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Overexpression of a membrane transport system MSMEG_1381 and MSMEG_1382 confers multidrug resistance in *Mycobacterium smegmatis*

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Abstract

Mycobacterium tuberculosis is a leading cause of human mortality worldwide, and the emergence of drug-resistant strains demands the discovery of new classes of antimycobacterials that can be employed in the therapeutic pipeline. Previously, a secondary metabolite chrysomycin A, isolated from *Streptomyces* sp. OA161 was shown to have potent bactericidal activity against drug-resistant clinical isolates of *M. tuberculosis* and different

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species of mycobacteria. The antibiotic inhibits the mycobacterial topoisomerase I and DNA gyrase leading to bacterial death, but the mechanisms that could cause resistance to this antibiotic are currently unknown. To further understand the resistance mechanism, using *M. smegmatis* as a model, spontaneous resistance mutants were isolated and subjected to whole-genome sequencing. Mutation in a Tet^R family transcriptional regulator MSMEG_1380 was identified in the resistant isolates wherein the gene was adjacent to an operon encoding membrane proteins MSMEG_1381 and MSMEG_1382. Sequence analysis and modeling studies indicated that MSMEG_1381 and MSMEG_1382 are components of the Mmp family of efflux pumps and over-expression of either the operon or individual genes conferred resistance to chrysomycin A, isoniazid, and ethambutol. Our study highlights the role of membrane transporter proteins in conferring multiple drug resistance and the utility of recombinant strains overexpressing membrane transporters in the drug screening pipeline.

Introduction

The emergence of antimicrobial resistance (AMR) in bacterial pathogens is a major health concern. Declaration by the World Health Organization (WHO) that AMR is a global health threat requiring urgent action is a testimony to this emerging crisis (1). *Mycobacterium tuberculosis* is the causative agent of tuberculosis and a leading cause of debilitation and mortality in humans. According to WHO, nearly $1/4^{th}$ of human population has latent tuberculosis infection and annually 1.2-1.5 million deaths occur due to the disease (2). Emergence of drug resistant TB strains is a major health concern, and further prolongs the duration and expenditure of treatment. The complex lipid-rich cell wall of mycobacteria serves as a natural barrier for entry of drugs, and the presence of efflux pumps further enhances the antibiotic resistance repertoire in *Mycobacterium*. Efflux pumps in bacteria are proteins embedded in the plasma membrane, whose function is to regulate the internal environment by removing toxic metabolites, antimicrobial agents and, quorum-sensing signal molecules. There are five major families of efflux transporters in prokaryotes based on their structural morphologies and similarities, energy sources, and substrates (3, 4).

Bioinformatic analysis of *M. tuberculosis* genome predicts the presence of at least 26 complete ATP-binding cassette superfamily (ABC) transporters and 16 major facilitator

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superfamily proteins that have been shown to be involved in the transport of antibiotics; many which are used in treatment of TB (5, 6). Among the five major families of efflux transporters, the Resistance-Nodulation-Division (RND) protein is found in all kingdoms of living organisms. It can mediate the transport of a wide range of substrates, including heavy metals, aliphatic and aromatic solvents, bile salts, fatty acids, detergents, and dyes. In *Escherichia coli* and *Pseudomonas aeruginosa*, the RND family proteins play an important role in antibiotic resistance (7).

Genes encoding the RND family of efflux pump are identified in *Mycobacterium* species and are named MmpL (**m**ycobacterial **m**embrane **p**rotein **l**arge) (8, 9). Several studies have demonstrated that the overexpression of efflux pump genes in MDR and XDR clinical strains increases resistance to antibiotics (10, 11). The Mmp family of proteins is often expressed as an operon and codes for a smaller protein (MmpS) and a larger protein (MmpL). They are found in close association with polyketide synthetic gene cluster and have been implicated in diverse functions such as exporting lipid molecules and siderophores across the bacterial envelope, and conferring antibiotic resistance (9, 12–14). The MmpS5 is a membrane fusion protein that works as a crucial auxiliary counterpart for MmpL5 by facilitating the homotrimeric assembly leading to drug resistance (15). The occurrence of the Mmp proteins in mycobacteria varies, with the lowest of 9 members reported in *M. leprae* to around 58 in *M. abscessus* (16). In mycobacteria, the *mmp* operon is often regulated by the Tet^R family of regulators (TFR). The TFRs are and often expressed divergently to the *mmp* operon and contain a Helix-turn-helix (HTH) DNA binding motif at the N-terminal. They generally function as repressors, but in a few exceptional cases can be activators of gene expression (17–19). Loss of function mutants of TFR has been linked to increased expression of efflux pumps and concomitant incidences of drug resistance (17, 20).

We have earlier reported that chrysomycin A, an antibiotic isolated from *Streptomyces* sp. OA161 possesses potent antimycobacterial activity and effectively killed drug-resistant strains of *M. tuberculosis* (21, 22). In the present study, we report a novel role for MmpL5 and MmpS5 complex in conferring resistance to chrysomycin A. Using *M. smegmatis* as a surrogate model for *M. tuberculosis*, we isolated chrysomycin A resistant mutants and identified mutations in the resistant colonies by whole genome sequencing. Our analysis

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identified mutation in MSMEG_1380, a Tet^R family of transcriptional regulator that controls a divergently transcribed operon MSMEG_1381 and MSMEG_1382 (*mmpS5* and *mmpL5*, respectively) involved in membrane transport in mycobacteria. Overexpression of *mmpS5* and *mmpL5* either alone or as an operon resulted in increased resistance to chrysomycin and other antitubercular drugs such as isoniazid, and ethambutol. Our finding has uncovered an auxiliary role of membrane transport protein in multiple antibiotic resistance, further expanding the list of candidates linked to AMR in mycobacteria.

Results

Isolation and whole-genome sequence analysis of chrysomycin resistant mutants of *M. smegmatis*

Chrysomycin A displays potent antimycobacterial activity, and our recent study has shown its bactericidal activity emanates from inhibition of topoisomerase I and DNA gyrase (22). It therefore, became necessary to further understand the mechanisms of resistance to this antibiotic. We employed an unbiased forward genetics approach by isolating spontaneous chrysomycin A resistant mutants and identified the mutations by whole-genome sequencing (WGS). A similar approach has been exploited to identify novel modes of resistance in mycobacteria and the specific antibiotic target (23). Randomly selected 50 chrysomycin A resistant colonies were repatched on fresh 7H10 chrysomycin A (2X the MIC) plates to reconfirm that the colonies were true resistant strains and could grow in the presence of the antibiotic. A set of ten isolated colonies (isolates 12, 18, 21-25, 42, 43, and 51) and the parental *M. smegmatis* strain were inoculated in plain 7H9 medium. No observable growth was seen in isolates 12, 18, 51, however the other isolates that managed to grow showed severe clumping and did not attain $O.D._{600}$ ~ 0.4. The genomic DNA was isolated from these cultures and subjected to WGS. The SNPs identified by WGS in the resistant isolates 21, 24 and 42 are included in supplementary table 2 excel sheet. The mutations identified in different isolates mapped to DNA binding protein (*bm3R1*), DNA polymerase IV (*dinB1*) and phosphoglucosamine mutase (*glmM*) (Table-1). Interestingly, mutation was observed in *bm3R1* among all the isolates and was therefore selected for further analysis.

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Table-1. WGS analysis of chrysomycin A resistant strains and the mutations identified.

Chrysomycin A mutants	Gene	Ref	Alt	Annotation	Amino acid change
Isolate 21	*bm3R1*	GTGAGTGCC	GTG...CCC	Deletion and missense variant	Ser2del and Ala3Pro
Isolate 24	*bm3R1*	GGCGTGGGCGTG	GGCGTGGGCGTGGGCGTG	Insertion	Gly49_Val50insValGly
Isolate 42	*dinB1*	G	A	Missense variant	Asp273Asn
	ilvI	A	T	Missense variant	Ile420Asn
	bm3R1	GGCGTGGGCGTG	GGCGTGGGCGTGGGCGTG	Insertion	Gly49_Val50insValGly
	glmM	G	C	Missense variant	Glu7Gln

Note: Ref - DNA sequence in the parental WT strain, Alt - alteration highlighted with grey.

Table-2. MIC values of the strains overexpressing *MSMEG_1381*, *MSMEG_1382*, or *MSMEG_1381~1382* (operon) in comparison with the *M. smegmatis* mc^2 155 WT strain as assessed by REMA assay.

Antibiotics	MIC (µg/ml)			
	M. smegmatis $mc^2$155 (WT)	WT ~P_{hsp}~MSMEG_1381	WT ~P_{hsp}~MSMEG_1382	WT ~P_{hsp}~MSMEG_1381-MSMEG_1382
Chrysomycin A	1.25	2.5	2.5	2.5
Ethambutol	0.5	1	1	2
Isoniazid	10	20	20	40
Rifampicin	3.9	3.9	3.9	3.9
Ciprofloxacin	0.625	0.625	0.625	0.625
Streptomycin	1	1	1	1

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Ethidium bromide	3	3	3	3

Resistant strains of chrysomycin A contain mutations in MSMEG_1380, a Tet^R regulator of *Mycobacterium smegmatis*

BLAST analysis of the DNA sequence corresponding to the DNA sequence annotated as *bm3R1* gene (Prokka database) was identified as *MSMEG_1380*. The gene is annotated as a Tet^R family transcriptional regulator and is adjacent to an operon consisting of two genes, *MSMEG_1381*, and *MSMEG_1382*. The organization of *MSMEG_1380*, *MSMEG_1381*, and *MSMEG_1382* in the *M. smegmatis* indicated that the *MSMEG_1380* is divergently transcribed compared to the other genes (Fig.1A). *In silico* modeling of MSMEG_1380 revealed that the protein shares structural homology to the Tet^R family of transcription regulators. Alignment of the DNA sequence of *MSMEG_1380* from the resistant colonies with the wild-type sequence revealed that isolate 21 had a deletion of three nucleotides and a missense variant, while isolates 24 and 42 had an insertion of 6 nucleotides within the open reading frame (Fig. 1B). Protein alignment of the translated sequence revealed that the nucleotide changes in isolate 21 resulted in loss of the amino acid Ser at position 2 and Ala3Pro substitution. In the case of isolates 24 and 42 the insertion of nucleotides resulted in duplication of amino acids Gly and Val at 49th and 50th positions.

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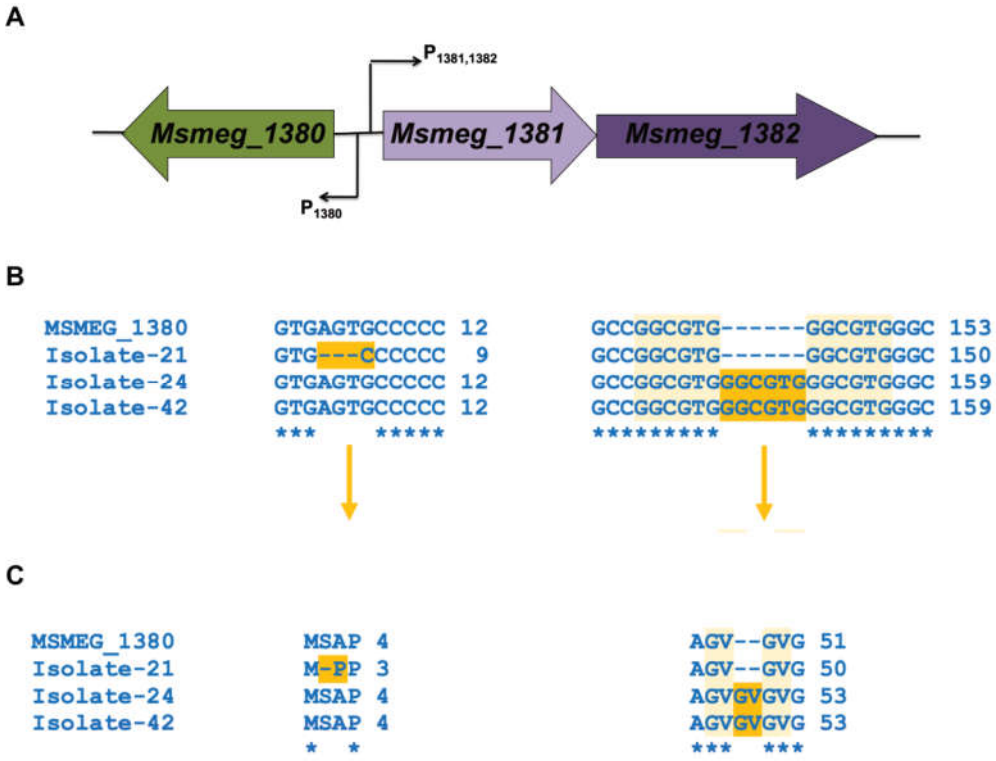

Figure 1. A) Genomic organization of the *MSMEG_1381~MSMEG_1382* operon with the Tet^R family transcriptional regulator MSMEG_1380 expressed divergently. B) Nucleotide changes detected in the transcriptional regulator MSMEG_1380 by WGS analysis in the chrysomycin A resistant isolates 21, 24, and 42 are shown in red adjacent to the corresponding sequence in the wild-type parental strain denoted by MSMEG_1380. C) ClustalOmega alignment of the amino acid residues of MSMEG_1380 of *M. smegmatis* mc^2 155 WT (MSMEG_1380) with the resistant isolates of chrysomycin A isolate 21, isolate 24 and isolate 42, respectively in which the isolates were showing mutations/indels. The observed changes in the protein are highlighted in dark color.

Interestingly, the modeling results predicted that the duplication of Gly and Val (observed in isolates 24 and 42) caused distortion in the protein structure (movement of the yellow and orange helices in the mutant protein (Fig. S1A, panel ii) in comparison to wild-type protein (Fig. S1A, panel i). As a consequence, the two helices failed to make contacts with the DNA sequence. We speculated that this distortion caused by duplication of residues 49 and 50 could negatively impact the DNA binding and affect regulatory functions of the protein. It is

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noteworthy to mention that insertion of amino acids in Rv0678, the transcriptional regulator of *mmpS5~mmpL5* operon in *M. tuberculosis* have been previously reported to result in de-repression of the operon (23, 24). We hypothesized that mutations observed in the Tet^R regulator *MSMEG_1380* could have similar effects on the expression of the adjacent genes *MSMEG_1381* and *MSMEG_1382*.

Overexpression of MmpL5 alone or as an operon causes growth defects

The open reading frames *MSMEG_1381* and *MSMEG_1382* are predicted to be involved in membrane transport and code for MmpS5 and MmpL5, respectively. Transmembrane domain analysis of the primary sequence indicated that the MmpL contains 11 transmembrane domains which is in agreement with its predicted membrane localization (Fig. S1B-C). The membrane proteins MmpS4/MmpL4 and MmpS5/MmpL5 constitute a family of transporters and function in exporting and recycling siderophores (13). Additionally, these transporters have been shown to contribute to drug resistance by efflux mechanism (9, 25). We postulated that the mutations mapping to MSMEG_1380 would result in de-repression of the operon, eventually conferring chrysomycin A resistance. The chrysomycin A resistant strains showed severe growth retardation, possibly attributed to the multiple mutations in the genome, which could be synthetically deleterious (Fig. 2A). Therefore, the antibiotic susceptibility assays could not be performed on the resistant colonies. We explored an alternate approach of expressing either the individual genes or the entire operon using a mycobacterial constitutive promoter P_{hsp}. Interestingly, transformants of *M. smegmatis* harboring either *MSMEG_1382* or the entire operon appeared late on 7H10 plates (Fig. 2B) indicating a potential growth defect in the strains over-expressing MmpL5 or the operon. This was further confirmed by performing growth curve analysis wherein the strain overexpressing the operon displayed the strongest growth defect followed by the MmpL5 overexpression strain while the presence of MmpS5 alone did not cause significant growth defect (Fig. 2C).

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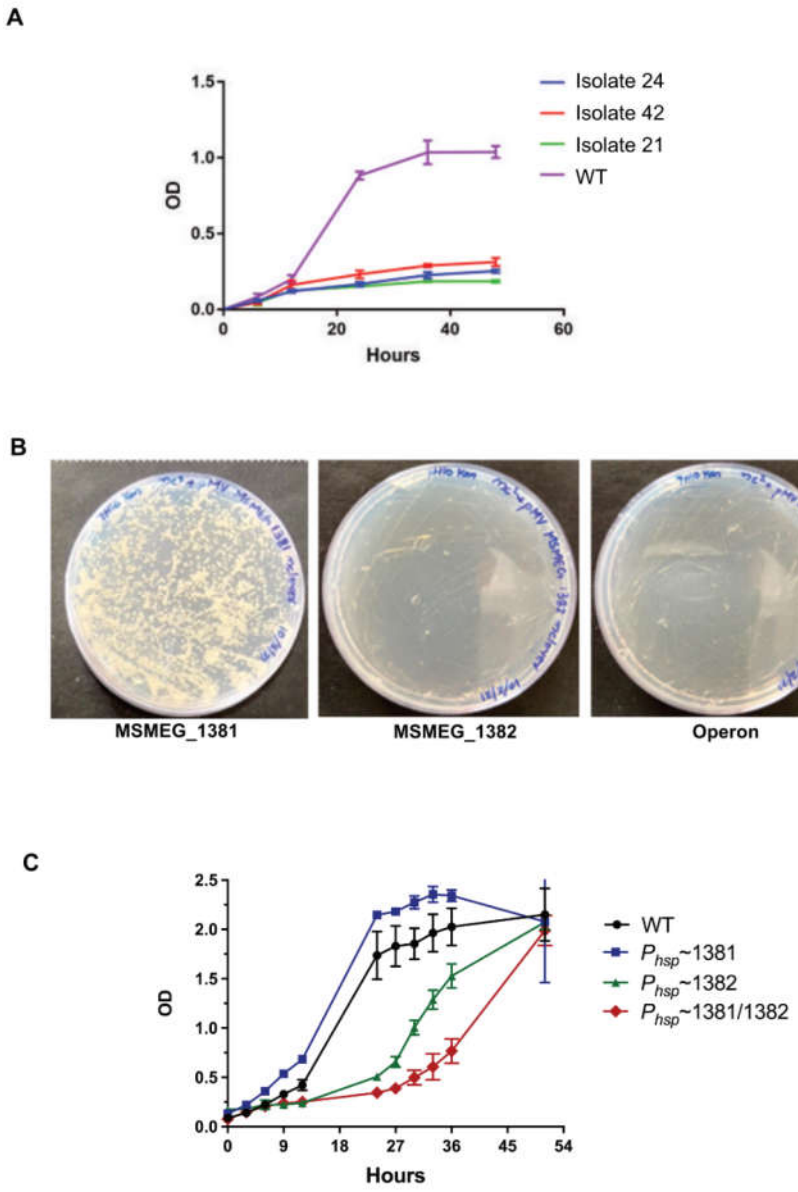

Figure 2. Over-expression of MSMEG_1382 alone or the entire operon affects growth in *M. smegmatis*. A) Freshly revived cultures of *M. smegmatis* mc^{2}155 and chrysomycin A resistant isolates 21, 24, and 42 was cultured in plain 7H9 medium at 37 °C. The increase in growth was monitored by measuring OD_{600} in a Bio-Rad plate reader every 4 hrs, and plotted as mean values ± SD were plotted using Graphpad Prism V8. Growth profile of chrysomycin A resistant strain was compared to *M. smegmatis* mc^{2}155. B) Electrocompetent cells of *M. smegmatis* mc^{2}155 were prepared and transformed with pMV261 expressing either i) MSMEG_1381, ii) MSMEG_1382, or iii) the entire operon (Method section) and plated on 7H10 kan plates and incubated at 37 °C. The appearance of colonies on the plate was monitored after 3 days. C) Freshly revived strains of wild-type *M. smegmatis* and strains

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expressing either MmpS5 or MmpL5 or the entire operon were cultured in 7H9 medium with appropriate antibiotics at 37 °C in an incubated shaker. The OD_{600} of the cultures were measured and mean values ± SD of five replicates for each strain was plotted using Graphpad Prism V8.

Expression of membrane transporters either individually or in operons confers multiple drug resistance

To further validate if the expression of the transporters is sufficient to confer resistance to chrysomycin A, we determined the minimal inhibitory concentration (MIC) of the strains expressing either of the genes individually or the entire operon. We observed that expression of either *MSMEG_1381* or *MSMEG_1382* alone was sufficient to increase the MIC of *M. smegmatis* to chrysomycin A by two folds (1.25 µg/mL to 2.5 µg/mL) (Table-2). Membrane transporters and efflux pumps often display the ability to transport a diverse group of molecules (26). Therefore, we tested if the recombinant strains expressing either MSMEG_1381, MSMEG_1382, or the entire operon confer resistance to other anti-TB drugs such as isoniazid, ethambutol, rifampicin, streptomycin and ciprofloxacin. Since the RND family of transporters efflux xenobiotic molecules, we also tested the strains for ethidium bromide resistance. Strains expressing MmpS5 or MmpL5 displayed increased resistance to ethambutol and isoniazid (1 µg/mL and 20 µg/mL) respectively which is an increase of ≥ 2X MIC of WT (0.5 µg/mL and 10 µg/mL) while there was no difference in the MIC to ciprofloxacin, rifampicin, and ethidium bromide (Table-2). Interestingly, the expression of the entire operon resulted in higher MIC to both isoniazid (40 µg/mL) and ethambutol (2 µg/mL) which is 4X MIC of WT strain. In comparison, the MIC of either the MSMEG_1381 or the MSMEG_1382 over-expression strains were 20 µg/mL or 1 µg/mL for isoniazid and ethambutol respectively (Table-2). In summary, over-expression of efflux pump components resulted in a multidrug resistance phenotype and over-expression of the entire operon enhanced the resistance phenotype in comparison to individual proteins.

Molecular interaction of antibiotics with MSMEG_1382

In silico modelling and analyzing of the structure of MSMEG_1382 revealed that the protein contains two distinct tunnels- a wider central tunnel spanning the region of the inner

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membrane and, a narrow-accessory tunnel facilitating the exit of molecules to periplasmic space (marked as yellow arrows in the structure of MSMEG_1382 in Fig. 3A). Molecular docking was performed using the site closer to the junction of the accessory tunnel and central one. The docking showed three different binding pockets to which different antibiotics (isoniazid, ethambutol, chrysomycin A, streptomycin , ciprofloxacin, and rifampicin) interact. Isoniazid and ethambutol bind at the start of the accessory tunnel (amino acid chains; 433-446; 159-160) (Fig. 3B and 3C), while ciprofloxacin and rifampicin interact with the amino acids of the central tunnel (amino acid chains; 443-449; 417-420; 164-167) (Fig. 3F and 3G). Chrysomycin A and streptomycin are found interacting near the central and accessory tunnel (amino acid chains; 159-160; 445-449; 762-763). Although chrysomycin A has the advantage of a planar coumarin ring and may get efluxed out, streptomycin may not pass through the accessory tunnel because of its bulkier side chain and deeper interacting pockets with the MmpL5 in the central tunnel (amino acid chain; 417-418) (Fig. 3D and 3E). Accordingly, the MmpL overexpressing strains showed 2-fold increased MIC against ethambutol, isoniazid, and chrysomycin A while the MIC remained unchanged against ciprofloxacin and rifampicin (Table-2). Thus, the docking results agree with the observed differences in drug susceptibility.

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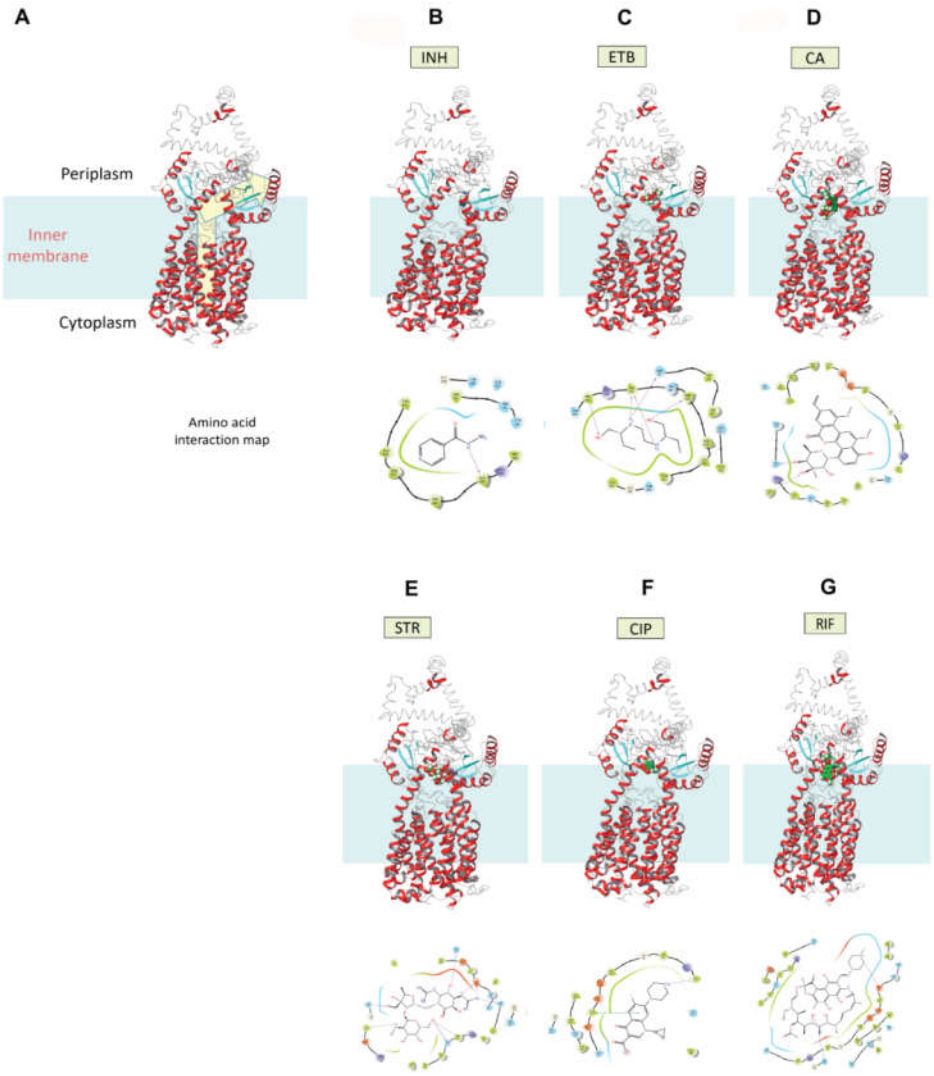

Figure 3. MmpL5 interactions with antibiotics. A) MmpL5 showing the predicted tunnel, and B) MmpL5 docked with Isoniazid (INH), C) Ethambutol (ETB), D) chrysomycin A (CA), E) Streptomycin (STR), F) ciprofloxacin (CIP), G) rifampicin (RIF). Representative amino acid interaction map for the respective antibiotics is also shown below each model.

MSMEG_1381 and MSMEG_1382 belong to the RND family of efflux transporters

Although uncharacterized, MSMEG_1381 and MSMEG_1382 are annotated to encode membrane transporter protein MmpS5 and MmpL5, respectively (https://mycobrowser.epfl.ch/). The MmpL group of proteins belongs to RND permease family and has been implicated in drug resistance (9, 15). While a single gene has been annotated for *mmpS5* (Rv0677c) and *mmpL5* (Rv0676c) in *M. tuberculosis*, there are three

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proteins annotated as *mmpS5* and *mmpl5* in *M. smegmatis*. To further investigate the potential relatedness of this multi-gene family, we performed a multiple sequence alignment of the four proteins of interest. The alignment demonstrates the strong similarity between the MSMEG_0226 and MSMEG_3495 encoding MmpS5 (91%), indicating a potential duplication. These proteins also shared higher sequence similarity with *M. tuberculosis* MmpS5 (Rv0677) ~60% (Fig. S2A). Similarly, in the case of MmpL5, the extent of similarity between MSMEG_0225 and MSMEG_3496 was greater (83%) and ~70% with *M. tuberculosis* protein Rv0676 (Fig. S2B). However, the MSMEG_1381 and MSMEG_1382 shared the least sequence similarity of 55% and 63%, respectively, with the annotated MmpS5 and MmpL5 protein of *M. tuberculosis*. Surprisingly, we observed a high degree of sequence divergence between the MSMEG_1382 and the other *M. smegmatis* proteins, indicating that MSMEG_1381 and MSMEG_1382 were more distantly related to the other *M. smegmatis* MmpS5 and MmpL5 proteins. This was further confirmed by phylogenetic tree analysis constructed using the amino acid sequences of the MSMEG_0225/0226, MSMEG_3495/3496, MSMEG_1381/1382 with Rv0676/0677, similar topologies were found in the two trees with somewhat congruent phylogenies. Both trees showed that *M. tuberculosis* protein H37Rv was more distantly related to MSMEG_0226 MSMEG_3495 than MSMEG_1382 (Fig. S2C and D).

Discussion

Previously, chrysomycin A has been reported to have potent bactericidal activity against drug-resistant clinical strains of *M. tuberculosis* and other Mycobacterium species (21, 22). The goal of the current study was to identify mechanisms that would contribute to drug resistance to chrysomycin A. We took a forward genetics approach and isolated spontaneous chrysomycin A resistant mutants in *M. smegmatis*. We identified mutations mapping to *MSMEG_1380* gene in all the resistant strains upon WGS analysis. Loss of function mutation of MSMEG_1380 results in de-repression of the Mmp complex resulting in increased resistance to multiple antibiotics (Fig. 4).

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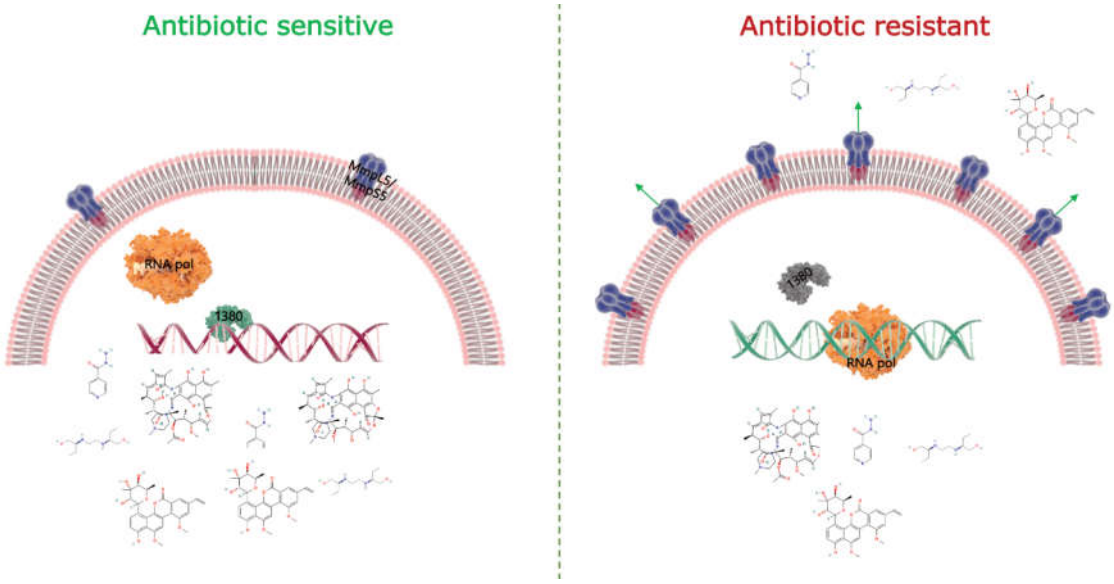

Figure 4. Mechanism of the evolution of chrysomycin A resistance. Under normal conditions, the TetR family transcriptional regulator MSMEG_1380 binds to the promoter of *mmpS/L5,* preventing transcription of the operon encoding the RND-family transporters MmpS5 (MSMEG_1381) and MmpL5 (MSMEG_1382) (left panel). Spontaneous mutants leading to loss of function in *MSMEG_1380* fail to bind to the promoter allowing transcription of the operon. The MmpS5 and MmpL5 form a functional efflux pump resulting in resistance to (isoniazid, ethambutol, and chrysomycin) in *M. smegmatis* (right panel)

All the sequenced isolates (21, 24, and 42) contained mutation in the transcriptional regulator MSMEG_1380, which has been reported to repress the expression of the divergent operon encoding MmpS5 and MmpL5 proteins(27) (Fig. 1B). Phenotypic analysis of the resistant colonies showed that the strains displayed reduced growth compared to the parental strain (Fig. 2A). While this apparent growth defect could be attributed to a synergistic effect of multiple mutations observed in the resistant colonies, the slow growth observed on solid or liquid media of transformants containing P_{hsp}~*MSMEG_1382* alone or the entire operon was surprising (Fig. 2B and 2C). Although the data presented in our study cannot explain the reduced growth of the over-expression strains, we believe that constitutive expression of the efflux system would burden the cell with unnecessary ATP expenditure compromising growth.

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Loss of function mutations in the divergently expressed transcriptional regulator of *mmpS5~mmpL5* operon has been reported to confer antimicrobial resistance to antimicrobial compounds such as bedaquiline and clofazimine in *M. tuberculosis* or to thiacetazone in *M. abcessus* (23, 28, 29). The orthologous protein in *M. smegmatis*, MSMEG_1380, functions similarly by acting as a repressor to the *mmpS5~mmpL5* operon and point mutation conferred resistance to Serine-Threonine kinase inhibitor to *M. smegmatis* strain (27). While Maslov et al. reported increased susceptibility of *M. smegmatis* to imidazo tetrazines upon overexpression of MSMEG_1380 (27), we performed the converse experiment of overexpressing either of the Mmp proteins in single or the entire operon through the constitutive promoter P_{hsp} and observed increased resistance to multiple antibiotics. Docking of different antibiotics to MmpL5 showed that isoniazid and ethambutol interact with MmpL5 near the exit to the periplasm, suggesting that they could easily pass through the tunnel because of their smaller size. The available structure of MmpL3 that is involved in the transport of trehalose monomycolate (TMM) suggests the presence of three exit gates named G_T, G_F and G_B are present at the top, front, and back of the protein, respectively (30). The authors further proposed that the front exit G_F containing hydrophilic residues could be accessed by the trehalose end of the TMM and could facilitate its movement to the periplasmic space. The accessory channel observed in our modeled structures resembles the G_F observed in MmpL3. In the case of chrysomycin A, the planar coumarin structure seems to support the travel across the central and accessory tunnel. However, the ease of passing through the tunnel would be only be next to isoniazid and ethambutol. Our docking studies indicated that streptomycin, rifampicin, and ciprofloxacin could be accommodated in deep binding pockets with higher binding affinity. However, the larger molecular size combined with a higher number of hydrogen bond acceptors and donors in these antibiotics would result in more interactions with the amino acid residues in the channel. (Fig. 3B-G). The planar structure of these molecules combined with the extensive molecular interactions with the protein would restrict the easy passage through accessory tunnel into the periplasmic space. We observed similar results in our drug susceptibility assays where the MmpL/MmpS5 overexpressing strain was most resistant to isoniazid and ethambutol (higher than even chrysomycin A). A meagre 2 fold increase in MIC was observed against chrysomycin A, suggesting that the efficiency of efflux is slightly lesser than isoniazid

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and ethambutol. It is noteworthy to mention that sensitivity to ciprofloxacin – a fluoroquinolone drug having similar activity as that chrysomycin A was unchanged when MmpL proteins were expressed either individually or as an operon because, the antibiotic is effluxed by another efflux pump LfrA (31).

Recently it has been demonstrated that co-expression of MmpS5 facilitates assembly of MmpL5 into a trimeric complex with other accessory proteins in the outer membrane that is competent in drug efflux function(15). However, in our study, expression of MmpS5 alone or MmpL5 alone also contributed to an increase in MIC from 0.5 µg/mL to 1 µg/mL (2-fold) but an expression of the complete operon resulted in the highest MIC to ethambutol up to 2 µg/mL (Table-2). We hypothesize that overexpression of either component of the operon (*mmpS5* or *mmpL5*) can form a heterocomplex with other annotated MmpL5 proteins in *M. smegmatis* (MSMEG_0226 or MSMEG_3496). However, only one copy of annotated *mmpS5* and *mmpL5* is present in the *M. bovis* BCG, as observed by Yamamoto and others (14). Although the resistant colonies were selected on agar plates with 6X MIC we observed a meagre 2 fold increase in MIC against chrysomycin A, suggesting that the efficiency of efflux is slightly lesser than isoniazid and ethambutol. It has been previously observed that the MICs observed in solid medium and liquid medium can vary and presence of tween in liquid media often enhances the bactericidal activity (23, 32, 33). The finding that overexpression of the entire operon resulted in a mere two-fold increase in MIC for chrysomycin A was surprising. However, a study by Maslov et al., deletion of MSMEG_1380, the regulator of efflux pump resulted in at least 2-fold increase in MIC (27). Further, we observed that the overexpression of efflux pump significantly reduces bacterial growth (Fig. 2C). This would impact the rate of resazurin reduction giving an apparent lower MIC than the actual ability of the recombinant strain to efflux the antibiotic. Due to the nature of assay, selective longer incubation times for the MmpL5 or the operon expression strain could not be achieved.

Antimicrobial compounds may themselves induce expression of efflux pump (27, 34) either *in vitro* or *in vivo*, negatively impacting the intended outcome of therapy. Therefore, efflux pump inhibitors as a part of combinatorial antibiotic therapy are attracting considerable interest to treat infections caused by *M. tuberculosis* and other Gram-negative bacteria (11, 35, 36). Molecules such as timcodar and tariquadar inhibit mammalian transporters leading

 doi:10.20944/preprints202204.0003.v2

to increased intracellular antibiotic concentration resulting in the efficient killing of intracellular bacteria(37, 38). Inhibiting mycobacterial efflux pump by verapamil or SQ109 in combination can promote antibiotic synergy (39–41), resulting in efficient bactericidal activity. The rationale for using *M. smegmatis* in drug screening is often debated as it is not a pathogen, and its lifestyle does not reflect that of *M. tuberculosis*. However, identification of mutations in clinical strains of *M. tuberculosis* associated with efflux pumps has been increasingly reported, particularly that of Rv0678, whose ortholog in *M. smegmatis*, which was identified in our study, shows the importance of this bacterium in drug discovery. Identifying a new class of molecules that can inhibit efflux pump will significantly increase the success rate of antibiotic therapy, and engineered strains that express efflux pump like the ones described in our study can be potentially used for deeper screening of lead molecules for their ability to resist being effluxed.

Material and Methods

Bacterial strains and culture conditions

The bacterial strains, plasmids, and oligonucleotides used in the study are listed in Supplementary Table-1. Strains of *M. smegmatis* were recovered on 7H10 Middlebrook supplemented with 0.5% v/v glycerol and 0.05% v/v Tween 80 plates from glycerol stocks using appropriate antibiotics. For culture in liquid condition, Middlebrook 7H9 medium supplemented with 0.1% v/v glycerol and 0.05% Tween 80 was used. When required, *M. smegmatis* cultures were selected by incorporating kanamycin (50 µg/mL). For cloning and plasmid DNA manipulation, *E. coli* TG1 or JM101 strains were used. *E. coli* strains were cultured in Luria Bertani (LB) broth or LB agar at 37 °C. For selection in *E. coli*, antibiotics with the following concentrations kanamycin (50 µg/mL) and ampicillin (100 µg/mL) were used. All media components were procured from Difco (Maryland, USA). Unless mentioned, all chemicals and reagents were procured from Sigma Aldrich (Missouri, USA).

Isolation of chrysomycin A resistant colonies in M. smegmatis

M. smegmatis mc^2155 was used as the fast-growing surrogate model to identify the mutations in spontaneous chrysomycin A resistant mutants. *M. smegmatis* revived on the 7H10 agar from the glycerol stock and incubated and inoculated into 20 mL 7H9 medium at

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an initial OD_{600} 0.01 and cultured at 37 °C in a shaken incubator till it reached mid-log phase. The culture was harvested, and the bacterial pellet was plated on 7H10 plates containing 12 µg/mL chrysomycin A (Abcam, USA) (6X the MIC) and incubated at 37 °C until resistant colonies appeared. Fifty random colonies were picked up and patched onto a fresh 7H10 agar plate containing chrysomycin A (12 µg/mL). Wild-type *M. smegmatis* and three random chrysomycin A resistant mutants were selected, and their genomic DNA was isolated as described previously described (42) and subjected to whole-genome sequencing.

Electrocompetent cell preparation and transformation

The freshly revived strain of *M. smegmatis* mc^2155 was cultured in 2 mL 7H9 medium with tween 80 at 37 °C in shaken condition till saturation. The culture was diluted 1:100 in 100 mL LB medium containing tween and incubated at 37 °C for 14-16 h till the OD_{600} reached ~0.6. The culture was cooled on ice, transferred to 2X50 mL sterile conical tubes, and harvested at 4 °C in an Eppendorf refrigerated centrifuge swing bucket 5804 rotor for 7 min at 4,200g. The culture supernatant was discarded, and the bacterial pellet was resuspended in with an equal volume of ice-cold 10% v/v glycerol. The bacterial suspension was harvested by centrifugation at 4,200g at 4 °C. The washing step was repeated once more, and the final pellet was resuspended in 2 mL of ice-cold 10% glycerol solution. The pellet was resuspended, and 0.1 mL of the suspension was aliquoted into prechilled 1.7 mL sterile microfuge tubes and mixed with 500 ng of pMV262 plasmid harboring either MSMEG_1381, MSMEG_1382, or MSMEG_1381~1382 operon. The mixture was transferred to a prechilled 2 mm sterile electroporation cuvette (BioRad). The electroporation was done in MicroPulser Electroporator (BioRad) using the following setting 2.5 kV, 25 µF, and 1000Ω. The transformed cells were immediately recovered by adding 1 mL of sterile, antibiotic-free 7H9 medium to the cuvette. The contents were transferred to a sterile 1.7 mL microfuge tube and incubated at 37 °C for 3 h. The contents were harvested by centrifuging at 13,000 rpm for 1 min at room temperature, and the pellet was resuspended in 0.1 mL of 7H9 medium and plated on 7H10 plates containing kanamycin. The plates were incubated till colonies appeared.

Homology modeling and molecular docking using Schrodinger

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The protein sequences of MSMEG_1380 (UniProt ID A0QS78) and MSMEG_1382 (UniProt ID A0QS80) were retrieved in FASTA format from Mycobrowser and submitted to the I-TASSER web server(43). At least 10 models were generated after considering at least 10 homology three-dimensional protein templates. The 3D models with the highest TM and C-scores values among the predicted structures were used for binding studies. The modeled structures of the protein were imported to Schrodinger software (version 2021-3) in PDB format, and the proteins were prepared using the Protein preparation tool. Briefly, the missing loops were added; over-lapping hydrogen bonds were optimized; the energy minimization was performed along with deletion of water molecules (if any). The prepared protein was analyzed for deep binding pockets using the Sitemap tool. The resulting maps with scores above 1 alone were selected, and a grid was generated with the same. In parallel, the ligands were prepared using LigPrep tool (LigPrep, Schrödinger, LLC, New York, NY, 2021) . The possible states of the ligands at pH 7.0 ± 0.2 were generated using Epik giving rise to 32 different combinations per ligand (44). The prepared ligands and protein were docked using the Glide Ligand docking tool at standard precision with default parameters. The docking poses were recorded along with their docking scores. For protein-DNA docking, 'Patchdock' (https://bioinfo3d.cs.tau.ac.il/PatchDock/php.php) was used, and the resultant structures were submitted to 'Firedock' (https://bioinfo3d.cs.tau.ac.il/FireDock/php.php) for further refinement of the structures (45, 46). To avoid false-positive docking results and to understand the passage of molecules out of the cell, the tunnels in the protein structures were predicted using Mole2.0 online tool (47).

Growth curve analysis

Freshly revived strains of *M. smegmatis* mc^2155 and the chrysomycin A resistant isolates 21,24 and 42 were inoculated in 5 mL 7H9 medium in triplicates, and the Optical density at 600 nm (OD_{600}) was adjusted to 0.025. The cultures were incubated in a shaker incubator at 37 °C at 175 rpm for 48 h. After every 4 h, 0.1 mL aliquot of the bacterial culture was transferred to a sterile optical bottom Nunc microtiter plates. The OD_{600} was measured in a BioRad microplate reader after normalization with an equal volume of sterile 7H9 medium as blank. The mean OD_{600} values ± SD were plotted for each strain using Graphpad Prism v8.0 software. In the case of Mmp overexpression, freshly revived bacterial strains were cultured in 7H9 medium in five replicates with appropriate antibiotics in optically transparent sterile

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culture tubes. The OD_{600} was monitored in Genesys30 Visible spectrophotometer (Thermo Fisher Scientific, USA) at different time intervals. The mean OD_{600} values ± SD were plotted for each strain using Graphpad Prism v8.0 software.

Whole-genome sequencing of the chrysomycin A resistant strains

Sequencing was performed using the Illumina platform of bridge amplification. Briefly, DNA was quantified by QUBIT 3 Fluorometer using dS DNA HS Dye. The intact DNA (200 ng) was enzymatically fragmented (enzyme in the Illumina sequencing kit was used), and 200-300 bp fragment size was chosen. The DNA fragments undergo end repair wherein the mix converts the overhangs resulting from fragmentation into blunt ends. The end-repair mix's 3' to 5' exonuclease activity removes the 3' overhangs, and polymerase activity fills in the 5' overhangs. To the blunt-ended fragments, adenylation was performed by adding a single 'A' nucleotide to the 3' ends. Loop adapters were ligated and cleaved with the uracil-specific excision reagent (USER) enzyme to the adenylated fragments. The samples were further purified using AM pure beads (New England Biolabs), and PCR then enriched the DNA with 6 cycles using NEB (New England Biolabs). Next, Ultra II Q5 master mix, Illumina universal primer, and sample-specific octamer primers. The amplified products were cleaned up using AM pure beads, and the final DNA library was eluted in 20 µLs of 0.1X TE buffer. One microlitre of the library was used for quantification by QUBIT 3 Fluorometer using dS DNA HS reagent. The fragment analysis was performed on Agilent 2100 Bioanalyzer by loading 1 µL of the library into Agilent DNA 7500 chip. The QC passed libraries were diluted to 2 nM and pooled together. As per the manufacturer's recommendation, the pooled library was further diluted to sequence it on Illumina Hiseq. The Hiseq Control Software was used to set up a 2X150 bp run on Hiseq, and data were demultiplexed using bcl2fastq v2.1.9.The sequence data quality was checked using FastQC and MultiQC software. The data was checked for base call quality distribution, % bases above Q20, Q30,% GC, and sequencing adapter contamination.

Assembly annotation and variant calling

The QC passed reads were used to assemble the genomes using the Hierarchical Genome Assembly tool (HGA). Contigs shorter than 100 bp were filtered from the assembly. The assembled genomes were annotated using Prokka v1.3 with default parameters (48). The QC

 doi:10.20944/preprints202204.0003.v2

passed reads were mapped to the assembled genome of the WT sample using the BWA MEM algorithm with default parameters. The PCR duplicates were marked using sambamamarkdup option. The BAM files were used to call variants using FreeBayes with the ploidy 1 option. The variants were annotated using the snpEff program by considering the prokka predicted protein annotations. The WGS data has been deposited at the Sequence Read Archive (SRA) server maintained by NCBI. The project code for the submission is PRJNA821497.

MIC determination by resazurin dye reduction assay

M. smegmatis mc^2155 *WT*, and strains harboring the constitutively expressing either MSMEG_1381, MSMEG_1382 or the operon (MSMEG_1381~MSMEG_1382) were freshly revived on 7H10 kanamycin plates. The strains were grown in 3 mL of plain 7H9 or 7H9 kanamycin broth for 48 h. The bacterial density was measured and adjusted to OD_{600} of 0.001 (measured and adjusted to OD_{600} of 0.1 and further diluted 20 times). In a sterile flat-bottom 96-well plate, 100 µl of appropriate 7H9 broth was dispensed in each well. In the last row, 200 µl of 7H9 media containing 2X concentration of individual compounds i.e., chrysomycin A (20 µg/mL), ethambutol (8 µg/mL), isoniazid (160 µg/mL), rifampicin (126 µg/mL), ciprofloxacin (10 µg/mL), Streptomycin and ethidium bromide was added. Serial dilution was performed to establish a continuous 2-fold dilution of the compound. The first well was left untreated and served as antibiotic free control. The diluted bacterial culture (100 µl) of each strain was dispensed into the wells to obtain a final volume of 200 µl/well and a concomitant dilution of antibiotic by 2X. In each plate, positive control and negative controls were kept. The plates were sealed in plastic bags and incubated at 37° C in a static incubator. After 36 h of incubation, 20 µl of freshly prepared resazurin (0.025%) solution was added to each well, and the plates were incubated in a static incubator at 37° C in a dark condition. The plates were observed after every hour for the reduction of blue-colored resazurin dye to pink-colored end-product. The MIC of the strain was calculated by calculating the lowest drug concentration that prevented the reduction of dye.

Generation of constitutive expression constructs of MSMEG_1381, MSMEG_1382, and MSMEG_1381~1382 operon in M. smegmatis

Oligonucleotides were designed to amplify *MSMEG_1381*, *MSMEG_1382*, or the entire operon (*MSMEG_1381~MSMEG_1382*). The primers were designed using the online Infusion primer design tool (Takara) to contain *BamH*I and *Hind*III in the forward and reverse primers, respectively, along with 10 nucleotides stretch to facilitate infusion cloning. The DNA sequence of *MSMEG_1381* (*mmpS5* ; 417 bp), *MSMEG_1382* (*mmpL5* ; 2904 bp), and the entire operon (3321 bp) were PCR amplified using Q5 DNA polymerase (NEB) in a 50 µl reaction containing 1X reaction buffer, 1X GC enhancer, 20 pmoles each of forward and reverse primers, 200 µM dNTP, ~300 ng of *M. smegmatis mc²155* gDNA and 1U of the enzyme. The PCR reaction involved denaturation at 98° C for 3 min followed by 29 cycles of denaturation at 98° C for 30 s, annealing 72° C for 30 s, amplification at 72°C for 1 min followed by a final extension for 5 min at 72° C. The PCR products were separated on 1% TAE gel, and the corresponding amplicons for the individual gene and to the entire operon were eluted from the gel, cloned into pMV261 plasmid previously digested with *BamH*I and *Hind*III by infusion cloning method according to manufacturer's protocol (Takara). The recombinant plasmids, pMV261~P_{hsp}-*MSMEG_1381,* pMV261~P_{hsp}-*MSMEG_1382*, and pMV261~P_{hsp}*MSMEG_1381~MSMEG_1382* were transformed into *E. coli* TG1 cells. The plasmids were isolated from the colonies selected on the LB agar containing kanamycin. The recombinant plasmids were confirmed for the presence of insert by restriction digestion analysis and were transformed into *M. smegmatis* mc²155 strain.

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