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MECHANISMS OF RESISTANCE

Novel Mutations Associated with Clofazimine Resistance in *Mycobacterium abscessus*

Yuanyuan Chen,[a] Jiazhen Chen,[b] Shuo Zhang,[c] Wanliang Shi,[c] Wenhong Zhang,[b] Min Zhu,[a] Ying Zhang[c]

[a]Tuberculosis Diagnosis and Treatment Centre, Hangzhou Red Cross Hospital, Hangzhou, Zhejiang, China

[b]Key Lab of Molecular Virology, Institute of Medical Microbiology, Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai, China

[c]Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA

ABSTRACT *Mycobacterium abscessus* is a major nontuberculous mycobacterial (NTM) pathogen and is responsible for about 80% of all pulmonary infections caused by rapidly growing mycobacteria. Clofazimine is an effective drug active against *M. abscessus*, but the mechanism of resistance to clofazimine in *M. abscessus* is unknown. To investigate the molecular basis of clofazimine resistance in *M. abscessus*, we isolated 29 *M. abscessus* mutants resistant to clofazimine and subjected them to whole-genome sequencing to identify possible mutations associated with clofazimine resistance. We found that mutations in the MAB_2299c gene (which encodes a possible transcriptional regulatory protein), MAB_1483, and MAB_0540 are most commonly associated with clofazimine resistance. In addition, mutations in MAB_0416c, MAB_4099c, MAB_2613, MAB_0409, and MAB_1426 were also associated with clofazimine resistance but less frequently. Two identical mutations which are likely to be polymorphisms unrelated to clofazimine resistance were found in MAB_4605c and MAB_4323 in 13 mutants. We conclude that mutations in MAB_2299c, MAB_1483, and MAB_0540 are the major mechanisms of clofazimine resistance in *M. abscessus*. Future studies are needed to address the role of the identified mutations in clofazimine resistance in *M. abscessus*. Our findings have implications for understanding mechanisms of resistance to clofazimine and for rapid detection of clofazimine resistance in this organism.

KEYWORDS *Mycobacterium abscessus*, clofazimine, mutations, resistance

Mycobacterium abscessus complex is a group of rapidly growing nontuberculous mycobacteria (NTM) that can cause severe human diseases, including respiratory, skin, and soft tissue disorders, particularly in cystic fibrosis and elderly patients (1–3). *M. abscessus* is a major NTM pathogen and is responsible for about 80% of all pulmonary infections caused by rapidly growing NTM. *M. abscessus* complex is notoriously resistant to standard antituberculous agents and most antimicrobial agents (1–3). It is also resistant to disinfectants and, therefore, can cause postsurgical and postprocedural infections (3, 4). *M. abscessus* has been classified into three subspecies on the basis of *rpoB* sequences: *Mycobacterium abscessus* subsp. *abscessus* (*sensu stricto*), *Mycobacterium abscessus* subsp. *massiliense*, and *Mycobacterium abscessus* subsp. *bolletii* (1–3). They have different susceptibilities to antimicrobial agents, and *M. abscessus* subsp. *abscessus* is the most prevalent and resistant and is called a "nightmare" bacterium (3). The treatment regimen for *M. abscessus* disease is a combination of amikacin, cefocetin, clarithromycin, and imipenem for the initial phase and oral antimicrobials for the continuation phase, for a total duration of 12 to 24 months (2). Even with such a complex regimen and long duration, the outcome of *M. abscessus* disease is still poor. It has been reported that up to 67% of patients' treatment failed (5).

Clofazimine (CFZ), a drug that is currently used for leprosy and multidrug-resistant

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Address correspondence to Ying Zhang, yzhang@jhsph.edu.

Y.C. and J.C. contributed equally to this article.

tuberculosis (MDR-TB) treatment (6), is also active against *M. abscessus* and shows synergistic activity when given with amikacin (7–10). Although CFZ has been shown to have therapeutic efficacy in animal models and in patients (11), it is not a front-line agent or routinely used to treat *M. abscessus* disease. Due to limited treatment options and poor treatment outcomes, CFZ has been increasingly used to treat *M. abscessus* disease in recent years (11, 12). Therefore, resistance to CFZ is expected to arise, but so far, no information on CFZ resistance in patients is available. In addition, intracellular redox cycling and membrane destabilization and dysfunction could be related to clofazimine-mediated antimicrobial activity (6), but its mechanism of action in *M. abscessus* remains to be established. Furthermore, the mechanism of resistance to clofazimine in *M. abscessus* is unknown.

To better understand the mechanism of clofazimine resistance and to develop more rapid molecular tests for detection of resistance, we characterized 29 clofazimine-resistant mutants isolated *in vitro* from *M. abscessus* ATCC 19977. We found several new genes that are distinct from the known resistance mechanisms in *Mycobacterium tuberculosis* (13) that are associated with clofazimine resistance in *M. abscessus*.

RESULTS

Clofazimine MIC for *M. abscessus* ATCC 19977. The MIC of clofazimine for the *M. abscessus* strain ATCC 19977 was determined as described in Materials and Methods. After 3 days of incubation, it was found that *M. abscessus* ATCC 19977 grew on plates containing 0.5 and 1 μg/ml of clofazimine but did not grow on plates containing 2, 4, and 8 μg/ml of clofazimine, indicating that the MIC of clofazimine was 2 μg/ml.

Isolation of *M. abscessus* mutants resistant to clofazimine. Approximately 2×10^7 cells of *M. abscessus* ATCC 19977 were spread on plates containing different concentrations of clofazimine for mutant isolation. After 5 days of incubation, 29 mutants were obtained on the plates containing 8 μg/ml of clofazimine. Based on the number of cells plated and the number of mutants obtained, the mutation frequency of resistant mutants in response to 8 μg/ml of clofazimine was about 5×10^{-6}. We then determined the MICs for all the mutants on 7H11 agar plates containing 2, 4, 8, 16, and 32 μg/ml of clofazimine with wild-type *M. abscessus* ATCC 19977 as a drug-susceptible control strain. Wild-type *M. abscessus* ATCC 19977 did not grow on plates containing 2, 4, 8, 16, or 32 μg/ml of clofazimine, while the clofazimine-resistant mutants grew on all clofazimine-containing plates, indicating that the MICs of clofazimine for these mutants were greater than 32 μg/ml, while wild-type *M. abscessus* grew only on the no-drug control plate and not on clofazimine-containing plates.

Whole-genome sequencing identified novel genes associated with clofazimine resistance in *M. abscessus*. To identify possible new mechanisms of clofazimine resistance, we subjected the 29 clofazimine-resistant mutants to whole-genome sequencing. Comparative genome sequence analyses revealed that 23 of 29 CFZ-resistant mutants had mutations in MAB_2299c, encoding the transcriptional repressor (Table 1). It is interesting that most (18 of 23) of the mutations in MAB_2299c are loss-of-function mutations due to indels or stop codons, with only 5 single nucleotide variations (SNVs) that caused amino acid substitutions (see Table 1). A search in a mycobacterial database revealed that there is a homolog of MAB_2299c in *M. tuberculosis* H37Rv, named Rv0452. There is 66.96% identity between MAB_2299c and Rv0452 at the protein level, but there is no significant homology between MAB_2299c and Rv0678 (only 14.54% identity). In fact, there is no homolog of Rv0678 in *M. abscessus*. Instead, there are two pairs of *mmpL* and *mmpS* genes downstream of MAB_2299c (Fig. 1). It is likely that the two sets of the membrane protein genes (MAB_2300 to MAB_2303) are regulated by the adjacent putative repressor MAB_2299c. MAB_2300 and MAB_2302 are predicted to encode putative MmpS with 48.12% and 41.35% and with 41.08% and 39.53% identities to MmpS4 and MmpS5 in *M. tuberculosis*, respectively. MAB_2301 and MAB_2303 are predicted to encode putative MmpL with 61.63% and 60.89% and with 54.91% and 52.59% identities to *M. tuberculosis* MmpL4 and MmpL5, respectively.

TABLE 1 Mutations identified from 29 clofazimine-resistant *M. abscessus* mutants by whole-genome sequencing[a]

Gene	Nucleotide mutation	Amino acid change	Mutation type	Gene product	Strain(s)
MAB_2299c	Ins of GGC (132)	Ins at position 44	Insertion	Possible transcriptional regulatory protein	N1, N5, N6, N12
	Ins of G (276)	FSC92	Insertion	Possible transcriptional regulatory protein	N2
	Del of G (322)	FSC108	Deletion	Possible transcriptional regulatory protein	N3, N8
	Ins of TT (344)	FSC115	Insertion	Possible transcriptional regulatory protein	N4, N7
	Ins of G (223)	FSC75	Insertion	Possible transcriptional regulatory protein	N10
	Del of C (215)	FSC72	Deletion	Possible transcriptional regulatory protein	N16
	C63A	E21STOP	SNV	Possible transcriptional regulatory protein	M19
	T518C	H173R	SNV	Possible transcriptional regulatory protein	M1, M2, M7
	C642A	A214S	SNV	Possible transcriptional regulatory protein	M8
	Ins of G (391)	FSC131	Deletion	Possible transcriptional regulatory protein	M3
	Ins of G (345)	FSC115	Insertion	Possible transcriptional regulatory protein	M11, M23, M29
	Ins of A (494)	FSC165	Insertion	Possible transcriptional regulatory protein	M14
	G19A	Q7STOP	Deletion	Possible transcriptional regulatory protein	N11
	C329T	C110Y	SNV	Possible transcriptional regulatory protein	N14
MAB_1483	Del of G (708)	FSC236	Deletion	Hypothetical protein	N1–N16
	G363A	W121STOP	SNV	Hypothetical protein	M1
	Del of TACCG (489)	FSC163	Deletion	Hypothetical protein	M84
	Ins of G (316)	FSC106	Insertion	Hypothetical protein	M29
	Ins of T (104)	FSC35	Insertion	Hypothetical protein	M11
	Del of A (343)	FSC115	Deletion	Hypothetical protein	M19
GlpK	Ins of GGC (3′ + 28 bp)		3′ intergenic insertion	Glycerol kinase	N11
MAB_0540	Del of G (−45)		5′ intergenic deletion	Hypothetical protein	N1–N16
MAB_0416c	C554T	A185T	SNV	Putative Crp/Fnr family transcriptional regulator	M65
MAB_4099c	T9776C	Y3259C	SNV	Probable nonribosomal peptide synthetase	M11
MAB_2613	T310C	V104A	SNV	Putative glucose/mannose:H^+ symporter GlcP	M7
MAB_0409	T(−88)G		5′ SNV	Putative transcriptional regulator WhiB	N13
MAB_1426	A649C	K217Q	SNV	Putative cytochrome P450	N3, N10
MAB_4605c	G122A	A41V	SNV	Probable aldehyde dehydrogenase	M1–M3, M7, M8, M11, M14, M19, M23, M29, M34, M65, M84
MAB_4323	Ins of C (1493)	FSC498	Insertion	Putative amino acid transporter	M1–M3, M7, M8, M11, M14, M19, M23, M29, M34, M65, M84

[a]Ins, insertion; Del, deletion; FSC, frame shift codon; STOP, stop codon; SNV, single nucleotide variation.

We also found 6 mutations in MAB_1483 in 20 mutants (N1 to N14, N16, M1, M11, M19, M29, and M84), including 5 indels causing frameshift mutations and 1 SNV that results in stop codons (Table 1). The other mutant, M65, had a C554T mutation (causing the amino acid change A185T) in the MAB_0416c gene, which encodes a putative

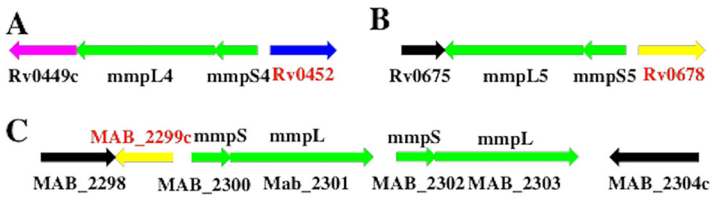

FIG 1 Genomic organization of membrane transport proteins in *M. tuberculosis* H37Rv (A and B) and *M. abscessus* ATCC 19977 (C). MAB_2299c is a homolog of Rv0452, which is a transcriptional repressor of the transmembrane MmpS4-MmpL4 efflux pump in *M. tuberculosis* (A). Rv0452 has a genomic organization similar to that of Rv0678, which is known to be a transcription repressor of the MmpS5-MmpL5 efflux pump (B) involved in clofazimine resistance in *M. tuberculosis*. The similarity of genomic structure of MAB_2299c and its adjacent two sets of *mmpS* and *mmpL* genes in *M. abscessus* (C) to Rv0452 and its adjacent MmpS4-MmpL4 efflux pump genes suggests that mutations in the putative repressor MAB_2299c may have a mechanism of CFZ resistance (by turning on the nearby efflux pump genes) similar to that in *M. tuberculosis*.

Crp/Fnr family transcriptional regulator (Table 1). In addition, two other mutants with mutations in MAB_4099c (encoding probable nonribosomal peptide synthetase) and MAB_2613 (encoding putative glucose/mannose:H^+ symporter GlcP) were identified, one having a T9776C mutation causing a Y3259C amino acid change and one having a T310C mutation causing a V104A amino acid change (Table 1).

Whole-genome sequencing indicated that a G122A mutation in MAB_4605c (encoding probable aldehyde dehydrogenase) and a C1493 insertion in MAB_4323 (encoding an amino acid transporter) were the most common mutations in 13 clofazimine-resistant mutants (Table 1). To confirm these two mutations to be genuine, PCR using MAB_4605c and MAB_4323 primers was performed on six individual mutants (M11, M23, M29, M34, M65, and M84) and a clofazimine-susceptible control strain (ATCC 19977), and Sanger sequencing of the PCR products indicated that the two mutations in the 6 mutants were correct.

DISCUSSION

Although clofazimine was discovered in 1957 as a riminophenazine drug for treating TB (14), it is mainly used for the treatment of leprosy (15) and also as a core second-line agent for the treatment of multidrug-resistant TB (MDR-TB) (16). In *M. tuberculosis*, the mechanisms of resistance to clofazimine are due mainly to mutations in the transcriptional repressor Rv0678, with concomitant upregulation of the efflux pump, MmpL5, and occasionally in Rv1979c (a permease) or Rv2535c (PepQ) (13, 17). However, the mechanisms of action and resistance of clofazimine in *M. abscessus* have not been reported so far. In this study, we isolated *M. abscessus* mutants resistant to clofazimine and found several genes (MAB_2299c, MAB_1483, MAB_0540, MAB_0416c, MAB_4099c, MAB_2613, MAB_0409, and MAB_1426) whose mutations are associated with clofazimine resistance through comparative genome sequence analyses.

It is worth noting that 23 of the 29 mutants had indels or SNVs in the MAB_2299c gene, encoding possible transcriptional regulatory protein. MAB_2299c is a homolog of *M. tuberculosis* Rv0452, a gene for a putative AcrR family transcription repressor of efflux genes *mmpL4* and *mmpS4* (Fig. 1). Rv0452 and *mmpL4-mmpS4* are organized in the same genomic structure as Rv0678 and *mmpL5-mmpS5 in M. tuberculosis* (Fig. 1). Therefore, mutations in the putative transcription repressor MAB_2299c in *M. abscessus* could be analogous to mutations in Rv0678 in *M. tuberculosis* in causing clofazimine resistance. Further studies are required to confirm this.

Twenty-one of the 29 mutants had loss-of-function mutations in MAB_1483, encoding a hypothetical protein with no significant homologs in *M. tuberculosis*. Sixteen of 29 mutants had a −45 G deletion in the upstream of MAB_0540, but its function is unknown. The role and function of MAB_1483 and MAB_0540 need to be explored with regard to clofazimine resistance. MAB_0416c encodes a putative Crp/Fnr family transcriptional regulator, and mutation in its homolog Rv3676 caused growth defect and is involved virulence attenuation in *M. tuberculosis* (18). MAB_0409 encodes putative transcriptional regulator WhiB, and its promoter region −88 mutation may affect the transcription of the WhiB homolog and lead to clofazimine resistance, though the mechanisms involved remain to be identified. MAB_4099c, encoding probable nonribosomal peptide synthetase, and MAB_2613, encoding putative glucose/mannose:H^+ symporter GlcP, are less likely causal in clofazimine-resistant *M. abscessus*. Although we found a G122A mutation in MAB_4605c (encoding probable aldehyde dehydrogenase) and a C1493 insertion in MAB_4323 (encoding an amino acid transporter) in all 13 clofazimine-resistant mutants (Table 1), we believe that these SNVs may not have a direct role in causing clofazimine resistance, as they are present in only one batch of clofazimine-resistant mutants (the M series) and not in the second batch of mutants (the N series).

Despite numerous studies, the mechanism of action of clofazimine has remained elusive. The drug is extremely hydrophobic, suggesting that its mode of action is closely associated with its effect on membranes (6). Clofazimine reduction by mycobacteria was noted in the first study (14) on the drug, which is supposed to react with molecular

O_2 to form reactive oxygen species (ROS), raising speculation that clofazimine antimycobacterial activity was related to the intracellular redox activity of the dye (6). Yano et al. proposed that clofazimine competes with the NDH-2 substrate menaquinone, which accepts electrons/H donated by NADH and delivered them to the electron transport chain (ETC), which, in turn, uses the electrons/H to reduce O_2, and that NDH-2 catalyzes clofazimine to a reduced form oxidized by molecular oxygen forming superoxide and H_2O_2 (19). However, in this study, the mutations we identified in *M. abscessus* (Table 1) do not map to redox or the ETC, which is similar to the situation in *M. tuberculosis*. Thus, the mutation approach may not always be useful for identifying drug targets needed for understanding the mode of action of the drug.

Despite our significant finding of several new candidate genes associated with clofazimine resistance in *M. abscessus*, their role in causing the resistance remains to be determined. However, genetic manipulation of *M. abscessus* has not been possible so far, and once the genetic tool is available, it would be of interest to determine if deletion or overexpression of the identified candidate genes could cause resistance to clofazimine in future studies.

In summary, we identified several new genes whose mutations are associated with clofazimine resistance in *M. abscessus*. Mutations in MAB_2299c, MAB_1483, and MAB_0540 seem to be major mechanisms of clofazimine resistance in *M. abscessus*. Mutations in MAB_2299c (encoding the transcriptional repressor of efflux pump) are analogous to Rv0678 mutations in causing clofazimine resistance in *M. tuberculosis*. Other new genes involved in clofazimine resistance in *M. abscessus* seem to affect transcription or transport functions. Our findings offer new insights about the mechanisms of resistance to clofazimine in *M. abscessus*. Identification of MAB_2299c, MAB_1483, and MAB_0540 mutations in most clofazimine-resistant mutants has implications for rapid molecular detection of clofazimine-resistant *M. abscessus*. Future studies are needed to assess the role of the identified mutations as new mechanisms of clofazimine resistance in *M. abscessus*.

MATERIALS AND METHODS

Clofazimine susceptibility testing and isolation of *M. abscessus* mutants resistant to clofazimine. *M. abscessus* ATCC 19977 was grown in 7H9 liquid medium (Difco) supplemented with 0.05% Tween 80 and 10% bovine serum albumin-dextrose-catalase (ADC) enrichment at 37°C for 3 days (exponential phase) and was diluted 100 times. Clofazimine (Sigma-Aldrich Co.) was dissolved in dimethyl sulfoxide (DMSO; Difco) at a stock concentration of 5 mg/ml and incorporated into 7H11 agar plates containing ADC supplement at appropriate concentrations. The MIC of clofazimine for *M. abscessus* ATCC 19977 was determined by the agar dilution method on 7H11 agar plates containing 0.5, 1, 2, 4, and 8 μg/ml of clofazimine. A log-phase ATCC 19977 culture grown in 7H9 medium was diluted 1,000 times, and 10-μl inocula (approximately 2×10^4 CFU) were added to plates containing different concentrations of clofazimine and also on 7H11 agar plate as a control. After 3 days of incubation at 37°C, the MIC was determined. For mutant isolation, aliquots of diluted ATCC 19977 (1:100 dilution) were spread on clofazimine-containing plates and also on 7H11 agar plates to confirm the CFU count. Mutants that grew on the plates containing 8 μg/ml of clofazimine after 5 days of incubation at 37°C were picked and grown in 7H9 liquid medium for confirming clofazimine resistance. The clofazimine susceptibility testing of the clofazimine-resistant mutants was performed on 7H11 agar plates containing 2, 4, 8, 16, and 32 μg/ml of clofazimine.

Whole-genome sequencing. Genomic DNA was isolated from bacterial cultures (3 ml) as described previously (20). Briefly, the bacterial cells were heat killed by incubating them at 80°C for 20 min followed by glass bead (diameter, 0.1 mm; Sigma) disruption by vortexing at high speed for 5 min. Bacterial lysates were extracted with phenol-chloroform-isoamyl alcohol (25:24:1). Genomic DNA was precipitated with 2 volumes of absolute alcohol, collected by centrifugation, washed with 70% alcohol, and then air dried. The genomic DNA samples from 13 clofazimine-resistant mutants were subjected to whole-genome sequencing using an Illumina HiSeq 2000 machine. Paired-end sequencing libraries for genomic DNA of each strain were barcoded and constructed with insert sizes of approximately 300 bp using TruSeq DNA sample preparation kits (Illumina, USA) according to the manufacturer's instructions. For each strain, 1.0 to 1.5 Gb (200-fold to 300-fold genome coverage) were generated after barcodes were trimmed. High-quality data were aligned with the reference sequence of *M. abscessus* subsp. *abscessus* ATCC 19977 (NC_010397.1) using SOAPaligner. Only reads for which both ends aligned to the reference sequence were used for single nucleotide variant (SNV) and insertion and deletion (indel) analysis. SNVs and indels ranging from 1 to 5 bp were sorted and called at minimum reads of 10.

PCR and DNA sequencing. The genomic DNA from clofazimine-resistant mutants of *M. abscessus* isolated *in vitro* was subjected to PCR amplification using MAB_4323 primers MAB_4323F (5′-CTATCCG AGCACACGCTTACC-3′; 331 bp before indel) and MAB_4323R (5′-GCGATGTCAATGAGGAGGAG-3′; 304 bp after indel). Qiagen HotStarTaq DNA polymerase was applied for PCR amplification with parameters as

follows: heat denaturation at 95°C for 15 min followed by 30 cycles of 94°C for 1 min, 51.5°C for 1 min, and 72°C for 1 min and then extension at 72°C for 10 min. The MAB_4605c gene was PCR amplified using primers MAB_4605cF (5′-GACAACACCGTCCGCCATT-3′; 349 bp before SNV) and MAB_4605cR (5′-GATC GGCTGGTCGGATACCT-3′; 363 bp after SNV). The MAB_4605c PCR products were obtained using the following parameters for PCR amplification: heat denaturation at 95°C for 15 min followed by 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min and then extension at 72°C for 10 min. All the PCR products were purified according to the instructions for the QIAquick PCR purification kit and then sequenced by Macrogen USA Corp. to confirm the mutations in those genes in selected mutants.

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