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Emergence of Macrolide Resistance Gene *mph*(B) in *Streptococcus uberis* and Cooperative Effects with *rdmC*-Like Gene▽

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***Streptococcus uberis* UCN60 was resistant to spiramycin (MIC = 8 μg/ml) but susceptible to erythromycin (MIC = 0.06 μg/ml), azithromycin (MIC = 0.12 μg/ml), josamycin (MIC = 0.25 μg/ml), and tylosin (MIC = 0.5 μg/ml). A 2.5-kb HindIII fragment was cloned from *S. uberis* UCN60 DNA on plasmid pUC18 and introduced into *Escherichia coli* AG100A, where it conferred resistance to spiramycin by inactivation. The sequence analysis of the fragment showed the presence of an *rdmC*-like gene that putatively encoded a protein belonging to the alpha/beta hydrolase family and of the first 196 nucleotides of the *mph*(B) gene putatively encoding a phosphotransferase known to inactivate 14-, 15-, and 16-membered macrolides in *E. coli*. The entire *mph*(B) gene was then identified in *S. uberis* UCN60. The two genes were expressed alone or in combination in *E. coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*. Analysis of MICs revealed that *rdmC*-like alone did not confer resistance to erythromycin, tylosin, and josamycin in those three hosts. It conferred resistance to spiramycin in *E. coli* and *E. faecalis* but not in *S. aureus*. *mph*(B) conferred resistance in *E. coli* to erythromycin, tylosin, josamycin, and spiramycin but only low levels of resistance in *E. faecalis* and *S. aureus* to spiramycin (MIC = 8 μg/ml). The combination of *mph*(B) and *rdmC*-like genes resulted in a resistance to spiramycin and tylosin in the three hosts that significantly exceeded the mere addition of the resistance levels conferred by each resistance mechanism alone.**

Streptococcus uberis is an environmental pathogen commonly responsible for a high proportion of cases of clinical and mostly subclinical mastitis in lactating cows (9). It is also the predominant organism isolated from mammary glands during the nonlactating period (2, 14). β-Lactams are the drugs of choice for therapy of *S. uberis* infections during lactation and at drying off (6, 27). However, lincosamides or macrolides may be alternatives (7). Spiramycin, a macrolide antibiotic, is used via intramuscular or intramammary routes to treat bovine mastitis due to *S. uberis* in a few countries (23). In France, it is also used orally or intravenously in humans for therapy of streptococcal or staphylococcal infections.

Spiramycin is a 16-membered lactone ring macrolide with two amino sugars, D-forosamine and D-mycaminose, and a neutral sugar, L-mycarose, attached via glycosidic bonds, whereas most other commercially available macrolides have a 14- or 15-membered ring structure (5). Macrolides act by inhibition of protein biosynthesis at the large subunit (50S) of ribosomes (10). Resistance to this class of antibiotics is conferred mainly by the following four different mechanisms: (i) modification of the 23S rRNA target site by different methyltransferases, (ii) base substitution in 23S rRNA, (iii) efflux of the drug mediated by ABC-type transport systems or by exporters belonging to the major facilitator superfamily, and (iv) inactivation of the molecule by different enzymes (24, 28).

There are two classes of macrolide-inactivating enzymes: erythromycin esterase (22) and macrolide 2′-phosphotransferase [MPH(2′)] (12, 16, 19). MPH(2′) inactivates macrolide antibiotics by O phosphorylation of the 2′-hydroxyl group of an amino sugar in the antibiotic molecule, where the phosphoryl group is derived from the γ-phosphate of ATP. Two groups of phosphotransferases, MPH(2′)-I and MPH(2′)-II, are distinguished on the basis of substrate specificity and primary amino acid sequence. MPH(2′)-I, encoded by the *mph*(A) and *mph*(D) genes, inactivates 14-membered ring macrolides more efficiently than the 16-membered ones, whereas MPH(2′)-II, encoded by the *mph*(B) and *mph*(C) genes, inactivates both groups of macrolides (3, 26).

The *mph*(B) gene has been evidenced only in gram-negative bacteria, first in *Escherichia coli* and then in other members of *Enterobacteriaceae* and *Pseudomonas* (11, 25) (http://faculty.washington.edu/marilynr/ermweb4.pdf). In *E. coli* BM2506, the gene is borne by two different plasmids, pTZ3721 and PTZ3723, which are conjugative and mobilizable, respectively. The *mph*(B) gene of pTZ3721 is carried by a transposable element of 39 kb belonging to the Tn*21* subgroup. This element has a Tn*21*-like transposition module at one end and a Tn*1721*-like transposition module at the other end (17). In both plasmids, the *mph*(B) gene was located a few base pairs downstream from an *rdmC*-like gene and an *acrR*-like gene (11). In this study, we report the presence of the *mph*(B) and *rdmC*-like genes in the spiramycin-resistant strain *S. uberis* UCN60. We also show that the *rdmC*-like gene participates in

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TABLE 1. Sequences of primers

Primer	Sequence (5′–3′)	Amplified DNA fragment
Acr-F	AGAGTTAGAAAAAGAAAAAATTCG	Internal to *acrR*-like
Acr-R	AACAGCTTCGAATAATAACTC	
MphB-F	ATTAAACAAGTAATCGAGATAGC	Internal to *mph*(B)
MphB-R	TTTGCCATCTGCTCATATTCC	
RdmC-F	ATATTTGCACTGAGAGTTTTGG	Internal to *rdmC*-like
RdmC-R	ATAAAGCATTAATAATTTCATCCC	
RdmC-KpnI-F	GTTAATAGGTACCCAAAAGGG	Entire *rdmC*-like gene
RdmC-KpnI-R	ATTAAACGGTACCGTTTCTTC	
MphB-KpnI-F	TGCTAGGTACCCTGGATGG	Entire *mph*(B) gene
MphB-KpnI-R	AATAGGTACCATAGTGGTAGG	
RdmC-KpnI-F	GTTAATAGGTACCCAAAAGGG	Entire *rdmC*-like and *mph*(B) genes
MphB-KpnI-R	AATAGGTACCATAGTGGTAGG	

resistance to spiramycin and that the *mph*(B) and *rdmC*-like genes have an enhanced effect on spiramycin resistance when combined in gram-negative and -positive hosts.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. uberis* UCN60 and *S. uberis* UCN61 were isolates obtained from clinical mastitis in cows and were resistant and susceptible to spiramycin, respectively. *E. coli* AG100A, *Staphylococcus aureus* RN4220, and *Enterococcus faecalis* JH2-2 were used in cloning experiments. *E. coli* AG100A is a mutant susceptible to lincosamides resulting from inactivation of the AcrAB pump responsible for the active efflux of lincosamides by transposon Tn*903*, harboring a kanamycin resistance gene (20). Strains were grown in brain heart infusion broth and agar incubated aerobically at 37°C.

Antibiotic susceptibility testing. Susceptibility to antibiotics was determined by the agar diffusion technique as recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (4). MICs were determined by the broth microdilution method (15) by use of Mueller-Hinton broth supplemented with 5% lysed horse blood for *S. uberis* and incubated at 35°C in ambient air for 24 h. Antibiotics were obtained from Sigma (Saint Quentin Fallavier, France) or from their manufacturers. Recommended CA-SFM breakpoints were used, including that for spiramycin (susceptible was defined as an MIC of ≤1 μg/ml; resistant was defined as an MIC of >4 μg/ml).

Spiramycin inactivation. Spiramycin inactivation by resting cells of *S. uberis* UCN60 and *E. coli* AG100A transformants was tested in liquid medium as previously described (13). Briefly, bacterial cells were suspended in 0.01 M phosphate buffer (pH 7) containing 6 μg of spiramycin per ml and were incubated at 37°C for 24 h. The pH of this suspension was monitored and remained constant. The inactivation of spiramycin was followed by a bioassay with *Micrococcus luteus* ATCC 9341 as an indicator organism.

PCR conditions. Oligonucleotide primers used in this study are given in Table 1. The PCR consisted of 30 cycles of denaturation (95°C, 30 s), annealing (50°C, 30 s), and extension (72°C, 30 s to 3 min).

Cloning and sequencing of a DNA fragment conferring resistance to spiramycin in *E. coli*. Chromosomal DNA from *S. uberis* UCN60 was digested with various restriction enzymes and ligated at 4°C to plasmid vector pUC18 digested with the corresponding restriction enzymes. Recombinant plasmids were introduced by electroporation (Gene Pulser; Bio-Rad, Ivry-sur-Seine, France) into competent *E. coli* AG100A cells. *E. coli* AG100A transformants were selected on media containing spiramycin (64 μg/ml), ampicillin (100 μg/ml), and kanamycin (20 μg/ml). DNA strands were sequenced in both directions. DNA analyses and nucleotide comparisons were carried out using the National Centre for Biotechnology Information server at http://www.ncbi.nlm.nih.gov/.

Cloning of the *mph*(B) and *rdmC*-like genes. A DNA fragment containing the *rdmC*-like and *mph*(B) genes and two other DNA fragments containing each gene alone were amplified by PCR using total DNA of *S. uberis* UCN60 as a template. Primers shown in Table 1 contained a KpnI site and were specific of DNA flanking the *rdmC*-like and/or the *mph*(B) gene. PCR fragments were digested with KpnI and cloned in the gentamicin-resistant shuttle vector pAT392 (1). The three recombinant plasmids bearing the *rdmC*-like and *mph*(B) genes alone or combined were then electroporated into *E. coli* AG100A, *S. aureus* RN4220, and *E. faecalis* JH2-2.

Nucleotide sequencing accession number. The nucleotide sequence of the *acrR*-like, *rdmC*-like, and *mph*(B) gene cluster has been deposited in the GenBank data library under accession no. EU727204.

RESULTS

Resistance to spiramycin in *S. uberis* UCN60. *S. uberis* UCN60 displayed an unusual resistance to spiramycin (the MIC for *S. uberis* UCN60 was 8 μg/ml, compared to 0.5 μg/ml for *S. uberis* UCN61). By contrast, *S. uberis* UCN60 was susceptible to the other tested macrolides and lincosamides, with MICs of clindamycin (0.03 μg/ml), erythromycin (0.06 μg/ml), azithromycin (0.12 μg/ml), josamycin (0.25 μg/ml), and tylosin (0.5 μg/ml) similar to those for *S. uberis* UCN61. Remarkably, among the three tested 16-membered macrolides, only spiramycin had a high MIC, whereas the two others, josamycin and tylosin, remained active. This dissociated phenotype contrasted with the usual phenotype of cross-resistance between macrolides, lincosamides, and type B streptogramins due to ribosomal methylation. The inactivation of spiramycin by resting cells of *S. uberis* UCN60 was checked by a microbiological technique. In the presence of nearly 10^9 cells of *S. uberis* UCN60, the concentration of native spiramycin (20 μg in a volume of 1 ml) decreased within 1 h to 2.5 μg (which is the limit of detection of the method). The spiramycin concentration remained constant with the negative control, *S. uberis* UCN61.

Cloning of resistance to spiramycin. Fragments from *S. uberis* UCN60 total DNA digested with various restriction enzymes were inserted into plasmid pUC18 DNA digested similarly and introduced into *E. coli* AG100A by electrotransformation. The plasmid contents of transformants resistant to ampicillin, kanamycin, and spiramycin were analyzed by agarose gel electrophoresis of crude bacterial lysates. One of the transformants harboring plasmid pUC18 with a 2.5-kb HindIII insert that conferred resistance to spiramycin (MIC = 200 μg/ml) in *E. coli* AG100A was studied further. The transformant was found able to inactivate spiramycin. The sequence of the insert was determined and its analysis revealed homology with known genes. The cloned DNA was nearly identical (>99%) to a fragment of plasmids pTZ3723 and pTZ3721 of *E. coli* BM2506 that contained, from 5′ to 3′, an *acrR*-like gene putatively encoding a protein that belonged to the AcrR/TetR

TABLE 2. MICs of macrolides and clindamycin for *E. coli* AG100A, *S. aureus* RN4220, and *E. faecalis* JH2-2 transformants

Bacterial strain	Plasmid	MIC (μg/ml) of:				
		Erythromycin	Josamycin	Tylosin	Spiramycin	Clindamycin
E. coli AG100A	pAT392	2	8	16	8	2
	pAT392::*rdmC*	2	8	16	200	2
	pAT392::*mph*(B)	64	16	128	400	2
	pAT392::*rdmC*+*mph*(B)	64	16	>128	1000	2
S. aureus RN4220	pAT392	0.25	1	1	4	0.06
	pAT392::*rdmC*	0.25	1	1	4	0.06
	pAT392::*mph*(B)	0.25	1	1	8	0.06
	pAT392::*rdmC*+*mph*(B)	0.25	1	16	32	0.06
E. faecalis JH2-2	pAT392	0.25	1	1	0.5	2
	pAT392::*rdmC*	0.25	1	1	2	2
	pAT392::*mph*(B)	0.25	1	1	8	2
	pAT392::*rdmC*+*mph*(B)	0.25	1	16	64	2

family, an *rdmC*-like gene, and the first 196 nucleotides of *mph*(B).

We designed various oligonucleotide primers from the sequence of plasmids pTZ3723 and pTZ3721 to amplify by PCR overlapping fragments of the *acrR*-like, *rdmC*-like, and *mph*(B) genes from total DNA of *S. uberis* UCN60. These PCR experiments and sequence determination confirmed the presence of the *acrR*-like and *rdmC*-like genes and revealed the presence of the entire *mph*(B) gene. However, genes present downstream and upstream from this gene cluster in plasmids pTZ3723 and pTZ3721 could not be amplified from *S. uberis* UCN60. The *mph*(B) gene from our strain was 100% identical to the *mph*(B) gene encoding a phosphotransferase known to inactivate 14-, 15-, and 16-membered macrolides in *E. coli* (11, 17). The deduced amino acid sequence of the *rdmC*-like gene displayed homology (from 34% to 50% identity) with a variety of proteins belonging to the alpha/beta hydrolase family, including RdmC, a methyl esterase involved in the biosynthesis of anthracyclines in *Streptomyces* species (29), streptothricin acetyltransferases, several carboxylesterases, and acetyltransferases described for *Bacillus* spp. and various species of enterobacteria.

No *mph*(B), *rdmC*-like, or *acrR*-like gene was detected by PCR in the spiramycin-susceptible strain *S. uberis* UCN61.

Effects of the combined *rdmC*-like and *mph*(B) genes. Cloning of the spiramycin resistance of *S. uberis* UCN60 into *E. coli* showed that the *rdmC*-like gene was sufficient to confer resistance to the macrolide, at least in this host. However, the presence of *mph*(B), adjacent to the *rdmC*-like gene, suggested that this gene might be also involved in resistance to spiramycin. Both genes were cloned, separately or combined, in shuttle vector pAT392 in order to determine the phenotype of resistance conferred in gram-negative and gram-positive hosts. Each construct was electroporated into *E. coli* AG100A, *S. aureus* RN4220, and *E. faecalis* JH2-2, and MICs of macrolides and clindamycin were determined (Table 2). MIC determinations revealed the following. (i) *rdmC*-like alone conferred resistance to spiramycin in *E. coli* and *E. faecalis* but not in *S. aureus*. This gene did not confer resistance to erythromycin, josamycin, or tylosin in those three hosts. (ii) *mph*(B) conferred resistance to all tested macrolides in *E. coli* but only to low levels of spiramycin in *E. faecalis* and in *S. aureus* (MIC = 8 μg/ml). (iii) Combination of *mph*(B) and *rdmC*-like genes contributed in a more-than-additive fashion to the high level of resistance to spiramycin and tylosin in *E. coli* (MICs > 128 μg/ml), *S. aureus* (MICs of 32 and 16 μg/liter, respectively), and *E. faecalis* (MICs of 64 and 16 μg/ml, respectively). No effect of the combination of genes against erythromycin and josamycin was observed. (iv) Clindamycin activity was not affected by *rdmC*-like or *mph*(B).

DISCUSSION

The *mph*(B) gene was initially reported for *E. coli* to inactivate macrolides by O phosphorylation of the 2′ hydroxyl group of the amino sugar of these molecules. For the first time, we report the presence of this gene in a gram-positive clinical isolate, *S. uberis* UCN60. It is interesting to note that the G+C content of *mph*(B) (38%) is more closely related to that of the *S. uberis* genome (34.8 to 37%) than to that of the *E. coli* genome (50%). Possibly, the gene might originate from *S. uberis*, or, alternatively, *S. uberis* UCN60 might have acquired the gene from an unknown gram-positive donor.

The *mph*(B) gene conferred resistance by inactivation to erythromycin, josamycin, tylosin, and spiramycin in *E. coli* AG100A but only to spiramycin in *E. faecalis* JH2-2 and in *S. aureus* RN4220. A similar expression of resistance has already been described by Noguchi et al. (18). It can be presumed that the activity of a given macrolide against a given bacterial strain producing an MPH-(2′)-II enzyme would result from a competition between the ribosome and the enzyme for binding the drug. Hypothetically, the difference between the various macrolides might reflect different affinities of the molecules for the ribosomes of gram-positive and gram-negative organisms and for the Mph(B) enzyme.

As in plasmids pTZ3721 and pTZ3723 of *E. coli* BM2506, an *rdmC*-like and a putative regulator *acrR*-like gene are located upstream from the *mph*(B) gene (11, 17). The product of *rdmC*-like is related to RdmC, a methyl esterase involved in the biosynthesis of aklavonone antibiotic that has never been reported as conferring antibiotic resistance, and to acetyltransferases conferring resistance to the streptothricin antibiotic (8, 11, 29). The product of the *acrR*-like gene belongs to the AcrR/TetR family of transcriptional repressor (21). Subcloning experiments confirmed that the *rdmC*-like gene was able to confer resistance to spiramycin by inactivation in *E. coli* and *E.*

faecalis but not in *S. aureus*. Combination of the *rdmC*-like gene and of *mph*(B) in the three recipients resulted in MICs of spiramycin and tylosin higher than those expected from just an additive effect. Clinically significant levels of resistance to both macrolides might be achieved only when the resistance genes are combined. Surprisingly, in the original host, *S. uberis*, the presence of the *rdmC*-like and *mph*(B) genes did not confer resistance to tylosin. Despite repeated attempts, we were unable to introduce the *rdmC*-like gene with or without *mph*(B) into a susceptible strain of *S. uberis* and therefore to confirm the difference of expression in this genetic background. The gene combination was already widespread in *S. uberis*, since we identified by PCR the presence of both genes in 10 isolates from bovine mastitis resistant to spiramycin by inactivation (data not shown). Other genes that were characterized in plasmids pTZ3721 and pTZ3723, in particular a transposase gene, were not found in our isolate.

The mechanism of inactivation of spiramycin by Mph(B) and RdmC-like was not characterized in this study. The function of Mph(B) should be similar to that previously reported, whereas the function of RdmC-like remained obscure (18). Dual modification of spiramycin molecules by RdmC-like and Mph(B) enzymes might explain the marked increase in MIC of the antimicrobial (≥32 μg/ml). However, this hypothesis remains to be confirmed.

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