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REVIEW ARTICLE

Resuscitation Promoting Factors: a Family of Microbial Proteins in Survival and Resuscitation of Dormant Mycobacteria

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Abstract *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is an extraordinarily successful pathogen of humankind. It has been estimated that up to one-third of the world's population is infected with *M. tuberculosis*, and this population is an important reservoir for disease reactivation. Resuscitation promoting factor (Rpf) is a secretory protein, which was first reported in *Micrococcus luteus*. There are five functionally redundant Rpf-like proteins found in *M. tuberculosis*. Rpf promotes the resuscitation of dormant bacilli to yield normal, viable colony forming bacteria. All Rpfs share a conserved domain of about 70 amino acids and possess a lysozyme-like activity. The structural studies of the conserved domain suggest that Rpfs could be considered as a c-type lysozyme and lytic transglycosylases. Recently a novel class of nitrophenylthiocyanates (NPT) inhibitors of the muralytic activity of Rpf were reported which opens a new approach in the study of cell-wall hydrolyzing enzymes. This review describes molecular and structural studies conducted on Rpf proteins, their role in the resuscitation of dormant bacteria, in the reactivation of latent infection and identification of low molecular weight inhibitors of resuscitation promoting factors.

Keywords Rpf · Latency · Dormancy · NPT · Antimicrobial compound

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Introduction

Tuberculosis (TB) is the world's longest running catastrophe, killing more than 200 people every hour and more than 5000 every day. More than 80% of all TB patients live in Sub-Saharan Africa and Asia [1]. Globally, India has more TB cases annually than any other country with one person dying of TB every minute [1]. An estimated 9.27 million new cases (15% amongst HIV positive) occurred in 2007; mostly in Asia (55%) and Africa (31%) with the maximum number of cases in India (2.0 million) [2]. TB was the most common cause of death among people living with HIV/AIDS in 2007, as HIV-positive people are about 20-37 times more likely to develop TB. Resurgence of TB has also been attributed to emergence of drug resistance. There were an estimated 0.5 million cases of multi-drug resistant TB (MDR-TB) in 2007 with the maximum number from India (0.13 million) [2]. In addition, majority of the persons infected with *M. tuberculosis* have clinically latent infection; that is, they are infected and PPD (purified protein derivative)-positive by skin test but do not present the clinical symptoms and are not contagious to others. In approximately 5–10% of latently infected persons, the infection can reactivate and cause active tuberculosis [3]. The majority of the population carrying latent *M. tuberculosis* poses the biggest obstacle to TB control efforts. The problem of latency, reactivation (or resuscitation), rapid emergence of multi drug-resistance, lack of a universally effective vaccine and synergy of TB with HIV has made it increasingly difficult to effectively treat infected individuals and eliminate tuberculosis in humans. Thus, the development of novel anti-tuberculosis therapeutics effective against replicating as well as latent or persistent bacilli is urgently desired.

Primary infection with *M. tuberculosis* leads to clinical disease in only approximately 10% of the cases. In the rest

of them bacteria enters into asymptomatic, non-infectious latent phase. The latent infection can reactivate to clinically symptomatic disease from clinically non-symptomatic state [4–6]. This represents a change in the immune response in which the host is no longer able to contain the infection as compared to latent infection where host response prevents active disease from occurring, and the bacterium avoids elimination. Most infected individuals are able to mount an effective immune response, which limits the proliferation of the bacilli and produces a long-lasting partial immunity. This immunity is directed to prevent new infections (also known as "exogenous reinfection") and reactivation of latent bacilli (also known as "endogenous reactivation") [6]. Although, a number of host factors such as immune status of the host and steroids are known to be responsible for reactivation/resuscitation of the latent bacilli, very little is known about the mycobacterial factors [6]. Recently, one such factor involved in reactivation/resuscitation of dormant bacteria belonging to a family of proteins named as resuscitation promoting factors (Rpfs) has been reported [7–9]. It has lysozyme like enzymatic activity [10]. However, how these proteins act in resuscitation of dormant bacteria or the signaling pathway that reactivates mycobacterial cell from dormant state is unresolved.

Resuscitation promoting factor (Rpf) was first reported in *Micrococcus luteus* as a secretory protein encoded by a single ORF [8]. Rpf from *M. luteus* promoted the resuscitation of dormant bacilli to yield normal, viable colony forming bacteria and was reported as an essential gene [7, 8]. The prototypes of Rpf were also present in other Gram-positive organism with high G+C content, like Mycobacterium spp., Corynebacterium spp. and Streptomyces spp. Homology searches revealed that several Mycobacterium spp. like *M. tuberculosis*, *M. smegmatis*, *M. bovis* (BCG) contain *rpf*-like genes (Table 1). *M. tuberculosis* contains five *rpf* like genes (*rpf* A–E) [9]. The five cognate proteins from *M. tuberculosis* have very similar properties characteristic to those of Rpf from *M. luteus*. They also stimulate bacterial growth at pico-molar concentrations. They too exert their activity from an extra cytoplasmic location, which suggests that they might be involved in intercellular signaling [11]. All the five different Rpf-like proteins of *M. tuberculosis* and *M. bovis* BCG have overlapping biological functions viz. as autocrine signaling molecules, stimulating the growth and multiplication of the cells that produce them. They also have paracrine, density dependant signaling (quorum sensing) function, where cells growing in close proximity may use envelope associated proteins for juxtacrine signaling [9]. Dormant cells require an exogenous source of these proteins for their resuscitation [7, 12, 13].

The role of five Rpf proteins in resuscitation of dormant BCG or *M. tuberculosis* has been experimentally demonstrated [9, 13, 14]. Reactivation of mycobacterium is an important event in the emergence of disease in immune compromised state as well as in latent infection. Rpfs represent attractive targets for development of new drugs preventing resuscitation of dormant *M. tuberculosis*. Such drugs will have a great potential for prophylaxis of latent TB reactivation. Study on this resuscitation promoting factor may help in finding new targets for development of vaccines and drugs, which may efficiently control all forms (latent as well as active) of tuberculosis.

The Rpf Domain

Bacterial genome sequencing projects have uncovered many genes whose products share a 70 amino acids residue segment with *M. luteus* Rpf, known as Rpf domain. This segment of *M. luteus* Rpf is both necessary and sufficient for biological activity, indicating that it corresponds to a

Table 1 Organisms containing *rpf*-like genes (modified and adopted from Ravagnani et al. [37])

Organism	Genome size (Mb)	No. of genes	Genome accession no.
Corynebacterium diphtheriae	2.5	3	NC_002935
Corynebavterium glutamicum	3.3	2	NC_003450
Corynebacterium efficiens	3.1	2	NC_004369
Micrococcus luteus	2.3	1	NC_012803
Mycobacterium avium	4.7	4	NC_002944
Mycobacterium bovis	4.3	5	NC_002945
Mycobacterium leprae	3.3	3	NC_002677
Mycobacterium marinum	6.5	4	NC_004506
Mycobacterium smegmatis	7.0	4	NC_002974
M. tuberculosis H37Rv	4.4	5	NC_000962
Streptomyces coelicolor	8. 7	5	NC_003888
Streptomyces avermitilis	9.0	6	NC_003155

functional protein domain [8]. SignalP [15] and TMHMM [16] predictions suggest that all of the Rpf-like gene products so far uncovered are either secreted, or membrane-associated, with the exception of one instance of an Rpf-like domain within a mycobacteriophage tape measure protein [17]. The Rpf domain also contains two highly conserved cysteine residues. Protein modeling has suggested that they lie in close proximity and may form a disulphide bridge [8].

Structural Analysis of *rpf*-like Genes in *M. tuberculosis*

All Rpf proteins structurally possess a c-type lysozyme-like domain (CAZy family: GH22) and glutamate is conserved in its active site [10]. Besides the Rpf-specific domain, peptidoglycan attachment sites (LysM domains), secretory signal sequences or transmembrane helices characterize some of these proteins. Although some proteins of the Rpf family show growth-stimulating activity on their own producers, their entire function remains unclear.

The SignalP and TMHMM servers were used to determine whether the five Rpf-like proteins of *M. tuberculosis* are also likely to be secreted (Fig. 1). Two of them, RpfA (Rv0867c, 407 aa) and RpfD (Rv2389c, 154 aa) have been predicted to be secreted proteins [18]. RpfA is a comparatively large protein in which the Rpf-like segment is followed by an extensive series (residues 146–320) of proline-alanine rich repeats with the consensus sequence APADLAPP. The RpfB protein (Rv1009, 362 aa) has its Rpf-like domain at the C-terminus. RpfB is probably anchored to the outer surface of the cell membrane by an N-terminal prokaryotic membrane lipoprotein lipid attachment site (Prosite PS00013). Residues 1–117 of RpfB share similarity with the N-terminal Mce domain (PF02470) that is found in all six predicted products of the multiple *mce* operons of *M. tuberculosis*, at least one of which (*mce1*, Rv0169) is involved in entry and survival in macrophages [19]. The status of the remaining two *rpf*-like gene products is less clear. Although RpfC (Rv1884c, 176 aa) is not predicted to contain a *trans*-membrane helix near its N-terminus, a secretory signal sequence was predicted using a neural network (http://www.cbs.dtu.dk/services/SignalP/) trained on Gram-positive signal sequences. RpfE (Rv2450c, 172 aa) has a weakly predicted *trans*-membrane helix close to its N-terminus, whereas the presence of a signal sequence was quite strongly predicted. Thus, while some of these five proteins are secreted, others are anchored in the cytoplasmic membrane [9].

The various *rpf*-like genes are scattered throughout the *M. tuberculosis* genome [20]. The *rpfA* and *rpfE* genes appear to comprise monocistronic operons; *rpf*A is located within a cluster of genes concerned with molybdopteroate biosynthesis. There is a 25 bp overlap between the 3' ends of *rpfB* and *ksgA*, which is predicted to encode a dimethyadenosine transferase. The *rpfD* gene lies downstream from a gene of unknown function. It is located between *hemN* and *nirA* encoding proteins involved in coproporphyrinogen III decarboxylation and nitrate reduction respectively. *rpfC* is the third gene in seven-gene operons containing a mycolyltransferase (*fbpB*) upstream and a probable dehydrogenase (Rv1882c), lipoprotein (Rv1881c) and cytochrome P450 (Rv1880c) downstream. The broadly differing contexts of the five *rpf*-like genes give no clear evidence for a common biological function and suggest that five Rpf proteins may act through distinct mechanism on or pathways [9].

Biological Significance of Rpf-like Genes in Mycobacteria

The function of Rpf proteins was assayed by incorporating them into the growth medium of *M. luteus* and *M. smegmatis* [9]. It was shown that when these fast growing organisms are inoculated at low cell density into a minimal medium, their apparent lag phase (time to detectable turbidity) is reduced in response to Rpf addition

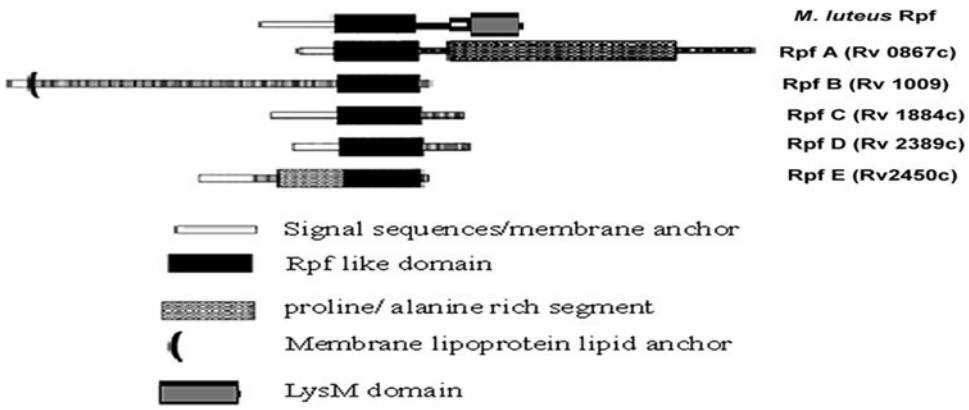

Fig. 1 Domain structure of *M. luteus* Rpf and the five Rpf-like genes of *M. tuberculosis* (Modified and adopted from Mukamolova et al. [9])

[9]. All four *rpf* A, C, D and E of *M. tuberculosis* also reduced the apparent lag phase of *M. luteus.* RpfA and RpfC were found to be the most potent. The former showed highest activity at fM (femtomolar) concentration whiles the latter at picomolar concentration. Similarly, all four proteins were active in reducing the apparent lag phase of *M. smegmatis*. At optimally active concentrations, all four proteins reduced the apparent lag phase to the same extent, with RpfA, RpfC and RpfE being maximally active at subpicomolar concentrations [9]. These profiles indicate that though there is an optimal concentration range for Rpf activity, above and below which there is reduced activity or no activity at all. Freshly purified samples were always used for the experiments, because these proteins lose biological activity during storage. Nevertheless, the possibility that differences in the proportion of biologically active molecules in different protein samples could account, at least in part, for the different potencies of the four proteins can not be ruled out.

In Vivo Role of Rpfs in Reactivation of Tuberculosis

Although latency and reactivation are central to pathogenesis of *M. tuberculosis*, many details of the reactivation process remain obscure at the cellular and molecular levels. The *M. tuberculosis* family of *rpf*-like genes has been suspected to play a role in regulating reactivation, based in part on in vitro growth promoting effects of the Rpf-like proteins on stationary phase bacilli [9]. The control of such a complex process is likely to be dependent upon multiple bacillary factors, as well as numerous host factors (tumor necrosis factor alpha [TNF-α], gamma interferon, NOS, interleukin-12 etc.). It was observed that there was significant effect on the kinetics of reactivation in mouse model when *rv*1009 gene was deleted from the *M. tuberculosis* chromosome. This was the first report of an *M. tuberculosis* mutant that exhibited a specific defect in the reactivation in murine model of infection. However, the growth and persistence in mice remained unaffected. The reactivation-deficient phenotype of the ΔRv1009 mutant provides a unique opportunity to characterize host and bacterial responses during reactivation [21]. The individual *rpf* deletion mutants had no attenuation of growth compared to wild type, in the organs of C57BL/6 mice through 16 weeks post infection. The immunopathology elicited by the *M. tuberculosis rpf* deletion mutants was also indistinguishable from that observed in tissues of wild-type-infected mice. Results of in vitro studies showed that the expression kinetics of *M. tuberculosis rpf* genes fall into distinct patterns that overlap to various degrees and these genes are expressed by the bacilli in extended-stationary-phase cultures. In vivo studies revealed that all five *rpf* genes were expressed in the lungs of mice acutely infected with *M. tuberculosis* [22]. The lack of a phenotype regarding in vitro or in vivo growth in acute and chronic persistent pulmonary infections of mice suggested that the function of the various *M. tuberculosis* Rpfs might be entirely or partially redundant. Hence, loss of a specific function because of disruption of a specific *rpf*-like gene may be compensated by continued expression of one or more of the remaining homologues. Analysis of the in vitro expression of these genes from early exponential phase through stationary phase revealed that the expression patterns of the five homologues were overlapping. *M. tuberculosis* possesses five *rpf* homologues, *rpfA-E*, all of which were expressed in vitro and in mice [22]. Expression of some of these genes has also been observed in human TB infection [23, 24]. Differential relative expression of all the five *rpf*-like genes during different stages of growth and physiological stress conditions revealed that during early resuscitation, all *rpf* genes were expressed with maximal expression ratio of *rpf*A and *rpf*D. *rpf*C was consistently expressed during all stages of growth and nutrient starvation. Acid stress induced higher relative expression of *rpf*D and *rpf*E and hypoxia of *rpf*C and *rpf*E, respectively [25]. These results therefore provide further evidence for the *rpf* genes performing distinct roles during cell growth and cell survival in different physiological stress and are consistent with the *rpf*-like genes being differentially regulated [25].

Individually, the *rpfA-E* genes are dispensable for growth of *M. tuberculosis* in vitro and in vivo [22, 26] suggesting functional redundancy. However, more recent evidence suggest some degree of functional specialization within this gene family. Tufariello et al., [21] found that an *rpf*B-defective mutant of *M. tuberculosis* Erdman displayed delayed kinetics of reactivation in a mouse model of dormancy. Downing et al. [27] found that two mutants of *M. tuberculosis* H37Rv lacking three *rpf*-like genes in different combinations were impaired for resuscitation from a 'non-culturable' state and were differentially attenuated for growth in mice.

Rpf proteins are known to be peptidoglycan glycosidases, the mechanism and role of Rpf in mediating reactivation remains unclear. Hett et al. [28] identified a potential interacting partner of RpfB using yeast two-hybrid system and reported the interaction between RpfB and a putative mycobacterial endopeptidase designated as Rpf-interacting protein A (RipA). RipA also interacts with RpfE but not with RpfA, RpfC or RpfD, suggesting that these Rpfs may act via distinct mechanisms and/or on different substrates. This interaction was confirmed in vitro and in vivo by co-precipitation assays [28]. RipA is a secreted, cell-associated protein, found in the same cellular compartment as RpfB. Both RipA and RpfB co-localize

within the septa of actively dividing bacteria. RipA is capable of digesting cell wall and is a peptidoglycan hydrolase. The interaction between these two peptidoglycan hydrolases at the septum suggests a role for the complex in cell division, possibly during reactivation. Mycobacteria, like all peptidoglycan-containing bacteria, must extend and cleave the surrounding structurally rigid layer of peptidoglycan to grow and divide. Hett et al. [29] demonstrated that RipA is essential for normal cell division in *M. smegmatis*, with its depletion resulting in long, branched filaments and increased susceptibility to a specific cell wall targeting antibiotic. Furthermore, RipA cleaves peptidoglycan and synergizes with RpfB. These data support a model where RipA is required in the final stage of cell division, when daughter cells are separated and has peptidoglycan hydrolytic activity that may be modulated by RpfB under certain conditions. Combination of RpfB and RipA resulted in enhanced hydrolysis of peptidoglycan in an in vitro assay, suggesting protein–protein interactions as one of the potential mechanism of regulation [29].

To further investigate the individual and collective roles of the Rpfs in *M. tuberculosis*, Kana et al. [30] constructed three quadruple mutants and a quintuple mutant of H37Rv lacking all the five genes. They demonstrated that although the *rpf*-like genes are required for virulence and resuscitation from dormancy, the entire *rpf*A-E family is dispensable for growth of this organism in vitro [30]. The five homologs of *rpf*-like genes in *M. tuberculosis* has presented major challenges for dissecting out the role of the individual genes and their encoded proteins in growth and culturability, their relationship to one another, and the mechanisms of regulation of their expression and activity. The results demonstrate that each of the *rpf*A-E genes is individually dispensable for growth in vitro and in vivo [22, 26, 30]. However, the two triple mutants ΔACB and ΔACD were defective for resuscitation in vitro and were significantly yet differentially attenuated for growth in vivo [27, 30]. The dispensability of RpfA-E for the growth of *M. tuberculosis* was rather surprising because of inhibitory effects of affinity-purified anti-Rpf antibodies on growth of avirulent Academia strain of *M. tuberculosis* and *M. bovis* BCG in vitro [9]. Deletion of the individual *rpf*B–E genes was previously shown to be accompanied by a modest upregulation of some or all of the remaining *rpf*-like genes [26]. However, these slight effects were reversed upon deletion of further *rpf*-like genes to a point at which the single *rpf*B, *rpf*D or *rpf*E gene remaining in the quadruple mutants was expressed at a level 1.3–1.8 fold lower than in the wild-type strain. These observations suggest that the remaining *rpf*-like genes do not compensate for the loss of the other genes by transcriptional upregulation and argue against a significant degree of regulatory cross talk within this gene family. Instead, the available data suggest that, *rpf*-like genes are likely to be regulated by other, distinct mechanisms. In this respect, it is worth noting that *rpfA* has been shown to be subject to regulation by the cAMP receptor protein [31], whereas *rpfC* is positively regulated by the alternate sigma factor, SigD [32] and the site two protease homologue, Rv2869c [33]. A key in vitro phenotype associated with progressive *rpf*-like gene loss in *M. tuberculosis* is the inability to resuscitate spontaneously from a 'non-culturable' state. Like their progenitor triple mutant [27], both quadruple mutants assessed in this model displayed this phenotype. Significantly, the resuscitation defects were partly reversed by genetic complementation and/or by the addition of culture filtrate (an exogenous source of Rpfs). Therefore, the failure of the multiple mutants to resuscitate cannot simply be attributed to poor survival of these strains in the Sauton's medium/sealed flask starvation model, but is attributable, at least in part, to a deficiency in Rpf function. Progressive *rpf*-like gene loss also differentially affected the colony-forming ability of *M. tuberculosis* on agar-solidified media. Whereas *rpfB* or *rpfE* alone was sufficient to support a normal rate of colony formation, the mutant retaining only *rpfD* and its quintuple mutant derivative were impaired in this regard. Although the precise reason(s) why mutant cells grown on agar plates showed delayed colony formation are not known, however, the complementation of mutant strain with *rpf*E or *rpf*B resulted in correction of delayed colony formation phenotype [30]. In *M. luteus*, which contains a single essential *rpf* gene, Rpf function is vital for survival under conditions that are inappropriate for bacterial growth, or when cells are exposed to stresses such as nutrient starvation [34]. In *M. tuberculosis*, which contains five non-essential *rpf*-like genes, the phenotype observed in vitro (delayed colony formation when transferred from a liquid to a solid medium) is less pronounced, although evident for mutant cells even in the absence of any stress. However, the greater challenge posed by prolonged starvation in stationary phase in vitro resulted in failure to grow on the plates for both the wild type and the mutant strains. Significantly, the same mutants that displayed delayed colony formation on 7H11 agar were also most sensitive to SDS. Together, the data therefore suggest that Rpf deficiency results in a cell wall defect that renders *M. tuberculosis* hypersensitive to stresses that affect the cell envelope, with the effect being most prominent in cells deficient in RpfB and RpfE. The ability of individual *rpf*-like genes to complement the delayed colony formation phenotype of the quintuple mutant provided a direct means of differentiating function and/or potency within the RpfA-E family. Importantly, the impression of a 'functional hierarchy' within this family inferred from the in vitro data was further supported by the in vivo data demonstrating that loss of *rpfD* from the

double mutant, ΔAC, had a lesser attenuating effect than loss of *rpfB* on growth in mouse lung [27] and loss of *rpfD* from the triple mutant, ΔACB, had a less pronounced effect than loss of *rpfE* on persistence [30]. These observations therefore suggest that RpfB and RpfE grade above RpfD and RpfC in the functional hierarchy. It is exciting to note that the Rpfs that rank highest in this hierarchy RpfB and RpfE correspond to the two Rpfs shown to interact with the partnering peptidoglycan hydrolase, RipA [28]. The quadruple mutants, ΔACBD and ΔACBE, were severely impaired for growth and persistence in mice. The data however suggested that progressive *rpf*-like gene loss was accompanied by progressive attenuation for growth in vivo, with the loss of *rpfE* or *rpfB* being more attenuating than loss of *rpfD* [27]. However, attempts to complement the in vivo phenotype of ΔACBE yielded equivocal results: during early infection, a partial restoration of virulence was observed, but the effect was lost at later stages.

In conclusion, the mutant studies by Kana et al. [30] described the collective dispensability of *rpfA-E* for growth of *M. tuberculosis* in broth culture, and have suggested a functional hierarchy within this multi-gene family. The fact that some Rpfs interact with other proteins in the cell to form protein complexes that may cleave distinct forms of peptidoglycan [28] further adds to the complexity of Rpf function and regulation. However, the collection of mutant strains reported in this and earlier studies [26, 27] have provided an important resource for future biochemical, microbiological and physiological studies on this enthralling family of proteins.

Future Perspective of Rpf Proteins as a Drug Targets

The importance of the Rpf proteins in the resuscitation of dormant (''non-culturable'') *M. tuberculosis* cells in vitro and for growth of viable cells in vivo was confirmed in the study of the Rpf knockout mutants [27, 30]. Despite the fact that these mutants were defective for reactivation from chronic tuberculosis [27, 30, 35] the possible significance of Rpfs in establishing and maintenance of latent tuberculosis remains yet to be clarified. Rpfs represent attractive targets for development of new drugs preventing resuscitation of dormant *M. tuberculosis*. Such drugs will have a great potential for prophylaxis of latent TB reactivation, provoked by the application of anti-TNF antibodies therapy [36]. Recent findings suggest that Rpfs are probably involved in the remodeling of bacterial cell envelope [8, 37]. According to NMR [38] and X-ray diffraction [39] studies of the conserved domain of Rpf, it is structurally close to the c-type lysozyme. However, functionally it is more similar to lytic transglycosylases [37]. Several experimental results confirmed that Rpf possesses cell wall hydrolysing activity. Moreover, a correlation between this enzymatic activity and the resuscitation activity of Rpf has been found [40]. Demina et al. reported several nitrophenylthiocyanates (NPT) with anti-Rpf activity. They have illustrated a novel class of 2-nitrophenylthiocyanates (NPT) compounds that inhibit muralytic activity of Rpfs with IC_{50} 1–7 μg/ml [41]. These low molecular weight compounds interact with the internal regions of the Rpf molecule and suppressed resuscitation of dormant cells of *M. smegmatis* at 1 μg/ml or delayed resuscitation of dormant *M. tuberculosis.* Therefore, a novel class of NPT inhibitors of the muralytic activity of Rpf opens a new way in the study of cell-wall hydrolyzing enzymes against mycobacteria. This explores a new search of antimicrobial compounds for the prevention of latent tuberculosis.

In the majority of the cases, *Mycobacterium tuberculosis* upon infection in human host enters into latent infection. Where latent bacilli reside and how and why it reactivates under immunocompromised conditions is still unclear. The resuscitation of dormant bacilli by resuscitation promoting factors has opened a new era in understanding how these proteins with lysozyme like activity can modulate the cell wall of dormant mycobacteria and cause peptidoglycan hydrolysis. The understanding of genes expressed in dormant and during transition to reactivation phase will not only help in understanding the pathogenesis of *M. tuberculosis* but also in the identification of new drug and vaccine targets against latent TB. It would be suggested to develop in vivo latency models, which mimic human infection for the screening of compounds active against Rpf proteins in demonstration of mechanism of reactivation and new TB drug active against persistent *M. tuberculosis* with shorter duration of therapy.

Conclusion

Persistence and reactivation play significant role in the pathogenesis of *Mycobacterium tuberculosis*. Only a proportion of persons infected with *M. tuberculosis* develop active tuberculosis while majority of them harbor the bacilli for extended period of time without producing clinical symptoms in form of latent infection. The protein known as resuscitation promoting factor was originally identified in *Micrococcus luteus* as ~16–17 kDa protein that is secreted by actively growing bacteria. The protein in picomolar concentration was able to resuscitate the dormant *M. luteus* or mycobacteria generated by prolonged incubation in spent culture medium [7, 14]. *M. tuberculosis* contains five Rpf homologues, which share sequence as well as functional homology with the *rpf* gene of *M. luteus* in their capacity to resuscitate dormant BCG or *M. tuberculosis* in vitro [9]. The five *rpf*-like genes of *M. tuberculosis* H37Rv are

distributed throughout the chromosome and are designated as *rpf*A (Rv0867c), *rpf*B (Rv1009), *rpf*C (Rv1884c), *rpf*D (Rv2389c), *rpf*E (Rv2450c) (http://genolist.pasteur.fr/tuberculist/). The presence of five *rpf*-like genes in *M. tuberculosis* is intriguing as all the five genes are expressed in vitro and in vivo in the lungs of mice acutely infected with *M. tuberculosis* [9, 22, 26]. Several experimental results confirmed that Rpf possesses cell wall hydrolysing activity but role of all the five *rpf*—like genes in *M. tuberculosis* remains speculative. A low molecular weight class of compounds, 2-nitrophenylthiocyanates (NPT), was investigated with anti-Rpf activity [41]. Inhibitors of Rpf activity open a new approach to attempt the problem of reactivation of tuberculosis.

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