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Phenotypic and Molecular Typing of Nosocomial Methicillin-Resistant *Staphylococcus aureus* Strains Susceptible to Gentamicin Isolated in France from 1995 to 1997

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Methicillin-resistant strains susceptible to gentamicin (Gm^s MRSA) have emerged since 1993 in several French hospitals. To study whether particular clones have spread in various French cities and whether some clones are related to gentamicin-resistant (Gm^r) MRSA strains, various methods (antibiotyping, phage typing, determination of *Sma*I macrorestriction patterns before and after hybridization with IS*256* transposase and *aacA-aphD* probes) were used to compare 62 Gm^s MRSA strains isolated from 1995 to 1997 in nine cities and 15 Gm^r MRSA strains. Eighteen major *Sma*I genotypes were identified, of which 11 included only Gm^s MRSA strains and 5 included only Gm^r MRSA strains. Each of the Gm^r MRSA strains contained 6 to 13 *Sma*I fragments hybridizing with the insertion sequence IS*256*, of which a single band also hybridized with the *aacA-aphD* gene. No such hybridizing sequences were detected in 60 of the 62 Gm^s MRSA strains. Thus, the divergence between Gm^r and Gm^s MRSA strains is revealed, not only by their distributions in distinct *Sma*I genotypes but also by the differences in hybridization patterns. Two of the 62 Gm^s MRSA strains had the uncommon feature of carrying several *Sma*I bands hybridizing with IS*256*, suggesting that they are possibly related to the Gm^r MRSA strains grouped in the same *Sma*I genotype. Five of the 11 *Sma*I genotypes including only Gm^s MRSA strains contained strains from diverse cities, isolated during different years and with different antibiograms, suggesting that some clones have spread beyond their cities of origin and persisted.

Until 1992, at least 99% of the French methicillin-resistant *Staphylococcus aureus* (MRSA) strains sent to the Reference Center were resistant to gentamicin, kanamycin, and related aminoglycosides. All the strains tested carried at least one chromosomal copy of a Tn*4001*-like structure delimited by two inverted copies of the insertion sequence IS*256* and including the *aacA-aphD* gene (10, 11, 17). Most of these strains also harbored multiple autonomous copies of IS*256* on several *Sma*I fragments of the cellular DNA (9, 17, 18).

In 1993, gentamicin-susceptible (Gm^s) MRSA strains emerged in several French hospitals (1, 15, 16, 19). In these hospitals, their incidence has increased from 2 to 5 to 20 to 60%. In one of these hospitals (1), the 97 Gm^s MRSA strains isolated from 1992 to 1996 were distributed into three unrelated pulsotypes. In another hospital (15), 16 of the 26 Gm^s MRSA strains isolated from 1993 to 1996 clustered in the same pulsotype whereas the 10 other strains were distributed in different and distant pulsotypes. In each of these hospitals, the Gm^s strains were unrelated to the epidemic Gm^r MRSA clones. We typed the Gm^s MRSA strains suspected to be responsible for very recent (1995) outbreaks in several other French hospitals (19); the spread of the same clone in each hospital was demonstrated.

In this study, we used several typing methods to analyze 62 Gm^s MRSA strains isolated from 1995 to 1997 in nine hospitals in nine French cities and 15 Gm^r MRSA strains which were representative of the Gm^r MRSA strains that spread in three of these hospitals in 1996 and in 1997. The aim of this study was to find out (i) whether some Gm^s clones are closely related to Gm^r MRSA, (ii) whether particular Gm^s MRSA clones have spread and persisted in various French cities, as is found for Gm^r MRSA strains (18), and (iii) if the strains grouped in the same pulsotype are easily traceable by antibiotyping and phage typing.

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MATERIALS AND METHODS

Bacterial strains and plasmids. A total of 77 MRSA strains isolated from 77 infected patients were studied (Table 1). They included 62 Gm^s strains isolated between 1995 and 1997 in nine hospitals in different French cities and 15 Gm^r strains isolated in three of the hospitals in 1996 and in 1997.

pSF815A (12) and pIP1551 (9) were used as probes. pSF815A is pUC8 plus a 1.5-kb *Alu*I insert carrying *aacA-aphD*, which encodes the bifunctional enzyme, AAC6′-APH2″, that inactivates gentamicin, kanamycin, and related antibiotics. pIP1551 consists of pUC18 carrying a 468-bp insert from the transposase gene of insertion sequence IS*256* (4).

Susceptibilities to antibiotics. Susceptibilities to antibiotics were determined by a disk diffusion assay (5) with commercially available antibiotic disks (Diagnostics Pasteur). Additional disks prepared in our laboratory contained pristinamycin IIA (20 μg) or pristinamycin IB (40 μg).

Phage typing. The strains were phage typed by the standard method of Blair and Williams (3) with the international set of 23 phages and an additional set of 9 phages: 616, 618, 620, 622, 623, 625, 626, 629, 630 (21). These phages were used at routine test dilution (RTD).

Pulsed-field gel electrophoresis of macrorestriction fragments and comparative analysis of banding patterns. The protocol used for the determination of *Sma*I restriction patterns was described previously (7). Concatemeric bacteriophage lambda DNA molecules (48.5 kb; Bio-Rad) and *Sma*I fragments of the cellular DNA from *S. aureus* NCTC8325 were used as size standards. Macrorestriction fingerprints were compared visually and were scanned with GelCompar software (Applied Maths, Kortrijk, Belgium). A similarity matrix was created by using the band-based Dice similarity coefficient (8). The unweighted pair group method with average linkages was used to cluster the strains on the basis of the *Sma*I patterns.

If the dendrograms revealed clusters that included strains with percentages of similarity of at least 80, the patterns of the strains in the same cluster were compared visually on the same gel. The strains were clustered according to the following criteria proposed by Tenover et al. (24): (i) strains were grouped in the same major genotype if their patterns differed by no more than three bands (such strains were considered to be closely related and monoclonal); (ii) if the number of band differences between patterns was between four and six, the strains were

TABLE 1. Relevant characteristics of the 77 MRSA strains typed by several methods

Genotypes[a]		Characteristics of MRSA strains				
*Sma*I	*Sma*I/IS*256*	Source	Yr of isolation	No. of strains	Phages used at RTD and giving at least 5 PFU	Drug resistance markers[b]
Ia	A	Clichy	1995	3	616	**Pc Ox Nm Sp MLSc Pf** (Sm Rf Fm)
Ib	B	Paris	1997	1	NT[c]	Gm Pc Ox Nm Sp MLSc Pf Fm
II	C	Blois	1996	1	53, 77, 85	Pc Ox Nm Pf Fm
		Montreuil	1995	1	84, 616	Pc Ox Nm Sm Sp MLSi Pf
		Toulouse	1997	1	53, 83A, 620, 622, 629, 630	Pc Ox Sm Sp Pf
III*	D#	Toulouse	1997	1	84, 616	Pc Ox Nm Sp MLSc Pf Fm
IV*	E#	Blois	1996	1	616	Pc Ox Nm Sm Sp MLSc Pf Fm Rf
		Toulouse	1996	1	84, 616	Pc Ox Nm Sm Sp MLSc Pf Fm
V	F	Paris	1997	1	NT	Gm Pc Ox Nm Sp MLSc Pf Fm
VIa†	G1	Paris	1997	1	85	Pc Ox Nm Sm Pf
VIb†	G2	Evry	1995	2	84, 616, 622	Pc Ox Nm Sm Sp Pf
		Colombes	1995	7	77, 84, 85, 616, 622	Pc Ox Nm Sm Sp Pf
		Clichy	1995	1	NT	Pc Ox Nm Sm Sp MLSc Pf Rf
		Toulouse	1997	4	NT	**Pc Ox Nm Pf** (Sm)
		Toulouse	1997	1	77	Pc Ox Nm Sm Pf Rf Fa
		SaintGermain	1995	1	NT	Pc Ox Nm Sm Sp Lc SgA Pf
VIc†	G3	Toulouse	1997	1	84, 85, 616, 622	Pc Ox Nm Sm Sp Pf
VId†	G4	Blois	1996	1	77, 616, 622	Pc Ox Nm Sp MLSc Pf Fm
		Toulouse	1997	1	77, 84, 85, 616, 622, 625	Pc Ox Nm Spe MLSc Pf
VIIa†	H	Paris	1997	1	616	Pc Ox Nm Sp MLSc SgA Pf Fm Tp
VIIa†	I	Paris	1997	1	84, 616	Pc Ox Nm Sp MLSc Pf Fm
VIIb†	J	Paris	1977	1	84, 616	Gm Pc Ox Nm Sp MLSc Pf Fm
VIIc†	K1	Toulouse	1997	1	84, 616	Pc Ox Nm Sm Sp MLSc Pf Fm
VIId†	K2	Nîmes	1995	9	616	Pc Ox Nm Sm Sp MLSc Pf Fm
				1	84, 616	Pc Ox Nm Sm Sp MLSc Pf Fm
				3	NT	Pc Ox Nm Sm Sp MLSc Pf Fm
		Toulouse	1997	4	84, 616	**Pc Ox Nm Sm Sp MLSc** (Pf SgA)
				1	84, 616, 622	Pc Ox Nm Sm Sp MLSc Pf
				1	616	Pc Ox Nm Sm Sp MLSc Pf
				1	616, 625	Pc Ox Nm Sm Sp MLSc Pf
		Clichy	1995	1	84, 616	Pc Ox Nm Sm Sp MLSc Pf
		Blois	1996	1	616	Pc Ox Nm Sp MLSc Pf Fm
VIIe†	L‡	Toulouse	1997	1	54, 630	Gm Pc Ox Nm Sm Sp MLSc Pf Rf Tc Mn
VIIf††	M‡	Paris	1997	1	77	Gm Pc Ox Nm Sm Sp MLSc Pf Rf
VIII	N	Blois	1996	2	47, 54, 75, 77, 83A, 85, 616, 618, 620, 622, 623, 629	Gm Pc Ox Nm Sm Sp MLSc Pf Rf
		Blois	1996	1	NT	Gm Pc Ox Nm Sm Sp MLSc Pf Rf
IX§	O‖	Paris	1997	1	84	Pc Ox Nm MLSi Pf
X§	P‖	Paris	1997	1	NT	Pc Ox Nm MLSi Pf
XI	Q	Toulouse	1997	1	84, 616, 622, 630	Pc Ox Nm Sm Sp MLSc Pf
XII	R	Blois	1996	1	3A, 3C, 6, 42E, 47, 54, 75, 77, 84, 94, 96, 81, 616, 618, 620, 622, 623, 629	Pc Ox Lc SgA Pf
				1	29, 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85, 81, 616, 620, 622, 623, 625, 626, 629, 630	Pc Ox Lc SgA
XIII	S	Toulouse	1997	1	77, 620, 630	Gm Pc Ox Nm Sm Sp MLSc Pf Rf Tc Mn Fm
XIV	T	Blois	1996	1	54, 77, 85, 630, 56B	Gm Pc Ox Nm Sm Sp MLSc Pf Rf Tc Mn Fm
XV	U	Blois	1996	1	NT	Pc Ox Sm Sp Pf
XVI	V	Toulouse	1997	4	29, 47, 54, 75, 77, 83A, 84, 85, 616, 620, 622, 623, 626, 629, 630	Gm Pc Ox Nm Sm Sp MLSi Pf Rf Tc Mn Fm Fa
				1	54	Gm Pc Ox Nm Sm Sp MLSi Pf Rf Tc Mn Fm Fa
XVII	W	Paris	1997	1	616	Pc Ox Nm Sp MLSc Pf Fm
XVIII	X	Paris	1997	1	NT	Pc Ox Nm MLSc Tc Mn

[a] Genotypes which include strains which are possibly related (less than seven band differences) are marked with same symbols (*, †, ‡, §, ‖, and #).

[b] All the strains resistant to neomycin or to gentamicin were also resistant to tobramycin and kanamycin. Antibiotic resistance markers in boldface are common to all the strains; those in parentheses vary between strains. Abbreviations: Fa, fusidic acid; Fm, fosfomycin; Gm, gentamicin; Km, kanamycin; Lc, lincosamides; MLSc, constitutive resistance to macrolides-lincosamides-streptogramin B; MLSi, inducible resistance to macrolides-lincosamides-streptogramin B; Mn, minocycline; Nm, neomycin; Ox, oxacillin; Pc, penicillinase production; Pf, pefloxacin; Rf, rifampin; SgA, streptogramin A; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; Tp, trimethoprim.

[c] NT, not typeable.

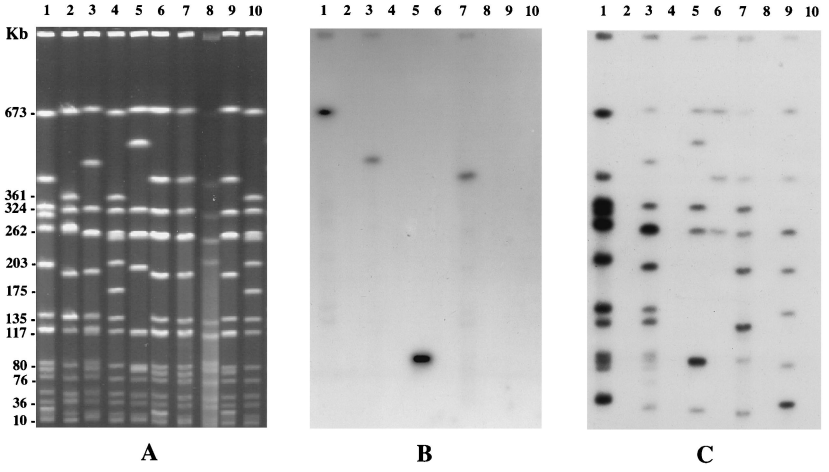

FIG. 1. Pulsed-field gel electrophoresis of *Sma*I-digested total DNA from eight MRSA strains. (A) *Sma*I macrorestriction patterns; (B) hybridization patterns with the *aacA-aphD*-probe; (C) hybridization patterns with the IS*256* probe. Lanes 1 to 3 and 5 to 9, *Sma*I-digested DNA from MRSA strains belonging to *Sma*I genotypes (Table 1) VIIf, VIa, Ib, V, VIIa, VIIb, XVII, and VIIg, respectively; lanes 4 and 10, *Sma*I-digested cellular DNA from strain NCTC8325 used as a standard.

scored as possibly related but were, nevertheless, classified into distinct genotypes to discriminate them clearly from the unambiguously closely related strains; and (iii) differences between patterns involving at least seven bands indicated different or unrelated strains. Major genotypes are designated by roman numerals. Strains with indistinguishable patterns were classified within the same subtype. Subtypes are designated by roman numerals with letter suffixes.

Within each major *Sma*I genotype, the *Sma*I patterns were also compared by analysis of the differences revealed by hybridization with an IS*256* probe (18). *Sma*I bands of the same apparent mobilities but differing by the presence or absence of hybridizing nucleotide sequences were considered to show an additional difference. The criteria used to cluster the strains into major genotypes and subtypes were the same as those used after analysis of *Sma*I patterns. The strains having the same *Sma*I pattern after hybridization with the IS*256* probe belong to the same subtype, and the patterns of the strains clustered in the same major genotype differ by three or fewer bands (bands of different sizes or distinguishable after hybridization with IS*256*). The genotypes characterized by IS*256* probing are designated by capital letters, and the subtypes are designated by capital letters with arabic numbers.

Blotting and hybridization. DNA was transferred from agarose gels to Hybond N^+ membranes (Amersham) with a Trans-Vac TE80 vacuum blotter (Hoefer Scientific Instruments, San Francisco, Calif.). Prehybridization and hybridization were performed under high-stringency conditions, as previously described (6). The plasmid used as a probe was labeled with [α-^{32}P]dCTP (110 TBq · mmol^{-1}) by random priming with the Megaprime DNA-labeling system (Amersham). The blots were exposed to Hyperfilm (Amersham) at −80°C.

Preparation of oligonucleotides and PCR. The following oligonucleotides were prepared by the phosphoramidite method with an Applied Biosystems model 380 B DNA synthesizer: oligonucleotide A, 5′-GGGATCATAGCGTCA TTATTC-3′; oligonucleotide B, 5′-AACGATTGTGACACGATAGCC-3′. Oligonucleotides A and B were used as primers in PCR to amplify a 529-bp sequence (20) from within the *mecA* gene (23). The forward primer (A) corresponds to nucleotide positions 516 to 536, and the reverse primer (B) corresponds to the complement of positions 1024 to 1044. The PCR was performed with a precycle of 5 min at 94°C and 5 min at 60°C; this was followed by 30 cycles of 1 min at 72°C, 1 min at 94°C, and 1 min at 60°C.

RESULTS

Analysis of antibiotypes. The 62 Gm^s MRSA strains studied were distributed into 19 antibiotypes (Table 1). Five antibiotypes were distinguished among the 15 Gm^r MRSA strains. The Gm^s strains exhibited heterogeneous and low-level resistance to oxacillin. This resistance was more easily detectable at 30°C than at 37°C and after a prolongation of the incubation time from 24 to 48 h. Fourteen of the 15 Gm^r strains exhibited high-level resistance to oxacillin, i.e., they grew at the contact of the disks containing 5 μg of oxacillin after 24 h of incubation at 37°C. All the strains exhibiting a low-level resistance to oxacillin were screened for the presence of the *mecA* gene by PCR, and an amplicon of the expected size (529 bp) was detected in all cases (results not shown).

There were several examples of strains from hospitals in different cities having the same antibiotype (Table 1).

Analysis of phage types. Sixty-two of the 77 MRSA strains could be typed with the phages used at RTD (Table 1). Despite the use of 9 phages in addition to the international collection of 23 phages routinely used to type *S. aureus* strains, 42 of the 62 typeable strains were susceptible to no more than three phages (RTD). Strains isolated in a single hospital in 1995 were all grouped in the same phage type. In 1996 and 1997, strains of diverse phage types were isolated in single hospitals. Note that some single phage types contained strains isolated in different cities between 1995 and 1997 and having different antibiograms.

Analysis of the *Sma*I patterns. There were 19 different *Sma*I patterns among the 62 Gm^s strains and 9 patterns among the 15 Gm^r MRSA strains. The patterns of eight of these strains are shown in Fig. 1, and the schematic representation of the 28 different patterns is reported in Fig. 2. A dendrogram of all *Sma*I patterns was constructed on the basis of the levels of similarity (Fig. 2). For patterns with no more than three fragment differences, the percentages of similarity were 88 to 100. An 88% similarity cutoff value gave 18 major *Sma*I genotypes of which 2 (genotypes I and VII [Fig. 2]) included both Gm^s and Gm^r MRSA strains. An 82% similarity cutoff value enabled the delineation of 15 *Sma*I genotypes, each including putatively related strains whose *Sma*I patterns differed by six or fewer bands. The 82% cutoff value was used to group the strains belonging to the following *Sma*I genotypes: III and IV, VI and VII, and IX and X (Fig. 2). Forty-five of the 62 Gm^s strains, including those of diverse origins and antibiograms, were grouped in *Sma*I genotypes VI and VII.

Analysis of *Sma*I patterns after hybridization with *aacA-aphD* and IS*256* probes. All the Gm^r MRSA strains hybridized with the *aacA-aphD* probe, whereas none of the Gm^s MRSA strains gave any detectable signal (Fig. 2). In the 15 Gm^r strains tested, the hybridizing nucleotide sequences were on a single *Sma*I fragment of ≈100 kb (1 strain) or >400 kb (14 strains).

Six to 13 *Sma*I bands hybridizing with IS*256* were present for each of the 15 Gm^r strains; these bands included the single band hybridizing with *aacA-aphD* (Fig. 2). Only 2 of the 62 Gm^s MRSA strains hybridized with IS*256*, and the hybridizing sequences were on three or seven *Sma*I bands (Fig. 2).

A comparative analysis of the 77 MRSA strains tested enabled the detection of 24 *Sma*I/IS*256* genotypes (Fig. 2). None

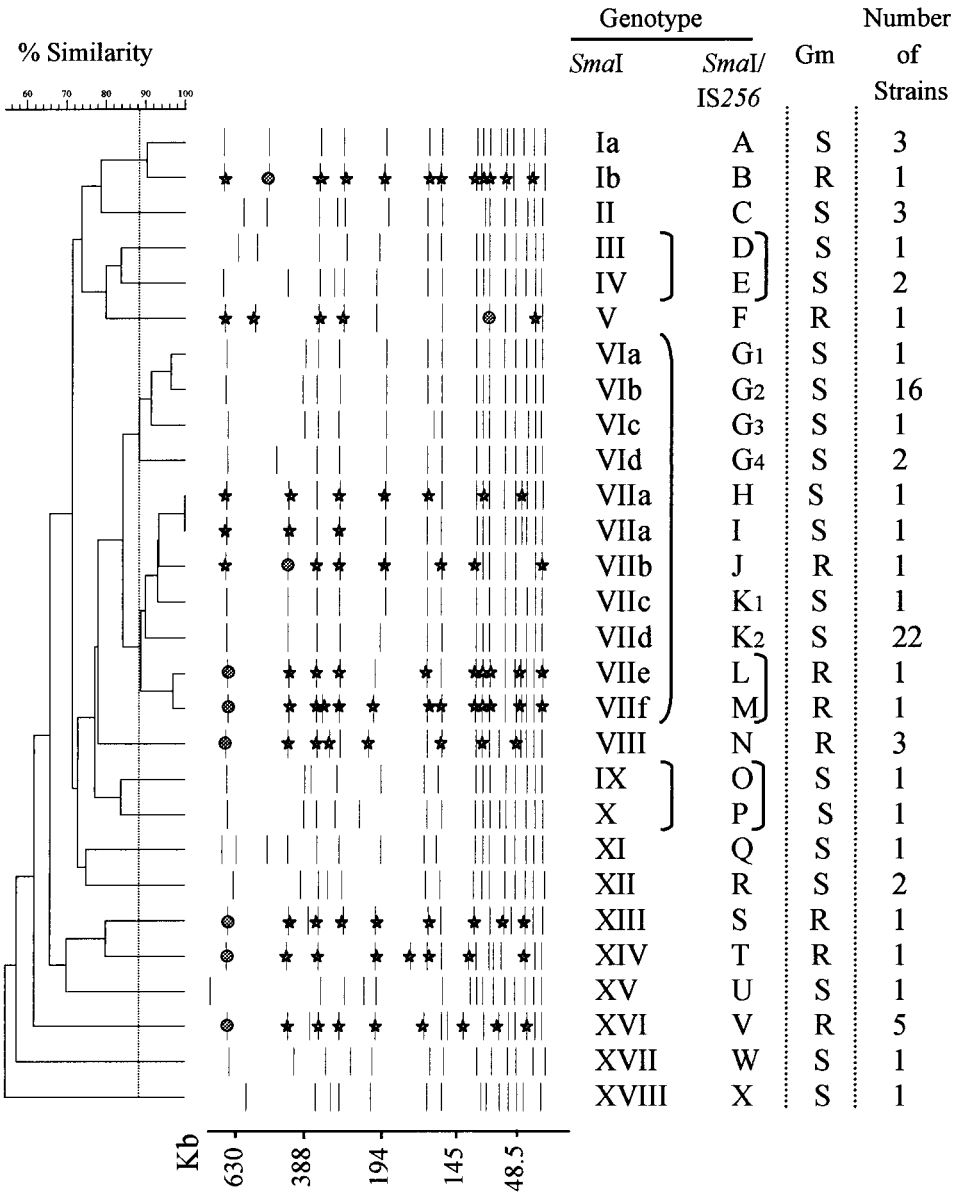

FIG. 2. Classification and schematic representation of the *Sma*I patterns of the 77 MRSA strains studied. The *Sma*I and *Sma*I/IS*256* genotypes assigned to the strains are reported, and the brackets linking some of them indicate that the strains, although in different genotypes, are possibly related (less than seven band differences between their patterns). The bands marked with a circle are those hybridizing with *aacA-aphD* and IS*256* probes, and the bands marked with a star are those hybridizing with the IS*256* probe only.

of these genotypes included both Gm^r and Gm^s MRSA strains. The strains belonging to genotypes D and E, L and M, or O and P were possibly related because their patterns differed by six or fewer bands.

Clustering of Gm^s MRSA strains on the basis of various typing methods. The seven strains isolated in 1995 in the hospital of Colombes were grouped in the same type, whatever the typing method used (phage typing, antibiotyping, comparison of *Sma*I patterns) (Table 1). The same was also observed for the 2 strains isolated in Evry in 1995 and for 10 of the 13 strains isolated in 1995 in Nîmes hospital. The three other strains isolated in Nîmes hospital had the same antibiotype and the same *Sma*I genotype, but they were not phage typeable. In the three hospitals, the Gm^s strains analyzed were those collected during the 6 months following their first emergence.

In contrast, for the strains isolated in the Toulouse (19 strains in 1997), Blois (7 strains in 1996), Paris (7 strains in 1997), and Clichy (4 strains in 1995) hospitals, there was no correlation between the clusterings obtained by using the different typing methods. In each of these four hospitals, in which the first Gm^s strains were isolated in 1993 to 1994, the strains were distributed in two to seven unrelated *Sma*I genotypes (seven or more band differences between the *Sma*I patterns).

DISCUSSION

None of the Gm^s strains tested contained nucleotide sequences hybridizing with the *aacA-aphD* gene. Thus, it is unlikely that these strains result from a modification in this gene abolishing its expression. Consequently, gentamicin and netil-

micin or any related aminoglycosides may be used for therapy. The divergence between Gm^s and Gm^r strains was revealed not only by their distribution into distant *Sma*I genotypes, but also by their differences in hybridization with the IS*256* probe. As shown in this study and other papers (9, 17, 26), the presence of multiple autonomous copies of IS*256* is common in Gm^r staphylococci but is rare in Gm^s staphylococci. The detection, in 2 of the 62 Gm^s MRSA strains studied, of three and seven *Sma*I bands hybridizing with the IS*256* probe suggested that these strains are possibly related to the Gm^r MRSA strains grouped in the same *Sma*I genotype (VII).

Gm^s MRSA strains considered closely or possibly related on the basis of the comparison of *Sma*I patterns were isolated in different French cities and at time intervals of up to 2 years (1995 to 1997). This suggests a capacity to disseminate beyond a single hospital or city and to persist. This phenomenon was most clearly observed among the strains in the *Sma*I genotypes VI and VII, which include possibly related strains (less than seven band differences). It would be worth looking for strains belonging to these two genotypes in other European countries to assess whether Gm^s MRSA strains have disseminated similarly to Gm^r MRSA strains (18). Unfortunately, phage typing and antibiotyping are not appropriate to trace such strains, not only because of the diversity observed among strains in the same genotype but also because some strains having the same phage type and/or antibiotype belong to distant genotypes. However, at the beginning of an outbreak in a hospital, these two phenotypic markers may be useful to screen closely related strains. Indeed, for such strains, our study revealed a good correlation between the results obtained by different typing methods.

This study and those previously published (1, 15) show that a multiplicity of unrelated Gm^s strains are isolated in hospitals. Diversity within a single hospital is mostly observed when the strains are collected several years after the emergence of the first Gm^s strains. In these cases, the diversity may be a result of rearrangements in persistent clones, which become endemic. However, we also detected unrelated clones among strains which have recently emerged in different hospitals or in the same hospital. Therefore, the increased incidence of Gm^s MRSA strains in several hospitals is not only attributable to the spread of derivatives of the same clone but also to the emergence of unrelated clones. The emergence of such clones in France coincided, in several hospitals, with a change in antibiotic prescribing patterns, including a decrease in gentamicin consumption, and with infection control programs (13, 15). The result has been a decrease in the proportion of Gm^r MRSA strains (15) and in particular of the endemic phage type 77 Gm^r MRSA strains (18).

The levels of resistance to β-lactams due to expression of the *mecA* gene carried by all the French Gm^s MRSA strains isolated since 1992 (this study) and before the emergence of the first Gm^r MRSA strain in 1975 (14) are very low and are expressed heterogeneously. Several genes other than *mecA*, including those of the *mecA* regulatory region (*mecI*, *mecR-I*) and promoter, are involved in the expression of *mecA* (2, 27). It would be valuable to compare the *Sma*I restriction patterns and the regulatory regions of *mecA* of the early and recent French Gm^s MRSA strains to determine whether they are related. If they are, it would mean that some of the old Gm^s MRSA clones have persisted with a low prevalence in the hospitals and have emerged with an increased frequency as the selective pressure exerted by the use of gentamicin has decreased. Data from each hospital about the consumption of antibiotics and the incidence of each antibiotic resistance marker among staphylococci would help in the evaluation of the contribution of the selective pressure exerted by antibiotics on the spread of clones. Note that other factors such as those involved in virulence and colonization (13, 22, 25) and/or resistance to environmental stress may also contribute to the spread and the persistence of some clones.

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