

Intrinsic Macrolide Resistance in Rapidly Growing Mycobacteria†

Kevin A. Nash,^{1*} Nadya Andini,¹ Yansheng Zhang,² Barbara A. Brown-Elliott,² and Richard J. Wallace, Jr.²

Department of Pathology, University of Southern California, and Saban Research Institute of Childrens Hospital Los Angeles, Los Angeles, California,¹ and Department of Microbiology, The University of Texas Health Center at Tyler, Tyler, Texas²

Received 31 March 2006/Returned for modification 17 May 2006/Accepted 14 July 2006

This study reports the discovery of *erm* genes in seven species of rapidly growing mycobacteria (RGM): *Mycobacterium boenickei*, *M. goodii*, *M. houstonense*, *M. mageritense*, *M. neworleansense*, *M. porcinum*, and *M. wolinskyi*. This study further substantiates the role of *erm* genes in intrinsic macrolide resistance in RGM.

Several rapidly growing mycobacteria (RGM) are significant human pathogens, with approximately 90% of RGM infections being caused by *Mycobacterium abscessus*, *Mycobacterium chelonae*, and *Mycobacterium fortuitum* (3, 4). Other members of the *M. fortuitum* and *Mycobacterium smegmatis* groups cause most of the remaining RGM infections. Although the incidence of infections caused by some species of slowly growing nontuberculous mycobacteria appears to be declining, there is evidence that RGM infections may be increasing or increasingly recognized (5). Many pathogenic RGM species are considered susceptible to the newer macrolides (e.g., clarithromycin), and these agents are important components in the treatment of RGM infections (13). However, several RGM species express intrinsic macrolide resistance, including *Mycobacterium goodii*, *Mycobacterium houstonense*, *Mycobacterium mageritense*, and *Mycobacterium wolinskyi* (2). Previous studies have shown that the novel rRNA methylase genes *erm*(38) and *erm*(39) confer inducible macrolide resistance on *M. fortuitum* and *M. smegmatis*, respectively (8, 9). Thus, the objective of this study was to determine if other clinically important RGM have similar *erm* genes.

Southern blot analysis with an *erm*(38)-derived probe (8, 9) indicated that *M. mageritense* strain 961635 had DNA similar to that of *erm*(38) of *M. smegmatis*. Genetic complementation of the *erm*(38) knockout variant *M. smegmatis* ermKO4, using methods described elsewhere (9), isolated a 2.8-kbp *M. mageritense* DNA fragment that conferred resistance (MIC \geq 128 μ g/ml) to clarithromycin, clindamycin, erythromycin, spiramycin, and the ketolide HMR3004, but not to quinupristin (streptogramin B). Sequencing of the *M. mageritense* DNA fragment identified a gene encoding a 251-amino-acid polypeptide that was 77% identical to Erm(39) of *M. fortuitum* (GenBank accession no. AAR92235) and 71% identical to the first 264 amino acids of Erm(38) of *M. smegmatis* (GenBank accession no. AAN86837). The *M. mageritense* *erm* gene sequence was submitted to the Nomenclature Center for MLS Genes maintained by Marilyn C. Roberts (11, 12) and was designated *erm*(40). Using *erm*(40)-specific primers (MM1 and MM2)

(Table 1) and methods described elsewhere (9), this gene was detected by PCR in 11/11 clinical isolates of *M. mageritense*.

The sequences of the *erm*(38), *erm*(39), and *erm*(40) regions (GenBank accession no. AY154657, AY487229, and AY570506) provided the basis for designing consensus primers (Table 1) targeting the RGM *erm* genes and flanking DNA. Using these primers, *erm* genes were detected by PCR in *Mycobacterium boenickei* (10 strains or isolates tested), *M. goodii* (1 strain or isolate tested), *M. houstonense* (3 strains or isolates tested), *Mycobacterium neworleansense* (1 strain or isolate tested), *Mycobacterium porcinum* (2 strains or isolates tested), and *M. wolinskyi* (4 strains or isolates tested). No evidence of a related *erm* gene was found for *M. abscessus* (7 strains or isolates tested), *M. chelonae* (2 strains or isolates tested), *Mycobacterium flavescens* (1 strain or isolate tested), *Mycobacterium immunogenum* (1 strain or isolate tested), *Mycobacterium peregrinum* (5 strains or isolates tested), *Mycobacterium senegalense* (4 strains or isolates tested), and *Mycobacterium thermoresistibile* (1 strain or isolate tested).

Sequencing of the *erm* genes and surrounding DNA of the *erm*-positive species was enabled by using different combinations of the consensus primers (Table 1). The encoded amino acid sequences of the *erm* genes of *M. boenickei*, *M. goodii*, *M. houstonense*, *M. neworleansense*, *M. porcinum*, and *M. wolinskyi*, along with the previously described *erm* genes of *M. fortuitum* (9) and *M. smegmatis* (8), were found to be highly conserved, with a mean identity of 77% (range, 68 to 99%) (see Table S1 in the supplemental material). Following the guidelines of Roberts (11), the *erm* alleles in different RGM fall into three existing Erm classes: Erm(38) for *M. goodii* and *M. smeg-*

TABLE 1. PCR primers used in this study^a

Primer	Sequence	Target
MM1	CGCCGGGTGCTGACCATCA	<i>erm</i> of <i>M. mageritense</i>
MM2	GGACCCACAATCTGCGCCACAC	<i>erm</i> of <i>M. mageritense</i>
CME-1Y	ACGTGGTGGTGGGCAAYCTG	<i>erm</i> consensus
CME-2	AATTGGAACCAACGCGCCACT	<i>erm</i> consensus
GERM-4	GTGACGCTGCCCGCACCGTTCA	3342 consensus
MAB-7	GGTGGCCTCGCGCAGCTACTG	3342 consensus
MM6	GCGTCGTACTIONGCGTTGAA	3342 consensus
GERM-1	CCTCGGGGTCACGTTCTCCAG	TR consensus
GERMTR-1	TCGATGCTGCGCCGATGCGTTC	TR consensus
MP-3	GATCGCGGCGAACTGTTCTTG	TR consensus
GERM-2	CCGGTGACGTCGCCCCCGAC	<i>folD</i> consensus
MAB-8	ACGTGGCCGAGGATGTCTGGGA	<i>folD</i> consensus
MFERM-2	CCCGTCAGGCCGACATCATCA	<i>folD</i> consensus
MSX-5L	GGTACC CGGACATGGTCAAG	<i>folD</i> consensus

^a Organized by target gene.

* Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, Saban Research Institute of Childrens Hospital Los Angeles, 4650 Sunset Blvd., Mailstop 103, Los Angeles, CA 90027. Phone: (323) 669-5670. Fax: (323) 666-7681. E-mail: kanash@usc.edu.

† Supplemental material for this article may be found at <http://aac.asm.org/>.

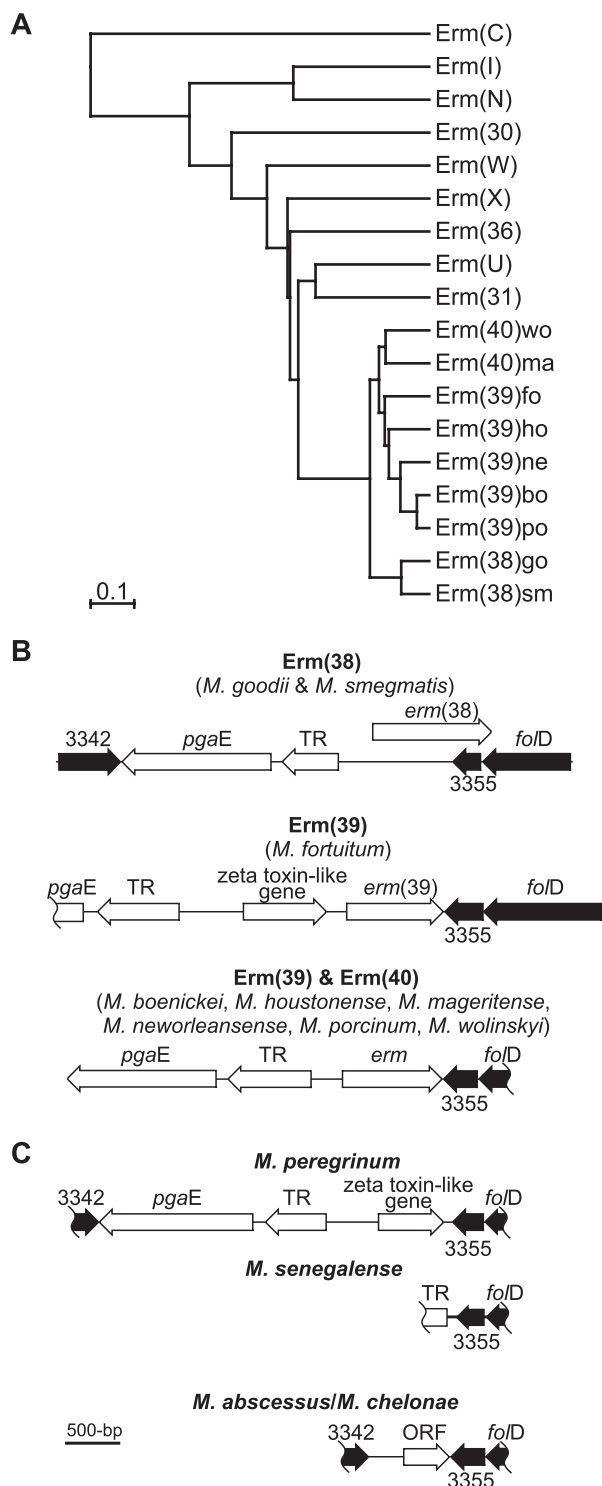


FIG. 1. (A) Phylogram showing the predicted relationships of the RGM Erm proteins, with the representative alleles of the eight most closely related Erm classes, and rooted on Erm(C) of *Staphylococcus aureus* (GenBank accession no. AAA20192). The Erm class representatives (with GenBank accession numbers) are Erm(30) of *Streptomyces venezuelae* (AAC69328), Erm(31) of *S. venezuelae* (AAC69327), Erm(36) of *Micrococcus luteus* (AAL68827), Erm(I) of *Streptomyces mycarofaciens* (A60725), Erm(N) of *Streptomyces fradiae* (CAA66307), Erm(U) of *Streptomyces lincolnensis* (CAA55770), Erm(W) of *Micromonospora griseorubida* (P43433), and Erm(X) of *Corynebacterium jeikeium* (AAK28910). For the RGM Erm proteins, Erm(38), Erm(39),

ermatis; Erm(39) for *M. boenickei*, *M. fortuitum*, *M. houstonense*, *M. neworleansense*, and *M. porcinum*; and Erm(40) for *M. mageritense* and *M. wolinskyi*. Furthermore, the predicted phylogenetic relationship between the amino acid sequences of the RGM *erm* alleles and other closely related Erm classes (Fig. 1A) showed that the RGM *erm* genes formed a distinct clade of the *erm* phylogenetic tree. This clade was consistent with the phylogeny of RGM predicted by molecular analysis of other genes (6). Furthermore, the RGM Erm node appears to have “emerged” more recently than the other Erm classes, which are largely derived from antibiotic-producing Actinomycetales (e.g., *Streptomyces*). This suggests that the RGM *erm* genes were acquired after the divergence of the RGM from other members of this order.

Conservation of the RGM *erm* genes was not only reflected in the coding regions, but also in the flanking genes (Fig. 1B), i.e., the *erm* gene regions of the RGM show synteny. Specifically, the *erm* genes were bound by the 3355 and *folD* genes downstream and the TR (putative transcriptional regulator) and *pgaE* genes upstream. Furthermore, PCR mapping indicated that the 3342 gene was upstream of the *pgaE* gene in all *erm*-positive RGM species. Using the consensus primers to amplify and sequence the 3342-*folD* regions of the *erm*-negative RGM species *M. peregrinum* (GenBank accession no. DQ435604) and *M. senegalense* (GenBank accession no. DQ435605), a gene organization similar to that of *erm*-positive RGM species, minus the *erm* gene, was revealed (Fig. 1C). Intriguingly, *M. peregrinum*, like *M. fortuitum*, has a zeta toxin-like gene absent in other RGM. Zeta toxin/antitoxin genes provide a mechanism for plasmid maintenance (7). Thus, the zeta toxin-like gene may be the “smoking gun” for the acquisition event of the *erm* gene (and possibly the *pgaE* and TR genes) from a plasmid.

Sequencing of the *folD* regions of *M. abscessus* (isolates MAB30 and MC719) and *M. chelonae* (strain ATCC 35752^T) revealed that the 3342 and *folD* genes were separated by 0.8 kbp of DNA (GenBank accession no. DQ144643 and DQ144644) (Fig. 1C). Although a possible open reading frame was identified in this region, BLAST analysis (1) revealed no convincing alignments with other sequences (all had <30% identity). In fact, BLAST searching of the current completely

and Erm(40), the allele (or RGM species) is indicated by the letters after the right parenthesis: bo, *M. boenickei*; fo, *M. fortuitum*; go, *M. goodii*; ho, *M. houstonense*; ma, *M. mageritense*; ne, *M. neworleansense*; po, *M. porcinum*; sm, *M. smegmatis*; and wo, *M. wolinskyi*. The scale bar shows the evolutionary distance (i.e., the amount of divergence, or time since splitting, from a theoretical common ancestor). (B and C) The genetic organization of the *folD* regions of *erm* gene-positive and *erm* gene-negative RGM, respectively. The shaded genes are similar (>64% amino acid identity) to *M. tuberculosis* strain H37Rv genes Rv3342 (GenBank accession no. CAA17114), Rv3355c (GenBank accession no. NP_217872), and *folD* (GenBank accession no. NP_217873). Other genes are those encoding TR, a putative *tetR* family transcriptional regulator, and *pgaE*, a putative polyketide synthase, and zeta toxin-like, containing a zeta toxin conserved domain (Pfam06414) and ~50% amino acid identity to conserved hypothetical genes of *Polaromonas* spp. (GenBank accession no. EAM36411) and *Xanthomonas oryzae* (GenBank accession no. AAW75612). ORF, open reading frame with a likely ribosome binding site (AAGGGA).

sequenced mycobacteria (through the NCBI website [http://www.ncbi.nlm.nih.gov/]) suggested that the *pgaE* and TR genes were found only in *erm*-positive RGM species and *M. peregrinum* and *M. senegalense*. This in turn suggested that *M. peregrinum* and *M. senegalense* originally had *erm* genes that were subsequently deleted.

As previously found for *M. fortuitum* and *M. smegmatis* (8, 9), *M. boenickei*, *M. houstonense* isolate 14133, *M. neworleansense*, and *M. porcinum* appeared susceptible to clarithromycin (MIC < 4 µg/ml) on standard susceptibility testing (10). However, as demonstrated with *M. houstonense* isolate 14133, overnight incubation in clarithromycin (0.1 µg/ml) increased the clarithromycin MIC to > 128 µg/ml. The clarithromycin MIC remained at ≤2 µg/ml for organisms incubated in the absence of macrolide. Thus, these results provide further evidence that macrolide resistance in RGM species can be inducible. Intriguingly, *M. houstonense* isolate 2014 and *M. mageritense* isolate 961635 were constitutively resistant to macrolide-lincosamide-ketolide agents (MIC > 128 µg/ml), yet reverse transcription-PCR analysis (using methods described elsewhere [8, 9]) indicated that the *erm* mRNA levels were ~10-fold higher in organisms preincubated for 2 h in 0.1 µg/ml clarithromycin or HMR3004 (data not shown). Thus, the results were consistent with RGM *erm* genes being inducible at the molecular level, even in organisms deemed constitutively resistant by standard susceptibility testing.

In conclusion, *erm* genes are present in a range of pathogenic RGM species, including species that are considered macrolide susceptible as currently defined by the Clinical and Laboratory Standards Institute (formerly NCCLS) (10). This, with the finding that all RGM *erm* genes are inducible at the molecular level, suggests that the results of routine susceptibility testing with macrolides should be interpreted with caution, especially for species of the *M. fortuitum* and *M. smegmatis* groups. Furthermore, induction of macrolide-lincosamide-ketolide resistance needs to be considered when assessing new macrolide-ketolide agents for potential antimycobacterial activity.

Nucleotide sequence accession numbers. The RGM *erm* regions and the *erm* gene-encoded polypeptides have been submitted to GenBank under the following accession numbers: *M. boenickei*, DQ144638 and AAZ76615; *M. goodii*, DQ144639 and AAZ76618; *M. houstonense*, DQ144640 and AAZ76622; *M. mageritense*, AY570506 and AAS76623; *M. neworleansense*,

DQ144641 and AAZ76625; *M. porcinum*, DQ447745 and ABE01848; and *M. wolinskyi*, DQ144642 and AAZ76628. The *fold* regions of *M. abscessus*, *M. peregrinum*, and *M. senegalense* have been submitted to GenBank under accession numbers DQ144643, DQ435604, and DQ435605, respectively.

Funding for this study was provided by National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases (NIAID) grant RO1-AI052291; the Department of Pathology and Laboratory Medicine, Childrens Hospital Los Angeles; and the Department of Microbiology, The University of Texas Health Center, Tyler, TX.

REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Brown, B. A., B. Springer, V. A. Steingrube, R. W. Wilson, G. E. Pfyffer, M. J. Garcia, M. C. Menendez, B. Rodriguez-Salgado, K. C. Jost, Jr., S. H. Chiu, G. O. Onyi, E. C. Bottger, and R. J. Wallace, Jr. 1999. *Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infections: a cooperative study from the International Working Group on Mycobacterial Taxonomy. *Int. J. Syst. Bacteriol.* **49**:1493–1511.
- Brown-Elliott, B. A., D. E. Griffith, and R. J. Wallace, Jr. 2002. Newly described or emerging human species of nontuberculous mycobacteria. *Infect. Dis. Clin. N. Am.* **16**:187–220.
- Brown-Elliott, B. A., and R. J. Wallace, Jr. 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clin. Microbiol. Rev.* **15**:716–746.
- CDC. 1999. Nontuberculous mycobacteria reported to the public health laboratory information system by state public health laboratories United States, 1993–1996. Centers for Disease Control and Prevention, Atlanta, Ga.
- Devulder, G., M. Perouse de Montclosand, and J. P. Flandrois. 2005. A multigenic approach to phylogenetic analysis using the genus *Mycobacterium* as a model. *Int. J. Syst. Evol. Microbiol.* **55**:293–302.
- Meinhart, A., J. C. Alonso, N. Strater, and W. Saenger. 2003. Crystal structure of the plasmid maintenance system epsilon/zeta: functional mechanism of toxin zeta and inactivation by epsilon 2 zeta 2 complex formation. *Proc. Natl. Acad. Sci. USA* **100**:1661–1666.
- Nash, K. A. 2003. Intrinsic macrolide resistance in *Mycobacterium smegmatis* is conferred by a novel *erm* gene, *erm*(38). *Antimicrob. Agents Chemother.* **47**:3053–3060.
- Nash, K. A., Y. Zhang, B. A. Brown-Elliott, and R. J. Wallace, Jr. 2005. Molecular basis of intrinsic macrolide resistance in clinical isolates of *Mycobacterium fortuitum*. *J. Antimicrob. Chemother.* **55**:170–177.
- NCCLS. 2003. Susceptibility testing of *Mycobacteria*, *Nocardia*, and other aerobic actinomycetes, 1st ed. Approved standard M24-A, vol. 23. NCCLS, Villanova, Pa.
- Roberts, M. C. 21 February 2005, accession date. Nomenclature Center for MLS Genes. [Online.] <http://faculty.washington.edu/marilynr/>.
- Roberts, M. C., J. Sutcliffe, P. Courvalin, L. B. Jensen, J. Rood, and H. Seppala. 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob. Agents Chemother.* **43**:2823–2830.
- Wallace, R. J., Jr. 1997. J. Glassroth, D. E. Griffith, K. N. Olivier, J. L. Cook, and F. Gordin. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. *Am. J. Respir. Crit. Care Med.* **156**:1S–25S.