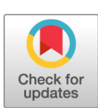

Emergence of Inducible Macrolide Resistance in *Mycobacterium chelonae* Due to Broad-Host-Range Plasmid and Chromosomal Variants of the Novel 23S rRNA Methylase Gene, *erm*(55)

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ABSTRACT Macrolides are a mainstay of therapy for infections due to nontuberculous mycobacteria (NTM). Among rapidly growing mycobacteria (RGM), inducible macrolide resistance is associated with four chromosomal 23S rRNA methylase (*erm*) genes. Beginning in 2018, we detected high-level inducible clarithromycin resistance (MICs of $\geq$16 μg/mL) in clinical isolates of *Mycobacterium chelonae*, an RGM species not previously known to contain *erm* genes. Using whole-genome sequencing, we identified a novel plasmid-mediated *erm* gene. This gene, designated *erm*(55)^P, exhibits <65% amino acid identity to previously described RGM *erm* genes. Two additional chromosomal *erm*(55) alleles, with sequence identities of 81% to 86% to *erm*(55)^P, were also identified and designated *erm*(55)^C and *erm*(55)^T. The *erm*(55)^T is part of a transposon. The *erm*(55)^P allele variant is located on a putative 137-kb conjugative plasmid, pMchErm55. Evaluation of 133 consecutive isolates from 2020 to 2022 revealed 5 (3.8%) with *erm*(55). The *erm*(55)^P gene was also identified in public data sets of two emerging pathogenic pigmented RGM species: *Mycobacterium iranicum* and *Mycobacterium obuense*, dating back to 2008. In both species, the gene appeared to be present on plasmids homologous to pMchErm55. Plasmid-mediated macrolide resistance, not described previously for any NTM species, appears to have spread to multiple RGM species. This has important implications for antimicrobial susceptibility guidelines and treatment of RGM infections. Further spread could present serious consequences for treatment of other macrolide-susceptible RGM. Additional studies are needed to determine the transmissibility of pMchErm55 and the distribution of *erm*(55) among other RGM species.

KEYWORDS *Mycobacterium chelonae*, clarithromycin, *erm* gene, macrolide resistance, mycobacterial plasmids, nanopore genomic sequencing, rapidly growing mycobacteria, whole-genomic sequencing

Macrolide antibiotics are a cornerstone in the treatment of infections caused by nontuberculous mycobacteria (NTM) (1–6). Point mutations in the 23S rRNA gene can confer constitutive resistance to macrolides, whereas recent studies of rapidly growing mycobacteria (RGM) have identified 23S rRNA methylase (*erm*) genes as key mediators of inducible macrolide resistance (7–9). The chromosomally located *erm*(38), *erm*(39), *erm*(40), and *erm*(41) genes are present in *Mycobacterium smegmatis*, the *Mycobacterium fortuitum* group, *M. mageritense*, and *M. wolinskyi*, and *Mycobacterium abscessus*, respectively (8, 9). Phenotypic detection of inducible macrolide resistance requires extended antimicrobial susceptibility testing times (up to 14 days of incubation) (10).

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The presence of *erm* genes has unequivocally impacted the utility of the macrolides in the treatment of infections caused by many species of RGM. A pivotal study in Korea showed that, when treated with a macrolide-containing regimen, only 25% of pulmonary patients infected with *M. abscessus* subsp. *abscessus* (with a functional *erm* gene) showed clinical improvement, compared with 75% of patients infected with *M. abscessus* subsp. *massiliense* (without a functional *erm* gene) (11). *M. abscessus* subsp. *abscessus* is the most commonly isolated RGM species in the United States, and approximately 80% of clinical isolates contain a functional *erm*(41) gene (9, 10).

The original studies of the *erm*(41) gene by Nash et al. found no evidence of inducible macrolide resistance among isolates of *M. chelonae* after 3 or 14 days of incubation (8, 9). Similarly, a large 2-year study by Hanson and colleagues of 45 isolates of *M. chelonae* also showed no inducible macrolide resistance after 14 days (12). In 2018, The Clinical and Laboratory Standards Institute (CLSI) recommended that extended incubation for detection of inducible macrolide resistance was no longer necessary for some RGM species, including *M. chelonae* (13).

However, beginning in 2018, the Mycobacteria/Nocardia Research Laboratory at the University of Texas Health Science Center at Tyler (UTHSCT) detected clinical isolates of *M. chelonae* that exhibited elevated MICs (1 to ≥16 μg/mL) to clarithromycin after 3 to 4 days of incubation and high-level resistance (≥16 μg/mL) after extended incubation for up to 14 days. Genomic approaches, including both short-read (Illumina) and long-read (Oxford Nanopore) platforms, were used to characterize these macrolide-resistant isolates of *M. chelonae*. Here, we describe three variants of a novel 23S rRNA methylase gene, *erm* (55), present both on a conjugative plasmid and on the *M. chelonae* chromosome.

MATERIALS AND METHODS

Collection, susceptibility testing, and selection of *M. chelonae* isolates. *M. chelonae* reference strain CIP 104535 (also known as ATCC 35752^T) was obtained from the Institute Pasteur (Paris, France) and was used as a control for all experiments. Clinical isolates of *M. chelonae* were obtained from culture collections at the Mycobacteria/Nocardia Research Laboratory at the UTHSCT (Laboratory A) and the Mayo Clinic in Rochester, Minnesota (Laboratory B). The clinical isolates were initially submitted for antimicrobial susceptibility testing. Species-level identification of *M. chelonae* was determined on all isolates by growth rate, colony morphology, pigmentation, and either targeted sequencing of the *rpoB* gene as previously described or matrix-assisted laser desorption ionization–time of flight mass spectrometry (14, 15). Susceptibility testing to clarithromycin was performed by broth microdilution with commercially available 96-well plates manufactured by ThermoFisher (Cleveland, OH) according to CLSI guidelines (13). Because extended incubation was not (and still is not) required according to current CLSI guidelines, screening for inducible resistance was only routinely performed in Laboratory A beginning in November 2020, upon recognition of inducible macrolide resistance in some isolates. The isolates from Laboratory B were tested for 14 days in 2021 to screen for possible resistant isolates.

Upon subsequent recognition that isolates with high-level inducible macrolide resistance at 14 days had high 3-day clarithromycin MICs of ≥1 μg/mL, isolates in Laboratory A were screened for possible macrolide-resistant isolates based on high 3-day MICs between 2018 (date of first known resistant isolate) and 2020. These isolates then underwent 14-day incubation.

Screening for the *erm*(41) gene and 23S rRNA (*rrl*) mutations. Isolates with high clarithromycin MICs were screened for the presence of the *erm*(41) gene and point mutations in the 23S rRNA gene (*rrl*) as previously described. (7, 9) The wild-type *M. chelonae* CIP 104535 (ATCC 35752^T) reference strain and isolates known to have the *erm*(41) gene or the 23S rRNA A2058 or 2059 mutations were used as controls.

Whole-genome sequencing and assembly. *M. chelonae* genomes were sequenced using a combination of Illumina and Oxford Nanopore platforms. For both, DNA was extracted using the EpiCentre Masterpure Complete DNA and RNA purification kit (Mandel Scientific, ON, Canada). Illumina Nextera XT libraries were sequenced on the Illumina MiSeq (San Diego, CA) instrument using the reagent kit v2 (300 cycles). MiSeq reads were assembled and annotated using the IRIDA pipeline (16).

Nanopore libraries were prepared using the Nanopore ligation sequencing kit with the native barcoding expansion kit and sequenced on a MinION device (Oxford Nanopore, Oxford, United Kingdom). Raw Nanopore reads were base-called and demultiplexed with Guppy (5.0.16) using the superaccuracy model with default settings. Additional read trimming was carried out using Porechop (0.3.2pre) to remove remaining adapter contamination. Filtlong (0.2.1) was used for light quality filtering, discarding the lowest quality 5% of reads and any reads shorter than 1,000 bp. The filtered reads were used as the input for long-read consensus assembly using Trycycler (0.5.3) with Flye (2.9-b1768), miniasm (0.3-r179) + Minipolish (0.1.2), and raven (1.7.0) as assemblers. Medaka (1.5.0), Polypypolish (0.4.3), and POLCA from MaSuRCA (3.4.2) were used to polish the initial assembly with a combination of long-read (Nanopore) and short-read (MiSeq) data.

Phylogenetic analysis. MEGA Molecular Evolutionary Genetics Analysis software (megasoftware.net) was used for construction of *erm*(55)^P, *erm*(55)^C, and *erm*(55)^T dendrograms. Default parameters for the

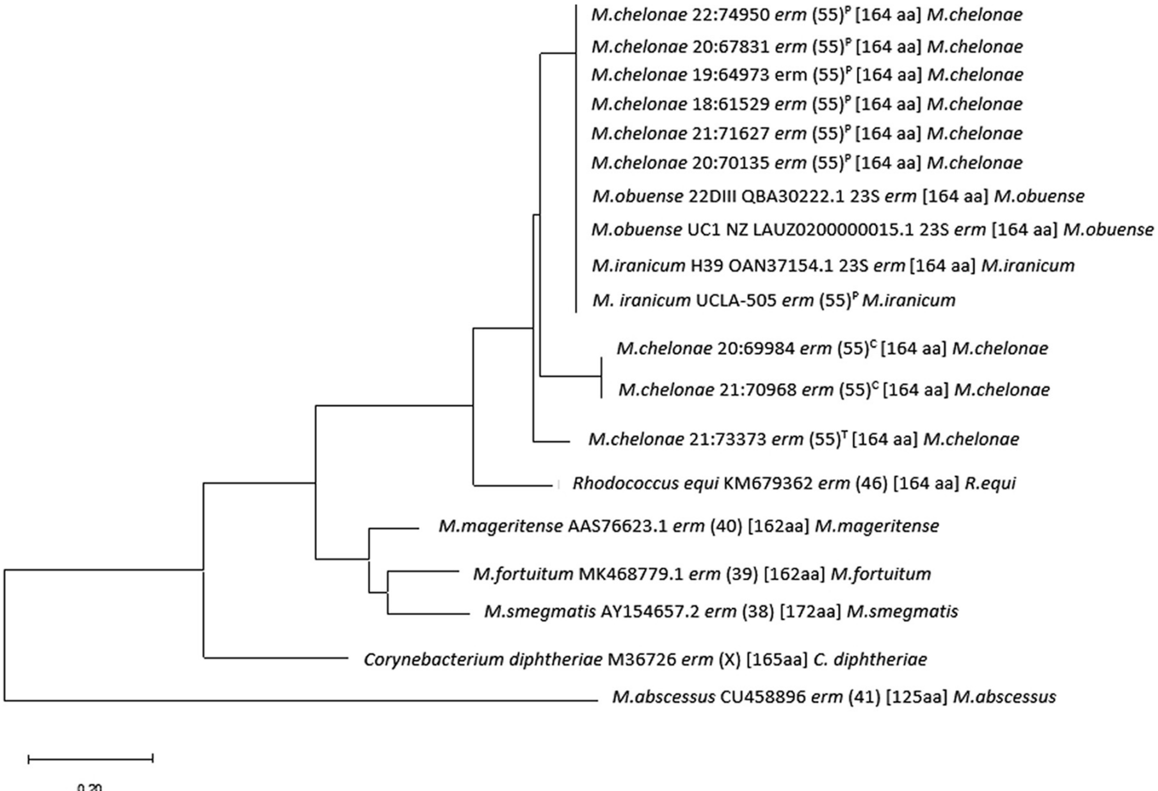


FIG 1 Neighbor-joining tree comparing the differences between the amino acid sequences of the *erm*(55)^{P}, *erm*(55)^{C}, and *erm*(55)^{T} genes to those in other mycobacterial species and notable bacterial species. The scale represents percentages, i.e., 0.20 equates to 20% difference between each sequence. *erm* genes of organisms that has <79% amino acid similarity to one another were considered different.

neighbor joining method were used for amino acids (Fig. 1). Sequences of *erm* genes from *M. fortuitum* subsp. *fortuitum*, *M. smegmatis*, *M. abscessus*, *M. mageritense*, *M. obuense*, *M. iranicum*, *Rhodococcus equi*, and *Corynebacterium diphtheriae* were used to illustrate *erm*(55) relatedness among *Mycobacterium* species and other relevant genera. GenBank numbers for reference sequences were included in the dendrogram.

Primer design and results for new *erm* gene PCR and gene sequencing. Primers for amplification of *erm*(55)^{P} were based on the sequence from *M. chelonae* strain 20:67831 designated *erm*55-F-1 (5′ CTT GAC TGA CCA ACC GAC GA 3′) and *erm*55-R-1 (5′ TGT CAT GAC CCC ACC TTT CG 3′) (Table 1). The utility of these primers for detection of *erm*(55)^{P} was evaluated by PCR-based screening of *M. chelonae* isolates with clarithromycin MICs of 4 to >16 μg/mL, including isolates with known 23S A2058 or A2059 mutational resistance to clarithromycin. Additional primers were designed to support PCR-based screening of the chromosomal *erm*(55)^{C} (primers *erm55*.2 Fa and *erm55*.2 Ra) and *erm*(55)^{T} (*erm55*.3 Fb and *erm55*.3 Rb) gene variants (Tables 1 and 2) .

Patient demographics. Information collected at the time of culture submission for patients found to be infected with *M. chelonae*, with intermediate susceptibility and high-level resistance, included the culture date, specimen source and geographic location, patient age, and type of infection if available (Table 2).

This study was approved by the Institutional Review Boards at the UTHSCT and the Mayo Clinic (Rochester, MN).

Data availability. Whole-genome sequencing data sets have been deposited in GenBank as BioProject PRJNA938130. Sequences for the three *erm*(55) gene variants have been deposited as GenBank accession numbers OQ656455, OQ656456, and OQ656457. The sequence for plasmid pMchErm55 from *M. chelonae* strain 20:67831 has been deposited as accession number CP118918.1.

RESULTS

Detection of macrolide-intermediate and macrolide-resistant isolates. A total of 133 *M. chelonae* isolates received in Laboratories A and B between November 2020 and March 2022 underwent extended, 14-day incubation. Five (3.8%) exhibited inducible, high-level resistance to clarithromycin (MIC of ≥16 μg/mL). In addition, five Laboratory A isolates obtained during January 2018 to October 2020 that had 3-day clarithromycin MICs of ≥1 μg/mL showed high-level inducible resistance with 14-day incubation. An additional six isolates were found to have intermediate clarithromycin MICs of 4 μg/mL at 14 days.

TABLE 1 Primers used for PCRs

Name[a]	Sequence	Direction	Target gene	Reference
*erm*F	5′ GAC CGG GGC CTT CGT GAT 3′	Forward	*erm*(41)	10
*erm*41-4*	5′ CCG GCC CGT AGC GTC CAA TG 3′	Reverse	*erm*(41)	10
*erm*R1	5′ GAC TTC CCC GCA CCG ATT 3′	Reverse	*erm*(41)	10
erm(55)^P-F-1	5′ CTT GAC TGA CCA ACC GAC GA 3′	Forward	*erm*(55)^P	Current study
erm(55)^P -R-1	5′ TGT CAT GAC CCC ACC TTT CG 3′	Reverse	*erm*(55)^P	Current study
erm(55)^C.2 Fa	5′ CAA CTA CCC TGT TCG CCG TA 3′	Forward	*erm*(55)^C	Current study
erm(55)^C.2 Ra	5′ CAT CGC CAA TTC CTC GAA CG 3′	Reverse	*erm*(55)^c	Current study
erm(55)^T.3 Fb	5′ CCA TCG TAG GAA ACC TGC CA 3′	Forward	*erm*(55)^T	Current study
erm(55)^T.3 Rb	5′ CGC GAG GCA AGG ATT GAT CT 3′	Reverse	*erm*(55)^T	Current study
*erm*fortF	5′ TCA CTT CTC TCG GAC CTT CC 3′	Forward	*erm*(39)	Current study
*erm*fortR	5′ CTC TAC ATC GCC TGG ACC AT 3′	Reverse	*erm*(39)	Current study
T1	5′ AAG GGT GAA GCG GAG AAT 3′	Forward	23S rRNA	7
R1	5′ TGA TTG CCG TCC AGG TT 3′	Reverse	23S rRNA	7
pA-F	5′ AGA GTT TGA TCC TGG CTC AG 3′	Forward	p16S rRNA	30
pF-R	5′ ACG AGC TGA CGA CAG CCA TG 3′	Reverse	p16S rRNA	30
pC-F*	5′ CTA CGG GAG GCA GTG GG 3′	Forward	p16S rRNA	30
pD-R*	5′ GTA TTA CCG CGG CTG 3′	Reverse	p16S rRNA	30
16S 830 F	5′-GTG TGG GTT TCC TTC CTT GG 3′	Forward	Full 16S rRNA	30
16S pH R	R-5′-AAG GAG GTG ATC CAG CCG CA-3′	Reverse	Full 16S rRNA	30

[a]Primer names followed by asterisks are those used for the BigDye reaction in Sanger sequencing that replaced the original PCR primers (36).

All *M. chelonae* isolates from Laboratories A and B were nonpigmented and grew upon subculture within 3 to 5 days at 30°C. Reference strain *M. chelonae* CIP 104535 (ATCC 35752^T) had a 14-day clarithromycin MIC of $\leq$2 μg/mL and was classified on initial testing (3 to 5 days of incubation) as clarithromycin susceptible, according to CLSI guidelines (17).

Identification of *erm*(55)^P in *M. chelonae*. Two *M. chelonae* isolates with distinct colony types were recovered from one patient. Isolate 20:68529 had a smooth colony morphology and was macrolide susceptible, whereas isolate 20:67831 had a rough granular morphology and was macrolide resistant (Table 1). Both isolates were sequenced on the Illumina MiSeq and Oxford Nanopore MinION platforms. Assembly of MinION data followed by polishing with MiSeq reads generated two complete, circular chromosomal sequences that were nearly identical in size (5,143,578 bp for 20:68529 and 5,143,566 bp for 20:67831). However, the assembly for the macrolide-resistant isolate 20:67831 genome included an additional 137,526 bp circular contig that was absent from the macrolide-susceptible isolate 20:68529 data set. Annotation of this second contig, a presumptive plasmid, revealed a putative 23S rRNA methylase gene with 72% amino acid sequence identity to *erm*(46) from *Rhodococcus* and <65% amino acid identity with the four previously described RGM *erm* genes: *erm*(38), *erm*(39), *erm*(40), and *erm*(41) (Fig. 1). The novel *erm* gene from *M. chelonae* was designated *erm*(55) by Marilyn Roberts at the Nomenclature Center for MLS Genes (https://faculty.washington.edu/marilynr/ermweb1.pdf). Here, we refer to this original allele as *erm*(55)^P, to highlight its presence on the plasmid contig, which we have named pMchErm55 (Fig. 2). The *erm*(55) sequence from *M. chelonae* strain 20:67831 was used to design oligonucleotide primers erm55-F-1 and erm55-R-1 for the *erm*(55)^P PCR assay (Fig. 3).

Description of pMchErm55. We refer to the 137-kb contig from *M. chelonae* 20:67831 as pMchErm55 because it encodes *erm*(55) as well as key features of conjugative mycobacterial plasmids, including type IV and type VII secretion system genes. A 137-kb contig with 99.9% identity to pMchErm55 is present in the genome assembly of the macrolide-resistant strain *M. chelonae* 18:61529. The plasmid is also present in other macrolide-resistant RGM species, as revealed by reference mapping of sequencing reads from *M. iranicum* strains H39 (SRA entry SRX1693165) and UCLA-505 (SRR21647431) and *M. obuense* strains UCI (GCF0009749252) and 22DIII(QBA30222.1) data sets (Table 3). Notably, a 12-kb region of pMchErm55 that encodes several integrase and recombinase genes is absent from both *M. iranicum* data sets (Fig. 2).

Identification of *erm*(55)^C and *erm*(55)^T in *M. chelonae*. Four macrolide highly resistant isolates of *M. chelonae* that tested negative with the *erm*(55)^P PCR screening were sequenced on the Illumina MiSeq platform. Contigs were assembled and annotated using

TABLE 2 Clinical and laboratory information on 10 clarithromycin-resistant (MIC, ≥16 μg/mL) PCR *erm*(55)[P]-positive isolates (one per patient) and 6 *erm*(55)-negative isolates with an intermediate clarithromycin MIC (4 μg/mL) of *Mycobacterium chelonae*[a]

Organism and patient no.	Age (yrs)	Geographic location	Strain	Culture date[b]	Source	Colony morphology	Clarithromycin MIC (μg/mL)		23S rRNA mutation	PCR result	*erm* (55)[P] aa sequence	PCR result		
							3-day	Extended (days)		*erm*(55)[P]		*erm*(55)[T]	*erm*(55)[C]	
M. chelonae, resistant[c]														
1	54	OH	18:60630	6/27/2018	Tissue, ankle wound	Smooth 7H10, rough irregular edges on TSA	>16	NA	−		−	82%	+	−
2	74	WA	18:62378	10/31/2018	Nasal septal cartilage	Rough on TSA or 7H10	1	(5), (8) 16, >16	−		+	100%	−	−
3	83	KY	19:64973	2/27/2019	Hand	Slightly smooth to smooth granular on 7H10, smooth intermediate with irregular edges on TSA	2	(6) >16	−		+	100%	−	−
4	5	MA	20:67831	3/13/2020	BAL	Smooth on TSA, rough on 7H10	4	(4), (5) 8 16	−		+	100%	−	−
4[c]	5	MA	20:68529	3/13/2020	BAL	Smooth on TSA or 7H10	0.25	(14) 1	−		−	NA	−	−
5	69	PA	MC6 22:74950	8/28/2020	Leg	Smooth on TSA or 7H10	1	>16 (×3)[d]	−		+	100%	−	−
6	71	NC	20:69984	12/03/2020	Synovial fluid	Smooth on TSA, smooth irregular edges on 7H10	>16	NA	−		−	86%	−	+
7	75	MA	21:70135	12/23/2020	Great toe pus	Smooth on TSA, smooth irregular edges on 7H10	2	(4) >16	−		+	100%	−	−
8	55	IA	21:70968	3/5/2021	Leg	Smooth on TSA, smooth irregular edges on 7H10	16	(4) >16	−		−	86%	−	+
9	49	MA	21:71627	5/06/2021	Knee biopsy	Rough on TSA or 7H10	2	(6) >16	−		+	100%	−	−
10	25	AL	21:73373	6/16/2021	Sputum	Smooth on TSA, smooth irregular edges on 7H10	>16	NA	−		−	82%	+	−
M. chelonae, intermediate[e]														
1	34	NY	21:71445	4/16/2021	Soft tissue, foot	Smooth on TSA; irregular edges on 7H10	0.5	4	NA		−	NA	−	−
2	60	MA	21:73597	9/30/2021	Sputum	Smooth irregular edges on TSA or 7H10	0.12	4	NA		−	NA	−	−
3[f]	62	TX	21:74200	11/26/2021	Skin	Smooth on TSA, smooth irregular edges on 7H10	0.25, 0.5	4, 4	NA		−	NA	−	−
4	76	NY	22:74948 MC4	2021	Sputum	Smooth on TSA7 or H10	0.5, 0.5	4, 4	NA		−	NA	−	−
5	9	PA	22:74954MC8	2021	Abdominal wound	Smooth on TSA, rough on 7H10	0.5, 0.25	8, 4	NA		−	NA	−	−
6	54	MO	22:74477	2/11/2022	Cornea	Smooth on TSA or 7H10	0.5	4	NA		−	NA	−	−

[a]The 10 resistant isolates (from 10 patients) were isolated in Laboratory A or B. Symbols and abbreviations: NA, not applicable; −, negative; +, positive; 7H10, Middlebrook 7H10 agar; TSA, trypticase soy agar; BAL, bronchoalveolar lavage; aa, amino acids.
[b]Isolates are presented in chronological order of isolation.
[c]Resistant (MIC ≥ 16 μg/mL; $n = 10$ patients); also includes one clarithromycin-susceptible isolate from mixed culture (number 4).
[d]Susceptible to clarithromycin (same patient as 20:67831).
[e]Intermediate (MIC = 4 μg/mL; $n = 6$ isolates from 6 patients).
[f]Same patient had two isolates.

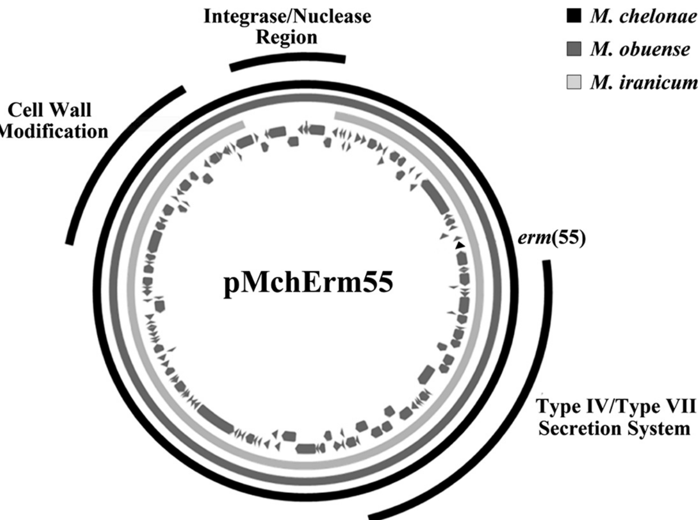


FIG 2 The 137,526-bp circular plasmid pMchErm55, encoding *erm*(55) (black triangle), is present in macrolide-resistant RGM, including *M. chelonae* strains 20:67832 and 18:61529 (black ring) and *M. obuense* strains UC1 and 22DIII (dark gray ring). In *M. iranicum* strains H39 and UCLA_505 (light gray ring), the ≈12-kb region encoding the integrase and nuclease region is absent.

the IRIDA pipeline. BLASTN analysis identified *erm*(55)-like sequences in all four assemblies (Fig. 1). The sequences from strains 20:69984 and 21:70968 were identical to each other but only 86% identical to *erm*(55)^P (Table 2 and Fig. 1). Genome comparison suggests that the *erm*(55) gene in isolates 20:69984 and 21:70968 is part of a 37-kb chromosomal insertion (Fig. 4A). To highlight the chromosomal location of this allele, we refer to it as *erm*(55)^C. The sequences from 18:60630 and 21:73373 were identical to each other but only 82%

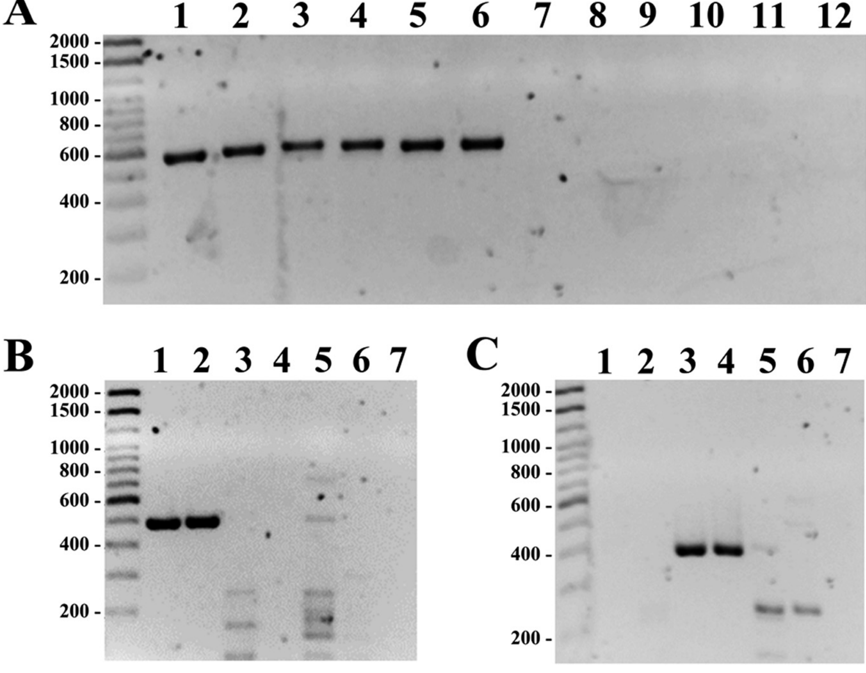


FIG 3 PCR assays targeting *erm*(55) genes. (A) PCR with the *erm*(55)^P primer set. Isolates encoding *erm*(55)^P (lanes 1 to 6) were positive, but isolates containing *erm*(55)^C (lanes 7 and 8) or *erm*(55)^T (lanes 9 and 10) were negative. Lane 11 is an isolate with no *erm*(55) gene. Lane 12 is the no-template (water) control. (B) PCR with the *erm*(55)^C primer set. Isolates encoding *erm*(55)^C (lanes 1 and 2) were positive, but isolates containing *erm*(55)^T (lanes 3 and 4) or *erm*(55)^P (lanes 5 and 6) were negative. Lane 7 is an isolate with no *erm*(55) gene. (C) PCR with the *erm*(55)^T primer set. Isolates encoding *erm*(55)^T (lanes 3 and 4) were positive, but isolates containing *erm*(55)^C (lanes 1 and 2) or *erm*(55)^P (lanes 5 and 6) were negative. Lane 7 is an isolate with no *erm*(55) gene.

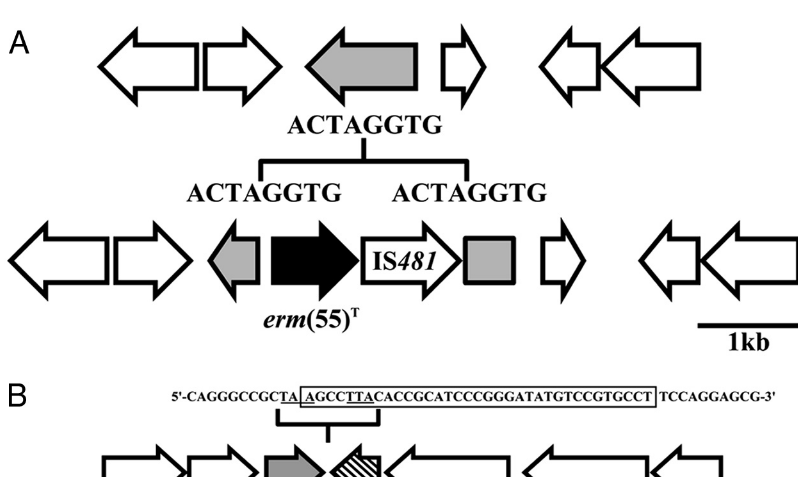
TABLE 3 Clinical and laboratory information from public databases on two isolates of *Mycobacterium iranicum* and two isolates of *Mycobacterium obuense*[a]

Organism and isolation yr	Strain	Patient age	Sequence accession no.	Geographic location	Source	Colony morphology	P16S CCUG 52297	P16S M05^T %	Clarithromyci MIC (μg/mL) 3 days	14 days	*erm*(55)^P PCR	*erm*(55)^P sequence %
M. iranicum												
2015	H39	NA	GCF_001650495.1	CA	Environ	NA	NA	100	NA	NA	NA	100
2022	UCLA-505	76	SRR21647431	CA	Blood	NA	NA	99.9	2	8 (7 days)	NA	100
M. obuense												
2008	UCI	NA	GCF_000974925.2	CA	Sputum	NA	100	NA	NA	NA	NA	100
2012	22DIII	NA	QBA30222.1	Portugal	Environ (sink)	NA	100	NA	16	NA	NA	100

[a]NA, not available; Environ, environmental.

identical to *erm*(55)^P. Genome annotation revealed that the *erm*(55) gene in 18:60630 and 21:73373 is immediately adjacent to a IS*481*-family transposase gene (Fig. 4B). This gene pair comprise a 2,029-bp insertion that disrupts a putative NADP oxidoreductase gene. The edges of the insertion are defined by 8-bp direct repeats (5′-ACTAGGTG-3′). These features are consistent with a transposon, such that we refer to this allele as *erm*(55)^T.

PCR-based screening for 23S rRNA gene mutations, the *erm*(41) gene, and *erm* (55) variants. Isolates with high-level resistance (MIC ≥ 16 μg/mL) or intermediate susceptibility (MIC = 4 μg/mL) to clarithromycin were screened by PCR. Previously described methods were used for detection of the *erm*(41) gene and mutations in the 23S rRNA (*rrl*) gene (7, 9). Novel assays were developed for detection of the *erm*(55)^P *erm*(55)^C, and *erm*(55)^T gene variants (Table 1). Verification experiments confirmed that the *erm*(55) assays were specific, with no cross-reactivity between the three variant genes. Similarly, strains that were susceptible to clarithromycin as well as strains containing *erm*(41) or 23S rRNA (*rrl*) gene mutations were negative when tested in the *erm*(55) assays.



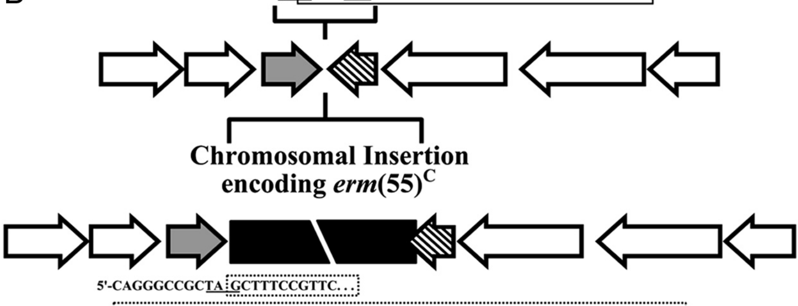


FIG 4 (A) Macrolide resistance in *M. chelonae* strain 21:73373 is associated with a ≈2-kb insertion that disrupts a NADP oxidoreductase gene (gray arrow). This putative transposon contains *erm*(55)^T (black arrow) and an IS*481* family transposase and is flanked by 8-bp direct repeats. (B) Macrolide resistance in *M. chelonae* strains 20:69984 and 21:70968 is associated with a chromosomal insertion (black box) that encodes *erm*(55)^C. The insertion replaces a 35-bp sequence (solid box) that overlaps the stop codons (underlined) of two open reading frames, an uncharacterized gene (gray arrow), and a polyketide cyclase (hatched). The 5′ end of the insertion mutates the stop codon of the uncharacterized gene (dotted box); however, this does not alter the coding sequence. In contrast, the 3′ edge of insertion does change the coding sequence of the polyketide cyclase and extends the open reading frame by 8 codons.

None of the 10 isolates from 10 patients with high-level clarithromycin resistance were PCR positive for *erm*(41) or 23S rRNA mutations, but six high-level macrolide-resistant *M. chelonae* isolates were positive for *erm*(55)^P. Genome sequencing of the four *M. chelonae* strains that were negative for *erm*(55)^P led to the discovery of the *erm*(55)^T and *erm*(55)^C gene variants (Tables 1 and 2).

The six *M. chelonae* isolates with intermediate susceptibility to clarithromycin tested negative for all PCR targets. The mechanism of elevated MICs in this intermediate susceptibility group is unknown at present.

Identification of *erm*(55)^P in *M. iranicum* and *M. obuense*. BLASTN and BLASTX analyses revealed identical matches to *erm*(55)^P in four GenBank entries: *M. iranicum* strains H39 recovered in 2015 (GCF_001650495.1) and UCLA-505, published in 2023 (SRR21647431), and *M. obuense* strains UC1 recovered in 2008 (GCF_000974925.2) and 22DIII recovered in 2012 (QBA30222.1) (Fig. 1 and 2 and Table 3).

Colony morphology. Macrolide-susceptible isolates of *M. chelonae* are typically described as smooth. Of the seven isolates of *M. chelonae* that were *erm*(55)^P positive, 3/7 were rough on both trypticase soy agar (TSA) and Middlebrook 7H10 (7H10), 2/7 were smooth on TSA with granular or rough margins on 7H10, and only one was smooth on TSA and rough on 7H10. All eight of the isolates with intermediate clarithromycin MICs were smooth on TSA; 3/8 had irregular smooth borders on 7H10, 3/8 had smooth borders on both media, and 1/8 was rough on 7H10 (Table 1). Notably, over time, the rough colony morphology was less apparent.

Demographics. The 10 *M. chelonae* patients with *erm*(55)-positive isolates were from eight states (Table 2). The isolates were from a sinus and sinus tissue (1), wounds and joints (7), sputum (1), and a bronchoalveolar lavage (1). Most (9/10) were adults and over the age of 50 years (7/10). The median age was 64 years. The dates of isolation are shown in chronological order in Table 2. Two were isolated in 2018, one in 2019, four in 2020, and three in 2021.

The six isolates with an intermediate clarithromycin MIC (4 μg/mL) were from six patients from five states (Table 2). Six were isolated in 2021 and one from 2022. Isolates were recovered from sputum (2), wounds (4), and cornea (1). One patient had two isolates from a skin wound. The median age of the patients was 60 years.

DISCUSSION

Since FDA approval of clarithromycin in 1990, macrolides have evolved as the standard of care, initially for *Mycobacterium avium* complex and subsequently for infections caused by many slowly growing mycobacteria (SGM) and RGM species, including *M. chelonae* (1–6, 18). Macrolides bind to the bacterial 23S ribosome to inhibit protein synthesis. Changes to the ribosome, due to mutation or enzyme-mediated modification (e.g., methylation), can prevent macrolide binding (7, 8). Early studies in 1996 by Wallace et al. revealed that point mutations in the A2058 and A2059 positions of the 23S rRNA gene conferred resistance to macrolides (7). This resistance is constitutive and is reliably detected by measuring MICs after 3 to 5 days of incubation (7). The subsequent discovery of inducible macrolide resistance, conferred by 23S rRNA methylase (*erm*) genes (especially *M. fortuitum* group and 80% of isolates of *M. abscessus* subsp. *abscessus*), has complicated antimicrobial susceptibility testing of RGM and compromised the usefulness of the newer macrolides, clarithromycin and azithromycin (8–10).

In contrast to *erm*(38), *erm*(39), *erm*(40), and *erm*(41) genes, which have only been found on the chromosomes of RGM, we describe here *erm*(55) gene variants located on an *M. chelonae* chromosomal insertion [*erm*(55)^C], a putative (chromosomal) transposon [*erm*(55)^T], and a putative 137-kb conjugative plasmid [*erm*(55)^P]. Antibiotic resistance due to plasmid-borne R factors has been recognized since the 1950s. However, it was not until 2014 that plasmid-mediated drug resistance was first demonstrated for mycobacteria, when plasmid pMAB01, which carries multiple resistance genes and confers resistance to kanamycin, was recovered from a strain of *M. abscessus* subsp. *massiliense* (then designated *M. bolletii*) responsible for a nationwide outbreak of surgical infections in Brazil (19). Additional studies are required to characterize the 137-kb pMchErm55 contig for *erm*(55)^P. In the current study,

annotation of the contig revealed several features of conjugative mycobacterial plasmids, including genes associated with type IV and type VII secretion systems, which appear essential for mycobacterial conjugation (20). Moreover, the presence of pMchErm55 sequences in data sets from *M. iranicum* and *M. obuense* suggest that this is a broad-host-range plasmid that can be transferred between RGM species (21). Except for *erm*(55)^{P}, pMchErm55 does not appear to contain any other antibiotic resistance genes. However, the plasmid does contain homologs to methoxymycolic acid synthase (*mma4*) and dimycocerosyl transferase (*papA5*). Cell wall modifications mediated by *mma4* or *papA5* may explain the rough colony morphology associated with *erm*(55)^{P} (i.e., pMchErm55-containing strains).

M. iranicum is a scotochromogenic (pigmented) RGM species and a recognized human pathogen first described in 2013 (22, 23). In 2017, Lymperopoulou et al. published the draft genome of *M. iranicum* strain H39, an environmental isolate collected in 2015 (24). Susceptibility testing of strain H39 was not described, but sequencing reads (SRA SRX1693165) mapped to 125 kb of pMchErm55 that includes *erm*(55)^{P}. The smaller plasmid size reflects a 12-kb region that includes recombinase and integrase genes which are missing (Table 3 and Fig. 2). In 2023, Ranson et al. reported a blood isolate of *M. iranicum* (strain UCLA-505) causing a central line infection (25). The isolate was identified by next-generation sequencing and was inducibly macrolide resistant using CLSI guidelines. Those authors were unable to detect any known *erm* gene. Subsequent sequencing reads (SRR21647431) mapped to pMchErm55 that included *erm*(55)^{P}. Interestingly, the same 12-kb plasmid fragment was missing, as in strain H39 (Fig. 2).

Mycobacterium obuense, also a scotochromogenic RGM that is a recognized human pathogen, was first characterized by Tsukamura and Mizune in 1971 (26). The first draft genome of an *M. obuense* strain (UCI), recovered in 2008 from sputum, was published in 2015 (21). The sequence of *M. obuense* strain 22DIII was reported in 2019 in a genomic study of NTM recovered from health care-associated environments in Portugal (27, 28). Strain 22DIII was the only isolate in the study that was macrolide resistant, with a clarithromycin MIC of 16 μg/mL. Although those authors noted that the strain possessed "a classical *erm* gene," additional details were not provided (27). BLAST analysis indicated that the UC1 and 22DIII strains both contained amino acid sequences identical to the translated sequence of *erm*(55)^{P}. Moreover, the draft genome of strain UC1 includes $>$83 kb of sequence with $>$99% identity to pMchErm55. This suggests that pMchErm55 is a conjugative plasmid that can be transferred between multiple RGM species. Comparison of *erm* genes and inferred amino acid sequences indicates that *erm*(55) is most closely related to *erm*(46), initially described in *Rhodococcus* species (Fig. 1) (29). The *erm*(46) gene appears to be quite promiscuous. It belongs to a mobile element that is present on a plasmid (pRErm46).

Horizontal gene transfer (HGT) in NTM has rarely been described except under laboratory conditions (30–33). A presumptive conjugative 23-kb mercury resistance plasmid, initially found in *M. marinum* strain ATCC BAA-535 (32), was also described in the genomic sequence of the type strain of *M. abscessus* subsp. *abscessus* (CIP 104536T), published by Ripoll et al. in 2009 (34). The first conjugative plasmid conferring antibiotic resistance in NTM was described by Leão et al. in 2013 (35) and further characterized by Matsumoto et al. in 2014 (19). This was a strain of *M. abscessus* (INCQS 00594) recovered in 2008 that was responsible for a nationwide epidemic of more than 2,000 surgical infections in Brazil. The 56,267-bp circular plasmid (pMAB 01) belonged to the broad-host-range Inc P-1β subgroup and contained a complete system for conjugative DNA to transfer and two genetic load regions carrying antimicrobial resistance genes. It was successfully transferred to a modified *Escherichia coli* strain but not to other NTMs. (19, 35). Characterization of the *M. chelonae* plasmid (pMchErm55) reported here is ongoing.

There are limitations to our current study. During the early part of the study period (2018 to late 2020), Laboratories A and B were following the current CLSI recommendation to not routinely perform extended (14-day) incubations for clarithromycin susceptibility testing if molecular identification was previously performed (13). As such, all strains with inducible macrolide resistance due to *erm*(55) were recovered from the subset of *M. chelonae* isolates that were incubated for 14 days; the complete collection of clinical isolates recovered

during 2018 to 2022 may also include additional isolates with *erm*(55). Although it is implied that all isolates with the *erm*(55)^P allele carry a plasmid, the current PCR-based screen only detects the *erm*(55)^P allele, not the plasmid sequence. However, of the two strains of *M. chelonae*, two strains of *M. obuense* (UCI, 22DIII) (Table 3), and two strains of *M. iranicum* (H39, UCLA-505) (Table 3) for which *erm*(55)^P-flanking region analysis is available, all had plasmid sequences. Similarly, additional experiments are required to demonstrate that the 137-kb contig really is a conjugative plasmid that can be transferred within and between RGM species. Those investigations are in progress.

Our current findings have major implications for antibiotic susceptibility testing of *M. chelonae* and the treatment of RGM infections. The discovery of *erm*(55) upends the 2018 CLSI recommendations regarding testing for inducible macrolide resistance in *M. chelonae* (13). Extended (14-day) clarithromycin susceptibility testing should be reinstated, pending CLSI evaluation and formal recommendation, at a minimum on isolates with clarithromycin MICs of $\geq$0.5 μg/mL after 3-day incubation, at least until a rapid and reliable method for detection of all three variant alleles of *erm*(55) becomes available. The *M. chelonae* isolates examined in the current study were collected during 2018 to 2022. However, the identification of *erm*(55)^P in *M. obuense* strain UC1, which was isolated in 2008, at least 10 years earlier (21), clearly indicates that plasmid-mediated macrolide resistance due to this *erm* allele is already widespread, albeit an underappreciated phenomenon among RGM.

Greater spread within *M. chelonae* strains and transmission to other macrolide-susceptible RGM pathogens, including *M. abscessus* subsp. *massiliense* and isolates of *M. abscessus* subsp. *abscessus* with a type II *erm*(41) gene (both of which are nonfunctional), will have dire consequences for antimicrobial therapy. These two subspecies of *M. abscessus* as well as *M. chelonae* are multidrug resistant, and the macrolides are the only proven effective oral antimicrobials. *M. abscessus* subspecies are also a major cause of chronic RGM lung disease, especially in the setting of cystic fibrosis and bronchiectasis. The potential spread of plasmids between RGM and SGM is highly likely, given the sequence identity of a 23-kb mercury resistance plasmid in *M. marinum* and *M. abscessus* subsp. *abscessus* (30–32, 34).

Our study is indeed a plea for newer antimicrobials which are designed to overcome these resistance mechanisms and better understanding of plasmids and their potential role in drug resistance in RGM and in NTM.

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