Infection and Drug Resistance

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

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

This article was published in the following Dove Press journal:
Infection and Drug Resistance

Qi Guo[1,2]
Jianhui Chen[1,2]
Shaoyan Zhang[1]
Yuzhen Zou[1,2]
Yongjie Zhang[1,2]
Dongdong Huang[1]
Zhemin Zhang[1]
Bing Li[1]
Haiqing Chu[1,3]

[1]Department of Respiratory Medicine, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai 200433, People's Republic of China; [2]Tongji University School of Medicine, Shanghai 200092, People's Republic of China; [3]Shanghai Key Laboratory of Tuberculosis, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai 200433, People's Republic of China

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, *rrl, rplC, rplD* and *rplV*, or in the *rrl* 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 *rrl* 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

Correspondence: Haiqing Chu; Bing Li
Department of Respiratory Medicine, Shanghai Pulmonary Hospital, Tongji University School of Medicine, No. 507 Zhengmin Road, Shanghai 200433, People's Republic of China
Tel/Fax +86 021 6511 5006
Email chu_haiqing@126.com; libing044162@163.com

submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/IDR.S239850

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 al[29] 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 (PaβN), 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; MIC_{50} and MIC_{90} 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

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

Note: [a]*E. 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	*M. tuberculosis* *M. smegmatis* *M. avium*	15,16
MAB_1409c	1412431-3702	Putative drug antiporter protein precursor	Rv1258c (*tap*) MAV_1406	*M. tuberculosis* *M. avium*	17,18
MAB_1846	1844817-6403	Putative ABC transporter ATP-binding protein	Rv2477c	*M. tuberculosis*	18,19
MAB_3142c	3180588-2105	Putative transmembrane efflux protein	Rv2846c (*efpA*)	*M. tuberculosis*	20,21
MAB_2807	2854903-6447	Major facilitator superfamily MFS_1	Rv1410c (*p55*)	*M. tuberculosis*	22,23
MAB_0970c	976402-8414	Probable drug resistance transporter	Rv1877	*M. tuberculosis*	20,21
MAB_1560	1587054-8727	Probable ABC transporter (macrolide-transport) ATP-binding protein	MAV_1695	*M. avium*	16

Note: [a]Complete nucleotide sequence in the *M. abscessus* genome NCBI database.

resistance exhibited by *M. abscessus*. The addition of efflux pump inhibitors PAβN, 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

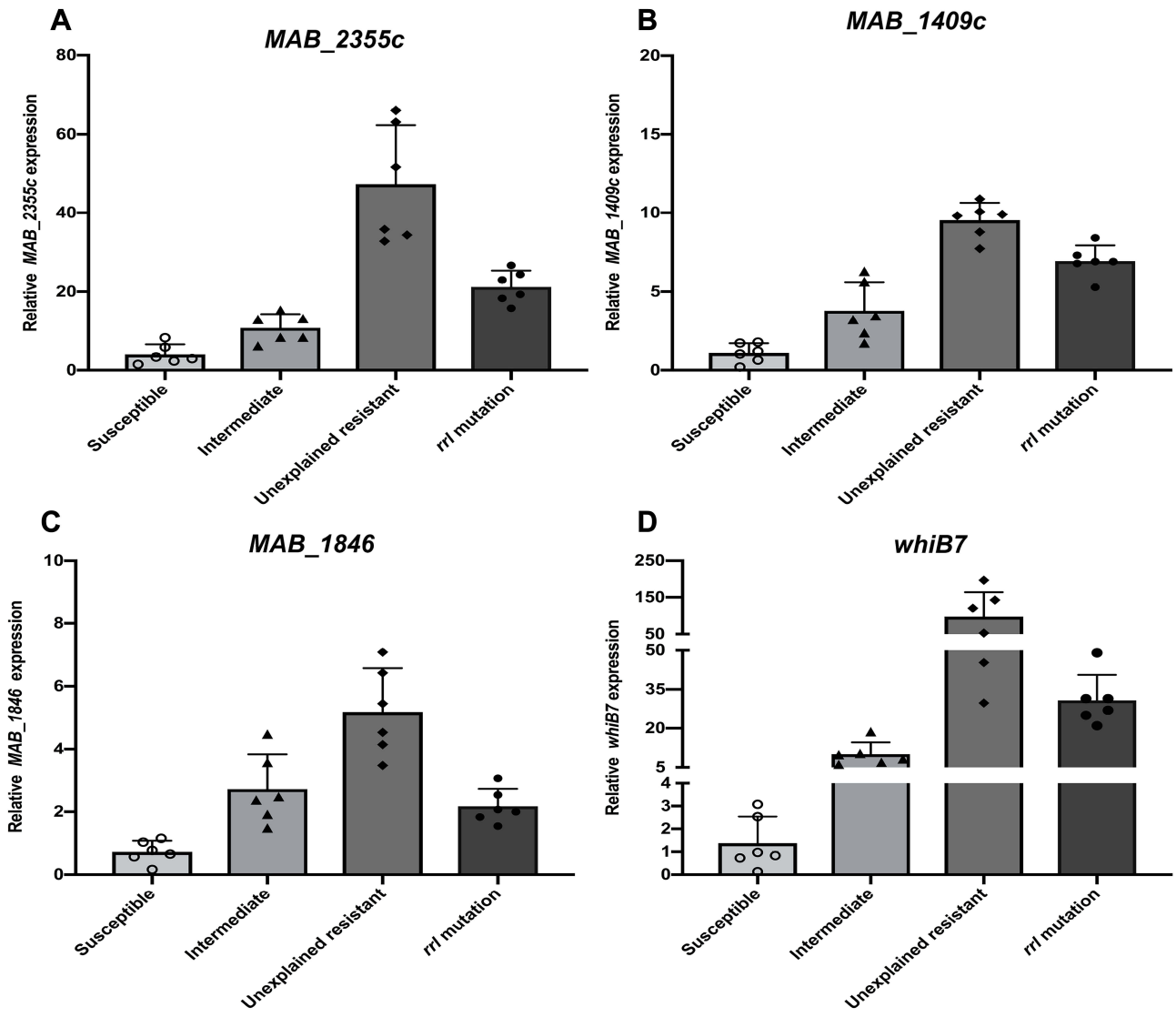

Figure 1 Efflux pump gene expression by clinical, *M. abscessus* isolates following exposure to CLA stress. Isolates that were CLA-susceptible, -intermediate and resistant [without (unexplained) or with a *rrl* 2270/2271 mutation] are shown. MAB_2355c (**A**), MAB_1409c (**B**), MAB_1846 (**C**) and *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 *rrl* Mutation (n =7)	Resistant Without *rrl* 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: [a]Isolates exhibiting MIC ≥4 mg/L after 3 days incubation were considered non-susceptible. [b]After 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 ≤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.

Acknowledgments

We sincerely thank Dr. Stephen H. Gregory (Providence, Rhodes Island, USA) who helped write and edit this manuscript.

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.

Funding

This work was funded by grants provided by the: National Natural Science Foundation of China (Nos. 81672063, 81971973 and 81800003), Natural Science Foundation of Shanghai Municipal Science and Technology Commission (Nos. 18ZR1431600 and 19ZR1442800), Medical Guide Program of Shanghai Science and Technology Committee

(Nos.18411970600 and 19411969600), New Frontier Technology Joint Project of Municipal Hospital, Shanghai Shenkang Hospital Development Center (No. SHDC12017113), Shanghai Health and Family Planning Commission Excellent Talents Training Program (No. 2018YQ55), General Project of Shanghai Health and Family Planning Commission (201940229), and Project of Top Clinical Medicine Centers and Key Disciplines Construction in Shanghai (No. 2017ZZ02012).

Disclosure

The authors report no conflicts of interest in this work.

References

1. Cowman S, van Ingen J, Griffith DE, Loebinger MR. Non-tuberculous mycobacterial pulmonary disease. *Eur Respir J*. 2019;54(1):1900250. doi:10.1183/13993003.00250-2019
2. Hoefsloot W, van Ingen J, Andrejak C, et al. The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: an NTM-NET collaborative study. *Eur Respir J*. 2013;42(6):1604–1613. doi:10.1183/09031936.00149212
3. Lin C, Russell C, Soll B, et al. Increasing prevalence of nontuberculous mycobacteria in respiratory specimens from US-affiliated Pacific Island Jurisdictions. *Emerg Infect Dis*. 2018;24(3):485–491. doi:10.3201/eid2403.171301
4. Lee H, Myung W, Koh WJ, Moon SM, Jhun BW. Epidemiology of nontuberculous mycobacterial infection, South Korea, 2007-2016. *Emerg Infect Dis*. 2019;25(3):569–572. doi:10.3201/eid2503.181597
5. Griffith DE. *Mycobacterium abscessus* and antibiotic resistance: same as it ever was. *Clinical Infectious Diseases*. 2019;69(10):1687–1689. doi:10.1093/cid/ciz071
6. Chen J, Zhao L, Mao Y, et al. Clinical efficacy and adverse effects of antibiotics used to treat *Mycobacterium abscessus* pulmonary disease. *Front Microbiol*. 2019;10:1977. doi:10.3389/fmicb.2019.01977
7. Nessar R, Cambau E, Reyrat JM, Murray A, Gicquel B. *Mycobacterium abscessus*: a new antibiotic nightmare. *J Antimicrob Chemother*. 2012;67(4):810–818. doi:10.1093/jac/dkr578
8. Bryant JM, Grogono DM, Rodriguez-Rincon D, et al. Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. *Science*. 2016;354(6313):751–757. doi:10.1126/science.aaf8156
9. Haworth CS, Banks J, Capstick T, et al. British Thoracic Society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). *Thorax*. 2017;72(Suppl2):ii1–ii64.
10. Griffith DE, Aksamit T, Brown-Elliott BA, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med*. 2007;175(4):367–416. doi:10.1164/rccm.200604-571ST
11. Wallace RJ Jr, Meier A, Brown BA, et al. Genetic basis for clarithromycin resistance among isolates of *Mycobacterium chelonae* and *Mycobacterium abscessus*. *Antimicrob Agents Chemother*. 1996;40(7):1676–1681. doi:10.1128/AAC.40.7.1676
12. Yoshida S, Tsuyuguchi K, Kobayashi T, et al. Discrepancies between the genotypes and phenotypes of clarithromycin-resistant *Mycobacterium abscessus* complex. *Int J Tuberc Lung Dis*. 2018;22(4):413. doi:10.5588/ijtld.17.0673
13. Mougari F, Bouziane F, Crockett F, et al. Selection of resistance to clarithromycin in *Mycobacterium abscessus* subspecies. *Antimicrob Agents Chemother*. 2017;61(1). doi:10.1128/AAC.00943-16
14. Lipworth S, Hough N, Leach L, et al. Whole-Genome sequencing for predicting clarithromycin resistance in *Mycobacterium abscessus*. *Antimicrob Agents Chemother*. 2019;63(1). doi:10.1128/AAC.00400-19
15. Zheng H, Liu D, Lu J, et al. Genetic correlation of antibiotic susceptibility and resistance genotyping for the *Mycobacterium abscessus* Group. *Antimicrob Agents Chemother*. 2019;63(1). doi:10.1128/AAC.00779-19
16. Duan W, Li X, Ge Y, et al. *Mycobacterium tuberculosis* Rv1473 is a novel macrolides ABC efflux pump regulated by WhiB7. *Future Microbiol*. 2019;14(1):47–59. doi:10.2217/fmb-2018-0207
17. Schmalstieg AM, Srivastava S, Belkaya S, et al. The antibiotic resistance arrow of time: efflux pump induction is a general first step in the evolution of mycobacterial drug resistance. *Antimicrob Agents Chemother*. 2012;56(9):4806–4815. doi:10.1128/AAC.05546-11
18. Sharma S, Kumar M, Sharma S, Nargotra A, Koul S, Khan IA. Piperine as an inhibitor of Rv1258c, a putative multidrug efflux pump of *Mycobacterium tuberculosis*. *J Antimicrob Chemother*. 2010;65(8):1694–1701. doi:10.1093/jac/dkq186
19. Miranda-CasoLuengo AA, Staunton PM, Dinan AM, Lohan AJ, Loftus BJ. Functional characterization of the *Mycobacterium abscessus* genome coupled with condition specific transcriptomics reveals conserved molecular strategies for host adaptation and persistence. *BMC Genomics*. 2016;17. doi:10.1186/s12864-016-2868-y
20. Daniel J, Abraham L, Martin A, Pablo X, Reyes S. Rv2477c is an antibiotic-sensitive manganese-dependent ABC-F ATPase in *Mycobacterium tuberculosis*. *Biochem Bioph Res Co*. 2018;495(1):35–40. doi:10.1016/j.bbrc.2017.10.168
21. De Rossi E, Arrigo P, Bellinzoni M, et al. The multidrug transporters belonging to major facilitator superfamily (MFS) in *Mycobacterium tuberculosis*. *Mol Med*. 2002;8(11):714–724. doi:10.1007/BF03402035
22. Li XZ, Zhang L, Nikaido H. Efflux pump-mediated intrinsic drug resistance in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother*. 2004;48(7):2415–2423. doi:10.1128/AAC.48.7.2415-2423.2004
23. Ramon-Garcia S, Martin C, Thompson CJ, Ainsa JA. Role of the *Mycobacterium tuberculosis* P55 efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. *Antimicrob Agents Chemother*. 2009;53(9):3675–3682. doi:10.1128/AAC.00550-09
24. Hohl M, Remm S, Eskandarian HA, et al. Increased drug permeability of a stiffened mycobacterial outer membrane in cells lacking MFS transporter Rv1410 and lipoprotein LprG. *Mol Microbiol*. 2019;111(5):1263–1282. doi:10.1111/mmi.2019.111.issue-5
25. Macheras E, Roux AL, Bastian S, et al. Multilocus sequence analysis and *rpoB* sequencing of *Mycobacterium abscessus* (Sensu Lato) strains. *J Clin Microbiol*. 2011;49(2):491–499. doi:10.1128/JCM.01274-10
26. Li B, Yang S, Chu H, et al. Relationship between Antibiotic susceptibility and genotype in *Mycobacterium abscessus* clinical isolates. *Front Microbiol*. 2017;8:1739. doi:10.3389/fmicb.2017.01739
27. Li B, Ye M, Guo Q, et al. Determination of MIC distribution and mechanisms of decreased susceptibility to bedaquiline among clinical isolates of *Mycobacterium abscessus*. *Antimicrob Agents Chemother*. 2018;62(7). doi:10.1128/AAC.00175-18
28. Machado D, Couto I, Perdigao J, et al. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. *PLoS One*. 2012;7:4.
29. Medjahed H, Singh AK. Genetic manipulation of *Mycobacterium abscessus*. *Curr Protoc Microbiol*. 2010;10:Unit 10D 12.
30. Viveiros M, Dupont M, Rodrigues L, et al. Antibiotic stress, genetic response and altered permeability of *E. coli*. *PLoS One*. 2007;2(4):e365. doi:10.1371/journal.pone.0000365
31. Ramis IB, Vianna JS, Silva L, et al. *In silico* and *in vitro* evaluation of tetrahydropyridine compounds as efflux inhibitors in *Mycobacterium abscessus*. *Tuberculosis*. 2019;118:101853. doi:10.1016/j.tube.2019.07.004

32. Ye MP, Xu LY, Zou YZ, et al. Molecular analysis of linezolid-resistant clinical isolates of *Mycobacterium abscessus*. *Antimicrob Agents Chemother*. 2019;63(2). doi:10.1128/AAC.00779-19
33. Guo Q, Chu HQ, Ye MP, et al. The Clarithromycin susceptibility genotype affects the treatment outcome of patients with *Mycobacterium abscessus* lung disease. *Antimicrob Agents Chemother*. 2018;62(5):e02360–17.
34. Doucet-Populaire F, Buriankova K, Weiser J, Pernodet JL. Natural and acquired macrolide resistance in mycobacteria. *Curr Drug Targets Infect Disord*. 2002;2(4):355–370.
35. Vianna JS, Ramis IB, Bierhals D, et al. Tetrahydropyridine derivative as efflux inhibitor in *Mycobacterium abscessus*. *J Glob Antimicrob Resist*. 2019;17:296–299. doi:10.1016/j.jgar.2018.12.020
36. Wu M, Li B, Guo Q, et al. Detection and molecular characterisation of amikacin-resistant *Mycobacterium abscessus* isolated from patients with pulmonary disease. *J Glob Antimicrob Resist*. 2019;19:188–191. doi:10.1016/j.jgar.2019.05.016
37. Vianna JS, Machado D, Ramis IB, et al. The contribution of efflux pumps in *Mycobacterium abscessus* complex resistance to clarithromycin. *Antibiotics*. 2019;8(3):153. doi:10.3390/antibiotics8030153