Population Studies of Methicillin-Resistant and -Sensitive *Staphylococcus aureus* Strains Reveal a Lack of Variability in the *agrD* Gene, Encoding a Staphylococcal Autoinducer Peptide

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The virulence of *Staphylococcus aureus* is controlled by the accessory gene regulator (*agr*) system, including an extracellular inducer encoded by *agrD*. Variable *agr* PCR restriction fragment length polymorphism (RFLP) patterns of unique *S. aureus* strains (*n* = 192) were determined for a region comprising *agrD* and parts of the neighboring *agrC* and *agrB* genes. Twelve unique RFLP patterns were identified among *S. aureus* strains in general; these patterns were further specified by sequencing. All sequences could be catalogued in the three current *agr* groups. A major proportion of the *S. aureus* strains belong to *agr* group 1, whereas only 6% of the methicillin-susceptible *S. aureus* strains and 5% of the methicillin-resistant *S. aureus* strains belong to *agr* groups 2 and 3, respectively. The homology between groups varied from 75 to 80%, and within groups it varied from 96 to 100%. Different levels of sequence variability were observed in the different *agr* genes. *agr*-related bacterial interference among colonizing *S. aureus* strains in the noses of persistent and intermittent human carriers was studied. *S. aureus* strains belonging to different agr groups were encountered in the same individual. This may suggest that the activity of the *agrD* gene product does not define colonization dynamics, which is further substantiated by the rarity of *agr* group 2 and 3 strains.

Staphylococcus aureus is a major human pathogen, causing a wide range of diseases including septicemia, meningitis, endocarditis, osteomyelitis, septic arthritis, toxic shock syndrome, and food poisoning (20). The pathogenic diversity of the bacterium reflects its ability to successfully survive in many different host tissues during infection. The pathogenic capacity of *S. aureus* is clearly dependent on its production of exoproteins. The synthesis of these virulence factors is globally regulated by an *S. aureus* quorum-sensing system called the accessory gene regulator (*agr*). The expression of genes in *agr* varies in response to changes in cell density (15). This global regulatory system utilizes a posttranscriptionally modified signal peptide that is excised from the AgrD peptide. This thiolactone-containing peptide accumulates in the external environment during postexponential growth of the *S. aureus* population. It is postulated that the signal peptide interacts with the transmembrane receptor protein (AgrC) of a classical two-component regulatory system (AgrC, AgrA) that in turn activates the transcription of the *agr* locus (5, 9). This induces the down-regulation of genes encoding surface proteins and the up-regulation of genes encoding secreted virulence factors (2, 5).

Ji et al. (4) recently described interference among different strains of staphylococci with regard to synthesis of their virulence factors and other extracellular proteins. These authors found that the AgrD peptide produced by a given strain of *S. aureus* activates its own *agr* locus but may inhibit the expression of *agr* in other strains. It is suggested that this inhibitory effect is correlated with the ability of a strain to compete with other strains for sites of colonization or infection. This phenomenon of AgrD-dependent cross-inhibition suggests the presence of

significant variability of the domain encoding for the AgrD signal peptide (4). Indeed, it was shown that the autoinducer (AgrD) and its modifying protein (AgrB) and receptor (AgrC) harbored sequence variation. This affects the specificity of the receptor-ligand interaction. On the basis of autoinducer-receptor specificity, *S. aureus* can be divided into at least three different *agr* groups (10).

Here, we report on the prevalence and nature of *agrD* polymorphism, as determined for a large collection of methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains. *agr* restriction fragment length polymorphism (RFLP) types were determined and classified into *agr* groups. The dissemination of *agr* RFLP types between MRSA and MSSA was studied. The potential relevance of *agrD* polymorphism in relation to colonization of distinct strain types in the noses of intermittent versus persistent *S. aureus* carriers was also analyzed.

MATERIALS AND METHODS

Strain collection. Strains of MRSA and MSSA (*n* = 192) were pooled from eight existing collections (Table 1). All strains were well typed by using a wide variety of phenotypic and genotypic procedures: pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), binary typing, and more. For cultivation, bacteria from glycerol stocks stored at −80°C were inoculated on Columbia III agar supplemented with 5% sheep blood (Becton Dickinson, Etten-Leur, The Netherlands) and incubated at 37°C for 24 h. All strains were identified as *S. aureus* by standard microbiological methods (6). MSSA strains from collection 1, isolated during the colonization studies, were derived from nine healthy nasal carriers of *S. aureus*. When 5 to 8 out of the 10 serial samples were positive, a person was identified as an intermittent carrier (*n* = 6). Persistent carriers (*n* = 3) had 9 or 10 positive cultures out of the 10 samples (17).

DNA isolation. Five to 10 colonies were suspended in a buffer containing 150 μl of 25 mM Tris-HCl (pH 8.0), 10 mM EDTA and 50 mM glucose. To prepare spheroplasts, 75 μl of a lysostaphin solution at a concentration of 100 μg/ml (Sigma Chemical Corporation, St. Louis, Mo.) was added. The mixture was incubated for 1 h at 37°C. DNA isolation was done as described by Boom et al. (3). Briefly, guanidine hydrothiocyanate was added for cell lysis and DNA was purified by affinity chromatography with Celite (Janssen Pharmaceuticals,

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TABLE 1. Characterization of the 192 *S. aureus* strains used in this study

Collection	Geographic origin	No. of strains	Description of the collection	Reference
1	Denmark	33	Nasal carriership (MSSA strains)	17
2	Worldwide	59	Geographically and temporally unrelated MRSA strains	7
3	US[a]	1	Type strain of a local MRSA outbreak in NYC[c] hospital	18
4	Spain and Portugal	11	Iberian (MRSA) clone	18
5	US	27	Community-acquired MRSA strains, isolated from outpatients from an NYC hospital	18
6[b]	US	49	Multicenter collection of MRSA ($n = 32$) and MSSA ($n = 17$) strains	16
7	The Netherlands	10	Representative MRSA ($n = 6$) and MSSA ($n = 4$) strains from 10 outbreaks in Dutch hospitals	19
8	US	2	MSSA strains obtained from 2 hospitalized patients (NYC)	18

[a] US, United States.
[b] Centers for Disease Control and Prevention, Atlanta, Ga.
[c] NYC, New York City.

Beerse, Belgium). DNA was eluted from the Celite particles with 100 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. The DNA concentration was estimated by electrophoresis in the presence of ethidium bromide (0.3 μg/ml) (13). Stock solutions of DNA were adjusted to a concentration of 50 ng/μl and stored at −20°C until further use.

PCR amplification of the *agrD* and partial *agrC* and *agrB* sequences. Approximately 50 ng of DNA was amplified in a 100-μl reaction mixture consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatin, and 0.1% Triton X-100. Deoxyribonucleotide triphosphates (0.2 mM; Amersham Pharmacia Biotech, Roosendaal, The Netherlands) as well as 0.2 U of *Taq* polymerase (SuperTaq; HT Biotechnology, Cambridge, United Kingdom) were present in the reaction mixture. The locations of the primers agrB1 and agrC2 within the *agr* locus are indicated in Figure 1. The National Center for Biotechnology Information (NCBI) code and sequences of the primers (50 pmol of primer per reaction) were as follows: agrC2, 5′-CTT GCG CAT TTC GTT GTT TGA-3′, SAAGRAB sequence, nucleotides (nt) 3208 to 3189 (GenBank accession no. X52543); agrB1, 5′-TAT GCT CCT GCA GCA ACT AA-3′, SAAGRAB sequence, nt 2142 to 2161. The PCR mixture was overlaid with 100 μl of mineral oil to prevent evaporation. Amplification of the DNA fragments was performed in a thermocycler (model 60; Biomed, Theres, Germany) with predenaturation at 94°C for 4 min, followed by 40 cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 74°C and ending with a postelongation step at 74°C for 3 min. Amplicons were purified by an additional ethanol precipitation step. The DNA pellet was lyophilized and redissolved in distilled water. The yield of amplicons was determined by electrophoresis in the presence of ethidium bromide (0.3 μg/ml) (13).

Restriction of the PCR products. Restriction analysis of the amplicons was performed with *Rsa*I (Roche Molecular Biochemicals, Almere, The Netherlands) and *Alu*I (Roche Molecular Biochemicals) according to the manufacturer's instructions. The *Alu*I and *Rsa*I digests were visualized on 3% NuSieve agarose gels (FMC Bioproducts, SanverTech, Heerhugowaard, The Netherlands) stained with ethidium bromide.

DNA sequencing and homology analysis. The 373 DNA-sequencing system (Perkin-Elmer, Foster City, Calif.) was used for sequencing those amplicons that generated a unique RFLP pattern. At first, amplicons were cloned with the TOPO-TA cloning kit (Invitrogen, Leek, The Netherlands). Dye terminator chemistry was applied (8, 14) using the manufacturer's cycle sequencing protocol

(Amersham Pharmacia Biotech). Briefly, DNA was amplified in the presence of a thermostable polymerase and primers agrB1 and agrC2. The extended, fluorescently labeled fragments were separated by polyacrylamide gel electrophoresis. Labels were excited by a laser as they passed the detector near the bottom of the gel. The first 100 nt were deleted because of unreliable sequencing results (GenBank; SAAGRAB sequence no. 2100–2200 and 3160–3300, SA502a sequence no. 300–415 and 1420–1550, and RN8462 sequence no. 280–415 and 1420–1550). Reliability of the sequencing was assessed by including different clones with the same RFLP pattern and checking the RFLP pattern versus the primary sequence. These comparisons confirmed the lack of PCR errors (results not shown). The sequences were compared with the data from the nucleotide and protein sequence database of NCBI and analyzed for similarity with BLAST (basic local alignment search tool) program (1). Sequence alignment and analysis of sequence polymorphism among the different *agr* genes were performed with Megalign Lasergene software (DNASTAR Inc., Madison, Wis.).

RESULTS

***agr* RFLP.** The *agr* restriction patterns of 192 epidemiologically and genetically unique *S. aureus* strains were determined. Only 12 unique-combination restriction patterns (*Alu*I and *Rsa*I digests) (Fig. 2) were detected, indicating a relatively high degree of sequence conservation. The frequency and distribution of the different RFLP patterns among the *agr* groups (see also next section) (4) are outlined in Table 2. Among the MRSA strains (71.4% of the total *S. aureus* collection), 10 distinct RFLP patterns (AA, CC, DD, DE, ED, FB, FE, GF, GG, and HE) could be identified. The most predominant *agr* RFLP types, AA and DE, represented 43.0 and 40.8% of the MRSA strains, respectively. Based on DNA sequencing (see also below) all types appeared to belong to *agr* group 1, except for *agr* type GG (prevalence of 5.1%; *agr* group 3). Seven different RFLP patterns (AA, BA, BB, DD, DE, ED, and FB)
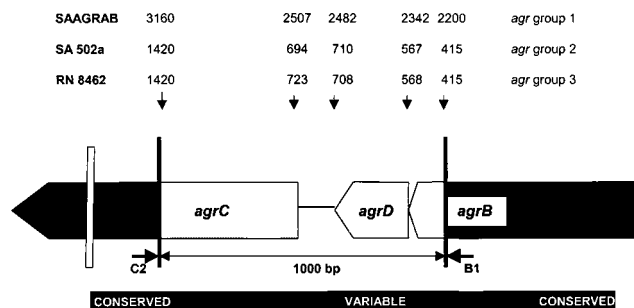

FIG. 1. Schematic map of the *S. aureus agr* locus showing the amplified region. The amplicon contained the variable sequence, defining the distinct *agr* groups (4). Primers C2 and B1 were selected from the conserved sequences. The GenBank nucleotide position numbers of the three *agr* sequences from the control strains, representing the three *agr* groups, are indicated above the corresponding positions on the map.
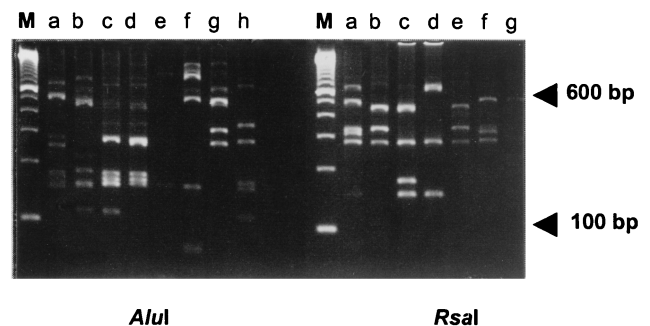

FIG. 2. RFLP analysis of the *agr* locus. Amplified DNA was digested with *Alu*I, yielding patterns a to h (left) and with *Rsa*I, producing patterns a to g (right). Twelve different combined *Alu*I-*Rsa*I combination patterns were detected. Lanes M, molecular size markers.

TABLE 2. Frequency and distribution of *agr* RFLP patterns within *agr* groups of the well-typed epidemiologically unrelated MRSA ($n = 137$) and MSSA ($n = 55$) strains[a]

agr group	No. of strains (MSSA/MRSA) with RFLP pattern:											
	AA	BB	BA	CC	DD	DE	ED	FB	FE	GG	GF	HE
1	13/59		3/0	0/2	8/2	2/56	20/7	1/1	0/1		0/1	0/1
2		8/0										
3										0/7		

[a] Strains were genetically typed using PFGE, RAPD analysis, binary typing, *mecA*/Tn*554* probing, or a combination thereof. For *Alu*I eight individual RFLP patterns (a to h; Fig. 2) were identified, whereas the use of *Rsa*I allowed for the identification of seven RFLP patterns (a to g; Fig. 2). Overall, 12 combined patterns were defined.

were found among the MSSA strains of which AA (prevalence, 23.6%) and ED (prevalence, 36.4%) appeared to be the most prevalent lineages. Apart from RFLP type BB (prevalence, 14.5%; *agr* group 2), all types were classified as belonging to *agr* group 1. One Dutch MRSA strain (Table 1, collection 7) generated an abnormally sized amplicon. Sequencing revealed that the Tn*4001* transposon was inserted in the 5′ part of the *agrC* gene (inserted between positions 2546 and 2547 of the SAAGRAB sequence). No phenotype analysis was performed for this strain, so we cannot discuss the activity of this deviating *agr* locus.

Sequence polymorphism in the *agr* locus. The nucleotide sequences of the 12 unique *agr* RFLP types were determined and compared with the GenBank data. The sequences of *agr* RFLP types BB, DD, DE, ED, FB, and GG were obtained for two or three different strains (Fig. 3 and 4). All *agr* RFLP types could be classified into the three previously known *agr* groups (Table 2). The vast majority (92%) of the *agr* RFLP types belong to group 1. Two distinct *agr* RFLP types (BB and GG) could be classified as groups 2 and 3, respectively. The frequency and positions of mutations in the *agr* locus for the unique RFLP types are displayed in Fig. 4. The positions and numbers of point mutations for strains D45 (RFLP type FB), K2-2 (RFLP type FB), and T11 (RFLP type CC) were similar. The sequences of RFLP types DD, DE, ED, and FE are closely related to the SAAGRAB sequence and rarely displayed a very small number of mutations. The nucleotide sequence of strain K1-65 (RFLP type HE) is identical to the SAAGRAB sequence (GenBank). Gain and loss of *Alu*I and *Rsa*I restriction sites are shown in Fig. 4. RFLP polymorphism cannot be deduced completely from the data presented in Fig. 4, since the *Alu*I and *Rsa*I restriction sites on both termini of the amplicon were not included in the sequence analysis. The inter- and intrasequence polymorphism of the *agr* groups is outlined in Fig. 3A. The sequence variability within an *agr* RFLP type ranged from 0 to 1% (types ED, DD, and FB). The distances between *agr* RFLP types within an *agr* group varied from 0 to 4%. The sequence diversity between *agr* groups 1 and 3 was 20%, while the *agr* group 2 cluster differed by 26.8% from the former two groups. The divergent variability among *agrD*, partial *agrC*, and *agrB* genes is displayed in Fig. 3B to D, respectively. The *agrD* sequence varied from 0 to 1% among the *agr* types within each *agr* group, except for *agr* group 2 (type BB), where the *agrD* gene varied from 5 to 7%. The first 600 nt of the *agrC* 5′ part varied from 0 to 6% among the different *agr* RFLP types. The intragroup variability of the *agrC* gene ranged from 22.6% for groups 1 and 3 to 28.8% for group 2. The last 140 bp from the *agrB* 3′ domain displayed a high level of similarity (99 to 100%) among the diverse RFLP types. The *agrB* sequence variability within the three distinct *agr* groups ranged from 14% for groups 1 and 3 to 24.8% for group 2.

The amino acid sequences of all *agr* types studied displayed variability values similar to those observed for the nucleotide

sequences (data not shown). The sequences of the amino acid residues of the AgrD propeptide within the unique *agr* RFLP types were compared and are displayed in Table 3. The group 1 AgrD propeptide sequence is completely conserved, except for types ED and CC. One amino acid, located downstream the signal peptide, was affected. A higher degree of variability was detected within the group 2 AgrD propeptide sequence, whereas only a single RFLP type was found. The AgrD signal peptide of this group consists of nine amino acids and displays homology with the AgrD nonapeptide sequence of *Staphylococcus epidermidis* Tü3298 (Table 3) (12). The AgrD propeptide sequences were compared and are outlined in Fig. 5. *agr* groups 1 and 2 displayed a high level of similarity upstream of the AgrD signal peptide. The AgrD propeptide sequences of *agr* groups 1 and 3 were conserved within and downstream of the signal peptide.

***agr* polymorphism among *S. aureus* strains of nasal carriers.** The *S. aureus* strains ($n = 3$), obtained from the volunteers were analyzed for *agr* polymorphism, and the results were compared with those obtained with other pheno- and genotyping methods (Table 4). Analysis of the *agr* polymorphism of the *S. aureus* strains from the persistent carriers reveals concordance with the results of the other typing techniques, except for protein A polymorphism. *S. aureus* strains isolated over several years from persistent carriers displayed an *agr* group switch in two of three carriers studied. Carrier 84 switched from *agr* group 1 to *agr* group 2, and carrier 126 switched from *agr* group 2 to *agr* group 1. Such switches are also observed among the intermittent carriers of *S. aureus* (see carriers 9 [switch from group 1 to 3] and 88 [switch from group 2 to 3]). The *agr* RFLP types within a given *agr* group of a carrier displayed the same level of variability as for RAPD analysis and coagulase (coag) gene typing. *agr* polymorphism within a single *agr* group of a persistent carrier was detected, as confirmed by a 3 to 4% divergence at the sequence level (data not shown). A predominant genotype could be identified. The pheno- and genotypically identical predominant strains (RAPD type BBB, coag gene type 14B; Table 4) displayed *agr* polymorphism (RFLP types AA and ED) but not *agr* group switching.

DISCUSSION

The synthesis of *S. aureus* virulence factors is controlled by a cell density-sensing system based on the action of a signal peptide secreted in the environment by the organism itself. When a strain enters the postexponential phase, it produces signal peptides that may inhibit or induce the expression of the *agr* operon in other strains, depending on the *agr* group of the strain. The amino acid sequences of the ligand (AgrD) and receptor (AgrC) for such mutually interfering strains differ considerably. This phenomenon suggests the presence of selectively variable domains (4) and points to intricate structure-function relationships at the protein level. Structure-activity
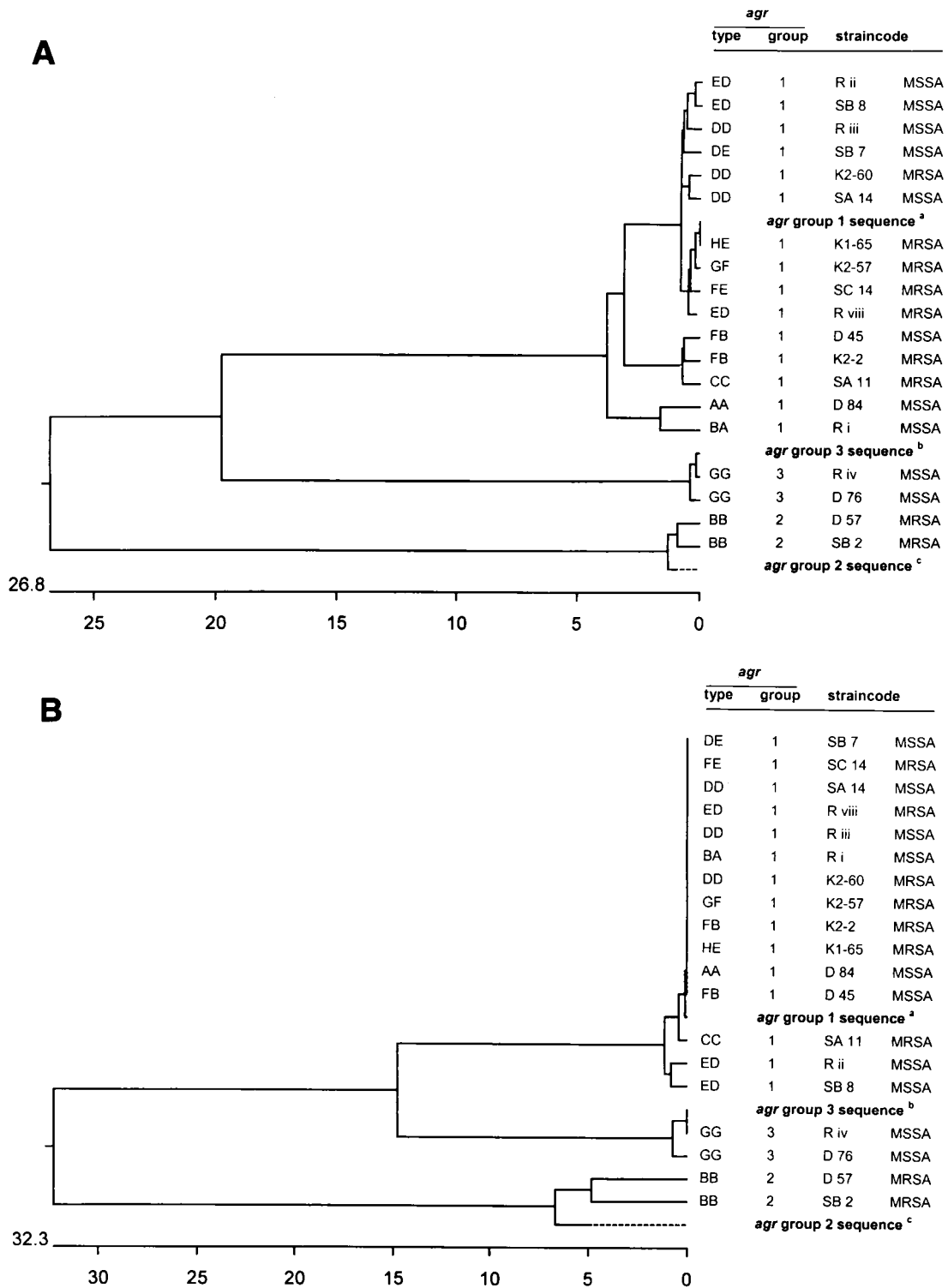
FIG. 3. Dendrograms displaying the cluster analysis of the intra- and extra-*agr* group sequence homologies from the 12 distinct *agr* RFLP patterns. The numbers on the horizontal axes display the percentages of similarity between the primary sequences of the distinct RFLP patterns. (A) Clustering of the total amplicon sequences; (B) clustering of the *agrD* sequences; (C) clustering of the *agrC* sequences; (D) clustering of the *agrB* sequences. *agr* group reference sequences were obtained from the data bank of the NCBI. aNCBI code SAAGRAB, accession no. X52543; bNCBI code SA502a, accession no. AF001782; cNCBI code RN8462, accession no. AF001783. The Tn*4001* insertion within the *agr* sequence of strain R viii was deleted for cluster analysis.

C

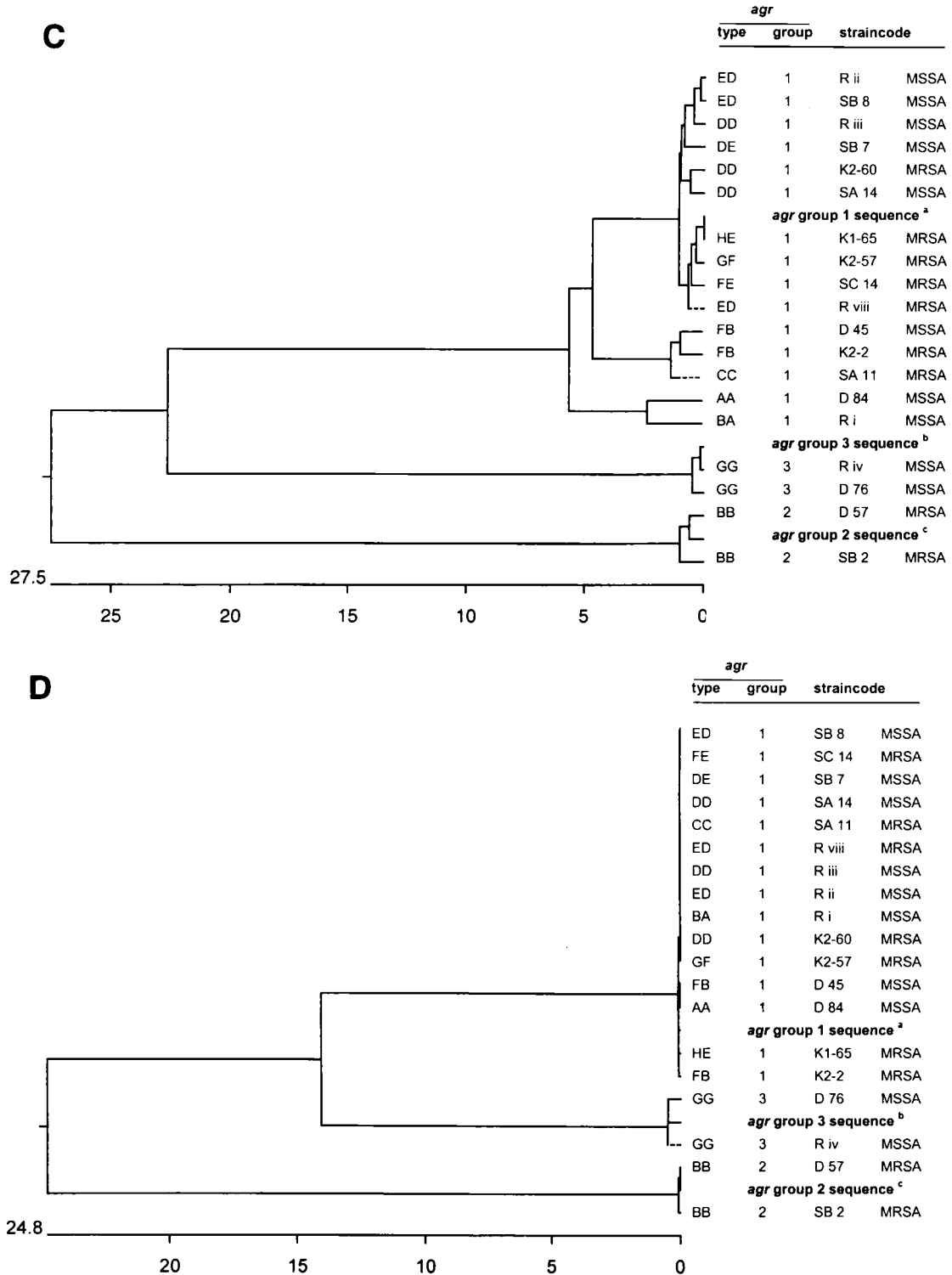| agr type	group	straincode	
ED	1	R ii	MSSA
ED	1	SB 8	MSSA
DD	1	R iii	MSSA
DE	1	SB 7	MSSA
DD	1	K2-60	MRSA
DD	1	SA 14	MSSA
agr group 1 sequence [a]			
HE	1	K1-65	MRSA
GF	1	K2-57	MRSA
FE	1	SC 14	MRSA
ED	1	R viii	MRSA
FB	1	D 45	MSSA
FB	1	K2-2	MRSA
CC	1	SA 11	MRSA
AA	1	D 84	MSSA
BA	1	R i	MSSA
agr group 3 sequence [b]			
GG	3	R iv	MSSA
GG	3	D 76	MSSA
BB	2	D 57	MRSA
agr group 2 sequence [c]			
BB	2	SB 2	MRSA

27.5

25 20 15 10 5 0

D

agr type	group	straincode	
ED	1	SB 8	MSSA
FE	1	SC 14	MRSA
DE	1	SB 7	MSSA
DD	1	SA 14	MSSA
CC	1	SA 11	MRSA
ED	1	R viii	MRSA
DD	1	R iii	MSSA
ED	1	R ii	MSSA
BA	1	R i	MSSA
DD	1	K2-60	MRSA
GF	1	K2-57	MRSA
FB	1	D 45	MSSA
AA	1	D 84	MSSA
agr group 1 sequence [a]			
HE	1	K1-65	MRSA
FB	1	K2-2	MRSA
GG	3	D 76	MSSA
agr group 3 sequence [b]			
GG	3	R iv	MSSA
BB	2	D 57	MRSA
agr group 2 sequence [c]			
BB	2	SB 2	MRSA

24.8

20 15 10 5 0

FIG. 3—*Continued.*

studies have demonstrated that the presence of a thiolactone group and the biochemical structure of the signal peptide (AgrD) are essential for stimulating biological activity (10). Cross-inhibition is less dependent on the structure of the signal peptide (12). Hypothetically, cross-inhibition of *agr* gene ex-

pression represents an example of bacterial interference that could be associated with colonization resistance or even competition of strains during infection.

***agr* clonality for MRSA and MSSA.** A major part (84%) of the MRSA strains harbored *agr* RFLP type AA or DE. The
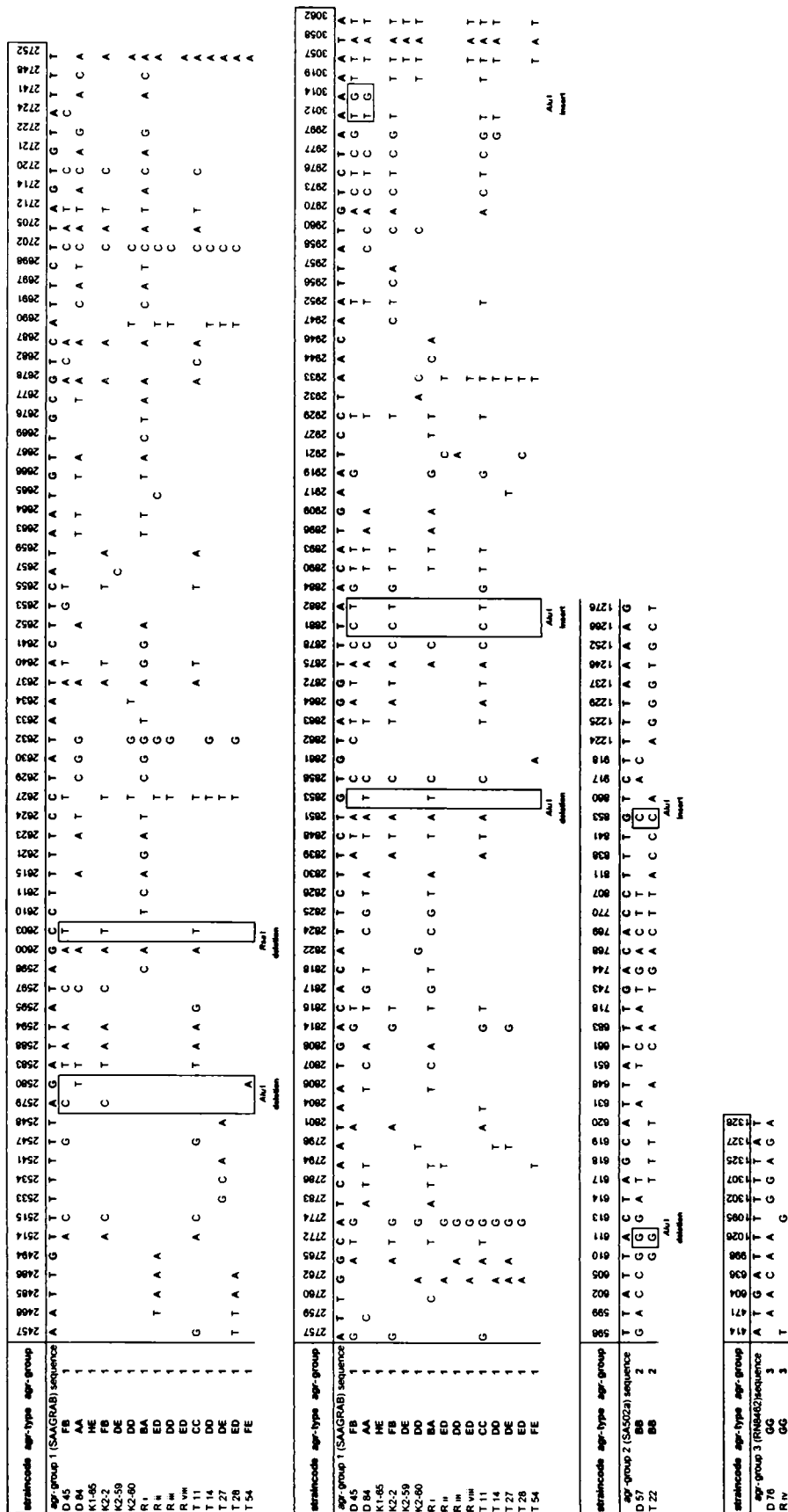
FIG. 4. Overview of the DNA sequences of the prevalent *agr* RFLP types subdivided into *agr* groups. The nucleotide positions of the detected sequence mutations, based on variation from the *agr* consensus sequences (three *agr* group control strains; GenBank), are indicated above. Identical residues are not indicated. Position numbers of the point mutations correspond with those indicated on the *agr* gene map (Fig. 1). Boxed areas, loss and gain of restriction sites for the enzymes *Alu*I and *Rsa*I.

TABLE 3. Comparative analysis of the amino acid sequence variability within the prevalent unique *agr* RFLP types and *agr* groups[a]

agr group	*agr* RFLP type	Strain code	Amino acid sequence of AgrD propeptide
1[b]			MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	FB	D45	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	AA	D84	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	HE	K1-65	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	FB	K2-2	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	DE	K2-59	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	DD	K2-60	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	BA	R i	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	ED	R ii	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	DD	R iii	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	ED	R viii	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPEELTQLHE
1	CC	T11	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	DD	T14	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	DE	T27	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVP_ELTQLHE
1	ED	T28	MNTLFNLFFDFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
1	FE	T54	
2[b]			MNTLVNMFFDFIIKLAKAIGIVG GVNACSSLF DEPKVPAELTNLYDK
2	BB	D57	MNTLVNMFFD_IIK_GNAIGIDG GVNACSSLF DEPKVPAELTNLYDK
2	BB	T22	MNTLVNMFFDFIIKWANFIGIVG GVNACSSLF DEPKVPAELTNLYDK
2[c]			GDSVCASYF
3[b]			MKKLLNKVIELLVDFFNSIGYRAA YINCDFLL DEAEVPKELTQLHE
3	GG	D76	MKKLLNKVIELLIDFFNSIGYRAA YINCDFLL DEAEVPKELTQLHE
3	GG	Riv	MKKLLNKVIELLVDFFNSIGYRAA YINCDFLL DEAEVPKELTQLHE

[a] The control sequences obtained from GenBank were as follows: *agr* group 1, SAAGRAB sequence, accession no. X52543; *agr* group 2, SA502a, accession no. AF001782; *agr* group 3, RN8462, accession no. AF001783. The sequences of the AgrD signal peptides are boxed. The sequences were aligned by MegAlign software (DNAStar). The signal peptide sequence of *S. epidermidis* Tü 3298 was derived from the study of Otto et al. (12). Sequence differences are underlined.

[b] Control sequence.

[c] *S. epidermidis* signal peptide sequence.

vast majority (98%) of RFLP type AA strains originated in the United States and were isolated in the 1980s. The other RFLP type (DE) is present in the majority (94%) of European strains isolated in the early 1960s. This geographical and temporal conservation of *agr* RFLP type among MRSA strains fits well with former observations regarding the clonal population structure of European and American MRSA (11). All MRSA strains belong to *agr* group 1, except for a small cluster of strains (*n* = 7) originating in Ontario, Canada (*agr* group 3). Ninety percent of the MSSA strains could be classified as *agr* group 1 as well. The remaining eight strains were indexed as *agr* group 2, and all had *agr* RFLP type BB. Strains that belong

FIG. 5. Comparative analysis of the AgrD propeptide sequences. Box, signal peptides; light grey, identical residues in all three sequences; dark grey, identical residues in two sequences.

TABLE 4. *agr* polymorphism among *S. aureus* strains isolated from both persistent and intermittent nasal carriers[a]

Person no.	Strain no.	Date of isolation (day-mo-yr)	*agr* type	*agr* group	RAPD type	No. of protA repeats	Coag gene type	Phage type
Persistent carriers								
53	44	09-03-92	DD	1	DDD	4	9C	641
53	45	31-08-92	FB	1	AAA	7	12A	793
53	47	01-11-93	DD	1	GGG	10	10F	693
53	48	15-08-94	DD	1	GGG	9	10F	542
84	54	09-03-92	AA	1	JIH	8	15I	692
84	55	18-04-93	AA	1	JIH	7	15I	692
84	56	01-11-93	ED	1	BBB	5	14B	811
84	57	15-08-94	BB	2	KJI	9	9C	292
126	58	19-10-92	BB	2	LKC	8	11N	793
126	59	18-04-93	ED	1	BBB	7	14B	812
126	60	15-08-94	ED	1	BBB	7	14B	811
Intermittent carriers								
85	61	24-02-92	AA	1	JIH	7	15I	311
85	62	09-03-92	ED	1	BBB	8	14B	812
85	63	06-01-93	ED	1	BBB	8	14B	232
85	64	15-08-94	AA	1	JIH	9	15I	362
2	69	25-06-92	ED	1	BBB	9	14B	813
2	70	19-10-92	ED	1	BBB	9	14B	691
2	71	18-04-93	AB	1	AAA	7	12A	793
2	72	14-02-94	ED	1	BBB	9	14B	811
9	73	25-06-92	ED	1	BBB	5	14B	811
9	74	19-10-92	BA	1	CCC	7	9C	292
9	75	01-11-93	BA	1	CCC	7	9C	233
9	76	15-08-94	GG	3	MLJ	5	—[b]	191
32	79	20-01-92	ED	1	BBB	3	14B	811
32	80	25-06-92	ED	1	BBB	3	14B	811
32	81	15-08-94	DD	1	NMK	9	9J	793
20	84	09-03-92	AA	1	PNC	8	10G	562
20	85	27-04-92	AA	1	CCC	10	9C	293
20	86	25-06-92	AA	1	BBB	10	14B	811
20	87	06-01-93	ED	1	AAA	7	12A	793
113	88	09-03-92	BB	2	JFH	7	10I	692
113	89	19-10-92	AA	1	BBB	10	14B	811
113	90	14-02-94	ED	1	OOL	9	9C	631

[a] Data on the carrier status and RAPD, protein A (protein A (protA), coag gene, and phage types are adapted from van Belkum et al. (17). The RAPD three-letter code summarizes the typing results for the primer used (first letter, primer AP-1; second letter, primer AP-7; third letter, primer ERIC-2). Numbers of protein A repeats were derived from an analysis of the so-called X region by PCR. Amplicons were digested with *RsaI*. The number of direct repeats in this region was determined after electrophoresis. Coag gene types were derived from a PCR-RFLP analysis of the coag gene and subsequent detection of the amplicon with *AluI*. Phage types are given in an abbreviated form according to standards applied in the Statens Seruminstitut, Copenhagen, Denmark.

[b] —, no restriction site for the amplicon.

to the latter group were geographically unlinked but genetically closely related. Strains appeared to be similar based on phage, ribo-, and multilocus enzyme electrophoresis typing. However, this strain cluster displayed diverged PFGE and RAPD patterns as if its members had evolved over time (data not shown). The index strain of a MRSA outbreak (strain collection 7) harbored part (approximately 1,000 bp) of the Tn*4001* transposon sequence, an aminoglycoside resistance determinant in *S. aureus* strains. Only the transposase gene was inserted in the 5′ part of *agrC*. The Tn*4001* sequence was absent in other outbreak strains. The potential effect of the transposon insertion on the activity of the *agr* operon was not analyzed and remains unclear, but it seems as if the 5′ part of *agrC* is not essential for staphylococcal viability.

agr polymorphism at the sequence level. The *agr* sequences from the agr RFLP types present in our *S. aureus* strains could be classified in one of the three different *agr* groups defined by Ji et al. (4). The main part (92.2%) of the *S. aureus* strains belong to *agr* group 1. Consequently, the evolutionary significance of the other *agr* groups is unclear. The amino acid sequences of AgrD from *agr* groups 2 and 3 are clearly different from that from *agr* group 1. Groups 2 and 3 both share do-

mains with group 1 but do not share domains with each other, except for sequences which are present in all three *agr* groups. The amino acid homology among the three different AgrD propeptide sequences suggests recombination.

Cluster analysis of the DNA sequences determined for the agr RFLP types demonstrated that *agrD* and parts of *agrB* and *agrC* have different evolutionary clock speeds. The observed stability of the *agrD* signal peptide sequence within each *agr* group confirms the results from the biochemical study with coagulase-negative staphylococci of Otto et al. (12) dealing with the relevance of the AgrD structure with respect to the ligand-receptor interaction specificity for *agr* activation. Overall, *agr* groups 1 and 3 are more closely related (80% sequence homology) than *agr* groups 1 and 2 (74% sequence similarity). Considering the *agr* sequences, there is no indication of major recombination events. It is interesting to see that AgrC sequence variation exceeds that of AgrD. Thus it could be speculated that the diversity of the AgrC receptor protein has a more profound influence than that of AgrD in the recognition reaction between noncanonical AgrD and AgrC proteins. This would imply that the reactivity of the peptides is largely defined by receptor polymorphism and not autoinducer peptide vari-

ability. However, this requires additional verification by biological experimentation.

***agr* incompatibility and colonization.** We studied the possible role of bacterial interference in relation to nasal *S. aureus* carriership. We therefore analyzed the *agr* locus polymorphism and the subsequent assignment to *agr* "incompatibility" groups (1, 2, and 3) among staphylococcal strains isolated from persistent and intermittent carriers. In both persistent and intermittent nasal carriers of *S. aureus*, strains with different genetic profiles, includng those identified by RAPD analysis, coag gene genotyping, and phage typing, and different *agr* groups alternate over time. Since carriers of *S. aureus* rarely carry two clones at the same time (17), *agr* grouping may well play a role. However, contrary to previous suggestions summarized above (4, 10), we here show that the carrier status is not restricted to a single *agr* group. RFLP analysis of the *agr* locus closely mimicked the results obtained with whole-genome typing. Although the numbers of individuals included in the present analysis are limited and although we did not determine biological *agr* activity, our data show both alternation and persistence of *agr* types. This is not in favor of a prominent biological function of the *agr* system in nasal colonization dynamics. This observation is further supported by the fact that the vast majority of *S. aureus* strains belong to *agr* group 1.

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