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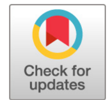
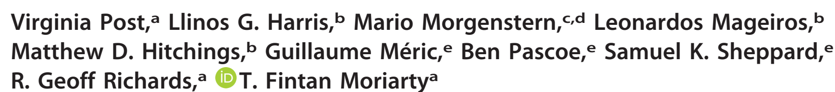


Comparative Genomics Study of *Staphylococcus epidermidis* Isolates from Orthopedic-Device-Related Infections Correlated with Patient Outcome



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ABSTRACT *Staphylococcus epidermidis* has emerged as an important opportunistic pathogen causing orthopedic-device-related infections (ODRI). This study investigated the association of genome variation and phenotypic features of the infecting *S. epidermidis* isolate with the clinical outcome for the infected patient. *S. epidermidis* isolates were collected from 104 patients with ODRI. Their clinical outcomes were evaluated, after an average of 26 months, as either "cured" or "not cured." The isolates were tested for antibiotic susceptibility and biofilm formation. Whole-genome sequencing was performed on all isolates, and genomic variation was related to features associated with "cured" and "not cured." Strong biofilm formation and aminoglycoside resistance were associated with a "not-cured" outcome ($P = 0.031$ and $P < 0.001$, respectively). Based on gene-by-gene analysis, some accessory genes were more prevalent in isolates from the "not-cured" group. These included the biofilm-associated *bhp* gene, the antiseptic resistance *qacA* gene, the cassette chromosome recombinase-encoding genes *ccrA* and *ccrB*, and the IS*256*-like transposase gene. This study identifies biofilm formation and antibiotic resistance as associated with poor outcome in *S. epidermidis* ODRI. Whole-genome sequencing identified specific genes associated with a "not-cured" outcome that should be validated in future studies. (The study has been registered at ClinicalTrials.gov with identifier NCT02640937.)

KEYWORDS *Staphylococcus epidermidis*, MRSE, virulence factors, antibiotic resistance, genotype, phenotype, orthopedic-device-related infections

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Staphylococcus epidermidis is a common member of the human skin microflora, predominant in moist sites such as nares or fossae and in sebaceous areas such as the facial skin. With the advent of implanted and indwelling medical devices, *S. epidermidis* has emerged as a prominent cause of nosocomial and device-associated infections (1, 2). The microorganism's ability to switch from a commensal to pathogenic lifestyle is facilitated by its ability to rapidly attach to, and form biofilms upon, medical devices. In the case of orthopedic-device-related infections (ODRI), *S. epidermidis* accounts for up to 43% of cases and is second only to *Staphylococcus aureus* as the most prevalent causative organism (1, 2).

Molecular epidemiological studies have begun to reveal information on both the population structure and genetic diversity within *S. epidermidis* populations (3–5). The complete *S. epidermidis* genome is estimated at approximately 2.5 Mb and comprises

80% core genes and 20% variable genes (3, 4, 6). Three distinct phylogenetic groups (clades) are evident in the population structure of *S. epidermidis* (3, 4, 6), with at least nine globally disseminated clonal complex (CC) lineages. The most common clonal complex (CC2) contains one particularly prominent sequence type (ST), ST2 (32% of all isolates) (5, 7).

In an attempt to identify the features that enable invasive infection in *S. epidermidis*, a number of studies have searched for features that may distinguish invasive from commensal *S. epidermidis* isolates on genotypic and phenotypic levels. Such studies have identified features such as IS*256*, the folate dehydrogenase gene, and copper remediation genes to be more common among invasive isolates (3, 6, 8, 9). However, clear separation between the two has proven to be difficult (9–11), perhaps indicating that the ability to invade the host and the ability to colonize it do not require significantly different genetically encoded features. One possibility, which has not been explored to date, however, is whether the genome/phenotype of the invasive isolate dictates the ultimate course of an infection, i.e., whether the patient eventually has a successful treatment outcome or a failed treatment outcome.

In the present study, *S. epidermidis* isolates were prospectively collected from patients with ODRI and were assigned a clinical outcome (either “cured” or “not cured”) after an extended patient follow-up (FUP). Clinical outcome was then related to genome variation and phenotypes believed to be important for *S. epidermidis* virulence.

RESULTS

Patient outcome and clinical parameters. A total of 104 patients with *S. epidermidis* ODRI were included in this study; complete demographic information is shown in Table 1. The lower-extremity cohort (70 patients) included only those patients with infection of the hip, knee, and upper ankle joints as well as the femur, tibia, and fibula. The majority of patients of the complete cohort study ($n = 85$, 81.7%) were considered to have had a “cured” clinical outcome at FUP.

Those considered to have a “not-cured” clinical outcome at FUP were statistically more likely to have had multiple revision surgeries in comparison with those with “cured” outcome isolates ($P < 0.067$) (Table 2). There was no association between outcome and any of the other monitored parameters, such as diabetes, chronic immunosuppression, or obesity (Table 2).

Patient outcome and phenotypic properties of isolates. (i) Antibiotic susceptibility. Antibiotic susceptibility testing of the 104 *S. epidermidis* isolates found that 74% (77/104) were multiply resistant isolates and 67.3% (70/104) were resistant to methicillin (Table 1). Rifampin resistance was also observed in 19.2% (20/104) of the isolates, which is notable due to the critical role of this antibiotic in treating ODRI. Resistance to aminoglycosides had a statistically significant influence on a “not-cured” clinical outcome ($P = 0.001$) (Table 3). Further antibiotic resistance (including resistance to aminoglycosides) had no statistically significant influence on any of the other prognostic parameters, such as chronic or acute ODRI. Although isolates from the group of chronic ODRI were more often resistant to aminoglycosides than isolates from the acute-ODRI group (42.7% of versus 31%), this was not statistically significant ($P = 0.276$). Furthermore, multidrug resistance also showed no statistically significant difference between chronic and acute ODRI (73.3% versus 75.9%).

(ii) Biofilm formation. As shown in Table 1, 70.2% (73/104) of the isolates formed a biofilm *in vitro*. The ability to form biofilm was subdivided into weak biofilm formers (37.5% [39/104] of the isolates), intermediate biofilm formers (21.2%, 22/104), and strong biofilm formers (11.5%, 12/104). The remaining isolates (29.8%, 31/104) were unable to form a biofilm under our *in vitro* conditions. A statistically significant association between biofilm-forming ability and clinical “cured” versus “not-cured” outcome was noted for the lower-extremity cohort ($P = 0.031$) (Table 3). Strong biofilm-forming ability also resulted in the highest percentage of “not-cured” outcome for the complete cohort (33.3%, $P = 0.059$) (Table 3). However, the strength of biofilm

TABLE 1 Patient health status, infection characteristics, bacteriology, clinical course, and outcome

Parameter	No. (%) of patients	
	Complete study cohort	Lower-extremity cohort
Total[a]	104 (100.0)	70 (100.0)
Clinical course and infection outcome		
Multiple revision surgeries	89 (85.6)	68 (97.1)
Clinical outcome, cured	85 (81.7)	53 (75.7)
Health status		
Obesity[b]	44 (42.3)	32 (45.7)
Smoking	28 (26.9)	17 (24.3)
Diabetes	14 (13.5)	8 (11.4)
Chronic immunosuppression	25 (24.0)	14 (20.0)
Infection characteristics		
Infection after fracture fixation	78 (75.0)	44 (62.9)
Prosthetic joint infection	26 (25.0)	26 (37.1)
Acute infection	29 (27.9)	20 (28.6)
Closed fracture[c]	53 (67.9)	25 (56.8)
Open fracture	25 (32.1)	19 (43.2)
Type of implant		
Internal fixator	6 (5.8)	0 (0)
Prosthetic joint	26 (25.0)	26 (37.1)
Nail	23 (22.1)	20 (28.6)
Plate	40 (38.5)	22 (31.4)
Screw	8 (7.7)	2 (2.9)
K-wire	1 (1.0)	0 (0)
Localization		
Spine	6 (5.8)	NA[d]
Upper extremity	7 (6.7)	NA
Pelvis	7 (6.7)	NA
Tibia	2 (1.9)	NA
Clavicle	3 (2.9)	NA
Hip joint	10 (9.6)	10 (14.3)
Femur	7 (6.7)	7 (10.0)
Knee joint	18 (17.3)	18 (25.7)
Lower leg, including upper ankle joint	35 (33.7)	35 (50.0)
Lower ankle joint, including foot	9 (8.7)	NA
Bacteriological evaluation		
Methicillin resistance	70 (67.3)	52 (74.3)
Multidrug resistance	77 (74.0)	56 (80.0)
Biofilm formation		
None	31 (29.8)	24 (34.3)
Weak	39 (37.5)	26 (37.1)
Intermediate	22 (21.2)	11 (15.7)
Strong	12 (11.5)	9 (12.9)

[a]Each patient had 1 *S. epidermidis* isolate.
[b]Defined as a BMI of >30.
[c]Prosthetic joint infection not included.
[d]NA, not applicable.

formation had no statistically significant influence on any of the prognostic variables, such as multiple revision surgery.

A description of biofilm-associated genes and their relative presence with respect to *in vitro* biofilm-forming ability is shown in Table 4. Among strongly biofilm-forming isolates, the intercellular adhesion (*icaA*) gene was more prevalent (83.3%) than the accumulation-associated protein-encoding *aap* gene (8.3%), the *bhp* (cell wall-associated biofilm protein) gene (16.7%), and the *embP* (extracellular matrix-binding protein) gene (66.7%).

TABLE 2 Association between prognostic factors and cure status for the complete study cohort

Parameter	No. (%) of patients in: "Not-cured" group	"Cured" group	Odds ratio for cured[a] (95% confidence interval)	P value[b]
Total no. of patients[c]	19 (18.3)	85 (81.7)		
Infection type			0.92 (0.30;2.86)	1.000†
FFI	14 (17.9)	64 (82.1)		
PJI	5 (19.2)	21 (80.8)		
Fracture			0.39 (0.12;1.27)	0.126†
Closed	7 (13.2)	46 (86.8)		
Open	7 (28.0)	18 (72.0)		
Acute infection			1.56 (0.44;7.08)	0.463††
No	15 (20.0)	60 (80.0)		
Yes	4 (13.8)	25 (86.2)		
Multiple revision surgery			0.12 (0.00;2.04)	0.067†
No	0 (0.0)	15 (100.0)		
Yes	19 (21.3)	70 (78.7)		
Obesity			0.78 (0.25;2.42)	0.621††
No	10 (16.7)	50 (83.3)		
Yes	9 (20.5)	35 (79.5)		
Smoking			0.76 (0.23;2.74)	0.613††
No	13 (17.1)	63 (82.9)		
Yes	6 (21.4)	22 (78.6)		
Diabetes			0.50 (0.14;1.81)	0.281†
No	15 (16.7)	75 (83.3)		
Yes	4 (28.6)	10 (71.4)		
Chronic immunosuppression			0.46 (0.16;1.34)	0.233†
No	12 (15.2)	67 (84.8)		
Yes	7 (28.0)	18 (72.0)		

[a]For calculation of odds ratios involving cells with 0 observations, the 0.5 zero-cell correction was applied.
[b]†, chi-square test; ††, Fisher's exact test.
[c]Each patient had 1 *S. epidermidis* isolate.

Patient outcome and pathogen genome variation. (i) Relationship between virulence-associated genes and patient outcome. The 104 genomes were analyzed for the presence of a selection of genes previously described as virulence factors in *S. epidermidis* (Fig. 1) (12–14). Within the population as a whole (i.e., "cured" and "not-cured" outcome isolates), *aae* (vitronectin), *gehC* (lipase), *gehD* (lipase), *hlb* (β-hemolysin), *sesB* (*S. epidermidis* surface protein), and *sesC* (*S. epidermidis* surface protein) were present in all 104 isolates. The methicillin resistance gene *mecA* was carried by 68.3% of the isolates, whereby 69/70 of the phenotypically confirmed methicillin-resistant *S. epidermidis* (MRSE) isolates and 2/34 of the phenotypically confirmed methicillin-susceptible *S. epidermidis* (MSSE) isolates possessed this gene. Figure 1 also shows the distribution of the known virulence genes between the 2 clinical outcome groups ("cured" and "not cured"). A trend for the presence of aminoglycoside resistance gene *aacA* [*aac*(6′)-*aph*(2″) and *mecA*] in the "not-cured" outcome isolates ($P = 0.076$ and $P = 0.099$, respectively) was observed. In addition, the presence of biofilm-associated *bhp* was statistically significantly associated with a "not-cured" clinical outcome in the lower-extremity cohort ($P = 0.023$).

(ii) *agr* types. Overall, accessory gene regulator (*agr*) type I was the most prevalent type (38.5%, 40/104) among the isolates, followed by *agr* type III (36.5%, 38/104). The distribution of the 3 different *agr* types within the "cured" and "not-cured" outcome groups are shown in Table 5; however, there were no statistically significant differences

TABLE 3 Association between bacterial phenotype and clinical cured status

Parameter	Complete cohort (n = 104)				Lower-extremity cohort (n = 70)			
	No. (%) of patients in:		Odds ratio for cured (95% confidence interval)	*P* value[a]	No. (%) of patients in:		Odds ratio for cured (95% confidence interval)	*P* value[a]
	"Not-cured" group	"Cured" group			"Not-cured" group	"Cured" group		
Biofilm formation				0.059†††				0.031†††
None	3 (9.7)	28 (90.3)			3 (12.5)	21 (87.5)		
Weak	7 (17.9)	32 (82.1)	0.49 (0.08;2.43)		6 (23.1)	20 (76.9)	0.48 (0.07;2.64)	
Intermediate	5 (22.7)	17 (77.3)	0.36 (0.05;2.19)		4 (36.4)	7 (63.6)	0.25 (0.03;1.96)	
Strong	4 (33.3)	8 (66.7)	0.21 (0.03;1.62)		4 (44.4)	5 (55.6)	0.18 (0.02;1.51)	
Antibiotic resistance[b]								
Methicillin			0.33 (0.06;1.29)	0.082††			0.54 (0.14;2.16)	0.529†
No	3 (8.8)	31 (91.2)			3 (16.7)	15 (83.3)		
Yes	16 (22.9)	54 (77.1)			14 (26.9)	38 (73.1)		
Aminoglycosides			0.17 (0.04;0.56)	<.001††				0.051††
No	5 (7.9)	58 (92.1)			5 (14.3)	30 (85.7)	0.32 (0.08;1.17)	
Yes	14 (34.1)	27 (65.9)			12 (34.3)	23 (65.7)		

[a]†, chi-square test; ††, Fisher's exact test; †††, Cochran-Armitage trend test.
[b]Not all antibiotic resistances are listed. Others tested showed no statistical significance.

($P \geq 0.05$). The only parameter associated with *agr* type was acute infection (Table 5). All other clinical parameters were not statistically associated with *agr* type.

(iii) MLST. Within the 104 isolates, 21 different sequence types (STs) were identified based on the 7-locus scheme for *S. epidermidis* (15) using the build-in multilocus sequence typing (MLST) function of BIGSdb linked with PubMLST databases (4) (Table 6). Thirty isolates could not be assigned to any known ST. While the majority of the ST2 (13/18, 72.2%) and ST5 (16/18, 88.9%) isolates were associated with a "cured" outcome, all ST57 (2/2, 100%), ST89 (1/1, 100%), and S110 (1/1, 100%) isolates were associated with a "not-cured" outcome (Table 6). However, more isolates would be needed to draw statistical conclusions. The identified STs belonged to previously described 7-locus MLST clonal complexes (CCs), of which the largest was CC2 (65/104, 62.5%) (4) (Table 6).

Patient outcome and accessory genomes. Further evaluation of the relative presence of accessory genes that were more prevalent in the "not-cured" outcome group than in the "cured" outcome group is shown in Table 7. *S. epidermidis* isolates from the "not-cured" outcome group carried the antiseptic resistance-coding gene *qacA* at a statistically significant higher percentage than isolates from the "cured" outcome group (89.5% versus 27.1%; $P = 0.023$). Furthermore, the presence of the cassette chromosome recombinase-encoding genes *ccrA* and *ccrB* (89.5% versus 23.6% and 89.5% versus 24.7%; $P = 0.042$ and $P = 0.034$, respectively) was significantly associated with the "not-cured" isolate genomes.

Core and accessory genome analysis. A pan-genome of all study isolates was used to compare the genomes of the 104 clinical *S. epidermidis* isolates. ClonalFrame was used to construct ancestral genealogies, free from recombination. In order to run ClonalFrame, a stringent approach to selecting core genes based on presence in 100% of the 104 isolates was applied. This resulted in a reduced core genome consisting of

TABLE 4 Biofilm-associated genes and biofilm formation

Biofilm-associated gene	No. (%) of isolates with gene and the indicated biofilm strength *in vitro*							
	Complete cohort study (n = 104)				Lower-extremity cohort study (n = 70)			
	None (n = 31)	Weak (n = 39)	Intermediate (n = 22)	Strong (n = 12)	None (n = 24)	Weak (n = 26)	Intermediate (n = 11)	Strong (n = 9)
icaA	7 (22.6)	7 (18.0)	8 (36.4)	10 (83.3)	7 (29.2)	5 (19.2)	6 (54.5)	8 (88.9)
aap	1 (3.2)	0 (0)	0 (0)	1 (8.3)	1 (4.2)	0 (0)	0 (0)	0 (0)
bhp	7 (22.6)	10 (25.7)	0 (0)	2 (16.7)	4 (16.7)	9 (34.6)	0 (0)	1 (11.1)
embP	28 (90.3)	31 (79.5)	15 (68.2)	8 (66.7)	21 (87.5)	20 (76.9)	6 (54.5)	6 (66.7)

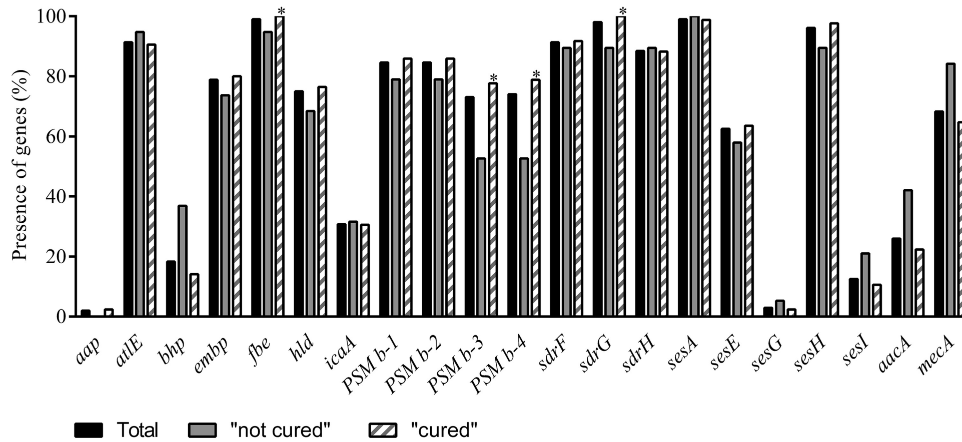

FIG 1 Percentages of genes present in the whole collection (black bars) and present in the two outcome groups. Hatched bars, "cured" outcome; gray bars, "not-cured" outcome. *, statistically significant ($P \leq 0.05$).

123 nontruncated genes. *S. epidermidis* isolates clustered into 3 clades (Fig. 2), with 86% of isolates (89/104) in clade A, 9.6% (10/104 isolates) in clade B, and 4.8% (5/104 isolates) in clade C (Fig. 2A).

Comparing patient outcome between the clades, a trend between clades A and B was observed. Clade B consisted of a comparatively higher percentage of "not-cured" outcome isolates (40%, 4/10) than clade A (15.7%, 14/89) (Fig. 2A and C). However, this trend did not quite reach statistical significance ($P = 0.08$; Fisher's exact test). Only 1/5 (20%) clade C isolates belonged to the "not-cured" outcome group, although the low numbers of isolates precluded reliable statistical analysis (Fig. 2B and D). Furthermore, clade B isolates also contained a higher percentage of moderately/strongly biofilm-forming isolates than clade A isolates (40% versus 31.5%) (Fig. 3A and C). In addition, the majority of clade B isolates (80%) belonged to CC2, while clade A possessed only 60.7% CC2 isolates (data not shown).

Clinical outcome, biofilm formation, and antibiotic resistance phenotypes were homogeneous compared to the clonal frame ($P \geq 0.05$). In addition, the permutation test revealed a strong association between lineage and biofilm formation ($P \leq 0.0001$) and resistance to methicillin ($P = 0.0002$), quinolones ($P = 0.0055$), erythromycin ($P < 0.00001$), clindamycin ($P < 0.00001$), tetracycline ($P < 0.00001$), trimethoprim-sulfonamide ($P = 0.02$), and fusidic acid ($P < 0.00001$). However, there was no association between lineage and outcome ($P = 0.09$) or resistance to penicillin ($P = 1$), aminoglycosides ($P = 0.3798$), fosfomycin ($P = 0.053$), and rifampin ($P = 0.151$).

DISCUSSION

S. epidermidis is a commensal microorganism that is also a frequent agent of ODRI (6, 8, 16, 17). However, little is known about the impact that genotypic and phenotypic features of the infecting pathogen can have on treatment outcome (3, 6, 8, 9). This

TABLE 5 Association between the *agr* types and clinical outcome

Parameter	No. (%) of isolates with *agr* type[a]: I	II	III	*P* value[b]
Clinical outcome				0.946
Not cured	7 (36.8)	5 (26.3)	7 (36.8)	
Cured	33 (39.8)	19 (22.9)	31 (37.3)	
Infection				0.002
Nonacute (chronic)	26 (35.1)	13 (17.6)	35 (47.3)	
Acute	14 (50.0)	11 (39.3)	3 (10.7)	

[a]Two isolates not belonging to any of the 3 *agr* groups were excluded for statistical reasons.
[b]Chi-square test.

TABLE 6 MLST of the 104 clinical *S. epidermidis* isolates

ST[a]	CC[b]	No. (%) of isolates		
		Total	"Cured" group	"Not-cured" group
2	2	18 (17.3)	13 (72.2)	5 (27.8)
5	2	18 (17.3)	16 (88.9)	2 (11.1)
7	2	1 (1.0)	1 (100)	0 (0)
23	2	4 (3.9)	4 (100)	0 (0)
57	2	2 (1.9)	0 (0)	2 (100)
59	2	6 (5.8)	5 (83.3)	1 (16.7)
73	2	1 (1.0)	1 (100)	0 (0)
83	2	1 (1.0)	1 (100)	0 (0)
87	2	4 (3.9)	2 (50)	2 (50)
88	2	1 (1.0)	1 (100)	0 (0)
89	2	1 (1.0)	0 (0)	1 (100)
130	2	6 (5.8)	6 (100)	0 (0)
184	2	1 (1.0)	1 (100)	0 (0)
384	2	1 (1.0)	1 (100)	0 (0)
19	147	2 (1.9)	2 (100)	0 (0)
32	S32	2 (1.9)	2 (100)	0 (0)
110	S110	1 (1.0)	0 (0)	1 (100)
167	S167	1 (1.0)	1 (100)	0 (0)
297	S297	1 (1.0)	1 (100)	0 (0)
490	S490	1 (1.0)	1 (100)	0 (0)
528	S528	1 (1.0)	1 (100)	0 (0)
NA[c]	NA	30 (28.9)	25 (83.3)	5 (16.7)
Total		104 (100)	85	19

[a]Sequence types determined using the built-in MLST function of BIGSdb, linked with PubMLST databases.
[b]Clonal complexes were obtained from previously described data (4).
[c]NA, possible truncation of a corresponding MLST locus at the end of a contig.

prospective study was designed to test the hypothesis that treatment outcome in patients with *S. epidermidis* ODRI may be influenced by phenotypic or genotypic features of the infecting pathogen. Against a background of scientific studies searching for features that distinguish commensal from invasive isolates (3, 6, 8–11), or for host factors that have an influence on patient outcome (8, 18), this study advances this line of investigation by looking for bacteria-retained features that distinguish infections that result in poor treatment outcome. After prospectively collecting 104 patients, with an average 2-year follow-up (FUP), and subjecting infecting pathogens to genome sequencing and a number of phenotypic assays, we have identified a number of features associated with poor treatment outcome. Those features include biofilm formation,

TABLE 7 Relative overrepresentation (>20% difference) of accessory genes in the "not-cured" outcome isolates

Locus	Description of product	"Not cured" group ($n = 19$)		"Cured" group ($n = 85$)		Difference (%) between "not cured" and "cured" groups	*P* value
		No. of isolates	Prevalence (%)	No. of isolates	Prevalence (%)		
SERP0915	IS*256*-like transposase	11	57.9	31	36.5	21.4	0.085
SERP1222	Transposase	9	47.4	16	18.8	28.5	0.008
SERP1586	Acetyltransferase, GNAT family	9	47.4	22	25.8	21.5	0.064
SERP2498	Cassette chromosome recombinase A (CcrA)	17	89.5	56	65.8	23.6	0.042
SERP2499	Cassette chromosome recombinase B (CcrB)	17	89.5	55	64.7	24.7	0.034
id1043_1239	Hypothetical protein	10	52.6	25	29.4	23.3	0.053
id1044_0888	Phage protein	8	42.1	15	17.6	24.5	0.02
id1044_0895	Phage antirepressor protein	8	42.1	17	20	22.1	0.041
id1044_1909	Antiseptic resistance protein QacA	17	89.5	53	62.4	27.1	0.023
id1044_2610	Unknown	8	42.1	15	17.6	24.5	0.02
id1048_0369	Replication-associated protein	11	57.9	29	34.1	23.7	0.054
id1632_0817	Zn-dependent hydroxyacylglutathione hydrolase	14	73.7	44	51.7	21.9	0.082

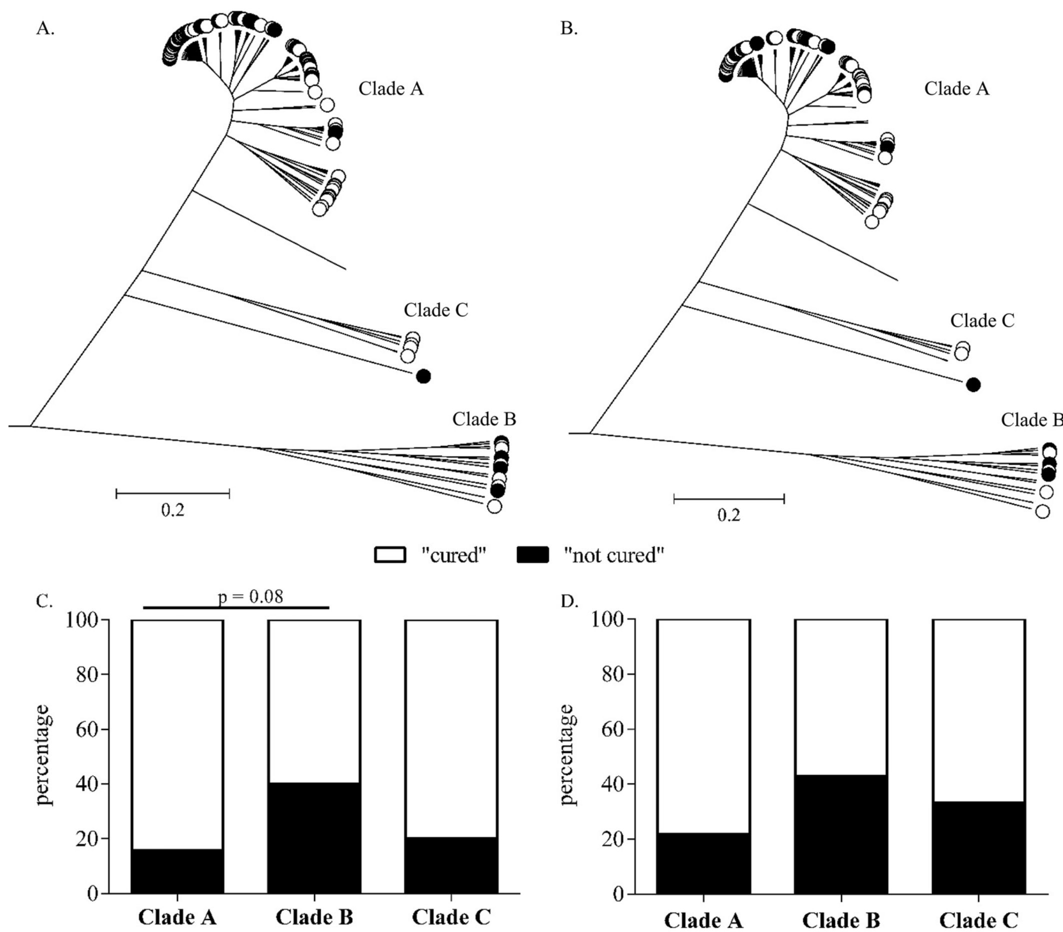

FIG 2 Population structure of *S. epidermidis* isolates constructed from 123 core genes and implemented in ClonalFrame. (A and B) All 104 isolates of the complete cohort (A) and all 70 isolates of the lower-extremity cohort (B) are labeled according to the clinical follow-up (FUP) outcome: "not cured" (black circles) or "cured" (white circles). (C and D) Percent distributions of "cured" and "not-cured" outcome in the three clades A, B, and C, showing the complete cohort (C) and the lower extremity cohort (D).

aminoglycoside resistance, the cassette chromosome recombinase encoding genes *ccrA* and *ccrB*, IS*256-like* and plasmid-borne *qacA* gene, as well as the biofilm-associated *bhp* gene.

Adhesion to and biofilm formation upon biomaterial substrates are widely believed to be the primary virulence factor enabling invasive *S. epidermidis* ODRI (6, 8, 16, 17, 19). The data from our study supports this by revealing that a "not-cured" clinical outcome was significantly associated with an increased ability to form biofilm *in vitro* ($P = 0.031$). Genomic analysis on the known biofilm-associated genes, such as *icaA*, *aap*, *bhp*, or *embP*, revealed that the only such gene found to be significantly associated with a "not-cured" outcome was *bhp* in the lower-extremity cohort ($P = 0.023$). Interestingly, *bhp* was most prevalent in the weakly biofilm-forming isolates (52.7%) indicating that its role may not be directly linked with biofilm-forming ability, at least *in vitro*. *bhp* has been reported to promote primary attachment to abiotic surfaces as well as intercellular adhesion during biofilm formation (20, 21). Thus, this protein might be important for rapid attachment to the implant rather than the amount of biofilm formed by the isolate *per se*. A rapid attachment clearly may be significant for early establishment of biofilm *in vivo* in "the race for the surface." This may partially explain its association with poor treatment outcome, despite the lack of association with *in vitro* biofilm-forming ability.

Antibiotic resistance is a second key challenge in treatment of ODRI. Previous studies have suggested that methicillin resistance is associated with a worse treatment

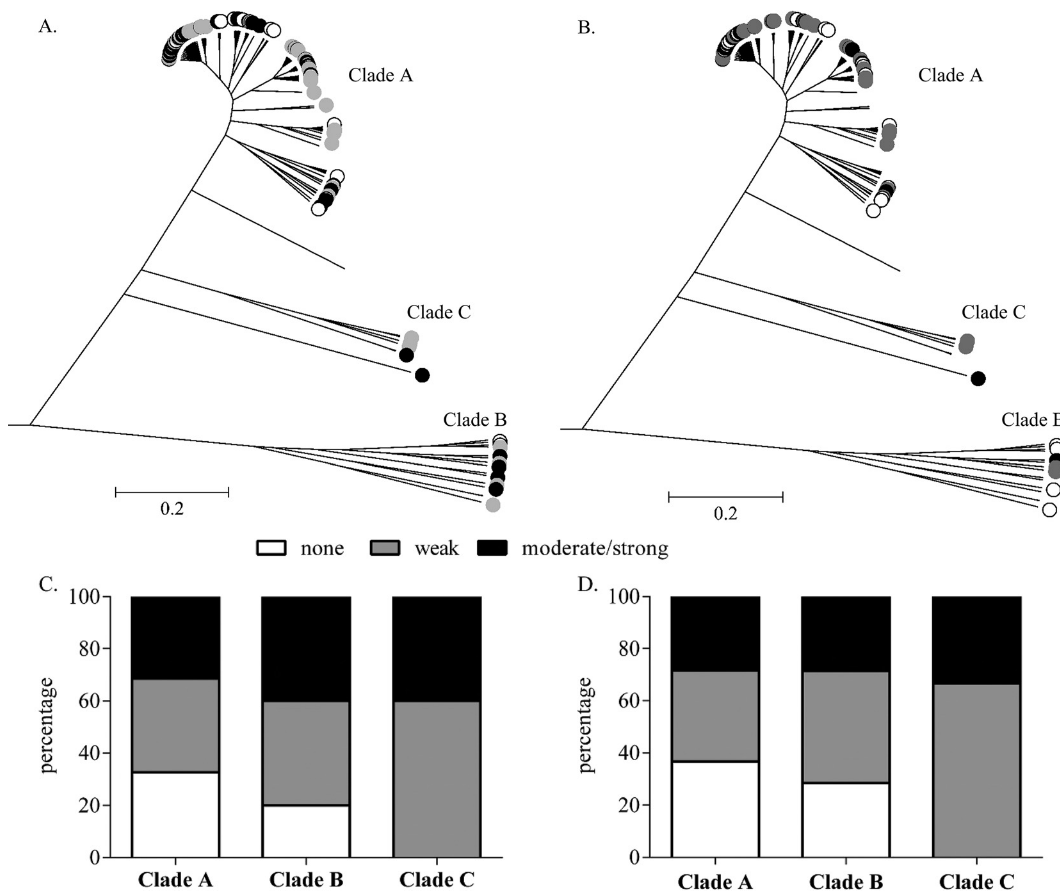

FIG 3 Population structure of *S. epidermidis* isolates constructed from 123 core genes and implemented in ClonalFrame. (A and B) All 104 isolates of the complete cohort (A) and of the lower-extremity cohort (B) are labeled according to the ability to form a biofilm: biofilm negative (white circles), weak biofilm formers (gray circles), and moderate to strong biofilm formers (black circles). (C and D) Percent distribution of the strength of biofilm formation in the three clades A, B, and C, showing the complete cohort (C) and the lower-extremity cohort (D).

outcome in staphylococcal ODRI (22–24), although a number of studies have provided contrasting findings (8, 25, 26). Methicillin resistance is due to the *mecA* gene. In the present study, resistance to methicillin showed a trend for a "not-cured" patient outcome ($P = 0.082$ based on phenotypic analysis and $P = 0.099$ for presence of the *mecA* gene), supporting previously reported trends (22–24). Furthermore, the chromosome recombinase A- and B-encoding genes *ccrB* and *ccrA* were significantly more prevalent in "not-cured" clinical-outcome isolates (89.5% versus 65.8% and 89.5% versus 64.7%; $P = 0.042$ and $P = 0.034$, respectively). These two genes are responsible for the chromosomal insertion of the genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*). The SCC*mec* mobile genetic island contains the *mec* gene complex, including the methicillin resistance gene *mecA*. In this study, 91.8% of the *ccrA/ccrB*-positive isolates possessed the *mecA* gene, indicative for the presence of the mobile element SCC*mec*. Of those 67 *ccrA/ccrB/mecA*-positive isolates, only 2 were not phenotypically resistant to methicillin, which might be due to mutations in the *mecA* gene. The 6 *ccrA/ccrB*-positive but *mecA*-negative isolates were not phenotypically resistant to methicillin, indicative of an absent SCC*mec* mobile element.

A second antibiotic class pertinent to the treatment of ODRI is the aminoglycosides (including gentamicin and tobramycin), which are commonly used in antibiotic-loaded bone cement (2, 16, 19, 27). Resistance to aminoglycosides in *S. epidermidis* isolated from patients with ODRI typically ranges from 40 to 65% (19, 27). In this study, 39.4% of the isolates were resistant to gentamicin/aminoglycoside, and we observed an

association between the "not-cured" outcome and isolates being phenotypically resistant to aminoglycosides ($P = 0.001$). The majority of the aminoglycoside-resistant isolates (65.8%) carried the *aacA* [*aac*(*6′*)-*aph*(*2″*)] gene, which confers resistance to all aminoglycosides. This gene was also observed in a higher prevalence in the "not-cured" group (42.1%, versus 22.4% in "cured"; $P = 0.076$). This correlates well with other studies in terms of prevalence of the *aacA* [*aac*(*6′*)-*aph*(*2″*)] gene among aminoglycoside-resistant isolates, ranging between 40 and 92% (16, 28, 29), although how this impacted treatment outcome was not described in these other studies.

Our data also revealed that the antiseptic (quaternary ammonium compound) *qacA* gene was statistically more prevalent in the "not-cured" outcome group ($P = 0.023$). The *qacA* gene is a plasmid-borne gene (pSK1 family plasmids) that confers resistance to antiseptics and disinfectants such as cetrimide, benzalkonium chloride, and chlorhexidine (30–32). Our observation that the *qacA* gene was present in 67.3% of isolates (89.5% of "not-cured" group isolates) within the complete cohort seems enriched compared to that in other studies for clinical (47% to 52%) and commensal (25%) *S. epidermidis* isolates (31, 32). Despite the relatively high presence in our collection and a moderate number of "not-cured" isolates, there is some statistical significance to associate the presence of this gene with a poor treatment outcome. Qac proteins are efflux pumps that protect bacteria not only from a variety of toxic substances but also from fluoroquinolones and β-lactams (30–32). The acquisition of such a gene/plasmid, possibly from antiseptic usage within the hospital, clearly provides the bacteria a survival advantage, especially in a clinical environment. Such resistant pathogens therefore not only are more difficult to clean within the hospital environment but, as we show, also are associated with a poor treatment outcome.

In addition, the IS*256*-like transposase was more frequently present in "not-cured" clinical-outcome isolates than in "cured" outcome isolates (57.9% versus 36.5%; $P = 0.085$). Previous studies have described an association between the presence of the IS*256* element, the *aac*(*6′*)-*aph*(2[dprime]) gene (33, 34), the *icaADBC* operon, and the ability to form biofilm (11, 35, 36). Furthermore, IS*256* has been suggested as a molecular marker for the molecular typing and identification of nosocomial, invasive *S. epidermidis* isolates (9–11, 36). This study provides further evidence that IS*256* not only is "enriched" within invasive isolates but is also more prevalent in isolates with a poor treatment outcome. The increased prevalence in the "not-cured" group indicates that it is not a marker for infection but rather potentially is one for poor outcome; however, this warrants further study with a larger set of isolates.

Previous genealogical reconstruction studies of *S. epidermidis* have shown that isolates clustered into 3 phylogenetic clades (4, 6), which is consistent with the observation in this study. To date, no study has associated genotypes with clinical outcomes in ODRI. In this study, a higher number of "not-cured" outcome isolates were found in clade B than in clades A and C. Clade B was also the lineage with the strongest biofilm-forming isolates. Harris et al. reported thick biofilm being 20% more common in CC2 isolates (6). In the present study, CC2 accounted for 80% of the 10 clade B isolates, with 50% of them being responsible for moderate/strong biofilm formation. Furthermore, of these moderately/strongly biofilm-forming CC2 clade B isolates, 75% (3/4) resulted in a "not-cured" outcome. This emphasizes that clade B CC2 isolates might be more likely to result in a poor clinical outcome. However, a greater number of isolates should be analyzed in a prospective manner in order to confirm this observation and determine whether it may be a prognostic molecular marker for poor treatment outcome.

A limitation of this study was that only a single *S. epidermidis* colony from each patient was analyzed, although the infection could, at least in theory, be polyclonal. A previous study has shown that only a minority of prosthetic joint infections (PJIs) (28.5% [4/14 patients]) were due to polyclonal *S. epidermidis* strains (37). Any future studies should consider analyzing several colonies from each patient. Furthermore, the morphology of colonies was not recorded in the present study, and so we do not know how

many, if any, small-colony variants (SCVs) were present in the current collection, but this should be considered in future studies.

In general, SCVs present phenotypic features such as low growth rate and small colony morphology (38–45). Additionally, SCVs are associated with increased biofilm-forming ability, antibiotic resistance, and ability to internalize and persist in osteoblasts, all of which may contribute to prolonged treatment or even treatment failure (38–45). In contrast to the case for *S. aureus* SCVs, very little information is available on *S. epidermidis* SCVs (38, 46). Only recently has the pathogenesis of PJIs been associated with *S. epidermidis* SCVs (38, 46, 47). Furthermore, SCV colonies from the same patient showed difference in growth rate, colony size, and levels of gentamicin resistance compared to each other (38). This highlights the importance of documenting and analyzing SCVs, as they may influence treatment outcome.

Patients with a "not-cured" clinical outcome were more likely to have had multiple revision surgeries than those with a with "cured" outcome ($P < 0.067$), which is to be expected as revision surgery is a standard intervention for failed treatment We have considered the final outcome to be "cured" or "not cured" at follow-up, regardless of the treatment steps taken in the interim period. Therefore, even though multiple revision surgeries occurred, if the patient was free of infection at FUP, we considered the patient to be cured. Of course, the need for multiple revisions is possibly an indicator that the infection was a greater challenge to treat; however, in a large patient population such as this, there is often a need for multiple revision surgeries to advance the healing of the fracture or replace the device, which may occur after infection has cleared, and so such patients have also had multiple surgeries.

Overall, genome sequencing is not absolutely required to determine some of the features identified in this study as being associated with poor outcome. For example, routine antibiotic susceptibility testing and conventional *in vitro* biofilm assays are readily available to provide this information. Nevertheless, whole-genome sequencing allowed us to test our hypothesis with greatest sensitivity and also identified features that are less easily measurable in a clinical laboratory. Finally, it should be mentioned that the treatment of ODRI is achieved by antibiotic therapy and surgical removal of infected tissue. Therefore, the outcome of ODRI treatment will be influenced by these factors in addition to the host defenses and not solely by the pathogen itself. The factors identified in this study therefore require prospective validation in further studies with larger patient cohorts in order to confirm their value as prognostic markers for ODRI treatment outcome.

MATERIALS AND METHODS

Ethics statement. Institutional Review Board approval was obtained from the local ethical committee Ethik-Kommission der Bayerischen Landesärztekammer under approval number 12063. The study was registered with https://clinicaltrials.gov with identifier NCT02640937. Only adult patients (>18 years) were included in this study, and all patients provided informed written consent prior to inclusion in the study.

***Staphylococcus epidermidis* collection.** This was a prospective study performed between November 2011 and September 2013 at the BGU Murnau, Germany, a level-one trauma center with a high volume, 70-bed unit for septic and reconstructive surgery. The phenotypic investigation of biofilm formation by a subgroup of these isolates has been previously described (8), although no genome sequence data for these isolates have been previously published.

Inclusion criteria were treatment for a confirmed *S. epidermidis* infection involving fracture fixation (FFI) or prosthetic joint infections (PJIs). Most of the primary surgeries for fracture fixation or implantation of an endoprosthesis were performed in other hospitals. In cases where the patient developed an infection that was not treated/treatable at the primary center, the patient was transferred to the study site which has a specialized unit for ODRI treatment. Bacterial growth in at least two biopsy specimens collected at the site of interest in combination with nonunion, implant loosening/failure, or local and systemic signs suggesting a surgical site infection were requirements for the diagnosis of ODRI, as per hospital standards.

In the previously described clinical study, patient data were analyzed as a complete study cohort but also as a cohort including only patients with infections associated with the lower limb (8). This is because there are numerous outcome measures for the lower extremity that are not available for other anatomical locations. These outcome measures include the lower-extremity functional score (LEFS) and the short form 12 (SF-12) score, as well as leg length discrepancy (8). The remaining patients, not included in the lower-extremity cohort, included patients with infections at other locations, such as

upper extremity, pelvis, and spine (Table 1). At the first surgical procedure after enrollment, bone biopsy specimens were taken from the interface between implant and affected bone. Samples were placed in a sterile container with thioglycolate liquid medium (bioMérieux, Hazelwood, MO, USA) and cultured for 10 days at 37°C. Any growth was inoculated onto a blood agar plate (bioMérieux, Hazelwood, MO, USA) for further growth and subsequent identification. All isolates were grown on tryptone soy agar (TSA) (Oxoid, Pratteln, Switzerland) and incubated overnight at 37°C. A single colony was then taken and resuspended in 1 ml tryptone soy broth (TSB) (Oxoid, Pratteln, Switzerland) containing 20% (vol/vol) glycerol for long-term storage at −80°C. Although the colony morphology of culture-positive samples was not described, we anticipate that SCV colonies had sufficient time to emerge under standard laboratory conditions and are not likely to have been missed in the clinical routine.

Clinical data collection. Clinical data were collected from each enrolled patient. The following surgical parameters were documented: affected bone or joint, type of implant, time between implantation of the device and onset of symptoms, and whether the fracture was open or closed (PJIs excluded).

Patients were assessed for treatment outcome after an average of 26 months of follow-up (FUP). Patients were assigned to have had a "cured" or a "not-cured" outcome at FUP. Patients had a "cured" clinical outcome if they were free of infection, surgical therapy and systemic antibiotic therapy had ceased, and function of the affected joint or limb was restored. Patients were considered to have had a "not-cured" clinical outcome if at least one of the above parameters was negative. Additional parameters were documented, such as acute/nonacute (chronic) infection (cutoff for onset of symptoms, 6 weeks), obesity (body mass index [BMI] of $\geq$30 kg/m^2), diabetes, smoking, chronic immunosuppressive conditions (diabetes mellitus, chronic alcoholism, Child's class C cirrhosis, neoplasia, transplantation, AIDS, and steroid medication), and whether multiple revision surgeries were required during treatment.

The clinical treatment strategies applied to these patients followed recent guidelines and recommendations, including guidance on antimicrobial stewardship. Therefore, treatment strategies differed between enrolled patients due to antibiotic resistance patterns, presence of implant (yes/no), and stage of treatment. The use of antibiotic-loaded bone cement was not extracted from the patient records; however, in all cases of infection with a gentamicin-resistant organism, any bone cement would have been loaded with vancomycin as the preferred alternative. Whether an implant was removed or retained was dependent upon the classification of the infection and the health status of the patient. In chronic infections, the implant was routinely removed in the first revision surgery whenever possible. In general, the implant was retained in acute infections if sufficient debridement was possible.

Antibiotic susceptibility testing. Susceptibility to 28 antibiotics was determined using a Vitek2 machine (bioMérieux Vitek Inc., Hazelwood, MO, USA). The antibiotics tested were amikacin, ampicillin-sulbactam, cefotaxime, cefoxitin, cefuroxime, ciprofloxacin, clindamycin, daptomycin, erythromycin, fosfomycin, fusidic acid, gentamicin, levofloxacin, linezolid, mezlocillin, moxifloxacin, netilmicin, ofloxacin, oxacillin, penicillin, piperacillin, rifampin, tetracycline, ticarcillin-clavulanate, tigecycline, tobramycin, trimethoprim-sulfamethoxazole, and vancomycin. Multiple antibiotic resistance was defined according to the definitions of the European Committee of Antimicrobial Susceptibility Testing (EUCAST). Oxacillin resistance was considered definitive for methicillin resistance status.

Biofilm formation. Biofilm formation was assayed as described previously (48, 49). Briefly, overnight cultures were grown in TSB and then subcultured in fresh TSB containing 1% glucose to approximately 1×10^6 CFU/ml. To achieve this, the bacterial density was adjusted to a target optical density (OD) of known concentration using a Multiskan Go microplate reader (Thermo Scientific, Zürich, Switzerland). A total of 200 μl of the bacterial suspension was incubated in flat-bottom 96-well tissue culture-treated polystyrene microtiter plates (Nuclon; Nunc A/S, Denmark) for 24 h at 37°C. Plates were rinsed with phosphate-buffered saline (PBS) (Sigma-Aldrich, Buchs, Switzerland) and stained with 150 μl of Gram's crystal violet solution (Sigma-Aldrich, Buchs, Switzerland). The dye bound to the attached cells was solubilized by addition of 150 μl of 95% ethanol. Optical density was measured as absorbance at 595 nm using the Multiskan Go microplate reader.

All isolates were tested in triplicate in three independent experiments. Each microtiter plate also consisted of negative controls (wells without bacterial inoculation). The average OD value (OD_a) was calculated for each isolate and the negative control. The results were evaluated using the scale described by Stepanovic et al. (49), whereby isolates may fall into the following four categories: biofilm nonproducer, weak biofilm producer, intermediate biofilm producer, and strong biofilm producer. Based on the OD_a values and the cutoff value (OD_c), which is defined as three standard deviations (SD) above the mean OD of the negative control, OD_c = average OD of negative control + (3 × SD of negative control). The strength of the biofilm production of each isolate was calculated as follows: $OD_a \leq OD_c$ = biofilm nonproducer, $OD_c < OD_a \leq 2 \times OD_c$ = weak biofilm producer, $2 \times OD_c < OD_a \leq 4 \times OD_c$ = intermediate biofilm producer, and $4 \times OD_c < OD_a$ = strong biofilm producer. *S. epidermidis* reference strain RP12 (ATCC 35983) was used as a control for strong biofilm production.

Genome sequencing and assembly. *S. epidermidis* isolates were cultured on TSA plates at 37°C for 24 h. Single-colony cultures were harvested, resuspended in 3 ml of TSB medium to minimize clumping, and incubated at 37°C with overnight shaking. Chromosomal DNA was extracted using a Qiagen QIAamp DNA minikit (Qiagen, Hilden, Germany) following the manufacturer's protocol, using 1 μg/ml lysostaphin (Sigma-Aldrich, Buchs, Switzerland) and 2 μg/ml lysozyme (Sigma-Aldrich, Buchs, Switzerland) to facilitate cell lysis. DNA was sequenced at the Swansea University Genome Centre using a MiSeq benchtop sequencer (Illumina, San Diego, CA, USA). Sequencing libraries were prepared using Nextera XT library preparation kits (v2) and paired-end 250-bp reads generated with the MiSeq run kit (v2). Short-read data were assembled using a *de novo* assembly algorithm within Velvet software (version 1.2.08) (50). Overall,

the average number of contiguous sequences (contigs) for all 104 genomes sequenced in this study was 439, which gave rise to an average total assembled genome size of 2,436,856 bp.

Genomes are archived using a gene-by-gene approach for genome alignment and comparison supported by the BLAST algorithm (51). A reference pan-genome was constructed from the clinical isolate genomes (all collected as part of this study) and the reference *S. epidermidis* RP62A (ATCC 35984) and ATCC 12228 genomes (52). Putative gene function was assigned to genes in the reference pan-genome list using RAST (Rapid Annotations using Subsystem Technology) (53) and the SEED database (54), which were cross-referenced with the *S. epidermidis* RP62A (ATCC 35984) and ATCC 12228 reference genomes before removing duplicate genes. The BLAST algorithm was used to scan all genomes for gene orthologs at each locus in the reference pan-genome. An ortholog was defined as a reciprocal best hit of the sequence with >70% nucleotide identity over at least 50% of the alignment length. MAFFT software (55) was used to align gene orthologs on a gene-by-gene basis, and these data were concatenated into contiguous sequence for each isolate genome, including gaps. A core genome of 123 genes was defined based on gene presence in all isolates (100%).

Estimating genealogies. ClonalFrame infers the clonal relationship of bacteria and the chromosomal position of homologous recombination events that disrupt a clonal pattern of inheritance (56). A stringent approach was used to estimate a reduced core genome for construction of a genealogy using ClonalFrame (version 1.2) on concatenated sequences of 104 *S. epidermidis* genomes with 100,000 iterations, half of which were discarded as burn-in. Substitution mutation and recombination regions were categorized from the output of ClonalFrame. The posterior probability of recombination and substitution at each site is calculated by ClonalFrame, and recombination events were defined with a probability of recombination of more than 75%, reaching 95% at any one site. The trees were visualized and annotated using MEGA6 (57).

Statistical analysis. Associations among and between the clinical parameters, bacterial phenotypes, clades, and presence/absence of genes were analyzed statistically using the chi-square test, Fisher's exact test, the Cochran-Armitage trend test, or the Kruskal-Wallis test as appropriate. The chi-square test was carried out to test the null hypothesis that the lineages are homogenous in their clinical outcome or resistance phenotypes. Permutation tests were performed to test the null hypothesis that there was no association between lineage and clinical outcome or resistance phenotype. Association between clinical outcome or antimicrobial resistance and lineage in the observed data was summarized by an association score. Statistical analyses were performed using SAS software (version 9.2; SAS, Cary, NC, USA) and SPSS (version 10; IBM, USA), and the level of significance was set at a *P* value of $\leq$0.05.

Data availability. Short reads are available from the NCBI Sequence Read Archive (SRA) associated with BioProject no. PRJNA382527. Assembled genomes are also archived in the publicly accessible Staphylococcal Bacterial Isolate Genome Sequence database (BIGSdb) (https://sheppardlab.com/resources/).

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