High Levels of Intrinsic Tetracycline Resistance in *Mycobacterium abscessus* Are Conferred by a Tetracycline-Modifying Monooxygenase

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ABSTRACT Tetracyclines have been one of the most successful classes of antibiotics. However, its extensive use has led to the emergence of widespread drug resistance, resulting in discontinuation of use against several bacterial infections. Prominent resistance mechanisms include drug efflux and the use of ribosome protection proteins. Infrequently, tetracyclines can be inactivated by the TetX class of enzymes, also referred to as tetracycline destructases. Low levels of tolerance to tetracycline in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* have been previously attributed to the WhiB7-dependent TetV/Tap efflux pump. However, *Mycobacterium abscessus* is ~500-fold more resistant to tetracycline than *M. smegmatis* and *M. tuberculosis*. In this report, we show that this high level of resistance to tetracycline and doxycycline in *M. abscessus* is conferred by a WhiB7-independent tetracycline-inactivating monooxygenase, MabTetX (MAB_1496c). The presence of sublethal doses of tetracycline and doxycycline results in a >200-fold induction of MabTetX, and an isogenic deletion strain is highly sensitive to both antibiotics. Further, purified MabTetX can rapidly monooxygenate both antibiotics. We also demonstrate that expression of MabTetX is repressed by MabTetR_x, by binding to an inverted repeat sequence upstream of MabTetR_x; the presence of either antibiotic relieves this repression. Moreover, anhydrotetracycline (ATc) can effectively inhibit MabTetX activity *in vitro* and decreases the MICs of both tetracycline and doxycycline *in vivo*. Finally, we show that tigecycline, a glycylcycline tetracycline, not only is a poor substrate of MabTetX but also is incapable of inducing the expression of MabTetX. This is therefore the first demonstration of a tetracycline-inactivating enzyme in mycobacteria. It (i) elucidates the mechanism of tetracycline resistance in *M. abscessus*, (ii) demonstrates the use of an inhibitor that can potentially reclaim the use of tetracycline and doxycycline, and (iii) identifies two sequential bottlenecks—MabTetX and MabTetR_x—for acquiring resistance to tigecycline, thereby reiterating its use against *M. abscessus*.

KEYWORDS *M. abscessus*, antibiotic resistance, drug inactivation, intrinsic resistance, tetracyclines

Mycobacterium abscessus is a rapidly growing environmental species of mycobacteria (RGM) that has emerged as an important human pathogen over the last 10 years (1–3). It causes bronchopulmonary infections in patients with underlying lung damage, such as bronchiectasis, prior tuberculosis, and cystic fibrosis (CF), which lead to either acute lung failure or a chronic disease with progressive decline in lung function (4–10). In addition, *M. abscessus* causes skin and soft tissue infections (SSTI) after surgery and trauma (11–13). *M. abscessus* stands out as one of the most antibiotic-resistant bacterial species known, making its infections incredibly difficult to treat (1, 14, 15). It is intrinsically resistant to the antituberculosis drugs rifampin, isoniazid, etham-

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butol, and pyrazinamide as well as to most of the FDA-approved antibiotics (16–18). The current treatment regimen recommends a combination of an oral macrolide in conjunction with amikacin and one or more of the injectibles cefoxitin, imipenem, and the recently introduced tigecycline for a period of several weeks to months (4, 19, 20). However, macrolide use is limited by inducible resistance such that strains that are initially antibiotic susceptible become resistant after extension of the testing period (21, 22). Despite such aggressive treatments, the average rate of eradication is only ~45% (23, 24).

Intrinsic resistance in mycobacteria is thought to be a consequence of the permeability barrier offered by its complex cell envelope as well as an antibiotic-induced transcriptional reprogramming that results in induction of drug resistance genes (25, 26). A number of effectors contributing to the intrinsic resistance of *M. abscessus* have been recently elucidated, including *erm41*, which methylates the erythromycin binding site in the ribosome, *Mab_Arr*, which ribosylates rifampins, *Mab_Bla*, which inactivates β -lactams, *eis2*, which acetylates amikacin, tobramycin, and kanamycin, and *Mab_APH*, which inactivates streptomycin (27–35). We recently demonstrated that some of these effectors, such as *erm41* and *eis2*, are induced via a *WhiB7*-dependent pathway (33). *Mab_WhiB7*, a transcriptional activator, is one of the earliest genes that are induced in response to several unrelated antibiotics, such as erythromycin, clarithromycin, amikacin, streptomycin, tetracyclines, and spectinomycin (33). The Δ *MabwhiB7* strain is highly sensitive to several of the antibiotics that induce its expression; however, its resistance to tetracycline remains largely unchanged.

Tetracyclines are one of the most successful group of antibiotics and have undergone several modifications since the original discovery of chlortetracycline to what are known as the semisynthetic (doxycycline, minocycline, etc.) and the glycylcycline (e.g., tigecycline) antibiotics (36). Resistance to the tetracycline group of antibiotics in bacteria is mediated primarily by ribosome protection proteins that release tetracycline from ribosomes and efflux pumps that exchange a H^+ for a molecule of tetracycline against a concentration gradient (37–46). A less prevalent resistance mechanism involves enzymatic modification of tetracyclines by flavin adenine dinucleotide (FAD)-requiring monooxygenases. The founding member, TetX, was identified in the transposons of the obligate anaerobe *Bacteroides fragilis* (47–49). Overexpression and purification of TetX from *Escherichia coli* demonstrated that TetX has a strict requirement for NADPH and O_2 and hydroxylates tetracyclines at C11a; the resultant product is unstable and decomposes into products that are not easily recognizable (47, 49–53). However, since *B. fragilis* is a strict anaerobe, the TetX gene does not confer tetracycline resistance in this species due to its requirement for O_2 . Subsequently, orthologues of *tetX* have been identified in another *Bacteroides* transposon, CTnDOT, and in soil microbiomes (54, 55). The clinical prevalence of *tetX*-mediated tetracycline resistance was not reported until the isolation of *tetX*-containing bacterial strains from a hospital in Sierra Leone in 2013 which were identified as members of *Enterobacteriaceae* and *Pseudomonadaceae* (56). More recently, a *tetX* homologue, *tet(56)*, was described from *Legionella longbeachae*, a causative agent of Pontiac fever and Legionnaires' disease (57). Homology searches have, however, failed to identify tetracycline-inactivating enzymes in mycobacteria.

Low levels of intrinsic resistance to tetracycline in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* have been previously attributed to the TetV/Tap efflux pump, which is included in the *WhiB7* regulon (58–60). *M. abscessus* is, however, ~500-fold more resistant to tetracycline and doxycycline than are *M. smegmatis* and *M. tuberculosis*, making these unavailable for therapy (18, 33, 61). *M. abscessus* is, however, susceptible to tigecycline *in vitro* and has demonstrated clinical efficacy (20, 62). Here we show that the high levels of tetracycline and doxycycline resistance in *M. abscessus* are conferred by a monooxygenase, MabTetX (MAB_1496c), whose expression is induced by the above-mentioned antibiotics via a *whiB7*-independent pathway. Further, we demonstrate that anhydrotetracycline (ATc) is an effective inhibitor of MabTetX *in vitro* and reduces the MICs of tetracycline and doxycycline *in vivo*. Finally, we show that

FIG 1 Identification of MAB_1496c as a possible *whiB7*-independent mechanism of tetracycline resistance in *M. abscessus*. (a) Ten-fold serial dilutions of wild-type (WT) *M. abscessus* ATCC 19977, the Δ whiB7 mutant, and the complemented strain were grown to an A_{600} of 0.7 and spotted onto Middlebrook 7H10 OADC containing 40 μ g/ml and 60 μ g/ml of tetracycline. (b) Ten-fold serial dilutions of *M. smegmatis* mc²155, the Δ whiB7 mutant, and the complemented strain were grown to an A_{600} of 0.7 and spotted onto Middlebrook 7H10 ADC containing 0.1 μ g/ml of tetracycline. (c) Transcriptomic changes in wild-type *M. abscessus* upon exposure to 16 μ g/ml of tetracycline for 30 min and 3 h. Genes induced >4-fold with a *q* value < 0.001 were analyzed, and the 20 most induced genes are represented as a heat map. Expression levels of the MAB_2780c, the TetV homologue, are also included in the heat map. MAB_1496c and MAB_1497c are the 2 most highly induced genes upon tetracycline exposure. (d) Genome organization of MAB_1496c and MAB_1497c. (e) Wild-type *M. abscessus* and the Δ whiB7 mutant were grown to an A_{600} of 0.7 and exposed to sublethal concentrations of tetracycline, and the amounts of MAB_1496c and MAB_1497c transcripts were determined by qPCR and plotted as fold induction over an unexposed control. Data represent means $\pm$ SDs (*n* = 3). Transcription of both genes is independent of WhiB7.

tigecycline is not only a poor substrate of MabTetX but is also incapable of inducing the expression of MabTetX, thereby affirming its use as an effective therapy against *M. abscessus*.

RESULTS

MAB_1496c and MAB_1497c are the earliest and most highly induced genes upon tetracycline exposure. In a recent study, we showed that although tetracycline is a strong inducer of *whiB7*, the tetracycline sensitivity of an *M. abscessus* Δ whiB7 mutant strain is not significantly altered from that of the wild-type parent (Fig. 1a) (33). This is in contrast to the tetracycline hypersensitivity of Δ whiB7 mutant strains of *M. tuberculosis* and *M. smegmatis* (Fig. 1b). However, we noted a mild sensitivity of the *M. abscessus* Δ whiB7 mutant when the tetracycline concen-

tration was increased to 60 $\mu\text{g/ml}$, a concentration at which the viability of the wild-type strain was reduced by 3 orders of magnitude (Fig. 1a). This suggests that the primary determinants for tetracycline resistance in *M. abscessus* lie outside the *whiB7* regulon. Transcriptome analysis by RNA sequencing (RNA-seq) of wild-type *M. abscessus* exposed to sublethal doses of tetracycline (16 $\mu\text{g/ml}$) for 30 min was performed in order to determine the genes that are immediately induced in response to tetracycline. Genes that were significantly (q value < 0.01) induced 3-fold or more compared to an unexposed control were selected for analysis (see Data Set S1 in the supplemental material). Using the above-described criteria, we identified 343 genes, of which 45 unequivocally comprise the *WhiB7* regulon, including MAB_2780c, which encodes a homologue of the efflux pump TetV (33). In addition, RNA-seq was also performed on *M. abscessus* exposed to tetracycline (16 $\mu\text{g/ml}$) for 3 h. Figure 1c shows the expression of 20 genes whose expression changed the most upon exposure to 16 $\mu\text{g/ml}$ for periods of 30 min and 3 h. Two adjacent genes, MAB_1496c and MAB_1497c, were the most highly induced genes under both conditions tested, and they lie outside the *WhiB7* regulon (Fig. 1d and e). These therefore constitute candidates that contribute to the *WhiB7*-independent tetracycline resistance of *M. abscessus*.

Sequence analysis of MAB_1496c and MAB_1497c suggests a role in tetracycline resistance. Sequence analysis using BLASTP revealed that the MAB_1496c product, MabTetX, shows similarity to FAD-binding monooxygenases. These comprise a highly diverse group of proteins that catalyze a range of chemical transformations, including inactivation of tetracycline and other antibiotics (63). Flavin monooxygenases are ubiquitous in mycobacteria; however, sequence diversity makes it challenging to ascribe functions to these genes. Nevertheless, MAB_1496c is 100% conserved within the *M. abscessus* complex and homologues with $>80\%$ sequence identity are found in *M. chelonae*, *M. immunogenum*, and *M. franklinii* and suggest a conserved function. Enzymatic inactivation of tetracycline is rare and is best described in *tetX* from *Bacteroides fragilis*. MAB_1496c, however, shows only 29% amino acid sequence identity to *B. fragilis* TetX. A protein sequence alignment of diverse monooxygenase representatives homologous to MAB_1496c and to TetX was performed and indicates that they all share a distant common origin, with the presence of an FAD binding consensus motif (Fig. S1) (64). A phylogenetic analysis showed two distinct groups of monooxygenases, with *M. abscessus* MAB_1496c and *B. fragilis* TetX being from different classes (Fig. 2).

Sequence analysis of MAB_1497c using BLASTP shows conservation with the TetR family of transcriptional regulators (TFTRs) that comprise a predominant one-component signal transduction system in bacteria. TFTRs are frequently repressors that bind as dimers to inverted repeat sequences in DNA (operators) and prevent transcription of an effector gene that is either divergently oriented, cotranscribed, or in the same orientation as the regulator (65–68). Ligand binding causes a conformational change in the regulator, thereby dissociating it from the operator DNA. Although initially identified as controlling the expression of the Tet efflux pump in *E. coli*, TFTRs have subsequently been shown to control a wide range of metabolic functions (69).

Deletion of MAB_1496c results in tetracycline sensitivity. We constructed an unmarked, isogenic deletion of MAB_1496c in *M. abscessus* ATCC 19977, using phage recombineering as described previously (33, 61). The unmarked deletion mutant was confirmed using PCR and sequencing. A complementing strain was constructed by transforming the deletion strain with pMH94-MAB_1496c, a phage L5-based integration vector in which MAB_1496c is expressed from the constitutive promoter P_{hsp60} . Wild-type *M. abscessus*, the $\Delta\text{MAB}_1496\text{c}$ mutant, and the complemented strain were tested for susceptibility to tetracyclines on Middlebrook 7H10 containing oleic acid-albumin-dextrose-catalase (OADC). Figure 3a to d show that the $\Delta\text{MAB}_1496\text{c}$ deletion strain exhibits ~ 20 -fold-greater sensitivity to tetracycline and doxycycline than do the wild-type parent and the ΔMAB_{whiB7} mutant. The growth of the $\Delta\text{MAB}_1496\text{c}$ strain, but not the ΔMAB_{whiB7} strain, was completely inhibited by ~ 3.75 $\mu\text{g/ml}$ of tetracy-

FIG 2 Maximum likelihood tree of MAB_1496c and *Bacteroides* TetX homologues. The phylogenetic tree was calculated with Phyml version 3. Numbers above each node show percent support of the branch configurations that occurred over 100 bootstrap trials. The scale bar shows the average number of amino acid residue changes per site.

cline and 2.5 $\mu\text{g/ml}$ of doxycycline (Fig. 3a and c); the wild-type strain showed robust growth even at 60 $\mu\text{g/ml}$ of both antibiotics, while the $\Delta\text{MAB}whiB7$ strain was mildly sensitive to high tetracycline and doxycycline concentrations (Fig. 3b and d). However, the sensitivity of the ΔMAB_{1496c} strain to tigecycline remained unchanged, whereas the $\Delta\text{MAB}whiB7$ strain was moderately sensitive to tigecycline (Fig. 3e). Further, not only was the sensitivity of the ΔMAB_{1496c} mutant restored in the complemented strain, but also the resistance of the complemented strain greatly exceeded that of the wild type (Fig. 3f). Therefore, MAB_1496c likely comprises the predominant mechanism for the high levels of resistance to tetracycline and doxycycline in *M. abscessus*, and the effectors contained within the *WhiB7* regulon constitute secondary, relatively insignificant, mechanisms for resistance to tetracycline but confer low levels of resistance to tigecycline.

Purified MAB_1496c protein catalyzes the modification of tetracycline and doxycycline by monooxidation. Tetracyclines show two absorption maxima, one at 260 nm and the other at 363 nm. TetX activity has previously been monitored using UV-visible (UV-Vis) spectroscopy as a disappearance in absorption maximum at 363 nm with time. The MAB_1496c protein was purified *in vitro* (Fig. S2), and its ability to inactivate tetracyclines was monitored by measuring the UV-visible spectrum upon addition of the protein to each of the antibiotics in a buffer containing Mg^{2+} and NADPH, over a period of 300 s. Since NADPH absorbs at 340 nm, the contribution of NADPH was subtracted from each of the reactions. Figure 4a and b show a temporal decrease in absorption at 363 nm of tetracycline and doxycycline upon addition of purified MAB_1496c protein. The results of control experiments show that the antibiotics are stable in the absence of the MAB_1496c protein and the activity of this protein is dependent upon the presence of NADPH (data not shown). Liquid chromatography-mass spectrometric (LC-MS) analysis showed that the disappearance of tetracycline and doxycycline ($m/z = 445.16$) was accompanied by the appearance of a product peak, P,

FIG 3 Deletion of MAB_1496c renders *M. abscessus* hypersensitive to tetracyclines. Ten-fold serial dilutions of *M. abscessus* ATCC 19977, the Δ whiB7 mutant, and the Δ MabTetX mutant were grown to an A_{600} of 0.7 and spotted onto Middlebrook 7H10 OADC containing the indicated concentrations of antibiotics. (a to d) The mutant is hypersensitive to tetracycline and doxycycline compared to the wild-type parent strain. A complementing strain containing an integrated copy of MAB_1496c expressed from the constitutive promoter P_{hsp60} restores antibiotic resistance. The sensitivity of the Δ whiB7 mutant to tetracycline and doxycycline is apparent only at high antibiotic concentrations. (e) Tigecycline sensitivity of the Δ MAB_1496c mutant is indistinguishable from that of the wild-type strain, but the Δ whiB7 mutant is more sensitive to tigecycline than the Δ MabTetX strain. (f) Overexpression of MAB_1496c in wild-type *M. abscessus* increases the resistance above that of the wild-type parent.

with an m/z value of 461.16 in a positive ion mode, corresponding to the addition of 16 Da and suggesting monooxygenation (Fig. 4c to f). The biochemical properties of the MAB_1496c protein were therefore similar to those of *Bacteroides* TetX, and we henceforth refer to the MAB_1496c protein as MabTetX. The absorption of tigecycline shifted minimally during the time frame of the experiment but was not comparable to that observed for tetracycline and doxycycline (Fig. S3a and b). Moreover, we could not detect appearance of significant products when MabTetX-treated tigecycline was analyzed using mass spectrometry, despite increasing the enzyme concentration and the incubation time to 3 h. The data therefore suggest that tigecycline is not a substrate of MabTetX and are consistent with the tigecycline sensitivity of *M. abscessus*.

MAB_1497c is a TFTR repressor that regulates the expression of MabTetX. The open reading frame (ORF) of MAB_1497c shows a 4-bp overlap with the downstream MAB_1496c, and the genes are likely cotranscribed. RNA-seq data indicate that transcription of MAB_1497c is initiated within the 31-nucleotide (nt) spacer separating MAB_1497c and MAB_1498c (Fig. S4). This spacer region also contains a 15-bp inverted repeat sequence flanking 5 bp of DNA and likely comprises the MAB_1497c binding site

FIG 4 MAB_1496c-mediated modification of tetracycline and doxycycline. (a and b) UV-visible spectra of tetracycline and doxycycline modification catalyzed by MAB_1496c. Purified MAB_1496c was added to 100 μ M tetracycline in a buffer containing 10 mM Tris (pH 8.0), 0.1 mM $MgCl_2$, and 0.4 mM NADPH. The UV-Vis spectrum of tetracycline and doxycycline was scanned for the indicated periods with a 30-s scan interval and shows a temporal decrease in the peak at ~ 370 nm. (c and d) Chromatographic separation of tetracycline and doxycycline and MAB_1496c protein-catalyzed products on an Acquity UPLC T3 (Waters, Milford, MA) column shows the appearance of an increasing product peak (P1) with time. (e and f) LC-MS was performed in a positive ion mode on a QTARXL (ABSCIEX) system. Analysis of tetracycline and doxycycline ($m/z = 445.2$) and their respective product peaks ($m/z = 461.2$) is consistent with monooxidation of each antibiotic.

FIG 5 MAB_1497c is a TFTR repressor that regulates the expression of MabTetX. (a) Genomic organization of MAB_1496c-MAB_1497c and the 35-nt palindromic sequence upstream of MAB_1497c. The 35-bp and 180-bp DNA substrates are shown. (b and e) DNA binding ability of purified MAB_1497c was analyzed using EMSA. DNA substrates were either 35 bp or 180 bp in length (as indicated) and were fluorescein labeled at the 5' end. Addition of tetracycline and doxycycline (but not tigecycline) either during or after MAB_1497c-DNA complex formation results in disruption of DNA-protein complex. (f) Wild-type *M. abscessus* was grown to an A_{600} of 0.7 and exposed to sublethal concentrations of tetracycline, doxycycline, and tigecycline, and the amount of MAB_1496c transcripts was determined by qPCR and plotted as fold induction over an unexposed control. Data represent means $\pm$ SDs ($n = 3$). Tetracycline and doxycycline, but not tigecycline, induced the expression of MAB_1496c (MabTetX).

(Fig. 5a; see also Fig. S4). Figure 5b shows that purified MAB_1497c protein binds to the 35-bp inverted DNA substrate to form a single protein-DNA complex; the 35-bp sequence is henceforth referred to as *tetO*. To rule out additional binding sites, electrophoretic mobility shift assays (EMSAs) were also carried out with larger DNA

substrates (180 bp) containing *tetO* and upstream portions of the DNA (Fig. 5c and d). However, a single protein-DNA species was observed using the 180-bp DNA substrate, indicating that *tetO* constitutes the primary binding site of MAB_1497c and presumably overlaps with the promoter. This observation is consistent with previously studied TFTRs where the operator sites coincide with promoter DNA (70). MAB_1497c is henceforth referred to as MabTetR_x.

Since MabTetR_x and MabTetX are tetracycline inducible *in vivo*, we expect that presence of tetracyclines would interfere with the binding of MabTetR_x to DNA. Figure 5c and d show that addition of tetracycline and doxycycline either to a preformed protein-DNA complex or during complex formation interferes with DNA binding. This suggests that binding of tetracycline and doxycycline to MabTetR_x prevents association with DNA as well as dissociates previously bound MabTetR_x from the DNA substrate. Moreover, addition of MabTetX to the above-mentioned reactions prevents antibiotic-dependent dissociation of MabTetR_x from substrate DNA. However, unlike for tetracycline and doxycycline, addition of tigecycline does not alter the ability of MabTetR_x to bind DNA and suggests that tigecycline would be a poor inducer of the MabTetR_x MabTetX operon (Fig. 5e). Real-time PCR shows that indeed tigecycline poorly induces MabTetX expression, in contrast to the high levels of induction elicited by tetracycline and doxycycline (Fig. 5f).

Anyhydrotetracycline inhibits the activity of MabTetX. Anhydrotetracycline (ATc) is a degradation product of tetracycline with poor antibiotic activity and was recently shown to be capable of inhibiting the activity of *L. longbeachae* Tet(56) as well as the *Bacteroides* TetX by competitively blocking substrate binding (57). In order to test the ability of ATc to inhibit the activity of MabTetX, we monitored the decrease in tetracycline and doxycycline absorption peaks in the presence of MabTetX and ATc using UV-Vis spectroscopy. As shown in Fig. 6a to f, addition of 50 μ M ATc completely inhibited the MabTetX-dependent inactivation of 100 μ M doxycycline, whereas 10 μ M ATc was sufficient to inhibit the degradation of 100 μ M tetracycline. Moreover, addition of 2 μ g/ml of ATc, a concentration >20-fold lower than the MIC of ATc, resulted in an ~4-fold decrease in the MICs of both tetracycline and doxycycline in wild-type *M. abscessus* (Fig. 6h; see also Fig. S5a). While ATc is itself not degraded by MabTetX and is not inhibitory to *M. abscessus* (Fig. S5a- to c), it is capable of inducing MabTetX expression by dislodging MabTetR_x from the operator DNA (Fig. S5d). The observed decrease in MIC is therefore despite an increase in ATc-induced expression of MabTetX.

DISCUSSION

Tetracycline and its derivatives have been among the most successful and widely used antibiotics since the discovery of tetracycline in 1948. However, tetracyclines are relatively stable in the environment and are not typically degraded, unlike most antibiotics. Their environmental accumulation combined with their indiscriminate use in the treatment of bacterial infections of humans and plants as well as in animal growth has led to a rapid emergence of resistant strains. *M. abscessus* is highly resistant to narrow- and expanded-spectrum tetracyclines but is susceptible to the broad-spectrum tigecycline (61). We have demonstrated that the high intrinsic resistance of *M. abscessus* to tetracycline and doxycycline can be attributed to a chromosomally encoded flavin monooxygenase, MabTetX, that inactivates tetracyclines by monooxygenation. Tetracycline inactivation by TetX enzymes is a relatively infrequent mechanism of resistance to tetracycline and has been detected in environmental DNA and commensal bacteria in the gut and intestines, such as *Bacteroides*, and more recently in clinical isolates belonging to the *Enterobacteriaceae* and *Pseudomonadaceae* (56). The infiltration of TetX genes in mycobacteria therefore presents a worrying trend. However, MabTetX is phylogenetically distinct from *Bacteroides* TetX, which suggests that the role of MabTetX in resistance evolved as a consequence of convergent evolution rather than through horizontal gene transfer. The biochemical properties of MabTetX closely resemble those of *Bacteroides* TetX, and both enzymes can inactivate the narrow- and

FIG 6 Anhydrotetracycline (ATc) is an inhibitor of MabTetX. (a to f) UV-visible spectra of tetracycline and doxycycline degradation catalyzed by MAB_1496c protein in the presence of indicated concentrations of ATc. The UV-Vis spectrum of tetracycline and doxycycline was scanned for indicated periods of time with a 30-s scan interval and shows an inhibition of the temporal decrease in the peak at ~ 370 nm in the presence of ATc. (g) Wild-type *M. abscessus* was grown in the presence of increasing concentrations of tetracycline (blue)/doxycycline (red) in either the presence or absence of 2 mM ATc. The presence of ATc resulted in a 2- to 4-fold increase in MICs of both tetracycline and doxycycline. Data represent means $\pm$ SDs ($n = 3$).

expanded-spectrum tetracyclines efficiently. However, in contrast to *Bacteroides* TetX, tigecycline does not appear to be a substrate of MabTetX.

We also demonstrate that the expression of MabTetX is negatively controlled by MabTetR_x, a TFTR repressor which binds to an operator sequence upstream of MabTetR_x and prevents cotranscription of MabTetR_x and MabTetX. Both tetracycline and doxycycline can bind to MabTetR_x and induce the operon; however, tigecycline is unable to interact with MabTetR_x and dislodge it from *tetO* and is therefore unable to induce MabTetX expression. Acquired resistance to tigecycline would therefore require not only mutations that increase the activity of MabTetX against tigecycline but also mutations in MabTetR_x that would allow effective tigecycline binding and subsequent induction of the MabTetR_x-MabTetX operon. Previous studies with *Bacteroides* TetX have shown that single amino acid substitutions are sufficient to greatly enhance its

activity against tigecycline (71). The presence of an additional level of control at the level of regulation of MabTetX expression therefore greatly decreases the probability of acquiring tetracycline resistance in *M. abscessus*.

Although tigecycline is highly effective against *M. abscessus in vitro* and demonstrates reasonable clinical efficacy (~50%), its use is commonly associated with nausea, vomiting, diarrhea, fever, and anorexia; serious side effects such as multiorgan failure, pneumonia, and respiratory failure are also not uncommon (61, 62). We show here that anhydrotetracycline (ATc) can potentially inhibit MabTetX and reduce the MICs of both tetracycline and doxycycline. It is noteworthy that the reduction in MIC is despite the fact that ATc can itself induce MabTetX expression. While the ability of ATc to induce MabTetX expression makes it less than an ideal candidate for use as an inhibitor of MabTetX, it can nevertheless serve as a starting point for the design of compounds that inhibit MabTetX. Synthesis of compounds similar to ATc that can inhibit MabTetX but no longer induce the operon can potentially reclaim the use of the better tolerated tetracycline and doxycycline that are presently ineffective against the *M. abscessus* complex.

MATERIALS AND METHODS

Media and strains. *Mycobacterium smegmatis* was grown at 37°C in Middlebrook 7H9 (Difco) supplemented with 10% ADC and 0.05% Tween 20. *Mycobacterium abscessus* was grown at 37°C in Middlebrook 7H9 (Difco) supplemented with 10% OADC and 0.05% Tween 20. Antibiotics were added as required. Gene replacement mutants were constructed using recombineering as described previously (72). The recombineering construct was generated by cloning in the multiple-cloning sites flanking the apramycin resistance cassette of pYUB854. Mutant clones were checked using F_{check} and R_{check} primers flanking the deletion site.

Antibiotic sensitivity assays. The wild type and mutant strains of *M. smegmatis* and *M. abscessus* were grown to an A₆₀₀ of 0.6 to 0.7. Cells were tested for susceptibility to tetracycline, doxycycline, and tigecycline by spotting a 10-fold serial dilution on Middlebrook 7H10 (Difco) plates containing the desired concentration of each drug. Antibiotic susceptibility in liquid media was assayed by inoculating the desired strain in a 2-fold dilution series of each antibiotic at an initial A₆₀₀ of 0.0004. Anhydrotetracycline was added where necessary. The cultures were incubated at 37°C, and the A₆₀₀ was measured after 72 h.

RNA preparation, quantitative PCR (qPCR), and RNA-seq analysis. Wild-type *M. abscessus* ATCC 19977 was grown to exponential phase (optical density [OD], 0.7) in Middlebrook 7H9-OADC, exposed to various concentrations of tetracycline for various periods (0 to 90 min), and evaluated for lethality. Total RNA was prepared from wild-type strains exposed to 16 µg/ml of tetracycline for a period of 30 min or 3 h, using the Qiagen RNA preparation kit followed by DNase I treatment. Unexposed samples were used as controls. Total RNA samples of approximately 5 µg were treated with the Ribo-Zero rRNA removal procedure (Illumina) to enrich for mRNA. Approximately 500 ng of RNA was used for library preparation using the Script Seq v2 RNA-seq kit and high-throughput sequencing on the Illumina NextSeq platform. The sequence data were analyzed using Rockhopper, in which the data are normalized by upper quartile normalization and transcript abundance is reported as reads per kilobase per million (RPKM). Differential gene expression is tested for each transcript, and q values are then reported that control the false-discovery rate (73, 74).

Wild-type *M. abscessus* was exposed to tetracycline (16 µg/ml), doxycycline (16 µg/ml), and tigecycline (1 µg/ml) for 30 min. Total RNA was prepared using the Qiagen RNA preparation kit followed by DNase I treatment. Primers for quantitative reverse transcription-PCR (qRT-PCR) were generated using Primer Quest software (IDT). cDNA was generated using random hexamers and Maxima reverse transcriptase (Fisher Scientific), and qRT-PCR was performed using the Maxima SYBR green qPCR master mix (Fisher Scientific) using the following primer pairs: MAB_1496c, 5'-CCTGTGGTTCGCGGAAA-3'/5'-CCCTGCTCAAGAATCTCACC-3'; MAB_1497c, 5'-GAGCAACAAGGCCGAAC-3'/5'-CGATGAGGTCTGTGCGTAATC; and MAB_SigA, 5'-CCAAGAACCATCTGCTGGAA-3'/5'-CCAGTTGCCTTCCTGAAT. The Applied Biosystems 7300 real-time PCR system was used with cycling conditions of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

Protein overexpression and purification. *M. abscessus* MAB_1496c and MAB_1497c were cloned into pET21a with a C-terminal His tag and transformed into BL21(DE3) pLysS grown to an A₆₀₀ of 0.4 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C. The cells were lysed in a buffer containing 100 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 5% glycerol, and the clarified lysate was loaded on a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen). Nonspecifically bound proteins were removed by washing with lysis buffer containing 40 mM and 60 mM imidazole, and the protein was eluted with 150 mM imidazole.

Phylogenetic analysis. Protein sequences were aligned using MAFFT version 7 (75) using the G-INS-i alignment strategy and all other default options. The phylogenetic tree was calculated with PhyML version 3.0 (76) using the LG + G + F model (gamma 4 categories with observed amino acid frequencies) and 100 bootstrap replicates. The optimal substitution model was determined using ProtTest3 (77).

Spectrophotometric analysis of MabTetX activity. Purified MabTetX was added at a final concentration of 0.286 μM to 100 μM tetracycline, doxycycline, or tigecycline in a buffer containing 10 mM *N*-Tris(hydroxymethyl)methylaminopropane sulfonic acid (TAPS) (pH 7.5), 0.1 mM MgCl_2 , and 0.4 mM NADPH. The total reaction volume was 300 μl . The decrease in absorbance at 370 nm was measured by monitoring the UV-visible spectrum over desired periods with a 30-s scan interval on a Thermo Scientific Evolution UV-Vis spectrophotometer. The contribution of NADPH was subtracted from each of the reactions, since NADPH absorbs at 340 nm.

LC-MS analysis. Liquid chromatography-mass spectrometry (LC-MS) analysis was performed on a QTARXL (ABSCIEX) configured with a Shimadzu UHPLC Nexera LC-30AD pumping system, a SIL-30AC autosampler, and a CTO-30A column oven. Briefly, the LC-MS system was operated under both Analyst 1.1 control (ABSCIEX) and LabSolutions software. Chromatographic separation of tetracycline, doxycycline, and tigecycline as well as their metabolites was achieved on an Acquity ultraperformance liquid chromatography (UPLC) T3 (Waters, Milford, MA) column, 50 by 2.1 mm (inside diameter), packed with 1.8- μm particles. Separation was performed using a flow of rate of 0.2 ml/min under the following gradient conditions: 10 to 15% solvent B in 1.5 min, 15 to 95% solvent B in 6 min, and 95 to 10% solvent B in 0.1 min, where solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. The column oven temperature was 40°C, and sample injection volume was 10 μl . The MS instrument was operated in a positive ion mode with a turbo V electrospray source. Mass spectra were acquired as metabolites eluted from the LC by scanning a mass range of m/z 350 to 700.

Electrophoretic mobility shift assays. The binding of MAB_1497c to the region upstream of MAB_1497c was analyzed by EMSA. Two DNA substrates were used. (i) A 180-bp DNA substrate was synthesized by PCR using the following primers: 5'-CCGATTGAACGGGTGCGCTCCCTGTTTC-3'/5'-6-carboxyfluorescein (FAM)/CGGTGCTATCCATCGCGTATCAGTACCTGCAATGC-3'. (ii) A 35-bp duplex DNA comprising of an inverted repeat sequence immediately upstream of MAB_1497c was synthesized by annealing primers 5'-TGACATAAGTGTACGGTATGCGTACACTTATGTCA-3' and 5'-FAM/TGACATAAGTGTA CGCATACCGTACACTTATGTCA-3'. The FAM-labeled DNA was incubated for 20 min with 1.2 μM purified MAB_1497c in a binding buffer containing 20 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl_2 , and 50% glycerol. Tetracycline, doxycycline, or tigecycline was added to a final concentration of 8.0 μM either during complex formation or after incubation of DNA substrate with MAB_1497c, followed by an additional incubation of 20 min. MabTetX was added to a final concentration of 0.2 μM where indicated. The samples were separated by 8% Tris-boric acid-EDTA native gel electrophoresis, and the DNA protein complex was visualized using a Typhoon Trio Plus imager (GE Health Sciences).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00119-18>.

SUPPLEMENTAL FILE 1, XLSX file, 0.5 MB.

SUPPLEMENTAL FILE 2, PDF file, 1.1 MB.

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