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Molecular basis of intrinsic macrolide resistance in clinical isolates of *Mycobacterium fortuitum*

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Objectives: Some clinical isolates of *Mycobacterium fortuitum* are naturally resistant to macrolides, e.g. clarithromycin. Thus, the aim of this study was to identify the gene(s) conferring this resistance.

Methods: *M. fortuitum* ATCC 6841[T] DNA libraries were screened for plasmids that complemented the macrolide-susceptible phenotype of *Mycobacterium smegmatis* variant ermKO4 [*erm*(38)-negative]. Macrolide-resistant *M. smegmatis* transformants were selected on agar containing 128 mg/L erythromycin.

Results: Genetic complementation identified an *M. fortuitum* rRNA methylase gene, termed *erm*(39), 69% identical to *erm*(38) of *M. smegmatis*. In addition, *erm*(39) was found to be in the same chromosomal location as *erm*(38) in their respective hosts. Like *erm*(38), *erm*(39) conferred resistance (MIC >128 mg/L) to macrolide–lincosamide (ML) agents, but not to streptogramin B. Analysis of *erm* gene expression in *M. fortuitum* showed that ML agents increased *erm*(39) RNA levels, reaching a steady state level ∼20-fold higher than baseline. Screening of 32 *M. fortuitum* clinical isolates by PCR showed that all were positive for *erm*(39), irrespective of clarithromycin susceptibility. A majority of clarithromycin-susceptible (MIC ≤2 mg/L) isolates were postulated to carry a disabled *erm*(39) gene as they had a GTG→CTG mutation in the putative initiation codon of the *erm*(39) gene.

Conclusions: The similarity of the *erm* genes of *M. smegmatis* and *M. fortuitum* suggests that they were inherited from a common ancestor. Although the clinical impact of *erm*(39) on the therapeutic utility of clarithromycin is unclear, induction of this gene is consistent with the trailing end-points commonly seen during susceptibility testing of *M. fortuitum* isolates against macrolides.

Keywords: clarithromycin, rapidly growing mycobacteria, methylase, *erm* gene

Introduction

Many clinically significant mycobacteria are susceptible to macrolides, such as clarithromycin.[1,2] Consequently, this class of agents has become the foundation for treating mycobacterioses caused by non-tuberculous mycobacteria. However, several mycobacteria are intrinsically resistant to macrolides, including the *Mycobacterium tuberculosis* complex, the *Mycobacterium smegmatis* group and some members of the *Mycobacterium fortuitum* group.[1–4] *M. tuberculosis* is undeniably the most important human pathogen of the Mycobacteriacae. Nevertheless, rapidly growing mycobacteria, such as *M. fortuitum*, are important causes of soft-tissue infections and abscesses, often

associated with trauma or surgery.[1,2] Thus, understanding the mechanisms of drug resistance in these organisms will aid the design and implementation of effective therapeutic regimens.

Macrolide antimicrobial agents act by binding to the 50S ribosome subunit near the catalytic site of the peptidyltransferase region. More specifically, the macrolide-binding site is believed to be in the exit channel from the peptidyltransferase centre for growing peptide chains.[5,6] This suggests that macrolides do not act as direct catalysis inhibitors, but rather as physical 'plugs' blocking the exit of the peptide chain. However, there is evidence that 16-member macrolides (e.g. spiramycin) may inhibit peptidyltransferase activity, and 14-member macrolides (e.g. erythromycin) may prevent translocation of tRNA and increase

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tRNA dissociation from the ribosome.[7] However, it is not clear whether these are primary or secondary effects of the binding of macrolides.

For mycobacteria that are normally susceptible to clarithromycin, clinically acquired resistance is conferred by mutation in the 23S ribosomal RNA (rRNA) gene.[8–10] However, the primary mechanism of acquired, clinically significant macrolide resistance in other pathogenic bacteria (e.g. *Streptococcus pneumoniae*) is the presence of an *erm* gene or an efflux pump [e.g. *mef*(A)].[11] Therefore, it is intriguing that novel *erm* genes have been described recently for *M. smegmatis*[12] and *M. tuberculosis*,[13] which are intrinsically resistant to macrolides.

The *erm* genes are a diverse collection of methylases that add one or two methyl groups to the adenine at position 2058 (*Escherichia coli* numbering) of the 23S rRNA; this modification impairs the binding of macrolides to ribosomes, and thus reduces the inhibitory activity of these agents.[11] In most organisms, expression of an *erm* gene confers resistance to macrolide–lincosamide–streptogramin B (MLS) agents. However, the mycobacterial *erm* genes seem to confer resistance limited to ML agents.[12] This phenomenon is possible because the binding site for streptogramin B in mycobacteria does not seem to overlap with the A2058 residue.[12]

One ancestral source of *erm* genes is undoubtedly the bacteria that synthesize macrolides (or related agents), such as *Streptomyces* species. Adenine rRNA methylases expressed by these organisms protect their ribosomes from the inhibitory effects of the drugs they make. Often these drug-producing bacteria inhabit complex ecological niches (e.g. soil), where there is competition with other bacteria and fungi for nutrients. It is interesting, therefore, that the *erm* genes of environmental organisms such as corynebacteria and *M. smegmatis* are closely related to the *erm* genes of *Streptomyces* (>50% identical).[12,14]

In a previous study,[12] Southern-blot analysis showed that *M. fortuitum* strain ATCC 6841[T] had DNA similar to *erm*(38) of *M. smegmatis*. Intriguingly, *M. fortuitum* strain ATCC 6841[T] appears to be macrolide susceptible (clarithromycin MIC ≤2 mg/L), unless it is pre-incubated in subinhibitory concentrations of macrolide, when it presents a resistant phenotype (clarithromycin MIC >128 mg/L). Thus, *M. fortuitum* strain ATCC 6841[T] expressed inducible ML resistance, similar to that of *M. smegmatis*.[12] The aim of the current study was to clone and characterize the ML resistance gene of *M. fortuitum*.

Materials and methods

Bacteria and susceptibility testing

M. fortuitum strain ATCC 6841[T] and *Mycobacterium peregrinum* strain ATCC 14467[T] were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Two clinical isolates of *M. fortuitum* (strains 9704-20 and 976218) were generously provided by A. E. Rosato (Virginia Commonwealth University, Richmond, VA, USA). Additional clinical isolates of *M. fortuitum*, *M. smegmatis* and *Mycobacterium mageritense*, used to test for incidence of resistance genes, were chosen from isolates submitted for susceptibility testing and/or identification to the Mycobacteria/Nocardia Laboratory at the University of Texas Health Center. They were identified to species level based on growth rate, growth and colony morphology on Middlebrook 7H10 agar, pigment production and PCR restriction enzyme analysis of the 441 bp Telenti fragment

of the *hsp65* gene, as previously described.[15–18] The *erm*(38)-knockout variant of *M. smegmatis* mc[2]155 (variant ermKO4) is described elsewhere.[12]

Susceptibility testing of clinical isolates of rapidly growing mycobacteria was based on a broth microdilution assay using cation-supplemented Mueller–Hinton (MH) broth, as described elsewhere.[19,20] MIC breakpoints for clarithromycin were those of the NCCLS for rapidly growing mycobacteria.[20] Breakpoints for the other test drugs (clindamycin, erythromycin, quinupristin and rifabutin) have not been established for rapidly growing mycobacteria. For the experiments with recombinant strains, the medium used was 7HSF broth, which comprised Middlebrook 7H9 broth supplemented with 1 g/L pancreatic-digest of casein (Difco), 0.05% Tween 80 and 10% oleic acid–albumin–dextrose–catalase (BD Diagnostic Systems, Sparks, MD, USA). This medium resulted in faster growth of these strains than with MH broth. To test for inducible resistance, organisms were incubated overnight in subinhibitory concentrations of clarithromycin (0.01 and 0.1 mg/L) or medium alone (as controls), prior to determining the clarithromycin MIC.

To distinguish inducible resistance from the selection of mutants with constitutive high-level resistance in susceptibility assays, organisms that had grown in 128 mg/L clarithromycin were washed twice with 10 volumes of sterile water (to remove residual drug), before being plated on 7H11 agar. Following a 3 day incubation, a sweep of the resulting colonies was used as the inoculum in a second susceptibility assay.

Cloning of the putative resistance gene(s)

Genomic DNA was isolated from mycobacteria by the method of Belisle & Sonnenberg.[21] Five μg of DNA isolated from *M. fortuitum* ATCC 6841[T] was digested for 2 h with 5–10 units of (1) *Bam*HI, (2) *Msc*I (an isoschizomer of *Bal*I) and *Bgl*II, or (3) *Bsp*DI (an isoschizomer of *Cla*I). The restricted DNA was size-selected to be between 1 and 20 kbp by preparative agarose electrophoresis. The DNA fragments were ligated to the vector pMV261, which is a kanamycin-selectable *E. coli*-mycobacterial shuttle vector.[22] This vector carries the *Mycobacterium bovis hsp65* promoter upstream from the multiple cloning site, allowing expression of promoterless cloned genes. The pMV261 constructs were used to transform *E. coli* strain XL1-Blue MRF′ (Stratagene, La Jolla, CA, USA) by electroporation. For each transformation, ∼20 000 colonies were pooled and the plasmid DNA isolated using the Qiaprep kit (Qiagen). Approx. 0.1 μg of this plasmid pool was used to transform *M. smegmatis* ermKO4 by electroporation.[23] The clarithromycin and erythromycin MICs for this organism were 0.125 and 4–8 mg/L, respectively. To isolate macrolide-resistant *M. smegmatis* transformants, the transformation reaction was plated on tryptic soy agar (TSA) containing kanamycin (50 mg/L), and the resulting colonies pooled in tryptic soy broth supplemented with 0.05% Tween 80 to a turbidity equivalent to that of a 1.0 McFarland standard. Aliquots (0.1 mL) of the suspensions were plated on TSA containing kanamycin (50 mg/L) and erythromycin (128 mg/L). The plasmids from 3–5 colonies per preparation were transferred to *E. coli* XL1-Blue MRF′ (Stratagene) by electroduction.[24] The resulting *E. coli* transformants provided a ready means of purifying the plasmids derived from the macrolide-resistant *M. smegmatis*.

PCR and Southern analysis

To screen for the presence of mycobacterial *erm* genes by PCR, the following primers were used: MFERM-7 (GCCCTCACCCTGCCG-TTACAGC), MFERM-8 (AGGATGGCGGTGGTCAGATGGA), MSX-1 (ACGAGCTCGGCCAGAACTTCCTGT), and MSX-3

(GGTGAGCGGGGCAGTGGGTAG). The former two primers target the *M. fortuitum erm* gene (between codons 45 → 108; GenBank acc. AY487229), and the latter two primers are specific for *erm*(38) of *M. smegmatis*[12] (between codons 9 → 102; GenBank acc. AY154657); the expected sizes of the two amplification products are 191 bp and 280 bp, respectively. *M. fortuitum* ATCC 6841[T] and *M. smegmatis* mc[2]155 were used as controls when testing the clinical isolates for resistance genes. The basic cycling conditions were 35 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 30 s. PCR additives, such as DMSO (5%–10%) or Qiagen Solution Q (1×), enhanced amplification product yields. Southern analysis methods are described in detail elsewhere.[12]

Expression analysis by real-time RT−PCR

For gene expression analysis, the RNA in bacterial suspensions was stabilized by the addition of two volumes of RNAProtect bacterial reagent (Qiagen). To isolate RNA, the Qiagen RNeasy system was applied, including a mechanical disruption step using lysing matrix B (QBiogene, Carlsbad, CA, USA) in a FastPrep FP120A instrument (speed setting 6.5 for 45 s). In addition, an on-column DNase-treatment (Qiagen) was included to remove residual DNA. Expression analysis was by real-time RT−PCR, using an iQ iCycler real-time PCR machine (Bio-Rad, Hercules, CA, USA) and the QuantiTect SYBR Green RT−PCR Kit (Qiagen) including solution Q PCR additive (Qiagen). The basic reaction conditions were 50°C for 30 min, then 95°C for 15 min (to activate the Taq DNA polymerase) followed by 35 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 30 s. Approx. 50–100 ng of RNA was added per amplification reaction. The primers used to analyse expression of the putative *erm* gene of *M. fortuitum* were MFERM-7 and MFERM-8 (see above). The results were normalized to the amount of 23S rRNA in each sample, assessed by RT−PCR using primers MS23S-1 (CGAATGGCGTAACGACTTCTCA) and MS23-3 (GTAGTGAAGGTCCCGGGGTC). Each experiment was set up in triplicate.

DNA sequencing and analysis

Plasmid DNA was sequenced using the BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA) and run on an ABI PRISM 3100 Genetic Analyzer. BLAST searching[25] of the DNA and protein databases (including unfinished prokaryote genomes) was through the National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/. The computer software MacVector version 7.2 (Accelrys, Burlington, MA, USA) was used for general sequence analysis (e.g. restriction site and open reading frame analysis) and ClustalW pairwise alignment.

Results

Cloning of the M. fortuitum macrolide resistance gene

The *M. fortuitum* DNA fragments containing the gene(s) conferring resistance to erythromycin (MIC >128 mg/L) were isolated from three independent genomic libraries expressed in *M. smegmatis* ermKO4 [an *erm*(38) gene knockout variant of strain mc[2]155]. The plasmids isolated from the erythromycin-resistant *M. smegmatis* transformants were termed pMVMF3, pMVMF5 and pMVMF10, and contained an ∼12 kbp *Bam*HI *M. fortuitum* DNA fragment, an ∼3 kbp *Msc*I-*Bgl*II fragment and an ∼7 kbp *Bsp*DI fragment, respectively. The resistance conferred by these plasmids was verified by transferring them back into

Table 1. Antimicrobial susceptibility of *M. smegmatis* ermKO4 expressing *erm*(39) in *trans*

Plasmid	MIC (mg/L)					
	CLI	CLR	ERY	Q	SPM	RFB
pMV261	32	0.125	8	64	4	0.5
pMVMF3	>1024	>512	>1024	64	>1024	0.5
pMVMF5	>1024	>512	>1024	64	>1024	0.5
pMVMF10	>1024	>512	>1024	64	>1024	0.5

CLI, clindamycin; CLR, clarithromycin; ERY, erythromycin; Q, quinupristin; SPM, spiramycin; RFB, rifabutin.

M. smegmatis ermKO4. In each case, the newly transformed *M. smegmatis* expressed high-level resistance (MIC >512 mg/L) to clindamycin, clarithromycin, erythromycin and spiramycin, but not streptogramin B (quinupristin) or rifabutin (Table 1). This phenotype was equivalent to that of the parental *M. smegmatis* mc[2]155, i.e. *erm*(38) positive.[12]

Southern analysis using a probe specific for *erm*(38) of *M. smegmatis* showed that plasmids pMVMF3, pMVMF5 and pMVMF10 contained DNA similar to this *erm* gene (data not shown). Restriction mapping and Southern analysis placed the *erm*(38)-like DNA within an estimated 1.5 kbp *Bam*HI−*Bsp*DI fragment of each plasmid. Figure 1 shows the alignment of the three plasmids based on this analysis. Since the overlap between the three plasmids is restricted to this 1.5 kbp fragment, this must define the location of the resistance gene.

Initially, a DNA sequence was obtained from plasmid pMVMF3 for ∼1 kbp of insert DNA proximal to the vector's *hsp65* promoter. Open reading frame and BLAST searching of the DNA sequence data revealed a putative adenine rRNA methylase or *erm* gene. A likely initiation codon (GTG) of this gene was 58 bp from the beginning of the cloned *M. fortuitum* DNA fragment and in the same orientation as the *hsp65* promoter of the vector. Thus, the location and orientation of this putative *erm* gene was consistent with it being expressed from the vector's *hsp65* promoter. Analysis of DNA sequence data obtained from pMVMF5 and pMVMF10 verified that the putative *erm* gene was common to all three plasmids.

The putative *erm* gene of *M. fortuitum* was predicted to encode a 246-amino-acid polypeptide. BLAST searching showed that this amino acid sequence was similar (>40% identical) to numerous rRNA methylases, including Erm(38) of *M. smegmatis* (GenBank acc. AAN86837), Erm(X) alleles of *Corynebacterium diphtheriae* (GenBank acc. NP_863178) and *Corynebacterium jeikeium* (GenBank acc. AAK28907 and AAK28910). The sequence for the *M. fortuitum erm* gene, designated *erm*(39) [or Erm(39) for the protein], has been registered with the Nomenclature Center for MLS Genes maintained by Dr Marilyn C. Roberts.[26]

ClustalW pairwise analysis aligned the complete 246-amino-acid Erm(39) protein with the first 259 amino acids of the *M. smegmatis* Erm(38) protein (GenBank acc. AAN86837) with 71% identity. Figure 2 shows the full alignment of the two proteins. The 386-amino-acid Erm(38) protein is longer than Erm(39), and has a unique C-terminal region that probably represents a fusion between the ancestral *erm* gene and its insertion
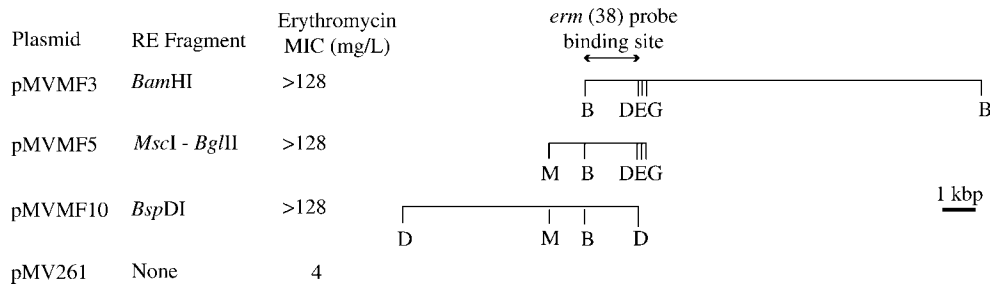
Figure 1. Summary of the cloned *M. fortuitum* DNA fragments that conferred high-level erythromycin resistance to *M. smegmatis* ermKO4. The common region that hybridized with an *erm*(38) derived probe is indicated by the arrows. The annotated restriction sites are: B, *Bam*HI; D, *Bsp*DI; E, *Eco*RV; G, *Bgl*II; M, *Msc*I [plasmids pMVMF3 and pMVMF10 had at least one additional *Bgl*II and *Msc*I site—the locations of these sites relative to the *erm*(38) probe binding site was not mapped in detail in this analysis]. The fragments are oriented so that RNA transcription from the *hsp65* promoter of the cloning vector (pMV261) proceeds from left to right.
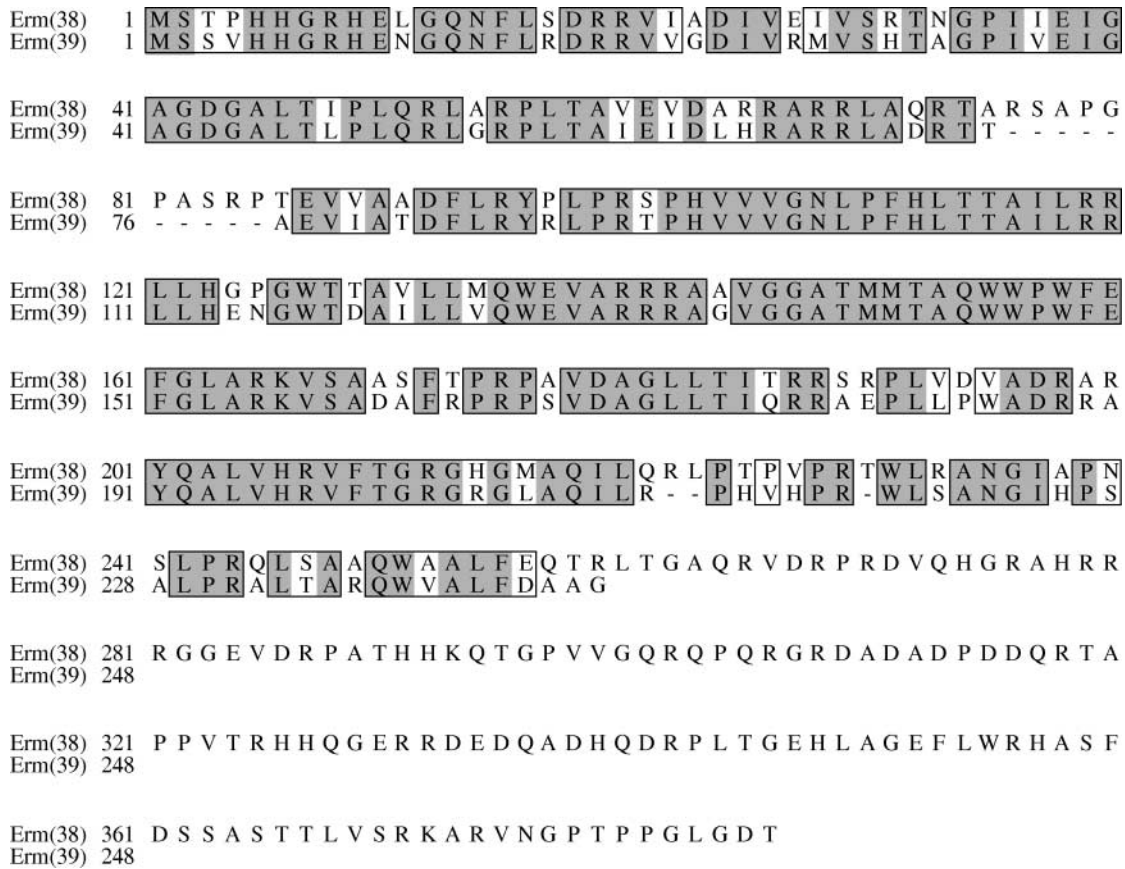

Figure 2. ClustalW alignment of the RNA methylases of *M. fortuitum* [Erm(39); GenBank acc. AY487229] and *M. smegmatis* [Erm(38); GenBank acc. AAN86837]. Dark-shaded amino acids are identical in the two proteins and light-shaded amino acids are functionally similar.

point within the *M. smegmatis* genome.[12] Thus, the C-terminus of Erm(39) aligns with what is believed to be the fusion site for Erm(38). Both Erm(38) and Erm(39) were found to be distinct (25% and 29% identity) from Erm(37) of *M. tuberculosis* (encoded by the gene Rv1988, GenBank acc. NP_216504.1).

Using all three plasmids as templates, the DNA sequence data were extended to 2.1 kbp upstream and 1.9 kbp downstream from the putative *erm* gene for a total of 4.8 kbp (GenBank acc. AY487229). Figure 3 shows the proposed genetic organization of this region. From this analysis, it was found that the inserts of plasmids pMVMF3, pMVMF5 and pMVMF10 had only two putative genes in common, *erm*(39) and 3355 (similar to Rv3355c of *M. tuberculosis* H37Rv, a hypothetical gene with unknown function). This further substantiates the role of *erm*(39) in the ML-resistance phenotype.

Downstream from *erm*(39) are several hypothetical genes with a high degree of identity to *M. tuberculosis* and *M. smeg-matis* (Figure 3). In particular, the 3355, *folD* and 3359 proteins are >64% identical to the comparable proteins in *M. tuberculosis* H37Rv. Upstream from *erm*(39) is a putative transcriptional
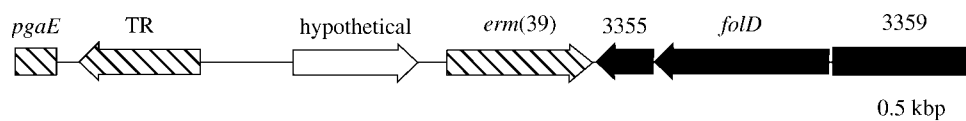
Figure 3. Genetic organization of the *M. fortuitum* chromosome in the region of *erm*(39) (GenBank acc. AY487229). Genes shown in black have a high degree of identity (≥65%) with *M. tuberculosis* H37Rv (the gene numbering in this figure is equivalent to the Rv gene index), and *M. smegmatis*. The genes shown with cross-hatching have a high degree of identity only with *M. smegmatis*. The hypothetical gene (shown in white) has no convincing homology (i.e. <30% identity) to any known sequence. Key: *pgaE*—putative polyketide oxygenase (65% amino acid identity with the N-terminus of PgaE of *M. smegmatis*;[12] 36% identity with N-terminus of oxygenase-reductase PgaM of *Streptomyces* species, GenBank acc. AAK57530.1); TR—putative transcriptional regulator [73% amino acid identity with TR associated with *erm*(38) of *M. smegmatis*;[12] 32% identical to CalR1, a calicheamicin synthesis regulator, GenBank acc. AAM94766]; *erm*(39)—adenine rRNA methylase [71% amino acid identity with Erm(38) of *M. smegmatis*, GenBank acc. AAN86837]; 3355—hypothetical gene with unknown function (65% identity with Rv3355c of *M. tuberculosis*, GenBank acc. NP_217872.1); *folD*—a tetrahydrofolate dehydrogenase/cyclohydrolase (82% amino acid identity with FolD or Rv3356c of *M. tuberculosis*, GenBank acc. NP_217873.1); 3359—probable NAD(H)-dependent flavin oxidoreductase (74% identity with N-terminus of Rv3359 of *M. tuberculosis*, GenBank acc. NP_217876.1).

regulator (TR) and a *pgaE* homologue, indicating that the gene organization surrounding *erm*(39) is largely conserved with respect to that of *erm*(38) of *M. smegmatis*. However, the DNA between *erm*(39) and TR shows no convincing homology (i.e. all BLAST alignments were <30% identical) to any known sequence in the GenBank databases.

Expression of erm(39)

The level of *erm*(39) RNA in *M. fortuitum* increased following exposure to 1 mg/L erythromycin (Figure 4a). By 20 min, the *erm*(39) RNA level was six-fold higher, and by 60 min, the level was 15-fold higher than baseline (i.e. time 0). After 60 min, the RNA levels entered a steady state phase, which at 120 min was ∼18-fold higher than baseline.

Consistent with the phenotypic analysis, *erm*(39) RNA levels also increased following exposure to sub-MIC concentrations of clindamycin (8 mg/L) and spiramycin (8 mg/L) but not quinupristin (16 mg/L) (Figure 4b). The differences in the expression of *erm*(39) induced by the three ML agents probably relates to concentration and/or kinetic effects. Thus, ML agents are effective inducers of *erm*(39), consistent with the resistance phenotype conferred by this gene.

Distribution of erm(39) in rapidly growing mycobacteria

A total of 32 clinical isolates of *M. fortuitum*, in addition to strain ATCC 6841[T], were screened for the presence of the *erm*(39) and *erm*(38) genes by PCR. The clarithromycin MIC of these strains was in the range 1–>32 mg/L, with 50% deemed as susceptible on routine susceptibility testing (clarithromycin MIC ≤4 mg/L). All 32 *M. fortuitum* strains were PCR positive for the *erm*(39) gene and negative for the *erm*(38) gene, irrespective of the susceptibility results. In contrast, the related species, *M. peregrinum* (one isolate, clarithromycin susceptible) and *M. mageritense* (13 isolates, all clarithromycin resistant)[17] were negative by PCR for both *erm*(38) and *erm*(39) genes. All 11 tested *erm*(38)-positive *M. smegmatis* isolates (all clarithromycin resistant) were negative for the *erm*(39)-specific PCR.

Although the presence of *erm*(39) in *M. fortuitum* did not appear to correlate with baseline clarithromycin MIC, some isolates may express a cryptic inducible resistance phenotype, i.e. that was not manifested in the original susceptibility assays. There was precedence for this, as our reference strain, *M. fortuitum* ATCC 6841[T], appeared to be clarithromycin-susceptible
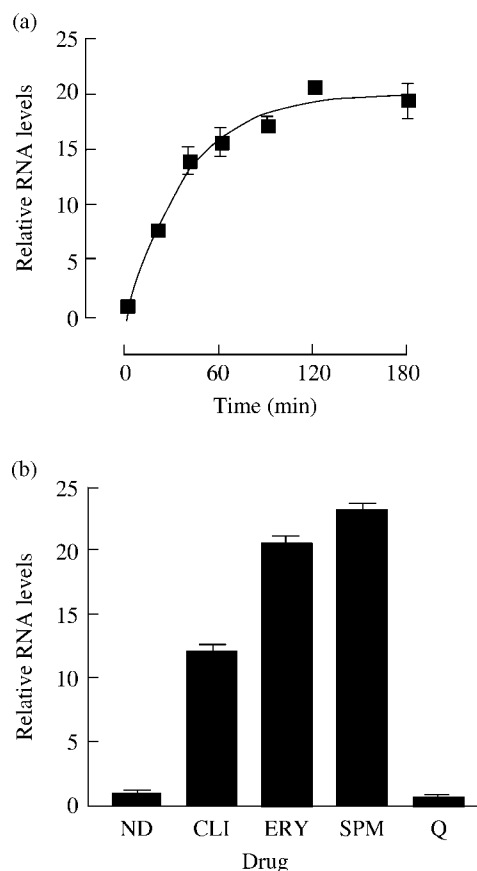

Figure 4. Real-time RT–PCR analysis of *erm*(39) expression. (a) A time course study following addition of erythromycin (1 mg/L). RNA levels are presented relative to baseline (time 0). (b) The effect of different antimicrobial agents on *erm*(39) expression after an incubation of 120 min. ND, control (no drug); CLI, clindamycin (8 mg/L); ERY, erythromycin (1 mg/L); SPM, spiramycin (8 mg/L); Q, quinupristin (16 mg/L). All data points represent the mean of three replicates.

(MIC ≤2 mg/L), unless it was pre-incubated in subinhibitory concentrations of macrolide, when it became clarithromycin-resistant (MIC >128 mg/L).

To investigate this 'cryptic inducibility' hypothesis, five clinical isolates (Mf1963, Mf1973, Mf1991, Mf2038 and Mf2169) were tested for inducible macrolide resistance (*M. fortuitum* strain ATCC 6841[T] was included as a positive control). Each of

these isolates was positive for *erm*(39) by PCR and with a clarithromycin MIC of ≤2 mg/L. Of the five isolates, only Mf1963 (20%) expressed an increased clarithromycin MIC (>128 mg/L) following overnight incubation in clarithromycin concentrations of 0.01 and 0.1 mg/L. Incubation in medium alone resulted in a clarithromycin MIC of ≤2 mg/L. The other four isolates (80%) demonstrated no evidence of phenotypic induction, i.e. clarithromycin MICs were ≤2 mg/L under all conditions. Thus, a proportion of the *erm*(39)-positive, 'susceptible' isolates could be explained by an inducible phenotype. Nevertheless, another mechanism must underlie the phenotype of the remaining *erm*(39)-positive, clarithromycin-susceptible isolates.

Possible alternative explanations for the clarithromycin-susceptible phenotype include a lack of molecular induction and/or a disabling mutation in the *erm*(39) gene. Molecular induction was assessed by real-time RT–PCR analysis of *erm*(39) RNA levels in organisms incubated for 3 h in either medium alone or 0.01 mg clarithromycin per L. This analysis showed that clarithromycin exposure increased *erm*(39) RNA levels by factors of 367 ± 32, 32 ± 4 and 96 ± 20 in isolates Mf1963, Mf1991 and Mf2169, respectively. Thus, RNA induction occurred in phenotypically non-inducible isolates, i.e. macrolide susceptibility in *erm*(39)-positive isolates was not explained by a lack of RNA induction.

In order to determine if an *erm*(39)-disabling mutation had occurred in isolates Mf1991 and Mf2169, the DNA sequences for the *erm*(39) gene and 800 bp upstream leader region (assumed to contain the promoter) were compared with that of our reference strain, ATCC 6841[T]. The *erm*(39) genes of Mf1991 and Mf2169 were found to be 100% identical to each other and have a 95% nucleotide (97% amino acid) identity with ATCC 6841[T]. Pairwise comparisons of the 800 bp upstream leader regions of the three isolates showed that they were 99% identical to each other. Thus, the largest difference between ATCC 6841[T] and the other two isolates was in the *erm*(39) gene.

Of the 11 codon discrepancies between the *erm*(39) genes of Mf1991/Mf2169 and ATCC 6841[T], all coded for either chemically or functionally similar amino acids, or were conserved with respect to *erm*(38) of *M. smegmatis*. That said, one of the divergent codons was the predicted initiation codon of *erm*(39). The initiation codon of the *erm*(39) gene of ATCC 6841[T] was GTG, whereas it was CTG in Mf1991 and Mf2169 (CTG is considered a valid initiation codon in some bacteria). Furthermore, a GTG initiation codon of *erm*(39) was found in three additional macrolide-resistant isolates (including Mf1963), whereas a CTG initiation codon was found in two additional susceptible and non-inducible isolates (Mf1973 and Mf2038). Thus, 5/5 resistant strains had a GTG initiation codon and 4/4 susceptible strains had a CTG initiation codon. These results suggested that a GTG → CTG mutation in the initiation codon of *erm*(39) was associated with the loss of macrolide resistance.

Discussion

With this study, three *erm* genes have been described in separate mycobacterial species, i.e. *erm*(37) of *M. tuberculosis* complex,[13] *erm*(38) of *M. smegmatis*[12] and *erm*(39) of *M. fortuitum*. The *erm*(38) and *erm*(39) genes are very similar, both in sequence (69% nucleotide identity) and in chromosome location (adjacent

to *folD*). These findings suggest that the *erm* genes of *M. fortuitum* and *M. smegmatis* are not recent acquisitions and the source gene probably originated in a common ancestor of these organisms. In contrast, *erm*(37) has a sequence distinct from the other two mycobacterial *erm* genes (and all other known *erm* genes), and it is in a different location within the *M. tuberculosis* chromosome. This is consistent with the evolutionary history of *erm*(37) being different to that of *erm*(38) and *erm*(39).

Usually, the acquisition of an *erm* gene by pathogenic bacteria is clinically significant, in that monotherapy with macrolides is unlikely to be efficacious. *M. tuberculosis* is intrinsically resistant to macrolides[3] and clarithromycin is not particularly useful in treating *M. tuberculosis* infections, at least in animal models.[4,27] Previous studies showed that expression of *erm*(37) of *M. tuberculosis* may explain, at least partially, the intrinsic macrolide resistance.[13] Interestingly, the RD2 deletion in the *M. bovis* BCG (Pasteur) chromosome[28] includes the *erm*(37) (or Rv1988) gene, and there is some evidence that erythromycin may be useful for treating *M. bovis* BCG infection,[29,30] although a recent review[31] suggests that antimicrobial treatment (including macrolide-containing regimens) of BCG lymphadenitis may not significantly affect the course of the disease.

An important issue is whether clarithromycin (or other macrolides) should be used for the treatment of *M. fortuitum* infections. The fact that ∼80% of *M. fortuitum* isolates have susceptible MICs of clarithromycin (MIC ≤4 mg/L)[32] suggests that empirical use of macrolides to treat infections caused by this organism would be efficacious in most cases. However, two important issues argue against such a use.

First, a significant proportion of macrolide 'susceptible' *M. fortuitum* may express cryptic inducible resistance, that routine susceptibility testing would miss. Although induction of *erm*(39) only occurs at very low drug concentrations, once induced these organisms are highly resistant to clarithromycin (MIC >64 mg/L). Furthermore, mutations could occur that derepress expression, leading to constitutive expression of high-level macrolide resistance. The presence of inducible β-lactamases (e.g. among *Enterobacter* species) has raised similar issues because of subsequent development of high-level constitutive enzyme production following mutation in the repressor gene.[33,34] This results in high-level resistance to agents such as ceftazidime, which are non-enzyme inducers and work initially in infected patients. *M. fortuitum* infections are chronic, so there is a prolonged time when derepression mutations can occur.

Second, truly macrolide-susceptible *M. fortuitum* isolates may carry an inactive form of the *erm*(39) gene (i.e. with a CTG initiation codon). Activation of *erm*(39) in such organisms may only require a single base transversion (CTG → GTG). This mechanism of clinically acquired macrolide resistance may occur in addition to 23S rRNA mutations that have been described in other mycobacteria.[8–10]

The presence of an inactive form of *erm*(39) in some clinical isolates of *M. fortuitum* is intriguing in terms of the evolution of this organism. It is likely that adenine methylation in the peptidyltransferase centre of the 23S rRNA will reduce growth rate, perhaps similarly to the effects of the 23S rRNA mutations that confer macrolide resistance in mycobacteria.[35] However, *erm*(39) expression is inducible and it is likely that there is minimal methylation without induction. Thus, it is not clear why organisms with disabling mutations should emerge. Furthermore, it is not known if the *erm*(39) disabling mutation occurred prior to or

during the human infection, or even during the subsequent isolation and culture.

As well as the impact *erm*(39) may have on the empirical treatment of *M. fortuitum* infections, the presence of this gene is clinically important for other reasons. It is documented that susceptibility testing of *M. fortuitum* isolates against clarithromycin can be problematic, often with trailing end-points.[20] The inducible ML resistance conferred by *erm*(39) in *M. fortuitum* provides an explanation for this phenomenon. Therefore, there is compelling evidence that MICs determined by routine susceptibility testing (i.e. without induction analysis) of *M. fortuitum* isolates against ML may be misleading and as such may be unwarranted.

It is intriguing that all tested isolates of *M. fortuitum* contain the *erm*(39) gene, whereas, the closely related rapidly growing species, *M. peregrinum* and *M. mageritense*, do not. Isolates of *M. mageritense* are known to be macrolide resistant,[17] and preliminary evidence suggests that *M. mageritense* carries another *erm* gene, distinct from *erm*(38) and *erm*(39) (K.A. Nash, unpublished data). These findings suggest that *erm* genes are widespread in the rapidly growing mycobacteria. With this said, and the ubiquitous distribution of mycobacteria in the environment, could the mycobacterial *erm* genes be a source of resistance genes to other organisms? This possibility seems unlikely as all of the known mycobacterial *erm* genes are chromosomal and none is associated with known or putative mobile elements.

In conclusion, given the ubiquitous occurrence of *erm*(39) in *M. fortuitum*, it is our opinion that clarithromycin should probably be used with caution with infections involving this organism (especially if the clarithromycin MIC is ≥ 4 mg/L), and probably never as a single agent for infections with a large burden of organisms. How often clarithromycin monotherapy for *M. fortuitum* is used, however, is unknown, as there are other potential therapeutic agents.[1,2]

Acknowledgements

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