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Susceptibilities of Oral and Nasal Isolates of *Streptococcus mitis* and *Streptococcus oralis* to Macrolides and PCR Detection of Resistance Genes

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The susceptibility of viridans group streptococci to macrolides was determined. Thirteen isolates (17%) were resistant to erythromycin. Five strains carried an *erm* gene that was highly homologous to that in Tn*917*. Four strains had *mefE* genes that coded erythromycin efflux ability.

Viridans group streptococci, commensal bacteria in the human oral and nasal cavities, are associated with systemic diseases, including infective endocarditis, bacteremia, and pneumonia (2, 5, 6, 10, 12). Macrolide resistance has spread in staphylococci, enterococci, and streptococci (7–9, 11), but little is known about the distribution of the resistance among oral streptococci (12, 19). In this work, clinical isolates of oral and nasal *Streptococcus mitis* and *Streptococcus oralis* have been tested for susceptibility to macrolides, and resistance genes have been characterized.

Eighty-four streptococcal isolates from patients who visited Tokushima University Hospital between June and December 1995 were studied. These strains were isolated from periodontal pockets, larynx, pharynx, maxillary sinus, and nasal secretion and identified as *S. oralis* (54 strains), *S. mitis* (29 strains), *Streptococcus sanguis* (1 strain), and *Streptococcus salivarius* (1 strain) by biochemical tests, including the API-20 Strep system (bioMérieux, La Balme des Grottes, France).

The following antibiotics were used: leucomycin and midecamycin (Meiji Pharmaceutical Co., Ltd., Tokyo, Japan), erythromycin and rokitamycin (Asahi Kasei Co., Ltd., Tokyo, Japan), clarithromycin (Dynabott), azithromycin (Pfizer Pharmaceuticals), and roxithromycin (Hoechst-Marion-Roussel).

MICs were determined by a broth microdilution method in anaerobic MIC broth (Difco, Detroit, Mich.). Microtiter plates were incubated at 37°C for 24 h in 5% CO_2.

Induction experiments for macrolide resistance were performed by preculture with a sub-MIC of erythromycin. Crude DNA of streptococci was prepared as previously described (13). PCR primers for *erm* and *mefE* were designed from published sequences (1) to provide specific PCR products of 530 and 1,218 bp, respectively. The *erm* primers were 5′-GAAATIGGIIIIGGIAAAGGICA-3′ and 5′-AAYTGRTTYTTGTRAA-3′, and the *mefE* primers were 5′-ATGGAAAAATACAACAATTGGAAACGA-3′ and 5′-TTATTTAAATCTAATTTTCTAACCTC-3′.

Macrolide susceptibility is shown in Table 1. The erythromycin MICs at which 90% of the isolates tested are inhibited (MIC_{90}s) for *S. oralis* and *S. mitis* were 8 and 32 μg/ml, respectively. MIC ranges of clarithromycin, azithromycin, and roxithromycin were similar to those of erythromycin. The MIC range of rokitamycin for *S. oralis* and *S. mitis* was narrow, with MIC_{90}s of 0.5 and 1 μg/ml, respectively. Among 13 erythromycin-resistant strains (MIC, ≥8 μg/ml), 6 strains were highly resistant to erythromycin; the MICs for them were ≥512 μg/ml (Table 2). All of the strains except O14 were also highly resistant to clarithromycin, azithromycin, and roxithromycin, but intermediately resistant to rokitamycin (MIC, ≥0.5 to ≤2 μg/ml). Strain O14 was intermediately resistant to azithromycin and roxithromycin (MICs, ≥0.5 to ≤2 μg/ml) and sensitive to rokitamycin (MIC, ≤0.25 μg/ml). Seven strains were intermediately resistant to 14- and 15-member macrolides.

As shown in Table 2, all erythromycin-resistant strains were more sensitive to rokitamycin than other macrolides. To determine whether resistance could be induced, MICs of 16-member macrolides for cells grown in medium with or without a sub-MIC of erythromycin were examined. Highly resistant strains SO12, SO13, O14, E2, and E21 were induced to develop resistance to rokitamycin, midecamycin, and leucomycin. Strain E3 was highly resistant to midecamycin and leucomycin, even when cultured without erythromycin, and rokitamycin resistance was not induced. The intermediately resistant strains, O24, E17, E11, E27, and E30, did not develop resistance to 16-member macrolides after erythromycin induction.

To clarify the mechanisms of resistance, PCR amplification of macrolide-resistant genes was performed. PCR primers for

TABLE 1. In vitro activity of macrolide antibiotics for *S. oralis* and *S. mitis*

Organism	Antibiotic	MIC (μg/ml)[a]		
		Range	50%	90%
S. oralis	Erythromycin	0.016–>512	0.125	8
	Clarithromycin	0.016–2048	0.031	8
	Roxithromycin	≤0.016–>512	0.25	8
	Azithromycin	≤0.016–>512	0.5	16
	Rokitamycin	0.031–4	0.125	0.5
S. mitis	Erythromycin	0.016–>512	0.125	32
	Clarithromycin	0.016–>512	0.031	32
	Roxithromycin	≤0.016–2048	0.125	64
	Azithromycin	≤0.016–2048	0.5	64
	Rokitamycin	0.031–8	0.125	1

[a] 50% and 90%, MIC_{50} and MIC_{90}, respectively.

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TABLE 2. MIC of macrolides in the presence and absence of inducer[a] for erythromycin-resistant strains

Strain	MIC (μg/ml) with or without inducer[b]											PCR product	
	Erythromycin		Rokitamycin		Midecamycin		Leucomycin		Clarithromycin[c]	Azithromycin	Roxithromycin		
	−	+	−	+	−	+	−	+	(−)	(−)	(−)	*erm*	*mefE*
S. oralis SO12	>1,024	>1,024	1	>256	4	>128	8	>1,024	>1,024	>1,024	>1,024	+	−
S. mitis SO13	>1,024	>1,024	2	>256	16	>128	16	>1,024	>1,024	>1,024	>1,024	+	−
S. mitis E2	512	512	1	256	4	>128	4	>512	>512	>512	>512	+	−
S. oralis O14	512	>512	<0.125	>256	<0.063	>128	<0.25	>512	256	1	2	+	+
S. oralis E21	512	>512	1	512	2	>128	4	>512	256	64	2	+	−
S. mitis E3	512	>128	8	16	>128	>128	>128	>256	64	>512	>512	−	+
S. mitis E11	32	16	<0.031	0.25	0.25	0.5	0.25	0.5	32	64	64	−	−
S. oralis E17	16	16	0.25	<0.25	0.125	0.125	0.125	0.25	8	32	32	−	+
S. mitis E27	16	8	<0.25	<0.25	<0.031	<0.063	<0.031	<0.125	32	>32	>32	−	−
S. oralis O24	8	8	<0.25	<0.25	0.125	<0.063	0.063	<0.125	4	32	16	−	+
S. mitis E10	8	ND[c]	0.5	ND	ND	ND	ND	ND	8	16	16	−	−
S. mitis E18	8	ND	0.25	ND	ND	ND	ND	ND	8	16	8	−	−
S. mitis E30	8	32	0.125	<0.25	0.25	0.5	<0.25	0.5	8	32	32	+	−

[a] The inducing concentration of erythromycin was 0.25 μg/ml.
[b] −, absence of inducer; +, presence of inducer. Note that the MICs of clarithromycin, azithromycin, and roxithromycin in the presence of inducer were not determined.
[c] ND, not determined.

the *erm* (23S rRNA methylase) gene were designed based on the sequence of corresponding genes from other organisms. The highly resistant strains gave a distinct band of the expected 530-bp size from position 103 to position 633 of the *erm* gene. PCR products from these strains and *S. salivarius* E37 were sequenced (Fig. 1). Sequences from strains E2, E21, and E30 were identical to those of the gene encoding the rRNA methylase on transposon Tn*917* (4). Those from other strains had ~98% homology to Tn*917*. The nucleotide changes in strains SO12, SO13, and E37 resulted in six, four, and two amino acid alterations, respectively, but did not affect the reading frame. Primer sets for the *mefE* gene were designed based on the sequence of that gene in *S. pneumoniae* (GenBank U83667). The intermediately resistant strains O24, E17, and E3 and highly resistant strain O14 gave the expected band (approximately 1,200 bp) for the *mefE* gene encoding the macrolide efflux pump (18). The sequences of DNA obtained from PCR amplification for *mefE* in strains O24, E3, and E17 were analyzed. These sequences were identical to those at positions 30 to 1190 of the corresponding *mefE* gene. Less erythromycin accumulated in *mefE*-positive strain E17 than in *mefE*-negative strain E1 15 min after the addition of [*N*-methyl-^{14}C]erythromycin (data not shown). Accumulation of erythromycin in the *mefE*-positive strain was restored by addition of proton conductors, suggesting that a macrolide efflux system exists in strain E17. For genes encoding macrolide-inactivating enzyme,

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                                                                                 *                              * *
E2/E21/E30       1 TACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAACATTCGT
E37                                                                              G
SO12                                                                                                            T C
SO13                                                                                                            T C

                                                                            *            *
E2/E21/E30     101 GTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAATATTCCTTACCATTTAAGCACACAAATTA
E37                                                                                      G
SO12                                                                        C
SO13                                                                        C

                       *           *       *
E2/E21/E30     201 TTAAAAAAGTGGTTTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTCACCGAACACTAGGGTT
E37
SO12                   G           A       G
SO13                                       G

                                               **
E2/E21/E30     301 GCTCTTGCACACTCAAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACC
E37
SO12
SO13                                           GG

                                        *                            *  *
E2/E21/E30     401 CGCCATACCACAGATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTGTTTACCAAAAACA
E37
SO12                                    A                            G
SO13                                                                    C

E2/E21/E30     501 ATT
E37
SO12
SO13
```

FIG. 1. Nucleotide sequences of PCR products obtained with oral streptococci by using an *erm* primer set. The sequences in strains E2, E21, and E30 were identical to that from the site of the *E. faecalis ermB* gene. In strains E37, SO12, and SO13, only the base changes are shown (highlighted by asterisks).

ereA, *ereB*, and *mphE* and macrolide efflux genes *msr* and *mefA* (17), PCR amplification was performed with DNA from macrolide-resistant strains; however, no PCR products have been detected.

Although *erm* genes encoding rRNA methylase are present in various organisms, such as *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*, and macrolide resistance is widespread in bacteria associated with humans (4, 15, 17), the potential reservoir for *erm* genes is unknown. It has been reported that the genes lie on various transposable elements or conjugal plasmids (4, 11, 14). In the present study, we showed that the nucleotide sequences of PCR products obtained with *erm* primers from some strains were identical to the rRNA methylase gene in *Enterococcus faecalis* transposon Tn*917*, while those from *S. oralis* SO12 and *S. mitis* SO13 were highly homologous with the *ermB* gene from conjugal plasmid pIP501 of *Streptococcus agalactiae* (3, 4). In the upstream region, the sequence homologous to LR, an internal sequence of Tn*917* has been found (16) (data not shown). *S. oralis* and *S. mitis* are major species in the oral and nasal normal flora. The results of this study suggest the transmission of macrolide-resistant genes between oral streptococci and other more virulent streptococci.

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