Staphylococcus aureus Isolates with Reduced Susceptibility to Glycopeptides Belong to Accessory Gene Regulator Group I or II

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We used multiplex PCR to determine the *agr* group membership of 18 European glycopeptide heterointermediate and intermediate-resistant *Staphylococcus aureus* strains. Of the 15 *agr* group I strains, 13 were resistant and 2 were susceptible to methicillin. The remaining three strains, like the United States and Japanese control strains, belonged to *agr* group II.

Glycopeptide intermediate-resistant *Staphylococcus aureus* (GISA) strains have been isolated in many countries since 1997 (2–4, 12, 17, 18, 20, 21, 23). They are defined by vancomycin MICs of 8 to 16 $\mu\text{g/ml}$ (13, 16). Glycopeptide heterointermediate *S. aureus* (hGISA) strains (11) are susceptible to vancomycin (as determined on the basis of conventional criteria) but contain subpopulations ($\sim 10^{-6}$) which grow in the presence of vancomycin at concentrations of $\geq 4 \mu\text{g/ml}$ (11). These two phenotypes of low-level glycopeptide resistance do not involve the *van* genes that confer vancomycin resistance in enterococci and in some rare *S. aureus* strains with high-level glycopeptide resistance (5) but seem to be related to antimicrobial sequestration by nonamidated mucopeptides within a thickened cell wall (6, 10).

To study whether the expression of this resistance mechanism could be related to global regulatory pathway of *S. aureus*, Sakoulas et al. analyzed hGISA and GISA strains for variations in the locus of the accessory gene regulator (*agr*) (19), a global regulon controlling the expression of about a hundred *S. aureus* genes involved in virulence, metabolism, transport, and degradation pathways (8). *Agr* comprises a quorum-sensing module producing an autoinducing peptide (AIP) (encoded by *agrD*) and AgrA-C, a two-component system in which AgrC is a response regulator sensing the AIP and AgrA is the effector. Variability in AIP and AgrC defines four mutually exclusive alleles of the *agr* system (groups I to IV) (15). Sakoulas et al. found that all hGISA and GISA strains from Japan and the United States belonged to *agr* group II (19). However, the exclusive representation of the *agr* group II in these strains reflected the predominance of the *agr* group II among methicillin-resistant *S. aureus* (MRSA) isolates from the Boston hospitals studied. In contrast, *agr* group I MRSA strains could predominate in other settings (22a), raising the

question of whether hGISA and GISA strains could belong to *agr* group I in these parts of the world.

We tested this hypothesis by analyzing 18 clinical hGISA and GISA strains isolated between 1998 and 2002 in France (17 strains, including LIM-2 [17] and MER-23 [2, 18]) or Belgium (1 strain [P1V44]) (7) (Table 1). The reference strains Mu3, Mu50, Michigan, and New Jersey (11, 12, 21) were also studied. The characteristics of these strains were compared to those of 35 non-hGISA and non-GISA MRSA strains recovered during the same period of time and in the same geographical area. Strain *S. aureus* ATCC29213 (which is susceptible to methicillin) was used as a control.

All strains were confirmed to be *S. aureus* strains by coagulase testing with rabbit plasma (bioMérieux, Marcy-l'Etoile, France) and a Staphyslide agglutination test (bioMérieux). MICs in Mueller-Hinton medium (bioMérieux) were determined using the broth macrodilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (16). Macromethod E-tests (AB BIODISK, Solna, Sweden), a heavy inoculum (adjusted to a McFarland standard of 2), brain-heart infusion agar (bioMérieux), and a 48-h incubation period were used as described by Wootton et al. (23) to detect hGISA. Population analysis was based on the method of Hiramatsu et al. (11).

The results we obtained with previously described strains were compatible with published data: the vancomycin MICs for strains Mu50, LIM-2, MER-23, Michigan, New Jersey, and P1V44 were determined by the NCCLS method to be $\geq 8 \mu\text{g/ml}$, and the strains were thus confirmed to be GISA strains. As expected, the vancomycin MIC for Mu3, a hetero-GISA strain, was $< 8 \mu\text{g/ml}$; however, the examined strain comprised subpopulations able to grow in the presence of vancomycin at concentrations higher than $> 4 \mu\text{g/ml}$ (22). The 15 remaining French strains had vancomycin MICs of $< 8 \mu\text{g/ml}$ and met the definition of hetero-GISA strains as determined by population analysis (Table 1). E-tests with the enhanced culture conditions described above (23) showed elevated MICs: the vancomycin MICs increased to 8 to 32 $\mu\text{g/ml}$ for GISA strains and to 4 to 16 $\mu\text{g/ml}$ for hGISA strains, and the teicoplanin MICs rose

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TABLE 1. Methicillin, vancomycin, and teicoplanin MICs for 22 *S. aureus* strains recovered from the United States, Japan, and Europe

Strain designation	Geographic origin	Methicillin, resistance phenotype	<i>agr</i> group	MIC by standard macrodilution ($\mu\text{g/ml}$)		MIC by modified E-test, method ($\mu\text{g/ml}$)		Glycopeptide resistance phenotype	Reference(s) or source
				Vancomycin	Teicoplanin	Vancomycin	Teicoplanin		
Mu3	Japan	MRSA	II	1	8	4	16	hGISA	11
Mu50	Japan	MRSA	II	8	16	12	24	GISA	12
LIM-2	France	MRSA	I	8	8	8	24	GISA	17
Michigan	United States	MRSA	II	8	16	24	48	GISA	21
New Jersey	United States	MRSA	II	8	4	12	12	GISA	21
P1V44	Belgium	MRSA	I	8	8	32	96	GISA	7
MER-23	France	MSSA	II	8	16	24	64	GISA	2, 18
Vers. A2	France	MRSA	I	2	4	6	16	hGISA	Present study
Vers. A1	France	MRSA	I	2	4	8	24	hGISA	Present study
LAC	France	MRSA	I	2	4	8	24	hGISA	Present study
MAS	France	MRSA	II	2	4	16	12	hGISA	Present study
AUP	France	MRSA	I	4	4	8	24	hGISA	Present study
PAD	France	MRSA	I	2	4	8	24	hGISA	Present study
PAR	France	MRSA	I	4	4	12	12	hGISA	Present study
FAN	France	MRSA	I	4	4	12	12	hGISA	Present study
DUR	France	MRSA	I	2	4	12	16	hGISA	Present study
RAB	France	MSSA	I	2	4	12	24	hGISA	Present study
PEY	France	MSSA	II	2	4	6	16	hGISA	Present study
GRA	France	MRSA	I	2	4	4	12	hGISA	Present study
PRO	France	MRSA	I	4	8	12	32	hGISA	Present study
BLA	France	MRSA	I	2	8	16	48	hGISA	Present study
PON	France	MSSA	I	2	4	12	64	hGISA	Present study
ATCC 29213	Reference strain	MSSA	II	2	1	2	2	Non-hGISA–non-GISA	

to 12 to 96 $\mu\text{g/ml}$ for GISA strains and to 12 to 64 $\mu\text{g/ml}$ for hGISA strains (Table 1). These differences (detected under NCCLS and enhanced E-test conditions) between strains with respect to levels of susceptibility to vancomycin and teicoplanin appear to be related to the selection of resistant subpopulations by the latter technique (2, 18, 23). The MICs of vancomycin and teicoplanin (as determined by the standard method for the 35 non-hGISA and non-GISA isolates and the reference *S. aureus* strain) were ≤ 2 $\mu\text{g/ml}$, and the isolates contained no subpopulations which grew in the presence of ≥ 4 μg of vancomycin/ml (11).

The *agr* groupings of the complete collection of strains were then determined by multiplex PCR with primers designed using the *agr* I to IV sequences as described elsewhere (15). Most (15 of 22) hGISA and GISA strains (13 MRSA and 2 methicillin-sensitive *S. aureus* [MSSA] strains, all from Europe) belonged to *agr* group I. Seven strains belonged to *agr* group II. They comprised five previously described (19) MRSA strains isolated in Japan or the United States (Mu3, Mu50, Michigan, and New Jersey) and also one MRSA strain and two MSSA strains from France. None of the strains belonged to *agr* group III or IV. The non-hGISA and non-GISA MRSA strains belonged mainly to *agr* group I (31 of 35 strains) and, less frequently, to *agr* group II (4 of 35). The 22 hGISA and GISA strains and the 35 non-hGISA and non-GISA MRSA strains were then analyzed by pulsed-field gel electrophoresis (PFGE) of *Sma*I-restricted chromosomal DNA (9). The *agr* group I hGISA and GISA strains (with the exception of strain PRO, which was distinct from the rest of the group) were closely related to one another. The 31 non-hGISA and non-GISA MRSA strains of *agr* group I had PFGE patterns closely related to each other (with an index of similarity of $>80\%$), reflecting the clonality of the hospital-acquired MRSA strains spreading in France. Surprisingly, the PFGE patterns

of these strains (except for that of the PRO strain) were not related to those of the *agr* group I hGISA and GISA isolates (Fig. 1). This suggests that (with the exception of strain PRO) French *agr* group I hGISA and GISA strains have mainly emerged from an *agr* group I background not detected in our collection.

The Japanese and United States strains (Mu3, Mu50, Michigan, and New Jersey), together with strain MER-23, all of which belonged to *agr* group II, showed distinct but related PFGE patterns (Fig. 1). One of the non-hGISA and non-GISA MRSA strains of *agr* group II (strain 58-1) had a PFGE pattern closely related to that of strain MER-23 (a GISA MSSA strain), suggesting that the same genetic background independently acquired either the *mecA* element (for strain 58-1) or the glycopeptide resistance (for strain MER-23). The three other non-hGISA MRSA strains of the *agr* group II (designated 104-1, 22-22, and 19-5) had different PFGE patterns not related to those of European hGISA and GISA strains (Fig. 1). The four methicillin-susceptible hGISA and GISA strains (designated RAB, MER-23, PEY, and PON) had clearly distinct PFGE patterns. Thus, European methicillin-susceptible and methicillin-resistant hGISA and GISA strains are unlikely to have arisen from a single clone; it is more probable that several distinct strains from preexisting MRSA or MSSA strains emerged independently.

Conflicting reports respecting this question have been published. A Korean study of 4,483 consecutive clinical MRSA and MSSA isolates showed that the macrorestriction patterns of glycopeptide-susceptible MRSA isolates were very different from those of GISA strains isolated during the same period (14). In contrast, a retrospective analysis of 457 German MRSA and MSSA isolates showed that the PFGE patterns of most GISA isolates resembled those of common epidemic MRSA strains (1).

FIG. 1. PFGE patterns and phylogenetic tree of 22 GISA and hGISA isolates and 35 non-GISA and non-hGISA *S. aureus* isolates from the United States, Japan, and Europe. Isolate designations in boldface characters indicate the GISA-hGISA phenotype. *Sma*I macrorestriction patterns were digitized and analyzed with Taxotron software (Institut Pasteur, Paris, France) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair group method using arithmetic averages (unweighted pair group method with arithmetic mean) clustering. The scale indicates the levels of pattern similarity. The fragment size (in kilobases) of reference strain NCTC8325 is indicated on the bottom lane.

In conclusion, almost all European hGISA and GISA strains appear to belong to *agr* group I or II. The results of macrorestriction analysis, and the fact that these strain groups contain both MSSA and MRSA strains, argue against a unique clonal origin. Moreover, with one exception, there was no obvious genetic relationship between our hGISA and GISA

strains and the predominant hospital MRSA strains from the same geographical area.

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