

# Methicillin-Resistant, Quinupristin-Dalfopristin-Resistant *Staphylococcus aureus* with Reduced Sensitivity to Glycopeptides

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Of 3,052 *Staphylococcus aureus* strains collected by the European SENTRY surveillance study, 35 were found to be nonsusceptible to quinupristin-dalfopristin (MIC of  $\geq 2$  mg/liter). These isolates originated from four hospitals in France and one in Spain. In isolates from two Parisian hospitals exhibiting the same *Sma*I macrorestriction pattern, streptogramin resistance was based on *vatA* and *vgaA*. One isolate from a hospital in Lyon and 22 from a hospital in Lille were of the *vatB vgaB* streptogramin A resistance genotype and possessed *ermA* and/or *ermC*. As deduced from the loss of either streptogramin A or streptogramin B resistance determinants in particular isolates, resistance to quinupristin-dalfopristin requires mechanisms conferring resistance to both compounds. The *Sma*I macrorestriction patterns of strains from hospitals in Lille and Lyon were different; however, similarity analysis suggested a relatedness of 20 methicillin-resistant *S. aureus* strains from the Lille hospital, a finding confirmed by PCR typing based on three different genomic polymorphisms. These groups of isolates were found to be hetero-glycopeptide-intermediate susceptible *S. aureus*. Information about the failure of glycopeptide chemotherapy has not been available.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become an important nosocomial pathogen in many countries. Glycopeptides have been the drugs of choice for treating MRSA infections. However, with the emergence of intermediate susceptibility to glycopeptides, first reported from sporadic cases in many countries (12, 20) and more recently also from an outbreak of infections (11), other treatment alternatives such as quinupristin-dalfopristin and linezolid have become important. Quinupristin-dalfopristin is a combination of streptogramin B and A compounds with a synergistic activity against most gram-positive bacteria (8, 14).

Resistance to the B component is mediated by methylation of 23S rRNA which confers macrolide-lincosamide-streptogramin B (MLS<sub>B</sub>) resistance when the *erm* genes are expressed constitutively (7). A second mechanism is hydrolysis of the drug by a lactonase encoded by the *vgaA* and the *vgaB* genes, respectively (1, 6). Two kinds of mechanisms are known to mediate resistance to the A compound: inactivation of the drug by acetylation and efflux. In staphylococci, three acetyltransferase genes, *vatA*, *vatB*, and *vatC* (3, 4, 6) and two genes for efflux pumps (ABC porters), *vgaA* and *vgaB* (2, 5), have been described. These streptogramin A and B resistance genes are often located on the same plasmids (6).

In this study we describe quinupristin-dalfopristin-resistant *S. aureus* strains collected during the course of the European SENTRY surveillance study in European countries (18). These strains were characterized in our laboratory with regard to streptogramin resistance genes, phenotypic resistance to other antibiotics, and genotype.

## MATERIALS AND METHODS

**Bacterial isolates.** A total of 35 isolates of *S. aureus* exhibiting insensitivity to quinupristin-dalfopristin originated from the strain culture collection of the European SENTRY study (18). *S. aureus* ES1767 (*vatA vgaA vgaB*), ES1768 (*vatB vgaB*), and ES1877 (*vatC vgaB*) were kindly provided by N. El Solh, Paris, France; *S. aureus* Mu 50 (13) was provided by K. Hiramatsu, Tokyo, Japan.

**Antimicrobial susceptibility testing.** Isolates were tested by broth microdilution assay performed according to the NCCLS standard (16) and by using Isosensitest broth from Oxoid. The prepared panels were immediately stored at  $-20^{\circ}\text{C}$ . *S. aureus* ATCC 25923 served as a control for the activity of the antibiotics tested and strains ES1767, ES1768, and ES1877 as controls for the expression of resistance to streptogramin compounds. The antibiotics tested included penicillin G, oxacillin, gentamicin, erythromycin, clindamycin, oxytetracycline, ciprofloxacin, moxifloxacin, sulfonamide-trimethoprim, fusidic acid, rifampin, quinupristin-dalfopristin (Synecid), quinupristin, dalfopristin, vancomycin, teicoplanin, and linezolid. Breakpoints were according to NCCLS standards (16), except for those antibacterials not included, such as phosphomycin (resistance,  $\geq 128$  mg/liter), fusidic acid ( $\geq 4$  mg/liter), and linezolid ( $\geq 8$  mg/liter).

**Genotyping and PCR.** *Sma*I macrorestriction patterns were obtained as described previously (24). The CHEF-III apparatus from Bio-Rad (Munich, Germany) was used with the following conditions: a charge of 6 V/cm, pulse times of 5 to 15 s for 7 h and 15 to 60 s for 19 h, an angle of  $120^{\circ}$ , and a temperature of  $14^{\circ}\text{C}$ . Image processing and cluster analysis for similarity were performed according to the method of Claus et al. (10): this program converts band positions into molecular masses by use of the patterns of *S. aureus* 8325 as an internal standard. Molecular mass patterns are analyzed for similarity by means of a matching algorithm. PCR typing by means of amplicon patterns for the 16S-23S rRNA gene spacer, the DNA stretches flanked by the transposon Tn916 attachment region and Shine-Dalgarno sequence (tar916-shida), and the ERIC-2 sequence were performed as described previously (24).

The following PCR primers were used for the detection of streptogramin resistance genes: the acetyltransferase gene *vatA* was amplified with primers *vatA1* (5'-CAA TGA CCA TGG ACC TGA TC) and *vatA2* (5'-AGC ATT TCG ATA TCT CC), *vatB* was amplified with primers *vatB1* (5'-CCT GAT CCA AAT AGC ATA TAT CC) and *vatB2* (5'-CTA AAT CAG AGC TAC AAA GTG), and *vatC* was amplified with primers *vatC1* (5'-TGG CAA AAT CAG CAA GG) and *vatC2* (5'-TCG TCT CTA TCT CTA GGT CC), the genes encoding efflux pumps *vgaA* were amplified with primers *vgaA1* (5'-AGT GGT GGT GAA GTA ACA CG) and *vgaA2* (5'-CTT GTC TCC TCC GCG AAT AC), and those encoding *vgaB* were amplified with primers *vgaB1* (5'-TCT CTC AAT TAG AAG AAC C) and *vgaB2* (5'-TTA TCT ATT CGT GTT TCC) (except for the primers for *vgaB* [3, 4, 6]). The streptogramin B resistance determinants *vgaA* and *vgaB* were amplified using primers *vgaA1* (5'-CCA GAT TCA GCA CCC

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TABLE 1. Origin, grouping according to genotypes, resistance phenotypes, and particular resistance genes in quinupristin-dalfopristin-resistant *S. aureus*<sup>a</sup>

Origin (hospital)	<i>Sma</i> I pattern	No. of isolates	Resistance phenotype	MIC (mg/liter)						Streptogramin resistance genes
				VAN	TEC	LNZ	Q-D	QUI	DAL	
Paris (Salpetriere)	A	1	PEN, OXA, CIP, MXF, Q-D	0.5	0.5	1.0	8	16	32	<i>vat</i> (A), <i>vgb</i> (A)
Paris (St. Joseph)	A	1	PEN, OXA, GEN, ERY, CLI, OTE, SXT, CIP, MXF, RIF	0.5	0.5	1.0	2	32	2	
	A	1	PEN, OXA, GEN, ERY, CLI, OTE, SXT, CIP, MXF, RIF, Q-D	0.5	0.5	1.0	8	64	32	<i>vat</i> (A), <i>vgb</i> (A)
Lyon	B	1	PEN, OXA, PHO, GEN, ERY, CLI, OTE, SXT, CIP, MXF, RIF, Q-D	0.5	0.5	0.5	8	64	32	<i>vat</i> (B), <i>vga</i> (B)
Lille	C	2	PEN, OXA, GEN, ERY, CLI, OTE, CIP, MXF, SXT, RIF, FUS, Q-D	2.0	2.0	0.5	8	64	32	<i>vat</i> (B), <i>vga</i> B
	D	6	PEN, OXA, GEN, ERY, CLI, OTE, CIP, MXF, SXT, RIF, FUS, Q-D	2.0	2.0	0.5	8	64	32	<i>vat</i> (B), <i>vga</i> (B)
	D1	1	PEN, OXA, GEN, ERY, CLI, OTE, CIP, MXF, SXT, RIF, FUS, Q-D	2.0	2.0	0.5	8	64	32	<i>vat</i> (B), <i>vga</i> (B)
	D2	1	PEN, OXA, GEN, ERY, CLI, OTE, CIP, MXF, SXT, RIF, FUS, Q-D	2.0	2.0	0.5	8	64	32	<i>vat</i> (B), <i>vga</i> (B)
	E	7	PEN, OXA, GEN, ERY, CLI, OTE, CIP, MXF, SXT, RIF, FUS, Q-D	2.0	2.0	0.5	8	64	32	<i>vat</i> (B), <i>vga</i> (B)
	E1	1	PEN, OXA, GEN, ERY, CLI, OTE, CIP, MXF, SXT, RIF, FUS, Q-D	2.0	2.0	0.5	8	64	32	<i>vat</i> (B), <i>vga</i> (B)
	F	2	PEN, OXA, GEN, ERY, CLI, CIP, MXF, Q-D	2.0	2.0	0.5	8	64	32	<i>vat</i> (B), <i>vga</i> (B)
	G	1	PEN, OXA, GEN, ERY, CLI, OTE, CIP, MXF, SXT, FUS, RIF, Q-D	2.0	2.0	0.5	8	64	32	<i>vat</i> (B), <i>vga</i> (B)
	G	2	PEN, OXA, GEN, OTE, CIP, MXF, SXT, FUS, RIF	2.0	2.0	0.5	2	8	32	<i>vat</i> (B), <i>vga</i> (B)
	G	2	PEN, OXA, GEN, ERY-ind., OTE, CIP, MXF, SXT, FUS, RIF	2.0	2.0	0.5	2	8	32	<i>vat</i> (B), <i>vga</i> (B)
	H	2	PEN, OXA, ERY, CLI, OTE, CIP, MXF, Q-D	0.5	0.5	0.5	2	8	32	<i>vat</i> (B), <i>vga</i> (B)
I	1	ERY, Q-D	0.5	0.5	0.5	4	16	16	<i>vat</i> (B), <i>vga</i> (B)	
Seville	J	1	PEN, ERY, CLI, CIP, MXF, Q-D	0.5	0.5	0.5	4	32	32	

<sup>a</sup> PEN, penicillin G; OXA, oxacillin; CIP, ciprofloxacin; MXF, moxifloxacin; Q-D, quinupristin-dalfopristin (Synercid); GEN, gentamicin; ERY, erythromycin; ERY-ind., inducible erythromycin resistance; CLI, clindamycin; OTE, oxytetracycline; SXT, trimethoprim-sulfamethoxazole; RIF, rifampin; FUS, fusidic acid; VAN, vancomycin; TEC, teicoplanin; LNZ, linezolid; QUI, quinupristin; DAL, dalfopristin.

TAC G) and *vgbA2* (5'-CCC CCC ATT CAG TAA ACC), as well as *vgbB1* (5'-CAG CAG TCT AGA TCA GAG TGG) and *vgbB2* (5'-CAT ACG GAT CCA TCT TTT CC) (1, 6). For *erm* genes the primers were used as described previously (9, 19). The PCR mixture consisted of ca. 20 ng of DNA, 100 pmol of each primer, a 200  $\mu$ M concentration of each of the deoxyribonucleotides, and 2.5 U of the Replithern polymerase (Biozym, Markoldendorf, Germany) with an appropriate buffer. Initial denaturation at 94°C for 5 min was followed by 30 cycles of amplification with 94°C for 1 min, annealing at 50 to 58°C for 1 min, and extension at 72°C for 1 min (except for the last cycle with an extension step of 4 min).

**Screening test for detection of GISA.** Brain heart infusion (BHI) agar plates containing 6 mg of vancomycin per liter were inoculated as described elsewhere (22). *S. aureus* ATCC 25923 was used as a control for the activity of vancomycin, and strain *S. aureus* Mu 50 was used as a control for the expression of the glycopeptide-intermediate susceptible *S. aureus* (GISA) phenotype. GISA strains are strains for which vancomycin MICs are 4 to 8 mg/liter (22).

**Population analysis for GISA.** This was performed on BHI agar as described previously (13). Each experiment was performed in triplicate.

## RESULTS

Of 3,052 *S. aureus* strains isolated during the European SENTRY surveillance study in 1997 and 1998 from 24 European hospitals, 32 isolates exhibited MICs of quinupristin-dalfopristin of  $\geq 2$  mg/liter: for 6 isolates, the MIC was 2 mg/liter, for 2 isolates it was 4 mg/liter, and for 24 isolates it

was 8 mg/liter. The results of characterization are shown in Table 1.

All 32 isolates have been typed by means of *Sma*I macrorestriction patterns separated by pulsed-field gel electrophoresis. Ten different patterns (A to J) could be discriminated when a difference in four and more fragments was used as a criterion (as described in reference 21).

Analysis of these patterns for similarity (Fig. 1A) leads to different lineages, which indicate but not necessarily document clonal relatedness. Therefore, three additional genomic polymorphisms were assessed by PCR (see Materials and Methods). PCR typing revealed five different, unrelated clusters (clonal groups I to V): for isolates with *Sma*I pattern I (PCR pattern I), I (PCR pattern III), J (PCR pattern IV), and H (PCR pattern V). Isolates with *Sma*I patterns B to G belonged to a group of ancestral relatedness because they all exhibit PCR pattern II (Fig. 1B).

The two isolates of *Sma*I pattern A were MRSA and originated from two Parisian hospitals. Both carried *vatA* and *vgbA*. It is of particular interest that the isolate from the Salpetriere Hospital lacked macrolide-lincosamide resistance and did not possess any of the tested *erm*(A/B/C) genes. The isolate from

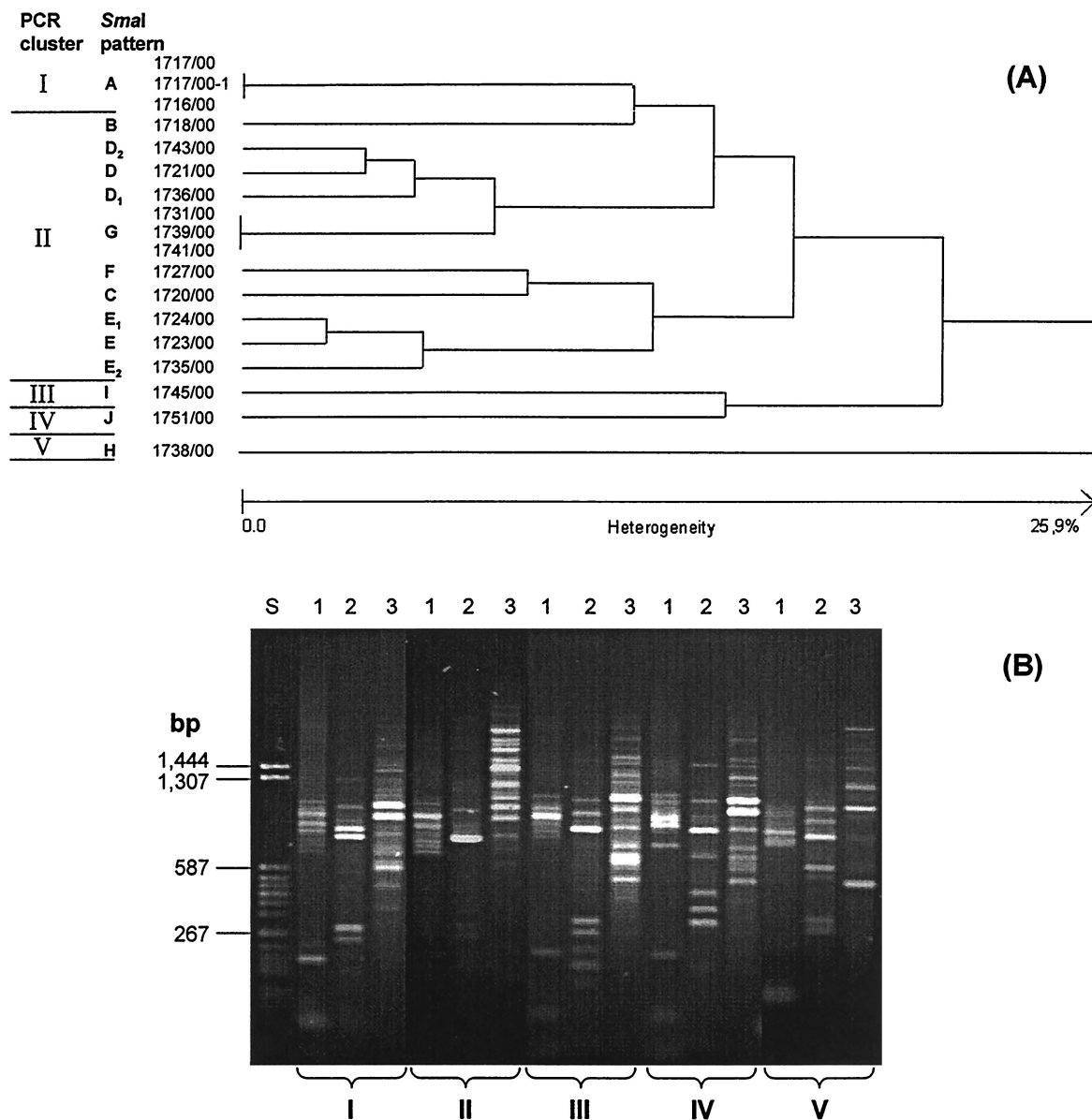


FIG. 1. Dendrogram of similarity of *SmaI* macrorestriction patterns of quinupristin-dalfopristin-resistant *S. aureus* strains in relation to patterns of PCR typing. (A) Similarity dendrogram. (B) PCR typing patterns found in quinupristin-dalfopristin-resistant *S. aureus*. Lanes: 1, rRNA gene spacer; 2, tar916-shida; 3, ERIC-2; S, molecular mass standard.

St. Joseph Hospital exhibited a broader resistance phenotype, including erythromycin-clindamycin resistance encoded by the *ermC* determinant. A spontaneous in vitro derivative of this isolate (1717/00-1) detected by lacking hemolysis on blood agar obviously was determined to be only intermediately susceptible to quinupristin-dalfopristin and has lost *vata* and *vgaA*.

Isolates of *SmaI* patterns B to G (PCR typing cluster II) are MRSA strains and originated from a hospital in Lille. These strains carried the *vatB* and *vgaB* streptogramin A resistance genes. Isolates belonging to patterns B, C, D, E, and F possessed *ermA* and partly also *ermC*. Of particular interest were the five isolates exhibiting pattern G. Isolate 1741/00 harbored *ermC* and was found to be constitutively resistant to erythro-

mycin and lincomycin and also resistant to quinupristin-dalfopristin (MIC, 8 mg/liter). Two isolates (1739/00 as an example in Fig. 1A) also possessed *ermC* but exhibited an inducible MLS<sub>B</sub> resistance, as demonstrated by disk diffusion (reduction of the inhibition zone of lincomycin by a neighboring erythromycin disk). The MIC of quinupristin-dalfopristin was 2 mg/liter. Two isolates (1731/00 as an example in Fig. 1A) had no *erm* determinant, were sensitive to erythromycin and to lincomycin, and exhibited an MIC of 2 mg/liter of quinupristin-dalfopristin.

All isolates of PCR cluster II exhibited an elevated MIC (2 to 4 mg/liter) of vancomycin and teicoplanin and grew on screening plates for assessing the GISA phenotype. When we performed a population analysis, these isolates were deter-

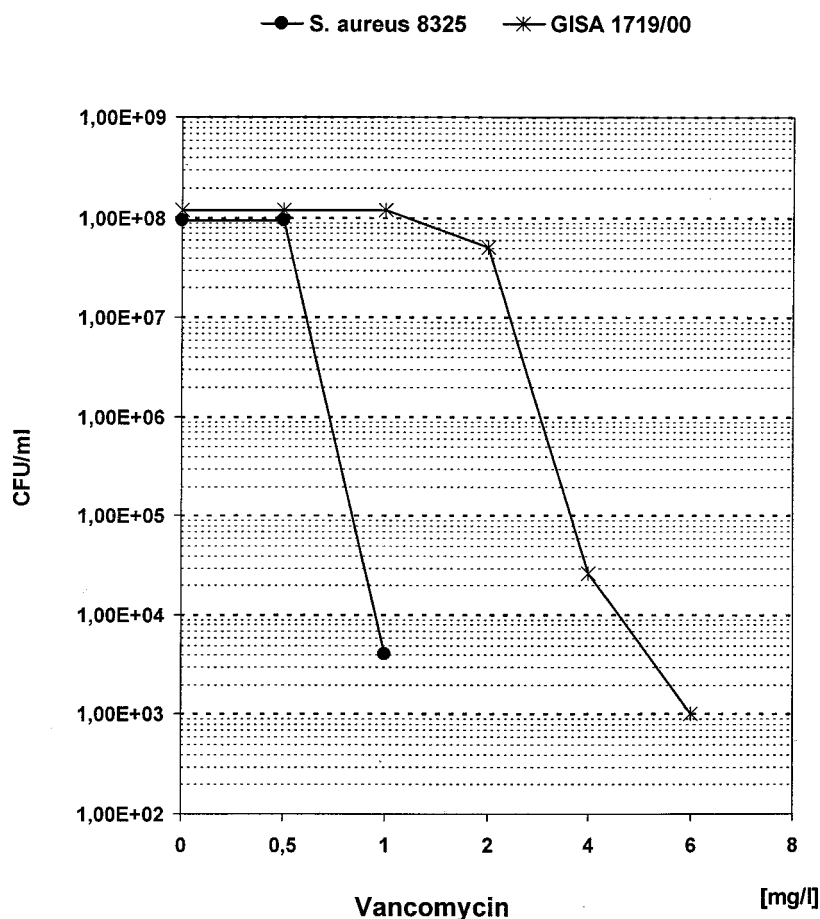


FIG. 2. Population analysis of GISA 1719/00 and *S. aureus* 8325.

mined to be hetero-GISA, as shown for strain 1719/00 as a representative in Fig. 2. For the antimicrobials checked, only MICs of phosphomycin and linezolid were in the sensitive range.

The two MRSA strains with *Sma*I pattern H (PCR cluster V) from Lille possessed *vatB* and *vgaB*, as well as *ermC*, but exhibited an MIC of quinupristin-dalfopristin of only 2 mg/liter. Another isolate from Lille which was not an MRSA (phenotypically sensitive, no *mecA* demonstrated) and belonged to a quite separate genotype (*Sma*I pattern I, PCR cluster III) had an MIC of quinupristin-dalfopristin of 4 mg/liter but also possessed *vatB*, *vgaB*, and *ermC*. One isolate from a hospital in Seville (1751/00) exhibited an MIC of 4 mg/liter of quinupristin-dalfopristin and resistance to both compounds when tested separately. It possessed *ermC* (constitutively expressed), but none of the known determinants conferring resistance to streptogramin A could be detected.

## DISCUSSION

Strain collection by the European SENTRY study in 1997 and 1998 was performed before quinupristin-dalfopristin was licensed in Europe. All but one streptogramin-resistant *S. aureus* strain originated from three French hospitals. This is most probably due to selective pressure by the use of pristinamycins

in France, although the consumption volume for this antibiotic has not been substantial. In the resistant strains, streptogramin A resistance genes have been detected as already described for *S. aureus* and other staphylococci from this country (15). The results from this study further show that different mechanisms of resistance to both compounds are essential for quinupristin-dalfopristin resistance in *S. aureus*. As evident from the *Sma*I pattern A strain originating from the Salpetriere Hospital, *S. aureus* can achieve quinupristin-dalfopristin resistance by the *vatA*-encoded acetyltransferase (inactivation of streptogramin A) and the *vgaA*-encoded lactonase (inactivation of streptogramin B). In other isolates resistance to the streptogramin A-streptogramin B combination is based on acetylation and efflux for streptogramin A resistance and *erm*-encoded 23S rRNA methylases for streptogramin B resistance. The loss of resistance either to streptogramin A or to streptogramin B leads to only intermediate susceptibility, as concluded from the data for isolates of *Sma*I patterns A (1717/00-1) and G (1731/00).

A cluster of related MRSA strains possessing *vatB* and *vgaB* originated from two hospitals, namely, in Lyon and Lille, with a broader dissemination in the latter one. The *vatB* and *vgaB* determinants have also been found in two *S. aureus* strains from the hospital in Lille; these strains exhibited other geno-

typical typing patterns, which suggests a dissemination between different strains of the same hospital. In both of the *SmaI* pattern H (PCR cluster V) isolates, the MIC of quinupristin-dalfopristin is only 2 mg/liter. Although they are fully resistant to erythromycin and lincomycin, the MIC of quinupristin was only 8 mg/liter.

*S. aureus* only possessing constitutively expressed *erm* genes is still not resistant to quinupristin-dalfopristin (breakpoint of  $\geq 4$  mg/liter). As known from earlier studies (15), resistance to both streptogramins needs additional streptogramin A resistance determinants. Prevention of further dissemination of streptogramin A resistance genes requires early detection in bacteriological routine. This might be problematic when quinupristin-dalfopristin is tested as a combination. Therefore, a separate testing of dalfopristin resistance is desirable as has already been suggested (4).

In one isolate from a hospital in Seville resistance to quinupristin-dalfopristin was found, although no streptogramin A resistance genes were detected. There are obviously further genes (mechanisms?) conferring streptogramin A resistance.

All but two of the quinupristin-dalfopristin-insensitive isolates are MRSA strains, and most of them are already resistant to other classes of antibiotics.

All isolates from PCR cluster II of the *SmaI* patterns C to G from the Lille hospital were determined to be hetero-GISA. They are only susceptible to phosphomycin and linezolid. We have no information about failure of glycopeptide chemotherapy in the patients affected. However, this has recently been described for infections with hetero-GISA also exhibiting an MIC of 2 mg/liter of vancomycin (23). Recently, an outbreak of MRSA strains which were also determined to be hetero-GISA has been reported from Boroussias Hospital in Paris (11), and GISA isolates have also been reported from two hospitals in Lyon (17). These isolates, however, have been found to be susceptible to quinupristin-dalfopristin.

PCR typing of the hetero-GISA by means of three independent DNA polymorphisms, with each of them covering a comparably small (PCR-detectable) region of the genome, revealed a genotypical relatedness, although these isolates clearly exhibited different *SmaI* macrorestriction patterns that are due to genomic rearrangements over the whole genome. This indicates that these particular MRSA strains were already endemic to the hospital in Lille for a longer period of time.

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