RESEARCH ARTICLE

Keto-Mycolic Acid-Dependent Pellicle Formation Confers Tolerance to Drug-Sensitive *Mycobacterium tuberculosis*

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ABSTRACT The chronic nature of tuberculosis (TB), its requirement of long duration of treatment, its ability to evade immune intervention, and its propensity to relapse after drug treatment is discontinued are reminiscent of other chronic, biofilm-associated bacterial diseases. Historically, *Mycobacterium tuberculosis* was grown as a pellicle, a biofilm-like structure, at the liquid-air interface in a variety of synthetic media. Notably, the most widely administered human vaccine, BCG, is grown as a pellicle for vaccine production. However, the molecular requirements for this growth remain ill defined. Here, we demonstrate that keto-mycolic acids (keto-MA) are essential for pellicle growth, and mutants lacking in or depleted of this MA species are unable to form a pellicle. We investigated the role of the pellicle biofilm in the reduction of antibiotic sensitivity known as drug tolerance using the pellicle-defective Δ*mmaA4* mutant strain. We discovered that the Δ*mmaA4* mutant, which is both pellicle defective and highly sensitive to rifampicin (RIF) under planktonic growth, when incorporated within the wild-type pellicle biofilm, was protected from the bactericidal activity of RIF. The observation that growth within the *M. tuberculosis* pellicle biofilm can confer drug tolerance to a drug-hypersensitive strain suggests that identifying molecular requirements for pellicle growth could lead to development of novel interventions against mycobacterial infections. Our findings also suggest that a class of drugs that can disrupt *M. tuberculosis* biofilm formation, when used in conjunction with conventional antibiotics, has the potential to overcome drug tolerance.

IMPORTANCE Two of the most important questions in tuberculosis (TB) research are (i) how does *Mycobacterium tuberculosis* persist in the human host for decades in the face of an active immune response and (ii) why does it take six months and four drugs to treat uncomplicated TB. Both these aspects of *M. tuberculosis* biology are reminiscent of infections caused by organisms capable of forming biofilms. *M. tuberculosis* is capable of growing as a biofilm-like structure called the pellicle. In this study, we demonstrate that a specific cell wall component, keto-mycolic acid, is essential for pellicle growth. We also demonstrate that a strain of *M. tuberculosis* that is both drug sensitive and pellicle defective exhibits commensal behavior and becomes drug tolerant by becoming part of a heterogeneous pellicle, a characteristic of multispecies biofilms. These observations could have important implications for identifying novel pathways for *M. tuberculosis* drug tolerance and the design of new modalities to rapidly treat TB.

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In the laboratory, bacteria are usually grown and studied as dispersed, planktonic, pure cultures. In nature, however, this is seldom the case. One preferred microbial lifestyle is that of a surface-adherent, multispecies microbial community called biofilm that is physiologically and phenotypically distinct from bacteria growing in a free-swimming planktonic state. The current definition of a bacterial biofilm is that of a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (1). Biofilms are strongly implicated in chronicity and transmission of several bacterial infections. Biofilm infections have been shown to occur on abiotic medical implants as well as living tissue, such as in the case of

endocarditis (1, 2). The ability of *Pseudomonas aeruginosa* to grow as a biofilm in patients with cystic fibrosis has been implicated in the chronic nature of cystic fibrosis pneumonia (3, 4). Uropathogenic *Escherichia coli*, which causes urinary tract infections, has been shown to grow as biofilm-like pods in mice (5). *Legionella pneumophila* in biofilms was shown to be involved in the transmission of Legionnaires' disease (6). *Vibrio cholerae*, streptococci, staphylococci, and several other bacterial pathogens have been shown to form biofilms that have been implicated in their pathogenesis and chronicity (1, 7–10). One of the most important characteristics of bacteria growing within biofilm is their ability to tolerate a wide variety of bactericidal compounds, and this contributes to the refractory nature of biofilm-associated infections to drugs and the chronicity of such infections. Biofilms are also thought to confer protection to bacteria growing within them to immune intervention (5, 11). Genetic experiments have led to the identification of several genes and pathways that are involved in biofilm formation in different bacteria, such as *P. aeruginosa*, *E. coli*, *Bacillus subtilis*, staphylococci, and streptococci (12–15).

Several members of the genus *Mycobacterium*, such as *M. ulcerans*, *M. avium*, *M. marinum*, and the saprophytic *M. smegmatis*, have been shown to form biofilms (16–19). The ability of *M. smegmatis* to form biofilms has been implicated in its resistance to antibiotics (20). Biofilms may also play a role in the transmission and pathogenesis of *M. ulcerans*, the causative agent of Buruli ulcer, which thrives in aquatic environments, and *M. avium*, an opportunistic environmental pathogen (17, 21, 22). Some aspects of genetic requirements and molecular events in mycobacterial biofilm formation have been determined. Mutants of *M. smegmatis* and *M. avium* with defects in glycopeptidolipid biosynthesis were unable to form biofilms (19, 23). In addition, *M. smegmatis* mutants altered in both undecaprenyl phosphokinase and mycolic acid (MA) biosynthesis have also shown to be defective in biofilm formation (24, 25). Taken together, mycobacterial pathways involved in cell wall biosynthesis seem to be important for biofilm formation.

It has recently been suggested that *M. tuberculosis* can form biofilms. These *in vitro* biofilms were shown to be rich in free mycolic acids that are likely released by the enzymatic hydrolysis of trehalose dimycolate (TDM) and shown to harbor drug-tolerant cells (26, 27).

Several aspects of *M. tuberculosis* biology and pathogenesis are reminiscent of biofilm behavior. For example, the ability of *M. tuberculosis* to grow as "cords," a correlate of virulence and ability to survive within the host, indicates the propensity of this bacterium to be part of a multicellular community (28–30). *M. tuberculosis* has also been described to be found in a biofilm-like structure in the acellular regions of certain types of human caseating lesions (31). In addition, studies using a guinea pig model of TB revealed that *M. tuberculosis* strains that survive extended drug treatment were primarily extracellular and confined to an acellular rim around the primary granuloma, which is morphologically similar to what has been described for human lung lesions (32). The chronic nature of TB, its requirement of a long duration of treatment, its ability to evade immune intervention, and its propensity to relapse after drug treatment is discontinued are all also reminiscent of other chronic, biofilm-associated bacterial diseases.

The air-liquid interface is an advantageous niche for aerobes due to increased accessibility to oxygen. Many aerobic bacteria exhibit an aerotactic response over a range of preferred oxygen tension, resulting in migration to the air-liquid interface and formation of a biofilm-like structure composed of cells and an extracellular matrix (ECM), known as a pellicle (33, 34). Historically, *M. tuberculosis* was predominantly grown as a pellicle at the liquid-air interphase in a variety of synthetic media, resulting in luxuriant, hypha-like growth at the surface (35). The name "mycobacteria," which means "fungus-like bacteria," in fact, reflects this observation (36). The hydrophobic nature of the mycobacterial cell wall, the preference for an aerobic lifestyle, and the presence of zinc ions (Zn^{2+}) have all been shown to contribute to this phenotype (37). However, the molecular basis of this mode of growth remains ill defined. Although a role for biofilms in *M. tuberculosis* pathogenesis remains unclear, the recent finding that *M. tuberculosis* pellicles harbor drug-tolerant cells, a trait commonly associated with biofilms (27), suggests that understanding the molecular events involved in the transition from planktonic to pellicle growth might provide useful insights into mechanisms by which *M. tuberculosis* acquires drug tolerance.

We hypothesized that *M. tuberculosis* pellicles may represent an adaptive diversification of the organism in response to certain stimuli, into a structured environment. Pellicles may hence be regarded as analogs of biofilms. In this study, we demonstrate that the ability to synthesize keto-mycolic acids (keto-MAs) is essential for bacterial organization into this ordered structure. We also demonstrate that growth within the pellicle confers drug tolerance to otherwise drug-susceptible bacilli.

RESULTS

M. tuberculosis pellicle is an organized structure distinct from cording. In the laboratory, several model systems have been used to study biofilms (38). Submerged biofilms such as those seen in aquatic environments are modeled using flow cells (39), biofilms that form on surfaces are assayed based on their ability to adhere to abiotic polyvinyl chloride or polystyrene surfaces (12, 40) or simply studied as bacterial colonies on agar plates (41), and biofilms that form at the air-liquid interface are studied as pellicles (42, 43).

Growth as pellicles requires a high degree of self-organization owing to the lack of a solid surface on which growth can be initiated. Scanning electron microscopy (SEM) of *M. tuberculosis* pellicles following processing with osmium tetroxide vapor, which allows preservation of the ultrastructure, showed that the bacteria in the pellicle are encased in an extracellular matrix (Fig. 1A). A more conventional processing of the pellicle using potassium ferrocyanide resulted in the stripping of the matrix and revealed a structured organization of the bacterial cells within this matrix (Fig. 1B). These observations are reminiscent of other bacterial biofilms, which have been shown to confer drug tolerance and contribute to persistence (1, 44). *M. tuberculosis* is known to display another form of organized growth referred to as cording. The ability to cord has been associated with the virulence of *M. tuberculosis*, and TDM has been identified to be the cord factor (29, 45). Modification of the mycolic acid constituents of TDM has been found to be required for cording, specifically cyclopropanation catalyzed by PcaA (30). However, the *M. tuberculosis* Δ*pcaA* mutant (Table 1) is fully capable of forming a pellicle (Fig. 1C). This suggests that the pellicle phenotype is distinct from cording. This was further confirmed by the observation that an unrelated cord-
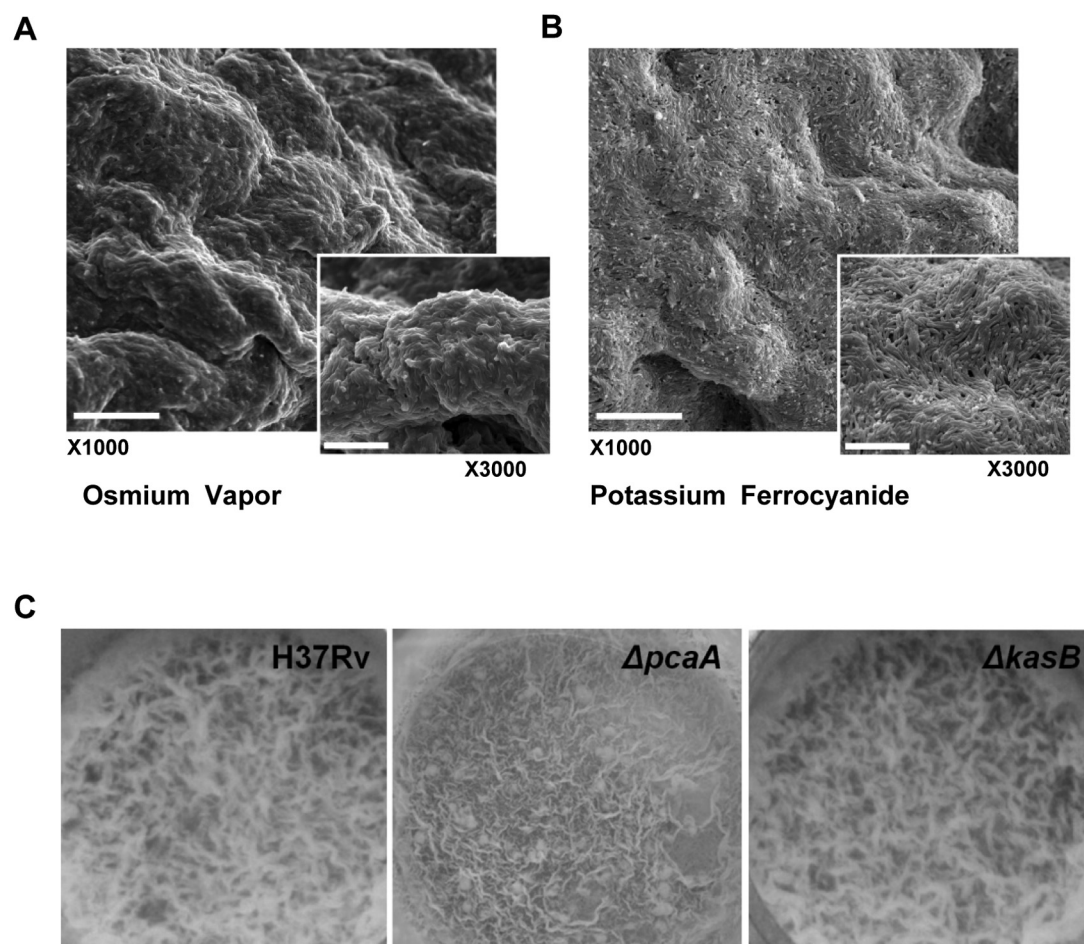
FIG 1 *M. tuberculosis* pellicle is an organized structure. SEM of *M. tuberculosis* pellicle using osmium tetroxide vapor (A) or potassium ferrocyanide (B) processing shows an organized bacterial community. Scale bars represent 10 μm in ×1,000 and 5 μm in ×3,000 magnification. (C) Pellicle phenotype is distinct from cording. Δ*pcaA* and Δ*kasB* mutants (both defective in cording) were inoculated into Sauton's medium without Tween 80 and incubated for 3 weeks under pellicle-promoting conditions. Both Δ*pcaA* and Δ*kasB* mutants are capable of growing as a pellicle, indicating that the ability to cord is not a prerequisite for pellicle growth.

ing mutant of *M. tuberculosis*, H37RvΔ*kasB* (46), is also competent for pellicle growth (Fig. 1C).

It has been recently demonstrated that the ability of *M. tuberculosis* to form a mycolate-rich extracellular matrix is central to the ability to form pellicles, of which a major constituent is free methoxy-MA (27). However, *M. bovis* BCG Pasteur, which is grown as a pellicle for vaccine production, is inherently defective in production of methoxy-MA due to a point mutation in the

methoxy-MA synthase *mmaA3* (47). This led us to hypothesize that other MA species may play pivotal roles in pellicle growth.

Construction of *M. tuberculosis* strains defective in specific mycolic acids. The defining feature of the mycobacterial cell envelope is the mycolic acids. They are among the most abundant components of the mycobacterial cell wall, comprising around 57% of the cell wall dry weight, and contribute to the pathogenicity of *M. tuberculosis* in a variety of ways (48, 49). Three major

TABLE 1 Plasmids and bacterial strains used in this study

Plasmid, strain, or phage	Description	Reference or source
pMV361-*mmaA3*	Hyg^r, single-copy integrating vector, overexpressing *M. tuberculosis* MmaA3	(75)
Bacterial strains		
mc^2 2	Wild-type H37Rv	Jacobs Lab, obtained from Trudeau Institute
mc^2 7051	H37RvΔ*mmaA4*	(60)
mc^2 7052	H37RvΔ*mmaA3*	(60)
mc^2 7053	H37Rv::pMV361::*mmaA3*	This study
mc^2 5859	H37RvΔ*kasB*	Jacobs Lab, Catherine Vilchèze
mc^2 7054	H37RvΔ*pcaA*	Gift from M. Glickman
mc^2 7055	H37RvΔ*mmaA4*::*mmaA4*	(60)

TABLE 2 Effect of genotype on mycolic acid production

	Production of:[a]		
Genotype	α-Mycolates	Keto-mycolates	Methoxy-mycolates
Wild type	+	+	+
ΔkasB	+	+	+
ΔpcaA	−	+	+
ΔmmaA3	+	+	−
ΔmmaA4	+	−	−
pMV361::mmaA3	+	−	+
ΔmmaA4::mmaA4	+	+	+

[a] +, produced; −, not produced.

structural classes of MAs are found in *M. tuberculosis*—alpha (α), methoxy, and keto, characterized by their functional groups. Mycolic acid methyltransferases are involved in the addition of functional groups that distinguish the three species of MAs (48), such that strains lacking these genes would be predicted to be lacking in specific MA species (Table 2).

To determine the role of specific MA species in pellicle growth, strains of *M. tuberculosis* H37Rv individually lacking specific methyl transferases were tested (Table 1). In addition, a strain defective in *pcaA*, which codes for a cyclopropane synthase required for α-MA synthesis, was also examined (30). Strains lacking the MA methyl transferases MmaA3 or MmaA4 had defects in MA synthesis (Fig. 2). The H37RvΔmmaA3 mutant did not produce methoxy-MA, whereas the H37RvΔmmaA4 mutant was unable to synthesize methoxy- and keto-MA. It has previously been shown that the overproduction of MmaA3 in a wild-type genetic background results in the loss of keto-MA without accumulation of the aberrant α-MA (50). We constructed a strain of H37Rv overexpressing MmaA3, and this strain was indeed deficient in keto-MA (Fig. 2). The availability of strains lacking individual MA

species now allowed us to determine the role of individual MA in pellicle growth.

Keto-MA is essential for pellicle growth. H37RvΔmmaA3 (Fig. 3) and H37RvΔpcaA (Fig. 1C) mutants were both capable of forming pellicles that were indistinguishable from those of the parental strain, suggesting that neither the loss of methoxy-MA, the major lipid constituent of the mycobacterial pellicle matrix (27), nor α-MA (30), the predominant MA species in *M. tuberculosis* under planktonic growth, interferes with pellicle formation. In sharp contrast, the H37RvΔmmaA4 mutant was defective in pellicle formation, and this defect was rescued upon genetic complementation of the deleted locus (Fig. 3). Taken together, these data suggest either both species of oxygenated MA or keto-MA alone is required for pellicle formation. Alternatively, the accumulation of aberrant α-MA (51) in the ΔmmaA4 mutant (Fig. 2) could be inhibitory. To distinguish these possibilities, the strain of H37Rv that overexpresses MmaA3, characterized by the production of α-MA and methoxy-MA only (Fig. 2), was examined for its ability to grow as a pellicle. This strain was indeed defective for pellicle formation, indicating that the lack of keto-MA, but not production of aberrant α-MA, is responsible for the inability to form pellicles (Fig. 3).

Furthermore, coculturing the two keto-MA-deficient strains (the ΔmmaA4 and H37RV::mmaA3 strains) did not restore pellicle formation, whereas coculturing a keto-MA-deficient strain with a keto-MA-producing strain (ΔmmaA4 and ΔmmaA3 strains; ΔmmaA4 and H37Rv strains) does restore pellicle formation, demonstrating the absolute requirement of keto-MA for pellicle formation and suggesting that the arrest in pellicle formation was not due to accumulation of repressive products in the mutants (Fig. 4).

Mycolic acid fluctuations under pellicle growth conditions. It has previously been demonstrated that growth under pellicle-
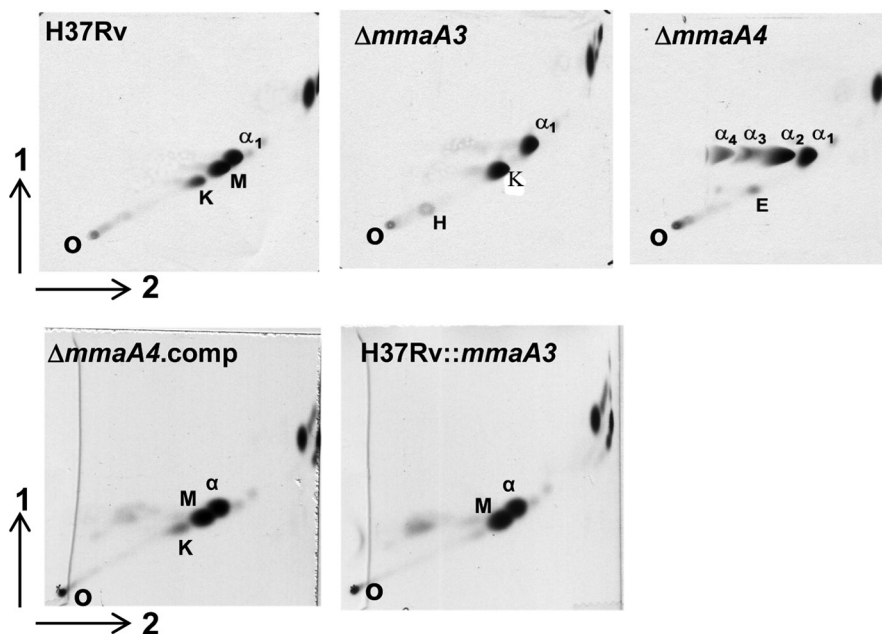
FIG 2 Mutants defective in specific MA species. Two-dimensional argentation TLC of mycolic acids from H37Rv, ΔmmaA4, ΔmmaA3, ΔmmaA4::Comp, and H37Rv::mmaA3 strains grown planktonically in Middlebrook 7H9 medium. Samples were run using hexane-ethyl acetate (95:5 [vol/vol]) in the first dimension (1) and petroleum ether-diethyl ether (85:15 [vol/vol]) in the second (2). O, origin; α_{1-4}, α-MA; K, keto-MA; M, methoxy-MA; H, hydroxy MA.
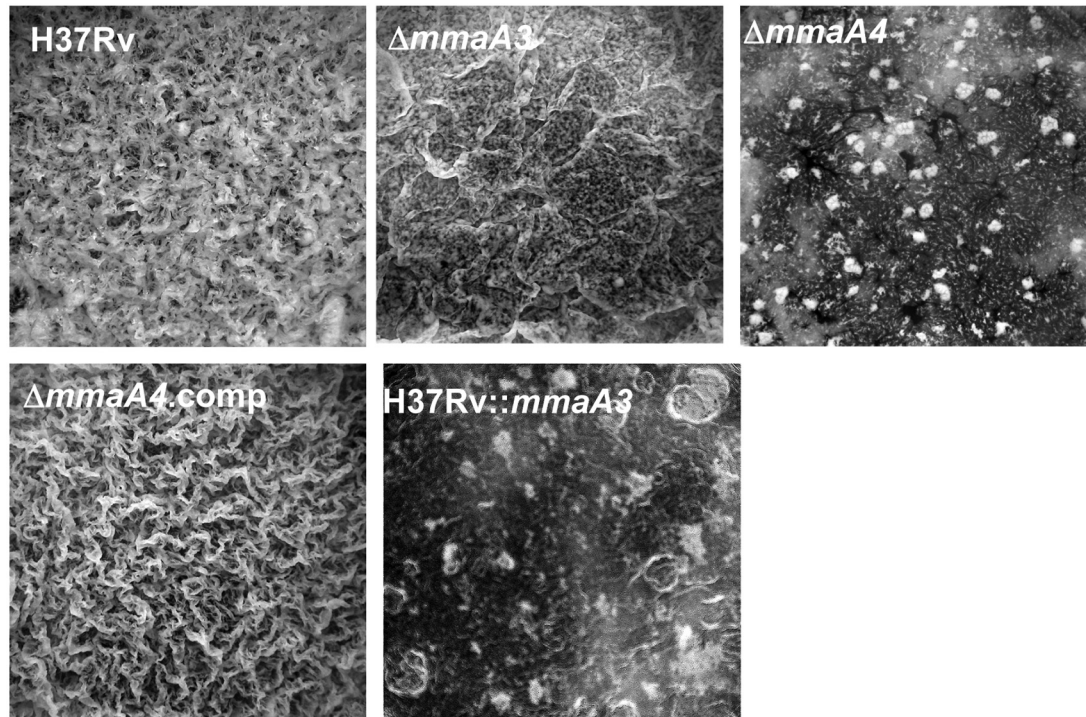
FIG 3 Keto-mycolates are essential for *M. tuberculosis* pellicle growth. H37Rv, ΔmmaA4, ΔmmaA4, ΔmmaA4::*Comp*, and H37Rv::*mmaA3* strains were inoculated into Sauton's medium without Tween 80 to assess pellicle formation. Pellicle formation was recorded at 4 weeks postinoculation.

promoting conditions results in formation of an extracellular matrix enriched in free methoxy-MA (27). Following the method described by Ojha et al. (27), apolar detergent-extractable lipids from wild-type and pellicle-defective strains, grown under pellicle-promoting conditions, were isolated, purified, and analyzed by chromatography. Indeed, wild-type *M. tuberculosis* accumulated methoxy-MA. The pellicle-defective *mmaA4* mutant, on the other hand, did not accumulate methoxy-MA, instead accumulating α-MA in the detergent-extractable fraction, and this defect was reversed in the complemented strain (Fig. 5A). However, the pellicle-defective H37Rv::*mmaA3* extract contained methoxy-MA at levels comparable to that of the wild type even
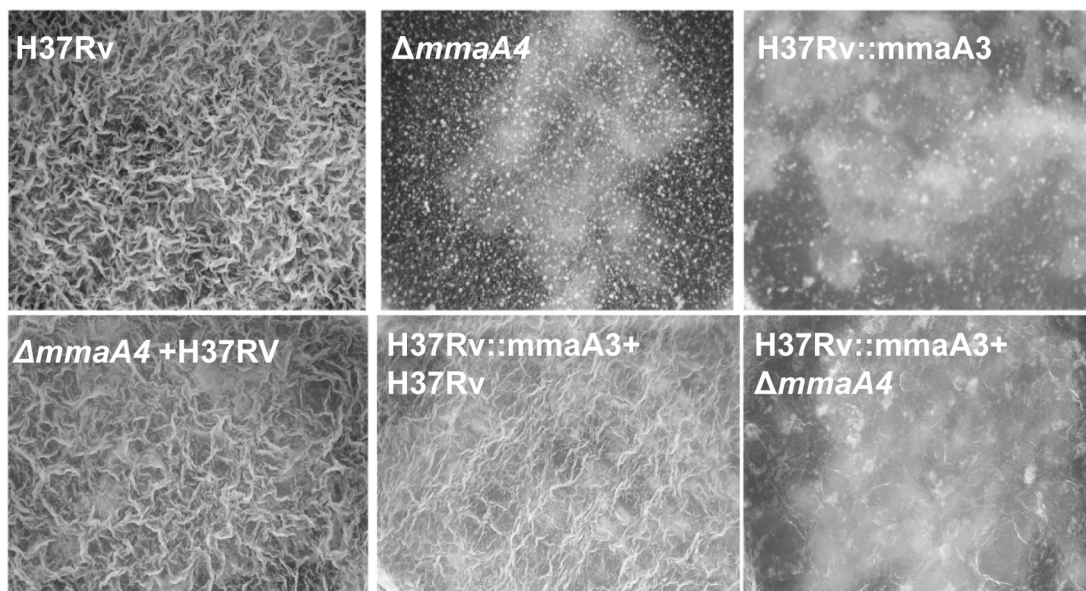
FIG 4 Coculturing keto-MA-deficient strains does not restore pellicle growth. H37Rv, ΔmmaA4, and H37Rv::*mmaA3* strains, individually and in combinations—ΔmmaA4 and H37RV coculture, H37RV::*mmaA3* and H37Rv coculture, H37RV::*mmaA3* and ΔmmaA4 coculture—were inoculated into Sauton's medium without Tween 80 and incubated for 3 weeks under pellicle-promoting conditions.

A

B
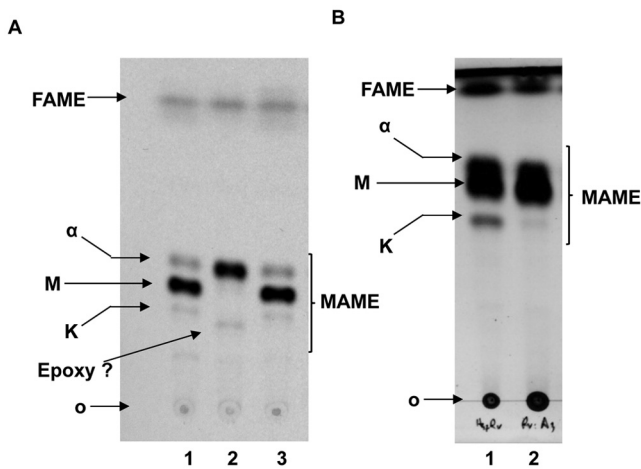
FIG 5 Analysis of mycolic acids under pellicle-promoting conditions. Major apolar lipids from cells grown as pellicles were derivatized as methyl esters and resolved on TLC using petroleum ether-acetone (95:5 [vol/vol]). (A) Methoxy-MA (M) is enriched in the wild-type pellicle (lane 1). The *mmaA4* extract lacks both keto-MA (K) and methoxy-MA (lane 2). This defect is relieved in the complemented strain (lane 3). (B) The H37Rv::*mmaA3* extract (lane 2) is deficient in keto-mycolates relative to the wild-type H37Rv (lane 1) but retains wild-type levels of methoxy-MA. O, origin; FAME, fatty acid methyl esters; MAME, mycolic acid methyl esters. This shows that methoxy mycolate, despite being a major constituent of mycobacterial pellicle, is dispensable for growth as a pellicle, and keto-mycolate, despite being a minor constituent, is essential.

though it does not form a pellicle (Fig. 5B). These results suggest that methoxy-MA accumulation is probably a function of the fluctuating levels of MA in response to a pellicle growth condition, and its accumulation in the wild-type pellicle is secondary to initiation of pellicle growth. Indeed, MAs are known to undergo fluctuations in relative abundance in response to changing environmental conditions, such as *in vivo* growth and growth under hypoxic conditions (50, 52). The common denominator in both the pellicle-defective strains was the lack of keto-MA, suggesting that keto-MA is a molecular determinant of *M. tuberculosis* pellicle growth.

Taken together, these genetic and biochemical studies demonstrate an essential role for keto-MA in *M. tuberculosis* pellicle growth. The fact that keto-MA, a minor MA component in both the planktonic and pellicle growth conditions assayed, dictates the ability to grow, as a pellicle confirms the vital role of this MA in organizing the mycobacterial outer membrane. Studies of the conformation adopted by the MAs have revealed keto-MA to be an extraordinarily stable W-shaped molecule in which the meromycolate chain is folded back on itself (53, 54). The stability of this conformation, which is not matched by the methoxy- and α-MA, may underpin the unique requirement for keto-MA for pellicle formation. Additionally, the presence of the nonbonded electron pair on the oxygen moiety could act as an anchoring point for hydrogen bonding of loosely associated cell wall components (55).

Growth within pellicle restores drug tolerance. Interestingly, the H37RvΔ*mmaA4* mutant exhibited a loss of tolerance to rifampicin (RIF) under planktonic growth conditions (Fig. 6A), and this decreased tolerance to a lipophilic drug (RIF) is likely not due to increased permeability (56). The availability of a mutant (the Δ*mmaA4* strain) that is not only defective in pellicle formation but

also inherently hypersensitive to a drug (RIF) provides us with a tool to address the question of pellicle-associated drug tolerance. First, wild-type H37Rv and the Δ*mmaA4* mutant were grown under pellicle-promoting conditions for three weeks, at which point the wild type forms a mature pellicle, and then the cultures were treated with RIF for seven days. As previously described by Ojha et al. (27), RIF was ineffective against wild-type H37Rv in the pellicle, whereas the bactericidal activity of the drug against the mutant was retained (Fig. 6B). This suggests that it is the growth within the pellicle and not merely the conditions of growth that results in tolerance to the drug. Second, we cocultured the wild-type and mutant strains under pellicle-promoting conditions. This resulted in the incorporation of the mutant into the wild-type pellicle, as observed by confocal microscopy (see Fig. S1 in the supplemental material). We then treated the mixed pellicle composed of H37Rv and the Δ*mmaA4* mutant with RIF for seven days, after which the pellicle was harvested and plated on solid medium containing hygromycin (Δ*mmaA4* selectable marker) or with no drug added. The Δ*mmaA4* mutant could be recovered from the treated mixed pellicle at proportions similar to those from the untreated control and at levels similar to those of the wild type from the treated mixed pellicles (Fig. 6C). These results imply that growth within the pellicle environment confers drug tolerance, and identifying pathways involved in this adaptation could result in the discovery of mechanisms contributing to tolerance and survival under stress.

DISCUSSION

Mycobacterial persistence—against drug and immune intervention—is the most significant challenge to the control of TB. Persistence is a complex phenotype that allows the cells to enter into a physiological state that can resist killing induced by drugs (57) or a bactericidal immune response (58). While persistence *in vitro* may be induced by a heterogeneous set of cues, such as hypoxia or starvation, this work has focused on the ability of *M. tuberculosis* to grow as a pellicle, a form of biofilm.

Our studies on *in vitro* growth of *M. tuberculosis* as a pellicle have demonstrated that keto-MA is required for *M. tuberculosis* to adapt to pellicle growth. An essential role for keto-MA in growth within the natural host cells has been described (50). Indeed, there is no known naturally occurring, slow-growing mycobacterial strain that is defective exclusively in keto-MA, suggesting an important role for this MA species in mycobacterial pathogenesis. This view is supported by the fact that *mmaA4*-knockout strains of *M. tuberculosis* are highly attenuated *in vivo* (56). Interestingly, previous studies on transcriptional profiling of *M. tuberculosis* in the lungs of human TB patients have shown that the *mmaA4* gene is upregulated *in vivo* (59). A recent study demonstrated that mycolic acid modification by MmaA4 renders *M. tuberculosis* capable of repressing interleukin 12 (IL-12) production, thereby assisting the bacterium in establishing chronic infection, since the attenuation depends on IL-12p40-mediated immunity (60). Taken together, these studies strongly suggest that identifying molecules targeting the keto-MA biosynthetic pathway may result in the development of new therapeutics against *M. tuberculosis*.

We also demonstrate that growth within the pellicle biofilm confers drug tolerance. The modality of this tolerance is still unclear. One possible mechanism for biofilm-mediated drug tolerance is the inability of antibiotics/drugs to penetrate the biofilm, which in itself is a formidable physical barrier (61). This has been
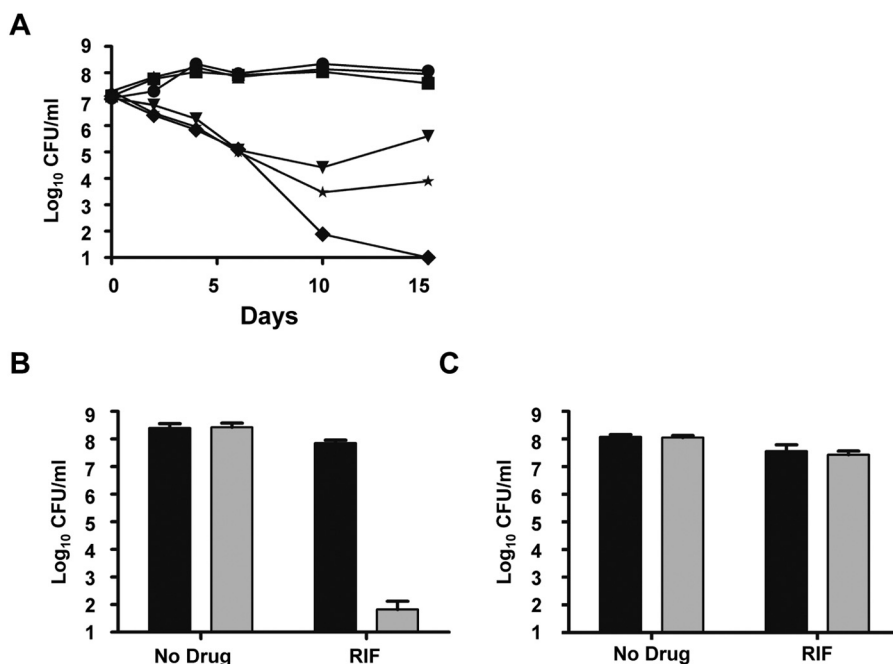
FIG 6 Growth within the pellicle confers protection to drug-intolerant *M. tuberculosis* strains. (A) H37Rv (upside down triangle), H37RvΔ*mmaA4* (diamond), and the complemented strain (star), grown planktonically in Middlebrook 7H9 medium containing RIF (2 μg/ml). Untreated controls of H37Rv (circle), H37RvΔ*mmaA4* (square), and the complemented strain (triangle) were included. CFUs were enumerated at the indicated time points by plating aliquots onto Middlebrook 7H10 agar plates. Results are representative of at least two independent experiments. (B) H37Rv (black bar) and H37RvΔ*mmaA4* (gray bar) strains were grown in Sauton's medium under pellicle-promoting conditions for 3 weeks, followed by RIF (5 μg/ml) treatment for 1 week. CFU were determined by plating aliquots onto Middlebrook 7H10 agar plates. (C) H37Rv (black bar) and H37RvΔ*mmaA4* (Gray bar) strains were grown in coculture in Sauton's medium under pellicle-promoting conditions for 3 weeks, followed by treatment with RIF (5 μg/ml) for 1 week. CFU proportions of H37Rv and H37RvΔ*mmaA4* strains in the mixed pellicle were determined by plating aliquots onto Middlebrook 7H10 agar plates with or without hygromycin (50 μg/ml), H37RvΔ*mmaA4* selectable marker. Experiments were performed in triplicate.

shown to be true in some cases, such as the penetration of piper-acillin into *P. aeruginosa* biofilm (62). However, in other cases, such as in *S. epidermidis* and *E. coli* biofilms, penetration of RIF, vancomycin, and tetracycline did not seem to be affected (63, 64). The ability of the biofilm environment to inactivate a drug at a rate that is faster than the diffusion rate of the drugs is another physical way by which the biofilm matrix can act as a barrier. Examples include the enhanced levels of beta-lactamases in *Klebsiella pneumoniae* biofilms, which results in the deactivation of beta-lactam antibiotics, such as ampicillin (65), and the inability of oxygen radicals to effectively penetrate the biofilm barrier (66, 67). Another model for biofilm drug tolerance proposes that bacteria within biofilms are in a nutrient-starved environment, causing the bacteria to slow down replication and enter a dormant state. As most antibacterial compounds are dependent on active replication and metabolism for their action, cells in a nongrowing state would therefore be refractory to such drugs (68). Indeed, it has recently been shown that active starvation response rather than passive growth arrest mediates antibiotic tolerance in *P. aeruginosa* biofilms (69). A third model for biofilm drug tolerance posits that the chemical and physiological heterogeneity within biofilms results in distinct bacterial populations with various levels of growth and metabolism (70). This would suggest that even though some of the bacteria within biofilms are in a state conducive to drug action and thus would be killed, some bacteria that exist in a state nonconducive to drug action would survive. Finally, it is thought that biofilms harbor specialized persisters, cells that are

phenotypically drug resistant, and that the biofilm environment may also enrich the formation of persister cells (71, 72). Recently, it has been shown that persisters are enriched in *P. aeruginosa* biofilms in patients with cystic fibrosis, strongly suggesting that the recalcitrance to drug and immune elimination of *P. aeruginosa* infections in these patients could be due to these persisters (73). It is also possible that phenotypic resistance to antibiotics exhibited by bacteria within biofilms is multifactorial and is due to more than one of the modalities discussed above.

Nevertheless, our finding that growth within the pellicle contributes to drug tolerance suggests that the *M. tuberculosis* pellicle biofilm phenotype can be a convenient surrogate to identify novel *in vivo*-active TB drugs that can kill drug-tolerant bacteria and may help identify targets and pathways relevant to drug tolerance and survival under stress.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Planktonic cultures of mycobacteria were grown at 37°C in Middlebrook 7H9 supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Difco), 0.5% glycerol, and 0.05% Tween 80 or in Sauton's liquid medium supplemented with 3.5 μM ZnSO$_4$. *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth (Difco). Selection of gene deletion mutants was carried out in the presence of 50 mg/liter hygromycin. Wherever necessary, mycobacteria were grown on solid Middlebrook 7H10 medium containing 10% OADC, 0.5% glycerol, and appropriate antibiotics and supplements. *E. coli* on solid medium was grown using Luria-Bertani agar (Difco). Mycobacterial pellicles were grown in polystyrene or polyvinyl chloride multiwell cell

culture plates (Corning) by inoculating Sauton's medium (without Tween 80) with the appropriate mycobacterial strain (with supplements where necessary) and incubating without shaking at 37°C under 5% CO_2 for 3 to 5 weeks (27).

Planktonic drug-killing assay. Exponentially growing cultures of mycobacteria were diluted in fresh medium to an optical density at 600 nm (OD_{600}) of 0.1 to 0.2. RIF (Sigma) was added at a final concentration of 2 μg/ml. The number of CFU at the start of the experiment was estimated by plating the appropriate dilution of the culture onto 7H10 agar plates. The effect of the drug treatment on viability was monitored by plating for CFU at the various indicated time points. All experiments were carried out in triplicates.

Pellicle biofilm drug-killing assay. Exponentially growing mycobacteria were used to inoculate biofilm medium (1:100). Pellicle biofilm was allowed to establish for three weeks, at which time RIF (5 μg/ml) was injected into the biofilm using a tuberculin syringe. CFU in pellicle biofilms were assessed by harvesting the pellicle, resuspending in 1 ml of phosphate-buffered saline (PBS) containing 0.5% (vol/vol) Tween 80, sonication, and plating appropriate dilutions on 7H10 agar plates after 7 days of treatment. All experiments were carried out in triplicate.

Mycolic acids analysis. Cultures of *M. tuberculosis* in exponential growth phase (OD_{600} of ~0.6) were labeled with [1-^{14}C]acetate (10 μCi; Moravek Biochemicals) for 24 h. Cells were centrifuged, washed twice with water, resuspended in 1 ml of water, and added to 1 ml of 40% of tetrabutylammonium hydroxide. The suspension was heated at 100°C for 20 h. After the suspension cooled, 100 μl of methyl iodide in 2 ml of methylene chloride was added. The mixture was stirred for 1 h at room temperature, and the organic phase was washed first with a 3 N aqueous HCl solution followed by water. The organic phase was dried. Along with the mycolic acids, the shorter-chain fatty acids are also extracted and methylated during this process (fatty acid methyl esters [FAMEs]). Samples were then resuspended in 200 μl methylene chloride (CH_2Cl_2), and equal cpm amounts of different samples were loaded onto a silica gel 60 F_{254} plate. The samples were run with hexane-ethyl acetate (95:5 [vol/vol]; 3 elutions). For two-dimensional separation of mycolic acids, TLC plates were impregnated with 10% silver nitrate solution and activated. Samples were spotted onto the activated plate and run in the first dimension using hexane-ethyl acetate (95:5 [vol/vol]) and in the second dimension using petroleum ether-diethyl ether (85:15 [vol/vol]), and the mycolic acids were visualized using autoradiography (74).

Apolar lipid analysis. Mycobacteria grown planktonically to an OD_{600} of 0.5 to 0.6 or as pellicle for 3 weeks were labeled with [1-^{14}C]acetate (Moravek Biochemicals) for 24 h (final concentration of 10 μCi), harvested by centrifugation, and washed in sterile water. Cells were resuspended in 2 ml of methanol-0.3% NaCl (10:1) and mixed with 1 ml of petroleum ether (PET) for 15 min at room temperature. The upper PET phase containing apolar lipids was collected in a glass vial. The residual solution was once again extracted of remaining apolar lipids by being mixed with PET for 15 min. The PET phase was pooled with the extract from the earlier step. The extract was dried under nitrogen at 50°C.

Apolar lipids from planktonic and pellicle cells were dissolved in 0.5 ml of dichloromethane and spotted onto a silica gel 60 F_{254} plate using a capillary tube. The major apolar lipids in biofilm cells which were further methylated to form methyl esters were spotted onto the TLC plates and resolved using PET-acetone (95:5 [vol/vol]).

Scanning electron microscopy. Pellicles were fixed for 24 h with 5% glutaraldehyde, 0.2 M sodium cacodylate, 0.4 M sucrose, and 10 mM $MgCl_2$ (pH 7.4) (mixed 1:1 with growth medium).

Osmium tetroxide vapor. Pieces of the fixed pellicles were lifted from the fixative and placed on carbon adhesive tabs (Electron Microscopy Sciences). The tabs were placed above 0.22-μm-pore-size filter paper and were allowed to air dry (~6 h). Samples were then placed in a closed chamber with 0.25 g osmium tetroxide for 16 h.

Potassium ferrocyanide. Pieces of the fixed pellicles were processed in suspension on a rotator. The pellicles were postfixed with 1% osmium tetroxide, 0.7% potassium ferrocyanide, 0.1 M sodium cacodylate, 0.2 M sucrose, and 5 mM $MgCl_2$ (pH 7.4) for 30 min. Samples were buffer rinsed and dehydrated through a graded series of ethanol. They were critical point dried with hexamethyldisilazane (HMDS) (Polysciences) (4 washes: 3 times 5 minutes and the 4th overnight under a hood) until all HMDS was evaporated.

The samples processed using the above-described methods were then placed on aluminum SEM stubs, sputter coated with gold-palladium in a Denton Vacuum desk-2 sputter coater (Cherry Hill, NJ), and examined under a JEOL JSM6400 scanning electron microscope (Peabody, MA), using an accelerating voltage of 10 kV.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00222-13/-/DCSupplemental.

Figure S1, TIF file, 4.1 MB.

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