

Efflux Pumps Contribute to Intrinsic Clarithromycin Resistance in Clinical, *Mycobacterium abscessus* Isolates

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Purpose: The emergence of clarithromycin resistance is a challenge in treating *Mycobacterium abscessus* infections. Known mechanisms that contribute to intrinsic clarithromycin resistance focus on *rrl* gene-related mutations, but resistant clinical isolates often exhibit an inconsistent *rrl* genotype.

Patients and Methods: In this study, 194 clinical *Mycobacterium abscessus* isolates were collected from patients with lung infections and the whole genome of each isolate was sequenced. A comprehensive examination of the molecular mechanisms underlying intrinsic clarithromycin resistance was performed, combining MIC determination, comparative genome sequence analysis and qRT-PCR.

Results: Of the 194 isolates, 13 (6.7%) were clarithromycin resistant; only seven of these harbored a *rrl* 2270/2271 mutation. The remaining six resistant isolates did not exhibit a specific resistance-associated mutation in the clarithromycin target-site genes, *rmlA*, *rmlB*, *rmlC*, *rmlD* and *rmlE*, or in the *rml* modification gene *erm(41)*. qRT-PCR analysis showed that the increased expression of the efflux pump genes, MAB_2355c, MAB_1409c and MAB_1846, as well as their positive regulatory gene *whiB7*, consistently correlated with increased clarithromycin resistance. The presence of efflux pump inhibitors significantly decreased the MIC of clarithromycin for nonsusceptible isolates, especially the intrinsic resistant isolates that exhibited no *rml* 2270/2271 mutation.

Conclusion: These findings indicate that efflux pumps play a prominent role in the intrinsic resistance of *M. abscessus* to clarithromycin, complementing other known resistance mechanisms.

Keywords: *Mycobacterium abscessus*, clarithromycin resistance, efflux pumps

Introduction

Infections caused by nontuberculous mycobacteria (NTM) have increased dramatically worldwide in recent years.¹⁻⁴ *Mycobacterium abscessus* is one of the most common NTM detected. *M. abscessus* infections are difficult to manage because *M. abscessus* is intrinsically resistant to a variety of antimicrobials currently available in clinical practice; infections that are refractory to antibiotic therapy frequently result in morbidity and mortality.⁵⁻⁷ Recently, human-to-human *M. abscessus* transmission was reported, making the problem even more disconcerting.⁸

Clarithromycin (CLA), which is effective in treating *M. abscessus* lung disease, is recommended as the core agent for treatment of infections although effective therapeutic options are evolving.^{9,10} The emergence of CLA resistance, however, is

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challenging. Currently, a 2270/2271 point mutation (*Escherichia coli* 2058/2059) in the 23S rRNA (*rrl*) gene and a full-length *erm(41)* gene with thymine located at position 28 [*erm(41)*full-T28 sequevar] are the best described mechanisms conferring CLA resistance. The *erm(41)*full-T28 sequevar confers induced CLA resistance and a *rrl* 2270/2271 point mutation confers intrinsic CLA resistance in the *M. abscessus* isolates assessed.¹¹ The *rrl* mutation, however, fails to account fully for the intrinsic resistance to CLA exhibited by *M. abscessus*.^{12–15} In addition to a *rrl* 2270/2271 mutation, the expression of a variety of efflux pump genes correlates with CLA resistance in mycobacterium.^{16–24} Almost all the homologous, efflux pump genes are found in *M. abscessus*; whether these efflux pump genes are involved in the resistance of clinical *M. abscessus* isolates to CLA remains to be determined.

In this study, the MIC of CLA was determined for 194 clinical *M. abscessus* isolates collected from patients with lung diseases. A comprehensive exploration of the molecular mechanisms of CLA resistance was performed by combining comparative genome sequence analysis and qRT-PCR; the efflux pump genes were a specific focus. In addition to mutations in the CLA target-site genes, efflux pump genes MAB_2355c, MAB_1409c and MAB_1846 were found to play an important role in CLA resistance. To our knowledge, this is the first comprehensive mechanistic investigation of intrinsic CLA resistance in a large number of clinical, *M. abscessus* isolates. This work extends our understanding of the factors that affect the resistance of *M. abscessus* to CLA and suggests novel approaches to treating CLA-resistant infections.

Materials and Methods

Bacterial Isolation and Identification

Between January 2014 and December 2017, 194 *M. abscessus* isolates were collected at Shanghai Pulmonary Hospital from sputum and bronchoalveolar lavage fluid samples of patients with *M. abscessus* lung infections. All isolates were preserved in the Clinical Microbiology Laboratory. Species were preliminarily screened for NTM by MGIT960 medium culture and the p-nitrobenzoic acid test, followed by molecular identification of *M. abscessus* by sequencing the *rpoB* and *erm(41)* genes.²⁵ Each isolate was then stored at -80°C until use.

Clarithromycin Susceptibility Test

CLA susceptibility was determined by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines; the breakpoints were interpreted according to (CLSI)-M24-A2, as follows: susceptible, MIC ≤ 2 mg/L; intermediate, MIC = 4 mg/L; and resistant, MIC ≥ 8 mg/L. The intrinsic resistance of *M. abscessus* to CLA was assessed after 3 days exposure. *Mycobacterium peregrinum* (ATCC 700686; American Type Culture Collection, Manassas, VA, USA) and *Staphylococcus aureus* (ATCC 29213; American Type Culture Collection, Manassas, VA, USA) served as control reference strains.

Whole-Genome Sequencing and Comparison of Resistance Genes

One hundred ninety-four isolates were sequenced. DNA extraction, library construction and sequencing were conducted by methods previously reported by us.²⁶ The full genome sequence of each isolate has been published and is available at DDBJ/ENA/GenBank (96 sequences found under bioproject PRJNA488058, 35 under bioproject PRJNA448987 and 63 under bioproject PRJNA398137).^{26,27} Sequence homologs were identified by BLAST and aligned with the homologous sequences of the *M. abscessus* reference strain ATCC19977 (NC_010397.1). Ribosome structural genes, *rrl*, *rplC*, *rplD* and *rplV*, and *rrl* modification gene *erm(41)* were also included in this analysis.

RNA Extraction and Quantitative Reverse Transcription PCR (qRT-PCR)

Seven homolog efflux pump genes and the regulatory gene *whiB7* (MAB_3508c) correlate with macrolides resistance in mycobacteria, were selected and analyzed. Relative expression of the genes was assessed by comparing the quantity of mRNA expressed by the organism cultured in the presence and absence of CLA using the same technical approach reported previously.²⁸ A culture incubated in the presence of half its MIC of CLA was shaken at 37°C for 3 h; the RNA was then extracted according to the protocols described by Medjahed et al²⁹ cDNA was synthesized using the HiScript III RT SuperMix with gDNA wiper (Vazyme Biotech Co., Ltd). qRT-PCR was performed using ChamQ Universal SYBR Master Mix (Vazyme Biotech Co., Ltd) on a QS6 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). SigA was chosen as the endogenous reference gene. All PCR primer pairs used for amplification are shown in

[Supplementary Table 1](#). Calculation of fold change was described previously in detail.³⁰ Reactions were repeated in triplicate; genes with expression levels ≥ 4 were considered overexpressed

Efflux Pump Inhibition Assay

The MIC of CLA used in combination with efflux pump inhibitors, phenylalanine-arginine β -naphthylamide (Pa β N), carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or verapamil (VP), was determined as previously described.^{31,32} A 2-fold decrease in MIC in the presence of an inhibitor was considered significant. The efflux pump inhibition test was performed in triplicate.

Results

Clarithromycin Susceptibility and Resistance Profiles

One hundred ninety-four clinical, *M. abscessus* isolates were collected; 148 isolates belonged to subsp. *abscessus* and 46 isolates belonged to subsp. *massiliense*. The MICs of CLA ranged from 0.06 to 256 mg/L for these isolates; MIC₅₀ and MIC₉₀ at 3 days were 0.5 and 2 mg/L, respectively.

A *rrl* 2270/2271 mutation confers intrinsic resistance to CLA (MIC ≥ 8 mg/L accessed after 3 days). Accordingly, a comprehensive analysis of the distribution of this resistant genotype and resistance profile among the 194 isolates was performed. A total of 13 (6.7%, 8 subsp. *abscessus* and 5 subsp. *massiliense*) resistant isolates were found (Table 1). Among these, only 7 (53.8%) of the isolates harbored a *rrl* 2270/2271 mutation while 6 isolates with no *rrl* 2270/2271 mutation also exhibited intrinsic resistance.

Gene Comparison of *M. abscessus* Isolates

CLA target-site genes *rrl*, *rplC*, *rplD* and *rplV*, encoding the ribosome structure, were further analyzed among all 194 isolates. Besides the 2270/2271 mutation, 23 additional mutations were observed in *rrl* although none specifically

presented in CLA-susceptible or -nonsusceptible isolates (Supplementary Table 2). Moreover, no meaningful mutation in ribosome protein genes *rplC*, *rplD* and *rplV* was found in the resistant isolates. The *rrl* modification gene *erm*(41) was also analyzed; no specific sequence changes related to CLA resistance were found.

Transcriptional Analysis of Efflux Pumps

The putative efflux pump genes in *M. abscessus* were identified by literature review and analysis of homology to genes found in other *Mycobacterium* strains (Table 2). Six CLA-resistant isolates (MIC ≥ 8 mg/L) harboring a *rrl* mutation and 6 resistant isolates with no *rrl* mutation, along with 6 CLA sensitive (MIC ≤ 2 mg/L) and 6 CLA intermediate (MIC ≤ 4 mg/L) isolates were selected at random. The expression of efflux pump genes was quantified by qRT-PCR. Among the 7 putative efflux pump genes analyzed, the transcriptional levels of MAB_2355c (Figure 1A), MAB_1409c (Figure 1B) and MAB_1846 (Figure 1C), as well as their positive regulatory gene *whiB7* (Figure 1D), showed consistent increases that correlated inversely with decreases in clarithromycin susceptibility (Figure 1). No significant changes in expression of the four remaining putative efflux pump genes were observed after CLA exposure (data not shown). The efflux pump genes in the unexplained (*rrl* mutation-negative) intrinsic resistant isolates showed the highest level of expression. Among the efflux pump genes, for example, MAB_2355c exhibited >40-fold and >20-fold increases in transcription in the *rrl* mutation-negative and *rrl* mutation-positive isolates, respectively.

Efflux Pump Inhibition Assay

Previously, three highly expressed genes (MAB_2355c, MAB_1409c and MAB_1846) were found to encode efflux pumps capable of extruding CLA in mycobacteria (15–19). An efflux pump inhibition test was conducted to evaluate the role of these efflux pumps in intrinsic CLA

Table 1 MIC of Clarithromycin for 194 Clinical *M. abscessus* Isolates

<i>M. abscessus</i>	No. of Isolates	Number of Isolates Exhibiting the MIC (mg/L) of CLA Indicated													No. of Resistant Isolates (%)	Resistant Isolates Harboring a <i>rrl</i> 2270/2271 Mutation ^a
		0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256		
subsp. <i>abscessus</i>	148	16	17	10	30	34	27	6	2	1	1	2	2	0	8 (5.4)	4
subsp. <i>massiliense</i>	46	23	10	3	2	2	1	0	0	0	0	2	1	2	5 (10.9)	3

Note: ^aE. coli numbering 2058 and 2059.

Table 2 Blast Search of the *M. abscessus* Genome with Reference Gene Sequences Corresponding to Efflux Pumps Conferring or Putatively Involved in CLA Resistance

Targeted Gene	Sequence in Reference Strain (NC_010397.1) ^a	Gene Description	Reference Gene		Reference(s)
			Homolog	Strain	
MAB_2355c	2410335-1993	Putative ABC transporter ATP-binding protein	Rv1473 MSMEG_3140 MAV_3306	<i>M. tuberculosis</i> <i>M. smegmatis</i> <i>M. avium</i>	15,16
MAB_1409c	1412431-3702	Putative drug antiporter protein precursor	Rv1258c (<i>tap</i>) MAV_1406	<i>M. tuberculosis</i> <i>M. avium</i>	17,18
MAB_1846	1844817-6403	Putative ABC transporter ATP-binding protein	Rv2477c	<i>M. tuberculosis</i>	18,19
MAB_3142c	3180588-2105	Putative transmembrane efflux protein	Rv2846c (<i>efpA</i>)	<i>M. tuberculosis</i>	20,21
MAB_2807	2854903-6447	Major facilitator superfamily MFS_I	Rv1410c (<i>p55</i>)	<i>M. tuberculosis</i>	22,23
MAB_0970c	976402-8414	Probable drug resistance transporter	Rv1877	<i>M. tuberculosis</i>	20,21
MAB_1560	1587054-8727	Probable ABC transporter (macrolide-transport) ATP-binding protein	MAV_1695	<i>M. avium</i>	16

Note: ^aComplete nucleotide sequence in the *M. abscessus* genome NCBI database.

resistance exhibited by *M. abscessus*. The addition of efflux pump inhibitors PABN, CCCP and VP significantly decreased the MIC of CLA 47.4%, 52.6% and 63.2%, respectively, for the nonsusceptible isolates while there was no significant effect on the CLA sensitive isolates (Table 3). This decrease was more pronounced in resistant isolates that did not harbor a *rrl* 2270/2271 mutation. Although the level of efflux pump gene expression was significantly elevated, the inhibitory effect of efflux pump inhibitors was nearly absent in resistant isolates characterized by a *rrl* 2270/2271 mutation.

Discussion

CLA remains the core antibiotic used to treat *M. abscessus* infections. Treatment options and outcomes differ significantly among patients infected with CLA-resistant versus -sensitive *M. abscessus* genotypes.³³ In a certain proportion of clinical *M. abscessus* isolates, resistance cannot be explained by the genotype,^{12–15} alternate mechanisms exist. In this study, 194 isolates were obtained from *M. abscessus* lung infection cases in mainland China. In addition to CLA-target site mutations, efflux pumps played an important role in the intrinsic resistance of *M. abscessus* to CLA.

Almost all known mechanisms of intrinsic resistance to CLA involve changes in the CLA binding site due to

mutations in *rrl* (i.e., 2270/2271) or adjacent sites.^{11–15}

The results reported provide additional support to indicate that the *rrl* genotype correctly predicts the CLA-resistant phenotype of most (181/194, 96.9%) clinical isolates. However, 6 (3.1%) intrinsic resistant isolates in this study exhibited an inconsistent, *rrl* mutation-negative, genotype. Recently, Lipworth and colleagues identified eight potential new resistance-conferring single nucleotide polymorphisms (SNPs) present only in the *rrl* and *erm(41)* genes of antibiotic-resistant, but not sensitive, isolates.¹⁴ None of the predicted SNPs or other resistance-conferring SNPs were found in *rrl* in the current study. Reportedly, mutations in ribosomal protein genes *rplC*, *rplD* and *rplV* are also associated with structural resistance to macrolides.³⁴ Mutations in these genes, however, were absent among all the CLA-resistant isolates collected in this study.

Efflux was an anticipated factor in the drug resistance exhibited by clinical *M. abscessus* isolates collected here. Multi-drug efflux pumps directly mediate the resistance of a number of mycobacteria species to CLA.^{16–24} Reportedly, efflux inhibitors reduce the MIC of CLA for *M. abscessus*.^{31,35} However, CLA-related efflux pumps have not been systematically demonstrated and validated in clinical, *M. abscessus* isolates specifically. In the present study, three putative efflux pumps (an ATP binding

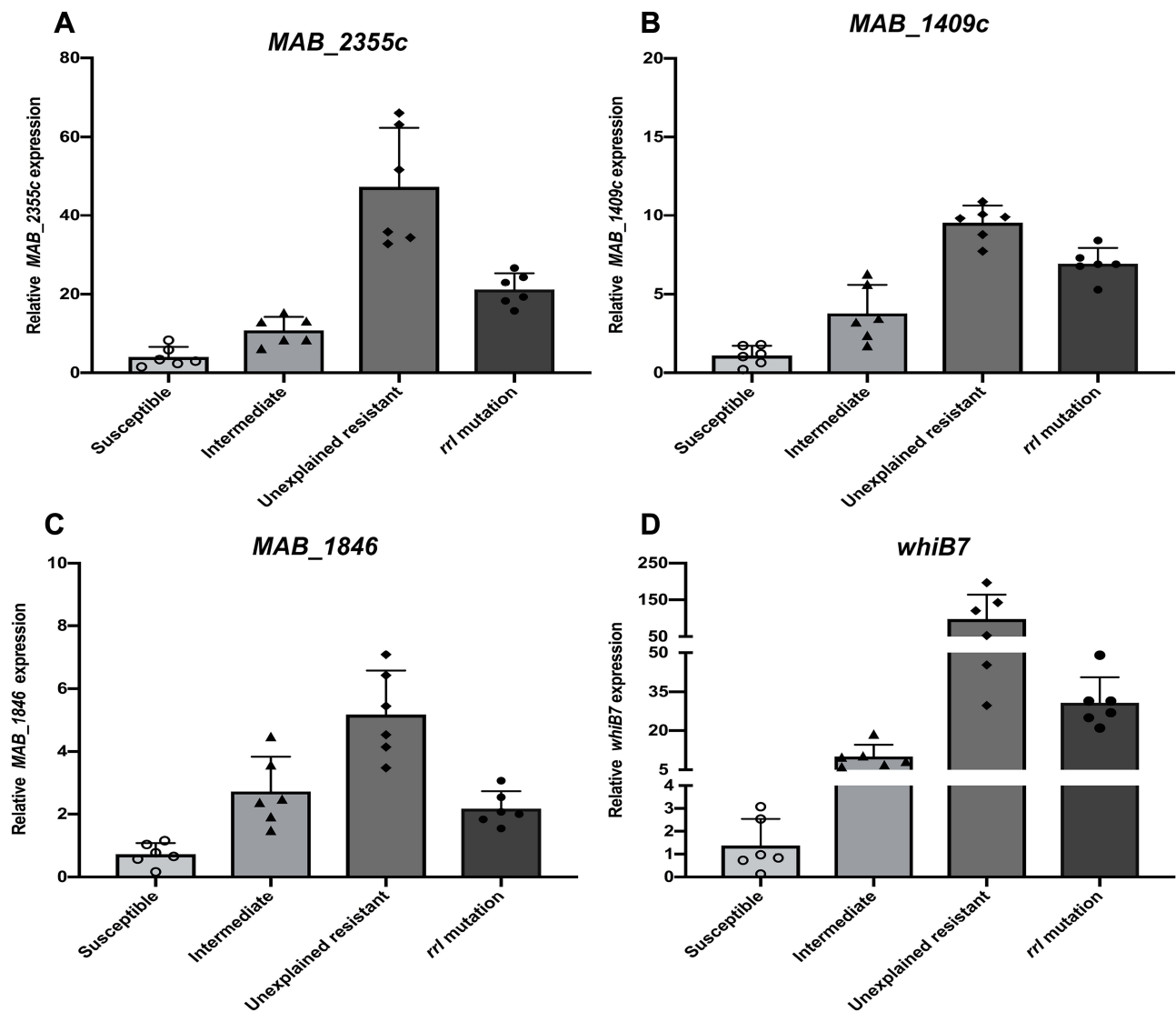


Figure 1 Efflux pump gene expression by clinical, *M. abscessus* isolates following exposure to CLA stress. Isolates that were CLA-susceptible, -intermediate and resistant [without (unexplained) or with a *rrl* 2270/2271 mutation] are shown. *MAB_2355c* (A), *MAB_1409c* (B), *MAB_1846* (C) and *whiB7* (D), message levels were quantified by qRT-PCR. Open circles, triangles, diamonds, and solid circles indicate the distribution of mRNA expression levels in isolates comprising the sensitive, intermediate, unexplained drug resistance, and *rrl* 2270/2271 mutation groups, respectively. Experiments were repeated >3 times for each isolate; error bars represent the standard error of the mean for each data set.

cassette efflux pump encoded by *MAB_2355c*, a tap like efflux pump encoded by *MAB_1409c*, and an ATP binding cassette efflux pump encoded by *MAB_1846*) contributed

to CLA resistance exhibited by *M. abscessus*. The ATP binding cassette efflux pump encoded by *MAB_2355c* was the most notable. *MAB_2355c* is a homolog of genes

Table 3 Efflux Pump Inhibition Decreases the MIC of Clarithromycin for Non-Susceptible Isolates^a

Treatment	Number (%) of Isolates Exhibiting a Significant Decrease in CLA Resistance ^b			
	Intermediate (n = 6)	Resistant with <i>rrl</i> Mutation (n = 7)	Resistant Without <i>rrl</i> Mutation (n = 6)	Total (n=19)
CLA + PaβN	4 (66.7)	0 (0)	5 (83.3)	9 (47.4)
CLA + CCCP	5 (83.3)	0 (0)	5 (83.3)	10 (52.6)
CLA + VP	5 (83.3)	1 (14.2)	6 (100)	12 (63.2)

Notes: ^aIsolates exhibiting MIC ≥ 4 mg/L after 3 days incubation were considered non-susceptible. ^bAfter the addition of the inhibitor, a decrease in MIC of ≥ 2 -fold was considered significant.

Abbreviations: PaβN, phenylalanine-arginine β -naphthylamide; CCCP, Carbonyl cyanide 3-chlorophenylhydrazone; VP, verapamil.

found in: *M. avium* (MAV_3306), *M. tuberculosis* (Rv1473) and *M. smegmatis* (MSMEG_3140). Schmalstieg and colleagues first reported that exposure to azithromycin over a 3-day period led to a 56-fold increase in expression of MAV_3306, which encodes the ATP binding cassette efflux pump in *M. avium*.¹⁷ They envisioned that this increase was the first step in a general pathway to drug resistance, which eventually leads to high-level, chromosome mutation-related resistance. Macrolide resistance mediated by the ATP binding cassette efflux pump encoded by Rv1473 was further characterized in 2019.¹⁶ Rv1258c (MAB_1409c homolog) is associated with multiple drug efflux in *M. tuberculosis*.¹⁸ We also found this efflux pump was closely related to amikacin resistance in *M. abscessus*.³⁶ Recently, Vianna and colleagues reported the association between MAB_1409c overexpression and CLA resistance in *M. abscessus*, a finding consistent with that reported herein.³⁷ In contrast to their results, however, we found no correlation between MAB_3142c and CLA resistance; presumably, sample bias contributes to this difference. Purportedly, Rv2477c (MAB_1846 homolog) was involved in mycobacterial protein translation, and resistance to tetracyclines and macrolides in *M. tuberculosis*.²⁰ This finding is consistent with our results showing MAB_1846 overexpression is associated with macrolides (CLA) resistance. Notably, MAB_2355c, MAB_1409c and MAB_1846 encode factors that belong to the intrinsic macrolides resistance network which is positively regulated by *whiB7*. *WhiB7* is a transcription factor that promotes intrinsic CLA resistance through self-activation and the activation of downstream genes involved in CLA efflux and the protection of ribosomes against antibiotic stress.¹⁶ Taken together, these findings are consistent with the results reported herein.

It is generally believed that the induction of efflux pumps is the first event leading to increased antibiotic resistance expressed by *Mycobacterium*, which usually exhibits low or moderate resistance prior to drug exposure.¹⁷ Similarly, most resistant isolates that lacked the *rrl* 2270/2271 mutation exhibited low or moderate resistance in the study reported here (ie, MIC \leq 16mg/L, [Supplementary Table 3](#)), but exhibit relative higher expression of efflux pumps than do resistant isolates with *rrl* 2270/2271 mutation. Moreover, efflux pump inhibitors exerted a greater effect on resistant isolates that lack a *rrl* 2270/2271 mutation. As such, we speculate that a change in the target-site (e.g., a *rrl* 2270/2271 mutation) is the ultimate event that culminates in a high level of

CLA resistance. Efflux pump overexpression, on the other hand, is a reversible, compensatory drug resistance mechanism making efflux pumps a potentially better target for clinical intervention. Whether efflux pump expression affects macrolide resistance induced by functioning *erm* (41) is currently unknown. Inasmuch as increases in both *erm*(41) and efflux pump expression reduce CLA sensitivity; however, we speculate that efflux pump expression can further enhance macrolide resistance dependent upon the degree of resistance induced by functional *erm*(41).

Conclusion

The present study identified three efflux pumps associated with the intrinsic resistance of *M. abscessus* to clarithromycin. Identification of these pumps adds to our current understanding of the factors that affect drug resistance, and suggests novel approaches to treating CLA-resistant *M. abscessus* infections.

Ethics Approval and Informed Consent

Ethical approval was not required because the isolates were collected for routine diagnostic testing.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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