Arabinosyltransferase C Mediates Multiple Drugs Intrinsic Resistance by Altering Cell Envelope Permeability in *Mycobacterium abscessus*

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ABSTRACT *Mycobacterium abscessus* is an emerging human pathogen leading to significant morbidity and even mortality, intrinsically resistant to almost all the antibiotics available and so can be a nightmare. Mechanisms of its intrinsic resistance remain not fully understood. Here, we selected and confirmed an *M. abscessus* transposon mutant that is hypersensitive to multiple drugs including rifampin, rifabutin, vancomycin, clofazimine, linezolid, imipenem, levofloxacin, cefoxitin, and clarithromycin. The gene *MAB_0189c* encoding a putative arabinosyltransferase C was found to be disrupted, using a newly developed highly-efficient strategy combining next-generation sequencing and multiple PCR. Furthermore, selectable marker-free deletion of *MAB_0189c* recapitulated the hypersensitive phenotype. Disruption of *MAB_0189c* resulted in an inability to synthesize lipoarabinomannan and markedly enhanced its cell envelope permeability. Complementing *MAB_0189c* or *M. tuberculosis embC* restored the resistance phenotype. Importantly, treatment of *M. abscessus* with ethambutol, a first-line antituberculosis drug targeting arabinosyltransferases of *M. tuberculosis*, largely sensitized *M. abscessus* to multiple antibiotics in vitro. We finally tested activities of six selected drugs using a murine model of sustained *M. abscessus* infection and found that linezolid, rifabutin, and imipenem were active against the *MAB_0189c* deletion strain. These results identified *MAB_0189c* as a crucial determinant of intrinsic resistance of *M. abscessus*, and optimizing inhibitors targeting *MAB_0189* might be a strategy to disarm the intrinsic multiple antibiotic resistance of *M. abscessus*.

IMPORTANCE *Mycobacterium abscessus* is intrinsically resistant to most antibiotics, and treatment of its infections is highly challenging. The mechanisms of its intrinsic resistance remain not fully understood. Here we found a transposon mutant hypersensitive to a variety of drugs and identified the transposon inserted into the *MAB_0189c* (orthologous *embC* coding arabinosyltransferase, EmbC) gene by using a newly developed rapid
and efficient approach. We further verified that the MAB_0189c gene played a significant role in its intrinsic resistance by decreasing the cell envelope permeability through affecting the production of lipoarabinomannan in its cell envelope. Lastly, we found the arabinosyltransferases inhibitor, ethambutol, increased activities of nine selected drugs in vitro. Knockout of MAB_0189c made M. abscessus become susceptible to 3 drugs in mice. These findings indicated that potential powerful M. abscessus EmbC inhibitor might be used to reverse the intrinsic resistance of M. abscessus to multiple drugs.

**KEYWORDS** Mycobacterium abscessus, intrinsic resistance, MAB_0189c, permeability

*Mycobacterium abscessus* is a rapidly-growing environmental mycobacterium causing local or disseminated lung, skin, and soft tissue infections in patients with underlying conditions such as cystic fibrosis and chronic obstructive pulmonary diseases (1). It is considered as an “incurable nightmare” due to its high-level intrinsic resistance to most antibiotics commonly used for bacterial infections, in particular, including nearly all antituberculosis drugs (2). Current therapeutic options, as recommended by the Infectious Disease Society of America, comprise the combination of amikacin (AMK), ceftoxitin (CEF), imipenem (IMP), and macrolides (3). However, partly due to the lack of appropriate treatment options, the average treatment success rate is only ~ 58% even with very long treatment duration (4). Thus, efforts to identify new therapeutic regimens should be emphasized.

Intrinsic resistance of *M. abscessus* to antibiotics is mediated by multiple factors. First, *M. abscessus* is known to produce numerous enzymes that can modify either the drug targets or the drugs themselves, leading to reduced antimicrobial effects. This is exemplified by the expression of ribosomal methylase encoded by *erm*(41) leading to macrolide resistance, rifampin (RIF)-inactivating ADP-ribosyltransferase, and β-lactam-hydrolyzing β-lactamase BlaMab (5–7). Second, genetic polymorphisms also play key roles in determining intrinsic resistance of *M. abscessus*. For instance, polymorphism in *atpE* confers resistance of *M. abscessus* to bedaquiline, a recently approved drug with high potency against *M. tuberculosis* (8). In addition to these mechanisms, the relatively low permeability of the cell envelope coupled with the presence of multidrug export systems contributes to the intrinsic antibiotic resistance by limiting intracellular concentrations of drugs (2, 9, 10). A recent study even demonstrated that enhanced cell envelope permeability resulting from compromised glycosylation of a lipoprotein renders *M. abscessus* more sensitive to β-lactams, vancomycin (VAN), and RIF (10).

A well-versed understanding of the genetic basis of intrinsic antibiotic resistance is prerequisite for the discovery and development of synergistic drug combinations, where one agent can restore the activity of a failing antibiotic by disrupting one of the above-mentioned mechanisms. A relatively good example in this regard is the identification of avibactam, a potent inhibitor of *M. abscessus* β-lactamase, to efficiently facilitate the activities of several β-lactams by 4- to 32-fold when used in combination against *M. abscessus* (11, 12).

Forward genetics approaches such as transposons (Tn) mutagenesis have been widely applied to generate random insertion mutants for subsequent identification of those with altered phenotypes. Antibiotic susceptibility screening of large collections of Tn mutants in combination with a Tn-Seq approach has the potential to identify mechanisms and factors associated with antibiotic resistance, which may further facilitate the development of new drugs. It has been recently applied in the discovery of a repertoire of previously unknown factors associated with resistance to oxazolidinones, fluoroquinolones, and aminoglycosides in pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (13–15).

Here, we generated a Tn mutant library of *M. abscessus* for screening hypersensitive mutants and identified rapidly and efficiently Tn insertion sites in target clones using a newly developed highly efficient method combining next generation sequencing (NGS) and multiple PCR. This allowed us to identify the MAB_0189c, a gene encoding a probable arabinosyltransferase C (EmbC) responsible for the polymerization of arabinose into the arabinan of lipoarabinomannan (LAM), as an important player in determining resistance of
M. abscessus to multiple antimicrobials. Disruption of the **MAB_0189c** gene markedly enhanced cell envelope permeability, which explained well the enhanced antibiotic susceptibilities to multiple drugs both in vitro and in vivo. Finally, we showed that ethambutol (EMB), a first-line antituberculosis drug inhibiting the arabinosyltransferases of *M. tuberculosis*, remarkably sensitized *M. abscessus* to a panel of existing antimicrobials.

**RESULTS**

Identification of a Tn mutant hypersensitive to multiple drugs. Using Himar1 mutagenesis (16), we first constructed a Tn mutant library containing approximately $1.25 \times 10^5$ mutant clones of *M. abscessus* GZ002 (MabWt), a clinical strain isolated in China and verified by 16s RNA gene sequencing and whole-genome sequencing (17). To identify genes associated with intrinsic antibiotic resistance of *M. abscessus*, we determined the growth of 5,000 randomly selected mutants on agar plates containing subinhibitory concentrations of RIF (4 μg/mL). This initial screening led to the identification of 10 mutants hypersensitive to RIF with 1 designated as MabZ6 (Fig. 1). In 7H9 liquid medium, the MIC of RIF against the MabZ6 strain was 1/32 of that of MabWt (Table 1), confirming the hypersensitive phenotype. The susceptibilities of MabWt and MabZ6 to other commonly used antibiotics were further tested. Interestingly, the MabZ6 strain was also more susceptible to linezolid (LIN), rifabutin (RFB), CEF, clofazimine (CLF), IMP, levofloxacin (LEV), VAN, and clarithromycin (CLA). However, on tigecycline (TGC) and AMK it was found partially less sensitive compared to other drugs (Fig. 1). In addition, the MIC of MabZ6 to TGC and AMK in liquid culture was observed similar to that of MabWt, whereas MabZ6 was hypersensitive to other drugs tested (Table 1). The MICs to MabZ6 decreased to 1/2 ~1/64 compared to those to MabWt. The detailed functional description of other transposon mutants is provided in Table S1. Most of the transposon disrupted genes belong to different functional categories, and the prime objective of the current study is to determine the key factor involved in intrinsic resistance in MabZ6.

Disruption of **MAB_0189c** in MabZ6. We developed a new method to efficiently identify the Tn insertion sites in the mutants (Fig. S1A in the supplemental material). The genomic DNAs mixture of 10 Tn mutants was subject to NGS, allowing for the mapping of 10 insertion sites in only one NGS reaction. Subsequently, multiple PCRs and sequencing were performed to identify the individual insertion site for each mutant. Using this efficient approach, we identified the Tn-disrupted gene in the MabZ6 strain to be the **EFV83_RS12000** gene of MabWt (= the **MAB_0189c** of *M. abscessus* ATCC 19977) (Fig. S1B and S1C) (17). The Tn inserted into the “TA” dinucleotide located 1,413 bases downstream of the starting codon of the 3,258-bp-long **MAB_0189c** (Fig. S1B).
Deletion of MAB_0189c resulted in hypersensitivity to multiple antibiotics in vitro. The amino acid sequence of MAB_0189 showed 68.40% identity to M. tuberculosis EmbC and 68.94% identity to M. smegmatis EmbC, indicating the role of MAB_0189 can be EmbC in M. abscessus (Fig. S2). A selectable marker-free, isogenic deletion of MAB_0189c in MabWt was constructed using recombineering and the Xer/dif system as described previously (18, 19) and designated as MabDembC (Fig. S3). MabDembC was complemented with pMV261-MAB_0189c expressing MAB_0189c or pMV261-embC_Mtb expressing M. tuberculosis embC under the strong mycobacterial promoter hsp60, to obtain strains MabDembC and MabDCMtb respectively. MabDembC grew slower than its parent strain MabWt in Middlebrook 7H9 medium, a phenotype partially restored in the complemented strain (Fig. S3D). However, MabDembC attained a similar growth peak to that of MabWt after 72 h of growth (Fig. S3D).

To confirm the role of the MAB_0189 in intrinsic antibiotic resistance in M. abscessus further, we tested the antibiotic susceptibilities of MabWt, MabDembC, MabDCMab, and MabDCMtb in vitro first. Corroborating the phenotype of the Tn insertion strain MabZ6, MabDembC exhibited a markedly enhanced sensitivity to Rif, CLR, CLF, VAN, IMP, LIN, LEV, CEF, and RFB compared to MabWt (Table 1 and Fig. 1). Expression of MAB_0189c in the complemented strain recovered the antibiotic resistance comparable to the resistance levels of MabWt (Table 1 and Fig. 1), thus confirming that MAB_0189c is critical for the intrinsic antibiotic resistance in M. abscessus. Importantly, expression of M. tuberculosis embC also restored M. abscessus drug resistance to the MabDCMtb levels (Table 1). Hence, based on mycobrowser (https://mycobrowser.epfl.ch/), the identity comparison, and the complementation experiments above, we propose that the function of MAB_0189 is the same as EmbC, i.e., participating in the synthesis of LAM as an arabinosyltransferase C.

Increased cell envelope permeability in MabDembC. In M. tuberculosis, EmbC is one of the three arabinosyltransferases required for the biosynthesis of LAM, an important structural component of mycobacterial cell envelope (20). Therefore, the effect of MAB_0189c on LAM was observed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) analysis. In agreement with a previous report (20), MabDembC strain retains lipomannan (LM) synthesis but is deficient in LAM (Fig. 2A, lane2), and complementation largely restored the normal phenotype (Fig. 2A, lane 3). Disruption of the LAM in M. smegmatis and Mycobacterium neoaurum was previously shown to increase cell envelope permeability (21, 22). Therefore, we reasoned that the hypersensitivity of MabDembC to multiple antibiotics was due to an increase in cell envelope permeability. To confirm this hypothesis, we observed that MabDembC accumulated ethidium bromide in a larger amount compared to MabWt. Complementation of MAB_0189c partially but significantly reduced the dye accumulation (Fig. 2B). We further compared the sensitivity of these strains to SDS,

### TABLE 1 MICs of various drugs determined in 7H9 medium

| Antibiotics | M. abscessus strains/MICs (µg/mL) |
|-------------|----------------------------------|
|             | MabWt  | MabZ6 | MabDembC | MabDCMab | MabDCMtb |
| Clarithromycin | >32    | 2     | 2        | >32      | >32      |
| Clofazimine   | 2-4    | 1     | 0.5      | 2        | 2        |
| Tigecycline   | 1      | 1     | 1        | 1        | 1        |
| Vancomycin    | 128    | 2     | 2        | 128      | 128      |
| Amikacin      | 8      | -     | 8        |          |          |
| Rifampin      | 128    | 4     | 4        | 128      | >128     |
| Imipenem      | 16     | 1     | 1        | 16       | 16       |
| Linezolid     | 64     | 2     | 2        | 64       | 32       |
| Levofoxacin   | 64     | 2     | 2        | 32–64    | 32–64    |
| Cefoxitin     | 64     | 16    | 16       | 32       | 16       |
| Rifabutin     | 16     | 1     | 1        | 16       | 8        |

*a*Broth microdilution method was used to determine the MICs. The MIC was defined as the lowest drug concentration that prevented visible bacterial growth. The experiment was performed in triplicate and repeated twice.

*b*Drugs for in vivo study are underlined.

To confirm the role of MAB_0189c in intrinsic antibiotic resistance in M. abscessus further, we tested the antibiotic susceptibilities of MabWt, MabDembC, MabDCMab, and MabDCMtb in vitro first. Corroborating the phenotype of the Tn insertion strain MabZ6, MabDembC exhibited a markedly enhanced sensitivity to Rif, CLR, CLF, VAN, IMP, LIN, LEV, CEF, and RFB compared to MabWt (Table 1 and Fig. 1). Expression of MAB_0189c in the complemented strain recovered the antibiotic resistance comparable to the resistance levels of MabWt (Table 1 and Fig. 1), thus confirming that MAB_0189c is critical for the intrinsic antibiotic resistance in M. abscessus. Importantly, expression of M. tuberculosis embC also restored M. abscessus drug resistance to the MabDCMtb levels (Table 1). Hence, based on mycobrowser (https://mycobrowser.epfl.ch/), the identity comparison, and the complementation experiments above, we propose that the function of MAB_0189 is the same as EmbC, i.e., participating in the synthesis of LAM as an arabinosyltransferase C.
malachite green, and crystal violet. Assessment of sensitivity to these compounds was widely used as an alternative methodology to determine cell envelope permeability (23). Consistent with the ethidium bromide uptake assay, both Mab\text{DembC} and Mab\text{Z6} were found to be more sensitive to the three compounds than Mab\text{Wt}. The complemented strain partially restored resistance to the compounds (Fig. 2C). Together, these results point to an increased cell envelope permeability of Mab\text{DembC}.

**EMB sensitizes *M. abscessus* to multiple antibiotics.** Given the important role of MAB\text{0189c} in intrinsic resistance of *M. abscessus* to multiple drugs (Table 1), we tested the possibility of sensitizing this bacterium by chemical inhibition of MAB\text{0189}. The homologous protein of MAB\text{0189} (i.e., Emb\text{C}) is known to be targeted by EMB, a first-line antituberculosis drug (24). We therefore tested whether EMB could increase the antibiotic susceptibility of Mab\text{Wt} as well as 3 other randomly selected *M. abscessus* clinical isolates (Table 2). *M. abscessus* has been reported naturally resistant to EMB, which was supposed be due to amino acid difference in Emb\text{B} (another target of EMB) between *M. abscessus* and *M. tuberculosis* (25). The MICs of EMB against the selected *M. abscessus* strains exceeded to 64 µg/mL due to substitution of glutamine at position 281 instead of isoleucine (I281Q) in Emb\text{B} in all isolates in this study (Fig. S4), as has been reported previously (25). Despite this, addition of sub-MICs of EMB (i.e., 4 and 8 µg/mL) resulted in a 2- to 8-fold decrease in MICs of RIF, CLR, CLF, VAN, IMP, LIN, LEV, CEF, and RFB (Table 2). In contrast, *M. abscessus* susceptibility to TGC and AMK did not change in the presence of EMB. We also detected the effect of EMB inhibition on LAM biosynthesis in *M. abscessus*. There were no significant changes in the content of LAM at 4 and 8 µg/mL of EMB (Fig. 2A, lanes 4–5), which may be because these concentrations of EMB are not enough to inhibit the synthesis of LAM efficiently as EMB cannot inhibit the growth of *M. abscessus* at such low concentrations. However, when we increased the concentration of EMB to 32 µg/mL, which is still much lower than its MIC to *M. abscessus*, we observed the clear inhibition of LAM synthesis (Fig. 2A, lane 6), indicating that Emb\text{C} activity was inhibited directly.

**Deletion of MAB\text{0189c} sensitizes *M. abscessus* to multiple antibiotics in vivo.** CLR, CLF, VAN, IMP, LIN, and RFB showed much lower MICs to Mab\text{DembC} than to Mab\text{Wt},

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**FIG 2** Disruption of MAB\text{0189c} in *M. abscessus* increases cell envelope permeability. (A) SDS/PAGE analysis of LAM from different strains. Lane M: protein MW standards. Lane 1: LAM and LM fraction from Mab\text{Wt}. Lane 2: LAM and LM fraction from Mab\text{DembC}. Lane 3: LAM and LM fraction from Mab\text{CMB}Mab. Lanes 4–6: LAM and LM fraction from Mab\text{Wt} when culturing bacteria adding the concentration of EMB at 4 µg/mL, 8 µg/mL, and 32 µg/mL. (B) Accumulation of ethidium bromide in different strains. (C) Sensitivity of *M. abscessus* strains to malachite green, crystal violet, and SDS. The experiments were performed at least thrice, and only one representative image is shown in each case.

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**MAB\text{0189c} Mediates Intrinsic Resistance in *M. abscessus***

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and the MICs of these drugs were also lower than blood peak concentrations in mice at normal dosage (26–29). We investigated their activities in mice infected with Mab\textsuperscript{WT}, Mab\textsuperscript{DembC}, and Mab\textsuperscript{D CMab} strains. Consistent with the data obtained from the in vitro study, the drugs did not significantly decrease bacterial burden in lungs of mice infected with Mab\textsuperscript{WT} or Mab\textsuperscript{D CMab} (Fig. 3B). However, in Mab\textsuperscript{DembC} infected mice, IMP, LIN, and RFB significantly decreased bacterial burden in lungs compared with sodium carboxymethyl cellulose (CMC-Na), the solvent control group ($P$, 0.05), at clinical relevant doses, while the other three drugs selected did not show obvious activities against Mab\textsuperscript{DembC} ($P$, 0.05) (Fig. 3B).

Hence, IMP, LIN, and RFB were active against Mab\textsuperscript{DembC} in vivo at the tested doses. Interestingly, bacterial burden in lungs of mice treated with CMC-Na in the Mab\textsuperscript{DembC} group at the treatment completion was significantly lower than that at treatment initiation ($P$, 0.001), while in the case of the Mab\textsuperscript{WT} group, the lung CFU at treatment initiation and completion were very close ($P$, 0.05) (Fig. 3B). The Mab\textsuperscript{DembC} load of mice decreased even in the CMC-Na group, which indicated its virulence attenuated compared to Mab\textsuperscript{WT}.

Unexpectedly, the bacterial burden in mice infected with the complemented strain was also decreased in the CMC-Na group ($P$, 0.01), thus suggesting that expression of MAB\textsubscript{0189c} could not fully restore its function for the virulence in vivo.

We further assessed the in vivo efficacy of the drug combinations containing EMB plus IMP, CLF, RFB, or CLA against Mab\textsuperscript{WT} in the same mouse model. Combining EMB with any drug selected did not enhance the drug activity in vivo compared to each drug alone (Fig. 3C). EMB may not penetrate inside M. abscessus enough to reach its target.

### DISCUSSION

M. abscessus is emerging as an important opportunistic pathogen responsible for severe infections (30, 31), having limited therapeutics options (32, 33). There is an urgent need for a better understanding of mechanisms of its intrinsic resistance to drugs available for new use of old drugs and discovery of potential new drugs. In this study, MAB\textsubscript{0189c} was found to play a key role in intrinsic resistance to many drugs by maintaining cell envelope impermeability in M. abscessus. Here we proved that MAB\textsubscript{0189c}-encoded EmbC as the embC gene from M. tuberculosis could complement Mab\textsuperscript{embC} for the drug susceptibilities (34). Interestingly, deletion of embC is tolerated in the fast-growing M. smegmatis (35), while its disruption is lethal in M. tuberculosis (36, 37). These observations indicate the differences in tolerability toward LAM defects between the two mycobacterial species. In this study, we demonstrated for the first time that embC (MAB\textsubscript{0189c}) is nonessential for viability in M. abscessus, which could be another main reason why M. abscessus is intrinsic to EMB even though EmbC is the target of EMB (24).

In this study, we found EMB increased the sensitivities of M. abscessus to RIF, VAN, CLF, LIN, RFB, IMP, LEV, CEF and CLA, which was very similar to that observed in the
Moreover, EMB also showed the interference in the synthesis of LAM here. This may be due to the inhibition of EmbC but not by the disturbance in the production of arabinogalactan through inhibiting EmbA/EmbB of *M. abscessus*. To our knowledge, this is the first study identifying the EmbC as a potentially viable drug target for counteracting the intrinsic antibiotic resistance of *M. abscessus*. Novel potent inhibitors targeting MAB_0189 (EmbC) might make multiple inactive drugs effective.

The Mab<sup>Wt</sup> load did not increase after 11 days of infection even in the dexamethasone (DEXA)-induced immunosuppression mice. A recent study showed that the *M. abscessus* burden in the lungs was unchanged after 1 week of infection using a similar DEXA-induced mouse model, but increased slightly after 2 weeks (27). This phenomenon indicates that it may need a longer period to observe a persistent uptrend in *M. abscessus* lung CFU even in immunosuppressed mice. Mab<sup>EmbC</sup> and Mab<sup>CMCmab</sup> burden of mice in the CMC-Na group significantly decreased compared with that at treatment initiation, which is possibly because of their attenuated virulence (*P* < 0.001). MAB_0189 is involved in the synthesis of cell envelope, which is an important determinant of immunogenicity and pathogenicity (38). Moreover, EMB showed the interference in the synthesis of LAM here. This may be due to the inhibition of EmbC but not by the disturbance in the production of arabinogalactan through inhibiting EmbA/EmbB of *M. abscessus*. To our knowledge, this is the first study identifying the EmbC as a potentially viable drug target for counteracting the intrinsic antibiotic resistance of *M. abscessus*. Novel potent inhibitors targeting MAB_0189 (EmbC) might make multiple inactive drugs effective.

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Deletion of *MAB_0189c* also resulted in hypersensitivity to RFB, IMP, and LIN in vivo, further demonstrating the possibility of using MAB_0189 as a drug target. The contribution of virulence attenuation to the drug hypersensitive phenotype was not studied in detail, which is one limitation of the study. Mab<sup>EmbC</sup> may cause fewer or looser granulomas, thereby allowing better penetration of these antibiotics in the lung tissue, thus showing better killing effect. However, according to a recently published study, treatment duration (11 days) in this study was not enough for granuloma formation and may require ≥15 days (39), and it is widely believed that no typical granuloma could form in mouse lung. Bactericidal activities by RFB, IMP, and LIN could be observed in Mab<sup>EmbC</sup> but not for sure in Mab<sup>Wt</sup> and Mab<sup>CMCmab</sup> infected mice if the CFU in lungs of these groups compared with that of the corresponding CMC-Na groups (Fig. 3B). The complementary strain also showed significant attenuated virulence, which can be observed from the CMC-Na group, but RFB, IMP, and LIN did not become hypersensitive to the complementary strain in vivo (Fig. 3B). Therefore,
hypersensitivities to RFB, IMP, and LIN, but not to other drugs, in Mab\textsuperscript{\textit{embC}}-infected mice are more likely contributed by \textit{MAB_0189c} knockout rather than its hypovirulence. Unlike IMP, RFB, and LIN, Mab\textsuperscript{\textit{embC}} was not more susceptible to CLA, CLR and VAN \textit{in vivo}, which indicated that the virulence attenuation cause by \textit{MAB_0189c} knockout alone is not enough to make \textit{M. abscessus} hypersensitive to drugs \textit{in vivo}. Anyway, even if there is contribution of virulence attenuation to the drug hypersensitivity \textit{in vivo}, the virulence was also led by the dysfunction of \textit{MAB_0189}. In other words, the potential inhibitors of \textit{MAB_0189} may help to kill \textit{M. abscessus} not only by enhancing the activities of multiple drugs, but also by enhancing the killing by the host through making \textit{M. abscessus} less virulent. The inactivity of CLF is likely due to the short course of treatment (10 days). It was reported that CLF had a delayed activity against \textit{M. abscessus}, which needs 2 weeks after treatment (40). Although CLA seemingly exhibited no activity, from the \textit{in vivo} data, we cannot exclude that it was potentially active against Mab\textsuperscript{\textit{embC}} since its bacteriostatic activity could be possibly covered up by the downward trend of bacterial loads due to virulence attention.

An intriguing result in Mab\textsuperscript{\textit{emb}}-infected mice is that the lungs CFU of RFB and RFB + EMB treated groups were even more than that of the CMC-Na treated group ($P < 0.01$ and $P < 0.001$, respectively; Fig. 3C). This observation was confined to RFB but not to others, which infers that the phenomenon was not a generalized response to drugs. The results seem to point out a possibility that RFB triggers a mysterious mechanism that promotes \textit{M. abscessus} growth \textit{in vivo} or further disrupts the host immune system. A previous study showed RFB exhibits potent bactericidal activity against \textit{M. abscessus} \textit{in vivo}. The RFB MIC to \textit{M. abscessus} used in our study is 16 $\mu$g/mL, whereas the MIC of the strain used in that study was 2.4 $\mu$M ($\approx$2.0 $\mu$g/mL) (26), which is close to the MIC (1 $\mu$g/mL) Mab\textsuperscript{\textit{embC}} here. Both studies showed the bactericidal activity of RFB (26), which indicated that \textit{in vitro} RFB activity could well predict \textit{in vivo} activity, though the mice were genetically modified in their study and DEXA induced in our study.

In conclusion, this study identified that \textit{MAB_0189c} played a key role in impermeability of cell envelope, a major determinant of \textit{M. abscessus} intrinsic antibiotic resistance and a cause of virulence attenuation. EMB can enhance the sensitivities of multiple drugs by inhibiting LAM synthesis, which might be due to inhibition of EmbC in \textit{M. abscessus}. Taken together, potential inhibitors targeting \textit{MAB_0189} could be attractive as they might make multiple drugs inactive against \textit{M. abscessus} effective and attenuate its virulence.

**MATERIALS AND METHODS**

**Strains, cells, and culture conditions.** 	extit{Escherichia coli} DH5$\alpha$ was grown at 37°C in Luria Bertani (LB) broth and on LB agar. \textit{M. abscessus} subsp. \textit{abscessus} GZ002 (NCBI GenBank accession numbers CP034181), a previously described clinical isolate (41), was grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 10% oleic acid albumin dextrose catalase (OADC, Difco) and 0.05% Tween 80, or on Middlebrook 7H10 agar (Difco) containing 10% OADC. It shows a smooth colony morphotype when grown on Middlebrook 7H11 agar. Where required, kanamycin and zeocin were used at final concentrations of 100 $\mu$g/mL and 30 $\mu$g/mL, respectively.

**Construction of Tn mutant libraries.** \textit{M. abscessus} GZ002 transposon libraries were constructed by \textit{Himar1} mutagenesis as described previously (42). Briefly, 100 mL of mid-log-phase \textit{M. abscessus} culture with an optical density at 600 nm ($OD_{600}$) of $\approx$0.7 to 1.0 was incubated with $1 \times 10^{11}$ to $2 \times 10^{11}$ PFU/mL of MycoMarT7 phage at 37°C for 4 h. Subsequently, culture was washed and plated on Middlebrook 7H10 plates containing kanamycin.

**Screening and identification of Tn mutants.** Tn mutants were replica plated on Middlebrook 7H10 agar supplemented with or without a subinhibitory concentration of RIF (4 $\mu$g/mL). Colonies showing defective growth in the presence of RIF were collected and subjected to MIC measurements. The identification of Tn insertion sites in the RIF-hypersensitive clones was performed by a simple, efficient, and highly adaptable approach. Briefly, a mixture of 10 Tn mutants was prepared for the extraction of genomic DNA, followed by NGS to identify Tn insertion sites in the genomes. The reads containing transposon sequence were mapped to the genome and only insertions with the read count >20 at specific sites were considered to be true insertions. To further verify the NGS results, the disrupted region was amplified by PCR with several mixed colonies as a template in one reaction using a pair of primers targeting Tn and the disrupted gene. Several designed permutations and combinations of the mutants were used for the first batch of PCR. Usually, the insertion location of 10 mutants can be identified using 2 runs of PCR.

**Deletion and complementation of \textit{MAB_0189c} in \textit{M. abscessus}**. Gene deletion in \textit{M. abscessus} was carried out as described earlier (18). Briefly, an allelic exchange substrate for generating \textit{MAB_0189c} gene deletion was prepared by amplification of the upstream and downstream flanking arms and...
subsequent cloning of them on either side of the a zeocin resistance gene and subsequently into the vector pBluescript II SK(+) . The allelic exchange substrate was then amplified from the vector backbone by PCR and directly electroporated into freshly prepared electrocompetent M. abscessus cells carrying pIV53. Plates were incubated at 37°C for 5 days, and colonies resistant to zeocin and kanamycin were screened by PCR for identifying correct gene replacement. The marker-free deletion clones, named Mab<sup>ΔembC</sup>, were verified by PCR and subsequent sequencing. Complementation of the MAB<sub>0189c</sub> gene was achieved by transforming pMV261-based plasmids expressing either MAB<sub>0189c</sub> or M. tuberculosis embC into Mab<sup>ΔembC</sup>. The complemented strains were referred to as Mab<sup>ΔembC</sup>ΔMab<sup>ΔembC</sup> and Mab<sup>ΔembC Δmab<sub>ΔembC</sub></sup>. Primers used are listed in Table S1.

**Antibiotic susceptibility testing.** M. abscessus strains were grown to an OD<sub>600</sub> of 0.6 to 0.7. Tenfold serial dilutions were spotted on Middlebrook 7H10 plates containing Rif (2 to 8 μg/mL), VAN (2 to 8 μg/mL), CLF (0.5 to 1 μg/mL), LIN (2 to 8 μg/mL), RFB (1 to 4 μg/mL), IMP (1 to 4 μg/mL), LEV (2 to 8 μg/mL), CEF (8 to 32 μg/mL), AMK (2 to 8 μg/mL), TCG (0.125 to 0.5 μg/mL), or CLA (0.5 to 1 μg/mL). Broth dilution method was used to measure the MICs. In this assay, cells were inoculated at 5 × 10<sup>5</sup> CFU/mL into 7H9 medium with 2-fold serial drug dilutions. Cells were incubated at 37°C for 14 days for CLA and 3 days for other drugs. The MIC was defined as the lowest antibiotic concentration that prevented visible bacterial growth. The experiment was performed in triplicate and repeated twice.

**Extraction and analysis of LAM.** The M. abscessus strains in logarithmic growth phase (OD<sub>600</sub> = 0.6 to 1.0) were diluted 100 times to subculture for 24 h. EMB existed in broth for subculture at the final concentrations (μg/mL) of 4, 8, and 32, respectively. Afterward, bacteria were collected by centrifugation and extracted with CHCl<sub>3</sub>/CH<sub>3</sub>OH (2:1) and CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O (10:10:3). The residue was further extracted with equal volumes of water and phosphate-buffered saline (PBS)-saturated phenol at 80°C for 2 h. The aqueous layer (containing LAM and LM) was separated by SDS-PAGE (10% to 20% gradient gel) and analyzed by periodic acid/Schiff staining (20).

**Ethidium bromide uptake assay.** Ethidium bromide uptake was monitored as described earlier (43). Mab<sup>Δmab<sub>ΔembC</sub></sup>, Mab<sup>Δmab<sub>ΔembC</sub>ΔMab<sub>ΔembC</sub></sup>, and Mab<sup>Δmab<sub>ΔembC Δmab<sub>ΔembC</sub></sup></sup> were grown to an OD<sub>600</sub> of 0.6–1.0. Bacteria were collected by centrifugation and resuspended in uptake buffer (PBS, 0.05% Tween 80, pH 7.0) to an OD<sub>600</sub> of 0.5, followed by the addition of 25 mM glucose for energization. Ethidium bromide (1 μg/mL) was added, followed by real-time measurement in a fluorescence spectrometer (PerkinElmer) with excitation and emission wavelengths set at 520 and 595 nm, respectively. Results were normalized against fluorescence of ethidium bromide and are depicted relative to the highest fluorescence measured. The experiment was performed in triplicate and repeated three times.

**Susceptibility to chemical compounds.** As reported previously (23), stationary-phase cells were diluted in PBS to an OD<sub>600</sub> of 0.5, and further 10-fold serial dilutions were prepared. One microliter of each dilution solution was spotted onto 7H10 containing 0.01% (vol/vol) SDS, 2 μg/mL malachite green, and 4 μg/mL crystal violet. Cells were incubated at 37°C for 3 days.

**Mouse infection and treatment.** All animal care and experimental protocols were approved by the Committee on Laboratory Animal Ethics of Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences. Five- to six-week-old, female BALB/c mice (Charles River) were infected with 10 mL M. abscessus culture at an OD<sub>600</sub> of 0.7–0.8 via aerosolization using a Glas-Col inhalation exposure system (Glas-Col, Terre Haute, Indiana) with inhalation time 45 min according to the manufacturer’s instructions.

To achieve adequate immunosuppression, mice were treated with DEXA. DEXA (D1756, Sigma-Aldrich) was dissolved in sterile 1× phosphate-buffered saline (PBS, pH 7.4) and administered via daily subcutaneous injection at 5 mg/kg/day, as described previously (27). Daily DEXA treatment began 2 weeks prior to infection and was continued throughout the duration of the experiment. Mice were sacrificed 24 h postinfection to determine the initial bacterial burden in the lungs.

**Antibiotics and regimens.** The mice were treated at doses (mg/kg) via oral gavage once daily with 0.4% CMC-Na as the solvent, CLR 250, CLF 50, LIN 100, RFB 20, and EMB 200, or via subcutaneous injection twice daily with either VAN 110 or IMP 100. Combination treatment groups were as follows: EMB + IMP, EMB + CLF, EMB + RFB, and EMB + CLA. Mice were sacrificed for CFU determination in lungs at 11 days after treatment initiation. Drug stocks (mg/mL) were dissolved in sterile 0.4% CMC-Na: CLR 25, CLF 5, LIN 10, RFB 2, EMB 20, or in 1× PBS: VAN, 11, IMP 10. Additionally, cilastatin was added to IMP preparation in a 1:1 dose ratio aimed to compensate for higher activity of murine renal dihydropeptidase-1 compared to humans, as described previously (44).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.5 MB.

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