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Efflux Pumps of *Mycobacterium tuberculosis* Play a Significant Role in Antituberculosis Activity of Potential Drug Candidates

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Active efflux of drugs mediated by efflux pumps that confer drug resistance is one of the mechanisms developed by bacteria to counter the adverse effects of antibiotics and chemicals. To understand these efflux mechanisms in *Mycobacterium tuberculosis*, we generated knockout (KO) mutants of four efflux pumps of the pathogen belonging to different classes. We measured the MICs and kill values of two different compound classes on the wild type (WT) and the efflux pump (EP) KO mutants in the presence and absence of the efflux inhibitors verapamil and L-phenylalanyl-L-arginyl-β-naphthylamide (PAβN). Among the pumps studied, the efflux pumps belonging to the ABC (ATP-binding cassette) class, encoded by *Rv1218c*, and the SMR (small multidrug resistance) class, encoded by *Rv3065*, appear to play important roles in mediating the efflux of different chemical classes and antibiotics. Efflux pumps encoded by *Rv0849* and *Rv1258c* also mediate the efflux of these compounds, but to a lesser extent. Increased killing is observed in WT *M. tuberculosis* cells by these compounds in the presence of either verapamil or PAβN. The efflux pump KO mutants were more susceptible to these compounds in the presence of efflux inhibitors. We have shown that these four efflux pumps of *M. tuberculosis* play a vital role in mediating efflux of different chemical scaffolds. Inhibitors of one or several of these efflux pumps could have a significant impact in the treatment of tuberculosis. The identification and characterization of *Rv0849*, a new efflux pump belonging to the MFS (major facilitator superfamily) class, are reported.

In earlier times, tuberculosis (TB) was described as the "white plague" and the "captain of death." Today, with a battery of drugs available to fight it, one would conclude that TB is almost eradicated, but the facts are grim, as TB has been consistently claiming a larger number of lives than any other infectious disease. There are 8 to 10 million new cases of tuberculosis per year with 2 million deaths annually, and TB remains a worldwide emergency mostly affecting poor and developing countries (12, 37). Multidrug-resistant (MDR) TB and the highly lethal form called extensively drug-resistant (XDR) TB are adding a new challenge to this old and persistent disease (7, 13). Antimycobacterial therapy has become less effective due to the emergence of drug resistance, and researchers are struggling to find novel approaches to tackle this ever-growing problem.

Drug resistance in *Mycobacterium tuberculosis*, as in any other bacterium, is an outcome of multiple mechanisms operating simultaneously. It could be due to the accumulation of mutations in the target genes over a period of time (21), exclusion of antibiotics by the highly impermeable cell wall (18, 22), the wide array of efflux mechanisms mediated by several ABC (ATP-binding cassette) transporters and major facilitator superfamily (MFS) proteins (9, 10), or antibiotic-modifying and -degrading enzymes (3), to name a few possibilities.

Quite often, investigational drugs that have excellent enzyme inhibition do not exhibit equally potent inhibition of bacteria, and this is a major challenge in the arena of TB drug discovery. Among other causes, efflux mechanisms contribute in a major way to intrinsic resistance to drugs. Efflux pumps of *M. tuberculosis* belonging to different classes have been described in the last few years, and some pumps have been well characterized (2, 6, 8, 11, 29, 30). They belong to the MFS, ABC superfamily, and SMR (small multidrug resistance) family.

Efflux-mediated drug resistance in *M. tuberculosis* could be due to one or more efflux pumps working alone or in coordination. This is possible because of the redundancy of their functions, which may overlap extensively (24, 27). In the current work, we have attempted to examine this by comparing the sensitivities of wild-type (WT) *M. tuberculosis* and the knockout (KO) mutants of four efflux pumps (encoded by *Rv1258c* and *Rv0849*, both belonging to the MFS class; by *Rv1218c* [6], belonging to the ABC transporter class; and by *Rv3065*, belonging to the SMR class) to different compound classes. We have also studied the effects of these compounds in the presence and absence of the efflux inhibitors verapamil and L-phenylalanyl-L-arginyl-β-naphthylamide (PAβN).

Rv0849 encodes a new efflux pump, which has been previously shown to belong to the MFS class by comparison of common sequence motifs (10). A deletion (KO) mutant was generated in the present investigation, and antimycobacterial drugs and different classes of investigational drugs were tested on this mutant. This efflux pump also appears to play an important role in mediating the efflux of investigational drugs, as can be seen from our findings. *Rv1218c*, encoding an ABC transporter, was described previously by our laboratory (6).

Our findings indicate that (i) the different chemical classes of

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
M. tuberculosis H37Rv	Virulent strain of *M. tuberculosis*	ATCC
ATCC 27294		
pAZI0290	Derived from pGOAL19 (15) by deleting the *lacZ* gene as a BamHI-BamHI fragment	Laboratory stock
pBAN0366	Truncated *Rv1218c* cloned into pAZI0290	6
pBAN0448	Truncated *Rv1258c* cloned into pAZI0290	This study
pBAN0447	Truncated *Rv0849* cloned into pAZI0290	This study
pBAN0449	Truncated *Rv3065* cloned into pAZI0290	This study
pMV261	Mycobacterial expression vector	35
pBAN0192	pMV261 vector with the intact *Rv1218c*	6
pBAN193	pMV261 vector with the intact *Rv0849*	This study
pPAZ11	Plasmid pSUM36 with intact Rv1258c insert	10
pMD31	Cloning vector	11
pMtb312	pMD31 containing the intact *Rv3065* gene	11
KO1	*M. tuberculosis* H37Rv with inactivated *Rv1258c*	This study
KO1(pPAZ11)	KO1 transformed with plasmid pPAZ11	This study
KO5	*M. tuberculosis* H37Rv with inactivated *Rv1218c*	6
KO5(pBAN192)	KO5 transformed with plasmid pBAN192	6
KO6	*M. tuberculosis* H37Rv with inactivated *Rv0849*	This study
KO6(pBAN193)	KO6 transformed with plasmid pBAN193	This study
KO7	*M. tuberculosis* H37Rv with inactivated *Rv3065*	This study
KO7(pMtb312)	KO7 transformed with plasmid pMtb312	This study

compounds studied are effluxed to different extents by the four pumps studied and (ii) irrespective of their modes of action, the efflux inhibitors studied act on multiple efflux pumps.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemicals. The strains used in this study are listed in Table 1. The *M. tuberculosis* strains and constructs were grown in 250-ml roller bottles (Corning Inc., Corning, NY) as smooth cultures to mid-log phase (optical density at 600 nm [OD_{600}] = 0.5) and stored frozen as 0.5-ml aliquots in screw-cap cryovials (Corning) at −70°C. Representative vials from the frozen lot were thawed and plated for enumeration of viable counts after 10 days and were found to contain approximately 8.0 $\log_{10}$ CFU/ml. For subsequent experiments, seed lot vials were thawed, and the cells were diluted to get the required CFU/ml. The media used for growth of *M. tuberculosis* were Middlebrook 7H9 broth and 7H10 agar (Difco Laboratories, Detroit, MI) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-catalase (ADC). Hygromycin B was purchased from Roche. When required, hygromycin or kanamycin was added to the cultures at final concentrations of 50 μg/ml and 20 μg/ml, respectively. Streptomycin, isoniazid (INH), rifampin, ethambutol, spectinomycin, kanamycin, gentamicin, amikacin, clofazimine, verapamil, and PAβN used in the antimicrobial susceptibility tests were all purchased from Sigma. Restriction enzymes were purchased from Bangalore Genei (India). Oligonucleotides were synthesized by Sigma for this work.

DNA amplification by PCR. Screening of single-crossover (SXO) and double-crossover (DXO) recombinants in *M. tuberculosis* was done by PCR using *Taq* DNA polymerase (Bangalore Genei). Single colonies were picked up from 7H9 plates, resuspended in 50 μl TE (10 mM Tris, 1 mM EDTA), and boiled for 20 min. Five microliters of the supernatant from the boiled samples was taken in 25 μl of PCR mixture. The denaturation and extension reactions were performed at 94°C and 72°C, respectively. The annealing temperature and the extension time for each PCR amplification were decided based on the melting temperature (T_m) of the primer pair and the length of the PCR product, respectively. The sequences of the primers used in this study will be made available upon request.

Plasmid constructs. The plasmids used in this study are listed in Table 1. The construction of the plasmids used for gene inactivation of *Rv1258c*, *Rv0849*, and *Rv3065* was outsourced to Syngene (India). The recombination substrate for creating the deletions in the respective genes of *M. tuberculosis* consisted of the mutant gene with flanking sequences cloned into a suicide vector, pAZI0290. Each gene was individually inactivated by introducing a markerless deletion in the gene and rendering the remaining part of the gene out of frame. The substrate for the KO1 gene (*Rv1258c*) was created by deleting 196 bp (from bp 245 to 440) of the gene and rendering the downstream part of the gene out of frame. The final construct, bearing 900 bp and 836 bp upstream and downstream, respectively, of the deleted region, was cloned into the NcoI-BglII sites of the suicide vector pAZI0290 to obtain pBAN0448. The DNA substrate for inactivating *Rv0849* consisted of 800 bp of the region upstream of *Rv0849* and a 298-bp deletion (from the initiation codon ATG onward), followed by 800 bp of the remaining part of the gene, which was rendered out of frame. The final construct was obtained by cloning the 1,600-bp fragment into the ScaI-SspI sites of pAZI0290 to obtain pBAN0447. The substrate for inactivating *Rv3065* consisted of 800 bp of region upstream of *Rv3065*, a 298-bp deletion (from the initiation codon ATG onward), and 26 bp of the remaining part of the gene, followed by 774 bp of the region downstream of *Rv3065*. The final construct was obtained by cloning the 1,574-bp fragment into the ScaI-SspI sites of pAZI0290 to obtain pBAN0449. The sequence of the cloned fragments was confirmed by DNA sequencing (Microsynth). The plasmids were electroporated into *M. tuberculosis* as described previously (23).

Inactivation of *Rv1258c*, *Rv0849*, and *Rv3065* in *M. tuberculosis*. All three genes were inactivated by 2-step homologous recombination as described previously (23). The genes were inactivated by making a deletion, which is not expected to affect the transcription of downstream genes. Out of 6 colonies of SXO recombinants screened, a small number of colonies had the plasmid integrated in the proper locus, as confirmed by PCR. One or two of the SXOs were grown in 7H9 broth in the absence of hygromycin for 3 weeks and then plated on 7H10 plates either with or without 2% sucrose. The colonies growing in the presence of sucrose were screened for the loss of the plasmid by PCR amplification of Hyg^r and *sacB* genes. In all cases, only 20 to 30 colonies grew on the sucrose plates. From these, 6 to 8 colonies were picked and analyzed by PCR with the respective primers. Among them, some colonies had retained the wild-type copy of the gene on the chromosome, while a few had the wild-type copy of the respective gene replaced by the truncated copy, thus rendering the gene nonfunctional. The absence of the wild-type copy in these mutants was further confirmed by specific PCRs (See Fig. S1 in the supplemental material) and Southern blotting using appropriate primers (data not shown).

Growth kinetics of the strains. The wild-type H37Rv strain, KO1, KO5, KO6, and KO7 were grown in Middlebrook 7H9 broth as described above. Growth profiles were obtained by aliquoting 200-μl samples every day and measuring the OD_{600} values in a spectrophotometer. This was repeated on three independent occasions.

Determination of antibiotic susceptibility. The MICs of standard drugs and compounds under investigation were tested by resazurin-based microplate assay on *M. tuberculosis* (6). Antibiotic susceptibility testing in the presence of the efflux inhibitors verapamil and PAβN was carried out by adding the respective inhibitors at subinhibitory concentrations (0.25× MIC) to the *M. tuberculosis* cultures in the assay. The MICs of

verapamil and PAβN were >200 μg (taken as 200 μg) and 32 μg/ml, respectively.

Studies to assess bactericidality. Drug susceptibility (MIC) assays were carried out to determine the sensitivities of the *M. tuberculosis* WT strain and the *Rv1258c* (KO1), *Rv1218c* (KO5), *Rv0849* (KO6), and *Rv3065* (KO7) deletion mutants to compounds belonging to different chemical classes (Tables 2 and 3) and also in the presence and absence of verapamil or PAβN (Table 3). The protocol was as described earlier (6) using the CFU-based evaluation. Briefly, MIC assays with relevant compounds were set up in a 96-well microplate format in 7H9 broth. Following exposure of the various strains to different concentrations of compounds for a period of 7 to 8 days, where appropriate, aliquots were plated on 7H10 agar plates for enumeration of survivors to assess the extent of kill (bactericidality) for the WT strain and the different efflux pump KO mutants. The plates were incubated for 21 to 28 days at 37°C, and colonies were scored. Since the MICs of the various drugs and compounds were different for the WT and the KO mutants, at least three concentrations of each compound were chosen for plating and comparison between the strains. The average bacterial number of the starting inoculum was 5.5 ± 0.2 $\log_{10}$ CFU/ml for the MIC assays. Control cultures of the WT and those of efflux pump mutants, as well as those of the WT and mutants to which the efflux inhibitors were added at subinhibitory (0.25× MIC) concentrations, grew to 7.5 to 8.9 $\log_{10}$ CFU/ml. The cultures to which the efflux inhibitors were added at subinhibitory concentrations did not have any difference in the final cell numbers, indicating that their growth was not affected in any way by the addition of the efflux inhibitors.

RESULTS AND DISCUSSION

Efflux pumps of *M. tuberculosis* display multiple substrate specificities. *M. tuberculosis* possesses a number of efflux pumps, but to date, only some of them have been shown to modulate susceptibility to agents used in the treatment of the disease (28, 33). The role of efflux in modulating drug susceptibility is both drug and bacterium dependent. These efflux pumps play an essential role aiding the survival of bacteria. Exposure of mycobacteria to antituberculous agents that are substrates of the various efflux pumps would contribute to the successful survival of *M. tuberculosis* within its human host, as well (25). In the present study, we have attempted to examine some of these by carrying out MIC and bactericidality assays on WT *M. tuberculosis* and KO mutants of efflux pumps (Table 1) belonging to different classes. In addition, the efflux inhibitors verapamil and PAβN were used in combination with different chemical classes of compounds under investigation (Tables 2 and 3).

Selection, construction, and growth properties of efflux pump KOs of *M. tuberculosis*. Four efflux pumps belonging to three different classes were selected to study their effects on the physiology of *M. tuberculosis*. We routinely screen investigational drugs (which have potent enzyme inhibition but poor MICs) using efflux inhibitors, such as verapamil and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). During these screens, it was observed that compounds belonging to the class of pyrazolones had decreased MICs for *M. tuberculosis* in the presence of these efflux inhibitors. Microarray data were generated for pyrazolones to understand their mode of action, which revealed 4- to 6-fold hyperexpression of a number of genes and certain putative ABC transporters (data not shown). We chose two of the transporter genes from this panel for our study: *Rv1218c*, coding for an ABC transmembrane transport protein (6), and *Rv0849*, coding for a protein of the multifacilitator superfamily. We also chose two other pumps reported previously by other research groups, namely, *Rv1258c* (Tap [2]) and *Rv3065* (Mmr [11]) belonging to the MFS and the SMR family, respectively. KO constructs were made as described in Materials and Methods, and their identities were confirmed by PCRs using forward and reverse primers encompassing the deleted region (see Fig. S1A in the supplemental material). All the KO constructs had smaller inserts than the WT, confirming the respective deletions (see Fig. S1B and C in the supplemental material). The growth profiles of the different KO strains in 7H9 broth were compared to that of the WT strain. No growth defects were detectable (data not shown).

Efflux pump hyperexpression studies and complementation of the efflux pump KOs. The susceptibilities of the WT *M. tuberculosis* strain, KO strains, complemented KO strains, WT transformed with vector plasmids, and WT transformed with recombinant plasmids (Table 1) were tested by MIC assays for a variety of antibiotics (Table 2).

(i) *Rv1258c*. Gentamicin and spectinomycin displayed a drop in MICs in the KO of the Tap efflux pump gene *Rv1258c* (KO1), and this was restored upon complementation of KO1 with the functional copy of the gene on a plasmid (pPAZ11). This plasmid, when inserted into WT *M. tuberculosis* (the hyperexpression strain), also displayed an increase in the MICs of these drugs (Table 2). These data are in agreement with what has been published (10, 36) and thus confirm the identity of KO1. The MIC of amikacin dropped only 2-fold in KO1 compared to the WT but increased more than 8-fold in the complemented KO and hyperexpression strains of *Rv1258c*.

(ii) *Rv3065*. The KO of the Mmr efflux pump gene *Rv3065* (KO7) did not exhibit any variation in the MICs of any known drugs tested (Table 2). KO7 also did not exhibit any drop in the MIC of ethidium bromide compared to the WT. However, the complementation strain of KO7 and the hyperexpression strain (both with plasmid pMtb312) had 8-fold increases in the MICs of ethidium bromide. This hyperexpression has also been reported by De Rossi et al. (11) for *Mycobacterium smegmatis* for the same gene. These findings confirm the correlation of the observed MIC changes with the identities of these two genes whose KO constructs have been described.

(iii) *Rv1218c* and *Rv0849*. Construction of and data for the KOs of *Rv1218c* (KO5) and *Rv0849* (KO6) have been reported by our laboratory for the first time (reference 6 and this work). KO5 had no remarkable decrease in the MICs of many reference drugs (6) (Tables 2 and 3). In KO6, a 2-fold drop was observed in the MIC of amikacin, which was restored to 8-fold-higher values in both the complemented KO6 and hyperexpression strains. In addition, KO6 had no remarkable decrease in the MICs of many reference drugs (Tables 2 and 3). All these efflux pumps exhibited only limited MIC shifts in the corresponding pairs, i.e., KO mutants and their corresponding hyperexpression strains; this may be a reflection of the "regulation" of these efflux pumps. Alternatively, efflux pump expression could make the bacterial strain undergo changes that alter the general drug susceptibility profile of the strain, and this has also been reported by other investigators (30).

Inactivation of *Rv1258c*, *Rv1218c*, *Rv0849*, and *Rv3065* in *M. tuberculosis* results in increased susceptibility to compounds belonging to various chemical classes. (i) Pyrrole class. MICs of the pyrrole class of compounds (AZI-533 and AZI-530) decreased 2- to 8-fold (Tables 2 and 3) in the four different KO mutants in comparison to that for the WT strain. Addition of the efflux inhibitor verapamil or PAβN caused a further decrease in the MICs

TABLE 2 Antimicrobial susceptibilities of *M. tuberculosis* WT, efflux pump KO mutants, complemented KO strains, and hyperexpression strains

Compound	MIC (μg/ml) for *M. tuberculosis* strain[a]:												
	WT	KO1[b]	KO1(pPAZ11)[c]	WT(pPAZ11)[d]	KO5[e]	KO5(pBAN192)[f]	WT(pBAN192)[g]	KO6[h]	KO6(pBAN193)[i]	WT(pBAN193)[j]	KO7[k]	KO7(pMtb312)[l]	WT(pMtb312)[m]
AZI-533 (pyrrole)	0.03	0.016	ND	0.03	0.008	0.03	0.03	0.016	0.016	0.02	0.008	0.016	0.03
AZI-219 (pyrazolone)	>256	256	256	>256	128	256	256	256	256	>256	256	256	>256
Clofazimine	0.125	0.125	0.06	0.125	0.125	0.06	0.125	0.125	0.125	0.06	0.06	0.06	0.06
Streptomycin	0.5	0.5	ND	ND	0.5	0.5	0.5	0.5	ND	ND	0.5	ND	ND
INH	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Rifampin	0.003	0.0016	ND	ND	0.0016	ND	ND	0.0016	ND	ND	0.006	ND	ND
Ethambutol	2	2	ND	ND	2	ND	ND	2	ND	ND	2	ND	ND
Gentamicin	4	2	4	8	4	4	4	ND	ND	ND	ND	ND	ND
Spectinomycin	64	8	32	64	ND	ND	64	ND	ND	64	ND	ND	64
Amikacin	1.0	0.5	>8	>8	1	ND	0.5	0.5	>8	>8	0.5	0.5	ND
Kanamycin	2	ND	>64	>64	4	>32	>32	2	>32	>32	2	>32	>32
Ethidium bromide	1	2	2	4	2	2	2	1	2	2	1	8	>8

[a] MICs were assayed over a range of 2-fold dilutions of antibiotics as discussed in Materials and Methods. MICs were determined by the resazurin-based microplate assay. Each reported value is the average of at least 2 independent assays. MICs of the WT strain containing the vectors pMV261 and pMD31 were the same as those of the WT; hence, they are not shown in the table. All the plasmids have kanamycin as the selection marker. ND, not determined.
[b] KO1 is *M. tuberculosis* with the *Rv1258c* gene inactivated.
[c] KO1(pPAZ11) is KO1 complemented with plasmid pPAZ11.
[d] WT(pPAZ11) is *M. tuberculosis* containing plasmid pPAZ11.
[e] KO5 is *M. tuberculosis* with the *Rv1218c* gene inactivated.
[f] KO5(pBAN192) is KO5 complemented with plasmid pBAN192.
[g] WT(pBAN192) is *M. tuberculosis* containing plasmid pBAN192.
[h] KO6 is *M. tuberculosis* with the *Rv0849* gene inactivated.
[i] KO6(pBAN193) is KO6 complemented with plasmid pBAN193.
[j] WT(pBAN193) is *M. tuberculosis* containing plasmid pBAN193.
[k] KO7 is *M. tuberculosis* with the *Rv3065* gene inactivated.
[l] KO7(pMtb312) is KO7 complemented with plasmid pMtb312.
[m] WT(pMtb312) is *M. tuberculosis* containing the plasmid pMtb312.

TABLE 3 Susceptibilities of *M. tuberculosis* WT and *Rv1258c*, *Rv1218c*, *Rv0849*, and *Rv3065* KO mutants to compounds in the presence and absence of efflux inhibitors

Compound	MIC (μg/ml) for *M. tuberculosis* strain:				
	WT	KO1	KO5	KO6	KO7
Pyrroles					
AZI-533	0.03	0.016	0.004–0.008	0.008–0.016	0.008
AZI-533 + verapamil	0.008	0.008	0.004	0.004	0.004
AZI-533 + PAβN	0.016	0.004	0.004	0.004	0.002
AZI-530	0.25	0.125	0.03	0.06	0.03
AZI-530 + verapamil	0.06	0.06	0.03	0.03	0.016
AZI-530 + PAβN	0.125	0.03	0.03	0.06	0.016
Pyrazolones					
AZI-219	256	128–256	64–128	128–256	128–256
AZI-219 + verapamil	16–32	16–32	8–16	8–16	16–32
AZI-219 + PAβN	32	32	32	128	16-32
AZI-335	1	1	0.5	0.5	0.5–1
AZI-335 + verapamil	0.5	0.5	0.25	0.25	0.25
AZI-335 + PAβN	0.5	0.5	0.125	0.25-0.5	0.5
Other drugs					
Clofazimine	0.125–0.25	0.06–0.125	0.06–0.125	0.06–0.125	0.03–0.06
Clofazimine + verapamil	0.03	0.03	0.016	0.016–0.03	0.008–0.016
Clofazimine + PAβN	0.016	0.03	0.016	0.06	0.016
Efflux inhibitors					
Verapamil	>200	>200	>200	>200	>200
PAβN	32	16	16	16	16

MICs were determined by the resazurin-based microplate assay. Each reported value is the average of at least 2 independent assays. The efflux inhibitors verapamil and PAβN were added at 50 μg/ml and 4–8 μg/ml (subinhibitory) concentrations where required.

of these pyrroles by 2- to 4-fold for the WT and the KO mutants. In the bactericidality assays, the WT and the four KO mutants (KO1, KO5, KO6, and KO7) displayed a concentration-dependent bactericidal response to AZI-533 (Fig. 1A and B). Bactericidal measurements of AZI-533 revealed killing values of 0.6, 1.0, 1.9, 0.8, and 2.1 $\log_{10}$ CFU/ml in the WT and the four KO mutants, respectively, at the highest concentration (0.03 μg/ml) tested (Fig. 1A). The best response was seen in KO5 and KO7, whose pumps are encoded by *Rv1218c* and *Rv3065*, respectively. The efflux inhibitor PAβN was selected for bactericidal studies. When PAβN was added to the assay, the killing values obtained were 1.7, 1.3, 1.6, 1.2, and 2.4 $\log_{10}$ CFU/ml, respectively, in the WT and the four KO mutants (Fig. 1B). It can be noticed that in the WT cells there is a 3-fold increase in the bactericidal values with PAβN compared to those of the KO mutants, which do not exhibit any significant increase in their bactericidal values. This finding argues in favor of the fact that an efflux inhibitor like PAβN could interfere with more than one efflux pump in WT *M. tuberculosis* cells. AZI-530 had a similar MIC profile but was not taken up for bactericidality studies (Table 3).

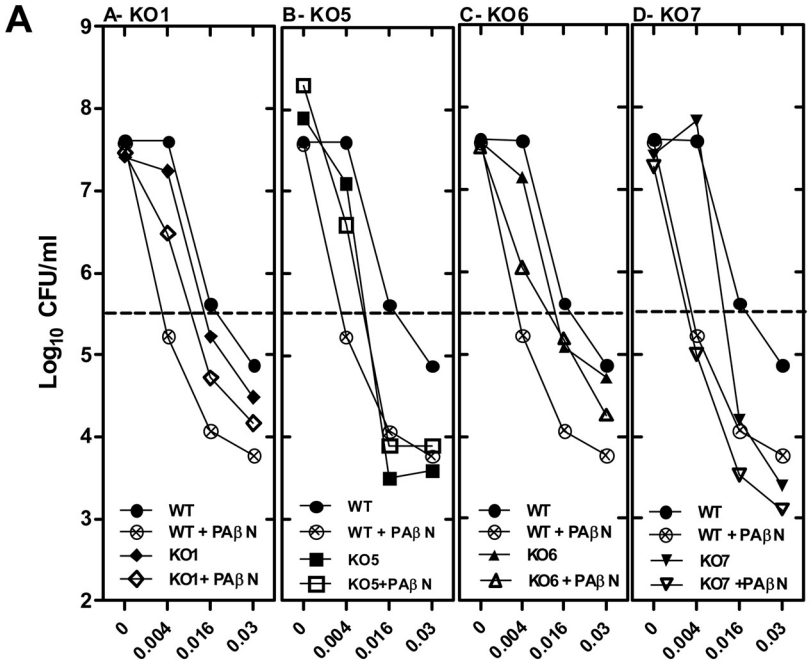
Rv1218c and *Rv3065* appear to be two important pumps responsible for the efflux of AZI-533, and *Rv1258c* and *Rv0849* are the other two pumps that are involved to a lesser extent. PAβN functions through a competitive-inhibition (peptidomimetic) mechanism, and the efflux pumps recognize PAβN as a substrate instead of the target compound (5). In the WT *M. tuberculosis* cells, all four pumps may compete, though to different extents, to efflux PAβN when it enters the bacterial cell along with the compound. Apart from these four pumps, there could be additional pumps that may compete for PAβN in the WT. In three of the four KOs, the kill values improved only marginally when the efflux inhibitor PAβN was added. This could imply that there are no additional pumps that could compete for PAβN apart from the three that were investigated in our study. We conclude from these data that all four of the pumps studied are involved to different extents in the efflux of AZI-533.

Even though PAβN was specifically shown to be an inhibitor of the RND transporters (31), our results demonstrate the affinity of PAβN for efflux pumps of *M. tuberculosis* belonging to classes other than the RND transporter class.

(ii) Pyrazolone class. The pyrazolone class of compounds presented a different picture. AZI-219, one of the pyrazolones tested, had an MIC of 256 μg/ml for WT *M. tuberculosis*. In three out of the four KO strains tested, the MICs decreased just 2-fold (Table 3). However, when verapamil was added as the efflux inhibitor in the assays along with AZI-219, there was a steep decrease of 8- to 16-fold in the MICs for all the strains (WT and the KO mutants) tested. This confirms the presence of an alternate efflux pump(s) that is susceptible to verapamil. Addition of PAβN caused 4-fold decreases in the MICs of the WT and the KO mutants, with the exception of KO6, which showed minimal (2-fold) to no decrease (Table 3). Again, this confirms the presence of an alternate efflux pump(s) that is susceptible to PAβN. Since verapamil appeared to be a better inhibitor, it was chosen for bactericidality studies.

Examining the bactericidal effects of these compounds, it is clear that the bactericidal effect of AZI-219 was not evident in the WT, KO1, KO6, and KO7 in the absence of verapamil (Fig. 2A and B), while the addition led to enhanced killing of the WT and, to different extents, of the KO strains. CFU values for survivors obtained from the bactericidality assays without verapamil recorded

A



B

AZI-533 (Log_{10} CFU values)										
concentration (μg/ml)	WT	WT + PAβN	KO1	KO1+ PAβN	KO5	KO5+ PAβN	KO6	KO6+ PAβN	KO7	KO7+ PAβN
0.004	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
0.015	0.0	1.4	0.3	0.8	2.0	1.6	0.4	0.3	1.3	2.0
0.03	0.6	1.7	1.0	1.3	1.9	1.6	0.8	1.2	2.1	2.4

FIG 1 Bactericidal profile of AZI-533 on WT *M. tuberculosis* and the efflux pump KO mutants. (A) Survival curves in the presence and absence of the inhibitor PAβN. Bactericidal assays were performed as described in Materials and Methods. Cultures were exposed to AZI-533 at the concentrations indicated and processed for enumeration of survivors. The three concentrations used cover the range of MICs for the different strains in the study: WT (0.03 μg/ml), KO1 and KO6 (0.016 μg/ml), KO5 (0.008 μg/ml), and KO7 (0.004 μg/ml). The vertical (*y*) axis represents the number of bacteria surviving under each concentration shown in the horizontal (*x*) axis after compound (AZI-533) exposure for 7 days. The dashed line indicates the cell number for all the strains at the start of the experiment, which was 5.5 ± 0.2 $\log_{10}$ CFU/ml. PAβN was used at 8 μg/ml for the WT and 4 μg/ml for the KO strains. (B) Log kill values for WT *M. tuberculosis* and the different efflux pump KO mutants deduced from the starting cell number of 5.5 $\log_{10}$ CFU/ml on day 0 and survivors at the end of the 7-day exposure to the compound. The results shown represent data from one of two or three independent experiments.

no kill values in the WT, KO1, and KO6 strains and a nominal value of 0.6 $\log_{10}$ CFU/ml in KO7. The addition of verapamil resulted in an increase in the kill values to 1.4, 0.9, >3.5, 1.8, and 3.1 $\log_{10}$ CFU/ml in the WT and the KO mutants, respectively. This increase in kill values ranged from 1.5- to 3-fold. In KO5, the values were below the limit of quantification. The kill values were quite significant for AZI-219 for KO5 even in the absence of verapamil. Thus, *Rv1218c* appears to play a major role in the efflux of AZI-219. These data seem to indicate that both the verapamil-sensitive efflux system and the efflux systems deleted in the specific mutants have various degrees of influence on the efflux of AZI-219.

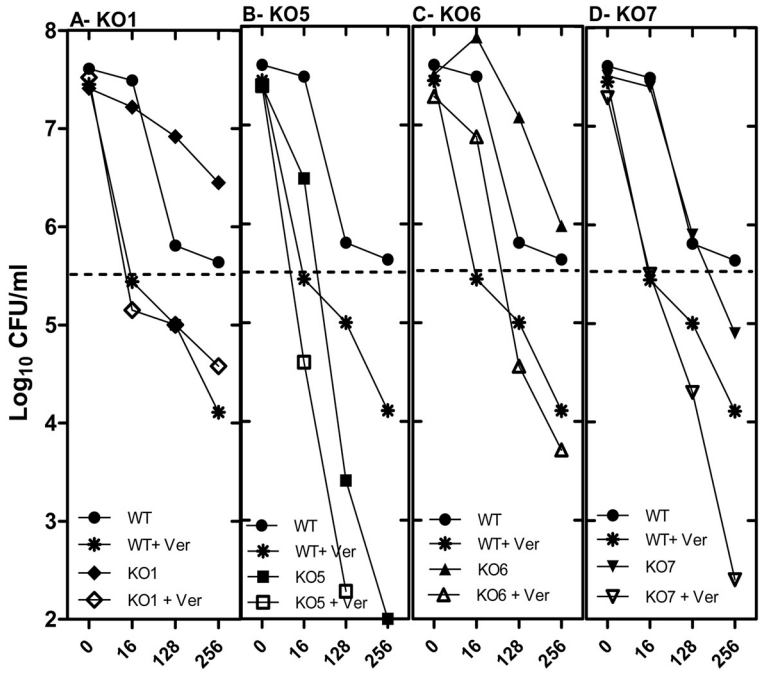
In the experiments with compound AZI-335, belonging to the same class, the decrease in MICs in the presence of either verapamil or PAβN was only 2- to 4-fold. This could be due to either or both of the following reasons: (i) the difference in the affinity of AZI-219 and AZI-335 for the target or (ii) the ability to be recognized by the efflux pumps under investigation. The latter reason seems more probable in the present context. AZI-335 was not profiled in bactericidality assays.

The MIC of clofazimine for the WT cells was 0.125 μg/ml, and it decreased to 0.06, 0.03, 0.06, and 0.03 μg/ml (2-, 4-, 2-, and 4-fold) in the mutants KO1, KO5, KO6, and KO7, respectively, compared to that for the WT (Table 3). Upon addition of the efflux inhibitor verapamil or PAβN, the MICs decreased 4- to 8-fold for the WT and a further 2- to 4-fold for the KO mutants. This was not profiled in the bactericidality assays.

INH consistently had an MIC of 0.03 μg/ml for the WT and the efflux pump mutants. The addition of the efflux inhibitor verapamil or PAβN at subinhibitory concentrations to the WT and the efflux pump mutants did not result in any change in the MICs of INH. Bactericidality assays did not reveal any reduction in cell numbers (data not shown).

Our studies indicate that among all four efflux pumps we studied and compared, the pumps encoded by *Rv1218c* (reference 6 and this work) and *Rv3065* appear to play major roles in mediating

A



B

AZI-219 (Log_{10} CFU values)										
concentration (μg/ml)	WT	WT + Ver	KO1	KO1+ Ver	KO5	KO5+ Ver	KO6	KO6+ Ver	KO7	KO7+ Ver
16 to 32	0.0	0.1	0.0	0.4	0.0	1.1	0.0	0.0	0.0	0.0
128	0.0	0.5	0.0	0.5	2.1	3.2	0.0	0.9	0.0	1.2
256	0.0	1.4	0.0	0.9	3.5	>3.5*	0.0	1.8	0.6	3.1

FIG 2 Bactericidal profile of AZI-219 on WT *M. tuberculosis* and the efflux pump KO mutants. (A) Survival curves in the presence and absence of the inhibitor verapamil. Bactericidal assays were performed as described in Materials and Methods. Cultures were exposed to AZI-219 at the concentrations indicated and processed for enumeration of survivors. The three concentrations used cover the range of MICs for the different strains in the study (WT [256 μg/ml] and KO1, KO5, KO6, and KO7 [128 to 256 μg/ml]) and the MICs of all cultures in the presence of 50 μg/ml of verapamil (16 to 32 μg/ml). The vertical (*y*) axis represents the number of bacteria surviving under each concentration shown in the horizontal (*x*) axis after AZI-219 exposure for 7 days. The dashed line indicates the cell number for all the strains at the start of the experiment, which was 5.5 ± 0.2 $\log_{10}$ CFU/ml. Verapamil was used at 50 μg/ml for the WT and KO strains. (B) Log kill values for WT *M. tuberculosis* and the different efflux pump KO mutants deduced from the starting cell number of 5.5 $\log_{10}$ CFU/ml on day 0 and survivors at the end of the 7-day exposure to AZI-219. The results shown represent data from one of two or three independent experiments. *, in KO5 at 256 μg/ml, the values were below the limit of quantification (no growth at 100 μl of the lowest dilution plated) and hence were marked as >3.5. Ver, verapamil.

the efflux of compounds belonging to the pyrrole and pyrazolone chemical classes (Table 4). The experiments with the efflux inhibitors verapamil and PAβN suggest the involvement of additional efflux pumps that could play a role against these classes of compounds. It is not unusual that a chemical/compound/drug is the substrate for several pumps, given the redundancy of these pumps and their broad substrate specificities.

Concluding comments. Drug resistance arising out of efflux pump overexpression has been an issue in clinical settings, as well (15, 16, 35), which poses serious problems in the treatment of tuberculosis; this needs to be addressed as a priority. Several efflux pumps and their regulators are also induced during macrophage infection (1, 20, 32, 34). In addition, efflux could be the rate-limiting step in the discovery of novel anti-TB compounds, as has already been recognized in the discovery of drugs for Gram-negative bacterial infections. The discovery of new pumps with multiple specificities in *M. tuberculosis* and the impact these pumps have on antitubercular therapy by conferring resistance to many of the new molecules discovered necessitate the study of efflux mechanisms as an important therapeutic target. Identification of an efflux inhibitor that could be used as adjunct therapy alongside the existent regimen of drugs will have a significant impact on drug discovery and tremendous value in the treatment of tuberculosis.

While the use and study of the KO mutants and their interaction with specific classes of anti-TB compounds have helped highlight the involvement of efflux, the use of efflux inhibitors has thrown some light on how the pumps can work in concert within *M. tuberculosis*. A recent elegant study has demonstrated that efflux pumps that are required for intracellular growth also mediate macrophage-induced drug tolerance (1), and this ascertains the effects exerted by these pumps in different milieus. Efflux inhibitors like verapamil have been shown to reduce this tolerance. Therefore, there is reason to infer that the study of efflux can have relevance to susceptibility and the clinical outcomes of treatment

TABLE 4 Log kill values of different efflux pump KO mutants in the presence and absence of efflux inhibitors

Strain	Log kill value (log_{10} CFU/ml)[a]			
	Pyrrole AZI-533	AZI-533 + PAβN	Pyrazolone AZI-219	AZI-219 + verapamil
WT Mtu[c]	0.6	1.7	0	1.4
KO1	1	1.3	0	0.9
KO5	1.9	1.6	3.5	>3.5[b]
KO6	0.8	1.2	0	1.8
KO7	2.1	2.4	0.6	3.1

[a] Log kill values for WT *M. tuberculosis* and the different efflux pump KO mutants at the highest concentration of the compounds tested.
[b] Below the LOQ (limit of quantification).
[c] *M. tuberculosis.*

with antimycobacterial agents. However, the overlap in substrate specificities among these pumps poses several challenges to this approach (4). On the other hand, our findings indicate that, depending on the mode of inhibition of the pump, an inhibitor like PAβN or verapamil would indeed add tremendous value in treating tuberculosis (5, 26, 33), since they seem to interact differently with each pump-substrate combination. Several investigators are actively involved in the search for such inhibitors (14, 19) to treat various bacterial infections. Recently, a third-generation P-glycoprotein inhibitor, tariquidar, has been reported to be a potent inhibitor of certain bacterial efflux pumps *in vitro* (17).

No efflux pump inhibitor has yet reached clinical practice, but it is clear that this area of drug development offers a lot of promise, as it will further enhance the effective use of several drugs that have previously been considered to be of great clinical value and also new molecules that are currently under development (24).

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M.B. conceptualized the work, designed the experiments, analyzed data, and wrote the paper. U.S. designed the KO constructs. S.K. was involved in the construction of the KOs and their characterization. A.V.N. performed some initial experiments and made the complemented constructs. N.D. and S.S. performed all the major experiments.

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