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Resuscitation promoting factors in bacterial population dynamics during TB infection

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***Mycobacterium tuberculosis* has five resuscitation promoting factors (Rpfs) which have been implicated in virulence and resuscitation from dormancy, possibly through cleavage of the β-1,4 glycosidic bond in peptidoglycan. Several possibilities exist for the role of these factors in influencing bacterial population dynamics, during active and latent tuberculosis infection, by the stimulation of bacterial growth through re-modeling of peptidoglycan. As such, the Rpfs may represent an interesting new class of cell wall targets for tuberculosis drug discovery.**

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Introduction

The success of *Mycobacterium tuberculosis* is inextricably linked to its ability to cause a spectrum of disease which ranges from subclinical, asymptomatic infection to chronic, progressive granulomatous disease. One-third of the world's population is estimated to be infected with the tubercle bacillus, the majority of which develop latent tuberculosis infection (LTBI [1]). Latently infected individuals have a finite risk of developing clinically active disease, with the risk being markedly increased by HIV co-infection [2]. Given the burden of future disease presented by the enormous reservoir of latently infected individuals, a multipronged approach that targets both active disease and LTBI is required to achieve the goal of eradicating tuberculosis (TB). The absence of culturable bacilli suggests a plausible link between LTBI and bacterial dormancy – 'a reversible state of low metabolic activity in which cells can persist for extended periods without division' [3] – and consequently, with bacterial factors associated with culturability. In this article, we review what is known about bacterial resuscitation promoting factors (Rpfs), and their proposed role in the physiology of *M. tuberculosis*.

Rpfs in bacterial growth and culturability

Rpf is an essential, secreted protein from *Micrococcus luteus* that increases the culturability (*i.e.* colony-forming ability) of dormant *Mi. luteus* cells, shortens the lag phase of cultures and allows for growth under suboptimal conditions [4]. Homologues of *Mi. luteus rpf* have been identified in other actinobacteria and *Firmicute* species, but, unlike *Mi. luteus* which encodes a single *rpf* gene [5], these organisms possess multiple *rpf* homologues (reviewed in [6]). *M. tuberculosis* has five such genes, designated as *rpfA-E* (*Rv0867c*, *Rv1009*, *Rv1884c*, *Rv2389c*, and *Rv2450c*) [7], which have been the subject of detailed investigation [6,8–18]. The key findings emanating from these and other studies on Rpf structure and function are summarized briefly below. For further information, the reader is referred to several articles in which this topic is reviewed in greater detail [3,6,19–22].

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Significant progress has been made in understanding the function of Rpf although detailed knowledge of the catalytic mechanism of this lytic enzyme is still lacking. In a crucial early insight, modeling of the conserved Rpf domain suggested that it adopts a fold similar to that of *c*-type lysozymes (reviewed in [6]). This suggestion was later confirmed by the NMR structure of the catalytic domain of RpfB from *M. tuberculosis*, which shares high similarity to soluble lytic transglyosylases (SLTs, reviewed in [6]). The crystal structure of a fragment of RpfB further revealed that the Rpf domain adopts a mini-lysozyme-like fold that is similar to *g*-type lysozymes and SLTs [15]. The associated G5 domain in RpfB has a novel super-secondary structure that confers adhesive properties on RpfB, consistent with the cell wall association of this protein (reviewed in [6]).

The SLT-like structure of the Rpf domain, combined with biochemical evidence demonstrating that Rpf from *Mi. luteus* cleaves a synthetic tri-*N*-acetylglucoseamine cell wall mimetic substrate and other gram positive cell-wall-like substrates [23], has led to the hypothesis that Rpfs cleave the β-1,4-glycosidic bond in peptidoglycan (PG [6,15,23]). While no direct biochemical evidence exists to support this hypothesis, our current understanding of the role of Rpfs in bacterial physiology is predicated firmly on the notion that these proteins are, indeed, associated with remodeling of the PG component of the cell wall through lytic activity. However, a key unresolved question in this regard is whether these enzymes function as lysozymes to generate a hydrolysis product, or rather, as SLTs, to generate a 1,6-anhydromuramyl product, which could serve in bacterial signaling [24]. SLTs cleave glycosidic bonds in PG to enable new PG units to be inserted during growth and to remodel the cell wall for insertion of protein secretary apparatus or flagella [25]. Therefore, in addition to stimulating resuscitation of non-culturable organisms, Rpfs may play an important role in essential growth processes. Consistent with this notion is the finding that *M. tuberculosis* RpfB and RpfE interact with an essential cell wall endopeptidase, RipA, and that RpfB synergizes with RipA in PG degradation [8,17]. Furthermore, both RipA and RpfB co-localize to the septa of dividing cells, suggesting a key role for these enzymes in cell division [8]. Depletion of RipA in *Mycobacterium smegmatis* results in growth defects due to incomplete cleavage of septa and the formation of bacterial chains [17]. Recent data suggest that the activity of the RipA–RpfB complex is negatively regulated by the *ponA*-encoding penicillin binding protein 1 (PBP1) [26]. PBP1 is a bifunctional protein that has both transglycosylase and transpeptidase domains and localizes to the cell poles and septa [26]. Together, these findings confirm that the RipA–RpfB complex is important for cell division and that its activity is regulated through protein interaction with PBP1, and possibly other proteins.

The ability of Rpf from *Mi. luteus* to enhance bacterial culturability, from an extracellular location, suggests that muralytic cleavage of PG is required for resuscitation from a dormant or 'non-culturable' state (reviewed in [6]). The fact that *Mi. luteus* Rpf stimulates the growth of aged cultures of *M. tuberculosis* and can dramatically enhance the recovery of tubercle bacilli from murine macrophages, suggests a conserved mechanism of action across species for these secreted proteins (reviewed in [6]). Recombinant forms of RpfA-E are active in enhancing the *in vitro* growth of stationary-phase *Mycobacterium bovis* BCG cells and reduce the apparent lag phase of *M. smegmatis* [14], thus recapitulating the function of the *Mi. luteus* protein. A collective role for the *M. tuberculosis* Rpfs in resuscitation of dormant tubercle bacilli was confirmed by the inability of mutants lacking three or four of the *rpfA*-E genes to resuscitate spontaneously from a non-culturable state [9,11]. The hallmark property of Rpfs to revive viable but non-culturable (VBNC) organisms has allowed these proteins to be used to probe the population of tubercle bacilli present in human, smear-positive sputum [18]. Interestingly, the majority of bacilli in sputum depend on Rpf for growth suggesting that they are in a physiological state analogous to that generated under *in vitro* models of dormancy, a conclusion consistent with the high proportion of lipid inclusion bodies contained in these organisms and the non-replicating persister-type transcriptional profile that they exhibit [27]. In addition to providing direct evidence for the relevance of Rpf to human infection, these findings raise the intriguing question of whether Rpf may, in fact, be required to establish growth in a new host following transmission.

Rpfs in *M. tuberculosis* infection

All five *rpf* homologues are expressed in *M. tuberculosis* in varying abundance, in a growth-phase-dependent fashion *in vitro* and in the murine model of TB infection [12,28]. Together with the observation that *rpfB* and *rpfC* are expressed during TB infection in rabbits and that some *rpf* genes are expressed during human disease, these findings, and those described above, convincingly implicate the Rpfs in disease pathogenesis (reviewed in [6]). Furthermore, recent studies have shown that the *rpf* genes in *M. tuberculosis* are differentially regulated under conditions encountered during infection such as acid stress, nutrient starvation, hypoxia and exposure to whole lung surfactant [28,29]. The expression of *rpfC* is also affected by regulated intramembrane proteolysis of anti-sigma factors and by treatment with phenanthroline, a metal chelator [30].

With the exception of RpfC, all the Rpfs from *M. tuberculosis* are immunogenic and demonstrate significant potential to serve as vaccine candidates (reviewed in [6]). Deletion of individual *rpf* genes has no effect on the viability of *M. tuberculosis in vitro* or on growth in mice, suggesting some level of functional redundancy within this family [10,12]. The collective roles of Rpfs in pathogenesis of *M. tuberculosis*