MAJOR ARTICLE

Nasal Carriage as a Source of *agr*-Defective *Staphylococcus aureus* Bacteremia

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Inactivating mutations in the *Staphylococcus aureus* virulence regulator *agr* are associated with worse outcomes in bacteremic patients. However, whether *agr* dysfunction is primarily a cause or a consequence of early bacteremia is unknown. Analysis of 158 paired *S. aureus* clones from blood and nasal carriage sites in individual patients revealed that recovery of an *agr*-defective mutant from blood was usually predicted by the *agr* functionality of carriage isolates. Many *agr*-positive blood isolates produced low levels of hemolytic toxins, but levels were similar to those of colonizing strains within patients, suggesting that introduction into the blood did not select for mutations with minor functional effects. Evidently, the transition from commensalism to opportunism in *S. aureus* does not require full virulence in hospitalized patients. Furthermore, *agr*-defective mutants were found in uninfected nasal carriers in the same proportion as in carriers who develop bacteremia, suggesting low correlation between virulence and infectivity.

Primary control of the staphylococcal virulon is mediated by the accessory gene regulator (*agr*) quorum-sensing system [1]. *agr*-defective mutants are attenuated for virulence in animal models of acute infection, and agents that block *agr* and quorum-sensing exhibit antiinfective properties [2]. However, *agr*-defective mutants are frequently recovered from patients with bacteremia, where the mutants are associated with persistent infection and poor outcome [3, 4]. The frequent recovery of *agr*− and mixtures of *agr*+ and *agr*− phenotypes from patients supports the idea that *agr* variation is selected for in vivo. However, it is unknown whether and to what extent loss-of-function mutations arise during the initial invasive stage of blood infection in human hosts.

In the present work, we sought to determine the frequency of a within-host shift in *agr* functionality during infection by screening for mutational changes in the *agr* locus among 158 *Staphylococcus aureus* clone pairs obtained from blood and nasal sites of individual patients [5]. Additionally, when a clonal *S. aureus* isolate was recovered from the clinically presumed focus of infection, strains were characterized to determine their role as a reservoir for *agr*-defective mutants. To address the directionality of colonization and infection, we included in the analysis carriage strains isolated both before and after the detection of bacteremia. Finally, to determine whether fully virulent, *agr*+ strains were more likely to cause invasive disease than *agr*− strains, we compared the frequency of *agr* dysfunction in colonizing strains from bacteremic patients and uninfected controls.

We report that *agr*− strains were frequently recovered from patients with *S. aureus* bacteremia and that clones having the same inactivating *agr* mutation were recovered from colonizing sites within the same individual. Furthermore, *agr*− strain frequencies were similar among carriers who developed bacteremia and controls who did not, providing evidence that

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variation in *agr* was not correlated with progression to disease. Taken together, these results support the importance of variables other than *agr* functionality, such as host risk factors, the mode of bloodstream invasion, and possibly inoculum size, in determining the invasiveness of *S. aureus* in hospitalized patients.

MATERIALS AND METHODS

Staphylococcus aureus Isolates

Staphylococcus aureus isolates were obtained from 2 patient populations as part of previous study [5]. The first population consisted of 158 pairs of genotypically isogenic blood and nasal isolates from single patients, collected in a German multicenter study that included general and intensive care units at 32 university and community hospitals. Nasal specimens were obtained for culture immediately after the isolation of *S. aureus* from the blood. Twenty-two pairs of isolates from an original set of 180 were lost. In 48 of the 158 patients, an additional clonal *S. aureus* isolate was recovered from the clinically presumed focus of infection. Sites and types of infection included intravenous catheter–related infections, osteomyelitis, skin and soft tissue infections, and lower-respiratory-tract infections (Supplementary Table 1). The second population consisted of 221 single-patient nasal isolates prospectively collected over a 6-year period at a single tertiary-care hospital in Germany (Supplementary Table 2). In addition to the nasal isolates, 12 clonal *S. aureus* blood isolates were collected from colonized patients who subsequently developed *S. aureus* bacteremia (Supplementary Table 1). None of the other 209 nasally colonized patients developed *S. aureus* bacteremia during the study. *Staphylococcus aureus* from 14 patients (8.2%) with bacteremia from the 2 parts of the study and from 6 uninfected nasal carriers (2.7%) harbored methicillin-resistant *S. aureus* strains, previously confirmed by testing for the *mecA* gene by polymerase chain reaction (PCR) [5].

Screening for Hemolytic Activity as an Indicator of *agr* Function

Hemolysin production in *S. aureus* can be used to approximate *agr* activity because δ-hemolysin is a translation product of *agr* RNAIII and because α-hemolysin and the phenol-soluble modulins (PSMs; a family of peptides that include δ-hemolysin) are upregulated by RNAIII and *agrA,* respectively [6, 7]. Production of δ-hemolysin and other PSMs can be semiquantitatively assayed on sheep blood agar (SBA) by virtue of their synergism with β-hemolysin [8, 9]. Individual colonies can be analyzed for these toxins by cross-streaking against RN4220, which produces only β-hemolysin. However, because cross-streaking of unfractionated cultures obscures heterogeneity, we first plated diluted cultures onto SBA and scored for α-hemolysin production, which causes direct hemolysis. In the case of α-hemolysin–negative populations, SBA plates were coated with a β-hemolysin–containing culture supernatant, as described elsewhere [10]. A total of 50–100 colonies per sample were surveyed from each culture to ensure the phenotypes reported were those that dominated in the original sample. Cultures were provisionally scored as hemolytic, nonhemolytic, or, when >30% of colonies were hemolytically distinct, mixed.

Exoprotein Profiles

Cultures were grown in trypticase soy broth (TSB), and 1.5-mL aliquots were centrifuged to remove bacteria. Culture supernatants were precipitated with a 10% volume of 50% trichloroacetic acid, and the pellet was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to the method of Laemmli [11].

RNA Extraction and Quantitative Nucleic Acid Sequence–Based Amplification (NASBA) Assay

Cultures were grown in TSB for 4 hours (optical density, approximately 0.9) at 37°C with shaking, and cell pellets were treated with lysostaphin (AMBI) for 30 minutes at 37°C. RNA was purified using the Qiagen RNeasy kit, and its integrity was checked by agarose gel electrophoresis [12]. RNAIII expression was quantified using a real-time NASBA assay, as previously described [13]. NASBA reactions were performed on a NucliSens EasyQ analyzer (BioMérieux), using the NucliSENS EasyQ Basic Kit Version 2 (BioMérieux). Standard curves were made using 10-fold serial dilutions of in vitro transcribed RNAIII and *gyrB* targets (Stratagene, Agilent Technologies). Time to positivity (TTP) was defined as the time at which the fluorescence signal rose 1.25-fold above the background emitted over the course of the first 5 measurements [13]. The RNAIII expression levels for RNA preparations were calculated from the standard curves and expressed as TTP ratios (TTP_{gyrB}/TTP_{RNAIII}). A TTP_{gyrB}/TTP_{RNAIII} ratio of 1.10 was used as a cutoff to define the lower limit of *agr* functionality, as previously described [13].

Genotyping

The relatedness of strains from the blood, nares, and presumed foci of the same patients, as well as variants from mixed *agr*+ and *agr*− cultures, was confirmed by DNA sequence analysis of the protein A gene variable repeat region (*spa* typing) [14]. *spa* types were used to identify multilocus sequence typing (MLST) clonal complexes, using the Ridom SpaServer database (available at: http://spa.ridom.de/mlst.shtml) [15].

Staphylococcus aureus isolates can be divided into 4 predominant *agr* groups on the basis of the specificity of the autoinducing peptide, encoded within *agrD*, for its membrane sensor (AgrC). All isolates were previously assigned to one of the 4 major *agr* groups by PCR-based assays [16].

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agr Sequencing

Nucleotide sequences were determined for the *agrA* and *agrC* genes and for the entire locus when mutations were found to be absent in these genes by PCR and automated DNA sequencing, as described elsewhere [10, 17]. Sequences were compared with the *agr* sequences of strains from the appropriate *agr* specificity group—NCTC 8325, D302, Not266, and RN9107 (*agr* group I); D61, JH1, and C126 (*agr* group II); MRSA252 and MW2 (*agr* group III); and RN4850 (*agr* group IV)—by means of a sequence analysis suite (DNAStar). When genotypically isogenic mixtures of *agr*+ and *agr*− organisms were recovered, we sequenced the *agr*+ component. When an *agr*− mutant was characterized by a single amino acid substitution resulting in a missense mutation and the strain was not from a mixed culture, *agr* sequences were compared to a genotypically isogenic *agr*+ clone from a different site in the same patient or from a different patient in the same collection. Missense substitutions that were predicted to affect protein function were identified by the SIFT (sorting intolerant from tolerant) algorithm [18].

Statistical Analysis

Analyses were performed in the R statistical environment (http://www.R-project.org). Categorical variables were compared using a 2-tailed Fisher exact test. *spa* type diversity was measured by the Simpson index of diversity (1-D), using the "vegan" package for R (http://CRAN.R-project.org/package=vegan) [19]. A P value of $< .05$ was considered statistically significant, and no adjustments were made for multiple comparisons.

RESULTS

When considering the within-host dynamics of *agr* function, we hypothesize that *agr*+ strains are more likely to initiate the change from benign colonization to active infection and that the subsequent state of infection, in this case bacteremia, provides the necessary milieu for the emergence of mutations in the *agr* locus. To test this hypothesis, we screened for hemolysin activity, which is widely used to distinguish *agr*+ from *agr*− staphylococci [3, 4, 10, 20, 21], among clones from multiple sites in individual patients with bacteremia. Two strain populations from a previous study were analyzed [5]. The first consisted of 364 *S. aureus* strains, isolated from 158 patients, from whom 158 strains were isolated from the blood, 158 from the anterior nares, and 48 from the clinically presumed focus of infection. The clonality of isolates from individual patients was confirmed in the original analysis by pulsed-field gel electrophoresis (PFGE) and here by *spa* typing (Supplementary Table 1). Strains were considered clonal if they had identical PFGE banding patterns and *spa* types. Fifteen of 158 blood isolates (9.5%) were found to be purely *agr* defective (ie, negative for synergistic hemolysis) (Table 1 and Supplementary Table 1). *agr* Dysfunction was detected with a similar prevalence among nares and foci isolates: 14 nares isolates (8.8%) and 4 foci isolates (8.3%) were either *agr* defective or, in one case, contained a mixture of *agr*+ and *agr*− organisms (patient 158, foci strain 652; Supplementary Table 1). In the case of the mixture, *spa* typing confirmed that subpopulations were naturally occurring variants from the same progenitor strain, indicating that the allelic split occurred very recently, probably within the patient.

Among the 158 patients with *S. aureus* bacteremia, only 11 were nasally colonized with a strain of *S. aureus* that differed in *agr* functionality, indicating that a shift in *agr* function was uncommon in hosts with *S. aureus* bacteremia. However, among the 15 patients with *agr*-defective bacteremia, 5 (33%) did not have an *agr*-defective isolate in their nares or in their foci (see patient 135), whereas of the 143 patients with *agr*-positive bacteremia, only 5 (3.5%) were nasally colonized with an *agr*-defective strain ($P < .001$ by the Fisher exact test). Thus, a shift to *agr* positivity in bloodstream isolates occurs less frequently than does the reverse.

Identity of mutations among *agr*-defective isolates from different sites implies a clonal origin. We therefore determined the basis of *agr* dysfunction in all hemolysin-negative strains by nucleotide sequencing of the *agrA* and *agrC* region of the locus. Previous work comparing functional and nonfunctional staphylococcal strains indicates that inactivating mutations are localized to these regions of the locus in most cases [10, 20, 21]. Indeed, putative inactivating mutations were identified in the coding region of these genes for all but 1 of the 15 *agr*− blood strains. The variants were characterized by frameshift indels (insertions/deletions; n = 6), nonsense mutations (n = 1), and nonsynonymous changes (n = 12). Thus, a variety of types of mutation, including a high frequency of insertion and deletion events, contributed to the production of the *agr* variants, consistent with the results of previous studies [20–22].

Examination of *agr*-defective clones from blood and nares indicated that in all but one case the putative inactivating *agr* mutation of isolates from the blood was the same as that from the nares of the same patients (7 of 8 *agr*− blood and nasal mutant pairs). Additionally, *agr* mutants were isolated from 3 foci; all 3 patients had the same mutations in the blood, and 2 patients had the same *agr* mutations at all 3 sites. Thus, although *agr* diversification occurs at a sufficient frequency in vivo to result in mixed infections, most patients possess a predominant population of 1 mutant (derived from single organisms).

It is possible that *agr* mutants originating during wound infection and subsequent bacteremia could result in subsequent self-inoculation of the nares. To address the issue, a second population was studied in which nasal swabs were prospectively collected from 221 patients (Supplementary Table 2), 12 of

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Table 1. Characteristics of *agr*-Defective *Staphylococcus aureus* Strains Recovered From Patients

		Genotype					Blood			
Group	Patient No.	*spa* Type	*spa* Motif	CC[a]	Reference Sequence	*agr* Group	Strain No.	δ-Hemolysin	*agr* Mutation[b]	Predicted Result
1	16	4	YHFGFMBQBLO	8	NCTC8325	1	4	–	126-IS*256*	Frameshift-truncated AgrC
1	24	21	UJGBBGGJAGJ	15	D61	2	13	–	g517a	Ala>Thr at aa 173 in AgrA[c]
1	30	532	WGKAKAOM	30	MRSA252	3	19	–	No mutation found	None
1	34	576	ZDGMDMGMMM	101	Not266	1	26	–	a409 (–1 bp), g52a	Frameshift-truncated AgrC, Ala>Thr at aa 18 in AgrA[d]
1	46	2	TJMBMDMGMK	5	JH1	2	46	Weak	No mutation found	None
1	53	363	YGFMBQBLO	8	NCTC8325	1	54	–	g1133a	Gly>Asp at aa 378 in AgrC[e]
1	60	385	TO2MBMDMGMK	5	JH1	2	61	–	c136t	Gln>Stop at aa 46 in AgrA
1	67	852	UJGJAGJ	15	D61	2	78	–	c771t, c583t	Pro>Pro at aa 316 in AgrC, Arg>Cys at aa 195 in AgrA[c]
1	73	33	WGKAKAOMQQ	30	MRSA252	3	85	–	t224a	Ile>Asn at aa 75 in AgrA[e]
1	77	755	UJGPLM	12	C126	2	91	–	t313 (+1 bp), a662 g	Frameshift-truncated AgrC, Tyr>Cys at aa 221 in AgrA[c]
1	84	1	YHGFMBQBLO	8	NCTC8325	1	99	+	…	…
1	110	33	WGKAKAOMQQ	30	MRSA252	3	130	–	t485c	Phe>Ser at aa 162 in AgrC[e]
1	117	44	WGMQ	30	MRSA252	3	143	–	955–985 (–30 bp)	Frameshift-truncated AgrC
1	119	16	WGKAKAOMQQQ	30	MRSA252	3	146	+	…	…
1	120	664	YC2BQBLO	8	NCTC8325	1	147	–	a86c	Asp>Ala at aa 29 in AgrA[e]
1	123	1365	ZFFGU2DMGGM	25	D302	1	150	+	…	…
1	135	53	YGFMBQBLQBLPO	8	NCTC8325	1	165	–	121-IS*256*	Frameshift-truncated AgrC
1	136	3	WGKAOMQ	8	NCTC8325	1	166	+	…	…
1	140	184	ZFGU2DMGGM	25	D302	1	171	–	t349 (+1 bp), g543t	Frameshift-truncated AgrC, Gln>His at aa 181 in AgrC[c]
1	146	385	TO2MBMDMGMK	5	JH1	2	180	–	g739a	Glu>Lys at aa 247 in AgrC[c]
2	**5**	**1405**	**UJJKBPE**	**30**	**MW2**	**3**	**14**	–	**c254a, a514g**	**Thr>Asn at aa 85 AgrC[c], Ile>Val at aa 172 AgrA[c]**
2	**7**	**43**	**WGKAKAOMQ**	**30**	**MRSA252**	**3**	**18**	–	**667–677 (–11 bp)**	**Frameshift-truncated AgrC**

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Table 1 continued.

		Nasal				Foci				
Group	Patient No.	Strain No.	δ-Hemolysin	*agr* Mutation[b]	Predicted Result	Strain No.	Specimen Source	δ-Hemolysin	*agr* Mutation[b]	Predicted Result
1	16	13	–	126-IS*256*	Frameshift-truncated AgrC	…	…	…	…	…
1	24	39	–	t826 (–1 bp)	Frameshift-truncated AgrC	…	…	…	…	…
1	30	54	–	No mutation found	None	…	…	…	…	…
1	34	75	–	a409 (–1 bp), g52a	Frameshift-truncated AgrC, Ala>Thr at aa 18 in AgrA[d]	…	…	…	…	…
1	46	138	–	g1177t	Gly>Cys at 393 aa in AgrC[e]	…	…	…	…	…
1	53	164	+	…	…	…	…	…	…	…
1	60	191	+	…	…	…	…	…	…	…
1	67	253	–	c771t, c583t	Pro>Pro at aa 316 in AgrC, Arg>Cys at aa 195 in AgrA[c]	…	…	…	…	…
1	73	277	+	…	…	…	…	…	…	…
1	77	295	–	t313 (+1 bp), a662 g	Frameshift-truncated AgrC, Tyr>Cys at aa 221 in AgrA[c]	296	Catheter	–	t313 (+1 bp), a662 g	Frameshift-truncated AgrC, Tyr>Cys at aa 221 in AgrA[c]
1	84	326	–	t1107 (+1 bp)	Frameshift-truncated AgrC	…	…	…	…	…
1	110	430	+	…	…	…	…	…	…	…
1	117	466	+	…	…	468	CSF	+	…	…
1	119	479	–	t47a	Lys>Met at aa 16 in AgrA	…	…	…	…	…
1	120	484	–	a86c	Asp>Ala at aa 29 in AgrA[e]	…	…	…	…	…
1	123	494	–	t2c	Val>Ala at aa 1 in AgrC[e]	…	…	…	…	…
1	135	545	+	…	…	546	Wound	–	121-IS*256*	Frameshift-truncated AgrC
1	136	549	–	78–84 (–7 bp)	Frameshift-truncated AgrA	550	Catheter	+	…	…
1	140	564	–	t349 (+1 bp), g543t	Frameshift-truncated AgrC, Gln>His at aa 181 in AgrC[c]	566	Catheter	–	t349 (+1 bp), g543t	Frameshift-truncated AgrC, Gln>His at aa 181 in AgrC[c]
1	146	595	–	g739a	Glu>Lys at aa 247 in AgrC[c]	…	…	…	…	…
2	**5**	**13**	–	**c254a, a514 g**	**Thr>Asn at aa 85 AgrC[c], Ile>Val at aa 172 AgrA[c]**	**…**	**…**	**…**	**…**	**…**
2	**7**	**17**	–	**667–677 (–11 bp)[g]**	**Frameshift-truncated AgrC**	**…**	**…**	**…**	**…**	**…**

Group 1 indicates retrospectively collected strain pairs from patients with bacteremia, and group 2 (in bold) indicates prospectively collected strain pairs from patients who subsequently developed bacteremia (see text).
Abbreviations: –, negative, +, positive; aa, amino acid; bp, base pair; CSF, cerebral spinal fluid.

[a] *spa*-type deduced clonal complex (CC).

[b] Designation corresponds to the region in *agrA* or *agrC* of the genome sequenced isolate of the appropriate *agr* group (Materials and Methods).

[c] Tolerated by the sorting intolerant from tolerant (SIFT) algorithm.

[d] Insignificant polymorphism found in closely related *agr*-positive clone.

[e] Not tolerated by the SIFT algorithm.

[f] Not tolerated by the SIFT algorithm (low confidence prediction).

[g] The indicated mutation was identified in a subpopulation of the culture, which contained additional *agr*-defective species.

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whom subsequently developed bacteremia (Table 1 and Supplementary Table 1). In the 2 cases of *agr*-defective bacteremia, nasal colonization with an *agr*-defective *S. aureus* preceded the recovery of a clonally identical mutant in the blood (Table 1). Thus, the results of the multicenter study were confirmed by the prospective study. Together the 2 showed that 70.6% of the patients with *agr*-defective *S. aureus* bacteremia were colonized in the anterior nares or infected at foci by the same mutant. Apparently, *agr* dysfunction primarily precedes new-onset bloodstream infection and therefore is not required for subsequent development of bacteremia.

The apparent lack of a within-host shift in *agr* function could have been due to inadequate screening. For example, closer comparison of colonizing and invasive organisms may reveal more subtle adaptive changes rather than discrete *agr*+ or *agr*− phenotypes, perhaps owing to partial loss-of-function mutations in the locus that reduce but do not eliminate *agr* activation. To address this possibility, hemolytic toxin levels were assayed among *agr*+ strain pairs from blood and nasal sites in the 2 study populations. Strikingly, in each case, toxin levels among strains were indistinguishable. Thus, selection for mutants with attenuated (or enhanced) virulence in the colonizing population did not correlate with the transition from commensal to pathologic habitats.

Analysis of hemolytic toxin levels revealed that production was weak but not absent in many strain pairs, the majority of which belonged to the clonal complex 30 (CC30) genotype (Supplementary Table 3). DeLeo and colleagues recently reported that contemporary CC30 strains have a mutation in *agrC,* resulting in an amino acid change at Gly55 (AgrC G55R) that attenuates *agr* function [23]. While this substitution was present in all CC30 strains with reduced hemolysin production, several CC30 strains in which it was present showed robust hemolysis and RNAIII expression (Table 2 and Figure 1). This distribution of phenotypes to strong and weak *agr* activity may be explained by plasticity of mutational

Table 2. Characteristics of Clonal Complex (CC) 30 Blood Isolates

Patient No.	Strain No.	*spa* Type	*spa* Motif	CC[a]	δ-Hemolysin	*agr* Mutation[b]	Predicted Result
167	211	43	WGKAKAOMQ	30	+	No mutation found	None
70	81	33	WGKAKAOMQQ	30	+	No mutation found	None
119	146	16	WGKAKAOMQQQ	30	+	No mutation found	None
56	57	19	XKAKAOMQ	30	+	No mutation found	None
122	149	1384	WGKAKAOLOMQQ	30	Weak	g272a	Ser>Asn at aa 91 AgrC[c]
163	204	43	WGKAKAOMQ	30	Weak	t487g	Ser>Ala at aa 163 AgrC[d]
33	22	43	WGKAKAOMQ	30	Weak	No mutation found	None
80	95	43	WGKAKAOMQ	30	Weak	No mutation found	None
89	109	43	WGKAKAOMQ	30	Weak	No mutation found	None
92	105	43	WGKAKAOMQ	30	Weak	No mutation found	None
15	3	33	WGKAKAOMQQ	30	Weak	No mutation found	None
72	84	33	WGKAKAOMQQ	30	Weak	No mutation found	None
75	88	33	WGKAKAOMQQ	30	Weak	No mutation found	None
87	102	33	WGKAKAOMQQ	30	Weak	No mutation found	None
94	112	33	WGKAKAOMQQ	30	Weak	No mutation found	None
103	122	33	WGKAKAOMQQ	30	Weak	No mutation found	None
109	129	33	WGKAKAOMQQ	30	Weak	No mutation found	None
118	144	33	WGKAKAOMQQ	30	Weak	No mutation found	None
131	159	33	WGKAKAOMQQ	30	Weak	No mutation found	None
154	190	33	WGKAKAOMQQ	30	Weak	No mutation found	None
40	40	16	WGKAKAOMQQQ	30	Weak	No mutation found	None
96	114	16	WGKAKAOMQQQ	30	Weak	No mutation found	None
157	194	19	XKAKAOMQ	30	Weak	No mutation found	None
30	19	532	WGKAKAOM	30	–	No mutation found	None
73	85	33	WGKAKAOMQQ	30	–	t224a	Ile>Asn at aa 75 in AgrA[c]
110	130	33	WGKAKAOMQQ	30	–	t485c	Phe>Ser at aa 162 in AgrC[c]

Abbreviations: –, negative; +, positive; aa, amino acid.

[a] *spa*-type-deduced CC. Primary grouping of strains is by *agr* functionality, followed by polymorphism of *spa*.

[b] Designation corresponds to the region in *agrC* or *agrA* of genome-sequenced CC30 prototype strain MRSA252, which has an AgrC G55R amino acid change that attenuates *agr* function (see text).

[c] Not tolerated by the sorting intolerant from tolerant (SIFT) algorithm.

[d] Tolerated by the SIFT algorithm.

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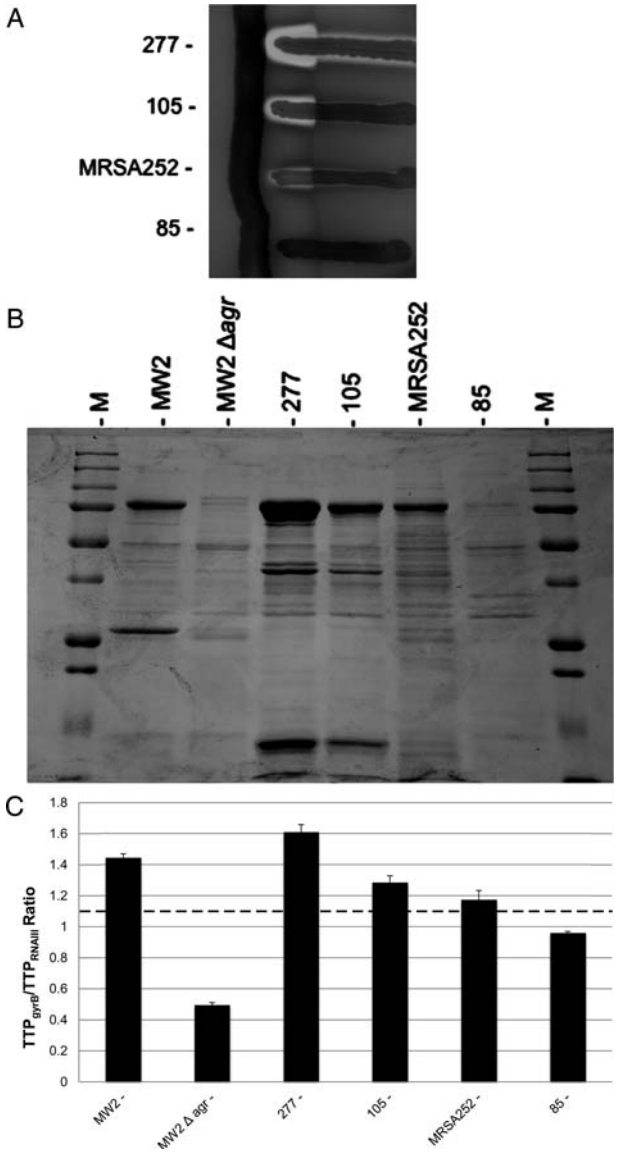

Figure 1. A representative subset of clonal complex 30 strains showing various levels of *agr* activity. *A*, Cross-streaking alongside the β-hemolysin producing strain RN4220; differentiation of the various hemolytic activities in *Staphylococcus aureus* can be scored on sheep blood agar by virtue of their synergism with β-hemolysin. Strain 277, strain 105, and weakly *agr*-positive reference strain MRSA252 have identical *agr* sequences, including an AgrC G55R amino acid change that attenuates *agr* function (Table 2) [23]. Strain 85 has the same G55R amino acid change but is *agr* defective, likely owing to an additional mutation in *agrA* (Table 1 and Table 2). *B*, Exoprotein profiles prepared from culture supernatants analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. M, protein ladder; MW2 and MW2 Δ*agr*, control strains. *C*, Relative quantitation of RNAIII by quantitative nucleic acid sequence–based amplification analysis and the time-to-positivity (TTP) ratio (TTP$_{gyrB}$/TTP$_{RNAIII}$). Data are representative of 3 separate experiments. Dotted line, TTP ratio of 1.10, indicating the cutoff for *agr* functionality; MW2 and MW2 Δ*agr*, control strains.

effects in different genetic backgrounds. In support of this hypothesis, phenotypically *agr*-defective CC30 strains did not consistently harbor secondary mutations that would be predicted to fully inactivate the locus (Table 2).

Although *S. aureus* strains with low virulence are apparently capable of invading host tissues, reduced virulence may slow the rate of invasion. To address this issue, we compared the frequency of *agr*-defective mutants among nasal isolates from bacteremic patients with nasal isolates from patients in the second study who never developed infection. *agr* dysfunction was present in 10.0% of nasal isolates (17 of 170) from bacteremic patients and in 9.6% (20 of 209) from uninfected carriers. Thus, *agr*-defective mutants were not more likely to be found in the nares of asymptomatic carriers than those of bacteremic patients, suggesting that *agr*-defective mutants possess the same infectivity as fully virulent strains. Comparison of *spa* type diversity among nasal isolates from bacteremic patients with those of uninfected patients demonstrated that both groups were nonclonal and equally diverse (Table 3). Thus, there was no evidence of clustering of genotypes by invasiveness, and infections did not reflect dissemination of an outbreak strain, which could skew results.

Because molecular typing can be used to reconstruct transmission networks, we sought evidence for transmission of *agr* mutations among clones from the 2 study populations. We found that identical *agr* variants were present in each of 2 groups of genotypically related strains selected from among all 329 patients. The first group of 2 strains, isolates W9 and 103, came from the colonized but uninfected study population (Supplementary Table 2). They were obtained from the nares of 2 patients from the same hospital. The second group of 4 strains, isolates 12, 78, 91, and 135, came from 4 patients, 3 of whom were uninfected *S. aureus* carriers from the same hospital (patients 12, 85, and 125; Supplementary Table 2), and 1 of whom was from a patient with bacteremia from a different hospital (patient 67; Table 1 and Supplementary Table 1). The recovery of identical *agr*-defective alleles in different hosts suggests nosocomial cross-infection, with secondary transmission indicated in the case of the *agr* mutant that was found in multiple cultures. Consistent with this hypothesis, *agr* alleles in both strains of the first group and in one of the strains from the second group (isolate 78) were able to circulate long enough to accumulate subsequent mutations. Such changes would be expected if *agr* genes are not transcribed and their sequences are no longer subjected to selection. Furthermore, strains in the second group were distinct but closely related to each other by *spa* repeat region organization, indicating genotypic diversification of the clones subsequent to acquisition of their respective *agr* mutations. Collectively, these results

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Table 3. Diversity of *Staphylococcus aureus* Genotypes Among 170 Bacteremic Patients and 209 Colonized Uninfected Controls

Major *spa* Type[a]	*spa* Motif	CC[b]	Bacteremic	Colonized Uninfected	*P* Value[c]
2	TJMBMDMGMK	5	4.7 (8)	1 (2)	.05
21	UJGBBGGJAGJ	15	9.4 (16)	9.1 (19)	1
43	WGKAKAOMQ	30	4.1 (7)	3.4 (7)	.79
33	WGKAKAOMQQ	30	7.6 (13)	7.2 (15)	1
73	XKAKBEMBKB	45	3.5 (6)	4.8 (10)	.62
1	YHGFMBQBLO	8	3.5 (6)	1.9 (4)	.35
201	ZDGMDMGMM	101	3.5 (6)	1.9 (4)	.35
184	ZFGU2DMGGM	25	3.5 (6)	4.3 (9)	.79
113	TJEJNCMOMOKR	22	0.6 (1)	2.4 (5)	.23
Other[d]	…	…	75.3 (128)	70.8 (148)	.35
SID	…	…	0.98	0.98	

Data are percentage (occurrence), unless otherwise indicated.

Abbreviations: CC, clonal complex; SID, Simpson index of diversity.

[a] Defined as ≥5 strains with the same *spa* type.

[b] Isolates broadly grouped according to their evolutionary relatedness.

[c] By the Fisher exact test.

[d] Minor *spa* types. Two colonizing isolates were non–*spa* typeable.

suggest that while continuous, indefinite circulation of specific *agr*-defective alleles in natural populations may be uncommon [20], they can circulate long enough to be detected in epidemiologically unlinked hosts.

DISCUSSION

agr is a global regulator of staphylococcal virulence that, in vitro, coordinates a switch from an establishment mode, in which genes for adhesins and protective surface proteins are expressed, to an invasive mode, in which genes for factors that promote cell and tissue destruction are activated [1]. However, *agr*-defective mutants are frequently isolated from clinical material and laboratory cultures [3, 10, 20, 21, 24, 25]. Recent reports indicate that recovery of *agr*-defective strains from bacteremic patients is associated with persistent infection and worse outcomes [3, 4], perhaps owing to increased production of cell surface proteins that facilitate biofilm formation and immune evasion. However, lack of knowledge regarding the timing of changes in *agr* function during or prior to bacteremic infections complicates the interpretation of such data.

We report that among patients with *S. aureus* bacteremia, the *agr* function of paired strains cultured from blood and nares was most frequently identical, regardless of whether the nasal culture was obtained before or after the detection of bacteremia. This result suggests that attenuation in *agr* during the transition to bloodstream infection occurs in a minority of patients. The results suggest that factors such as hospitalization and intravenous catheter use permit *S. aureus* lacking full virulence to cause infection. Bacteria such as these express fewer and different virulence factors than fully virulent organisms, such as community-acquired MRSA strains, which are almost always *agr*+ [26, 27] and able to infect structurally and functionally immune competent hosts. The data also show that *agr*+ colonization frequencies were not greater in the noses of infected patients than in those of uninfected controls. Thus, lack of secreted toxins did not adversely affect invasiveness.

In a previous report, we showed that genotypic diversification subsequent to *agr* inactivation is uncommon among *S. aureus* isolated from clinical infections, indicating that *agr*-defective mutants are often short-lived [20]. At the same time, the success with which the CC30 clone has established itself in natural populations of *S. aureus* indicates that mutations with more moderate or weak functional effects, such as the amino acid change at Gly55 [23], can demonstrate distinctive population dynamics. Indeed, the differential populational stability of *agr* mutations appears to be inversely proportional to the degree of functional effect induced. This pattern may result from functional trade-offs, wherein completely inactivating mutations may promote survival in certain host niches in the short term but represent a liability to clones over the longer term.

The main strategy adopted for silencing the *agr* locus usually relies on mutations in *agrCA* genes. However, the ability of the G55R amino acid change to inactivate the locus appears to depend on an appropriate genetic background, either through direct effects on *agr* genes themselves or the "fine-tuning" of the interaction between genetic background and *agr* response. This interaction may provide the CC30 lineage a unique mechanism to resolve adaptive conflicts that arise from having alleles under negative selection in one environment but under positive selection in others.

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Our study has a number of limitations. First, study data may not be generalizable to nonstudy areas. Second, correlations between *agr* functionality and bacteremia may reflect factors we did not account for, such as duration of hospitalization. Third, our data are limited to bacteremia, so *agr* functionality patterns between colonizing and infecting sites in different syndromes might be different. Finally, the data do not capture cases of undiagnosed bacteremia. Antibiotic treatment prior to sample collection would both decrease the rate of overall disease detected and potentially increase the proportion of *agr*-positive strains that may be linked to more severe disease.

In summary, the results shed light on the role of *agr* dysfunction in the causal pathway leading to complicated disease and poor outcomes with which these strains are associated [3, 4]. A better understanding of the biological detail of such pathways is needed before assuming the general applicability of attempts to block infection by interfering with *agr*-mediated virulence. A critical question is to determine which steps during infection involve *agr* evolution. In this connection, future work will determine whether complicated *S. aureus* bacteremia is more likely to have developed as a consequence of being infected with an *agr*− isolate or whether an *agr*− isolate is more likely to develop in a patient who presents with bacteremia complicated by deep-seated infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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