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Distribution of *mef*(A) in Gram-Positive Bacteria from Healthy Portuguese Children

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We screened 615 gram-positive isolates from 150 healthy children for the presence of the *erm*(A), *erm*(B), *erm*(C), *erm*(F), and *mef*(A) genes. The *mef*(A) genes were found in 20 (9%) of the macrolide-resistant isolates, including *Enterococcus* spp., *Staphylococcus* spp., and *Streptococcus* spp. Sixteen of the 19 gram-positive isolates tested carried the other seven open reading frames (ORFs) described in Tn*1207.1*, a genetic element carrying *mef*(A) recently described in *Streptococcus pneumoniae*. The three *Staphylococcus* spp. did not carry *orf1* to *orf3*. A gram-negative *Acinetobacter junii* isolate also carried the other seven ORFs described in Tn*1207.1*. A *Staphylococcus aureus* isolate, a *Streptococcus intermedius* isolate, a *Streptococcus* sp. isolate, and an *Enterococcus* sp. isolate had their *mef*(A) genes completely sequenced and showed 100% identity at the DNA and amino acid levels with the *mef*(A) gene from *S. pneumoniae*.

The normal flora is thought to act as a reservoir for many bacterial antimicrobial resistance genes, including those that confer macrolide resistance (12). In 1999, there were 20 different rRNA methylases described in the literature, which coded for macrolide-lincosamide-streptogramin B resistance, and 24 efflux and inactivating genes, which coded for one or more of the macrolide-lincosamide-streptogramin B complex of antimicrobials (14). However, relatively few of these 44 genes are found in the majority of macrolide-resistant gram-positive bacteria (1, 2, 13). Resistance to macrolides in the absence of resistance to lincosamides and streptogramin B has been associated with the presence of the *mef*(A) gene in *Streptococcus pneumoniae* (17, 18). The *mef*(A) gene has become more common than *erm*(B) in macrolide-resistant *S. pneumoniae* isolates from North America (7, 15). We have shown that the *mef*(A) gene is present in macrolide-resistant oral *Streptococcus* spp. and *Enterococcus* spp. isolated in Seattle, Wash., and *Micrococcus luteus* and *Corynebacterium* spp. isolated in the United Kingdom (5), as well as in gram-negative *Acinetobacter junii* and *Neisseria gonorrhoeae* (6). All of these species have been able to conjugally transfer the *mef*(A) genes to a variety of recipients. Recently, two genetic elements, Tn*1207.1* (16) and mega (3), have been characterized from macrolide-resistant *S. pneumoniae*. A highly related gene has been sequenced from *Streptococcus pyogenes*, while related genes have been identified in Lancefield group C and G streptococci from Finland (4).

In this study, we examined randomly selected gram-positive isolates collected from healthy Portuguese children for the presence of the common macrolide resistance genes, *erm*(A), *erm*(B), *erm*(C), *erm*(F), and *mef*(A). Representative *mef*(A) genes were sequenced, and the presence of the other seven open reading frames (ORFs) from Tn*1207.1* was investigated.

(The data in Table 2 were presented in part at the First Annual Symposium on Resistant Gram-Positive Infections in San Antonio, Tex., 3 to 5 Dec. 2000.)

MATERIALS AND METHODS

Bacterial isolates. A total of 615 randomly chosen isolates were included in the study: 392 oral and 223 urine gram-positive isolates collected from 150 healthy children enrolled in a randomized study of amalgam versus composite fillings. The children were 73 girls and 77 boys; 81% were Caucasian, 17% were of African descent, and 2% identified themselves as Asian or Pacific Islander. From the children's records, we found that during the collection period, five or six children per year received some type of medication from doctors. This included both antibiotics and nonantibiotics. The oral isolates were isolated from samples collected from the gingiva and buccal mucosa using the BBL CultureSwab Plus Transport System (Becton Dickinson, Sparks, Md.), while the urine isolates were collected from cultured urine. Individual colonies were identified using standard biochemical protocols (9) and frozen at −70°C until needed. Two *Corynebacterium* sp. isolates, one *Corynebacterium jeikeium* isolate, one *A. junii* isolate, and two *S. pneumoniae* isolates were tested for the presence of the seven different *orf* genes from Tn*1207.1*. These six isolates had previously been shown to carry the *mef*(A) gene (5–7).

Determination of antibiotic resistance phenotype. Susceptibilities were determined by disk diffusion assay, following NCCLS protocols, using the control organisms *Staphylococcus aureus* ATCC 25923 and *S. pneumoniae* ATCC 49619 (10).

Identification of resistance genes. For the initial testing, we used bacterial dot blots and radiolabeled internal oligonucleotide probes to screen the isolates for the presence of *erm*(A), *erm*(B), *erm*(C), *erm*(F), and/or *mef*(A) genes, as previously described (8). Positive and negative controls were included in each assay. The results were confirmed using PCR assays with hybridization of the PCR products as previously described (1, 2, 8). Representative isolates carrying the *mef*(A) gene were examined by DNA-DNA hybridization using seven radiolabeled oligonucleotide probes for the presence of the other seven ORFs described in Tn*1207.1* (Table 1).

PCR assays. The PCR assay for the *erm*(F) gene was done as previously described (1, 2). The PCR assay for the *mef*(A) gene used MF4 and MF6, both internal to the ends of the gene as previously described (5, 6). For sequencing the *mef*(A) gene, PCR with primers MEFF and MEFR, which are at the ends of the *mef*(A) gene, were used. The assay used 30- to 100-ng genomic DNA as a template, 2 U of *Taq* polymerase in a thermal cycler from Perkin-Elmer Cetus

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TABLE 1. Oligonucleotide probes and primers used to identify the *mef* and *orf* genes

Oligonucleotide	Sequence (5′-3′)	Gene[a]	Reference
ORF1	TGA TGA AGA GGA AAA TTA G	*orf1*	This study
ORF2	GCA TCA GGA ACA TCA ATC	*orf2*	This study
ORF3	GGT ATT GTT CAG GTA GGT C	*orf3*	This study
MEFF	ATG GAA AAA TAC AAC AAT TGG AAA C	*mef*(A)	This study
MEFR[a]	TTA TTT TAA ATC TAA TTT TCT TAA	*mef*(A)	This study
MF4	ACC GAT TCT ATC AGC AAA G	*mef*(A)	6
MF4AR	TTC TTT GCT GAT AAA ATC GGT GT	*mef*(A)	This study
MF6	GGA CCT GCC ATT GGT GTG	*mef*(A)	This study
MF6R	GCA CAC CAA TGG CAG GTC C	*mef*(A)	This study
MF7	ATG CAG ACC AAA AGC CAC AT	Upstream from *mef*(A)	6
ORF5	CAT CTG GGT GAA CTT GCC	*orf5*	This study
ORF5FRev	GGC AAG TTC ACC CAG ATG	*orf5*	This study
ORF6	CAT GTT GGA GTA GCG GTA G	*orf6*	This study
ORF7	GAA ATC TTT GGT CAG ACT TGG	*orf7*	This study
ORF8	CCC TCC AAT CCA CCA GCG	*orf8*	This study

[a] Taken from Tn*1207.1*. All the primers except MEFF, MEFR, and MF7 are within the genes listed. MEFF is the first 25 bp of the *mef*(A) gene; MEFR contains the last 6 bp of the *mef*(A) gene and 15 bp downstream of the gene.

(Norwalk, Conn.), 200 μM deoxynucleoside triphosphates, 1× PCR buffer (1.5 mM $MgCl_2$), and 100 ng of each primer. The reactions consisted of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 2 min for 35 cycles. To finish sequencing the beginning of the gene, we used the MF7 primer, which is 102 bp upstream of the start of the *mef*(A) gene in Tn*1207.1*, and we used the Orf5FRev primer, which is 1,240 bp downstream of the *mef*(A) gene in Tn*1207.1* and internal to the *orf5* gene, to sequence the end of the *mef*(A) gene. We used ORF5FRev and MF4AR primers for one PCR assay. The reaction mixture included 30 to 100 ng of genomic DNA as a template, 2 U of *Taq* polymerase (Perkin-Elmer Cetus), 200 μM deoxynucleoside triphosphates, 1× PCR buffer (1.5 mM $MgCl_2$), and 100 ng of each primer. The reactions were carried out by denaturing at 96°C for 1 min, annealing at 42°C for 1 min, and elongation at 72°C for 2 min for 35 cycles. The PCR using the MF7 and MF6 primers used 30 to 100 ng of genomic DNA as a template, 2 U of *Taq* polymerase (Perkin-Elmer Cetus), 200 μM deoxynucleoside triphosphates, 1× PCR buffer (1.5 mM $MgCl_2$), and 100 ng of each primer. The reaction mixtures were denatured at 96°C for 1 min, annealed at 54°C for 1 min, and elongated at 72°C for 2 min for 35 cycles. The PCR products were dried, resuspended in 1/10 volume of sterile water, and separated on a 1.5% agarose gel with 0.5× TBE running buffer. The bands were visualized by ethidium bromide staining. Positive and negative controls were run with each assay. All primers are listed in Table 1.

Sequencing. We sequenced the PCR products from selected strains as previously described (2, 8, 11). The complete *mef*(A) genes were sequenced and compared to the previously sequenced *mef*(A) genes from *S. pneumoniae* (U83667) and *S. pyogenes* (U70055) and to AF227520 from Tn*1207.1* and AF376746 from the mega element. The *mef*(A) sequences AF227520 and U70055 have 100% DNA and amino acid identity with each other and 91% DNA and amino acid identity with U83667 and AF376746. The *mef*(A) sequences from U83667 and AF376746 have 100% DNA and amino acid identity with each other. The PCR product for *erm*(F) was sequenced and compared to the GenBank sequence accession no. M1712. The sequences were compared using the Biological Information Resource software at the University of Washington.

Mating procedure. Matings were done with *Enterococcus faecalis* JH2-2 as the recipient and *mef*(A)-positive isolates, including *S. aureus* 5, *Streptococcus intermedius* 424, *Enterococcus* sp. strain 130, and *E. faecalis* 2, as the donors. Matings were done as previously described (1, 2, 5, 6, 8, 13). We also used the *S. aureus* 5 and *S. intermedius* 424 donors with *Kingella denitrificans* 87.023461 and *Neisseria mucosa* CTM1.1 as recipients as previously described (6).

RESULTS

Detection of the five macrolide genes. We found that 375 (61%) of the isolates were susceptible to macrolides and did not hybridize with the five oligonucleotide probes used. This included 278 of the 392 (71%) oral isolates and 97 of the 223 (44%) urine isolates examined. Susceptible isolates from both the oral and urine sites included enterococci, coagulase-negative staphylococci, and *S. aureus*, while the oral isolates also included *Streptococcus* spp., *S. intermedius*, *Streptococcus mutans*, and *Streptococcus salivarius* (data not shown). Of the remaining isolates, 223 (36.3%) hybridized with one or more of the five genes used (Table 2).

The *mef*(A) gene was found in 20 isolates, including *Enterococcus* sp. (1 isolate), *E. faecalis* (1 isolate), *S. aureus* (1 isolate), *Staphylococcus haemolyticus* (1 isolate), *Staphylococcus* spp. (2 isolates), *S. intermedius* (2 isolate), and *Streptococcus* spp. (12 isolates), either alone (16 isolates) or with *erm* genes (4 isolates) (Table 2). Five of the isolates were urine isolates, and 15 were from oral samples; the oral *Streptococcus* spp. were the most prevalent with 11 isolates (Table 2). To verify the presence of the *mef*(A) genes, all 20 isolates were tested with the *mef* PCR assay. Each isolate gave PCR products of the appropriate size which hybridized with an internal probe (data not shown).

The *mef*(A) sequences were determined from the isolates *S. aureus* 5, *S. intermedius* 424, *Streptococcus* sp. strain 6, and *Enterococcus* sp. strain 130. These sequences had 100% DNA and amino acid identity with the U83667 and AF376746 sequences from GenBank and 91% identity with the AF227520 and U70055 sequences from GenBank (data not shown).

S. aureus 5, *S. intermedius* 424, *Enterococcus* sp. strain 130, and *E. faecalis* 2 were used as donors in mating experiments with the recipient *E. faecalis* JH2-2. All isolates were able to transfer the *mef*(A) gene to JH2-2 at frequencies ranging from 2.9×10^{-5} to 6.2×10^{-8} per recipient (data not shown). Similar frequencies were found when *S. aureus* 5 and *S. intermedius* 424 donors were mated with *K. denitrificans* 87.023461 or *N. mucosa* CTM1.1 as recipients.

Of the remaining 213 isolates, 1 enterococcus hybridized with the *erm*(F) probe. The PCR sequence had 99% base pair identity with the *erm*(F) GenBank sequence M1712 (data not shown). The *erm*(A) gene was found in three isolates: two staphylococci and one streptococcus. The *erm*(B), *erm*(C), and *erm*(F) genes were commonly found in the different genera examined, with 90% carrying a single determinant (Table 2). Of the 240 erythromycin-resistant isolates, 18 (7.5%) isolates (15 urine and 3 oral) were macrolide resistant but did not

TABLE 2. Distribution of macrolide resistance genes found in oral and urine isolates

Organism	no. of urine isolates	No. of isolates with gene(s)	No. of oral isolates	No. of isolates with gene(s)
Enterococcus spp.	38			
		34 *erm*(B)		
		1 *erm*(C)		
		1 *erm*(B), *erm*(C)		
		1 *erm*(B), *erm*(F)		
		1 *mef*(A)		
E. faecalis	2	1 *erm*(B)		
		1 *erm*(B), *mef*(A)		
S. aureus	11	4 *erm*(B)	6	1 *erm*(B)
		4 *erm*(C)		1 *erm*(C)
		2 *erm*(F)		1 *erm*(B)
		1 *erm*(C), *erm*(F), *mef*(A)		1 *erm*(B), *erm*(C)
				1 *erm*(C), *erm*(F)
				1 *erm*(B), *erm*(C), *erm*(F)
Staphylococcus spp.	52	1 *erm*(A)	9	
		15 *erm*(B)		
		12 *erm*(C)		6 *erm*(C)
		11 *erm*(F)		3 *erm*(F)
		7 *erm*(B), *erm*(C)		
		3 *erm*(B), *erm*(F)		
		1 *erm*(B), *erm*(C), *erm*(F)		
		2 *mef*(A)		
Staphylococcus epidermidis	3	1 *erm*(A)		
		2 *erm*(B)		
S. haemolyticus	2	1 *erm*(C)		
		1 *mef*(A)		
Staphylococcus hominis	1	1 *erm*(B)		
Staphylococcus saprophyticus	2	1 *erm*(B)		
		1 *erm*(B), *erm*(C)		
S. intermedius			6	1 *erm*(C)
				3 *erm*(F)
				1 *erm*(F), *mef*(A)
				1 *mef*(A), *erm*(B), *erm*(C), *erm*(F)
Streptococcus spp.			90	1 *erm*(A)
				53 *erm*(B)
				13 *erm*(C)
				7 *erm*(F)
				1 *erm*(B), *erm*(C)
				2 *erm*(B), *erm*(F)
				1 *erm*(C), *erm*(F)
				11 *mef*(A)
				1 *mef*(A), *erm*(C)
Total	111		111	

hybridize with the five probes used. Of these, 13 were staphylococci, which have been known to carry other macrolide resistance genes, including *erm*(Y) and *msr*(A) (14).

Detection of the seven *orf* genes from Tn*1207.1*. Recently Santagati and colleagues (16) described a genetic element (Tn*1207.1*) carrying the *mef*(A) gene and seven other ORFs in an *S. pneumoniae* isolate that was not able to transfer the *mef*(A) gene by conjugation. However, more recently, a new genetic element has been described (Tn*1207.3*) which carried Tn*1207.1*, and this element has been shown to be conjugative (M. Santagati, F. Iannelli, C. Messina, M. R. Ogginoi, S. Stefani, and G. Pozzi, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2014, 2001). The *mef*(A) gene is *orf4* in the Tn*1207.1* element. All six of these strains carried *orf1* to *orf8* (Table 3). We also tested *S. intermedius* (two isolates), *Streptococcus* spp. (seven isolates), *E. faecalis* (one isolate), *Enterococcus* sp. (one isolate), and three species of *Staphylococcus* (Table 3) with the seven other *orf* probes. The nine streptococcal and two enterococcal isolates hybridized with probes specific for each of the seven *orf* probes. In contrast, the three staphylococcal isolates did not hybridize with *orf1*, *orf2*, or *orf3* probes (Table 3) but did hybridize with *orf5* to *orf7* probes (Table 3).

Nucleotide sequence accession numbers. The GenBank accession numbers are AY064721 for the *S. aureus* 5 isolate, AY064722 for the *S. intermedius* 424 isolate, AY071835 for the *Streptococcus* sp. strain 6 isolate, and AY071836 for the *Enterococcus* sp. strain 130 isolate.

DISCUSSION

A collection of 615 normal-flora gram-positive isolates, taken from healthy children, were screened for susceptiblity to macrolides and for the presence of the five most common macrolide resistance genes. Only five or six children in the study received any doctor-approved medication during the col-

TABLE 3. Presence of the ORF regions from Tn*1270.1* in representative isolates

Isolate	Strain no.	Presence of gene[a]							
		*orf*1	*orf*2	*orf*3	*mef*(A)[d]	*orf*5	*orf*6	*orf*7	*orf*8
Gram positive									
Corynebacteria sp.[b]	274	+	+	+	+	+	+	+	+
Corynebacteria sp.[b]	214	+	+	+	+	+	+	+	+
C. jeikeium[b]	388	+	+	+	+	+	+	+	+
S. intermedius[e]	424	+	+	+	+	+	+	+	+
S. intermedius	357	+	+	+	+	+	+	+	+
Streptococcus sp.[e]	6	+	+	+	+	+	+	+	+
Streptococcus sp.	14	+	+	+	+	+	+	+	+
Streptococcus sp.	18	+	+	+	+	+	+	+	+
Streptococcus sp.	281	+	+	+	+	+	+	+	+
Streptococcus sp.	65	+	+	+	+	+	+	+	+
Streptococcus sp.	68	+	+	+	+	+	+	+	+
Streptococcus sp.	215	+	+	+	+	+	+	+	+
S. pneumoniae[b]	163	+	+	+	+	+	+	+	+
S. pneumoniae[b]	970147	+	+	+	+	+	+	+	+
Enterococcus sp.[e]	130	+	+	+	+	+	+	+	+
E. faecalis	2	+	+	+	+	+	+	+	+
S. haemolyticus	6	−	−	−	+	+	+	+	+
Staphylococcus saprophyticus	167	−	−	−	+	+	+	+	+
S. aureus[e]	5	−	−	−	+	+	+	+	+
Gram negative									
A. junii[c]	329	+	+	+	+	+	+	+	+

[a] +, present; −, absent.
[b] Described in reference 5.
[c] Described in reference 6.
[d] *mef*(A) is *orf4* in Tn*1270.1*.
[e] *mef*(A) sequenced.

lection period. Thus, the number of children who had exposure to antibiotics was low; however, 29% of the oral and 56% of the urine isolates were macrolide resistant. The urine isolates were primarily staphylococci and enterococci, while the oral isolates were predominately streptococci. This could explain why the urine isolates were twice as likely as the oral isolates to be macrolide resistant. In addition, these isolates were commonly multidrug resistant, with enterococci being the most commonly multidrug-resistant genus. Many of the macrolide resistance genes were associated with conjugative elements and thus could act as reservoirs of these genes for pathogenic bacteria.

The *mef*(A) gene was found in 9% of the macrolide-resistant isolates. This is the first description of the *mef*(A) gene in the genus *Staphylococcus*. The complete *mef*(A) gene was sequenced for the first time from *S. aureus*, *Streptococcus* sp., *S. intermedius*, and *Enterococcus* sp. isolates. All four of these sequences were identical to each other at the DNA and amino acid levels and identical with the GenBank U83667 and AF376746 sequences. Seventeen of the isolates in Table 3 hybridized with *orf1* to *orf8* from Tn*1207.1*, and seven of the isolates have been shown here or previously (5, 6) to transfer the *mef*(A) gene to related and/or unrelated recipients. Whether these strains carry transposons similar to Tn*1207.3*, a recently described conjugal element from *S. pyogenes* (Santagati et al., 41st ICAAC), needs to be investigated. The three *Staphylococcus* spp. tested hybridized with *orf5* to *orf8* but not with the *orf1*, *orf2*, and *orf3* probes, suggesting that these strains may have transposons related to the recently described *S. pneumoniae* mega element (3). One of the most interesting findings was that the gram-negative *A. junii* isolate hybridized with all seven *orf* genes, suggesting that it carries a transposon related to Tn*1207.1* from gram-positive isolates. This is the first indication that this family of transposons may be transferred and maintained in some gram-negative species. The presence of the *mef*(A) gene in other gram-negative bacteria from the Portuguese collection is being determined.

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