Detection of Erythromycin Resistance by the Polymerase Chain Reaction Using Primers in Conserved Regions of *erm* rRNA Methylase Genes

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Genes belonging to different *erm* DNA hybridization classes were selectively amplified by polymerase chain reaction with a pair of oligonucleotides that corresponded to conserved amino acid motifs in known ERM methylases. Identification of the resistance mechanism was possible despite substantial nucleotide sequence diversity among the erythromycin resistance genes.

Resistance to macrolide, lincosamide, and streptogramin B-type (MLS) antibiotics is widespread and generally due to production of rRNA methylases that dimethylate a specific adenine residue of 23S rRNA (13, 23, 24). The sequences of 13 related *erm* genes encoding rRNA methylases have been determined (2, 7). The genes were isolated in gram-positive and gram-negative human pathogens, soil organisms, and MLS antibiotics producers. Despite these extensive data, epidemiological surveys have indicated that these genes account for only part of the diversity of MLS resistance determinants (1, 14, 22). This genetic diversity precludes detection of MLS resistance by rRNA methylation with classical DNA hybridization techniques. We have developed an assay based on the polymerase chain reaction (PCR) that appears universal for the detection of *erm* genes disseminated in gram-positive cocci.

Design of oligonucleotide primers. Alignment of rRNA methylase sequences has revealed the presence of regions of high homology with invariant amino acids (2, 7). Two conserved motifs were selected for their respective locations near the NH₂ and COOH ends of the methylases (Fig. 1A). Genes *ermA* (*Staphylococcus aureus*) (17), *ermBC* (*Escherichia coli*) (4), *ermC* (*S. aureus*) (10), and *ermG* (*Bacillus sphaericus*) (16) belong to different classes, as defined by hybridization under stringent conditions by using total DNA bound to membranes as target and intragenic restriction fragments (>200 base pairs [bp]) to generate the probes (14). Genes *ermA*, *ermBC*, and *ermC* were selected as representatives of the three hybridization classes that are disseminated in gram-positive cocci and members of the family *Enterobacteriaceae* (1, 2, 11, 14, 19, 22, 25). Gene *ermG* was included because of its close relationship with *ermC* (16).

Oligonucleotides E1 and E2 were designed to hybridize, not only to the structural genes encoding ERMA, ERMBC, ERMC, and ERMG methylases, but also to every sequence encoding motifs I and II, respectively. Because of degeneracy of the genetic code and of variation in position four of motif I, a large number of synonymous sequences can code

for these amino acid motifs (Fig. 1B). When two alternate codons differed at the third position, the two alternate nucleotides were provided in equimolar amounts during the corresponding synthesis cycle (Fig. 1C). Deoxyinosine was used for all other variable positions, since this nucleotide can potentially pair with any DNA base (5, 6, 9, 12, 18). Oligonucleotides E1 and E2 can theoretically act as universal primers for the amplification of an internal segment of every *erm*-related gene encoding motifs I and II (Fig. 1C).

Amplification procedure. Amplification was performed with crude total DNA (1 to 5 ng) by using 400 ng of each oligonucleotide in a final volume of 100 μl, as previously described (15). DNA was denatured for 3 min at 95°C. Two units of *Taq* DNA polymerase (Perkin Elmer-Cetus, Norwalk, Conn.) was added during the first oligonucleotide-template annealing step at 37°C. After 35 amplification cycles (elongation at 72°C for 90 s, denaturation at 93°C for 30 s, annealing at 37°C for 2 min), a last elongation step was performed at 72°C for 5 min. The oligonucleotides were synthesized by the methoxy phosphoramidite method with an Applied Biosystems 380B DNA apparatus (Applied Biosystem, Foster City, Calif.); the oligonucleotides were not purified. A negative control in which DNA was omitted was included in each run.

Amplification of known *erm* genes and characterization of the amplification products. PCR was performed on total DNA of six reference strains harboring *erm* genes in low- or high-copy-number plasmids or in the chromosome (Table 1). DNA bands with indistinguishable electrophoretic mobilities were revealed by ethidium bromide staining after agarose gel electrophoresis. No amplification product was observed with MLS-susceptible strains *S. aureus* 80CR5 (8) and *E. coli* C1a (21). The size of the amplification products corresponded with those of the DNA segments which extend from motif I to motif II (531 bp in *ermA*, *-BC*, and *-G*; 528 bp in *ermC*) (Fig. 1A). A ca. 530-bp fragment was also observed for *E. coli* BM2576. An *erm*-related gene, designated *ermC*-like, was detected in this clinical isolate by using an *ermC* probe under moderately stringent conditions (14).

To confirm that the DNA bands observed were the expected segments of the *erm* genes, nucleotide sequencing was performed for two isolates by the chain termination technique (20). Amplified DNA was used as a template in double-stranded DNA sequencing (15) with oligonucleotides

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                                 motif I                                                          motif II
A) ERMA      NH2---35--- GLU ILE GLY SER GLY LYS GLY HIS----163----PHE THR LYS ASN GLN PHE---31---COOH
   ERMBC     NH2---34--- GLU ILE GLY THR GLY LYS GLY HIS----163----FHE THR LYS ASN GLN PHE---34---COOH
   ERMC      NH2---35--- GLU ILE GLY SER GLY LYS GLY HIS----162----:HE THR LYS ASN GLN PHE---32---COOH
   ERMG      NH2---35--- GLU ILE GLY ALA GLY LYS GLY HIS----163----PHE THR LYS ASN GLN PHE---32---COOH


B) Conserved amino acids      GLU ILE GLY     GLY LYS GLY HIS         PHE THR LYS ASN GLN PHE

   Corresponding codons       GAA ATA GGA     GGA AAA GGA CAC         TTC ACA AAA AAC CAA TTC
                              GAG ATC GGG     GGG AAG GGG CAT         TTT ACG AAG AAT CAG TTT
                                  ATT GGC     GGC     GGC                 ACC
                                      GGT     GGT     GGT                 ACT


                            G                           G
C) Oligonucleotide E1   5' GAA ATI GGI III GGI AAA GGI CA ─────────────────────────►
   Oligonucleotide E2       ◄───────────────────────────── AAA TGI TTC TTA GTT AA 5'
                                                            G       T   G   C
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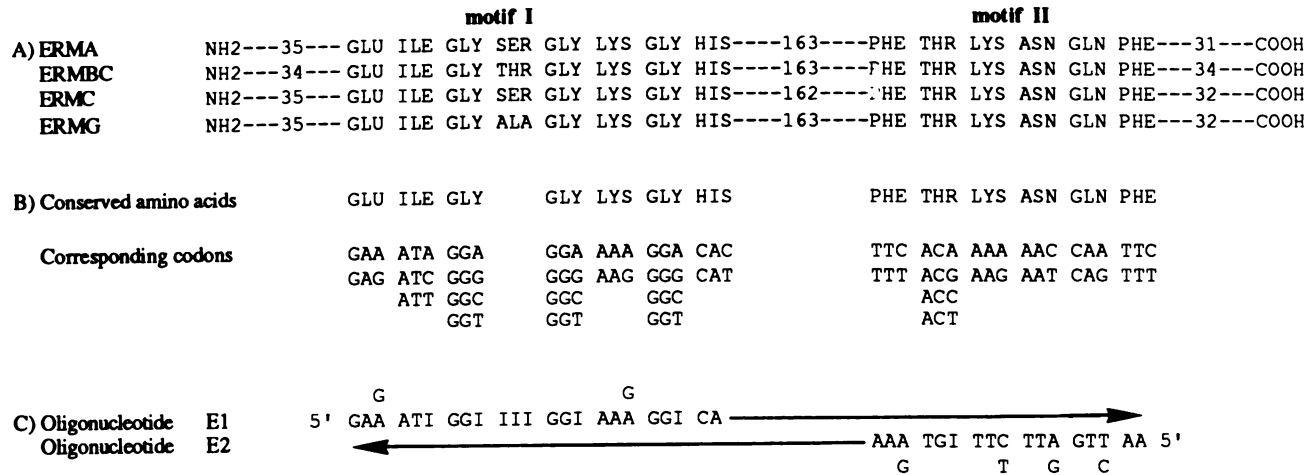
FIG. 1. Strategy used to amplify *erm*-related genes by the polymerase chain reaction. (A) Sequence of motifs I and II of rRNA methylases. The numbers of amino acids between the NH_2 terminus and motif I, motifs I and II, and motif II and the COOH terminus are indicated. (B) Amino acids common to the four sequences and corresponding codons. (C) Nucleotide sequence of oligonucleotides E1 and E2. At positions where two nucleotides were incorporated, the alternative nucleotide is indicated above (E1) or below (E2) the sequence. I, Deoxyinosine. Arrows indicate direction of DNA synthesis.

E1 and E2 as primers. With strain BM2576 (*ermC*-like), we obtained sequence data adjacent to motif I (70 bp) and motif II (153 bp) that were homologous (90%) to the leading and lagging strands of *ermC*, respectively. The 163 bp sequenced from BM2570 harboring *ermBC* was identical to the published sequence (4).

Amplification of *erm* genes from clinical isolates and comparison of detection by PCR or hybridization. The DNA amplification assay was performed on genomic DNA of 21 MLS-resistant gram-positive cocci. This group represents the *erm* genetic diversity observed by DNA hybridization under stringent conditions (14) using probes specific for genes *ermA*, *ermBC*, and *ermC* (Y. Corfmat, R. Leclercq, C. Mabilat, and P. Courvalin, unpublished results). Eleven *Staphylococcus* isolates were assigned to hybridization classes *ermA* (seven), *ermC* (two), and *ermA*+*ermC* (two), and two *Streptococcus* isolates were assigned to hybridization class *ermBC*. Eight strains gave negative results with all probes under high stringency conditions. The ca. 530-bp amplification product was observed with all isolates.

These results indicate that in the 21 isolates tested, MLS resistance was due to production of rRNA methylases encoded by *erm*-related genes. The resistance genes that did not hybridize must belong to one or several hybridization classes for which no probe is yet available. These results also suggest that MLS resistance by rRNA methylation may be detected by amplification in all clinical isolates of *Streptococcus* and *Staphylococcus* species.

Nucleotide sequence diversity is one of the major limita-

tions in the detection of a gene compared with detection of a gene function. On the basis of sequence analyses of a few variants of the *erm* gene family, we have shown that one can design oligonucleotides that prime the amplification of other members of the family. The presence of ERM methylases of motifs with six conserved amino acids was sufficient to ensure specificity of the assay.

Theoretical considerations indicated that the oligonucleotides used in this study are likely to anneal nonspecifically to sequences other than *erm*-related genes, and oligonucleotides E1 and E2 were therefore not used as probes. A sequence complementary to that of oligonucleotide E1 with a single mismatch should, on average, be present once in a random sequence with the complexity of the *E. coli* genome (data not shown). In addition, screening of the Los Alamos gene bank revealed a perfect match between oligonucleotide E2 and a sequence of *Klebsiella pneumoniae* that does not encode a protein with motif II. No match was detected with E1 (data not shown). Thus, use of oligonucleotides E1 and E2 in PCR allowed specific amplification of *erm* genes, whereas specific detection would not have been obtained by DNA hybridization by using the same oligonucleotides as probes. This difference is not surprising, since specificity of the amplification reaction results from two factors. (i) Two specific hybridization targets must be present on opposite DNA strands. (ii) The distance between the targets must be compatible with the amplification reaction, i.e., less than approximately 5 kilobases. By contrast, there are fewer requirements when two oligonucleotides are used separately

TABLE 1. Origin and characteristics of reference strains

Organism	*erm* gene	Location and copy no.	Source or reference
S. aureus RN1389	*ermA*	Tn*554* in the chromosome	17
S. aureus RN4932	*ermA*	Tn*554* on multicopy plasmid pT181cop615	E. Murphy, unpublished data
E. coli BM2570	*ermBC*[a]	Low-copy plasmid pIP1527	3
E. coli BM694(pAT77)	*ermC*	1660-bp *Cla*I fragment of pE194 on multicopy plasmid pUC1318	C. Mabilat, unpublished data
B. sphaericus 33	*ermG*	One or two copies per chromosome	16
B. subtilis BD1146	*ermG*	Multicopy plasmid pBD364	16

[a] The nucleotide sequence of *ermBC* is nearly identical to those of *ermAM* of pAM77 (10-bp difference) and *ermB* of Tn*917* (5-bp difference) from enterococci (4).

as probes, since hybridization could occur with target sequences located at any relative position.

LITERATURE CITED

1. **Arthur, M., A. Andremont, and P. Courvalin.** 1987. Distribution of erythromycin esterase and rRNA methylase genes in members of the family *Enterobacteriaceae* highly resistant to erythromycin. Antimicrob. Agents Chemother. **31:**404–409.

2. **Arthur, M., A. Brisson-Noël, and P. Courvalin.** 1987. Origin and evolution of genes specifying resistance to macrolide, lincosamide and streptogramin antibiotics: data and hypotheses. J. Antimicrob. Chemother. **20:**783–802.

3. **Arthur, M., and P. Courvalin.** 1986. Contribution of two different mechanisms to erythromycin resistance in *Escherichia coli.* Antimicrob. Agents Chemother. **30:**694–700.

4. **Brisson-Noël, A., M. Arthur, and P. Courvalin.** 1988. Evidence for natural gene transfer from gram-positive cocci to *Escherichia coli.* J. Bacteriol. **170:**1739–1745.

5. **Chamberlin, M. J., and D. L. Patterson.** 1965. Physical and chemical characterization of the ordered complexes formed between polyinosinic acid, polycytidylic acid and their deoxyribo-analogues. J. Mol. Biol. **12:**410–428.

6. **Cruse, W. B. T., J. Aymani, O. Kennard, T. Brown, A. G. C. Jack, and G. A. Leonard.** 1989. Refined crystal structure of an octanucleotide duplex with I.T. mismatched base pairs. Nucleic Acids Res. **17:**55–72.

7. **Dubnau, D., and M. Monod.** 1986. The regulation and evolution of MLS resistance, p. 369–387. *In* S. B. Levy and R. P. Novick (ed.), Banbury report 24. Antibiotic resistance genes: ecology, transfer, and expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

8. **Engel, H. W. B., N. Soedirman, J. A. Rost, W. J. Van Leeuwen, and J. D. A. Van Embden.** 1980. Transferability of macrolide, lincomycin, and streptogramin resistances between group A, B, and D streptococci, *Streptococcus pneumoniae,* and *Staphylococcus aureus.* J. Bacteriol. **142:**407–413.

9. **Gregoire, L., M. Arella, J. Campione-Piccardo, and W. D. Lancaster.** 1989. Amplification of human papillomavirus DNA sequences by using conserved primers. J. Clin. Microbiol. **27:**2660–2665.

10. **Horinouchi, S., and B. Weisblum.** 1980. Posttranscriptional modification of mRNA conformation: mechanism that regulates erythromycin-induced resistance. Proc. Natl. Acad. Sci. USA **77:**7079–7083.

11. **Horinouchi, S., and B. Weisblum.** 1982. Amino acid conservation in two MLS resistance determinants, p. 159–161. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.

12. **Kawase, Y., S. Iwai, H. Inoue, K. Miura, and E. Ohtsuka.** 1986. Studies on nucleic acid interactions. I. Stabilities of miniduplexes (dG$_2$A$_4$XA$_4$G$_2$ · dC$_2$T$_4$YT$_4$C$_2$) and self complementarity d(GGGAAXYTTCCC) containing deoxyinosine and other mismatched bases. Nucleic Acids Res. **14:**7727–7736.

13. **Lai, C.-J., and B. Weisblum.** 1971. Altered methylation of ribosomal RNA in an erythromycin-resistant strain of *Staphylococcus aureus.* Proc. Natl. Acad. Sci. USA **68:**856–860.

14. **Mabilat, C., and P. Courvalin.** 1988. Gene heterogeneity for resistance to macrolides, lincosamides and streptogramins in *Enterobacteriaceae.* Ann. Inst. Pasteur Microbiol. **139:**677–681.

15. **Mabilat, C., S. Goussard, W. Sougakoff, R. Spencer, and P. Courvalin.** 1990. Direct sequencing of the amplified structural gene and promoter for the extended-broad-spectrum β-lactamase TEM-9 (RHH-1) of *Klebsiella pneumoniae.* Plasmid **23:**27–34.

16. **Monod, M., S. Mohan, and D. Dubnau.** 1987. Cloning and analysis of *ermG,* a new macrolide-lincosamide-streptogramin B resistance element from *Bacillus sphaericus.* J. Bacteriol. **169:**340–350.

17. **Murphy, E.** 1985. Nucleotide sequence of *ermA,* a macrolide-lincosamide-streptogramin B determinant in *Staphylococcus aureus.* J. Bacteriol. **162:**633–640.

18. **Ohtsuka, E., S. Matsuki, M. Ikehara, Y. Takahashi, and K. Matsubara.** 1985. An alternative approach to deoxyoligonucleotides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions. J. Biol. Chem. **260:**2605–2608.

19. **Ounissi, H., and P. Courvalin.** 1982. Heterogeneity of macrolide-lincosamide-streptogramin B-type antibiotic resistance determinants, p. 167–169. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.

20. **Sanger, F., and A. R. Coulson.** 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. **94:**441–448.

21. **Sasaki, I., and G. Bertani.** 1965. Growth abnormalities in Hfr derivatives of *Escherichia coli* strain C. J. Gen. Microbiol. **40:**365–376.

22. **Thakker-Varia, S., W. D. Jenssen, L. Moon-McDermott, M. P. Weinstein, and D. T. Dubin.** 1987. Molecular epidemiology of macrolides-lincosamides-streptogramin B resistance in *Staphylococcus aureus* and coagulase-negative staphylococci. Antimicrob. Agents Chemother. **31:**735–743.

23. **Thakker-Varia, S., A. C. Ranzini, and D. T. Dubin.** 1985. Ribosomal RNA methylation in *Staphylococcus aureus* and *Escherichia coli:* effect of the "MLS" (erythromycin resistance) methylase. Plasmid **14:**152–161.

24. **Weisblum, B.** 1985. Inducible resistance to macrolides, lincosamides and streptogramin type B antibiotics: the resistance phenotype, its biological diversity, and structural elements that regulate expression—a review. J. Antimicrob. Chemother. **16**(Suppl. A):63–90.

25. **Weisblum, B., S. B. Holder, and S. M. Halling.** 1979. Deoxyribonucleic acid sequence common to staphylococcal and streptococcal plasmids which specify erythromycin resistance. J. Bacteriol. **138:**990–998.