Involvement of efflux pumps in the resistance to peptidoglycan synthesis inhibitors in *Mycobacterium tuberculosis*.

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Running title: efflux pumps and inhibitors of cell-wall synthesis

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Abstract:

We evaluated the contribution of *Mycobacterium tuberculosis* efflux pumps towards intrinsic resistance to different classes of peptidoglycan synthesis inhibitors (PSI). Our study indicates that the efflux pump knockouts are more susceptible to PSI than wild type. Vancomycin and ceftriaxone exhibited up to 3-log increased kill on efflux pump mutants when compared to wild type strain, strongly suggesting an important role for efflux pumps in 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 β-lactamase coded by *blaC* (1, 2, and 3). It is concerning that although β-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).

Intrinsic resistance of Mycobacteria to PSI could also be attributed to the presence of multiple drug efflux pumps (5). We have constructed knock-out (KO) mutants of the different classes of efflux pumps of *M. tuberculosis* as follows; Rv1258c (KO1) and Rv0849 (KO6), which are Major Facilitator Superfamily (MFS) efflux pumps, Rv1218c (KO5) belongs to the ATP-binding Cassette (ABC) superfamily and Rv3065 (K07) belongs to the small multidrug resistance (SMR) family (6, 7). We investigated the contribution of these efflux pumps of *M. tuberculosis* towards intrinsic resistance to various PSI by comparing the in vitro activity of selected drugs on wild type *M. tuberculosis* and the efflux pump KO mutants. Minimum Inhibitory Concentration (MIC) was determined by the Resazurin-based method as previously described (8). The bactericidal values were evaluated using the CFU-based method as previously described (6).

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

Either the KO strains complemented with the respective genes on plasmids as previously described, (7) or the hyper-expression strains were used to verify if the MICs were restored to the WT values. The MIC for compounds which showed 2 to 4-fold drop on the KO strains, reverted closer to wild type values, when a functional copy of the respective efflux pump gene was present (data not shown).

Measurement of bactericidal (log kill) values revealed a slightly different picture (Fig 1). Cidal values for ampicillin in KOs 1, 5 and 7 showed good increase (1.8, 2.2 and 2.4 log₁₀ CFU/ml respectively) compared to the WT and KO6 showed marginal kill of 0.5 log₁₀ CFU/ml. Meropenem did not have enhanced cidality in any of the efflux knock-outs tested. Ceftriaxone had increased kill values in the four KOs ranging from 1- 3 log₁₀ cfu/ml. Vancomycin also had remarkable increase in the kill values compared to the WT on all the four KOs which ranged from 2.8 -3.5 log₁₀ CFU/ml. The cidality values for INH remained the same across the WT and KOs (6, 7), and is therefore not shown here.

These results indicate the involvement of efflux in the intrinsic resistance of *M. tuberculosis* to the β-lactam class of antibiotics and a few other drugs (vancomycin and bacitracin) acting on the peptidoglycan synthesis of *M. tuberculosis*. The differences in the fold-drop in MICs could imply that depending upon the structure of the β-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 \textit{M. tuberculosis} (Rv0194) in resistance to ampicillin which further corroborates the role of efflux in resistance to the \beta-lactam antibiotics in this bacterium. Using efflux pump KO mutants to study the efflux in bacteria is an artificial situation because in the infected individual, all the efflux pumps work in concert depending upon which class of compounds they need to efflux. It will not be possible to mimic such a situation in the in vitro assays using either the efflux inhibitors or the KO mutants. However, the in vitro studies conducted here give us valuable information on the potential role of specific efflux pumps in modulating the activity of different PSI.

It is interesting that although the MIC of meropenem is modulated by the efflux activities of various pumps, cidal 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 \textit{M. tuberculosis} is shown to be associated with intrinsic or inducible expression of efflux systems (5, 6, 7, and 12). Our findings show that the different \beta-lactams are effluxed by the three classes of pumps studied, to varied extents. 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 knock-out strains when compared to the wild type. Our findings suggest that efflux is one of the mechanisms which make the \beta-lactams and other PSI ineffective on \textit{M. tuberculosis}. Additional work in this direction will add significant value towards our understanding of the mechanism of intrinsic drug resistance of \textit{M. tuberculosis} to this class of antibiotics.
References


Table 1: MICs of cell wall inhibitors on WT and Efflux Pump knock-outs of *Mycobacterium tuberculosis*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MICs (µg/ml)</th>
<th>WT</th>
<th>KO1</th>
<th>KO5</th>
<th>KO6</th>
<th>KO7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td></td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>Ampicillin</td>
<td></td>
<td>16</td>
<td>4 to 8</td>
<td>8</td>
<td>8</td>
<td>4 to 8</td>
</tr>
<tr>
<td>Meropenem</td>
<td></td>
<td>2 to 4</td>
<td>1</td>
<td>1</td>
<td>2 to 4</td>
<td>2</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td></td>
<td>2 to 4</td>
<td>0.5 - 1</td>
<td>0.5 - 1</td>
<td>0.5 - 1</td>
<td>0.5 - 1</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td></td>
<td>2 to 4</td>
<td>0.5 - 1</td>
<td>0.5 - 1</td>
<td>1 to 2</td>
<td>0.5</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>2</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Bacitracin</td>
<td></td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>32</td>
<td>&gt;128</td>
<td>128</td>
</tr>
<tr>
<td>INH</td>
<td></td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
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</tr>
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</table>

Table 1: MICs were determined by the resazurin-based microplate assay. Each reported value is the average of at least 3 independent assays.
Figure 1. Bactericidal effect of β-lactams on *M. tuberculosis* WT and efflux pump knock-out mutants. The three concentrations used cover the range of MICs for the different KO strains in the study (WT, KO1, KO5, KO6, and KO7). The vertical (y) axis represents the number of bacteria surviving under each concentration shown in the horizontal (x) axis after exposure to antibiotics 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.