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Presence of *mefA* and *mefE* Genes in *Streptococcus agalactiae*

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Eighteen unrelated clinical isolates of *Streptococcus agalactiae* with the M phenotype harbored an *mef* gene. DNA sequencing showed that one of nine strains contained *mefA* (producing one amino acid substitution), whereas the remaining eight carried *mefE* (identity, 100%). Restriction analysis of PCR products indicated that the nine other strains also contained *mefE*.

Streptococcus agalactiae (group B streptococcus) is mainly responsible for meningitis and septicemia in newborns; beta-lactam agents are the treatment of choice of these infections, but macrolides and related drugs are useful alternate therapy in allergic patients (2, 13). Until recently, the only known mechanism of resistance to macrolides in streptococci was target site modification by 23S rRNA methylases, encoded by the *erm* genes. The Erm enzymes convey cross-resistance to macrolides, lincosamides, and the streptogramin B compounds (MLS), which is referred to as MLS_B phenotype (23). Since the late 1980s, a new phenotype, designated M and consisting of resistance to 14- and 15-membered macrolides but susceptibility to 16-membered macrolides, lincosamides, and streptogramins, has been recognized in group A streptococci and pneumococci isolated in a number of countries (6, 8, 9, 15–17, 19, 21, 24, 25). The mechanism of this resistance is a proton-dependent efflux system (4, 11, 21, 22), encoded by *mef* genes: *mefA* in group A streptococci (4) and *mefE* in pneumococci (22), which have 90% identity. *mef* genes have also been detected very recently in group C streptococci (10). Preliminary studies indicated that they might be widespread among other streptococcal species, including *S. agalactiae* (22). In addition, a novel efflux system, encoded by *mreA*, distinct from the Mef pump, has been characterized in a single strain of *S. agalactiae* which displayed resistance to 14-, 15-, and 16-membered macrolides (5). The aim of the present study was to determine the macrolide resistance mechanism in 18 clinical strains of *S. agalactiae* bearing the M phenotype.

These 18 nonredundant isolates (SB1 to SB18) were collected from distinct patients in several laboratories in the southwest of France, from 1993 to 1998. They were mainly recovered from genital samples of adults and gastric fluid of neonates. Three clinical strains of *S. agalactiae*, susceptible or expressing inducible or constitutive MLS_B phenotypes, were also used as controls. Identification was done by conventional tests (13). MICs of 12 MLS drugs were determined by an agar dilution method in Mueller-Hinton medium supplemented with 5% horse blood, with a final inoculum of 10^5 CFU per spot (7). The strains showed low-level resistance to 14- and 15-membered macrolides while being fully susceptible to 16-membered macrolides, lincosamides, and pristinamycin II (group A) and pristinamycin I (group B), even after prolonged incubation at 37°C in aerobiosis (Table 1). MICs were slightly lower than those obtained for the strains of group A streptococci (8, 15, 17, 25) or pneumococci (9, 18) with an M phenotype (MICs of erythromycin at 4 to 8 mg/liter). Moreover, subinhibitory concentrations of erythromycin (0.005 to 8 μg/ml) or lincomycin (0.0002 to 0.5 μg/ml) did not induce MLS resistance in the first nine erythomycin-resistant strains of *S. agalactiae* that we tested (SB1 to SB9). Finally, the possibility that an *erm* gene was present was excluded by two experiments: dot blot DNA hybridization under conditions of low and high stringencies performed by using radioactively labelled *ermAM* probe, and amplification by PCR by using degenerate oligonucleotides corresponding to conserved amino acid motifs in Erm methylases (1).

Then, DNA amplification by PCR was performed with genomic DNA of the 18 isolates, using primers specific for the *mef* genes, i.e., mef1 (5′-ATGGAAAATACAACAATTGGAAAC-3′; 5′-ATG translation start codon is underlined) and mef8 (5′-TTATTTAAATCTAATTTTCTAACCTC-3′; TAA-3′ translation stop codon is underlined). Amplification products of 1.2 kb were obtained in all cases, in contrast with negative controls (DNA of a susceptible strain or strains with an MLS_B phenotype). The presence of an erythromycin resistance gene was further characterized in half of the strains (SB1 to SB9). Mating between the nine isolates and a streptomycin-resistant mutant of *S. agalactiae* BM132 as the recipient strain was carried out on filters (7). No transfer was observed, in contrast to the positive control (*S. agalactiae* BM132 harboring an *ermAM* gene on a conjugative plasmid; transfer frequency, 10^{-5}). Furthermore, plasmid DNA was not detected after extraction and analysis by electrophoresis on agarose gel (14). Erythromycin resistance in *Streptococcus pyogenes* was found in one case to be transferable by conjugation to streptococci or *Enterococcus faecalis* recipients, although no plasmid DNA could be detected (15). However, no information has been provided on the transferability of *mef* genes (22).

For the same nine strains, the PCR products were directly sequenced on both strands by an automated fluorescent sequencing method. The analysis showed that one of these amplimers (pSB1) was identical to *mefA* (4), except for two substitutions: one silent mutation at position 471 (T→C) and one mutation at position 239 (A→C) that led to the replacement of a methionine by a valine at position 77. This is the first description of the *mefA* gene in *S. agalactiae*. Hybridization experiments, using this amplified product (pSB1) radioactively labelled and used as a probe, gave a weaker signal with the eight remaining amplified products (pBS2 to pBS9) than with the positive control (pBS1). Indeed, all nucleotide sequences of pBS2 to pBS9 exhibited 100% identity with the original

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TABLE 1. Susceptibility of *S. agalactiae* clinical strains to MLS antibiotics

Antimicrobial agent	MIC (mg/liter)[b] for:				
	18 clinical strains studied	Control strains			
		Susceptible strain	MLS_Bi[a] strain at:		MLS_Bc[a] strain
			24 h	48 h	
14-membered macrolides					
Erythromycin	0.1–1	0.02	4	>512	>512
Clarithromycin	0.1–0.5	0.005	2	>512	>512
Dirithromycin	2–16	0.1	32	>512	>512
Roxithromycin	0.5–4	0.05	8	>512	>512
Ketolide HMR 3647	0.1–0.2	0.02	0.005	0.02	0.1
15-membered macrolides					
Azithromycin	1–4	0.1	64	>512	>512
16-membered macrolides					
Spiramycin	0.1–0.2	0.2	4	4	>512
Josamycin	0.1–0.2	0.2	2	4	8
Lincosamides					
Lincomycin	0.02–0.1	0.1	0.5	256	256
Clindamycin	0.005–0.02	0.005	0.2	32	128
Streptogramins					
Pristinamycin	0.1–0.2	0.2	0.2	0.2	0.1
Pristinamycin II	0.2–1	0.5	0.5	1	0.2
Pristinamycin I	1–4	2	4	4	8

[a] MLS_Bi, MLS_B inducible phenotype; MLS_Bc, MLS_B constitutive phenotype.
[b] All data except those for the MLS_Bi resistant strain were read at 24 h.

mefE gene (GenBank accession no. U83667) (22). There was no apparent clustering of our strains. However, as a confirmation, epidemiological typing by randomly amplified polymorphic DNA was performed, since this method has been found to be discriminant for this species (3). Using primer AP12h (5′-CGGCCCCTGT-3′; 80% G+C content) and the method described elsewhere (3), the SB1 to SB9 strains of *S. agalactiae* were found to exhibit different patterns and thus were concluded to be multiclonal isolates.

Finally, the PCR products obtained with the nine other strains (SB10 to SB18) were analyzed after restriction by four endonucleases (*Acc*I, *Cla*I, *Hin*dIII, and *Hha*I) designed to digest differentially *mefA* and *mefE*. The nine amplimers gave restriction patterns consistent with *mefE* rather than with *mefA*. These findings confirmed the predominance of *mefE* among *S. agalactiae* strains with an M phenotype. The distribution of the *mef* genes has been studied only in pneumococci, where *mefE* was exclusively found (18). Enzymatic restriction combined with PCR amplification should be helpful to determine the relative prevalence of the *mef* genes among various streptococcal species.

In the late 1980s, a sudden increase in the frequency of erythromycin-resistant streptococci was observed in several countries (including the United Kingdom, Finland, and the United States) (6, 15, 16, 19, 25), and this was probably related to the increased prescription of this antibiotic or its then newly available semisynthetic derivatives (16). In these countries, the prevalence of the M phenotype (and/or *mef* genes) among the erythromycin-resistant streptococci is very high: 38 to 97% in group A streptococci (8, 17, 20), 95% in group C streptococci (10), and 41 to 85% in pneumococci (9, 18, 20). In France, no national data are available. In our area, between 1996 and 1997, the percentages of erythromycin-resistant strains at different hospitals ranged from 31 to 56% for pneumococci, from 0 to 20% for group A and group C streptococci, and from 8 to 16% for group B and group G streptococci (12). MLS_B phenotype remains widely predominant since only two strains of group A streptococci, one strain of group C streptococci, and one strain of *S. pneumoniae* containing an *mef* gene (data not shown) have been collected, compared to 18 isolates of *S. agalactiae* that were collected during the same period. Our findings emphasize the need to perform cultures and susceptibility testing whenever streptococcal infections are suspected; antibiograms should include 16-membered macrolides or lincosamides, since these drugs may remain active against erythromycin-resistant streptococci.

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