains were seen, including a positive correlation between *tst* and *agr* class III and negative correlations between *tst* and *lukE-splB* and between *lukE-splB* and *seg-sei*. This suggests that the core genome or the presence of accessory genetic elements within a strain may influence acquisition and loss of other elements encoding virulence genes.

*Staphylococcus aureus* is an important nosocomial pathogen causing a variety of clinical infections including septicemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis, and postsurgical toxic shock syndrome (7, 56). It is also a significant cause of community-acquired disease. Genetic variation among *S. aureus* strains has been shown to be associated with pathogenic potential (5, 16). Variation occurs at the level of both core genes (present in >95% of isolates [31]) and accessory (variable) genes.

Many *S. aureus* accessory genes encode virulence factors such as enterotoxins A through I (*sea* to *sei*, respectively), toxic shock syndrome toxin 1 (*tst*), exfoliative toxins A and B (*eta*, *etb*), as well as leukocidins and surface-associated proteins, for example, collagen binding protein (*cna*) (39, 43). These genes are often carried on mobile genetic elements, such as phages and pathogenicity islands (SaPIs), which transfer horizontally between strains (*sea*, *tst*, *eta*) (4, 35, 57, 58) or which are suspected to do so (*etb*, *seb*) (24, 29). Transfer of *tst* can occur at an extremely high frequency (35) in vitro, although it is not known if this occurs in the clinical setting. There is also evidence that mobile accessory virulence genes are not distributed uniformly among strains. For example, *seb* and *tst* are never seen in the same strain (5), and it has been suggested that carriage of *tst* is correlated with *agr* class III strains and with phage group I strains (28, 30). Furthermore, some virulence

genes can be carried on more than one element. For example, *tst* has been described on at least six related SaPIs that can be distinguished by Southern blotting (20, 34, 35).

Virulence gene expression in *S. aureus* is controlled by regulators such as *agr* (accessory gene regulator) (43), and four different mutually inhibitory classes have been described (26, 28). *agr* is a potential target for therapy, as the response can be modulated by synthetic peptides (37). However, these responses are *agr* class specific, and the distribution of *agr* types in clinical strains is poorly characterized.

The purpose of the present study was to investigate the distribution of genomic differences in clinical strains of *S. aureus*, particularly those that might have an effect on pathogenicity. We studied epidemiologically unrelated strains, collected from a 1,200-bed teaching hospital in southwest London, United Kingdom, chosen to reflect the spectrum of *S. aureus*-associated disease seen in our hospital. In order not to bias our collection with known clones of isolates, we studied only methicillin-sensitive *S. aureus* (MSSA) isolates. Our results confirm that hospital strains of MSSA comprise a heterogeneous population, although certain distinct lineages appear to be more prevalent. Within these lineages, horizontal movement of genetic elements encoding virulence factors is occurring, possibly in vivo. Furthermore, this horizontal movement is not random and may be restricted between certain genetic lineages.

## MATERIALS AND METHODS

**Isolates.** We characterized 38 strains of MSSA (Table 1). The strains were isolated by the Department of Medical Microbiology at St. George's Hospital and were chosen to reflect the spectrum of hospital *S. aureus* isolates. No isolates were known to be epidemiologically related, and the isolates comprised strains

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isolated from different patients on different wards and taken at different times. No strains were part of an outbreak, and none were determined to be from patients with cross infection by our Hospital Infection Control team. The isolates comprised 21 isolates from blood cultures, 9 isolates from sputum samples, 4 nasal isolates, 2 cerebrospinal fluid isolates, 3 lung isolates obtained postmortem from babies, 1 throat isolate obtained postmortem, 1 hip septic arthritis isolate, and 1 sternal osteomyelitis isolate. No isolates were collected from patients with scalded skin syndrome, food poisoning, or toxic shock syndrome. A second isolate from each of four patients was tested to determine the genetic stabilities of strains.

Reference strains used for comparison were 8324-5 (*agr* class I) (provided by S. Foster) and SA502A (class II), RN8463 (class III), and RN4850 (class IV) (all provided by R. P. Novick). The Public Health Laboratory Service (PHLS), Colindale, United Kingdom, provided enterotoxin-carrying strains American strain 578, 96/ST/31504, and 96/ST/31503 as templates for probe amplification. 8325-4 *maIII::lacZ* was provided by S. Foster for use in the characterization of the *agr* phenotype.

**Selection of genes and primer design.** *agr* class-specific primers were designed for each of classes I, II, and III (Table 2). The primers specific for *agr* classes II and III spanned the variable region of the *agrC* gene, whereas the *agr* class I-specific primer spanned the *agrD* region. The regulator *sarA* was investigated, as was the *agr* response effector, *maIII*. Ten other core genes comprising those for hemolysins, surface binding proteins, and heat shock protein 70 (*hla*, *hly*, *hlg*, *icaA*, *ssp*, *spa*, *sbi*, *fnbA*, *efb*, and *hsp70*, respectively) were studied, as were 14 accessory genes comprising those for enterotoxins A through I, toxic shock syndrome toxin 1, leukocidin E, a serine protease-like gene, collagen binding protein, and capsular types 5 and 8 genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *ist*, *lukE*, *spb*, *cna*, *cap5*, and *cap8*, respectively). The genes were chosen because they are known or potential virulence factors and are listed in Table 2. A *16SrRNA* probe was used for ribotyping. *sea* and *see* primers were predicted to amplify PCR products with 78% nucleotide sequence homology with published sequences (GenBank accession numbers M18970 and M21319), but no other significant homologies (>40%) were predicted.

**Southern blotting.** Probes were prepared by PCR amplification. Initial denaturation was for 3 min at 94°C, during which the *Taq* polymerase was added, followed by 32 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s), and extension at 72°C (45 to 90 s, determined at 1 min/kb). The probes were then gel purified on a QIAquick mini column (Qiagen), according to the manufacturer's instructions.

*S. aureus* genomic DNA was obtained after lysis of the cells with lysostaphin (25 µg/ml; Sigma) and extraction with Nucleobond Ax tips (Clontech). DNA was digested with *ClaI* (Promega) for 6 h at 37°C, electrophoresed on 0.7% (wt/vol) agarose gels, and transferred to nylon membranes for Southern blotting. The membranes were hybridized overnight with probes labeled with horseradish peroxidase (Amersham enhanced chemiluminescence), according to the manufacturer's instructions. The membranes were washed, and the signal was detected on X-ray film. As the signal fades after several hours, sequential probing of the same membrane without stripping was possible, and membranes were reused up to 10 times with no loss of signal. A restriction fragment length polymorphism (RFLP) value for each gene was ascribed by estimating visually the size (in kilobases) of the *ClaI* fragment hybridizing to the probe by comparison to a bacteriophage  $\lambda$  *HindIII* ladder. To confirm the robustness of the methods used, chromosomal DNA was extracted from a second subculture of the stored bank for selected isolates (isolates PM2, PM12, PM19, PM80, PM83, PM94, PM116, PM122, PM150, and PM152) and analyzed with a variety of accessory gene probes, as described above; identical results were obtained.

***agr* class designation.** Reaction with one of the *agr*-specific probes was used to assign each strain to a class. However, *agr* class IV strain RN4850 cross-reacted with the *agr* class I probe on Southern blotting, hybridizing to a *ClaI* band of 1.4 kb instead of the band of 2.3 kb typical of an *agr* class I strain. Strains of *agr* classes I and IV have a high degree of genetic homology (26). The *agr* class for strains of classes I through III was therefore ascribed by the presence of a 2.3-kb *ClaI* digestion product that reacted with the class-specific probe, whereas *agr* class IV strains were denoted by a 1.4-kb *ClaI* band that reacted with the *agr* class I-specific probe.

*agr* class designation was confirmed phenotypically as either class I or not class I by using an *maIII::lacZ* fusion reporter strain in an 8325-4 (*agr* class I) background (18). This strain was constructed as described by Chan and Foster (9). Test strains were grown for 6 h in brain heart infusion broth, and supernatant containing peptide was prepared as described by Ji et al. (28). The 8325-4 *maIII::lacZ* strain was grown in brain heart infusion broth to an optical density at 600 nm of 1, test supernatant was added, and the cultures were sampled at 2 and 3 h. The P3 promoter activity of the *maIII::lacZ* strain was measured by

4-methylumbelliferyl- $\beta$ -D-galactopyranoside (MUG) assays (9). Supernatant from *agr* class I strains enhanced  $\beta$ -galactosidase activity by at least 30% over that for the control, as measured by the amount of luminescence of the MUG product; non-class I strains suppressed MUG production by at least 30% compared to that for the control.

**Typing methods.** To confirm that strains were distinguishable, strains were kindly phage typed by the Staphylococcal Reference Laboratory (PHLS). Isolates were assigned to phage group I if they had any reaction, lytic or inhibitory (lack of growth), with phages of group I (phages 29, 52, 52A, 79, 80, 95); as phage group II if they reacted with phages of group II (phages 3A, 3C, 55, 71) and not with other groups; as phage group III if they reacted with phages of group III (phages 6, 42E, 47, 53, 54, 5, 75, 77, 83A, 84, 85, 88A, 90, 83C, 932) and not with phages of group I; and as phage group V if they reacted only with phages 94 and/or 96 (1).

Pulsed-field gel electrophoresis (PFGE) typing of selected strains was also kindly performed by the Staphylococcal Reference Laboratory. This method involves digestion of chromosomal DNA with *SmaI* and comparison of the size of the resultant bands (54).

**Membrane arrays.** Since characterization of an individual strain by Southern blotting for each gene in turn is a lengthy procedure, we developed a rapid method of screening for *agr* class and the presence of several accessory virulence factors simultaneously using membrane arrays. Each Hybond-N nylon membrane (Amersham) was robotically spotted with 100 ng of DNA of each of the gene probes listed in Table 2 by using a MicroGrid (BioRobotics) instrument. The membrane was sitting on Whatman paper soaked in membrane denaturation buffer (0.5 M NaOH, 1.5 M NaCl). The DNA on the membrane was then neutralized (1.5 M NaCl, 1 M Tris [pH 7.4]) and UV fixed.

A total of 0.3 µg of genomic DNA from each test strain was extracted and digested with *Sau3A* for 6 h and cleaned on a QIAquick mini column (Qiagen), according to the manufacturer's instructions. DNA was labeled, hybridized, and detected with the ECL kit (Amersham). The resulting pattern of spots (positive or negative for each gene) was compared to the results from the Southern blots.

**Statistics.** Fisher's exact test for approximate two-sided probability was used for comparisons of accessory gene distributions. Calculations used 38 strains and omitted the second strain of each pair. The Fisher's exact calculation for phage groups with *agr* groups used the statistics package Stata 5.0 (Stata Corp, College Station, Tex.).

BURST analysis (<http://www.mlst.net/BURST/burst.htm>) (16) was performed by using each of seven core gene locus RFLP values for genes *maIII* (including *agr* class), *sarA*, *ssp*, *spa*, *icaA*, *fnbA*, and *hlg*. Strains were included in a cluster if they had at least five of seven loci identical to at least one other strain (16).

## RESULTS

***agr* class distribution.** By using the *agr*-specific probes to assign each strain to a class, 23 of 38 isolates (61%) were *agr* class I, 10 (26%) were *agr* class II, and 5 (13%) were *agr* class III (Table 1). No isolate was found to be *agr* class IV. We confirmed the results of our genotypic assays with a phenotypic assay because the original description of the *agr* classes was of mutual inhibition of *agr* function between strains by the *agrD*-encoded octapeptide. The results of the phenotypic assays ascribed isolates as *agr* class I or not class I. Our phenotypic results matched those of the genotypic assays, supporting our *agr* class assignments.

**Diversity of *S. aureus* genes and evidence of clusters.** Of the 12 core genes present in all strains, only 3 were always found on the same-sized *ClaI* fragment. Probes for *hla*, *sbi*, and *hsp70* always hybridized to fragments of 0.5 and 0.7 kb (double band), 6 kb, and 5 kb, respectively. In contrast, nine genes (*maIII*, *sarA*, *ssp*, *spa*, *icaA*, *fnbA*, *efb*, *hly*, *hlg*) were found on variably sized *ClaI* fragments in some strains, and their distributions are shown in Table 1. Hybridization with a *16SrRNA* probe (ribotyping) identified 10 different patterns of between three and six bands of between 2 and 10 kb. The distribution of accessory genes is also shown in Table 1. Of the accessory genes, *see*, *eta*, *etb*, and *mecA* were not carried by any isolate (data not shown).

TABLE 2. Gene probes<sup>a</sup>

Probe	Gene product or function	5' primer sequence (5' to 3')	3' primer sequence (5' to 3')	Probe size (bp)	Source(s) of sequence <sup>b</sup>	Template <sup>c</sup>	Reference <sup>d</sup>
<i>agr</i> I	<i>agrCDB</i> of class I	5'-CAC TTA TCA TCA AAG AAG C-3'	5'-CGA CTA ATT ATTA GCT GG-3'	351	X52543	8325-4	28
<i>agr</i> II	<i>agrC</i> of class II	5'-GTA GAG CCG TAT TGA TTC C-3'	5'-GTA TTT CAT CTC TTT AAG G-3'	464	AF001782	SA502A	28
<i>agr</i> III	<i>agrC</i> of class III	5'-GTG CAT TTA TTA GTG GAA TAC G-3'	5'-GTT TCA TTT CTT TAA GAG-3'	558	EMRSA-16	RN8463	28
<i>malIII</i>	Mediator of the <i>agr</i> response	5'-CAG AGA TGT GAT GGA AAA TAG TTG-3'	5'-AAT GAA GTA GAA CAG CAA CGC G-3'	824	X52543	8325-4	43
<i>spa</i> I	Staphylococcal accessory regulator A	5'-GGC AAA TGT ATC GAG CAA GAT G-3'	5'-GGT TCA GTG ATT CAT TTA TTT ACT C-3'	398	U46541	8325-4	10
<i>16S</i> rRNA	16S rRNA	5'-GTA GGT GGC AAG CGT TAT CC-3'	5'-CGC ACA TCA GCG TCA G-3'	228	Reference 38	8325-4	45
<i>hsp70</i>	Heat shock protein 70	5'-GAC TTA GGT GGC GGT ACA TTT G-3'	5'-GAC AGC TTC TTG TAC TGC-3'	468	D30690	8325-4	44
<i>ssp</i>	Serine protease (V8)	5'-GGA GGT TTT TAG ATG AAA GG-3'	5'-GCG CAT TGT CTG GAT TAT CAG G-3'	988	Y00356	8325-4	8
<i>spa</i>	Protein A. Immunoglobulin binding	5'-GGT GTA GGT ATT GCA TCT G-3'	5'-CGA CGT CCA GCT AAT AAT CAG G-3'	1303	U54636	8325-4	36
<i>sbi</i>	Immunoglobulin M and β <sub>2</sub> -glycoprotein I binding	5'-CAC AGA GGA ACA ACG TAA CC-3'	5'-GAT TTA GCT AAG TAG CCG-3'	957	AF027155	8325-4	59
<i>icaA</i>	Intercellular adhesion	5'-GTG GAT GAA TTA GAA GGC-3'	5'-TTA GCG TTG GGT ATT CCC-3'	1113	AF086783	8325-4	15
<i>fibA4</i>	Fibrinogen binding protein	5'-CAC AAA CTG CAC AAC CAG C-3'	5'-GGA TTT GAT TCC TCA GAG GAC-3'	1522	J04151	8325-4	51
<i>efb (fib)</i>	Extracellular fibrinogen binding protein	5'-AAC ATT AGC GGC AAT AGG-3'	5'-ATT CGC TCT TGT AAG ACC-3'	432	X72014	8325-4	13
<i>clu</i>	Collagen binding protein	5'-AAG CAT TTG CAG CAC GAG-3'	5'-ATA TGA CCC ATA GCC TTG TGG-3'	740	M81736	RN8463	40
<i>cap5</i>	Capsule type 5	5'-ATG ACG ATG AGG ATA GCG-3'	5'-CTC GGA TAA CAC CTG TTG C-3'	881	U81973	8325-4	33
<i>cap8</i>	Capsule type 8	5'-ATG ACG ATG AGG ATA GCG-3'	5'-CAC TTA ACA TAA GGC AAG-3'	1148	U73374	RN8463	49
<i>lukE</i>	Leukocidin E	5'-GAC TGA TTG CAC CTT TAG G-3'	5'-GGA ATT GAT GAG GCA ACT GAT G-3'	953	Y13225	8325-4	21
<i>spb</i>	Serine protease-like B gene	5'-CCA TAT ACT GGT GTA GTT G-3'	5'-GGG TGT GGA TAA CCA ATC-3'	352	U60589	8325-4	46
<i>hla</i>	α-Hemolysin	5'-CAC GTA TAG TCA GCT CAG-3'	5'-CTG AAG AAC GAT CTG TCC-3'	909	X01645	8325-4	22
<i>hly</i>	β-hemolysin	5'-AGT TGG TGC ACT TAC TGA C-3'	5'-TAC TAT AGG CTT TGA TTG GG-3'	920	S72497	8325-4	42
<i>hlg</i>	γ-Hemolysin leukocidin	5'-CCA ATC CGT TAT TAG AAA ATG C-3'	5'-CCA TAG ACG TAG CAA CCG AT-3'	937	X81586	8325-4	41
<i>tsf</i>	Toxic shock syndrome toxin 1	5'-ATC GTA AGC CCT TTG TTG-3'	5'-GTG GAT CCG TCA TTC ATT G-3'	578	Reference 35, U93688	RN8463	35
<i>sea</i>	Enterotoxin A	5'-CAT TGC CCT AAC GTT GAC-3'	5'-CGA AGG TTC TGT AGA AGT ATG G-3'	619	M18970	RN8463	3
<i>seb</i>	Enterotoxin B	5'-CTA AAC CAG ATG AGT TGC AC-3'	5'-CCA AAT AGT GAC GAG TTA GG-3'	489	M11118	PM452	29
<i>sec</i>	Enterotoxin C	5'-GTC TGT AGA TAA ATT TTT GGC AC-3'	5'-TAT GTC TAG TTC TTG AGC TTG-3'	367	X05815	PM58	23
<i>sed</i>	Enterotoxin D	5'-GTG GTG AAA TAG ATA GGA CTG C-3'	5'-ATA TGA AGG TGC TCT GTG G-3'	385	M28521	SA502A	2
<i>see</i>	Enterotoxin E	5'-GCT TTA AGC AAT CTT AGG C-3'	5'-CTA TGC ACA AGT TAA TTG GTA C-3'	531	Reference 38	American strain 578	14
<i>seg</i>	Enterotoxin G	5'-TCT TTA TAC GTC TCC ACC-3'	5'-GTC TAT TGT CGA TTG TTA CC-3'	326	Reference 38	96/ST/1504	39
<i>seh</i>	Enterotoxin H	5'-AGA GTG ATG AAA TAA GTG G-3'	5'-TTT GAA TAC CAT CTA CCC-3'	270	O'Neill	96/ST/1503	53
<i>sei</i>	Enterotoxin I	5'-CAA CTC GAA TTT TCA ACA GGT AC-3'	5'-CAG GCA GTC CAT CTC CTG-3'	466	Reference 38	96/ST/1504	39
<i>etx</i>	Exfoliative toxin A	5'-GTA GGA GGT AGT GCA TTT G-3'	5'-GGT CTC TAT CAA GAT GAG AC-3'	721	M17357	RN4850	32
<i>etb</i>	Exfoliative toxin B	5'-CCT TAC CTG TCA TTC CTT TTG-3'	5'-ATC AAC CGA ATA GAG TGA AC-3'	719	M17348	RN4850	24
<i>mecA</i>	Methicillin resistance (PBP 2 <sup>a</sup> )	5'-GTG GAA TTG GCC AAT ACA GG-3'	5'-TGG ATA GCA GTA CCT GAG CC-3'	575	Y14051	PM59	48

<sup>a</sup> All probes were prepared by PCR amplification (see Materials and Methods) with the primer pairs listed.

<sup>b</sup> All primers were designed for this study by using the sequence from the GenBank accession number listed, unless indicated otherwise. The EMRSA-16 sequence is found at [www.sanger.ac.uk/Projects/S\\_aureus](http://www.sanger.ac.uk/Projects/S_aureus).

<sup>c</sup> The strain from which genomic DNA used as the template for PCR amplification was derived.

<sup>d</sup> Reference to gene description or use in typing.

<sup>e</sup> Designed by G. O'Neill, personal communication.



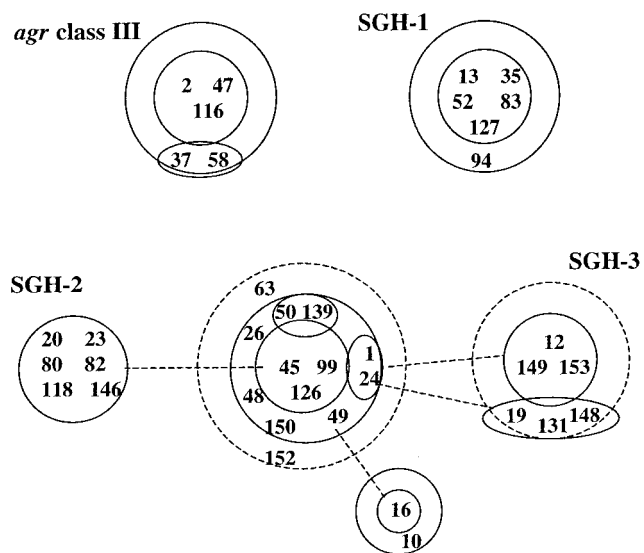


FIG. 1. BURST analysis of core genotypes of MSSA isolates. The relatedness of strains was determined by BURST analysis, based on core gene RFLP values (16). Numbers refer to the MSSA isolate numbers listed in Table 1. Core genotypes were defined by RFLP values for core genes *maIII* (including *agr* class), *sarA*, *ssp*, *spa*, *icaA*, *fnbA*, and *hlg*. Isolates in each central ring cluster have identical core genomes. Isolates in the first concentric ring have a core genome RFLP fingerprint that differs by one of the seven core gene RFLPs compared to those for isolates in the central ring. Isolates in the second concentric ring (dotted circle) differ by two core gene RFLPs from those for isolates in the central ring. Similarly, the solid lines connect rings of isolates that differ by a single RFLP, whereas dotted lines connect rings of isolates that differ by two RFLPs. At least four clusters of related strains can be identified and are called *agr* class III, SGH-1, SGH-2, and SGH-3 (Table 1).

In Table 1 four clusters of isolates, each with unique and virtually identical core RFLP profiles and ribotypes, could be clearly differentiated, and these are illustrated by BURST analysis (Fig. 1). The first of these clusters included all five *agr* class III isolates. *sarA*, *ssp*, and *spa* were conserved on unique *ClaI* fragments in these isolates, and the cluster had a unique ribotype (Table 1). We have called the second cluster of six strains the SGH-1 (St. George's Hospital) cluster; it includes strains PM13, PM35, PM52, PM83, PM127, and PM94. The strains carried *sarA*, *ssp*, *spa*, *icaA*, and *hlg* on unique and identical *ClaI* fragments and had a unique ribotype. Another cluster of strains identified within *agr* class I, comprising strains PM20, PM23, PM80, PM82, PM118, and PM146 (SGH-2), were identical across all core genes. The fourth cluster of isolates identified (SGH-3) comprised strains PM12, PM149, PM153, PM19, PM131, and PM148. These isolates were *agr* class II and were identical across all core and accessory genes analyzed with the exception of *fnbA*. Surprisingly, phage typing did not group strains into clusters apart from cluster SGH-3.

**Multiple accessory virulence genes may be mobile.** The clusters of *S. aureus* strains within our collection of hospital MSSA isolates are likely to represent conserved core lineages. However, within the conserved backgrounds of the first three clusters there appeared to be much variation in the carriage of accessory virulence genes. For example, four of the five *agr* class III strains carried *seg* and *sei*, three carried *cna*, and only two carried *sec* or *seh*. Notably, the *ClaI* fragments carrying

*cna*, *seg*, and *sei* were unique to *agr* class III isolates. Furthermore, *tst* was carried on three different genetic elements (11, 34, 35) including SaP12, as judged by a *tst ClaI* fragment of 16 kb. None carried the SaPI1 *int* (integrase) gene (data not shown).

Of the six SGH-1 strains, one carried *lukE* and *splB*, three carried *sec*, and one carried *seb*. One isolate had a unique *hlyB* RFLP profile, and the *hlyB* gene is a known insertion site for toxin-carrying phages (12). Strains of SGH-2 varied in their carriage of *sea* and *sed*, with different *ClaI* fragments carrying *sea* within this group. Variation was seen not only in *hlyB* but also in *efb*. SGH-3 strains carried few accessory genes, and no variation was seen in this cluster.

Because these results suggested that horizontal transfer of accessory virulence genes occurred frequently, we examined a second isolate of *S. aureus* from four of the patients to see if transfer might be occurring in the hospital setting (Table 1). One pair of isolates, taken from a baby who died with staphylococcal sepsis, appeared to be identical, except that the nasal strain (PM122) did not carry *sea*, whereas the lung isolate (PM116) taken 6 days later at postmortem examination was *sea* positive. The *hlyB* band sizes in these strains also varied, and *sea* is known to be carried on a phage that inserts into the *hlyB* gene, thus splitting the gene into two *ClaI* fragments. To confirm that the *hlyB* gene had been disrupted, we amplified *hlyB* by PCR with the primers listed in Table 2. We found that amplification was possible for PM122, which showed the expected band of 0.92 kb, but PM116 *hlyB* could not be amplified. To further investigate these two strains, we compared their PFGE profiles, which showed no band differences, although their phage types differed by two strong lytic and seven inhibitory reactions. We concluded that an *sea*-carrying phage was inserted into *hlyB* in one of two otherwise identical isolates from a patient.

A second pair of strains (PM153 and PM154) taken from the throat and lung of a baby at postmortem examination could not be differentiated by our RFLP or ribotyping methods but showed variable phage typing patterns, with three strong lytic differences. For the remaining two sets of pairs, neither our methods nor phage typing could differentiate the linked strains.

***lukE* and *splB* may be carried on the same genetic element.** We found that the gene encoding *splB* always co-occurred with *lukE*. Both were present in 68% of *agr* class I strains and 100% of *agr* class II isolates. The genomic sequence of strain 8325 ([www.genome.ou.edu/staph](http://www.genome.ou.edu/staph)) shows that in this strain *lukE* and *splB* are 12.6 kb apart. Between these two genes are two *ClaI*-cut sites, explaining why the RFLP band sizes are different. Downstream of *lukE* is *lukD* and then an open reading frame (ORF) with 44% amino acid homology to the *Staphylococcus epidermidis epiB* gene. *epiB* and the next six ORFs are predicted to be involved in lantibiotic synthesis (50), a known variable trait among *S. aureus* isolates. Farther downstream is a series of six serine protease-like genes in tandem, showing homology to *splA* through *splF* (GenBank accession number AF271715). The fact that *lukE* and *splB* are either present or entirely absent suggests that all these genes may be carried on the same accessory genetic element.

***seg* and *sei* are on the same genetic element.** *seg* and *sei* always co-occurred and were usually carried on the same-sized *ClaI* fragment, as expected (25, 27). They are 1.9 kb apart and within a cluster of seven enterotoxin-like genes (27). Down-

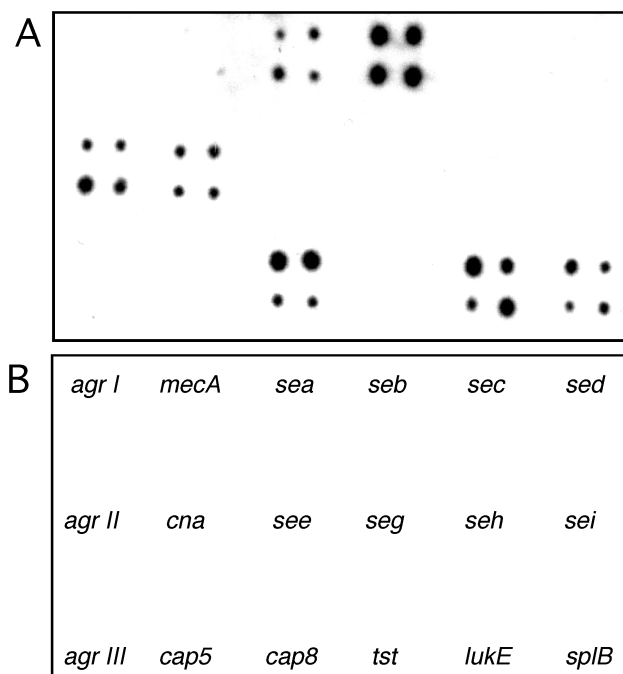


FIG. 2. Membrane array results for PM152. The array was manufactured as described in Materials and Methods. Each gene was spotted four times. (A) PM152; (B) order of genes spotted onto the membrane. *agr* I, II, and III refer to the *agr* class I-, II-, and III-specific probes listed in Table 2, respectively. The results illustrate the ability of membrane arrays to identify differences in accessory gene carriage in different strains. We have no explanation for the variable sizes of the spots on the arrays.

stream (2.2 kb) from *seg* is an ORF with 49% amino acid homology in the terminal 141 amino acids to a putative *Escherichia coli*  $\phi$ 27 phage protein (Shiga toxin 2e converting phage; GenBank accession number CAB93761). Downstream is a group of *spl* homologs similar to those downstream of *lukE* and *splB*.

**Carriage of genetic elements is influenced by *agr* class and other genetic elements.** We confirmed the previously reported association between *agr* class III and *tst* ( $P < 0.00001$ ). We were unable to demonstrate a correlation between *tst* carriage and phage group I strains ( $P = 0.21$ ). As expected, *seb* was not seen with *tst*. We discovered a significant inverse correlation between *lukE-splB* and *tst* ( $P = 0.003$ ), with *lukE-splB* being found only in *tst*-negative strains and, therefore, in *agr* class I and II strains ( $P = 0.003$ ). We also found a significant negative correlation between *lukE-splB* and *seg-sei*, such that they were rarely found in the same strain ( $P = 0.0005$ ). However, we did not demonstrate a significant correlation between *seg-sei* and *tst* ( $P = 0.28$ ).

In contrast to Ryding et al. (47) and Booth et al. (6), we did not find a correlation between *cna* and *cap8*; however, we did find a negative correlation between *cna* and *lukE* ( $P = 0.003$ ).

**Membrane arrays.** We developed membrane arrays as a rapid method for screening for the presence of several accessory virulence genes as well as *agr* class simultaneously. The patterns generated with membrane arrays correlated with the Southern blot results exactly, confirming their accuracy (Fig. 2). The entire collection could be characterized in 3 days.

## DISCUSSION

Our results demonstrate that hospital strains of MSSA are genetically diverse; however, within this diversity, some strains are clearly related and probably represent successful lineages. These conclusions are based upon the results generated by looking at core genes alone. Altered *Cla*I fragment sizes are presumably the result of a mutation in a nearby *Cla*I restriction site or a reorganization such as a deletion or an inversion. In addition, horizontal transfer of DNA between strains and recombinational replacement in the new host occurs in *S. aureus* (17, 19), which is probably mediated by generalized transducing phages. Therefore, we can predict that strains that differ in the *Cla*I fragments encoding one core gene are more closely related than strains that differ in the *Cla*I fragments encoding several core genes. By this approach, it is clear that at least four "clusters" of strains are present in our collection. Furthermore, two of these clusters are quite unrelated to the remaining strains, as they have multiple unique *Cla*I fragments encoding core genes. These lineages appear to be successful in our hospitals, which could be due to increased virulence. Our results are consistent with the findings of Day et al. (16), who were also able to group their *S. aureus* strains into related clusters and showed that conserved ancestral clones of isolates were more virulent.

The isolates of one of the successful lineages that we identified were all *agr* class III. However, van Leeuwen et al. (55) screened a collection of 55 MSSA isolates mostly taken from healthy nasal carriers and did not find any *agr* class III isolates. Our *agr* class III cluster may therefore be associated with virulence in hospitals, although since this cluster appears to be genetically distinct from the other isolates, it is not necessarily due to *agr* class per se. van Leeuwen et al. (55) also reported that only 15% of their strains were *agr* class II, while 26% of the strains in our collection were *agr* class II. Therefore, it is possible that *agr* class II strains are also more frequently associated with disease in hospitals, and this also needs to be investigated further.

Accessory virulence genes appear to be widely distributed in hospital strains, and all our strains carried at least one. Our results by RFLP analysis with *Cla*I also indicate that the same gene can be carried on a variety of elements; for example, *sea* can probably be carried on four distinct genetic elements.

More importantly, by focusing on three clusters of strains (SGH-1, SGH-2, and *agr* class III strains) we can see that the loss and/or acquisition of accessory genes *tst*, *sea*, *seb*, *sec*, *sed*, *seg-sei*, *seh*, *cna*, and *lukE-splB* is occurring in otherwise stable backgrounds. The data strongly suggest that vertical transmission of accessory genes to daughter cells does not account for all of this variation and that horizontal transfer of these genes between strains is occurring.

By looking at pairs of isolates taken from the same patient, we can predict that some of this variation may even be occurring in patients. In one pair of isolates, we have evidence that the *sea* phage either has been acquired by a nasal isolate that has gone on to cause infection in the lung or has been lost by an isolate residing in the nose. An alternative explanation is that the loss of the *sea* phage in the nasal isolate occurred during collection, storage, or laboratory growth. Any of these possibilities has important implications. First, if *sea* phage ac-

quisition occurs in patients in vivo, we need to consider a possible source of this phage, what conditions promote phage acquisition, the frequency at which this occurs, and the effect of the phage on virulence. Alternatively, if phage can be lost in vivo, understanding the mechanism of loss may be beneficial for the treatment of infections. Finally, if phage is easily lost in the laboratory, we may need to reassess the reliability of published data on the frequency and distribution of accessory virulence genes in *S. aureus* populations. However, we found the *sea* gene to be stable after storage and subculture of PM116 in vitro (data not shown).

We examined a second isolate from only four patients and found variation in two, which suggests that horizontal transfer or loss of genetic elements may be frequent. The second pair of isolates showed an altered phage susceptibility profile, although we were unable to identify a difference using probes specific for selected accessory genes. Phage profiles are known to alter when a new phage or genetic element is integrated into the bacterial chromosome (4, 35), although the cause of this "phage immunity" is unknown. Furthermore, new *S. aureus* accessory virulence genes are continuously being described. Therefore, it seems likely that these two strains are closely related and that one has acquired or lost a phage or accessory genetic element, which may or may not carry virulence genes.

Increasing numbers of known and novel virulence genes have been shown to be encoded on mobile genetic elements. Our results suggest that *lukE-splB*, *seg-sei*, and *seh* may also be on genetic elements that can be acquired or lost. Some of these elements and putative elements were not evenly distributed between groups of strains. Vertical transfer could explain the nonrandom distribution of accessory genes; however, we believe that our evidence supports frequent horizontal transfer as well. If so, this suggests that acquisition of a mobile element may be restricted by host background (such as *agr* class) or the presence of other mobile elements. Mechanisms of restriction are under investigation in our laboratory.

The relatedness of diverse populations of *S. aureus* strains has been poorly characterized until recently due to a lack of appropriate methods. Epidemiological typing methods, such as phage typing and PFGE, are unsuitable for comparison of strains that are not epidemiologically linked because they rely on a comparison of patterns, while the causes of pattern variation are complex and poorly understood. Furthermore, our results and those of others (52) show that both methods are oversensitive to horizontal movement of mobile elements. Recently, the powerful technique of multilocus sequence typing, which involves the sequencing of seven core housekeeping genes and comparison of nucleotide differences, has been applied to invasive and carriage strains of *S. aureus* (16, 17). This approach also identified several clusters of related strains. However, since many known and putative virulence genes may be highly mobile, we believe that screening for these genes will enhance any such studies. Our rapid membrane array method is ideally suited for this and can easily be adapted to include more or different combinations of genes. It will also be appropriate for the screening of large numbers of clinically relevant isolates for their carriage of *agr*-class and genetic elements.

In conclusion, our results illustrate the genetic diversity of hospital MSSA strains and show that horizontal transfer of mobile genetic elements encoding virulence genes occurs fre-

quently, probably in the hospital environment, and possibly in vivo. However, factors in the core genome of the recipient strain or other accessory genetic elements may restrict this transfer. These phenomena need to be investigated further, as they may have significant impact on the evolution and success of *S. aureus* strains in hospitals, as well as on the treatment of infections. This includes the potential for epidemic hospital-associated strains, including methicillin-resistant *S. aureus* strains, to acquire accessory virulence genes.

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