ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2000, p. 967–971 Vol. 44, No. 4
0066-4804/00/$04.00+0

Macrolide Resistance Genes in *Enterococcus* spp.

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Received 15 July 1999/Returned for modification 10 November 1999/Accepted 30 December 1999

Seventy-eight isolates of different *Enterococcus* species (*E. faecalis*, *n* = 27; *E. faecium*, *n* = 23; *E. durans*, *n* = 8; *E. avium*, *n* = 6; *E. hirae*, *n* = 9; *E. gallinarum*, *n* = 3; and *E. casseliflavus*, *n* = 2) with a variety of erythromycin resistance phenotypes were examined for the presence of macrolide resistance genes (*ermA*, *ermB*, *ermC*, *ermTR*, *mefA*/*E*, and *msrA*). Positive PCR amplifications of *ermB* were obtained for 39 of 40 highly erythromycin-resistant *Enterococcus* isolates (MICs, >128 μg/ml) of different species; the remaining highly resistant *E. faecium* isolate was positive for PCR amplification of *ermA* but was negative for PCR amplification of the *ermB* and *ermC* genes. For all enterococcal strains for which erythromycin MICs were ≤32 μg/ml PCRs were negative for *erm* methylase genes. For all *E. faecium* isolates PCR amplified products of the expected size of 400 bp were obtained when *msrA* primers were used, with the results being independent of the erythromycin resistance phenotype. All the other enterococcal species gave negative results by *msrA* PCRs. Sequencing of the *msrA* PCR products from either erythromycin-susceptible, low-level-resistant, or highly resistant *E. faecium* strains showed that the amplicons did not correspond to the *msrA* gene described for *Staphylococcus epidermidis* but corresponded to a new putative efflux determinant, which showed 62% identity with the *msrA* gene at the DNA level and 72% similarity at the amino acid level. This new gene was named *msrC*.

Over the last few years, *Enterococcus* has emerged as an important bacterial pathogen in nosocomial infections (13). The acquisition of specific mechanisms of resistance to different antibiotics, especially for the species *Enterococcus faecium*, has rendered infections with these microorganisms difficult to treat (8, 25); in just 10 years, antibiotic resistance has spread rapidly among enterococci and has become an important public health concern (11, 14). Macrolide-lincosamide-streptogramin (MLS) antibiotics constitute an alternative therapy for the treatment of insidious enterococcal infections. Three different mechanisms account for the acquired resistance to MLS antibiotics in gram-positive bacteria: modification of the drug target, inactivation of the drug, and active efflux of the antibiotic. In the first case, a single alteration of the 23S rRNA confers broad cross-resistance to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics, whereas the inactivation mechanism confers resistance only to structurally related MLS antibiotics. Regarding the pump mechanisms, the *mefA* (4), *mefE* (34), *msrA* (29), and *mreA* (5) genes have been involved in the active efflux of macrolides in gram-positive bacteria. The *mef* and *mreA* genes have been associated with macrolide resistance, and the *msrA* gene has been associated with macrolide and streptogramin B resistance. Erythromycin resistance by *erm* methylases of the *ermB-ermAM* hybridization class has been described in *Enterococcus* isolates (3, 15, 19). However, even though some reports indicate the presence of a putative erythromycin efflux pump in this bacterial genus (21; H. Fraimow and C. Knob, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., 1997, abstr. A-125, p. 22, 1997), little is known of the presence of such resistance determinants in enterococci.

The work described here was designed to study the presence of different erythromycin resistance genes in *Enterococcus* isolates of different species and with a variety of erythromycin susceptibility patterns. A novel intrinsic gene that encodes a putative ABC transporter was identified in all *E. faecium* isolates and presumably accounts for the higher macrolide MICs for this species in comparison with those for other enterococci (27).

(This work was presented in part at the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., 26 to 29 September 1999.)

MATERIALS AND METHODS

Bacterial isolates. Seventy-eight isolates of different *Enterococcus* species with a variety of erythromycin susceptibility patterns were included in this study (see Table 1): *E. faecalis*, *n* = 27; *E. faecium*, *n* = 23; *E. durans*, *n* = 8, *E. avium*, *n* = 6; *E. hirae*, *n* = 9; *E. gallinarum*, *n* = 3; and *E. casseliflavus*, *n* = 2. Sixty-three isolates were obtained from human clinical samples from the Hospital San Millán of Logroño, Spain; 2 *E. faecium*, 8 *E. hirae*, and 2 *E. gallinarum* isolates were of animal origin; and 1 *E. hirae* isolate (CECT 302), 1 *E. gallinarum* isolate (CECT 970), and 1 *E. casseliflavus* isolate (CECT 969) were from the Spanish Culture Type Collection. Species identification was based on the biochemical API 20 Strep system (BioMerieux, la Balme, France) and was also carried out according to the biochemical scheme of Facklam and Collins (10).

Susceptibility testing. Susceptibility testing was performed by the agar dilution method in Mueller-Hinton (MH) agar (Difco, Detroit, Mich.) by the standard method of the National Committee for Clinical Laboratory Standards (26). The antibiotics tested were erythromycin and spiramycin (Sigma Chemical Co.), azithromycin (Hoechst Marion Roussel, Romainville, France), and pristinamycin I (Rhône Poulenc Rorer, Paris, France).

Antibiotic inactivation test. A bioassay for the detection of the erythromycin inactivation mechanism was performed (12, 35). *Enterococcus* strains were incubated in brain heart infusion broth (Difco) with 40 μg of erythromycin per ml for 48 h. After centrifugation, 25 μl of the supernatant was deposited on sterile disks over MH agar plates previously seeded with *Micrococcus luteus* ATCC 9341. The plates were incubated for 24 h, and the zone sizes around the disks, which indicate the antibiotic remaining in the culture medium after incubation with cells, were measured. The zone sizes were compared with those produced when the antibiotic was incubated with either medium alone or with the erythromycin-susceptible *E. faecium* AR10 (erythromycin MIC, 0.5 μg/ml).

PCR analysis of erythromycin resistance genes. The presence of genes involved in MLS resistance with a methylation mechanism was determined by PCR amplification of known *erm* genes by using primers specific for *ermA*, *ermB*, and *ermC* (33) and for *ermTR* (17, 32). The presence of genes involved in antibiotic efflux systems was determined by PCR with gene-specific primers and conditions for the amplification of the *mefA*/*E* (33) and *msrA* (35) genes. The low-level

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TABLE 1. Macrolide resistance genes in *Enterococcus* sp. isolates with different macrolide resistance phenotypes.

Enterococcal species	No. of strains	MIC range (μg/ml)[a]				PCR amplification				
		ERY	AZM	SPY	PRI-I	*msrA*	*ermA*	*ermB*	*ermC*	*mefA/E*
E. faecium	12	>128	>128	64–>128	64–>128	+	−	+	−	−
	1	>128	>128	>128	>128	+	+	−	−	−
	1	32	64	0.5	1	+	−	−	−	−[b]
	9	≤0.125–16	≤0.125–64	0.5–4	1–2	+	−	−	−	−
E. faecalis	14	>128	>128	>128	64–>128	−	−	+	−	−
	13	≤0.125–8	≤0.125–32	0.25–2	1–4	−	−	−	−	−
E. durans	2	>128	>128	>128	≥128	−	−	+	−	−
	6	≤0.125–0.25	≤0.125	0.25–2	≤0.125–1	−	−	−	−	−
E. avium	2	>128	>128	>128	≥128	−	−	+	−	−
	4	0.25–16	2–64	1–4	0.5–2	−	−	−	−	−
E. hirae	8	>128	>128	>128	4–16	−	−	+	−	−
	1	≤0.5	0.5	4	1	−	−	−	−	−
E. gallinarum	1	>128	>128	>128	>128	−	−	+	−	−
	2	≤0.5–1	2	4	1	−	−	−	−	−
E. casseliflavus	2	4	32	1	2–4	−	−	−	−	−

[a] ERY, erythromycin; AZM, azithromycin; SPY, spiramycin; PRI-I, pristinamycin I.
[b] A PCR product of 450 bp (larger than expected) was obtained.

erythromycin-resistant *Enterococcus* isolates that gave negative results in the PCR experiments described above were analyzed by using degenerate *erm* primers (2). Positive and negative controls were included in all experiments. Genomic DNA for PCR analysis was obtained with the Instagene matrix system (Bio-Rad) according to the manufacturer's instructions.

DNA isolation and Southern blot hybridization. Plasmid and genomic DNAs from *Enterococcus* isolates were extracted by alkaline lysis as described previously (31) by lysozyme treatment for 1 h. Southern blotting was performed with total DNAs and plasmid DNAs from erythromycin-resistant and erythromycin-susceptible *E. faecium* isolates by using an *msrA*-like PCR fragment (obtained from erythromycin-resistant isolate *E. faecium* E134) as the probe. This probe was labeled with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim). The homology of *ermTR* PCR amplicons with the *ermTR* gene was also analyzed by Southern blotting. An *ermTR* fragment from a group G *Streptococcus* strain, strain S211, which contained the *ermTR* gene, was labeled with digoxigenin and was used as a probe. Hybridizations were carried out at 50°C, and in all cases, positive and negative controls were included.

DNA sequencing. The amplicons were obtained with *msrA*-specific primers (35) by PCR analysis of genomic DNAs of the following strains: *E. faecium* AR10 (erythromycin MIC, 0.5 μg/ml), *E. faecium* E136 (erythromycin MIC, 32 μg/ml) and *E. faecium* E134 (erythromycin MIC, >128 μg/ml). The amplicons were then purified and sequenced. The amplicon obtained with *ermA*-specific primers (33) by PCR analysis of genomic DNA of *E. faecalis* E307 was also purified and sequenced. Automatic sequencing (ABI 310 gene sequencer; Perkin-Elmer) was carried out by using the same primers used for the PCRs. Analysis of the sequences was performed with the aid of Wisconsin Package (version 9.1; Genetics Computer Group, Madison, Wis.).

Nucleotide sequence accession number. The nucleotide sequence of the *E. faecium msrC* gene has been assigned GenBank accession no. AJ243209.

RESULTS AND DISCUSSION

The MICs of erythromycin, azithromycin, spiramycin, and pristinamycin I were determined for 78 strains belonging to seven different *Enterococcus* species. Two groups of strains (highly resistant and susceptible) could be distinguished among all *Enterococcus* species, depending on the MICs obtained. One *E. faecium* strain (strain E136) with a low level of resistance to the macrolides was also found. All the *Enterococcus* isolates included in this study were classified according to their species and their erythromycin susceptibility patterns, and a variety of erythromycin resistance mechanisms was investigated by PCR (Table 1). These groups proved to be homogeneous: all strains that belonged to the same group had the same antibiotic resistance determinants.

The inactivation tests described above were performed with 19 strains of the seven different enterococcal species included in this study with a variety of erythromycin susceptibility patterns. No significant differences in zone sizes were observed between susceptible and resistant strains, nor were significant differences in zone sizes observed when they were compared with the zone sizes for erythromycin incubated under the same conditions but without the presence of bacterial cells. These results indicate that the strains that were analyzed do not express a detectable mechanism of erythromycin inactivation under these conditions.

Presence of *erm* methylase genes. The *Enterococcus* isolates were analyzed for the presence of *erm* methylase genes by PCR by using specific conditions for detection of the *erm* genes characterized in gram-positive bacteria (see Materials and Methods). When PCR analysis was carried out with specific primers for the amplification of the *ermB* gene, a band with the expected molecular size (639 bp) was obtained for 39 of the 40 highly erythromycin-resistant *Enterococcus* isolates (MICs, >128 μg/ml), independently of the species involved (12 *E. faecium*, 14 *E. faecalis*, 2 *E. durans*, 2 *E. avium*, 8 *E. hirae*, and 1 *E. gallinarum* isolates) (Table 1). For all these strains, the MICs of azithromycin, spiramycin, and pristinamycin I were always ≥64 μg/ml. The remaining highly resistant *E. faecium* isolate was positive by PCR with the *ermA*-specific primer and negative by PCR with *ermB*- and *ermC*-specific primers. This *ermA* amplicon was sequenced and was found to have 100% homology with the *ermA* gene described for *Staphylococcus aureus* (24); this gene has been frequently associated with macrolide resistance in *S. aureus* and coagulase-negative staphylococci (20). To our knowledge, this is the first description of the *ermA* gene in enterococci. No PCR fragment of the expected size was obtained from any of the enterococcal isolates for which erythromycin MICs were ≤32 μg/ml (10 *E. faecium*, 13 *E. faecalis*, 6 *E. durans*, 4 *E. avium*, 1 *E. hirae*, 2 *E. gallinarum*,

and 2 *E. casseliflavus* isolates) with either *ermA*-, *ermB*-, or *ermC*-specific primers. The same results were obtained when PCRs were carried out with degenerate *erm*-specific primers. These results indicate that *erm* genes could be present only in highly macrolide-resistant strains of *Enterococcus*.

The presence of the *ermTR* gene was also investigated by PCR. Several DNA amplification fragments (some of them of the expected size) were obtained when *ermTR*-specific primers were used with DNAs from 19 enterococcal isolates (7 *E. faecium*, 4 *E. faecalis*, 4 *E. avium*, 3 *E. durans*, and 1 *E. gallinarum* isolates). The homologies of those amplicons with the *ermTR* gene were analyzed by Southern blotting (at 50°C) with an *ermTR*-specific probe from a group G *Streptococcus* strain, strain S211, which contained the *ermTR* gene. Under conditions in which the positive control produced a strong hybridization signal, none of the amplicons hybridized with the probe. These results indicate that, despite the PCR analysis, the enterococcal isolates did not contain sequences homologous to *ermTR*. Erythromycin resistance is frequently associated with the *ermTR* gene in *Streptococcus pyogenes* (33) and in group G *Streptococcus* (17); however, our data indicate that this gene does not play a role in the acquisition of macrolide resistance in *Enterococcus*.

The *ermB* gene has previously been demonstrated to be involved in macrolide resistance in different gram-positive bacteria, such as *Enterococcus* (15), *Streptococcus pneumoniae* (16), *S. pyogenes* (18), and *S. aureus* (9). All our data taken together indicate that the *ermB* gene is most frequently found among the highly resistant *Enterococcus* isolates tested in our study, irrespective of the species. Thus, its acquisition could have a predominant role in the development of high-level erythromycin resistance in *Enterococcus* spp.

Presence of erythromycin efflux *mef* genes. The presence of erythromycin-efflux genes in *Enterococcus* isolates was analyzed by PCR with primers specific for the *mefA* and *mefE* genes. *mef* efflux pump genes have been detected in *S. pyogenes* (4), *S. pneumoniae* (34), and *Streptococcus agalactiae* (1), as well as in *Micrococcus luteus*, *Corynebacterium jeikeiium*, *Corynebacterium* spp., and viridans group streptococci (21). Previous reports have indicated that *mefE* might have an important role in erythromycin resistance in *E. faecium*; according to studies carried out in the United States (Fraimow and Knob, Abstr. 97th Gen. Meet. Am. Soc. Microbiol. 1997), 42% of the resistant strains have been reported to carry this determinant. In the same way, very recently, Luna et al. (21) have reported on the presence of *mef* genes in *Enterococcus* spp. However, we were unable to detect amplification with any of the enterococcal isolates when the *mefA/E*-specific primers were used in PCR analysis. This result may reflect a different geographical distribution of *mef* genes in *E. faecium*. In one of our samples (*E. faecium* E136), a faint band of 450 bp, which was larger than expected (348 bp), was obtained. *E. faecium* E136 was the only isolate with a low-level erythromycin resistance phenotype (MIC, 32 μg/ml) (Table 1). The homology of the 450-bp amplicon obtained from *E. faecium* E136 was analyzed by Southern blotting with a *mefA*-specific probe (obtained from *S. pyogenes* S2). The amplicon gave a positive signal upon hybridization at 50°C (data not shown). The positive results by both PCR analysis and Southern hybridization indicated the presence of a DNA sequence related to the *mef* sequence in this isolate (A. Portillo, A. Alonso, F. Ruiz-Larrea, M. Zarazaga, J. L. Martinez, and C. Torres, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-122, 1998). However, the different size of the PCR fragment indicated that it was not the same *mef* gene so far described.

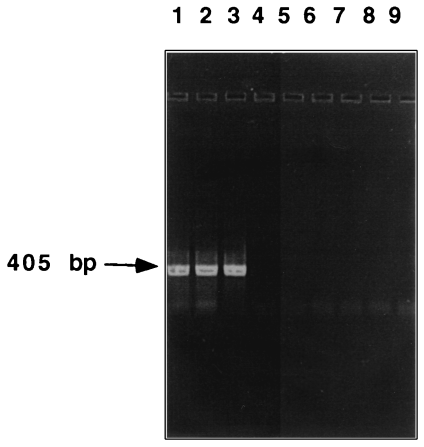

FIG. 1. PCR amplification with *msrA*-specific primers. Lanes: 1, *E. faecium* E134 (erythromycin MIC, >128 μg/ml); 2, *E. faecium* E136 (MIC, 32 μg/ml); 3, *E. faecium* AR10 (MIC, 0.5 μg/ml); 4, *E. faecalis* E121 (MIC, >128 μg/ml); 5, *E. durans* AR23 (MIC, >128 μg/ml); 6, *E. avium* E402 (MIC, >128 μg/ml); 7, *E. hirae* P9 (MIC, >128 μg/ml); 8, *E. gallinarum* AR45 (MIC, >128 μg/ml); 9, *E. casseliflavus* C85 (MIC, 4 μg/ml). The DNA band of 405 bp is marked with an arrow.

***msrC* gene.** PCR analysis for determination of the presence of *msrA* produced an unexpected result. The *msrA* gene, first identified in *Staphylococcus epidermidis* (29), confers resistance by an efflux system after induction with erythromycin, and an *msrA*-related gene, *msrB*, has been described in *Staphylococcus xylosus* (23). All our *E. faecium* isolates gave a PCR amplification product with the expected molecular size with *msrA*-specific primers (Fig. 1), irrespective of their MLS resistance phenotypes. Nevertheless, no *msrA* gene was found in any of the other *Enterococcus* species by the same PCR protocol. Similar results were obtained by hybridization at 50°C by using the *msrA*-like PCR fragment from *E. faecium* E134 as a probe; positive results were obtained for *E. faecium* strains, and negative results were obtained for the other enterococcal species. The *msrA*-like amplicons obtained from susceptible strain *E. faecium* AR10 (erythromycin MIC, 0.5 μg/ml), low-level-resistant strain *E. faecium* E136 (MIC, 32 μg/ml), and highly resistant strain *E. faecium* E134 (MIC, >128 μg/ml) were sequenced. No differences were observed among the sequences of the three fragments. However, even though the amplicons were of the expected molecular size, the sequence obtained was different from that of *msrA*. When compared with *msrA*, this novel gene showed an identity of 62% at the DNA level and a similarity of 72% at the amino acid level, with an overlap of 135 amino acids. This new gene was named *msrC* (GenBank accession no. AJ243209). The homology was extremely high for the regions that contained the nucleotide binding motifs and the signature sequence included in the ABC transporter domain described for other MLS efflux determinants from gram-positive bacteria (erythromycin, tylosin, carbomycin, pristinamycin, virginiamycin) (Fig. 2). This analysis strongly suggests that *msrC* also belongs to this efflux pump gene family. Figure 2 shows the two nucleotide-binding motifs of the *msrC* gene: motif A, which corresponds to a P loop, and motif B, which, together with the signature pattern for this class of ABC transporters, is located between the A and the B motifs of the ATP-binding site (30).

The ubiquity of the *msrC* gene among *E. faecium* isolates might indicate that it is an indigenous gene present in the chromosomes of all isolates of this bacterial species or that it is located in an epidemic plasmid present in all *E. faecium* iso-

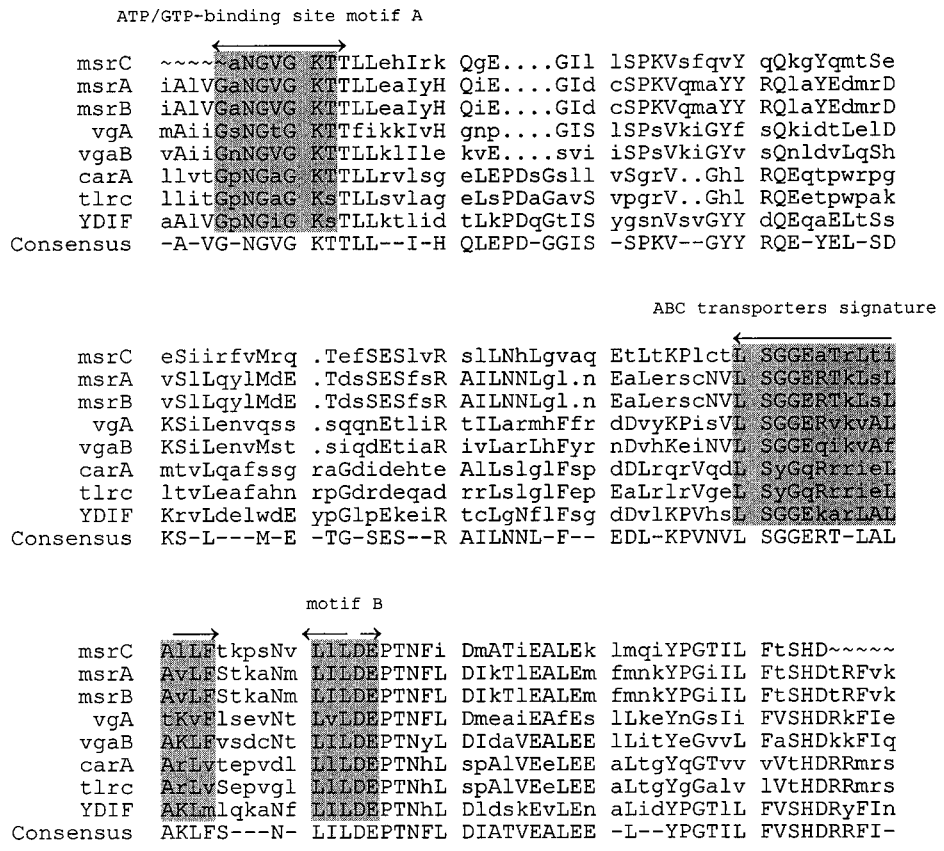

FIG. 2. Comparison of MsrC amino acid sequence with other sequences of ABC transporter. MsrC, AJ243209 efflux pump in *E. faecium*; MsrA, P23212 erythromycin resistance protein in *S. epidermidis*; MsrB, M81802 erythromycin resistance protein in *S. xylosus*; VgA, JC1204 putative ATP-binding protein involved in resistance to virginiamycin in *S. aureus*; VgaB, AAB95639 pristinamycin resistance protein in *S. aureus*; CarA, AAC32027 carbomycin resistance protein in *Streptomyces thermotolerans*; TlrC, P25256 tylosin resistance protein in *Streptomyces fradiae*; YDIF, O05519 ABC transporter (ATP-binding protein) in *Bacillus subtilis*. Nucleotide binding motifs and the ABC transporter signature (PS00211) are indicated by shaded areas.

lates in this collection. To distinguish between the two possibilities, Southern blotting hybridization was carried out with plasmid and genomic DNAs from erythromycin-resistant and -susceptible *E. faecium* isolates by using an *msrC* PCR fragment from *E. faecium* E134 as a probe. Positive signals were obtained for the chromosomal DNAs but not for the plasmids, indicating that *msrC* is an indigenous gene in *E. faecium* but not in other *Enterococcus* species (data not shown). It is worth noting that previously described *msrA* and *msrB* genes are inducible and are located in large plasmids, in contrast to this novel *msrC* gene. Lynch et al. (22) refers to an active efflux of antimicrobial agents from wild-type strains of enterococci that pumped out norfloxacin and chloramphenicol. Other macrolide efflux pump genes, such as *mreA* in *S. agalactiae* (5), which contain no ABC transporter domain in their structures, have been described. The presence of this new putative efflux pump determinant in all *E. faecium* isolates indicates that it was not acquired as a response to antibiotic selective pressure but is an intrinsic gene that could constitute an advantage for the species; generally, *E. faecium* has been reported to be more resistant to macrolides (MICs at which 50% of isolates are inhibited are 3 dilutions higher) than other enterococci (27). The function of this novel *msrC* gene, which encodes a putative efflux pump of the ABC transporter family, is probably other than macrolide resistance. However, it could affect the efflux of antibiotics in a way so far described for many other indigenous efflux pump systems (28).

In summary, PCR analysis with the *msrA*-specific primers designed for *S. aureus* gives a DNA fragment of the expected size for all *E. faecium* strains; since it shows 62% identity at the nucleotide level with the *msrA* gene, it has been named *msrC*. Detection of the *aac*(*6′*)-*Ii* gene, which codes for a chromosomal aminoglycoside acetyltransferase specific for *E. faecium* (7), has been efficiently used for identification of *E. faecium* species (6). Primers specific for *msrC* could also be useful for detection and identification of *E. faecium* species. This topic is under study in our laboratory.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Ministerio de Salud y Consumo of Spain (grant FIS 98/0282). Aránzazu Portillo has an FPI fellowship from the Ministerio de Educación y Ciencia, and Ana Alonso is a recipient of a fellowship from the Gobierno Vasco of Spain.

We thank M. Lantero and M. J. Gastañares as well as to the Spanish Culture Type Collection for providing us with some of the enterococcal strains used in this study.

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