The Pup-Proteasome System Protects Mycobacteria from Antimicrobial Antifolates

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ABSTRACT Protein turnover via the Pup-proteasome system (PPS) is essential for nitric oxide resistance and virulence of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Our study revealed components of PPS as novel determinants of intrinsic antifolate resistance in both *M. tuberculosis* and nonpathogenic *M. smegmatis*. The lack of expression of the prokaryotic ubiquitin-like protein (Pup) or the ligase, PafA, responsible for ligating Pup to its protein targets, enhanced antifolate susceptibility in *M. smegmatis*. Cross-species expression of *M. tuberculosis* homologs restored wild-type resistance to *M. smegmatis* proteasomal mutants. Targeted deletion of *prcA* and *prcB*, encoding the structural components of the PPS proteolytic core, similarly resulted in reduced antifolate resistance. Furthermore, sulfonamides were synergistic with acidified nitrite, and the synergy against mycobacteria was enhanced in the absence of proteasomal activity. In *M. tuberculosis*, targeted mutagenesis followed by genetic complementation of *mpa*, encoding the regulatory subunit responsible for translocating pupylated proteins to the proteolytic core, demonstrated a similar function of PPS in antifolate resistance. The overexpression of dihydrofolate reductase, responsible for the reduction of dihydrofolate to tetrahydrofolate, or disruption of the Lonely Guy gene, responsible for PPS-controlled production of cytokinins, abolished PPS-mediated antifolate sensitivity. Together, our results show that PPS protects mycobacteria from antimicrobial antifolates via regulating both folate reduction and cytokinin production.

KEYWORDS tuberculosis, mycobacterium, proteasome, antifolates

Tuberculosis (TB) remains a leading cause of mortality and morbidity worldwide (1), killing two million people annually (2). Standard regimens prescribed for drug-susceptible TB patients often includes 3 to 4 drugs taken daily for a duration of 6 to 9 months. The widespread emergence of drug-resistant *Mycobacterium tuberculosis* strains compounds global TB control as existing drugs become less potent against the disease. Repurposing non-TB, ineffective, and old drugs, such as β -lactams and antifolates, for TB treatment has become a promising strategy for tackling drug-resistant TB (3, 4).

Antifolates such as sulfonamides and trimethoprim (TMP) were among the earliest antibacterials developed to treat bacterial infections, including TB (5, 6). The use of sulfonamides only started to decline in the 1960s, when more effective antibiotics were introduced (7, 8). The basic issues of sulfonamides, namely, bacterial resistance and cytotoxic side effects, were partially addressed through the introduction of trimethoprim, which cotargets the folate pathway, thereby creating synergy. While sulfonamides inhibit dihydropteroate synthase (DHPS), TMP suppresses dihydrofolate reductase (DHFR), which is responsible for converting dihydrofolate to the metabolically active form, tetrahydrofolate. Cotrimoxazole, a combination of trimethoprim and sulfamethoxazole, has been used to treat urinary tract infections and shigellosis. Interestingly, recent studies

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show that cotrimoxazole may also be effective against *M. tuberculosis*, including multi-drug-resistant strains, indicating that these antibiotics could be further boosted for use as anti-TB drugs (9–11). In fact, cotrimoxazole is currently the most common prophylactic medicine for preventing bacterial infections in HIV-positive individuals, who are most vulnerable to TB due to compromised immunity (12). On the one hand, this widespread and effective use of cotrimoxazole strongly suggests that antifolate combinations could be optimized to provide cost-effective therapies for global TB control. On the other hand, the worldwide use of the antifolate combination has raised concerns of increasing resistance. For antifolates to become more effective and lasting anti-TB drugs, novel antifolate resistance targets druggable to sensitize *M. tuberculosis* to existing antifolates need to be determined (11).

We recently initiated the identification of genome-wide antifolate resistance determinants in mycobacteria, also called the mycobacterial antifolate resistome, by performing a chemogenomic screen in *M. smegmatis* (13). This study identified 50 genetic determinants responsible for *M. smegmatis* innate antifolate resistance, many of which showed homologs in *M. tuberculosis* and other pathogenic mycobacteria (13, 14). One of the intrinsic antifolate resistance pathways identified from our screen is further evaluated in this report.

The proteasome is an energy-dependent multisubunit proteolytic complex responsible for degradation of unwanted or damaged cellular proteins (15), thereby regulating the turnover of proteins essential for DNA replication, transcription, cell division, and other cellular processes (15). Proteasomes are found in eukaryotes, archaea, and only two bacterial phyla, *Nitrospira* and *Actinobacteria*, which includes the *Mycobacterium* taxon (16). Proteasomal degradation is required for the persistence of *M. tuberculosis* in host macrophages, presumably through regulating the turnover of several virulence factors (17–19). For example, the proteasome protects *M. tuberculosis* from nitric oxide (NO) and other reactive nitrogen intermediates (RNI) generated in activated host macrophages (17, 18, 20, 21) by regulating the turnover of Lonely Guy (Log; Rv1205) (22). Lonely Guy is a phosphoribohydrolase that catalyzes the final step in the production of cytokinins, which, when broken down into aldehydes such as *para*-hydroxybenzaldehyde (pHBA), kill mycobacteria in the presence of NO (23). Inhibitors specific for mycobacterial proteasome were identified that sensitize *M. tuberculosis* to acidified nitrite *in vitro* and to killing by macrophages *ex vivo* (24).

Unlike the eukaryotic proteasome that uses ubiquitin to label target proteins (25), mycobacteria utilize Pup. Pup is an intrinsically disordered protein encoded immediately upstream of *prcB* and *prcA* that, together, encode the 20S proteasomal subunit (16, 26, 27). Furthermore, the enzymes and chemistry involved in pupylation also differ from those of eukaryotic ubiquitination. During pupylation, the C-terminal glutamine residue of Pup is deamidated to glutamate by Dop (deaminase of Pup). Proteasome accessory factor A (PafA) then activates Pup through the phosphorylation of the C-terminal glutamate and catalyzes the formation of an isopeptide bond between Pup and a lysine residue of a substrate protein. Pup-tagged protein is then recognized by the proteasome ATPase Mpa, resulting in unfolding and translocation of pupylated substrate into the 20S core for degradation.

Here, we show that the Pup-proteasome system (PPS) is required for mycobacterial intrinsic resistance to sulfonamides and TMP, antifolates that were once commonly used to treat bacterial infections, including TB (5, 6). Defects in Pup production (*pup*), its ligation (*pafA*) to targeted proteins, the translocation (*mpa*) of Pup-tagged proteins through the proteasomal core, or the ultimate degradation (*prcAB*) of the targeted proteins is each sufficient to impair antifolate resistance. We further demonstrate synergy of antifolates and an acidified nitrite used as an NO donor, which both become more potent against mycobacteria in the absence of proteasomal activity. Mechanistic studies suggest that PPS mediates antifolate resistance through two pathways: (i) DHFR, an enzyme required for the reduction of oxidized folate intermediates, and (ii) Lonely Guy, an enzyme responsible for the production of cytokinins. Our data support targeting

PPS by specific inhibitors as a therapeutic strategy to treat mycobacterial infections, including TB.

RESULTS

Protein pupylation is required for antifolate resistance in *M. smegmatis*. Our study of the *M. smegmatis* antifolate resistome (13, 14) revealed two transposon mutants, 64D12 and 23G4, that were more susceptible to antimicrobial antifolates. Chromosomal mapping revealed *Himar1* insertion into two separate genes encoding two components of the pupylation process that label cellular proteins for PPS degradation (16, 28, 29).

In 64D12, *Himar1* inserted into the dinucleotide TA^{172–173} of the open reading frame *msmeg_3896* (Fig. 1A), which putatively encodes a homolog of the prokaryotic ubiquitin-like protein, or Pup (27). The insertion disrupted the translation of the last seven amino acids at the C terminus, where conjugation of Pup to protein substrates occurs (Fig. 1A and 2A). Since the genes in this chromosomal locus are likely translationally coupled (30), the *Himar1* insertion in 64D12 may also cause polar effects, diminishing the expression of the *prcB* and *prcA* genes located downstream (Fig. 1A). To confirm the mapping data, PCR was performed using primers c1 and c2, flanking the insertion site (Fig. 1A), followed by sequencing. Whereas the PCR products amplified from wild-type genomic DNA exhibited a molecular weight of 1,861 bp, those produced from 64D12 displayed a molecular weight of 4,060 bp. Therefore, the mobility shift of the PCR products from 64D12 was caused by an extra DNA fragment of 2,199 bp (Fig. 1A, top right), exactly the molecular weight of *Himar1* (31). Cloning of PCR products and sequencing confirmed the insertion of *Himar1* into the *M. smegmatis* *pup* gene (Fig. 1A, top left).

Similarly, mapping of *Himar1* in 23G4 revealed its insertion at TA^{376–377} of *msmeg_3890* (Fig. 1A), which encodes a homolog of proteasome accessory factor A, or PafA, responsible for ligating Pup to protein substrates. PCR amplification of *pafA* using primers c3 and c4 visualized the insertion of *Himar1* on an agarose electrophoresis gel (Fig. 1A, bottom left). While the PCR products amplified from wild-type *M. smegmatis* genomic DNA showed a molecular weight of 1,379 bp, the insertion of *Himar1* (2,199 bp) resulted in the molecular weight of 3,578 bp seen with the PCR products amplified from 23G4 (Fig. 1A, bottom left).

To assess the consequences of *Himar1* insertions at a translational level, Western blotting followed by immune detection using anti-Pup or anti-PafA antibodies was performed with the mutant cell lysates. The lack of either Pup in 64D12 or its ligase, PafA, in 23G4 abolished cellular pupylation activity (Fig. 1B), demonstrated by a smear ranging from 35 to 100 kDa in the wild-type cell lysates (Fig. 1B, left). Disrupted expression of Pup and PafA and the consequent protein pupylation were most likely caused by *Himar1* insertions, because expression of 5,10-methenyltetrahydrofolate synthase (MTHFS) (14), an enzyme of the folate pathway used as an internal loading control, was identical in all cell lysates tested (Fig. 1B, lower).

The absence of pupylation activity in *M. smegmatis* mutants likely caused increased susceptibility to antifolates. Both 64D12 and 23G4 displayed impaired resistance to sulfonamides (Fig. 1C and Table 1). Interestingly, resistance was restored when pABA or folic acid was supplemented extracellularly (Fig. 1C), suggesting that the mutants were fully capable of metabolizing these two metabolites to bypass their genetic defects. However, extracellular supplementation of folic acid, which requires reduction by DHFR, failed to restore sulfonamide resistance (Fig. 1C), suggesting that the connection of PPS with intrinsic antifolate resistance is mediated through DHFR activity.

Cross-species expression of *M. tuberculosis* Pup restores pupylation and antifolate resistance. Sequence alignment analyses revealed that the Pup proteins from *M. smegmatis* and *M. tuberculosis* are almost identical, especially at the C-terminal end, where *Himar1* inserted in 64D12 (Fig. 2A). This suggested that protein tagging through pupylation is interchangeable between these two mycobacterial species. To address this, plasmids that allow in *trans* cross-species expression of *M. tuberculosis* proteasomal

FIG 1 Proteasome components as determinants of *M. smegmatis* antifolate resistance. (A) *Hmar1* insertion in *pup* (64D12; top) and *pafA* (23G4; bottom) and their chromosomal locations relative to genes encoding other components of *M. smegmatis* PPS (middle). Bar, 1 kb.

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genes were introduced into the 64D12 mutant. Plasmid pVN1012 was engineered to express *M. tuberculosis* Pup alone, while pVN1013 expressed PrcA and PrcB and pVN1014 expressed all three proteins combined (Fig. 2B). Transformation was followed by Western blotting of cellular pupylation patterns and antifolate susceptibility testing as described below.

Immunodetection using Pup antibody showed that transformation of either pVN1012 or pVN1014 restored both Pup expression and protein pupylation activity to 64D12, whereas those activities were unchanged in cells transformed with pVN1013 (Fig. 2C). These patterns of Pup expression and cellular pupylation correlated with antifolate resistance profiles observed with *M. smegmatis* strains. While pVN1012, expressing Pup alone, and pVN1014, expressing all three proteins, restored antifolate resistance to 64D12, plasmid pVN1013, expressing only *prcB* and *prcA*, exhibited unchanged sensitivity (Fig. 2D, Table 1). These results confirmed that the lack of Pup expression or its ligation to protein substrates is enough to abolish intrinsic antifolate resistance in *M. smegmatis*. This also indicated that the expression of *prcB* and *prcA* was not affected in 64D12. Most importantly, these results suggest that PPS-mediated antifolate resistance is a common function shared among mycobacteria, including *M. tuberculosis*.

Pup ligation to protein substrates via PafA is required for *M. smegmatis* antifolate resistance. The genomes of both *M. smegmatis* and *M. tuberculosis* encode at least three homologs of proteasome accessory factors, *pafA*, *pafB*, and *pafC*, which together form an operon (32). Recent reports show that *pafC* is required for mycobacterial resistance to fluoroquinolones (33) and that *pafBC* is involved in mycobacterial regulation of DNA repair (34, 35). This suggests that impaired antifolate resistance in 23G4 is due to failed *pafBC* expression by a polar effect of *Himar1* insertion but not *pafA*. To clarify this, we constructed a plasmid for *in trans* expression of *M. smegmatis* *pafA* (pVN1016). Transformation of pVN1016 into 23G4 resulted in PafA overexpression, as shown by a Western blot using a PafA antibody (Fig. 3A, left). Restoring PafA expression brought protein pupylation activity back to 23G4. Compared to 23G4 transformed with plasmid vector alone, transformation with pVN1016 restored pupylated proteins to levels observed with wild-type *M. smegmatis* (Fig. 3A, right).

However, *in trans* overexpression of PafA from pVN1016 only partially reinstated 23G4's antifolate resistance (Fig. 3B, Table 1). While confirming a requirement of PafA for *M. smegmatis* intrinsic antifolate resistance, a role for PafB and/or PafC was not excluded. This kind of functional coordination has been observed in previous studies, which showed that PafA, PafB, and PafC share roles in mycobacterial resistance to reactive nitrogen intermediates (32) and that PafB and PafC are involved in regulating the transcription of PafA (36). In addition, the *in trans* expression of PafA from pVN1016 in the complemented strain was driven by the P_{SOD} promoter, which might not be coordinated well with the other PPS components in response to antifolates, leading to the incomplete restoration of antifolate resistance.

Proteasomal core proteins PrcA and PrcB are required for antifolate resistance. To assess if pupylation-dependent antifolate resistance in *M. smegmatis* (Fig. 1 and 3) requires that Pup-tagged proteins are degraded by proteolytic core proteins (37), *prcA* and *prcB* were deleted by homologous recombination. Using a recombineering approach (38, 39), allelic exchange substrates were first constructed by PCR cloning the homologous DNA sequences flanking the two genes, *prcBA* (Fig. 4A), into plasmid

FIG 1 Legend (Continued)

The number below each gene indicates the accession code (*msmeg_*). Filled arrowheads indicate the precise TA dinucleotides where *Himar1* inserted. Agarose electrophoresis gels demonstrate transposon insertions as shifts in molecular weight. Genomic DNAs from *M. smegmatis* and 64D12 or 23G4 were used as a template for PCR with primers flanking the *pup-prcBA* locus (c1 and c2) or *pafA* (c3 and c4), respectively. PCR products from the mutants generated larger fragments corresponding to *Himar1* insertion. Chromatograms illustrate the sequences at chromosome-*Himar1* junctions. (B) Immunodetection of Pup and pupylated proteins (left) and PafA (right) in lysates of *M. smegmatis*, 64D12, and 23G4 cells growing at exponential phase. Expression of 5,10-methenyltetrahydrofolate synthase (MTHFS) was used as a loading control. Both 64D12 and 23G4 exhibited a lack of protein pupylation. (C) Antifolate susceptibility and chemical complementation profiles of 64D12 and 23G4. Pictures were taken directly from replicated plates of the previously described antifolate resistance screen in *M. smegmatis* (13). Sulfachloropyridazine (SCP) was used at 10.5 $\mu\text{g}/\text{ml}$, while supplements were used at 0.3 mM.

FIG 2 Protein pupylation is required for *M. smegmatis* intrinsic antifolate resistance. (A) Amino acid sequence alignment of *M. smegmatis* and *M. tuberculosis* Pup homologs. The unstructured N-terminal sequence that engages with the proteasomal pore is highlighted in yellow, whereas the flexible C-terminal sequence, where conjugation to substrates occurs, is highlighted in red. The sequence responsible for binding to the Mpa coiled-coil domain, which partially overlaps the docking region for PafA, is highlighted in blue (26). The filled arrowhead indicates the position of *Himar1* insertion in 64D12. (B) Construction of plasmids for in *trans* cross-species expression of *M. tuberculosis* *pup* (*rv2111c*, pVN1012), *prcB-prcA* (*rv2110c-rv209c*, pVN1013), or all three, *pup-prcB-prcA* (pVN1014), in *M. smegmatis* mutants. Ab, antibody. (C) Expression of Pup and patterns of pupylated proteins in the cell lysates of *M. smegmatis*, 64D12 carrying the empty vector, and its derived strains expressing different gene combinations from the *pup-prcB-prcA* locus, as analyzed by Western blotting and immunodetection using Pup antibody. (D) Antifolate susceptibility tested by disc

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TABLE 1 Susceptibility of *M. smegmatis* and *M. tuberculosis* strains^a

Strain	MIC (mg/liter)		
	SCP	TMP	SMZ
<i>M. smegmatis</i>			
mc ² 155	12	20	ND
23G4	4	5	ND
23G4/ <i>pafA</i>	10	10	ND
64D12	4	5	ND
64D12/ <i>pup-prcB-prcA</i>	12	20	ND
Δ BA2	4	5	ND
Δ BA2/ <i>pup-prcB-prcA</i>	12	15	ND
Δ BA2 Δ log	12	10	ND
<i>M. tuberculosis</i>			
H37Rv	10	ND	10
Δ <i>mpa</i>	2.5	ND	2.5
Δ <i>mpa/mpa</i>	10	ND	10

^aSCP, sulfachloropyridazine; TMP, trimethoprim; SMZ, sulfamethoxazole; ND, not determined.

pYUB854, flanking a hygromycin cassette. After checking the orientation by sequencing, the allelic exchange substrates were excised and DNA fragment transformed directly into *M. smegmatis* mc²155 cells, previously induced to express the recombining system. Cells were selected for resistance to hygromycin. To confirm the replacement of *prcBA* genes by the hygromycin cassette, PCR was used to amplify the chromosomal locus with primers conf1 and conf2 located outside of the homologous sequences of the allelic exchange substrates (Fig. 4A). While PCR products amplified from wild-type *M. smegmatis* genomic DNA migrated as 3,441 bp, those amplified from the Δ BA1 hygromycin-resistant mutant displayed a mobility shift corresponding to 3,801 bp (Fig. 4B). The difference in gel migration corresponded exactly to the molecular mass of the hygromycin cassette against the combined molecular mass of *prcB* and *prcA* (Fig. 4B). The hygromycin cassette in the Δ BA1 mutant was then removed by expressing a resolvase that excised specific sites flanking the cassette (40), creating an unmarked Δ BA2 *prcBA* mutant strain. PCR products recovered from the Δ BA2 mutant migrated at around 1,900 bp, showing the removal of the hygromycin cassette (Fig. 4B).

Antifolate susceptibility testing showed that the Δ BA2 mutant was more sensitive to both trimethoprim and sulfonamides (Table 1, Fig. 4C) than wild-type *M. smegmatis* and that its sensitivity levels were comparable to those observed with 64D12 and 23G4. These results indicated that the proteolytic core was equally important for mycobacterial antifolate resistance. Indeed, genetic complementation by transformation of pVN1011 or pVN1014 (*pup-prcBA*) restored the wild-type resistance to the Δ BA2 mutant (Fig. 4C), confirming the requirement of PrcBA for *M. smegmatis* antifolate resistance.

Sulfonamides and acidified nitrite exhibit synergy that is potentiated in the absence of PPS. Our recent study established the role of the vitamin B₁₂-dependent methionine synthase (MetH) in the methylfolate trap, which induces thymineless death in mycobacteria exposed to antifolates (13). Because MetH is hypersusceptible to NO (41, 42), which is neutralized by mycobacterial PPS (22), we reasoned that PPS-mediated antifolate resistance may be affected by NO. Indeed, we found synergy between sulfonamides and acidified nitrite. Double disc synergy tests at pH 5.5 exhibited bridging of inhibition zones when discs containing sulfonamides and sodium nitrite were placed in close proximity (Fig. 5), suggesting synergy between these two antimicrobial agents. In the absence of either Pup (64D12) (Fig. 5A) or PafA (23G4) (Fig. 5B), *M. smegmatis* became more susceptible to sulfonamides, acidified nitrite, or their combination.

FIG 2 Legend (Continued)

diffusion. Cells of *M. smegmatis* strains were seeded onto the surface of NE plates. Paper discs embedded with 0.075 mg SCP or 0.025 mg TMP were then placed at the center of the plates for diffusion. Susceptibility, visualized as the zone of inhibition surrounding the discs, was recorded at day 5 of incubation at 37°C. Bar, 1 cm.

FIG 3 PafA-mediated ligation of Pup to protein substrates is required for *M. smegmatis* intrinsic antifolate resistance. *M. smegmatis*, 23G4, and a 23G4-derived strain in *trans* expressing *M. smegmatis* *pafA* were analyzed by Western blotting and antifolate sensitivity tests. (A) Western blotting followed by immunodetection of PafA (left, anti-PafA antibody) and pupylation (right, anti-Pup antibody) confirmed the role of PafA in ligating Pup to protein targets. MTHFS was used as a loading control. (B) Antifolate susceptibility testing of *M. smegmatis*, 23G4, and 23G4/*pafA*. Cells were seeded on the surface of NE medium, and paper discs embedded with SCP were placed at the center of the plates for diffusion. Plates were incubated at 37°C for 5 days before inhibition zones were recorded. Bar, 1 cm.

Mpa protein is required for intrinsic antifolate resistance in *M. tuberculosis*. To establish if the PPS system has a role in intrinsic antifolate resistance in *M. tuberculosis*, the gene encoding the proteasome-associated ATPase (*Mpa*) was deleted in the tubercle bacillus using specialized transduction. An allelic exchange substrate was constructed by cloning the DNA regions flanking the *mpa* gene to pYUB854 flanking the hygromycin cassette (Fig. 6A). The constructed plasmid was then subcloned into the genome of the temperature-sensitive mycobacterial phage, Phae87, to form a recombinant phage, which lyses mycobacterial cells at permissive temperatures. Specialized transduction was carried out at a nonpermissive temperature. Successful mutants, in which the hygromycin cassette replaced *mpa* (Fig. 6A), were confirmed by PCR using primer *mpa*-c1, which annealed to a chromosomal region beyond the homologous sequences of the allelic exchange substrates, and primer P2, which annealed to the hygromycin cassette. The formation of the expected PCR products amplified from the genomic DNA of the mutant candidates indicated that targeted deletion was successful (Fig. 6B). The result was further supported by cloning PCR products followed by sequencing (Fig. 6C).

Wild-type *M. tuberculosis* H37Rv, *mpa* mutant (Δ *mpa*), and an *mpa* complemented strain (Δ *mpa*/*mpa*) were subjected to susceptibility testing with sulfamethoxazole (SMZ) and sulfachloropyridazine (SCP) (Table 1, Fig. 6D). On agar plates, at a concentration of 5 mg/liter sulfonamides, the Δ *mpa* mutant displayed a growth defect that was compensated for when *mpa* was supplied in *trans* from plasmid pVN1015 (Fig. 6D).

FIG 4 Proteolytic core component PrcBA is required for *M. smegmatis* antifolate resistance. (A) Targeted deletion of *prcBA* in *M. smegmatis* by recombineering approach. The *prcBA* allelic exchange substrate was constructed by PCR cloning the left and right flanking DNA regions in the right orientation into pYUB854, flanking the built-in hygromycin cassette. As a result of homologous recombination, both *prcB* and *prcA* were replaced by the hygromycin cassette in the Δ BA1 mutant. The hygromycin cassette was removed by $\gamma\delta$ resolvase-expressing plasmid pGH542, resulting in the unmarked Δ BA2 mutant. (B) Confirmation of *prcBA* deletion by PCRs using primers conf1 and conf2. PCR products amplified from the *M. smegmatis* genome exhibited a molecular weight of 3,441 bp, whereas PCRs amplified from Δ BA1 and Δ BA2 mutants displayed molecular weights of 3,801 bp and $\sim$ 1,900 bp, respectively. (C) *prcBA* is required for *M. smegmatis* intrinsic antifolate resistance. Targeted deletion of *prcBA* resulted in increased SCP susceptibility, as visualized by larger inhibition zones, while in *trans* expression of *pup-prcBA* (pVN1014) restored antifolate resistance. Bar, 1 cm.

These results indicate that every component of the PPS system, including proteasome-associated ATPase (Mpa), is required for antifolate resistance in mycobacteria, including *M. tuberculosis*.

Effects of DHFR overexpression on PPS-mediated antifolate resistance. First, we tested the effect of DHFR on PPS-mediated antifolate resistance and folic acid utilization in *M. smegmatis* strains. The encoding gene in *M. tuberculosis* (*dfrA*) was PCR cloned to vector pVN747 (Table 2), coupling its expression to the SOD promoter. The constructed plasmids (pVN1019; Table 2) were then introduced to *M. smegmatis* strains by electroporation for *trans* expression. Strains carrying the plasmids together with control strains were subjected to antifolate susceptibility testing and chemical complementation assays. As shown in Fig. 7A, in *trans* cross-species expression of DHFR enhanced folic acid utilization and antifolate resistance in *M. smegmatis*, bypassing defects caused by a lack of PPS activity.

Effects of cytokinin production on PPS-mediated antifolate resistance. To examine if cytokinins affect *M. smegmatis* antifolate resistance, the gene encoding Lonely Guy (*log*, *msmeg_5087*), a cytokinin production protein previously found to be regulated by mycobacterial PPS (22), was deleted in both wild-type *M. smegmatis* and the Δ BA2 mutant (unmarked *prcBA* double mutant, Fig. 4), using the recently

FIG 5 Synergy of acidified nitrite and sulfonamides and role of PPS. (A) Synergy in the presence or absence of Pup. Cells of *M. smegmatis*, 64D12, or 64D12/pup were seeded onto NE agar plates (pH 5.5). Plates were treated with a disc containing 12.5 mg NaNO₂ alone or placed in proximity (4 cm) with another disc containing 0.075 mg SCP. Inhibition zones were recorded after 5 days of growth at 37°C. Bar, 1 cm. (B) Synergy in the presence or absence of PafA. Cells of *M. smegmatis*, 23G4, or 23G4/pafA were seeded onto NE agar plates (pH 5.5) and tested as described for panel A. While synergy between NaNO₂ and SCP was observed in all strains, it became profound in the absence of pupylation activity. Drawn circles are to distinguish original inhibition zones from extended inhibition areas caused by synergy (bridging of zones of inhibition). Bar, 1 cm.

developed oligonucleotide-mediated recombineering followed by Bxb1 integrase targeting (ORBIT) method (43). Cells were first transformed with Che9c-RecT annealase-producing plasmid pKM444 (Table 2). Thereafter, cells were cotransformed with a synthetic targeting oligonucleotide (Log-Oligo) (Table 3), which contains two *log*-homologous sequences, flanking a Bxb1 integration sequence (Int), and the payload plasmid pKM464 (43). Mutant candidates were selected for resistance to both kanamycin and hygromycin, and successful *log* disruption was validated by PCR using combinations of chromosomal specific primers (P1 and/or P2; Table 3) and pKM464-specific primers (P3 and/or P4; Table 3). PCRs using P1 and P2 amplified a 585-bp DNA fragment from the genome of wild-type *M. smegmatis* or the Δ BA2 mutant but not their derived *log* mutants (Fig. 7B, white arrows). In contrast, PCRs using combinations of a chromosomal specific primer and pKM464-specific primers (P2 and P4 or P1 and P3) resulted in DNA amplification only from the derived *log* mutants (250 bp or 745 bp, respectively) but not their parental strains (Fig. 7B, white arrows).

FIG 6 Role of Mpa in *M. tuberculosis* antifolate resistance. (A) Targeted deletion of *mpa* in *M. tuberculosis* using phage transduction. Left and right flanking DNA regions of *mpa* were cloned into pYUB854, flanking the built-in hygromycin cassette. The plasmid was subcloned into the genome of temperature-sensitive Phae87, followed by transduction into *M. tuberculosis* H37Rv at nonpermissive temperature. Homologous recombination generated the Δ*mpa* strain. Bar, 1 kb. (B) Agarose gel electrophoresis verifies the replacement of *mpa* with the hygromycin cassette in the Δ*mpa* strain. PCR using primers mpa-c1 and P2, and genomic DNA from the Δ*mpa* strain as a template, generated a 1,106-bp band, which was absent from PCR using wild-type genomic DNA. (C) Chromatogram shows sequences of the junction of the hygromycin cassette and genomic DNA in the Δ*mpa* strain. (D) Antifolate susceptibility testing of *M. tuberculosis* strains. Approximately 10⁷ cells of *M. tuberculosis* and Δ*mpa* and Δ*mpa*/*mpa* strains were spread on 7H10 medium containing 5 mg/liter SMZ or SCP and incubated at 37°C for 4 weeks. Bar, 2 cm.

Next, the antifolate sensitivity of the obtained *log* mutants and their parental strains was tested by a culture spotting method. The disruption of *log* restored antifolate resistance to the Δ*BA2* mutant (Fig. 7C), indicating that Lonely Guy's role in cytokinin production also plays a part in PPS-mediated antifolate resistance in mycobacteria.

DISCUSSION

In a previous screen of 13,500 *Himar1*-transposon mutants, representing approximately 2-fold coverage of the *M. smegmatis* genome, we identified 50 chromosomal loci representing the whole-genome antifolate resistance determinants (13). Further characterization of this antifolate resistome uncovered a novel mechanism that is described in this paper.

The resistance mechanism is mediated by the prokaryotic Pup-proteasome system (PPS), which is functionally homologous to the ubiquitin-proteasome system (UPS) in

TABLE 2 Plasmids and strains used in this study

Plasmid or strain	Relevant feature(s) ^a	Reference or source
Plasmids		
pMV361	<i>Mycobacterium</i> integrative vector, Kan ^r , P _{hsp60}	65
pYUB854	<i>E. coli</i> plasmid (Ωhyg) for constructing AESs	58
pVN701B	Plasmid expressing <i>Che9c</i> 60-61, <i>ts</i> -Ori, <i>sucA</i>	39
pGH542	<i>Mycobacterium</i> vector expressing the γδ resolvase	40
pVN747	<i>Mycobacterium</i> replicative vector, Hyg ^r , P _{SOD}	39
pKM444	Plasmid expressing <i>Che9c</i> phage RecT, Kan ^r	43
pKM464	ORBIT plasmid for gene knockout	43
pVN1009	pMV361 derived, expressing <i>M. tuberculosis</i> <i>pup</i> from P _{hsp60}	This study
pVN1010	pMV361 derived, expressing <i>M. tuberculosis</i> <i>prcB-prcA</i> from P _{hsp60}	This study
pVN1011	pMV361 derived, expressing <i>M. tuberculosis</i> <i>pup-prcB-prcA</i> from P _{hsp60}	This study
pVN1012	pVN747 derived, expressing <i>M. tuberculosis</i> <i>pup</i> from P _{hsp60}	This study
pVN1013	pVN747 derived, expressing <i>M. tuberculosis</i> <i>prcB-prcA</i> from P _{hsp60}	This study
pVN1014	pVN747 derived, expressing <i>M. tuberculosis</i> <i>pup-prcB-prcA</i> from P _{hsp60}	This study
pVN1015	pMV361 derived, expressing <i>M. tuberculosis</i> <i>mpa</i> from P _{hsp60}	This study
pVN1016	pVN747 derived, expressing <i>M. smegmatis</i> <i>pafA</i> from P _{SOD}	This study
pVN1017	pYUB854 derived, carrying <i>M. tuberculosis</i> Δ <i>mpa</i> ::Ωhyg AES	This study
pVN1018	pYUB854 derived, carrying <i>M. smegmatis</i> Δ <i>prcBA</i> ::Ωhyg AES	This study
pVN1019	pVN747 derived, expressing <i>M. tuberculosis</i> <i>dfrA</i> from P _{hsp60}	This study
Strains		
mc ² 155	<i>M. smegmatis</i> wild type	56
64D12	mc ² 155 derived, <i>Himar1</i> transposon <i>pup</i> mutant (TA ¹⁷²⁻¹⁷³)	This study
64D12/ <i>pup</i>	64D12 expressing <i>M. tuberculosis</i> <i>pup</i> from pVN	This study
64D12/ <i>prcBA</i>	64D12 expressing <i>M. tuberculosis</i> <i>prcB-prcA</i> from pVN	This study
64D12/ <i>pup-prcBA</i>	64D12 expressing <i>M. tuberculosis</i> <i>pup-prcB-prcA</i> from pVN	This study
23G4	mc ² 155 derived, <i>Himar1</i> transposon <i>pafA</i> mutant (TA ³⁷⁶⁻³⁷⁷)	This study
23G4/ <i>pafA</i>	23G4 transformed with pVN1016	This study
Δ <i>BA1</i>	mc ² 155 derived, Δ <i>prcBA</i> ::Ωhyg	This study
Δ <i>BA2</i>	mc ² 155 derived, unmarked Δ <i>prcBA</i>	This study
Δ <i>BA2/pup</i>	Δ <i>BA2</i> expressing <i>M. tuberculosis</i> <i>pup</i> from pVN1012	This study
Δ <i>BA2/prcBA</i>	Δ <i>BA2</i> expressing <i>M. tuberculosis</i> <i>prcB-prcA</i> from pVN1013	This study
Δ <i>BA2/pup-prcBA</i>	Δ <i>BA2</i> expressing <i>M. tuberculosis</i> <i>pup-prcB-prcA</i> from pVN1014	This study
H37Rv	<i>Mycobacterium tuberculosis</i> laboratory strain	66
<i>RvΔmpa</i>	H37Rv derived, targeted <i>mpa</i> deletion mutant	This study
<i>RvΔmpa/mpa</i>	<i>RvΔmpa</i> strain expressing <i>M. tuberculosis</i> <i>mpa</i> from pVN1015	This study

^aAES, allelic exchange substrates.

eukaryotes. We show that many steps of the PPS process are required for antifolate resistance in both *M. smegmatis* and *M. tuberculosis*. Genetic disruption of genes encoding (i) the prokaryotic ubiquitin-like protein (Pup; Fig. 1 and 2), (ii) the ligase responsible for tagging Pup to protein substrates (PafA; Fig. 1 and 3), (iii) the proteasome associated ATPase responsible for unfolding and translocating Pup-tagged proteins through the 20S proteolytic core (Mpa; Fig. 6), and (iv) the structural proteolytic core proteins themselves (PrcA and PcrB; Fig. 4) all resulted in increased antifolate susceptibility. Genetic evidence is supported by complementation and phenotypic characterization as well as immunodetection of protein expression and pupylation, substantiating the hypothesis that PPS protects mycobacterial cells from the cytotoxicity of antimicrobial antifolates. This establishes PPS as a novel antifolate resistance determinant in mycobacteria.

Our further studies suggest that the role of PPS in mycobacterial antifolate resistance is mediated through its regulatory function in at least two downstream pathways.

First, PPS, through its specialized proteolytic activity, may control the half life of an unknown inhibitory protein that suppresses folate reductases, such as dihydrofolate reductase (DHFR) or RibD (44). Increased proteasomal degradation of such an inhibitor would enhance DHFR activity, allowing more oxidized folates to recycle to the reduced, metabolically active tetrahydrofolate, undermining the growth-inhibitory activity of antifolates. In the absence of PPS, the inhibitor would be more available to inhibit DHFR, boosting the antimycobacterial activity of antifolates. This hypothesis is supported by our observation that the impaired sulfonamide resistance of *pup* (64D12)

FIG 7 Possible mechanisms underlying PPS-mediated antifolate resistance in mycobacteria. (A) Effects of *trans* DHFR overexpression on folic acid assimilation and antifolate resistance in wild-type *M. smegmatis* and its derived PPS mutants. The DHFR-encoding gene from *M. tuberculosis* (*dhfrA*) was cloned to couple its expression to the SOD promoter and transformed into *M. smegmatis* PPS strains. Cultures were spotted on NE medium supplemented with antifolates (5 μ g/ml SCP or 2.5 μ g/ml TMP) in the absence or presence of folic acid (0.3 mM) or folinic acid (0.3 mM). Growth was recorded after 5 days of incubation at 37°C. (B) PCR confirmation of *log* disruption by *Int*-mediated insertion of pKM464 plasmid. *log*-specific primers (P1 and P1) resulted in 585-bp PCR products (white arrows) from parental strains, *M. smegmatis*, or the $\Delta B A 2$ strain but not their derived *log* mutants. In contrast, combinations of a chromosome-specific primer (P1 or P2) and a pKM464-specific primer (P3 or P4) resulted in PCR products from *log* mutants but not their parents. (C) Antifolate susceptibility of *M. smegmatis* strains. A total of 10^5 cells of *M. smegmatis*, $\Delta B A 2$, Δlog , or $\Delta B A 2 \Delta log$ strain were spotted onto NE plates containing 5 μ g/ml SCP. Growth was recorded after 5 days of incubation at 37°C.

and *pafA* (23G4) mutants was compensated by exogenous pABA or folinic acid but not folic acid (Fig. 1C and 7A), which requires folate reductase activity. In addition, in *trans* overexpression of DHFR bypassed PPS, allowing both the proteasome mutants to utilize folic acid and restore intrinsic sulfonamide resistance (Fig. 7A, left). Interestingly, the eukaryotic ubiquitin-proteasome system UPS was also reported to regulate DHFR activity under oxidative conditions through direct degradation (45). Future studies will focus on identifying the inhibitory protein(s) of DHFR and related PPS-mediated regulatory mechanism(s).

Second, the function of PPS in mycobacterial antifolate resistance might be mediated through its role in regulating cytokinin production (23). This hypothesis is based on the observation that both cytokinins (23) and antifolates (Fig. 5) enhance the antimycobacterial activity of acidified nitrite. We show that disruption of the *M. smegmatis* gene (*log*) encoding Lonely Guy, the phosphoribohydrolase that catalyzes the biosynthesis of cytokinins and an established target of mycobacterial PPS (23), abolished the increased antifolate sensitivity caused by the absence of PPS (Fig. 7B and C). This result suggests that Lonely Guy functions downstream of PPS in regulating antifolate sensitivity in mycobacteria. Although the precise molecular mechanism underlying cytokinin-mediated antifolate resistance remains largely unknown, previous studies support the involvement of cytokinins in folate production (46). Interestingly, *log* (*msmeg_5087*,

TABLE 3 Oligonucleotides used in this study

Primer	Sequence (5'–3')
Del1	ACTAGTGATGGGCACTGGTGACGACGAC
Del2	AAGCTTCTCAGAACGCTCTGACTACCCA
Del3	TCTAGAGCTATTGATACCGAGTCCAACCTCG
Del4	GGTACCGGCTTGCCGGGCAACGT
mpa-c1	GGTCTGCCTAGACAATTGCCAGC
P2	CTGCACGACTTCGAGGTGTTCTGA
Compl1	GAATTCATATGGGTGAGTCAGAGCGTTCTGAG
Compl2	GGATCCAAGCTTCTACAGGTACTGGCCGAGGTTGG
MTBpup1	GAATTCATGGCGCAAGAGCAGACCAAGCGT
MTBpup2	AAGCTTGATCGGGCAACGGCCAGGTCA
MTBprcB1	GAATTCATGACCTGGCCGTTGCCGATC
MTBprcA2	AAGCTTCGTCGGACTTTCGGACTCAGC
c1	GAATTCATATGGCTCAGGAGCAGACCAAGCG
c2	GGATCCAAGCTTCTATTTCGGTGGGTTTGTGCGCTGA
c3	GAATTCATATGCAGCGACGAATCATGGGCATC
c4	GGATCCAAGCTTCACATACTGGCGATCAGCCGCTT
MSPupBA1	GAATTCATATGGCTCAGGAGCAGACCAAGCG
MSPupBA2	GGATCCAAGCTTCTATTTCGGTGGGTTTGTGCGCTGA
ms-Del1	ACTAGTGGTCACCGAGCAGCAAGTGC
ms-Del2	AAGCTTGAGAACGAGGACAGGTCCAC
ms-Del3	TCTAGACCGAATAGTCGCGTCAGGACG
ms-Del4	GGTACCGATCCTGAGCCTGGACTCCGAT
conf1	GCTGCAACGCATCTACCTCGAC
conf2	TGTGGTTGCTGCTGCGCTTCGA
dfrA1	GAATTCATATGGTGGGGCTGATCTGGGCTC
dfrA2	AAGCTTCTCATGAGCGGTGGTAGCTGTAC
P1	GTTGGAAGCTGCGGCTCA
P2	GCCGGTGGCCAAACACCTAAC
P3	CCTGGTATCTTTATAGTCTGTCG
P4	GAGGAAGTGGCGAGTTCCTCTGG
Log-Oligo ^a	ATGTGAAAGAAGGACAGGACCGCCAGTGGGCGGTGTGTGTACTGCGCGTCGGGA CCGACGCATGGTTTGTCTGGTCAACCAACCGCGGTCTCAGTGGTGTACGGTACAAACC CCATGGACAGCTGGTGGTGGTCGACAACGTGAGGCCGCGCTCGAGGCGTGC GCG CCCGAGTAG

^aInt sequence is underlined.

rv1205) is located next to *folP2* (*msmeg_5085*, *rv1207*), which encodes one of the two dihydropteroate synthase homologs (DHPS) (47), in *M. smegmatis* and *M. tuberculosis* genomes. It is possible that cytokinins or their degradation products function as storage molecules or regulators of the folate pathway in mycobacteria. Future studies will focus on elaborating this hypothesis and determining the regulatory molecule derived from the cytokinin pathway that affects PPS-mediated antifolate resistance. Therefore, the synergy between sulfonamides and acidified nitrite boosted in the absence of PPS (Fig. 5) might be mediated through the dual effects of cytokinins on both the folate pathway and NO cytotoxicity (23).

Although our work indicates the PPS regulation of the mycobacterial folate pathway is mediated through controlling activities of DHFR, other folate-dependent enzymes and pathways are likely impacted as well. For example, one or both DHPS proteins (FolP1 or/and FolP2) in mycobacteria may be controlled directly through PPS proteolytic activity or indirectly via cytokinin-mediated enzymatic regulation. In eukaryotes, several folate-dependent enzymes have also been shown to be targets of the UPS (45, 48, 49). 10-Formyltetrahydrofolate dehydrogenase, which converts 10-formyltetrahydrofolate to tetrahydrofolate, is degraded by UPS during S-phase, increasing the cellular level of 10-formyltetrahydrofolate required for *de novo* purine synthesis and cell proliferation (48). Thymidylate synthase, the activity of which is associated with thymineless death and the bactericidal activity of antimicrobial antifolates (13, 50), is regulated by the proteasome in eukaryotic cells (49).

Our finding of the three-way interaction of antifolates, acidified nitrite, and PPS in mycobacterial antifolate resistance (Fig. 5) is intriguing for possible clinical use. Antifolates, NO, and PPS inhibitors each have recently emerged as novel or potentially repurposed antimycobacterial therapies. Indeed, antifolates such as cotrimoxazole have been repeatedly shown to be effective against *M. tuberculosis* isolates, including drug-resistant strains (9, 10, 51, 52), while inhibitors selective for mycobacterial PPS were found to kill nonreplicating *M. tuberculosis* in macrophages (24). NO inhalation has also been suggested as a treatment against mycobacterial infections (53), with two separate clinical trials testing the use of inhaled gaseous NO (160 ppm) to treat non-TB mycobacteria and *M. abscessus* complex infections (54, 55). Our findings suggest that combination therapies using sulfonamides, NO, and PPS inhibitors represent a novel therapeutic approach for treating mycobacterial infections in this era of increasing drug resistance.

MATERIALS AND METHODS

Special chemicals and reagents. All chemicals were of the highest available quality. Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Plasmids and oligonucleotides. Plasmids and oligonucleotides used in this study are listed in Tables 2 and 3, respectively. Oligonucleotide primers were purchased from Eurofins MWG Operon (Huntsville, AL). PCRs were performed using the expand long template PCR kit (Roche Molecular Biochemicals). QIAquick gel extraction and QIAprep spin miniprep kits were purchased from Qiagen (Valencia, CA). DNA sequences were verified by cloning PCR products into pGEM-T Easy vector (Promega, Madison, WI), followed by sequencing (ACGT Inc., Wheeling, IL).

Bacterial strains, media, and growth conditions. All strains used in this study are listed in Table 2. Difco media and components were obtained from Fisher Scientific. Wild-type parental strain *M. smegmatis* mc²155 (56) and its derived mutants were grown in 7H9 or Luria-Bertani (LB) medium supplemented with glucose and 0.5% Tween 80 at 37°C. Strains were propagated on supplemented 7H10 or LB agar medium. Drug susceptibility was performed on NE medium (14, 57). pH was adjusted to 5.5 when acidified nitrite sensitivity was tested (22). *M. tuberculosis* strains were grown using 7H9 or 7H10 supplemented with 0.05% Tween 80 and oleic acid-albumin-dextrose-catalase (OADC) (BD) at 37°C. Kanamycin was used at 50 µg/ml. Hygromycin was used at 150 and 100 µg/ml for *M. smegmatis* and *M. tuberculosis* strains, respectively. Transformation and genetic manipulations were carried out as described previously (58). Sulfonamide drugs and NaNO₂ solutions were made fresh the day of use in dimethyl sulfoxide and distilled H₂O, respectively.

Genetic screen for *Mycobacterium smegmatis* antifolate-sensitive mutants. The genetic screen for *M. smegmatis* whole-genome antifolate resistance determinants was described previously (13). Briefly, a library of 13,500 *Himar1* *M. smegmatis* mutants was first constructed using the temperature-sensitive pMycMar plasmid (31, 57, 58). Transformants were recovered overnight at 30°C and then plated on LB agar plates containing 50 µg/ml kanamycin. After 5 days of incubation at 39°C, individual kanamycin-resistant colonies retaining the transposon were selected and cultured separately in 96-well plates with LB medium containing 50 µg/ml kanamycin for 2 days. A kanamycin-resistant *M. smegmatis* mc²155 strain (pMV361) was used as a growth control. Cultures from these 96-well plates were then replicated onto solid NE medium in the presence of various concentrations of SCP (0, 10, 15, 20, 25, or 50 µg/ml) or TMP (0, 1.25, 1.5, 2, 2.5, or 3 µg/ml) using a sterilized 96-prong replicator (14). After 5 days of growth at 37°C, colonies that grew on NE control plates but failed to grow on plates supplemented with antifolates were selected. These mutants were subjected to two additional rounds (each from a different colony) of replication to confirm drug susceptibility profiles.

Mapping chromosomal *Himar1* insertion sites. Chromosomal locations of *Himar1* transposition in antifolate-sensitive mutants were mapped using an arbitrary PCR approach (39, 57, 59). Sequences around the chromosome-*Himar1* junctions were first PCR amplified using a random-sequenced primer (ARB1 or ARB6) paired with a *Himar1*-specific primer (Mar-Ext1 or Mar-Ext2). PCR products from the first round were then used as templates for a second round of PCR using primers that recognize sequences of the first-round primers (ARB2 and Mar-Int1 or Mar-Int2). Second-round PCR products were purified using a Qiagen PCR purification kit, followed by sequencing (ACGT, Inc.) with primers Mar-Int1 or Mar-Int2. Sequences were searched against the *M. smegmatis* genome sequence deposited at the National Center for Biotechnology Information. Transposon insertions were confirmed by PCRs using primers that recognize chromosomal sequences outside the insertion sites, followed by sequencing, repeated BLAST searches, and sequence alignments.

Targeted gene deletion. Specialized transduction was used to delete the *mpa* gene (*rv2115c*) in *M. tuberculosis*, as previously described (58). Briefly, the 654-bp 3' DNA region of *mpa* was PCR amplified using primers Del3 and Del4 (Table 3). PCR products were cloned into pGEM-T Easy and sequence confirmed by sequencing. The cloned DNA sequence was then subcloned into pYUB854 (Table 2) at XbaI and KpnI sites, followed by sequencing to confirm correct orientation. Thereafter, the 719-bp 5' DNA region of *mpa* was similarly PCR cloned using primers Del1 and Del2 (Table 3) and then subcloned into the SpeI and HindIII sites on pYUB854:3'-*mpa* to create pVN1017 (Table 2) (58). The orientation of the cloned DNA fragments was confirmed by sequencing using primers annealing to the hygromycin

cassette in pYUB854. The temperature-sensitive mycobacteriophage pVN1017 (Table 2) was generated by cloning pVN1017 into the unique PacI site of the TM4-derived temperature-sensitive phAE87 genome as previously reported (58). Briefly, PacI-digested pVN1017 was ligated to the PacI-digested concatemeric phAE87 genomic DNA and *in vitro* packaged to the λ phage heads using λ packaging extracts (GIGAPack III gold kit; Stratagene). After transduction, *E. coli* NM554 was plated on hygromycin-containing LB agar plates. Plasmid DNA prepared from a pool of hygromycin-resistant transductants was electroporated into *M. smegmatis* mc²155. Plaques that grew at permissive temperature (28°C) were purified, checked for temperature sensitivity, and then used to transduce *M. tuberculosis* H37Rv as previously described (58). *RvΔmpa* transductants were selected on 7H10-OADC plates supplemented with 100 μ g/ml hygromycin at 37°C. The replacement of *mpa* by the hygromycin cassette in the *RvΔmpa* mutant was confirmed by PCR using primers mpa-c1 and P2 (Table 3), which annealed to the chromosomal sequence outside the allelic exchange substrate and to the hygromycin cassette in pYUB854, respectively, followed by sequencing.

Both the *prcB* and *prcA* genes in *M. smegmatis* (*msmeg_3895* and *msmeg_3894*) were simultaneously deleted using the recombineering method as previously described (39). The 659-bp 5' DNA region of *prcB* (left flank) was PCR amplified using primers ms-Del1 and ms-Del2 (Table 3). Similarly, the 557-bp 3' DNA region of *prcA* (right flank) was similarly PCR amplified using primers ms-Del3 and ms-Del4 (Table 3). PCR products were cloned into pGEM-T Easy vector, and the sequences were confirmed by sequencing. pVN1018 was constructed by subcloning the 5'-*prcB* at SpeI/HindIII sites and the 3'-*prcA* at XbaI/KpnI sites of pYUB854 (58). The *MsΔprcBA::Ωhyg* linear allelic exchange substrate was excised from pVN1018 by SpeI/KpnI digestion and transformed into *M. smegmatis* mc²155 cells that had been induced to express the recombineering system from pVN701B (Table 2) (39). Transformed cells were plated on 7H9 medium containing hygromycin. Successful recombination in mutant candidates (Δ BA1) was confirmed by PCR using primers conf1 and conf2 (Table 3), which anneal to chromosomal sequences, extending the homologous sequences of the allelic exchange substrate. Next, pVN701B was removed by shifting cultures of the Δ BA1 mutant to 39°C in 7H9 medium containing hygromycin and sucrose (1%, wt/vol). The hygromycin resistance cassette was then excised by transforming pGH542 (Table 2) expressing $\gamma\delta$ resolvase into the Δ BA1 mutant. The unmarked mutants (Δ BA2) were selected on 7H10 with tetracycline (2.5 μ g/ml) at 37°C and confirmed by PCR using primers conf1 and conf2 (Table 3).

The *M. smegmatis* gene encoding Lonely Guy (*log*, *msmeg_5087*) was disrupted using the oligonucleotide-mediated recombineering followed by Bxb1 integrase targeting (ORBIT) method (43). Log-Oligo was purchased from IDT (Coralville, IA) as an ultramer at a concentration of 100 μ M. *M. smegmatis* strains were first transformed with plasmid pKM444 expressing Che9c-RecT annealase and selected for kanamycin resistance (20 μ g/ml). Cells were grown to an optical density at 600 nm (OD₆₀₀) of 0.5 and induced with 500 ng/ml anhydrotetracycline for about 3 h, when the OD₆₀₀ reached 1. Competent cells were then prepared by washing with cold 10% glycerol and transformed with a mixture of 200 ng pKM464 and 1 μ g Log-Oligo (Table 3). Transformants were selected on LB agar supplemented with 150 μ g/ml hygromycin and 20 μ g/ml kanamycin.

Plasmid constructions for *in trans* expression. The *M. tuberculosis mpa* gene (*rv2115c*; 1,830 bp) was PCR cloned from *M. tuberculosis* genomic DNA using primers Compl1 and Compl2 (Table 3). *mpa* was then subcloned to pMV316 at EcoRI/HindIII sites, coupling its expression to the built-in promoter P_{hsp60}, creating pVN1015. Plasmid pVN1015 was transformed into the *RvΔmpa* strain by electroporation. Transformants were selected for resistance to both kanamycin and hygromycin.

The *M. tuberculosis pup* gene (*rv2111c*) was PCR cloned from genomic DNA using primers MTBpup1 and MTBpup2 (Table 3) and ligated to pMV361 at EcoRI/HindIII sites, coupling its expression to the P_{hsp60} promoter, to create pVN1009. The XbaI/SpeI P_{hsp60}-*pup* fragment from pVN1009 was then subcloned into pVN747 cut with SpeI to create pVN1012. Similarly, the two-gene DNA fragment for expressing both *M. tuberculosis* *PrcB* and *PrcA* (*rv2110c-rv2109c*) was PCR cloned using primers MTBprcB1 and MTBprcA2 (Table 3) and ligated to pMV361 at EcoRI/HindIII sites (pVN1010). The XbaI/SpeI P_{hsp60}-*prcB-prcA* fragment was then subcloned into SpeI-digested pVN747 to create plasmid pVN1013. Using the same approach, the DNA fragment carrying all three *M. tuberculosis* genes, *pup-prcB-prcA* (*rv2111c-rv2110c-rv2109c*), was first PCR cloned using primers MTBpup1 and MTBprcA2 and ligated to pMV361 at EcoRI/HindIII sites (pVN1011). The XbaI/SpeI P_{hsp60}-*pup-prcB-prcA* fragment was then subcloned into pVN747 cut with SpeI to create pVN1014. Plasmid pVN1012, pVN1013, or pVN1014 was transformed into *M. smegmatis* 64D12 and Δ BA2 strains by electroporation, and transformants were selected for hygromycin resistance. Plasmid pVN1009, pVN1010, or pVN1011 was also each transformed to Δ BA1 and Δ BA2 strains and transformants selected for kanamycin resistance.

For *in trans* expression of *M. smegmatis pafA* (*msmeg_3890*), the gene was PCR cloned using primers c3 and c3 (Table 3) and ligated to pVN747 at NdeI/HindIII sites, coupling its expression to the P_{SOD} promoter, to create pVN1016 (Table 2). Plasmid pVN1016 was then transformed into *M. smegmatis* 23G4 by electroporation. Transformants were selected by kanamycin and hygromycin resistance.

Similar procedures were used to construct pVN1019 using primers dfrA1 and dfrA2 (Table 3) for *in trans* expression of *M. tuberculosis dfrA*.

Disc diffusion assays. Single colonies of *M. smegmatis* strains were grown to an OD₆₀₀ of 1. Aliquots (50 μ l) of each culture were cast in soft NE agar (0.5%, pH 5.5) and placed on top of NE medium plates (pH 5.5). Paper discs embedded with 0.075 mg SCP, 0.025 mg TMP, and/or 12.5 mg NaNO₂ were placed 4 cm apart on the surface of the culture plates. Growth inhibition was visualized as the inhibition zone surrounding the disc after 5 days of incubation at 37°C. Synergy was visualized as the bridging of the existing inhibition zones or the formation of an additional inhibition zone between the discs (60, 61).

Susceptibility assays. MICs of *M. tuberculosis* strains were determined using the broth dilution method (13, 62). Approximately 10^4 *M. tuberculosis* cells were inoculated in 7H9-S medium (7H9 plus 0.1% trypsin digest of casein, 0.5% glycerol, and OADC), supplemented with serially diluted concentrations of SMZ or SCP. Cultures were grown for 7 days, followed by growth detection using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method (13, 62). The MIC was recorded as the lowest concentration of SMZ or SCP that prevented growth-mediated conversion of MTT (yellow) to formazan (violet). For visualizing *M. tuberculosis* susceptibility (Fig. 6D), approximately 10^7 cells of wild-type H37Rv, Δ mpa, and Δ mpa/mpa strains were spread onto 7H10-OADC medium in the absence or presence of 5 mg/liter SCP or SMZ and incubated at 37°C for 4 weeks. MICs of *M. smegmatis* strains were determined using the agar dilution method (63, 64). Briefly, 10^4 *M. smegmatis* cells were spotted on plates containing serial dilutions of drugs, and plates were incubated at 37°C for 5 days. The MIC was defined as the lowest drug concentration resulting in complete growth inhibition or growth of fewer than 10 colonies (<10% inoculum). Experiments were performed in triplicates to ensure reproducibility.

Western blot analyses. Single colonies of *M. smegmatis* strains were picked and grown in LB with Tween 80 and appropriate antibiotics to an OD₆₀₀ of 1.6 to 1.8. Cell pellets were harvested at 4°C and 4,000 rpm for 15 min and washed twice with Tris-HCl-buffered saline (TBS) containing a protease inhibitor cocktail (Roche Molecular Biochemicals). Cells were resuspended in 2 ml TBS buffer with protease inhibitors and disrupted by sonication (5 times for 10 s on ice with 1-min cooling intervals), and the lysate was spun for 20 min at 10,000 rpm and 4°C to remove the insoluble material. The obtained supernatant was treated with SDS loading buffer and boiled at 95°C for 10 min. Samples containing 20 µg proteins were separated on 15% SDS-polyacrylamide electrophoretic gel. Proteins were transferred to nitrocellulose membranes using a semidry apparatus at 0.8 mA/cm² for 2 h. The membranes were blocked for 1 h at 4°C in TBS containing 0.05% Tween 80 and 5% nonfat dry milk (TBS-T). Blocked membranes were then incubated in a primary antibody for 1 h. Anti-Pup and anti-PafA antisera were obtained from Eyal Gur (Ben-Gurion University of the Negev) and used at a 1:20,000 dilution. After washing thrice with 1× TBS-T, the membranes were incubated with a peroxidase-conjugated secondary antibody for 1 h at room temperature. Signals were detected using the digital chemiluminescent ImageQuant LAS 4000 machine (GE Healthcare Bio-Sciences, Pittsburgh, PA), and images were analyzed using ImageQuant LAS 4000 control software (GE Healthcare Bio-Sciences, Pittsburgh, PA).

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