M.tuberculosis, the causative agent of tuberculosis, resists killing by many antibiotics. This resistance arises partly from the unique mycobacterial long-chain mycolic acids that are covalently bound to the cell wall peptidoglycan by intermediate arabinogalactan polymers, encasing the bacterium in a poorly permeable, hydrophobic shell. Bacteria regulate growth and division using many classes of peptidoglycan-modifying enzymes to remodel the cell wall, including, in M. tuberculosis, the five resuscitation-promoting factors (RPFs; rpfA to -E), which are thought to correspond to peptidoglycan lytic transglycosylases in Gram-negative organisms. Like lytic transglycosylases, RPFs are not essential for in vitro growth. Thus, deletion of all five RPF homologues (H37RvΔrpfACDEB, here RPFnull) in M. tuberculosis has no effect on bacterial viability in broth culture, although on solid agar delayed colony formation has been observed (5). In contrast, the RPFs are essential for growth and persistence in a mouse model of M. tuberculosis infection (5). Since cell wall growth and division can apparently proceed in vitro in the absence of RPFs, it remains unclear what their essential role is in vivo.

Homology studies, demonstrated enzymatic activity (8), and interactions with other cell wall-modifying enzymes (3) all suggest that RPFs can function to modify peptidoglycan. Because of the covalent interaction between the peptidoglycan and the mycolic acids, alterations to peptidoglycan may affect the outer membrane, the integrity of which contributes to hallmark mycobacterial characteristics, including clumping of cells in culture, impermeability, and broad antibiotic tolerance. RPFs may therefore contribute to these characteristics.

In order to elucidate further roles of the RPFs in M. tuberculosis, we performed a high-throughput chemical screen of 26,000 compounds to identify molecules that preferentially inhibit growth of an M. tuberculosis mutant lacking RPFs over wild-type bacilli and found that the mutant has enhanced sensitivity to the β-lactam class of antibiotics. By monitoring β-lactam diffusion across the mycobacterial outer membrane, we found that the RPFs are required to maintain the outer membrane integrity, as their deletion results in an increase in outer membrane permeability.

Myobacterial resuscitation-promoting factors (RPFs) have been of great interest since the discovery that they promote the growth of nonculturable Mycobacterium tuberculosis cells. Yet, their precise role in mycobacterial survival and infection has remained elusive. We performed a chemical screen to identify molecules that show preferential killing of a Mycobacterium tuberculosis mutant lacking RPFs over wild-type bacilli and found that the mutant has enhanced sensitivity to the β-lactam class of antibiotics. By monitoring β-lactam diffusion across the mycobacterial outer membrane, we found that the RPFs are required to maintain the outer membrane integrity, as their deletion results in an increase in outer membrane permeability.

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TABLE 1 Additional hits from RPF\textsuperscript{null} screen

\textsuperscript{a} ChemBank is a public cheminformatics resource available at http://chembank.broad.harvard.edu/ (7).
RPF<sup>null</sup> verified that observed effects on OD<sub>600</sub> arose from bacterial activity (Fig. 1).

To determine which RPFs contributed to RPF<sup>null</sup>'s increased β-lactam sensitivity, we measured the cefotaxime IC<sub>50</sub> against a range of mutants lacking one or more RPFs (Table 2). No individual RPF gene was singlehandedly responsible for RPF<sup>WT</sup>-level cefotaxime resistance. The biggest increases in sensitivity came with the loss of <i>rpfA</i> and <i>rpfB</i>. In general, with the progressive loss of RPFs, cefotaxime sensitivity gradually increased. The additive effects observed suggest functional redundancy of the RPFs. Restoration of <i>rpfC</i>, <i>rpfD</i>, and <i>rpfE</i> by integration of a plasmid carrying <i>attB</i> suppressed cefotaxime resistance in RPF<sup>null</sup>.

To test whether increased β-lactam sensitivity in RPF<sup>null</sup> could be due to loss of β-lactamase activity with sequential deletion of the RPFs, we measured the cefotaxime IC<sub>90</sub> of RPF<sup>WT</sup> and RPF<sup>null</sup> in the presence of 1 µM clavulanate, a β-lactamase inhibitor. If differences in β-lactamase activity account for the differences in cephalosporin sensitivity in the two strains, its inhibition by clavulanate should cause the cefotaxime IC<sub>90</sub> values for the RPF<sup>WT</sup> and RPF<sup>null</sup> to converge. In fact, treatment with clavulanate did not cause this convergence; instead, a small divergence was observed (Table 2), thus demonstrating that loss of β-lactamase activity does not account for the increased β-lactam sensitivity in RPF<sup>null</sup>. Since RPF<sup>null</sup> is also hypersensitive to SDS and macrolide antibiotics relative to RPF<sup>WT</sup> (5), we hypothesized that it may have a general permeability defect underlying both these phenotypes as well as cephalosporin sensitivity. To test this, using previously reported methods (9), we measured the relative rates of cefamandole hydrolysis by intact RPFWT and RPF<sup>null</sup> cells (Fig. 2). When these rates are normalized to their respective rate of hydrolysis in lysates, they provide a comparison of the relative rates of diffusion across the intact outer membrane and of their relative permeabilities (provided that the concentration of cefamandole internal to the permeability barrier is below the order of the Km<sub>l</sub>). In highly permeable cells, intracellular hydrolysis of cefamandole is assumed to be rate determining, while in poorly permeable cells, diffusion through the permeability barrier is assumed to be rate limiting.

We calculated a <i>v<sub>intact</sub>/v<sub>lysed</sub></i> ratio of 1.09 ± 0.04 for RPF<sup>null</sup>, compared to an RPF<sup>WT</sup> <i>v<sub>intact</sub>/v<sub>lysed</sub></i> ratio of 0.07 ± 0.09. We also found that the loss of cefamandole sensitivity observed with the
restoration of rpfC, rpfD, and rpfE in RPFnull was reflected in a correspondingly lower $v_{\text{intact}}/v_{\text{lysed}}$ ratio of 0.19 ± 0.04, indicative of decreased permeability. These results show that RPFnull has increased permeability to cefamandole.

We have found that the deletion of RPFs in the M. tuberculosis H37Rv strain results in increased sensitivity to cephalosporins because of a resulting increased permeability in its outer membrane. While this work provides a clear demonstration of the change in permeability with respect to cephalosporins, it is possible that the reported sensitivity of RPFnull to other small molecules, including SDS (5), the macrolide class of antibiotics, vancomycin, and rifampin (6), may also be due to this same permeability defect. This finding suggests that changes in mycobacterial cell physiology at the peptidoglycan level may have far-reaching effects on the essential mycolic acid permeability barrier and that such alterations may affect the ability of the mutant bacilli to survive in vivo. Specifically, loss of particular cell wall-modifying enzymes may decouple the rates of peptidoglycan and outer membrane synthesis, as suggested in a model by Heidrich et al. (2), thus causing defects in the outer membrane that permit a wide range of molecules to diffuse in and out of the cell.

Our work shows that genetic ablation of RPF activity can potentiate a number of small molecules against mycobacteria, including known antibiotics presently unused in therapy. The use of RPF inhibitors should have similar effects. Recently, Demina et al. (1) have identified several small molecules with promising RPF-inhibitory activity, while more generally bulgecin is a known inhibitor of lytic transglycosylases. β-Lactams are among the most successful antibiotic classes, and the use of the β-lactamase inhibitor clavulanate to potentiate them against mycobacteria has recently been proposed (4). This work provides a mechanistically orthogonal approach to potentiating β-lactam antibiotics and other antibiotics.

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REFERENCES