

# Involvement of Efflux Pumps in the Resistance to Peptidoglycan Synthesis Inhibitors in *Mycobacterium tuberculosis*

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**We evaluated the contributions of *Mycobacterium tuberculosis* efflux pumps towards intrinsic resistance to different classes of peptidoglycan synthesis inhibitors (PSI). Our study indicates that the efflux pump knockout strains are more susceptible to PSI than the wild type. Vancomycin and ceftriaxone exhibited up to 3 log increased kill on efflux pump mutants compared to the wild-type strain, strongly suggesting an important role for efflux pumps in the intrinsic resistance of *M. tuberculosis* to PSI.**

Intrinsic resistance of *Mycobacterium tuberculosis* to peptidoglycan synthesis inhibitors (PSI) is generally attributed to the poor permeability of the mycobacterial cell wall and to the presence of the  $\beta$ -lactamase encoded by *blaC* (1, 2, 3). It is of concern that although  $\beta$ -lactam antibiotics are the most successful and widely used antibacterial agents; they have little or no use in the treatment of mycobacterial infections, including tuberculosis (4).

The intrinsic resistance of mycobacteria to PSI could also be attributed to the presence of multiple drug efflux pumps (5). We have constructed knockout (KO) mutants lacking the different classes of efflux pumps of *M. tuberculosis* as follows. Rv1258c (KO1) and Rv0849 (KO6) are major facilitator superfamily (MFS) efflux pumps, Rv1218c (KO5) belongs to the ATP-binding cassette (ABC) superfamily, and Rv3065 (KO7) belongs to the small multidrug resistance family (6, 7). We investigated the contributions of these efflux pumps of *M. tuberculosis* towards intrinsic resistance to various PSI by comparing the *in vitro* activities of selected drugs on wild-type (WT) *M. tuberculosis* and the efflux pump KO mutants. MICs were determined by the resazurin-based method as previously described (8). Bactericidal activities were evaluated by the CFU-based method as previously described (6).

The  $\beta$ -lactams exhibited increased potency against all four of the efflux pump KOs tested compared to the WT (Table 1). The MIC of penicillin was 64  $\mu$ g/ml for WT *M. tuberculosis*. KO5 and KO7 were the most sensitive to penicillin, as measured by the MICs, which dropped 4-fold (16  $\mu$ g/ml), while KO1 and KO6 exhibited a 2-fold drop (32  $\mu$ g/ml) in the MIC. WT *M. tuberculosis* had an ampicillin MIC of 16  $\mu$ g/ml; KO1, KO5, KO6, and KO7 were equally susceptible to this  $\beta$ -lactam, displaying 2- to 4-fold drops in the MICs (8  $\mu$ g/ml). The MICs of meropenem for KO1 and KO5 dropped 2- to 4-fold, KO7 showed a 0- to 2-fold drop, and the MIC for KO6 remained the same as that for the WT, which was 2 to 4  $\mu$ g/ml. Ceftriaxone had an MIC of 2 to 4  $\mu$ g/ml for WT *M. tuberculosis*. The drop in the MICs for all four of the KOs was uniformly 4-fold (0.5  $\mu$ g/ml). Cefotaxime had an MIC of 2 to 4  $\mu$ g/ml for WT *M. tuberculosis*. The MICs for KO1, KO5, and KO7 dropped 2- to 4-fold, and that for KO6 dropped 0- to 2-fold. Vancomycin had an MIC of 2  $\mu$ g/ml for WT *M. tuberculosis*. The MICs for KO1 and KO7 dropped 4-fold, while a MIC decrease of only 2-fold for the KO5 and KO6 strains was observed. Interestingly, the decrease in the bacitracin MIC was noticeable only for KO5 and not for any of the other KOs studied.

Either the KO strains complemented with the respective genes on plasmids as previously described (7) or the hyperexpression

TABLE 1 MICs of PSI for WT and efflux pump KO mutant strains of *M. tuberculosis*

Antibiotic	MIC ( $\mu$ g/ml) <sup>a</sup>				
	WT	KO1	KO5	KO6	KO7
Penicillin G	64	32	16	32	16
Ampicillin	16	4–8	8	8	4–8
Meropenem	2–4	1	1	2–4	2
Ceftriaxone	2–4	0.5–1	0.5–1	0.5–1	0.5–1
Cefotaxime	2–4	0.5–1	0.5–1	1–2	0.5
Vancomycin	2	0.5	1	1	0.5
Bacitracin	>128	>128	32	>128	128
Isoniazid	0.03	0.03	0.03	0.03	0.03

<sup>a</sup> MICs were determined by the resazurin-based microplate assay. Each value is the average of at least three independent assays.

strains were used to verify if the MICs were restored to the WT values. The MIC of compounds that showed 2- to 4-fold drops for the KO strains reverted closer to WT values when a functional copy of the respective efflux pump gene was present (data not shown).

Measurement of bactericidal activities (log kill) revealed a slightly different picture (Fig. 1). The bactericidal activities of ampicillin for KO1, KO5, and KO7 showed good increase (1.8, 2.2, and 2.4 log<sub>10</sub> CFU/ml, respectively) compared to that for the WT, and KO6 showed marginal kill of 0.5 log<sub>10</sub> CFU/ml. Meropenem did not have an enhanced bactericidal effect on any of the efflux KOs tested. Ceftriaxone had increased kill values against the four KOs, ranging from 1 to 3 log<sub>10</sub> CFU/ml. Vancomycin also had remarkable increases in its kill values, compared to that for the WT, for all four of the KOs that ranged from 2.8 to 3.5 log<sub>10</sub> CFU/ml. The bactericidal activities of isoniazid remained the same across the WT and the KOs (6, 7) and are therefore not shown here.

These results indicate the involvement of efflux pumps in the

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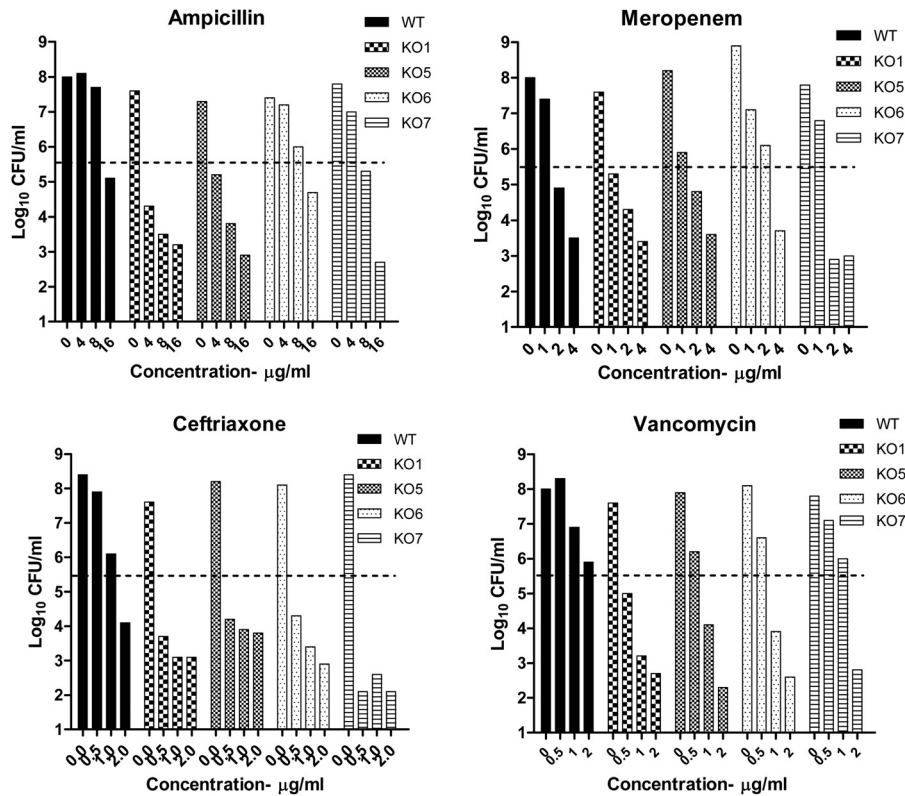
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**FIG 1** Bactericidal effects of  $\beta$ -lactams on WT and efflux pump KO mutant *M. tuberculosis* strains. Shown are the bactericidal activity profiles of PSI for WT *M. tuberculosis* and the efflux pump KO mutants. The three concentrations used cover the range of MICs for the different KO strains in this study (WT, KO1, KO5, KO6, and KO7). The y axis represents the number of bacteria surviving under each concentration shown on the x axis after exposure to antibiotics for 7 days. The dashed line indicates the cell number for all of the strains at the start of the experiment, which was  $5.5 \pm 0.2 \log_{10}$  CFU/ml.

intrinsic resistance of *M. tuberculosis* to the  $\beta$ -lactam class of antibiotics and a few other drugs (vancomycin and bacitracin) that act on the peptidoglycan synthesis of *M. tuberculosis*. The differences in the  $n$ -fold drops in the MICs could imply that, depending upon the structure of the  $\beta$ -lactam studied, the recognition and extent of efflux by the different pumps could vary. An earlier study by Danilchanka et al. (9) had shown the involvement of an ABC transporter of *M. tuberculosis* (Rv0194) in resistance to ampicillin, which further corroborates the role of efflux in the resistance of this bacterium to  $\beta$ -lactam antibiotics. Using efflux pump KO mutants to study drug efflux in bacteria is an artificial situation because in an infected individual, all of the efflux pumps work in concert, depending upon which class of compounds they need to pump out. It will not be possible to mimic such a situation in *in vitro* assays by using either efflux inhibitors or KO mutants. However, the *in vitro* studies conducted here give us valuable information on the potential roles of specific efflux pumps in modulating the activities of different PSI.

It is interesting that although the MIC of meropenem is modulated by the efflux activities of various pumps, the bactericidal effect of the drug is not altered. This could be due to the fact that carbapenems are poor substrates for BlaC and therefore are more stable as a class (10, 11).

Multiple-drug resistance in *M. tuberculosis* has been shown to be associated with the intrinsic or inducible expression of efflux systems (5, 6, 7, 12). Our findings show that different  $\beta$ -lactams are effluxed to various extents by the three classes of pumps stud-

ied. Vancomycin and bacitracin, which also target the cell wall of bacteria, are effluxed by these pumps, as indicated by the increased potency of these drugs on the efflux pump KO strains than against the WT. Our findings suggest that efflux is one of the mechanisms that make  $\beta$ -lactams and other PSI ineffective against *M. tuberculosis*. Additional work in this direction will add significantly to our understanding of the mechanism of the intrinsic resistance of *M. tuberculosis* to this class of antibiotics.

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M.B. conceptualized the work and designed the experiments. N.D. and S.S. carried out all of the experiments. M.B. and N.D. wrote the manuscript.

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