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Mycobacterium abscessus WhiB7 Regulates a Species-Specific Repertoire of Genes To Confer Extreme Antibiotic Resistance

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ABSTRACT *Mycobacterium abscessus* causes acute and chronic bronchopulmonary infection in patients with chronic lung damage, of which cystic fibrosis (CF) patients are particularly vulnerable. The major threat posed by this organism is its high intrinsic antibiotic resistance. A typical treatment regimen involves a 6- to 12-month-long combination therapy of clarithromycin and amikacin, with cure rates below 50% and multiple side effects, especially due to amikacin. In the present work, we show that *M. abscessus whiB7*, a homologue of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis whiB7* with previously demonstrated effects on intrinsic antibiotic resistance, is strongly induced when exposed to clinically relevant antibiotics that target the ribosome: erythromycin, clarithromycin, amikacin, tetracycline, and spectinomycin. The deletion of *M. abscessus whiB7* results in sensitivity to all of the above-mentioned antibiotics. Further, we have defined and compared the *whiB7* regulon of *M. abscessus* with the closely related nontuberculous mycobacterium (NTM) *M. smegmatis* to demonstrate the induction of a species-specific repertoire of genes. Finally, we show that one such gene, *eis2*, is specifically induced in *M. abscessus* by *whiB7* and contributes to its higher levels of intrinsic amikacin resistance. This species-specific pattern of gene induction might account for the differences in drug susceptibilities to other antibiotics and between different mycobacterial species.

KEYWORDS *Mycobacterium*, *abscessus*, antibiotic resistance, intrinsic, *whiB7*

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The *Mycobacterium abscessus* group is a rapid-growing, nontuberculous species of mycobacteria (NTM/RGM) comprised of three subspecies, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *abscessus massiliense*, and has emerged as an important human pathogen over the last 10 years (1–8). *M. abscessus* is one of the most prominent causes of bronchopulmonary infections in patients with underlying lung damage, such as bronchiectasis, prior tuberculosis, and cystic fibrosis (CF), and leads to either acute lung failure or chronic disease with a progressive decline in lung function. *M. abscessus* infections comprise 80% of all RGM-associated pulmonary infections, with an incidence of ~1 per 100,000 of the general population and a prevalence of 6 to 13% in CF patients (4, 9–13). In addition, *M. abscessus* causes skin and soft tissue infections (SSTI) postsurgery and posttrauma (14, 15). In rare cases, *M. abscessus* can also cross the blood-brain barrier to cause meningitis and cerebral abscesses in immunocompetent individuals (16, 17).

Mycobacteria are intrinsically drug resistant; nontuberculous mycobacteria (NTM) are particularly drug resistant, of which *M. abscessus* stands out as one of the most resistant bacterial species known. *M. abscessus* is resistant to most antimicrobial agents, including antituberculosis drugs (rifampin, isoniazid, ethambutol, and pyrazinamide), and it presents a challenge in public health settings (18, 19). Only a few antibiotics, clarithromycin (0 to 38%), cefoxitin (15%), and amikacin (7.7%), exhibit activity against

M. abscessus (with low rates of acquired resistance shown in parenthesis) (20, 21). A particularly vexing aspect of *M. abscessus* therapy is that there is poor correlation between *in vitro* antibiotic susceptibility and *in vivo* efficacy; a large part of macrolide resistance is attributed to inducible *erm*(41) expression (20, 22–25). The current regimen of treatment typically involves an extended (12-month) combination therapy of a macrolide (clarithromycin or azithromycin) and intravenous amikacin and cefoxitin/imipenem. Despite such aggressive treatments, the average rate of eradication is only 45% (26).

Intrinsic resistance in mycobacteria is thought to be a synergistic action of a waxy and impermeable cell envelope and internal defense mechanisms (27). Diffusion of antibiotics into the bacterial cytosol, albeit at a reduced rate, induces a massive transcriptional reprogramming that results in changes in growth rate, metabolism, and induction of genes facilitating drug resistance, such as those encoding efflux pumps and enzymes that modify either the antibiotic or its target. The existence of ~190 transcription regulators which include two-component systems, protein kinases, as well as >100 transcription activators and repressors in the mycobacterial genome suggests an exceptionally intricate and flexible system of gene regulation (28); however, the hierarchy and topology of molecular networks in the antibiotic-induced global reprogramming of gene expression are poorly understood. One of the best studied regulators of this reprogramming circuit is WhiB7, a transcriptional activator that belongs to the WhiB family of transcriptional regulators conserved in actinomycetes (29, 30). WhiB7 proteins have a variable N terminus and a conserved core sequence characterized by four iron-binding conserved cysteine residues, a G/Y-rich motif, and a positively charged AT-hook that binds an AT-rich region in DNA (31). *whiB7* is induced in the presence of several structurally unrelated antibiotics, such as tetracycline, macrolides, and aminoglycosides, as well as compounds that perturb respiration, redox balance, and iron starvation (30–34). A deletion of *whiB7* in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* results in multidrug sensitivity (34). Whole-genome sequencing of *M. abscessus* subsp. *abscessus* ATCC 19977 (referred to as *M. abscessus* in the current work) reveals the presence of a transcription factor, MAB_3508c, which is 75% identical to *M. smegmatis* and *M. tuberculosis whiB7*.

In the present study, we have investigated the effect of MAB_3508c deletion on the sensitivity of *M. abscessus* to six unrelated antibiotics: erythromycin, clarithromycin, streptomycin, spectinomycin, amikacin, and tetracycline. We establish that MAB_3508c is the *M. abscessus whiB7* and is required for intrinsic resistance of *M. abscessus* to all antibiotics tested; this effect is specific, and a deletion of MAB_3508c (here referred to as Mab*whiB7*) does not influence rifampin or isoniazid resistance. Further, we show that although *whiB7* is required for multidrug resistance in both *M. abscessus* and *M. smegmatis*, the *whiB7* regulon shows minimal overlap between the two species. The species-specific repertoire of *whiB7* gene induction between different mycobacterial species might account for the observed differences in their drug susceptibilities. This is supported by the *whiB7*-dependent induction of *eis2* exclusively in *M. abscessus* and a direct role of *eis2* in high levels of amikacin resistance.

RESULTS

MAB_3508c is highly induced by ribosome-targeting antibiotics. *M. abscessus* and *M. smegmatis* are two fast-growing NTMs with a high conservation in their genes and genetic organization. However, *M. abscessus* and *M. smegmatis* have strikingly distinct susceptibilities to amikacin, tetracycline, spectinomycin, and streptomycin (Table 1 and Fig. 1) while showing comparable tolerance to the macrolides erythromycin and clarithromycin. In an ongoing RNA sequencing (RNA-Seq) study of antibiotic-induced genes in *M. abscessus* (data not shown), we found that MAB_3508c is the earliest and the most highly induced gene upon exposure to sublethal concentrations of all ribosome-targeting antibiotics tested (Fig. 2A and S1). These results were subsequently confirmed using quantitative PCR (Fig. 2B). The extent of induction of MAB_3508c is a function of the time of exposure as well as the antibiotic used. As seen

TABLE 1 Survival of wild-type *M. abscessus* ATCC 19977 and *M. smegmatis* mc²155 in a 2-fold dilution series of antibiotics in Middlebrook 7H9 medium

Antibiotic	MIC_{99} (μg/ml)[a]	
	M. abscessus WT	*M. smegmatis* WT
Erythromycin	0.5–1	2.5
Clarithromycin	0.25–0.5	0.25–0.5
Amikacin	8	0.625
Spectinomycin	>256	50
Tetracycline	16	0.2
Streptomycin	16	0.3

[a]MIC_{99}, the minimum concentration of antibiotic required to inhibit 99% of growth.

in Fig. 2A and B, sublethal doses of erythromycin, clarithromycin, tetracycline, and spectinomycin were the strongest inducers of MAB_3508c (induced >100-fold within 30 min of exposure). The induction of MAB_3508c by amikacin is much weaker than that by the above-mentioned antibiotics. We tested a range of amikacin concentrations and found comparable levels of induction using 16 μg/ml; even at this concentration, the induction of MAB_3508c was low at early times of exposure but increased upon extension of the exposure time (Fig. 2 and S2). However, the related aminoglycoside kanamycin is a stronger inducer of MAB_3508c. Finally, streptomycin was the weakest inducer of MAB_3508c.

Deletion of MAB_3508c causes varied levels of susceptibility to ribosome-targeting antibiotics. MAB_3508c is 75% identical to *M. smegmatis* and *M. tuberculosis whiB7*, suggesting a conserved role of MAB_3508c in *M. abscessus* drug resistance (Fig. 3A). We constructed an isogenic deletion of the gene in *M. abscessus* ATCC 19977 using phage recombineering (Fig. 3B) (35). Following transformation with the knockout construct in the recombineering strain, we obtained 130 apramycin-resistant (Apr^r) colonies, of which 20 were screened using PCR using the flanking F_{check} and R_{check} primers. Three colonies were found to contain the expected fragment size corresponding to a double-crossover event (~15%). The frequency correlated with previously reported recombineering efficiencies (36). The insertional mutant was unmarked by recombination between the *loxP* sites mediated by the Cre recombinase, as shown in Fig. 3B and C. The unmarked deletion mutant was also confirmed using PCR and sequencing. A complemented strain was constructed by transforming the deletion strain with pMH94-MAB_3508c, a phage L5-based integration vector, in which MAB_3508c is expressed from the constitutive promoter P_{hsp60}.

Wild-type *M. abscessus*, the ΔMAB_3508c mutant, and the complemented strain were tested for their susceptibilities to erythromycin, clarithromycin, tetracycline, ami-

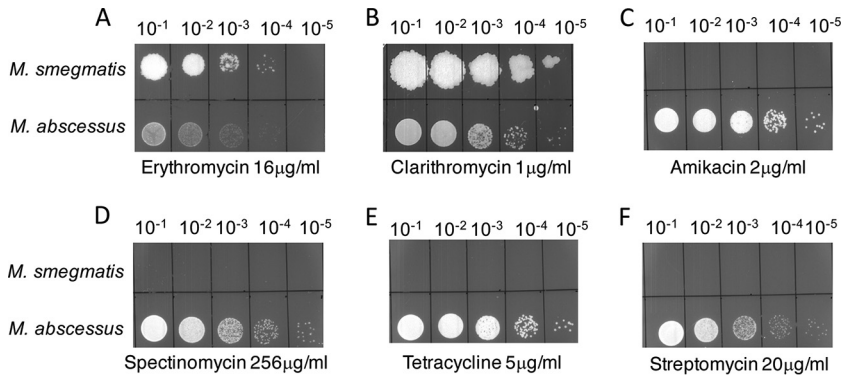

FIG 1 Differences in antibiotic sensitivities between *M. abscessus* ATCC 19977 and *M. smegmatis* mc²155 (A to F). Ten-fold serial dilutions of *M. abscessus* ATCC 19977 and *M. smegmatis* mc²155 grown to A_{600} of 0.7 and spotted on Middlebrook 7H10 containing indicated concentrations of antibiotics. *M. abscessus* is more resistant to amikacin, tetracycline, streptomycin, and spectinomycin than *M. smegmatis* but the sensitivities to clarithromycin and erythromycin are comparable.

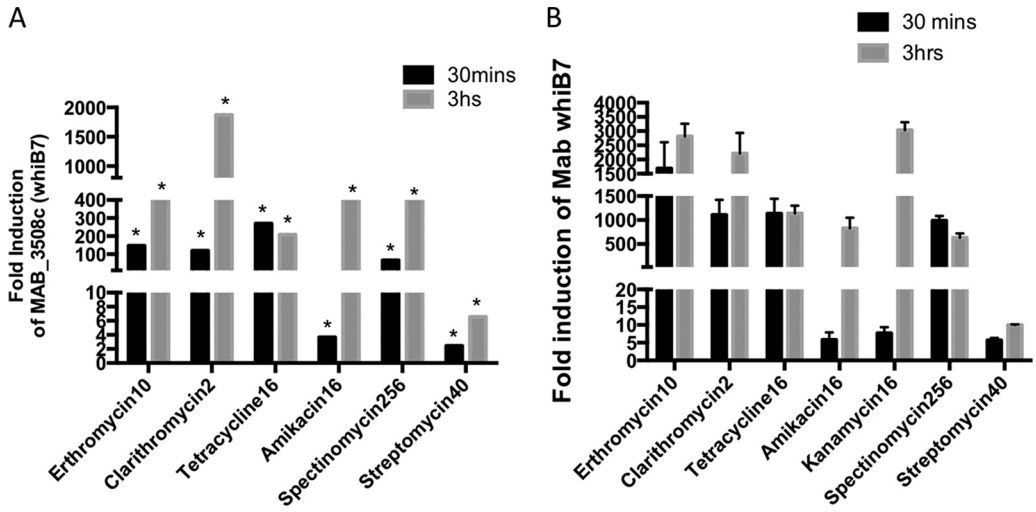

FIG 2 Time course of induction of *whiB7* upon antibiotic exposure. (A) The fold induction of transcript of MAB_3508c in wild-type *M. abscessus* ATCC 19977 exposed to amikacin (16 μg/ml), clarithromycin (2 μg/ml), erythromycin (10 μg/ml), tetracycline (16 μg/ml), streptomycin (40 μg/ml), and spectinomycin (256 μg/ml) over unexposed samples was determined in previous RNA-Seq studies. The asterisks (*) indicate q values of <0.001 (B) The results were verified by quantitative PCR (qPCR) and expressed as a fold overexpression over unexposed samples. Data represent the mean $\pm$ standard deviation (SD), $n = 3$. *sigA* was used as an endogenous control.

kacin, streptomycin, and spectinomycin by spotting a 10-fold serial dilution of each strain on Middlebrook 7H10 containing oleic acid-albumin-dextrose-catalase (OADC) and the indicated concentrations of antibiotics (Fig. 4). MICs were also determined in liquid media as described in Materials and Methods and Table 2. The deletion of MAB_3508c resulted in sensitivity to all the antibiotics tested and is reminiscent of the behavior of *M. tuberculosis* and *M. smegmatis* Δ*whiB7* mutants. Moreover, this effect was specific, and a deletion of MAB_3508c did not influence resistance to rifampin or isoniazid (data not shown). We here refer to MAB_3508c as Mab*whiB7*. The deletion of Mab*whiB7* resulted in a spectrum of effects on antibiotic resistance that were not entirely predictable from the induction profile. The ΔMab*whiB7* mutant is hypersensitive to erythromycin, clarithromycin, and spectinomycin compared to the wild-type parent strain and correlates with the high levels of induction of Mab*whiB7* upon exposure to these drugs. The sensitivity of the mutant is restored in the complemented strain (Fig. 4). Streptomycin is an ineffective inducer of Mab*whiB7*, and the ΔMab*whiB7* mutant shows a modest defect in streptomycin sensitivity (Fig. 4). However, although amikacin is a weak inducer, the ΔMab*whiB7* mutant is highly sensitive to amikacin. In contrast, tetracycline is a potent inducer of Mab*whiB7*, but the deletion mutant is only mildly more sensitive to tetracycline than the wild type (Fig. 4). Together, these findings suggest that the role of Mab*whiB7* is complex and that it plays a varied role in the intrinsic resistance to different antibiotics. Moreover, there is little correlation between the level of induction of Mab*whiB7* and the extent of resistance conferred. While Mab*whiB7* is perhaps the primary regulator of intrinsic resistance toward erythromycin, clarithromycin, and spectinomycin, additional WhiB7-independent determinants may be required to confer resistance to tetracycline and streptomycin.

WhiB7 regulates species-specific repertoire of genes in *M. smegmatis* and *M. abscessus*. Despite a central contribution of WhiB7 in drug resistance of *M. abscessus* and *M. smegmatis*, the level of resistance between the two species differs by 20- to 200-fold. We speculated that the *whiB7* regulon of *M. abscessus* may be different from that of *M. smegmatis* and includes genes that confer increased levels of intrinsic drug resistance. We used RNA-Seq to determine the *whiB7* regulon of *M. abscessus* and *M. smegmatis* using the Δ*whiB7* mutant strains of the two species complemented with respective *whiB7* genes expressed from a constitutive promoter, P_{hsp60}. (see RNA-Seq in

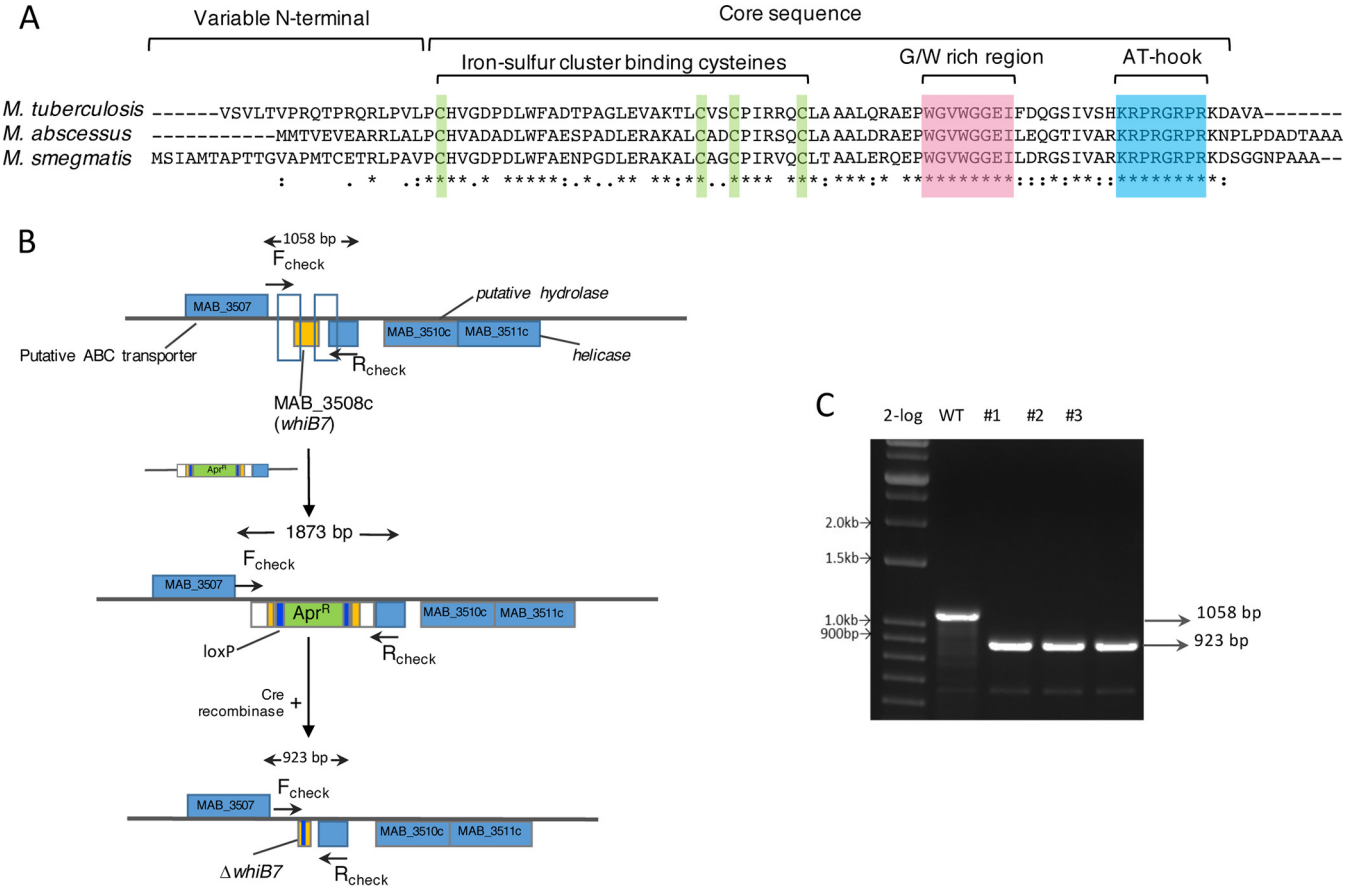

FIG 3 Deletion of *M. abscessus whiB7*. (A) Multiple-sequence alignment of WhiB7 from *M. abscessus*, *M. smegmatis*, and *M. tuberculosis* showing identical residues (*) and conserved motifs. (B) Schematic representation of creating a deletion of MAB_3508c (*whiB7*) using phage recombineering and unmarking using the Cre-lox system. (C) Three clones were selected (no. 1 to 3), and the *whiB7* gene was amplified using the F_{check} and R_{check} primers, followed by confirmation by Sanger sequencing.

Data Set S1 in the supplemental material). We identified 128 and 96 genes comprising the *whiB7* regulon of *M. abscessus* and *M. smegmatis*, respectively, using the criteria of >4-fold induction in the *whiB7*-overexpressing strain compared to the Δ*whiB7* mutant and *q* values of <0.01. Exceptions for statistical significance were made for highly

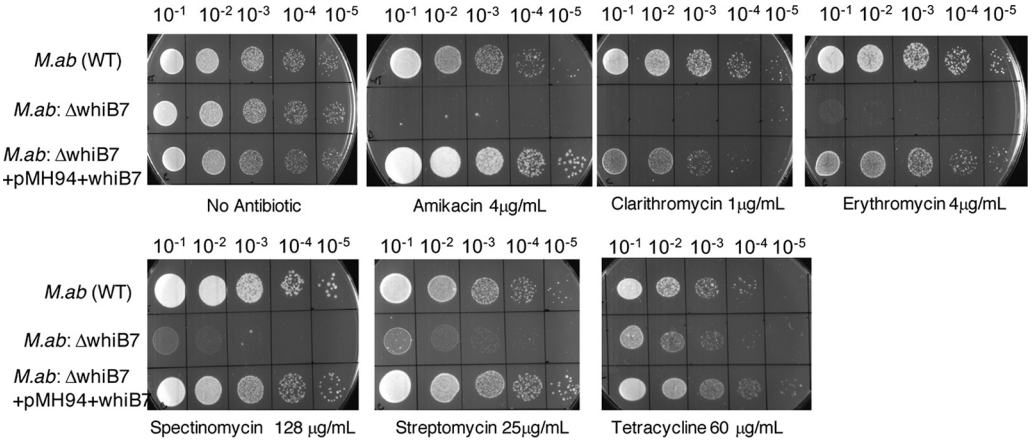

FIG 4 Deletion of MAB_3508c (*whiB7*) renders *M. abscessus* hypersensitive to multiple antibiotics. Ten-fold dilutions of the Δ*whiB7* mutant cells were spotted on Middlebrook 7H10/OADC containing indicated concentrations of antibiotics. The mutant is hypersensitive to clarithromycin, erythromycin, amikacin, streptomycin, and spectinomycin but marginally more sensitive to tetracycline than the wild-type parent strain. A complementing strain containing an integrated copy of *whiB7* expressed from a constitutive promoter P_{hsp60} restores antibiotic resistance. *M.ab*, *M. abscessus*.

TABLE 2 Survival of wild-type *M. abscessus* ATCC 19977 and ΔMAB_3508c in a 2-fold dilution series of antibiotics in Middlebrook 7H9 medium

Antibiotic	MIC_{99} (μg/ml)[a]	
	M. abscessus WT (μg/ml)	*M. abscessus* Δ*whiB7* mutant (μg/ml)
Amikacin	8	2
Clarithromycin	0.25–0.5	0.0625
Erythromycin	0.5–1	0.0625
Spectinomycin	>256	32–64
Tetracycline	16	8
Streptomycin	16	8

[a]MIC_{99}, the minimum concentration of antibiotic required to inhibit 99% of growth after 96 h.

induced genes. Surprisingly, we found an overlap of only 16 genes in the *whiB7* regulons of the two species (Fig. 5, Table 3, and RNA-Seq in Data Set S1). Of the remaining 111 genes in the *M. abscessus* WhiB7 regulon, 56 genes were unique to *M. abscessus* (Fig. 5, shaded in green). Notably, 55 genes, despite having clear orthologues in *M. smegmatis*, did not comprise the *M. smegmatis* WhiB7 regulon (Fig. 5, shaded in yellow).

MAB_4532c confers amikacin resistance in *M. abscessus*. Amikacin is the front-line drug used in combination with clarithromycin against *M. abscessus* infections. However, intrinsic resistance to amikacin is ~10-fold higher in *M. abscessus* than in *M. smegmatis*, despite the fact that *whiB7* is upregulated in both species (more strongly in *M. smegmatis*) upon amikacin exposure (Fig. 6B and D). This could be due to one of two possibilities: (i) the *M. abscessus*-specific *whiB7* regulon contains genes uniquely induced in *M. abscessus* but not in *M. smegmatis* which contribute to the enhanced amikacin resistance in *M. abscessus*, or (ii) amikacin induces genes outside the *whiB7* regulon in *M. abscessus* that contribute to its increased tolerance to amikacin. In order to identify such determinants of *M. abscessus* that contribute to the 10-fold greater resistance to amikacin, we generated and introduced a partial *M. abscessus* genomic DNA library into *M. smegmatis* and screened for the appearance of growth on Middlebrook 7H10/OADC containing 8 μg/ml amikacin, a concentration that is lethal to *M. smegmatis*. We obtained 500 amikacin-resistant colonies (compared to none when using a control vector), of which 40 were amplified by PCR using junction primers and sequenced. All sequenced amikacin-resistant clones were found to contain MAB_4532c. MAB_4532c was recloned into an integrating vector and was confirmed to increase the

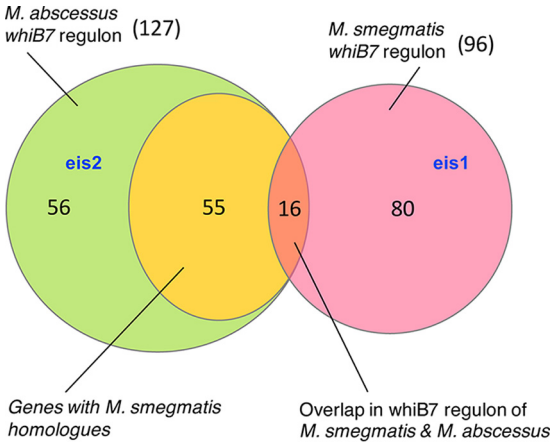

FIG 5 Species-specific regulons of WhiB7 in *M. smegmatis* and *M. abscessus*. *M. smegmatis* and *M. abscessus whiB7* were expressed from constitutive promoters in respective Δ*whiB7* mutant strains. RNA-Seq analysis was performed to determine the regulon of WhiB7 in each species, and the overlap in the regulon is represented. *eis2* is WhiB7 regulated exclusively in *M. abscessus*, whereas *eis1* is exclusively induced in *M. smegmatis* by WhiB7. Numbers refer to the number of genes in each category.

TABLE 3 Overlapping genes in the *whiB7* regulons of *M. smegmatis* and *M. abscessus*

M. abscessus gene	*M. smegmatis* homologue	Product[a]
MAB_1341	MSMEG_5087	Hypothetical protein
MAB_1342	MSMEG_5086	Probable fatty-acid-CoA ligase FadD
MAB_1395	MSMEG_5047	Multidrug resistance transporter, Bcr/CflA family
MAB_1846	MSMEG_5102	ABC transporter ATP-binding protein
MAB_2273	MSMEG_2795	Putative MFS transporter
MAB_2355c	MSMEG_3140	ABC transporter ATP-binding protein
MAB_2396	MSMEG_2306	Acetyltransferase
MAB_2595	MSMEG_6576	Pyridoxamine 5′-phosphate oxidase
MAB_2736c	MSMEG_3140	ABC transporter
MAB_2780c	MSMEG_5187	TetV efflux pump
MAB_3042c	MSMEG_2736	GTP-binding protein
MAB_3078	MSMEG_0091	TetR transcriptional regulator
MAB_3467c	MSMEG_5611	18-kDa antigen (HSP 16.7)
MAB_3508c	MSMEG_1953	*whiB7*
MAB_3762	MSMEG_1530	Hypothetical protein
MAB_4294	MSMEG_0688	Aspartate aminotransferase

[a]CoA, coenzyme A; MFS, major facilitator superfamily.

amikacin resistance of wild-type *M. smegmatis* from 0.4 μg/ml to 8 μg/ml, as well as kanamycin resistance from 1 μg/ml to 8 μg/ml (Fig. 6A). However, resistance to streptomycin was unaffected (data not shown). This is in agreement with observations of Rominski et al., who recently showed that a deletion of MAB_4532c results in amikacin and kanamycin sensitivity (37).

Kanamycin resistance of *M. tuberculosis* has been previously attributed to a member encoding GNAT family acetyltransferases, *eis1* (38). Sequence analysis suggests that MAB_4532c belongs to the GNAT family of acetyltransferases with 29% homology to *M. abscessus eis1* (MAB_4124) and is referred to as *eis2* (37). Interestingly, both paralogs of *eis*, *eis1* (MSMEG_3513) and *eis2* (MSMEG_4540), are found in *M. smegmatis*. MAB_4532c (*eis2*) is induced ~100-fold upon amikacin exposure in *M. abscessus* in a *whiB7*-dependent manner (RNA-Seq in Data Set S1 and Fig. 6C), whereas MAB_4124 (*eis1*) is not amikacin inducible (Fig. 6C and S2). In contrast, while MSMEG_3513 (*eis1*) comprises the *M. smegmatis* WhiB7 regulon and is induced to low levels by amikacin, MSMEG_4540 (*eis2*) remains largely unresponsive and is also not a part of the *M. smegmatis* WhiB7 regulon (Fig. 6E and RNA-Seq in Data Set S1). Overexpression of MSMEG_4540 (*eis2*) from a constitutive promoter also does not increase amikacin or kanamycin resistance of *M. smegmatis* to the levels displayed by *eis2* of *M. abscessus*, suggesting that although *eis2* from the two species share ~30% identity at the level of protein sequence, they are not functionally equivalent (Fig. 6A).

DISCUSSION

The influx of antibiotics into the mycobacterial cytosol induces a massive transcriptional reprogramming that results in changes in growth rate, metabolism, and induction of drug resistance genes. One of the best studied regulators of this reprogramming circuit is *M. tuberculosis* WhiB7, a transcriptional activator that regulates the expression of several genes involved in resistance to tetracycline, macrolides, and aminoglycosides (30, 31, 33, 34). Consistent with previous findings, our study here demonstrates an important role of WhiB7 in the intrinsic resistance of *M. abscessus* to several ribosome-targeting antibiotics. Similar to *M. tuberculosis*, *whiB7* is the earliest gene induced in *M. abscessus* and *M. smegmatis* in response to ribosomal antibiotics and plays a critical role in the expression of downstream drug resistance effectors. Previous work by Ramón-García et al. demonstrates that the disruption of *whiB7* from three different actinomycetes (*M. smegmatis*, *Streptomyces lividans*, and *Rhodococcus jostii*) results in different resistance profiles despite WhiB7 being functionally equivalent in these bacteria and is presumably due to the regulation of distinct sets of genes (31). In the present study, we have defined the *whiB7* regulons of *M. abscessus* and *M. smegmatis*,

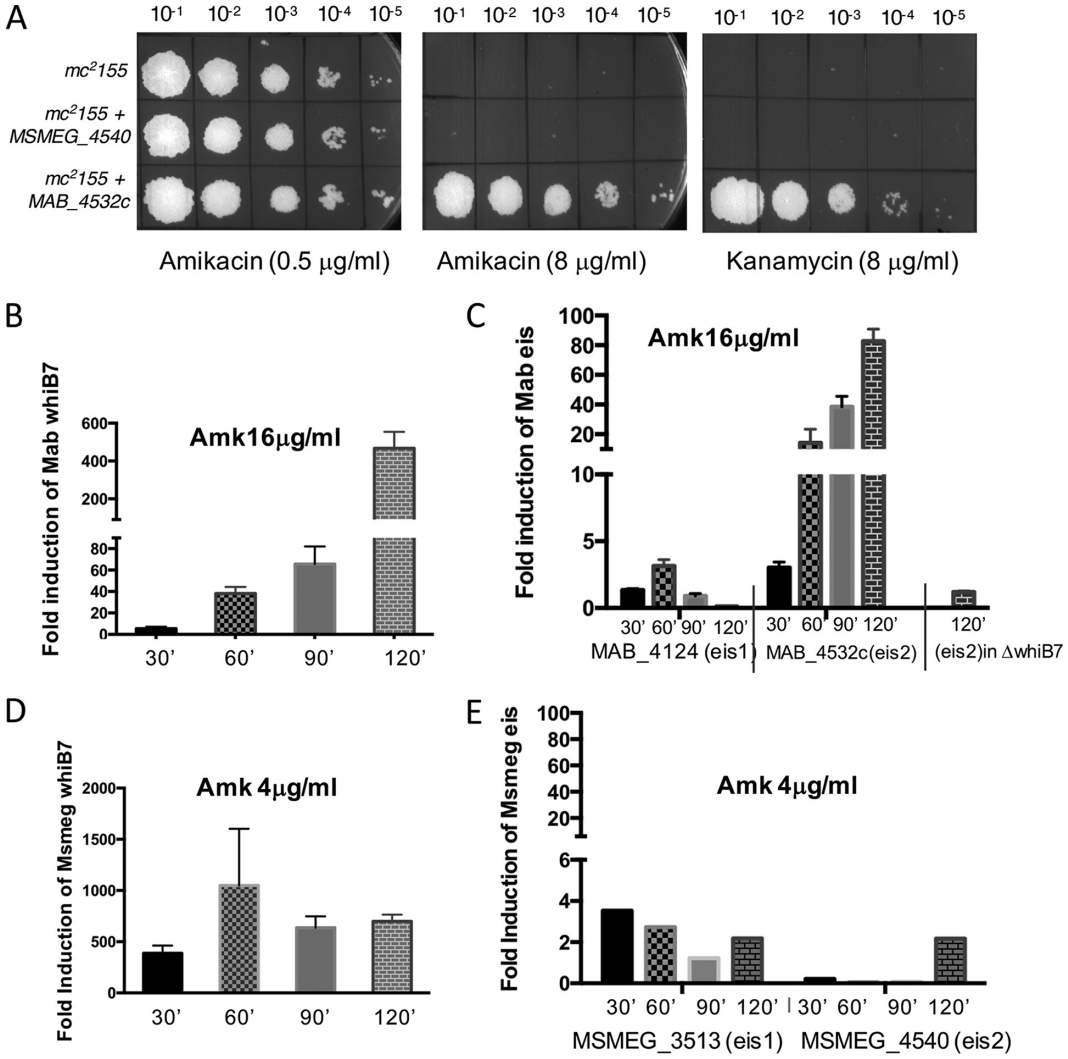

FIG 6 MAB_4532c is induced exclusively in *M. abscessus* as part of the *whiB7* regulon and confers amikacin (Amk) resistance. (A) Heterologous expression of MAB_4532c (*eis2*) from a constitutive promoter integrated at the L5 attB site increases amikacin and kanamycin resistance of *M. smegmatis* from 0.4 μg/ml to 8 μg/ml. Similar overexpression of MSMEG_4540 (*eis2*) is insufficient to enhance amikacin/kanamycin resistance in *M. smegmatis*. (B to E) Wild-type *M. smegmatis* and *M. abscessus* and the Δ*Mabwhi7* mutant were grown to an A_{600} of 0.7, exposed to sublethal concentrations of amikacin, as indicated, and the amount of *whiB7*, *eis1*, and *eis2* transcripts were determined by qPCR and plotted as fold induction over an unexposed control. Data represent the mean ± SD, $n = 3$. Although *whiB7* is induced in both bacteria, *eis2* is induced only in *M. abscessus* in a *whiB7*-dependent manner and confers species-specific amikacin sensitivity.

which reveals a minimal overlap of 16 genes. This suggests that although WhiB7 is the master regulator in both species, the set of genes regulated by WhiB7 is specific to a given mycobacterial species. We hypothesize that the existence of species-specific repertoires of genes likely forms the basis of varying antibiotic susceptibilities within mycobacterial species. The extreme antibiotic resistance of *M. abscessus* can therefore be attributed to two classes of genes within the *whiB7* regulon: (i) genes that lack orthologues in *M. smegmatis*, and (ii) genes that have orthologues in *M. smegmatis* but are not *whiB7* inducible in *M. smegmatis*. This idea is further supported by the involvement of *eis2* in amikacin and kanamycin resistance of *M. abscessus*. Amikacin and kanamycin induce *eis2*, but not *eis1*, via the *whiB7* pathway in *M. abscessus*. *M. smegmatis eis2* is not a part of the *whiB7* regulon, even though amikacin is a fairly strong inducer of *whiB7* in *M. smegmatis*. The differential amikacin sensitivity between the two species therefore occurs due to the species-specific inclusion of a gene with unique enzymatic properties, MAB_4532c (*eis2*), in the *whiB7* regulon of *M. absces-*

TABLE 4 Strains and plasmids used in the present study

Lab ID[a]	Strain	Strain description
M. abscessus	*M. abscessus* subsp. *abscessus* ATCC 19977	Wild-type ATCC strain
MABPG1	*M. abscessus* subsp. *abscessus* ATCC 19977 ΔMAB_3508c	Isogenic deletion in type strain
MABPG2	*M. abscessus* subsp. *abscessus* ATCC 19977 ΔMAB_3508c/pMH95hspMab3508c	MABPG1 overexpressing MAB_3508c from P_{hsp60} integrated at L5 locus
MSPG1	*M. smegmatis* ΔMSMEG_1953	Isogenic deletion of MSMEG_1953 in type strain
MSPG2	*M. smegmatis* ΔMSMEG_1953/ pSJ25hspMSMEG_1953	MSPG1 overexpressing MSMEG_1953 from P_{hsp60} integrated at Bxb1 locus

[a]ID, identification.

sus. An in-depth study of species-specific patterns of gene induction could additionally account for the differences in drug susceptibilities to other antibiotics and between different mycobacterial species.

Our results further demonstrate that WhiB7 contributes differentially to resistance to different antibiotics. For example, the macrolides clarithromycin and erythromycin are potent inducers and cause near-saturating levels of induction of *whiB7* within the first 30 min of exposure. Consistent with this rapid induction, a *whiB7* mutant loses the distinctive resistance to these antibiotics and is suggestive of macrolide resistance being determined entirely by genes within the *whiB7* regulon. In contrast, the deletion of Mab*whiB7* results in a moderate reduction in streptomycin and tetracycline resistance, suggesting that genes outside the *whiB7* regulon also play a role in the resistance to these antibiotics. Furthermore, induction of *whiB7* by the synthetic aminoglycosides amikacin and kanamycin is gradual and only achieves high levels of expression upon extending the exposure time and concentration. This delayed induction of *whiB7* and its downstream drug resistance determinants possibly overestimates the amikacin sensitivity of *M. abscessus* in *in vitro* drug susceptibility assays. The efficacy of amikacin *in vivo* therefore may not reflect *in vitro* conclusions. Moreover, our data suggest that the timing and rate of induction of *whiB7* by clinically relevant antibiotics can affect the efficacy of the treatment regimen. The use of macrolides early in treatment would cause a rapid induction of *whiB7* and compromise the outcome of amikacin therapy for *M. abscessus* infections. However, an initial amikacin treatment that is slow to induce Mab*whiB7*, followed up by macrolide treatment, is likely to enhance the effectiveness of this combined regimen and concomitantly reduce the duration of therapy.

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 (Table 4). Antibiotics were added as required to the indicated amounts. Gene replacement mutants were constructed using recombineering, as described previously (35). The recombineering construct was generated by cloning in the multiple-cloning sites flanking the apramycin cassette of pYUB854. The left arm and right arms were generated using the primer pairs 5′-GTGCCTTTGTCGTCTTAAGCCGATCGC-3′/5′-GTGGAGGCCTCTAGATCT-3′ and 5′-AAGCTTCCCACTGCCCGA-3′/5′-GGCCAAAGCGGTCTGACTAGTAATCCATCACCTG-3′, respectively. Mutant clones were checked using F_{check} (5′-CGGAGACACCTTGTGGCGTGATGCC-3′) and R_{check} (CCCGGACAGCTGAACGTCCGG).

Antibiotic sensitivity assays. Wild-type and *whiB7* mutant strains of *M. smegmatis* and *M. abscessus* were grown to an A_{600} of 0.6 to 0.7. Cells were tested for their susceptibility to various antibiotics by spotting a 10-fold serial dilution initially on Middlebrook 7H10 (Difco) plates containing a range of each drug: tetracycline, 0.05 to 120 μg/ml; clarithromycin, 0.01 to 1.0 μg/ml; erythromycin, 4 to 40 μg/ml; spectinomycin, 1 to 256 μg/ml; streptomycin, 0.2 to 20 μg/ml; amikacin, 0.05 to 8 μg/ml; and kanamycin, 0.05 to 8 μg/ml. The concentration of antibiotic showing the best dynamic range in each case was then used in subsequent experiments. Antibiotic susceptibility in liquid media was assayed by inoculating the desired strain in a 2-fold dilution series of each antibiotic at an initial A_{600} of 0.0004. The cultures were incubated at 37°C, and the A_{600} was measured after 48 h for *M. smegmatis* and after 96 h for *M. abscessus*.

RNA preparation, quantitative PCR, and RNA-Seq analysis. Total RNA was prepared from wild-type *M. abscessus* ATCC 19977, wild-type *M. smegmatis* mc²155, and the corresponding Δ*whiB7* mutant strains containing a chromosomally integrated copy of *whiB7* expressed from a constitutive promoter and grown to exponential phase in Middlebrook 7H9-ADC/OADC using the Qiagen RNA preparation kit, followed by DNase I treatment. Approximately 5 μg of total RNA samples was 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 ScriptSeq version 2 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 (39, 40).

Wild-type *M. abscessus*, *M. abscessus* Δ*whiB7* mutant, and wild-type *M. smegmatis* were exposed to different concentrations of antibiotics for either 30 min or 3 h. 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: for MAB_3508c, 5′-CCTGTGGTTCGCGGAAA-3′/5′-CCCTGCTCAAGAATCTCACC-3′; for MAB_4532c, 5′-GAGCTTCATGTGCAAG AGGT-3′/GCGCCGTGATACTTGATCTT; for MAB_4124, 5′-CCCGTCAAGCCTTATGTAGTG-3′/5′-CAAGATCGTCAA CGGATATGGT-3′; for MSMEG_4540, 5′-GCGACGATCGAAGTGGATG-3′/5′-GCCGGTTGTTGGTGTAGAT-3′; and for MSMEG_3513, 5′-GAACCCGAACAGACACAGG-3′/5′-GCTGCATGTCCAGGTACAG-3′. 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.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01347-17.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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