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Molecular Characterization of Endocarditis-Associated *Staphylococcus aureus*

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Infective endocarditis (IE) is a life-threatening infection of the heart endothelium and valves. *Staphylococcus aureus* is a predominant cause of severe IE and is frequently associated with infections in health care settings and device-related infections. Multilocus sequence typing (MLST), *spa* typing, and virulence gene microarrays are frequently used to classify *S. aureus* clinical isolates. This study examined the utility of these typing tools to investigate *S. aureus* epidemiology associated with IE. Ninety-seven *S. aureus* isolates were collected from patients diagnosed with (i) IE, (ii) bloodstream infection related to medical devices, (iii) bloodstream infection not related to medical devices, and (iv) skin or soft-tissue infections. The MLST clonal complex (CC) for each isolate was determined and compared to the CCs of members of the *S. aureus* population by eBURST analysis. The *spa* type of all isolates was also determined. A null model was used to determine correlations of IE with CC and *spa* type. DNA microarray analysis was performed, and a permutational analysis of multivariate variance (PERMANOVA) and principal coordinates analysis were conducted to identify genotypic differences between IE and non-IE strains. CC12, CC20, and *spa* type t160 were significantly associated with IE *S. aureus*. A subset of virulence-associated genes and alleles, including genes encoding staphylococcal superantigen-like proteins, fibrinogen-binding protein, and a leukocidin subunit, also significantly correlated with IE isolates. MLST, *spa* typing, and microarray analysis are promising tools for monitoring *S. aureus* epidemiology associated with IE. Further research to determine a role for the *S. aureus* IE-associated virulence genes identified in this study is warranted.

Infective endocarditis (IE) is a life-threatening infection of the endocardial surfaces of the heart and heart valves (1, 2). Antibiotic therapy is often difficult due to causation by multidrug-resistant bacteria and the capacity of infecting bacteria to form biofilms on the heart endothelium or implanted medical devices (3). *Staphylococcus aureus* is the predominant cause of the most severe cases of IE and is the most common pathogen isolated from cases related to intravenous drug use, health care environments, and medical devices such as prosthetic heart valves and intravenous catheters (4–8). The potential of *S. aureus* to colonize the heart endothelium and cause IE is thought to be closely associated with the genotype of infecting strains (9–11).

Genotyping methods, such as multilocus sequence typing (MLST) and *spa* typing, have been developed to classify and monitor the *S. aureus* population. MLST involves assigning strains a sequence type (ST) based on allelic variations in seven genes in the core genome (*arc*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqi*). Strains that diverge at no more than one of the seven MLST loci are considered to belong to the same clonal complex (CC). Double-locus variants (dlvs) are included in the CC if the linking single-locus variant (slv) is present in the MLST database (http://eburst.mlst.net). *S. aureus* isolated from patients with IE have generally been classified into CC1, CC5, CC8, CC15, CC30, and CC45 (10–12), with the majority belonging to CC30 (10). It has indeed been suggested that CC30 is more closely associated with IE than with other types of *S. aureus* infections (10). However, other studies have found no association between CC and invasive disease (12–14).

spa typing involves amplification and sequencing of the hypervariable region of the *spa* gene, which encodes the surface-exposed staphylococcal protein A, a major *S. aureus* virulence factor (15). A *spa* type is assigned to a *S. aureus* strain according to the number and combination of variable short repeats (24 bp) which are located in the C terminus of the *spa* gene and are flanked by well-conserved regions. Associations between *spa* type and IE have not been reported.

Bacterial pathogenicity and the development of IE are underpinned by the presence of virulence genes that encode factors such as adhesins, toxins, and exoproteins. Fibronectin-binding protein A and B (FnBPA/FnBPB) and Clumping Factor A (ClfA) are well-characterized Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) that mediate attachment and colonization of *S. aureus* to the heart endothelium and medical devices (16–19). The capsular polysaccharide is another important virulence factor expressed by most clinical strains of *S. aureus*. The capsule prevents phagocytosis of the bacterium by host immune cells, thus facilitating the persistence of *S. aureus* within the host and development of disease (20, 21).

A recent analysis of individual *S. aureus* virulence genes and *S. aureus* IE strains found no correlation between *S. aureus* genotype

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and IE (12). However, complex bacterial phenotypes such as the capacity to cause IE are likely to be underpinned by multiple genetic determinants. This study therefore examined associations of CC, *spa* type, and carriage of multiple virulence-associated genes with *S. aureus* IE. We identify CCs and *spa* types that are significantly associated with IE isolates and define a set of virulence-associated genes and allelic variants that, when combined, are significantly correlated with *S. aureus* IE isolates compared to non-IE isolates.

MATERIALS AND METHODS

Bacterial isolates. Ninety-seven *S. aureus* clinical isolates were collected between 2003 and 2007 from patients admitted to metropolitan hospitals in Brisbane, Australia. These hospitals included the Royal Brisbane and Women's Hospital, Ipswich Hospital, and Princess Alexandra Hospital. Isolates were categorized according to the clinical outcome associated with the respective patients: (i) IE ($n = 24$), (ii) bloodstream infection related to a medical device (BSI-D; $n = 17$), (iii) bloodstream infection not related to a medical device (BSI-ND; $n = 32$), and (iv) skin or soft-tissue infection (SSTI; $n = 24$). *S. aureus* endocarditis was defined according to the modified Duke Criteria (22).

Preparation of genomic DNA. Bacteria were grown overnight on Columbia blood agar. To prepare genomic DNA, six to eight colonies were suspended in 100 μl of lysis solution, which contained 0.05 mg lysostaphin (Sigma, Steinheim, Germany), 2 mg lysozyme (Sigma), 2 mg RNase A (Sigma), 2 μl 20 mM Tris-HCl (pH 8.0), 2 μl 2 mM EDTA, and 1 μl Triton X-100. The suspension was incubated at 37°C for 45 min with aeration (300 rpm). Proteinase K (10 μl) and buffer AL (100 μl) (DNeasy kit; QIAgen, Hilden, Germany) were added, and the suspension was incubated at 56°C for 45 min. The DNA was then processed using a QIAgen EZ1 device according to the manufacturer's tissue lysis protocol.

Multilocus sequence typing and eBURST analysis. MLST was performed as previously described (23). Briefly, primers were designed to PCR amplify seven defined *S. aureus* genes (*arc*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqi*). The PCR products were purified using a PCR purification kit (QIAgen), and both strands were sequenced using a CEQ 8000 genetic analysis system (Beckman Coulter, High Wycombe, United Kingdom). The sequences were aligned and analyzed using BioNumerics software (Applied Maths, St-Martens-Latem, Belgium). Allelic profiles and STs were assigned, and an MLST tree was constructed using the MLST database (http://www.mlst.net). STs were analyzed and assigned into CCs by the use of eBURST software (http://eburst.mlst.net). Strains that diverged at no more than one of the seven MLST loci were considered to belong to the same CC. Double-locus variants were included in the CC if the linking single-locus variant was present in the MLST database. The STs for strains SA4, SA6, SA7, and SA18 were assigned based on >99% sequence conservation for *yqi*, *tpi*, *aroE*, and *tpi* alleles, respectively. The isolates were compared with each other as well as with the entire *S. aureus* population available in the MLST database at the time of the study.

***spa* typing.** The hypervariable region of the *spa* gene was amplified using *Taq* DNA polymerase (QIAgen) according to the manufacturer's protocol and using primers designed as previously described (24): 1095F (5′-AGACGATCCTTCGGTGAGC) and 1517R (5′-GCTTTTGCAATGT CATTTACTG). PCR cycling conditions were as follows: an initial 3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and a final 10 min at 72°C. The PCR product was purified using a Wizard SV Gel and PCR Clean Up system (Promega, Madison, WI). The forward and reverse strands of the purified PCR product were sequenced using BigDye Terminator v3.1 according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The sequences were analyzed and *spa* types assigned using the *spa*Type Finder (http://fortinbras.us/cgi-bin/spaTyper/spaTyper.pl) and the Ridom *Spa*Server database (http://spa.ridom.de).

DNA microarray analysis. The DNA microarray and reagents were obtained from Alere Technologies (Jena, Germany). The principle of the assay and related procedures have been described previously (25, 26). Briefly, the assay employs a linear PCR-like approach to amplify and label target DNA. In this approach, the array is stained by horseradish peroxidase-based tetramethylbenzidine precipitation for 10 to 30 min, after which the resulting pattern is recorded and analyzed using an ATR01 reader (CLONDIAG) and IconoClust software (CLONDIAG). The raw data were interpreted as previously described (25, 26).

The DNA array targets, primers, and probes used in the study have been previously published (27). The array covered 352 target sequences consisting of a variety of species markers, markers for accessory gene regulator (*agr*) alleles and capsule types, virulence factors, resistance genes, staphylococcal superantigen-like and exotoxin-like genes (*set* and *ssl*), and genes encoding adhesins. Depending upon the nomenclature used, the target sequences correspond to 185 distinct genes and their allelic variants. All probes were specific to their target gene, were not self-hybridizing, and were similar with respect to GC content, length, and melting temperature. The probes were analyzed against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) to eliminate probes that might cause false-positive or false-negative reactions.

Statistical analysis of CC, *spa* type, and microarray data. To determine the correlation between the presence of each CC, *spa* type, and IE isolates, a null model with 1,000 permutations was used. In each permutation, each CC/*spa* type was randomly assigned to a strain. The difference between the percentage of IE strains with that CC/*spa* type and the percentage likely to be obtained by chance was calculated. The percentages obtained in the 1,000 permutations were then used to calculate the likelihood of the observed difference occurring by chance. A correlation matrix was built to explore the correlation between the presence of each CC and each *spa* type.

Permutational analysis of multivariate variance (PERMANOVA) was used to determine if there were genotypic differences between IE and non-IE strains. A principal coordinates (PCO) analysis was used to represent the genotype in a two-dimensional graphical space. To identify groups of genes that were present in IE isolates, the correlation coefficient values corresponding to the presence or absence of each gene and the first two axes of the PCO were calculated. Groups of genes or alleles with correlations that corresponded to regions associated with IE isolates in the multivariate space were then identified.

TABLE 1 Characteristics of 97 patients diagnosed with *S. aureus* IE, BSI-D, BSI-ND, and SSTI[a]

Characteristic	Values			
	IE ($n = 24$)	BSI-D ($n = 17$)	BSI-ND ($n = 32$)	SSTI ($n = 24$)
Median age in yr (range)	55 (28–83)	58 (0–88)	67 (0–92)	34 (4–86)
No. (%) of female patients	3 (13)	9 (53)	10 (31)	15 (63)
No. (%) of patients with MRSA infection	0 (0)	2 (12)	3 (9)	0 (0)

[a] BSI-D, bloodstream infection related to a medical device; BSI-ND, bloodstream infection not related to a medical device; SSTI, skin or soft-tissue infection; MRSA, methicillin-resistant *S. aureus*.

RESULTS

Patients and isolates. Ninety-seven *S. aureus* clinical isolates were collected from patients representing four disease categories: IE, BSI-D, BSI-ND, and SSTI. A summary of the characteristics of patients for each type of infection is presented in Table 1. There was no significant difference in the median ages of patients and the clinical outcomes. More males were diagnosed with IE than females (7:1). Only five of the 97 *S. aureus* isolates were methicillin-resistant *S. aureus* (MRSA) isolates. All five MRSA isolates were from bloodstream infections not associated with IE.

Analysis of CCs associated with IE *S. aureus* isolates. Previ-

TABLE 2 Characteristics of MSSA clinical isolates associated with IE[a]

Isolate	Sex of patient	Age of patient (yr)	ST	Capsule
1	M	66	12	8
2	M	59	15	8
3	M	35	72	5
4	M	83	12	8
5	M	74	1	8
6	M	35	8	5
7	F	73	78	8
8	M	77	20	5
9	M	62	641	5
10	M	27	5	5
11	M	31	20	5
12	F	33	20	5
13	M	21	97	5
14	M	69	1273	8
15	M	70	30	8
16	M	49	78	8
17	M	76	101	8
18	M	56	188	8
19	M	53	795	8
20	M	39	30	8
21	M	45	5	5
22	M	28	8	5
23	M	30	291	5
24	F	65	8	5

[a] F, female; M, male; ST, sequence type; Capsule, capsular serotype.

ous studies have suggested an association between *S. aureus* CC and the type and severity of infection (9–11). The ST of all 97 *S. aureus* isolates was therefore determined, and 27 different STs were identified. Sixteen different STs were identified among the 24 IE isolates (Table 2).

An eBURST analysis was conducted to group STs into CCs and to determine if particular CCs were associated with IE. Strains that diverged at no more than one of the seven MLST loci were considered to belong to the same CC. The clustering of STs into CCs allowed visualization of how the isolates were distributed among the members of the global *S. aureus* population (Fig. 1). The IE isolates were distributed across several CCs, and isolates with different clinical outcomes were assigned the same CC. We found that CC12 and CC20 were significantly positively associated with IE isolates and that CC45 was negatively associated with IE isolates in this study (Table 3).

Analysis of *spa* types associated with IE *S. aureus* isolates. Two IE isolates contained novel *spa* sequences and were therefore designated new *spa* types from the Ridom *Spa*Server: t11474 for isolate 22 and t11475 for isolate 24. The prevalence of IE and non-IE isolates within each *spa* type was analyzed, and a significant association was found between *spa* type t160 and IE isolates ($P < 0.05$). A significant correlation was also found between CC12 and *spa* type t160 ($P < 0.05$). However, most CCs in this study were associated with a number of *spa* types (Fig. 2).

Analysis of virulence genes associated with IE *S. aureus* isolates. A number of genes, including the protease *sspA*, *sspB*, and

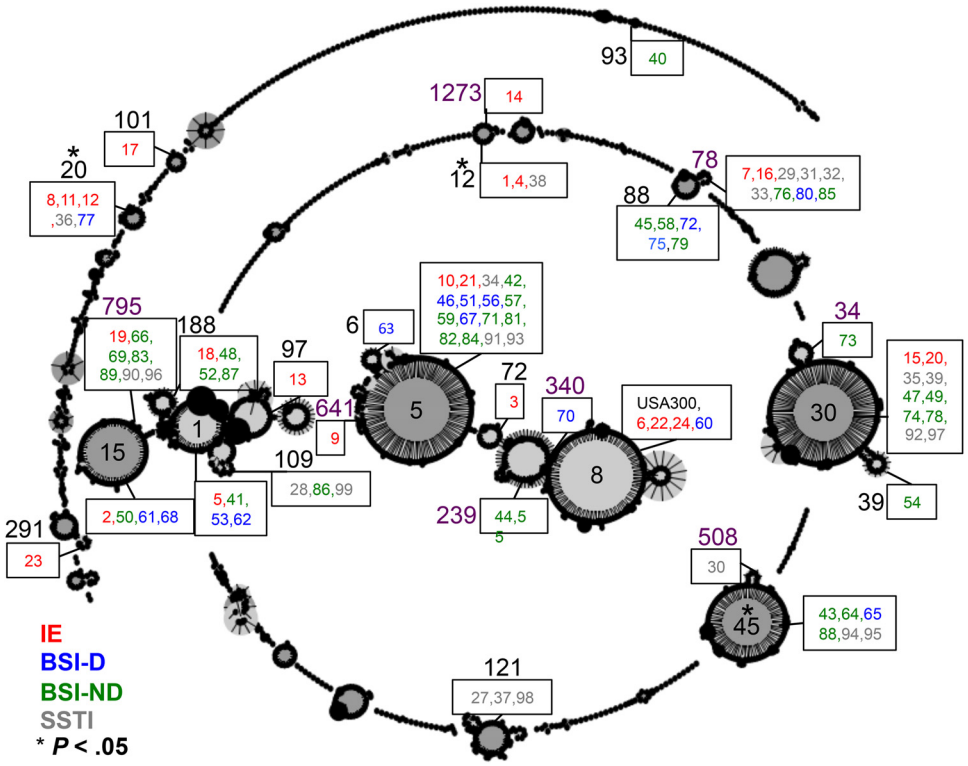

FIG 1 eBURST analysis showing phylogenetic relatedness of the 97 clinical *S. aureus* isolates with the *S. aureus* population. The number of individual isolates is written in a box next to the respective ST. The isolates are color coded according to clinical outcome: red for IE, blue for BSI-D, green for BSI-ND, and gray for SSTI. The ST number is provided beside the respective box or within the founder group. The CC number is shown in black, whereas a ST belonging to the CC is shown in purple. CCs were defined as all isolates within the group having at least six of the seven alleles identical with those of at least one other isolate in the group (http://eburst.mlst.net). CC12 and CC20 are significantly positively associated with IE, and CC45 is significantly negatively associated with IE ($P < 0.05$).

TABLE 3 Relationship between CC and *spa* type with IE strains[a]

CC	% difference	*P*	*spa* type	% difference	*P*
CC12	11.13	0.007	t160	6.96	0.003
CC20	9.76	0.012	t021	4.167	0.08
CC8	7.02	0.06	t024	4.167	0.08
CC101	4.167	0.27	t164	4.167	0.08
CC291	4.167	0.27	t267	4.167	0.08
CC72	4.167	0.27	t537	4.167	0.08
CC97	4.167	0.27	t888	4.167	0.08
CC1	0.057	0.49	t937	4.167	0.08
CC188	0.057	0.49	t1333	4.167	0.08
CC39	**−1.37**	**0.43**	t1987	4.167	0.08
CC6	**−1.37**	**0.43**	t11474	4.167	0.08
CC93	**−1.37**	**0.43**	t11475	4.167	0.08
CC15	**−4**	**0.241**	t002	2.91	0.238
CC30	**−4**	**0.241**	**t037**	**−4.11**	**0.14**
CC109	**−4.11**	**0.24**	**t371**	**−4.11**	**0.14**
CC121	**−4.11**	**0.24**	**t186**	**−5.48**	**0.06**
CC5	**−6.68**	**0.18**			
CC88	**−8.11**	**0.07**			
CC45	**−9.59**	**0.04**			

[a] % difference values represent the difference between the percentage of IE strains with that CC/*spa* type and the percentage likely to be obtained by chance. *P* values represent the probability that the difference between the percentages of IE and non-IE strains associated with each type was obtained by chance. Negative correlations are indicated with boldface characters.

sspP genes, the biofilm-associated *icaA*, *icaC*, and *icaD* genes, the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) staphylococcal protein A (*spa*), clumping factor A (*clfA*), clumping factor B (*clfB*), enolase (*eno*), and fibronectin-binding protein A (*fnbA*) genes, and immune evasion transferrin-binding protein A (*isdA*) and type I site-specific DNase subunit (*hsdSx*) genes, were identified in all 97 isolates. The genes that showed variation between the isolates were analyzed to determine if any gene or gene set was more closely associated with the IE isolates than with the non-IE isolates. PERMANOVA results suggest that there is a marked difference between IE and non-IE strains (Sheudo-F = 3.0241; $P = 0.01$) The PCO shows the ordination of the different genes and alleles in a compressed two-dimensional space (Fig. 3). Isolates that clustered shared similar genotypes, whereas isolates that were spatially separated had greater genotypic variation.

The majority of the IE isolates ($n = 21$; 88%) clustered on the right of the plot and contained the *ssl03*, *ssl7-set1*, *ssl8-set12*, *ssl08*, *ssl9-set5*, and *setB3* genes, the fibrinogen binding protein (*fib*) gene, and the leukocidin/hemolysin toxin family protein *lukY-var1* gene (Fig. 3). In contrast, a largely non-IE isolate cluster on the left of the plot (which included IE isolate 15) did not contain these genes or contained allelic variants of these genes. A different set of genes and alleles, including *ssl9-set5* (*MRSA252*), *setB3* (*MRSA252*), *lukY-var2*, type I site-specific DNase subunit, 2nd locus (*hsdS2*) (*MRSA252*), *ssl4-set9* (*MRSA252*, *SAR0425*), *ssl5-set3* (*MRSA252*), and *setB2*, was present in this largely non-IE isolate cluster. The gene set *ssl03*, *ssl7-set1*, *ssl8-set12*, *ssl08*, *ssl9-set5*, *setB3*, *fib*, and *lukY-var1* was significantly associated with IE isolates compared with the non-IE isolates ($P < 0.05$).

Variation was also observed in the genes that encode serotype-specific capsular polysaccharides. The *S. aureus* isolates in this study contained either *cap5* genes ($n = 32$; 33%) or *cap8* genes ($n = 65$; 67%). Among the 24 IE isolates, the *cap5* and *cap8* genes were equally distributed. Four of the *cap5* IE isolates clustered into a tight subgroup (Fig. 3; dotted oval), the majority of whose members were IE isolates ($n = 4$; 80%). The other isolate within this subgroup was from a patient with a bloodstream infection related to a hemodialysis intravascular device. The differentiation of this subgroup from the other IE *cap5* isolates is attributable to subspecies allelic variations between several genes, including those encoding glutamyl endopeptidase (*sspA*) and bone sialoprotein-binding protein (*bbp*), *clfA*, *clfB*, *fnbA*, *fnbB*, and the gene encoding von Willebrand factor-binding protein (*vwb*).

DISCUSSION

In this study, we determined the CC, *spa* type, and virulence-associated gene content of 24 *S. aureus* isolates associated with IE and compared these profiles to those of a set of *S. aureus* strains isolated from patients with different clinical syndromes from the same geographic location. Two CCs, one *spa* type, and a discrete set of virulence-associated genes and allelic variants displayed significant correlations with IE.

Although some studies have found no significant associations between CC and invasive disease (12, 13), it has also been suggested that IE *S. aureus* isolates generally belong to CC1, CC5, CC8, CC15, CC30, and CC45 (9, 10, 12). In this study, a total of 19 CCs were identified from the 97 clinical *S. aureus* isolates; these included 13 CCs containing IE isolates. Only 11 of the 24 IE isolates belonged to CC1, CC5, CC8, CC15, CC30, or CC45. Two IE isolates (8%) compared with nine non-IE isolates (12%) belonged to CC30; thus, there was no significant association between CC30 and IE in our study set. In contrast, we found that CC12 and CC20 were significantly positively associated with IE isolates and that CC45 was negatively associated with IE. The difference between this study and previous studies is most likely due to the different strain sets analyzed; for example, genetic variation exists between strains from different geographic locations (10).

As no previous study has reported a correlation between *spa* type and IE, *spa* typing was performed in this work to determine if particular *spa* types were associated with IE. *spa* type t160 was significantly associated with IE. A significant correlation was also found between CC12 and *spa* type t160. Most CCs in this study were associated with a number of *spa* types.

A DNA microarray and PERMANOVA were also performed to determine if known *S. aureus* virulence genes were associated with IE isolates. Analysis of the DNA microarray data clustered isolates into three main groups. Isolates between clusters differed in the presence of the genes and allelic variants *ssl03*, *ssl7-set1*, *ssl8-set12*, *ssl08*, *ssl9-set5*, *setB3*, *fib*, and *lukY-var1* and the presence of *cap5* or *cap8* genes. The set of genes and allelic variants was significantly associated with IE isolates compared with non-IE isolates ($P < 0.05$). This result differs from those of a recent study by Tristan et al. in which no difference between IE and non-IE *S. aureus* isolates in the prevalences of individual virulence-associated genes was found. A two-tailed Fisher's exact test was used in this recent study, which analyzed the results obtained with each gene independently (12). However, the PERMANOVA used in the current work was able to identify correlations between gene sets and clinical outcome. As multiple genes are likely to contribute to complex phenotypes such as the capacity to cause bacterial IE, the PERMANOVA is better suited to predict a gene repertoire that correlates with disease.

The identified *S. aureus*-IE gene set contains a number of genes

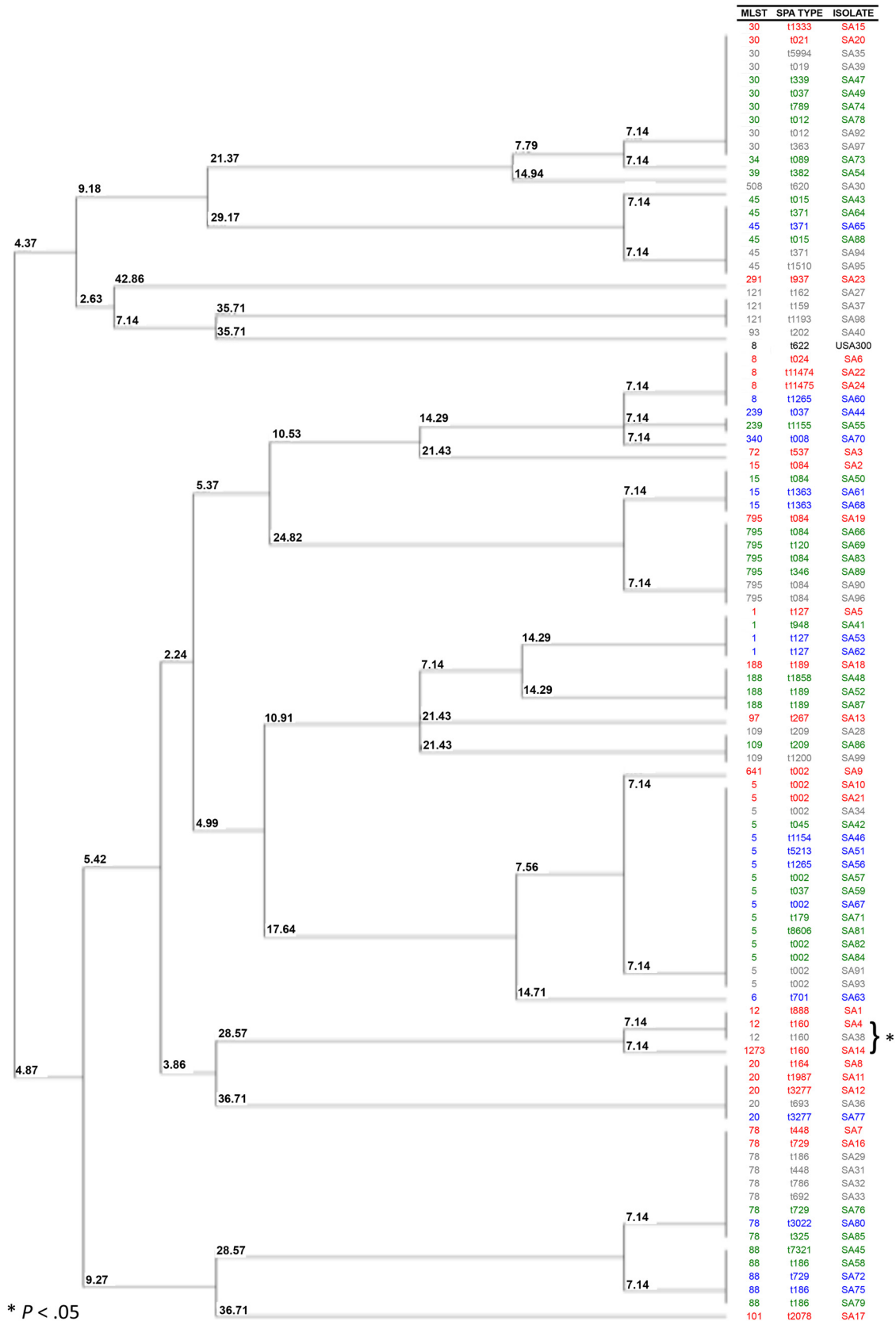

FIG 2 A phylogenetic tree based on the ST of the 97 clinical *S. aureus* isolates and USA300. The *spa* type and isolate number are shown next to the respective ST. Isolates are color coded according to clinical outcome: red for IE, blue for BSI-D, green for BSI-ND, and gray for SSTI. The tree was constructed using the MLST database (http://www.mlst.net). CC12 and t160 were significantly associated with IE ($P < 0.05$).

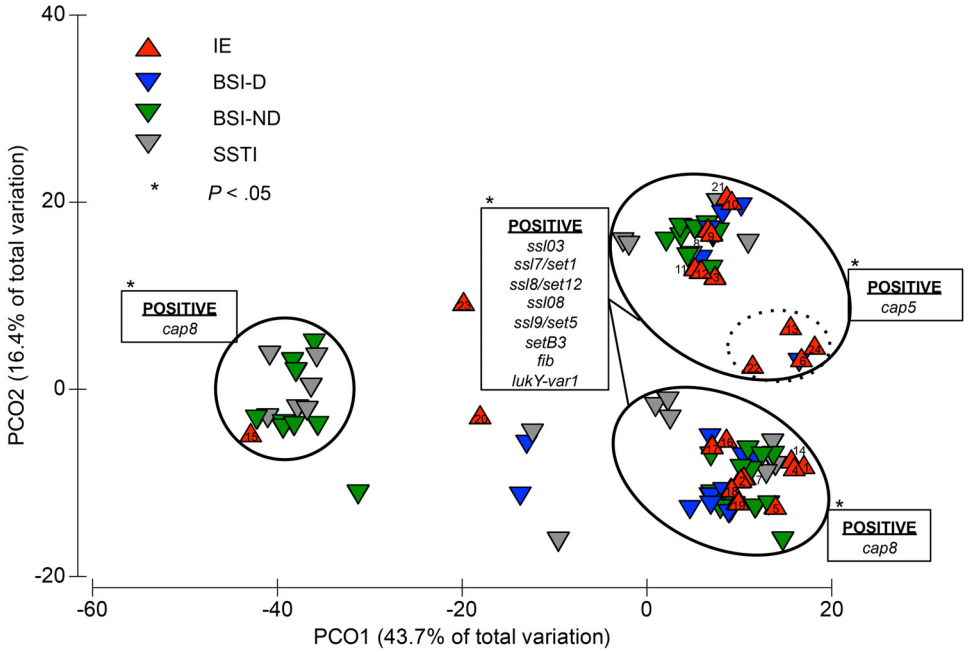

FIG 3 Ordination of the 97 clinical *S. aureus* isolates based on the first two axes of a PCO corresponding to the presence or absence of 352 target sequences. The presence of the genes and allelic variants was determined using a DNA microarray, and the 265 targets showing variation among the isolates were analyzed by a PERMANOVA and distributed in a PCO. Isolates are color coded according to clinical outcome: red for IE, blue for BSI-D, green for BSI-ND, and gray for SSTI. Genes are listed in the boxes next to the cluster containing isolates positive for the respective genes. The genes in the set consisting of *ssl03*, *ssl7-set1*, *ssl8-set12*, *ssl08*, *ssl9-set5*, *setB3*, *fib*, and *lukY-var1* are significantly associated with the IE isolates ($P < 0.05$). The isolates within the dotted cluster differ from other *cap5*-positive IE isolates by subspecies allelic variations in several genes (*lukS*, *sspA*, *ssl1-set6*, *ssl01*, *ssl11-set2*, *bbp*, *clfA*, *clfB*, *fnbA*, *fnbB*, *sdrC*, *sasG*, *hsdS3*, *Q2FXCO*, and *vwb*).

that encode superantigen-like proteins (Ssls), previously known as staphylococcal exotoxin-like proteins (Sets). All known *ssl-set* genes are located on mobile genetic elements, with *set6–15* located in a 19-kb region on staphylococcal pathogenicity island 2 (28, 29). Ssl/Set proteins are structurally homologous to staphylococcal superantigens but lack superantigenic properties such as mitogenicity, pyrogenicity, and inducement of endotoxic shock (28, 30). Although defined functions have not yet been assigned to every Ssl/Set protein, the members of this family of proteins are collectively known to mediate immune evasion by inhibiting complement activation and binding to IgA and IgG and preventing the recruitment and function of neutrophils (31).

The *fib* gene encodes an extracellular fibrinogen binding protein (Efb), which is secreted by *S. aureus* during infection (32). Efb plays a number of important roles during pathogenesis. The protein binds to host fibrinogen (Fg) and platelets and therefore blocks the binding of Fg to neutrophils and inhibits platelet aggregation (33–35). Efb also binds to C3b, which minimizes B and T cell responses, and inhibits complement-mediated opsonization and phagocytosis (36, 37).

The *lukY* gene encodes a leukocidin subunit which is not associated with the well-characterized Panton-Valentine leukocidin (PVL) *lukF-PV* and *lukS-PV* genes. It is important to highlight that, according to our analysis (as partially supported by previous results [12]), the gene sets identified as positively or negatively related to IE appear to have a synergistic effect. It could therefore be misleading to infer that each independent member of each gene subset has a direct independent effect on IE.

The PCO analysis revealed that some isolates from different CCs clustered, such as isolate 12 (CC97) clustering with isolates 6, 22, and 24 (CC8). This observation is not surprising, as virulence-associated genes are readily passed horizontally between unrelated strains via homologous recombination or acquisition of mobile elements (38). In contrast, genes in the core genome, such as the seven MLST genes, are vertically acquired from a common ancestor and evolve more slowly. The CC or *spa* type alone does not indicate the potential of an isolate to cause disease (13).

It is likely that there are other *S. aureus* virulence determinants associated with IE that were not identified due to their absence from the DNA microarray. In addition, all IE strains analyzed in this study were methicillin-susceptible *S. aureus* (MSSA), and it is possible that there are differences between MSSA and MRSA strains in IE-associated genes. For example, MSSA and MRSA utilize different mechanisms for biofilm growth (39). MRSA strains typically utilize protein adhesins such as FnBP to form biofilms, whereas biofilm formation by MSSA strains is generally dependent on production of the polysaccharide adhesin polymeric *N*-acetylglucosamine. In addition, a combination of host factors (e.g., age, gender, and immune status) may contribute to the capacity of *S. aureus* to cause IE in an individual.

In conclusion, we have determined that CC12, CC20, and t160 are significantly associated with the IE-related *S. aureus* isolates used in this study. These two CCs and this *spa* type could be used to differentiate *S. aureus* strains which have the potential to cause IE from less virulent *S. aureus* strains. We have also identified *S. aureus* genes and allelic variants, namely, *ssl03*, *ssl7-set1*, *ssl8-set12*, *ssl08*, *ssl9-set5*, *setB3*, *fib*, and *lukY-var1*, which are significantly associated with IE. These IE-associated genes may provide a

framework for further study to investigate the virulence of *S. aureus* strains associated with IE.

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