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A Novel Erythromycin Resistance Methylase Gene (*ermTR*) in *Streptococcus pyogenes*

HELENA SEPPÄLÄ,[1]* MIKAEL SKURNIK,[2] HANNA SOINI,[3] MARILYN C. ROBERTS,[4] AND PENTTI HUOVINEN[1]

Antimicrobial Research Laboratory[1] and the Mycobacterial Reference Laboratory,[3] National Public Health Institute, 20520 Turku, Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, 20521 Turku,[2] Finland, and Department of Pathobiology, School of Public Health and Community Medicine, University of Washington, Seattle, Washington 98195[4]

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Erythromycin resistance among streptococci is commonly due to target site modification by an rRNA-methylating enzyme, which results in coresistance to macrolide, lincosamide, and streptogramin B antibiotics (MLS_B resistance). Genes belonging to the *ermAM* (*ermB*) gene class are the only erythromycin resistance methylase (*erm*) genes in *Streptococcus pyogenes* with MLS_B resistance that have been sequenced so far. We identified a novel *erm* gene, designated *ermTR*, from an erythromycin-resistant clinical strain of *S. pyogenes* (strain A200) with an inducible type of MLS_B resistance. The nucleotide sequence of *ermTR* is 82.5% identical to *ermA*, previously found, for example, in *Staphylococcus aureus* and coagulase-negative staphylococci. Our finding provides the first sequence of an *erm* gene other than *ermAM* that mediates MLS_B resistance in *S. pyogenes*.

Three principal mechanisms have so far been found to be responsible for the acquired erythromycin resistance in bacteria: target site modification, enzymatic inactivation of erythromycin, and active efflux of erythromycin (20, 21). In streptococci as well as in many other gram-positive bacteria, target site modification is a common resistance mechanism (45). It is due to the presence of an rRNA methylase that mono- or dimethylates the N^6 amino group of an adenine residue in 23S rRNA. Methylation probably results in a conformational change in the ribosome, leading to reduced binding of macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics to their target site in the 50S ribosomal subunit. MLS_B resistance can be divided into constitutive resistance, when the methylating enzyme is produced continuously, and inducible resistance, when the presence of an inducing antibiotic is required for production of the enzyme. Different types of erythromycin resistance methylases, encoded by *erm* genes, are produced by different bacteria. The *erm* genes have been divided into at least 12 different classes on the basis of hybridization studies and sequence comparisons (45).

In addition to MLS_B resistance, active efflux has recently been shown to be a common mechanism of erythromycin resistance, at least in *Streptococcus pyogenes* and *Streptococcus pneumoniae* (41). In some of these bacteria, resistance to 14- and 15-membered macrolides is due to the *mefA* gene, which encodes a membrane-associated protein (9).

In streptococci MLS_B resistance has commonly been due to genes belonging to the *ermAM* (*ermB*) gene class. The *ermAM* gene was first sequenced from plasmid pAM77 of *Streptococcus sanguis* (13). Thereafter, genes of the same class have been sequenced, for example, from *S. pneumoniae* (43), *S. pyogenes* (7, 8), and *Streptococcus agalactiae* (5). In *S. pyogenes*, genes belonging to the *ermAM* class are actually the only *erm* genes that have been sequenced. In this study, we have characterized a novel *erm* gene, designated *ermTR*, from an erythromycin-resistant clinical strain of *S. pyogenes* isolated in Finland. Our data provide the first sequence of an *erm* gene other than *ermAM* that mediates MLS_B resistance in *S. pyogenes*.

* Corresponding author. Mailing address: Antimicrobial Research Laboratory, National Public Health Institute, Kiinamyllynkatu 13, 20520 Turku, Finland. Phone: 358-2-2519255. Fax: 358-2-2519254. E-mail: helena.seppala@utu.fi.

MATERIALS AND METHODS

Bacterial strains and determination of antimicrobial resistance. *S. pyogenes* A200 of serotype T11 is an erythromycin-resistant clinical skin isolate. Its erythromycin resistance phenotype was determined by the double-disk test and MIC determinations (36). The MICs of different antimicrobial agents were determined by the plate dilution method (30).

S. agalactiae 90-30-591 was used as a positive control for the *ermAM* gene in PCR. *S. pyogenes* 13 234 containing the 17.5-MDa MLS_B resistance plasmid pERL1 (25) was used as a control strain for plasmid isolation.

DNA extraction and dot blot hybridization. Streptococcal DNA was extracted by the cell lysis method described by Anderson and McKay (1), with the following modifications. The cells were grown in 3 ml of Todd-Hewitt broth (BBL, Cockeysville, Md.) with 1% yeast extract (Oxoid, Basingstoke, United Kingdom). Instead of lysozyme alone, a combination of lysozyme (10 mg/ml) and mutanolysin (300 U/ml) (Sigma, St. Louis, Mo.) was used to enhance cell lysis during an incubation for 2 h at 37°C. After cell lysis DNA was extracted once with 1 volume of phenol and once with 1 volume of chloroform, and after the addition of 1/10 volume of 2 M sodium acetate (pH 6.2), the DNA was precipitated with 2 volumes of ethanol. The DNA was dissolved in 20 μl of water, and 1 μl of the solution was used as template in PCRs.

Dot blot hybridization was performed by standard techniques. The probes used are shown in Table 1. The probes were labelled with two ^{32}P-labelled nucleotide triphosphates and two unlabelled nucleotides by nick translation as described previously (40). Filters underwent three 30-min washes at 52°C in 0.1% sodium dodecyl sulfate, 0.015 M NaCl, and 0.0015 M sodium citrate followed by three 15-min washes at 52°C in 0.015 M NaCl and 0.0015 M sodium citrate. Under these high-stringency conditions, none of the probes cross-hybridized. The plasmids that are the original sources of the genes were used as controls (Table 1).

PCR and sequencing experiments. The oligonucleotide primers used in PCR and sequencing are shown in Table 2. Primers III_1 and III_2 were designed from the sequence of *ermAM* found in *S. pyogenes* (8), *S. sanguis* (13), and *S. pneumoniae* (43). Primers III_9 and III_{13} were directly from the sequence of *ermA* of *S. aureus* (28). The rest of the primers were from newly sequenced regions of strain A200. Primers III_{14} and III_{15} were used in the ligation PCR after self-ligation of A200 DNA digested with *Taq*I restriction endonuclease. *Taq*I was chosen because there were no *Taq*I recognition sites in the newly sequenced DNA. The oligonucleotides were synthesized by the Applied Biosystems 391 DNA synthesizer (PCR-Mate, Foster City, Calif.). DNA amplification was performed by using a DNA thermocycler (HB-TR1; Hybaid Ltd., Middlesex, United Kingdom). The PCR mixture of 100 μl contained 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% (vol/vol) Triton X-100, 200 μM (each) deoxyribonucleotides, 20 pmol of oligonucleotides, 1 U of DynaZyme DNA polymerase (Finnzymes Oy, Espoo, Finland), and approximately 50 ng of template DNA

TABLE 1. Probes and controls used in dot blot hybridization to detect known *erm* genes in *S. pyogenes* A200

Gene	Source or control	Fragment	Vector or probe	Reference(s)
ermA	pEM9592	0.7-kb *Ssp*I	pBR328	29
ermBP	pJIR229	0.8-kb *Pst*I-*Eco*RI	pUC18	3, 33
ermC	pBR328::33EV	0.9-kb *Hpa*I	pBR328	31
ermFS	pFD292	0.75-bp *Pvu*II	pUC19	37
ermQ	pJIR745	1.0-kb *Eco*RI-*Hin*dIII	pUC18	34

extracted from A200. A total of 35 cycles, with denaturation at 93°C for 30 s, annealing at temperatures adjusted for each primer pair for 1 min, and extension at 72°C for 90 s, were carried out in the thermal reactor. A 20-μl volume of the reaction mixture was run in a 1.0% agarose gel (FMC BioProducts, Rockland, Maine). A 100-bp ladder (Gibco-BRL, Gaithersburg, Md.) was run in the gel as a size marker. After staining with ethidium bromide, the PCR products were visualized under UV light and were photographed with type 667 Polaroid film.

Sequencing was done directly from the PCR products. Purification of the PCR products and sequencing according to Sanger's dideoxynucleotide chain termination method were performed as described previously (39). The sequences of the regions corresponding to the positions (Table 2) of the primers derived from the *ermAM* and *ermA* genes (primers III_1, III_2, III_9, and III_{13}) were determined from PCR products that were produced with other primers.

Comparison of sequences. The nucleotide and amino acid sequence similarities of different *erm* genes and their predicted products were determined by the programs GAP and PILEUP of the Genetics Computer Group Program Package (11). The output of PILEUP includes a dendrogram, constructed by the neighbor joining method, and an ordered gapped listing of sequences.

Plasmid isolation experiments. Two methods were used to detect plasmids in strain A200. The procedure of Anderson and McKay (1) was used, but with the modifications in the cell lysis procedures described earlier in this paper. In addition, a 125-ml overnight culture (Todd-Hewitt broth with 1% yeast extract) was analyzed with the Wizard Plus Maxipreps DNA Purification System (Promega, Madison, Wis.) according to the instructions of the manufacturer, except that half of the volumes were used and mutanolysin (300 U/ml) and pronase (500 μg/ml) were added in the cell resuspension solution and the mixture was incubated for 1 h at 37°C.

Nucleotide sequence accession number. The nucleotide sequence of *ermTR* and its leader sequence has been assigned GenBank accession number AF002716.

RESULTS AND DISCUSSION

Resistance phenotype and antimicrobial susceptibilities of *S. pyogenes* A200. Strain A200 expressed an inducible type of MLS_B resistance. This was indicated by resistance to erythromycin and susceptibility to clindamycin in MIC determinations (the MICs were 8 and 0.25 μg/ml, respectively) and by a reduced clindamycin inhibition zone proximal to the erythromycin disk in the double disk test. A200 was susceptible to all other antimicrobial agents tested; the MICs were as follows: penicillin, 0.016 μg/ml; cephalothin, 0.125 μg/ml; tetracycline, 0.5 μg/ml; chloramphenicol, 4 μg/ml; ciprofloxacin, 0.5 μg/ml; and vancomycin, 0.5 μg/ml.

Dot blot hybridization. Hybridization of strain A200 gave negative results with the probes listed in Table 1, indicating that the *ermA*, *ermBP* (which belongs to the *ermAM* gene class), *ermC*, *ermF*, or *ermQ* gene was not present in A200.

Sequence analysis of *ermTR* and upstream sequences and comparison to other *erm* genes. To identify the resistance gene in strain A200 a PCR-based approach was used. Primers III_1 and III_2 successfully amplified an approximately 530-bp region of the methylase gene from the genome of *S. agalactiae* 90-30-591 used as a positive control. A PCR product of the same size was also amplified from A200. The nucleotide sequence of the 530-bp PCR product of A200 was determined. Unexpectedly, the sequence that was obtained shared 82% homology with the *ermA* gene of *S. aureus*. The *ermA* gene has not previously been found in *S. pyogenes*. Therefore, in sequencing of the A200 gene, the sequence of *ermA* was used to help the primer design, when applicable (Table 2).

The completely sequenced 942-bp region of strain A200 aligned with the *S. aureus ermA* gene region is presented in Fig. 1. Three potential open reading frames (ORFs) were detected. The longest ORF consists of the same number of nucleotides as is present in the *ermA* gene, that is, 732 nucleotides. The sequence of this ORF is 82.5% identical to the *ermA* sequence, and it is predicted that it encodes a polypeptide of 243 amino acids (Fig. 2). This strongly suggests that the polypeptide is also a methylase conferring resistance to erythromycin by methylating an adenine residue in the 23S rRNA. The structural gene for the methylase was designated *ermTR*.

The G+C content of the coding sequence of *ermTR* is 30% and that of *ermA* is 32.5%; these are equally close to the G+C contents of 32 to 36% and 34.5 to 38.5% of the chromosomes of *S. aureus* and *S. pyogenes*, respectively (38). It is possible that these genes share a common origin and have relatively recently diverged from each other. The native gene may have been a staphylococcal or a streptococcal gene, or it may have been transferred to these species from another gram-positive organism. In addition to *S. aureus*, the *ermA* gene has been found in coagulase-negative staphylococci and in the gram-negative organisms *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* (32, 42, 44).

Upstream of *ermA*, there is a leader sequence with two ORFs (28). One is predicted to encode a 15-amino-acid peptide and the other is predicted to encode a 19-amino-acid peptide. This situation resembles that of the *ermTR* gene, in which a leader sequence potentially encodes two small peptides of the same size (Fig. 1), with the identity to the *ermA* ORFs being 87.5 and 85.0%, respectively. The regulation of the expression of *ermA* in *S. aureus* has been shown to depend on the leader sequence upstream of *ermA* (28). It has been

TABLE 2. Oligonucleotide primers used in PCR and sequencing

Primer	Sequence (5′ to 3′)[a]	Gene used for primer design	Position in *ermTR*	Primer used with primer
III_1	GAA ATT GG(A/C) ACA GGT AAA GGG CA	*ermAM*	316–338	III_2[b]
III_2[b]	AAA (C/T)TG ATT TTT AGT AAA	*ermAM*	828–846	III_1
III_6	GAA GTT TAG CTT TCC TAA	*ermTR*	468–485	III_2[b]
III_7	TGC TGT TAA TGG TGG AAA	*ermTR*	641–658	III_2[b]
III_8[b]	GCA TGA CAT AAA CCT TCA	*ermTR*	393–410	III_9
III_9	ACA TAA GGA GGT TTC AAT	*ermA*	1–18	III_8[b]
III_{10}	AGG TTA TAA TGA AAC AGA	*ermTR*	204–221	III_8[b]
III_{13}[b]	TTA GTG AAA CAA TTT GTA	*ermA*	925–942	III_7
III_{14}	TCT CCT TGC CGG TTA TAA	*ermTR*	186–203	III_{15}[b]
III_{15}	ATC AAT TAA GAC AGG TGC TGA AGC	*ermTR*	839–862	III_{14}[b]

[a] Nucleotides in parentheses indicate that during synthesis either of the nucleotides was incorporated into the oligonucleotide.
[b] The primer was biotinylated at the 5′ end for sequencing purposes.

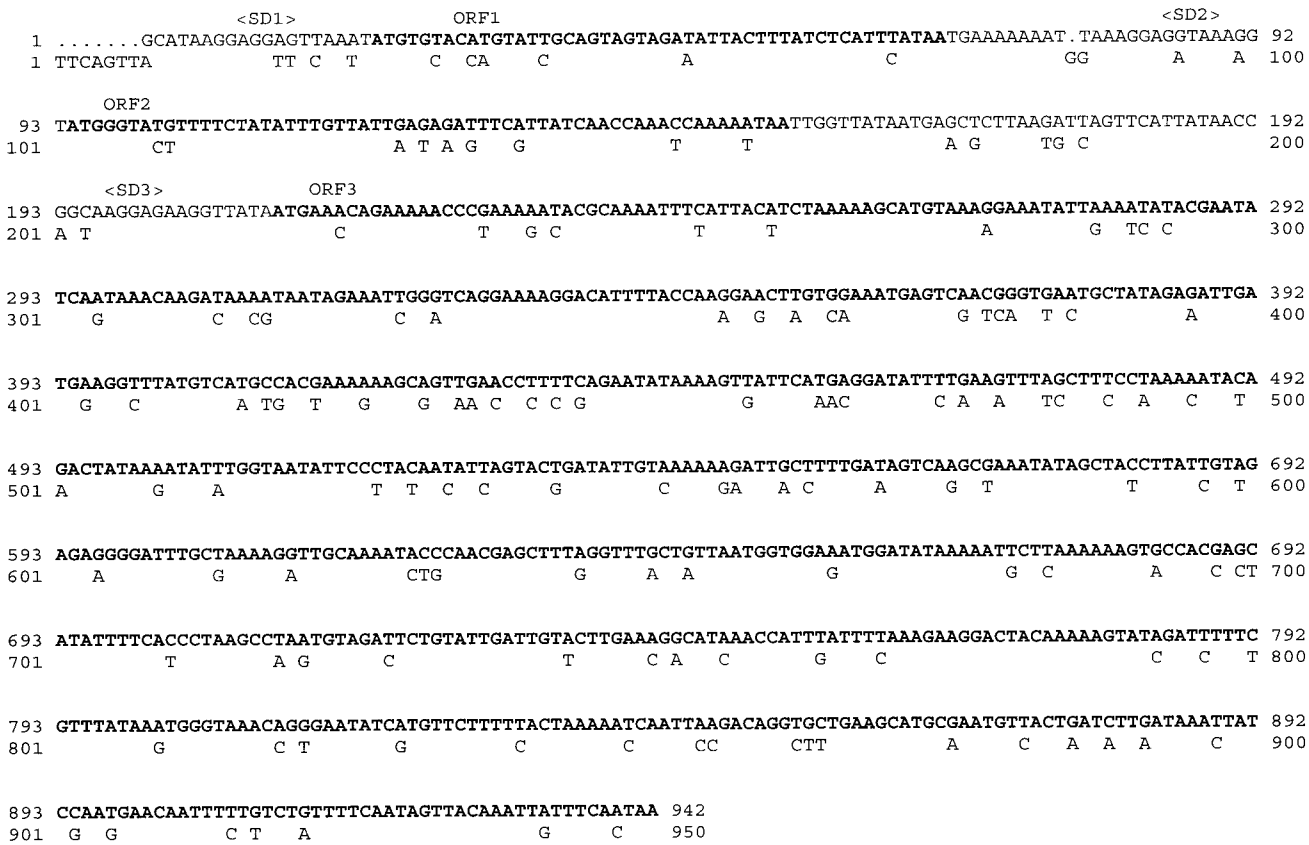

FIG. 1. Nucleotide sequence of the *ermTR* gene of *S. pyogenes* A200 and its leader sequence. The ribosome-binding sites (Shine-Dalgarno sequence) and the ORFs (boldface) are indicated. The *ermTR* gene has been aligned with the *ermA* gene, and only those nucleotides of *ermA* that differ from the *ermTR* sequence are shown.

suggested that in the presence of inducing concentrations of erythromycin, translation of peptide 1 by a ribosome that has bound to erythromycin would result in a ribosome stall, allowing translation of peptide 2. In turn, stalling of ribosomes translating peptide 2 would lead to the translation of *ermA*. On the basis of the similarities also found between the leader sequences of *ermA* and *ermTR* (Fig. 1), regulation of the expression of *ermTR* probably occurs via a similar mechanism.

Comparison of the *ermTR* gene to other *erm* gene classes indicated that the sequence of *ermTR* is about 61 to 64% identical to the sequences of *ermC*, *ermG*, and *ermGT*, 58% identical to the sequence of *ermAM* (*ermB*), which is the only *erm* gene in *S. pyogenes* previously sequenced, 56% identical to the sequence of *ermQ*, and 48 to 49% identical to the sequences of *ermF* and *ermD* (which is similar to those of *ermJ* and *ermK*). The conserved regions encoding conserved amino acid motifs of the rRNA-methylating enzymes that are present in the different *erm* genes were also found within the *ermTR* gene sequence (data not shown).

Comparison of amino acid sequences. The alignment showing the similarities between the predicted amino acid sequences of the *ermTR* and *ermA* gene products is shown in Fig. 2. Altogether, 197 (81.1%) of the encoded 243 amino acids are identical, but the similarity between the two gene products reaches 90.1%. A dendrogram showing the potential evolutionary relationships between the methylase encoded by *ermTR* and other rRNA-methylating enzymes is shown in Fig. 3.

Plasmid isolation experiments. Attempts to detect extrachromosomal DNA in strain A200 were unsuccessful, although the methods did detect the control plasmid in *S. pyogenes* 13 234. In general, antibiotic resistance genes in streptococci are carried by the chromosome, and they are often associated with conjugative transposons (12, 14, 35, 44). However, plasmids carrying determinants for MLS_B resistance have

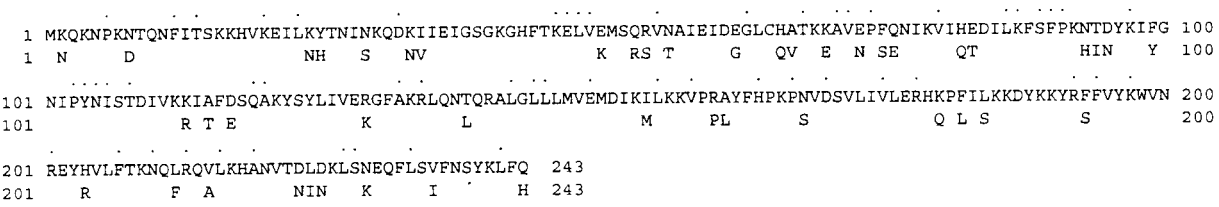

FIG. 2. Predicted amino acid sequence of the polypeptide encoded by the *ermTR* gene of *S. pyogenes* A200. The sequence has been aligned with the predicted amino acid sequence of the polypeptide encoded by the *ermA* gene, and only those amino acids that differ are shown below the sequence. The 56 identical amino acids that are encoded by different codons are indicated by black spots above the sequence.

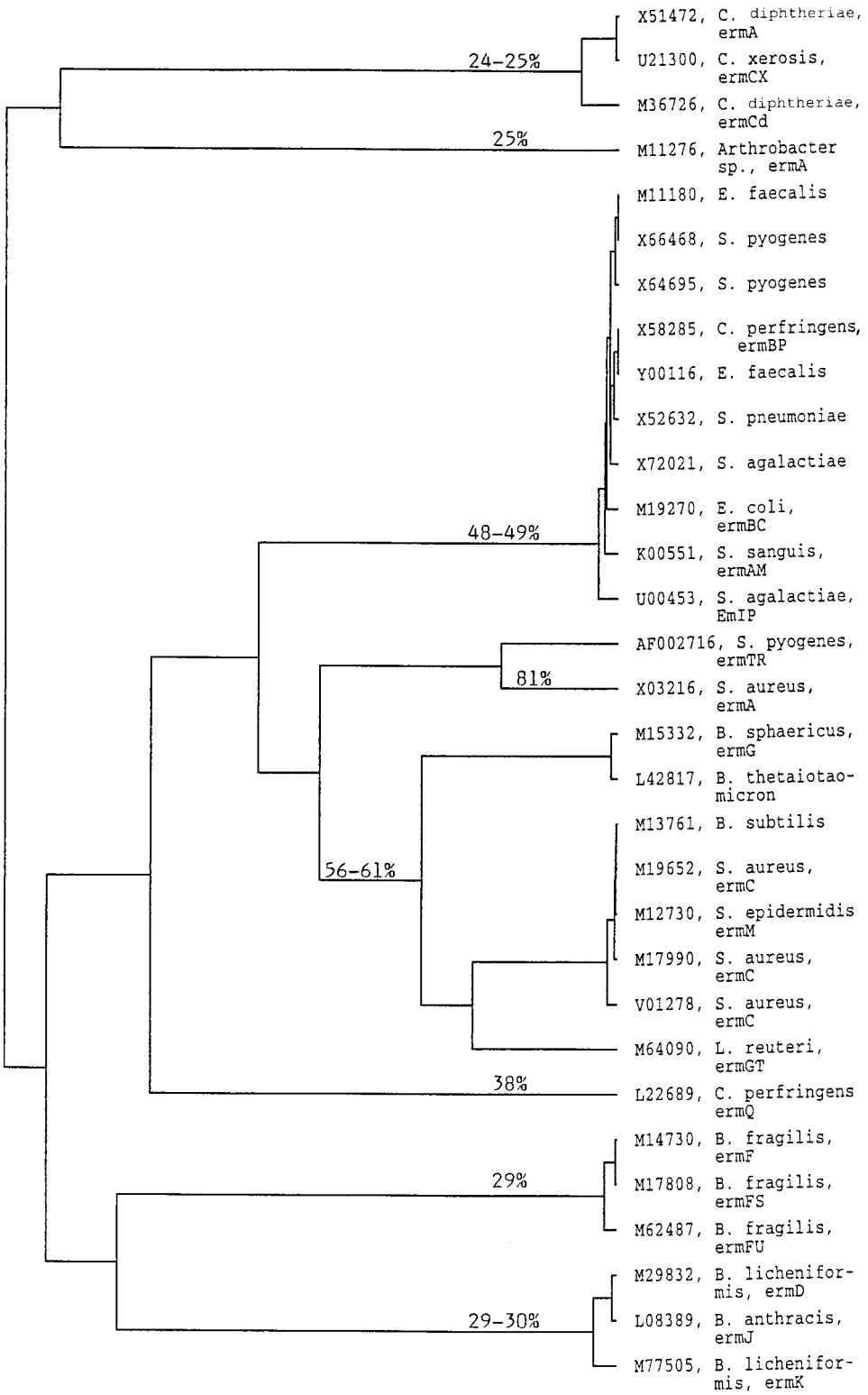

FIG. 3. Dendrogram of methylases encoded by different *erm* genes. The source of the amino acid sequence is indicated by the GenBank accession number, the bacterial species, and the name of the gene, if given. The percent identity of the predicted amino acid sequences of the polypeptides encoded by different *erm* genes to the predicted amino acid sequence of the polypeptide encoded by the *ermTR* gene of *S. pyogenes* A200 is marked on the dendrogram. Note that the most remote methylases associated with resistance to MLS_B antibiotics were not included in this analysis.

previously been isolated from *S. pyogenes* (2, 7, 8, 10, 22, 24–27, 34). Most streptococcal plasmids carrying antibiotic resistance genes are conjugative and have been shown to transfer by conjugation between streptococcal species (4, 6, 15, 17) and especially among *S. pyogenes* strains by transduction (16, 23).

Epidemiology of the *ermTR* gene. We have carried out epidemiological investigations to study the distribution of different *erm* genes, including *ermTR*, in clinical isolates of streptococci collected from different parts of Finland by use of a methodology consisting of PCR and digestion of the PCR products (18, 19). Twenty-four *S. pyogenes* isolates representing five different serotypes and 29 group G streptococcal isolates, all expressing the inducible type of MLS_B resistance, were studied, and all contained the *ermTR* gene (18, 19). Although the drug efflux gene *mefA* is at present a predominant erythromycin resistance determinant in *S. pyogenes* and probably also in other streptococci (41), these studies suggest that *ermTR* may be widely distributed among streptococci with MLS_B resistance.

We conclude that *ermTR*, characterized in this study, is the first sequenced *erm* gene that mediates MLS_B resistance in *S. pyogenes* but that does not belong to the *ermAM* gene class.

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