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A product of RpfB and RipA joint enzymatic action promotes the resuscitation of dormant mycobacteria

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Resuscitation-promoting factor proteins (Rpfs) are known to participate in reactivating the dormant forms of actinobacteria. Structural analysis of the Rpf catalytic domain demonstrates its similarity to lysozyme and to lytic transglycosylases – the groups of enzymes that cleave the β-1,4-glycosidic bond between *N*-acetylmuramic acid (MurNAc) and GlcNAc, and concomitantly form a 1,6-anhydro ring at the MurNAc residue. Analysis of the products formed from mycobacterial peptidoglycan hydrolysis reactions containing a mixture of RpfB and resuscitation-promoting factor interacting protein (RipA) allowed us to identify the suggested product of their action – *N*-acetylglucosaminyl-β(1→4)-*N*-glycolyl-1,6-anhydromuramyl-L-alanyl-D-isoglutamate. To identify the role of this resulting product in resuscitation, we used a synthetic 1,6-anhydrodisaccharide-dipeptide, and tested its ability to stimulate resuscitation by using the dormant *Mycobacterium smegmatis* model. It was found that the disaccharide-dipeptide was the minimal structure capable of resuscitating the dormant mycobacterial cells over the concentration range of 9–100 ng·mL^{-1}. The current study therefore provides the first insights into the molecular mechanism of resuscitation from dormancy involving a product of RpfB/RipA-mediated peptidoglycan cleavage.

Introduction

Tuberculosis (TB) is one of the most life-threatening infectious diseases in the world. *Mycobacterium tuberculosis* kills ~ 2 million people each year, and is thought to latently infect one-third of the world's population. One of the most remarkable features of *M. tuberculosis* is its ability to survive for decades in humans, as well as in hypoxic and nutrient-depleted media, although the bacteria do not form spores. A possible response to such stress among nonsporulating bacteria is the development of dormant forms, which are often characterized by the 'nonculturability' (NC) state, even though cells remain viable for a long period of time. NC is an operational term describing the temporal inability to recover colony-forming units by plating them on standard nutrient agar [1]. For reasons that are not fully understood, latent TB infection can transition into the active state at any time. Immunocompetent individuals who are latently infected with *M. tuberculosis* are estimated to have a higher risk of developing active disease, which can occur several years after the primary infection [2–4].

Bacterial proteins of the resuscitation-promoting factor protein (Rpf) family are known to participate in

Abbreviations
anhydroGMDP, *N*-acetylglucosaminyl-β(1→4)-*N*-acetyl-1,6-anhydromuramoyl-L-alanyl-D-isoglutamate; CFU, colony-forming unit; Dap, *meso*-diaminopimelic acid; MS, mass spectrometry; MurNAc, *N*-acetylmuramic acid; NC, nonculturability; PG, peptidoglycan; RipA, resuscitation-promoting factor interacting protein; Rpf, resuscitation-promoting factor protein; TB, tuberculosis.

resuscitating the dormant mycobacteria *in vitro*. *M. tuberculosis* encodes five paralogues of Rpf [2]. Recombinant mycobacterial Rpf can facilitate the growth of freeze-dried *Mycobacterium bovis* BCG [5], and increase the recovery of *M. tuberculosis* from the sputum of the patients with active TB [6]. The importance of the Rpfs for *M. tuberculosis* resuscitation was shown *in vitro* with multiple *rpf* null mutants of *M. tuberculosis* [7]. The role of Rpfs in TB infection establishment was demonstrated by comparing the wild-type strain with the knockout strains in the mouse model of TB infection; *rpfB* gene deletion resulted in the delayed reactivation of chronic TB infection following immune suppression in mice [8]. Triple and quadruple *rpf* mutants [2] showed an inability to resume bacterial growth upon immune suppression in the chronic TB mouse model, which clearly indicates the involvement of Rpfs in resuscitating the dormant bacteria *in vivo* [9].

The conserved domain structure of Rpfs includes a c-type lysozyme-like fold, and the protein was predicted to cleave a glycosidic bond between *N*-acetylmuramic acid (MurNAc) and GlcNAc in the peptidoglycan (PG) from the mycobacterial cell wall [10,11]. The muralytic activity of Rpfs was proven experimentally by their ability to cleave cell wall-like substrates [12–14].

RpfB does not act alone, but in a complex with another extracellular protein, resuscitation-promoting factor interacting protein (RipA) [15–18]. RipA is a 472-residue protein encoded by the *M. tuberculosis* rv1477 gene, and has been identified as a binding partner of RpfB and RpfE. Depletion of the RipA orthologue in *Mycobacterium smegmatis* caused defective septum formation [16]. Both proteins were shown to be colocalized at the septum of the dividing cells [16]. RpfB cleaves the glycoside bonds of the cell wall PG [10], whereas RipA is predicted to be an endopeptidase that cleaves the stem peptide [D-iGlu-*meso*-diaminopimelic acid (Dap)] [17,18] (Fig. 1). Interaction between these cell wall hydrolases results in synergistic hydrolysis of mycobacterial PG [17]. However, the mechanisms underlying their cooperativity remain unclear, as their cooperativity may be associated either with the allosteric activation of one of these proteins, or with their enhanced ability to release free muropeptides by acting on both the glycan and peptide moieties. In addition, the question of how PG hydrolysis is related to the reactivation of the dormant cells remains to be elucidated.

The PG of the dormant bacteria shows unique chemical features in both its polysaccharide chains and peptide crosslinks. For instance, MurNAc is often replaced by *N*-glycolylmuramic acid (substitution of the *N*-acetyl moiety with the *N*-glycolyl residue), resulting in an increase in the number of hydrogen bonds in the PG layers [19]. Similarly, the peptide

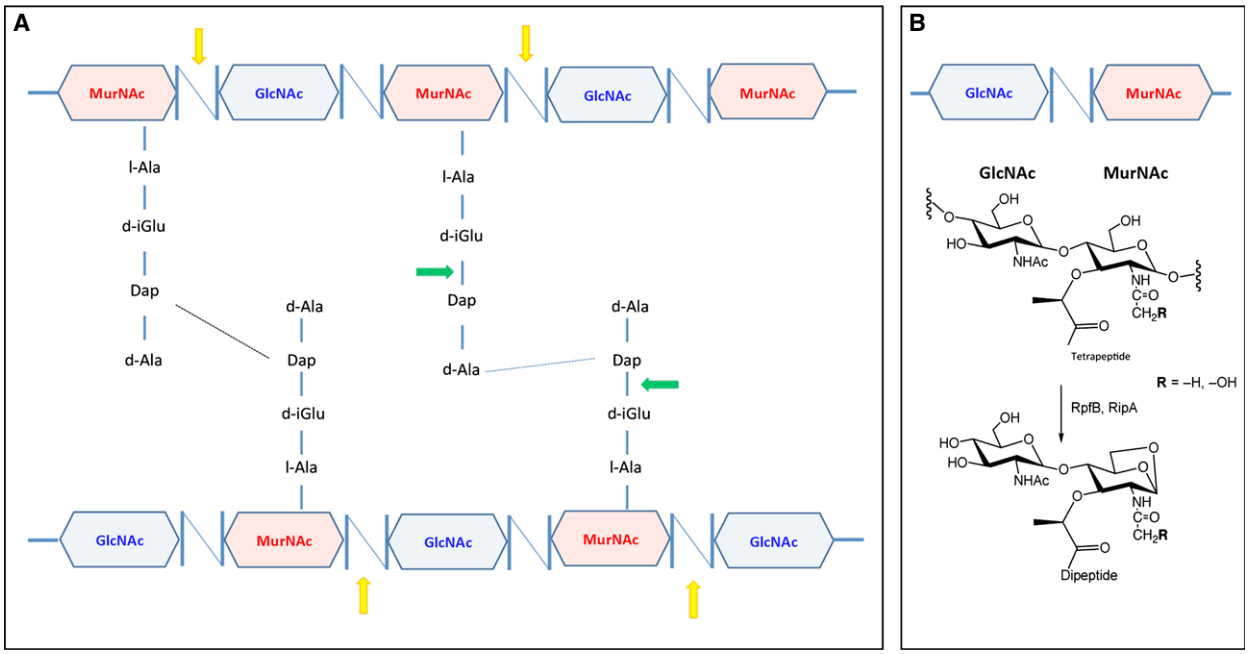

Fig. 1. Mycobacterial PG structure and proposed cleavage sites of Rpfs and RipA. (A) RpfB is predicted to cleave the polysaccharide moiety (yellow arrows), and RipA cleaves the peptide stems (green arrows). (B) Chemical structures of each muropeptide monomer and the presumed product of the hydrolysis reaction.

moieties of PG are heavily crosslinked in the dormant state; for example, up to 80% of peptide chains are crosslinked in mycobacteria [20], whereas the amount of crosslinking in *Escherichia coli* is only ~ 30–50% [21], although both species contain a rather similar amino acid composition (L-Ala, D-Glu, Dap, and D-Ala) in their peptide 'tails'. Remarkably, for most bacteria, the formation of Dap-D-Ala crosslinks is rather typical, whereas, in *M. tuberculosis*, Dap-Dap forms covalent linkages (3,3-crosslinks), particularly in the nutrient depletion-induced or antibiotic-induced dormant state [20,22]. These Dap-Dap bonds are vital for the long-term survival of the pathogen within the host macrophages [23]. This peculiar amino acid arrangement has evolved to confer resistance towards the conventional proteases [20,24]. The recruitment of hydrolytic enzymes during cell division at the septal region and PG remodelling are essential for division into the daughter cells [25,26].

In most Gram-positive bacteria, the septum is formed and then cleaved along a middle line. Together with amidases, the function of the lytic transglycosylases is to split the septum, thereby permitting the separation of the dividing cells [27]. In addition, enzymatic modification of the PG structure through the partial hydrolysis of its crosslinks could be necessary, either for facilitating the diffusion of low molecular weight compounds into the cells, or for an impulse transition for metabolic reactivation. On the other hand, it is also probable that both proteins, acting cooperatively, release low molecular weight secondary messengers (muropeptides) that act as signalling molecules [6,28,29].

In this study, we elucidated the chemical processing events that were associated with PG hydrolysis and subsequent resuscitation. We observed that these events revealed the action of RpfB as a lytic transglycosylase and not as a lysozyme-a, like *N*-acetyl-β-D-muramidase, a finding that was previously unclear, despite the large number of detailed studies on this enzyme [10,30]. Moreover, the combined actions of RpfB and RipA produce the proper molecular entity for the resuscitation and growth stimulation of nonculturable mycobacteria *in vitro*. Our data elucidate the molecular determinants of *M. tuberculosis* resuscitation from the dormant state.

Results

The RpfB and RipA catalytic domains synergistically hydrolyse PG

We performed PG degradation experiments with the catalytic domains of $RpfB_c$ (residues 280–362) and $RipA_c$ (residues 332–472), with the aim of elucidating the mechanism of the previously observed synergistic PG hydrolysis by these two enzymes. This allowed us to eliminate the contribution of the noncatalytic domains to PG degradation synergy. Additionally, RipA structural studies have demonstrated that the noncatalytic domains are responsible for the enzyme's self-inhibition as such, through a regulatory mechanism that requires proteolytic activation [15]. This hypothesis was subsequently confirmed *in vivo* [31].

Cell wall degradation experiments were performed on purified PG from *M. smegmatis* that was labelled with FITC (see Experimental procedures). Both recombinant proteins, $RpfB_c$ and $RipA_c$, alone and in combination, were incubated with the labelled mycobacterial PG. The hydrolysing activity of the proteins was evaluated by estimating the amount of FITC-labelled products released in the supernatant. Fluorescence measurements of supernatant samples at different time points demonstrated a slow increase in the release of the labelled products up to 100 h of incubation (results not shown). The amount of the released products was dependent on the concentration of the proteins added to the reaction mixture (Fig. 2). The results clearly showed that the mixture of $RpfB_c$ and $RipA_c$ gave the highest PG hydrolysis efficiency. It was evident that, at any concentration point (on the abscissa axis), the summation of the particular hydrolysis-dependent fluorescence value (the value of fluorescence units on the ordinate axis) of either protein (either $RpfB_c$ or $RipA_c$) was less than the same effect produced by the mixture of both proteins ($RpfB_c$ + $RipA_c$) at the same concentration (Fig. 2). This result corroborates the fact that PG degradation synergy does not require the noncatalytic domains of the enzymes.

In contrast to the results published by Hett *et al.* [17], we were able to detect hydrolytic activity of *M. tuberculosis* RpfB towards *M. smegmatis* PG. However, the level of this hydrolytic activity was fairly low, especially as compared with the enzymatic activity of RipA and the mixture of the two enzymes (Fig. 2). We have noticed that expression of the recombinant RpfB in relatively low concentrations from the producing strain (*E. coli* with a maximal induction time of ~ 2 h) is crucial for achieving maximal enzymatic activity, and, therefore, for effective PG hydrolysis in the hydrolysis assay and in resuscitation experiments. This methodological approach implies that recombinant RpfB should be obtained at initial concentrations of $< 200\ \mu g \cdot mL^{-1}$. Expression of the protein at higher concentrations leads to aggregation and consequent loss of activity (Fig. 3).

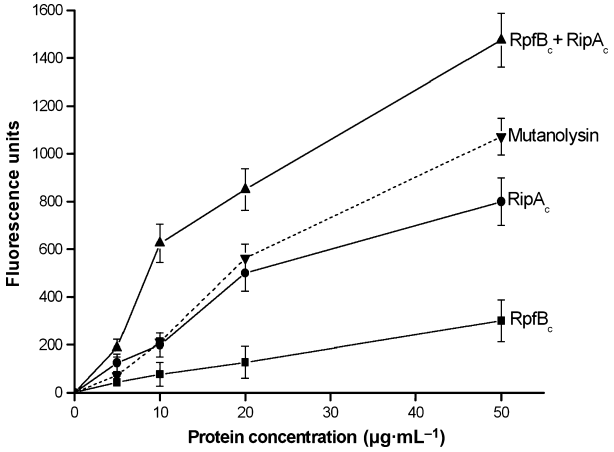

Fig. 2. Enzymatic hydrolysis of mycobacterial PG. FITC-labelled PG from *Mycobacterium smegmatis* was dissolved in reaction buffer (see Experimental procedures). Recombinant $RpfB_c$ and $RipA_c$ were added to the samples at varying concentrations, and were incubated for 96 h at 37 °C. The mixture contained equal amounts of each protein (e.g. 20 μg·mL^{-1} each protein). The level of hydrolysis was assessed by measuring the unbound fluorescent dye in the supernatant after centrifugation (13 000 ***g*** 10 min). For comparison, the hydrolysis curve in the presence of mutanolysin is shown. Each point on the graph represents the fluorescence value corrected for control fluorescence. The error bars represent the standard error of the mean from three repetitions with similar results. A typical result from one independent experiment is shown.

Identification of low molecular weight products of PG synergistic digestion

To identify the product of PG synergistic digestion, we utilized MALDI-TOF mass spectrometry (MS). MS analysis of filtered PG hydrolysates (3-kDa cut-off filter) from different, parallel experiments revealed the presence of an m/z 716.6 peak (Fig. 4). To identify the chemical nature of this product, we obtained fragmentation spectra (MS/MS). Fragmentation pattern analysis allowed us to identify the product of PG hydrolysis in the presence of both proteins, which appeared to be *N*-acetylglucosaminyl-β(1→4)-*N*-acetyl-1,6-anhydromuramoyl-L-alanyl-D-isoglutamate (anhydroGMDP) (Fig. 4B). The fragmentation pattern of the identified product matched (Fig. 4B, top) the fragmentation pattern of the synthesized product (Fig. 4B, bottom). In both cases, the fragments with the following m/z ratios were detected: GlcNAc −244; dipeptide −312; disaccharide fragments −411 and 425; and monosaccharide-dipeptide −513.3 and 497.3. The observed mass differences in the initial compounds could be explained by the fact that natural PG from mycobacteria consists of a large number of glycolyl-modified fragments of muramic acid.

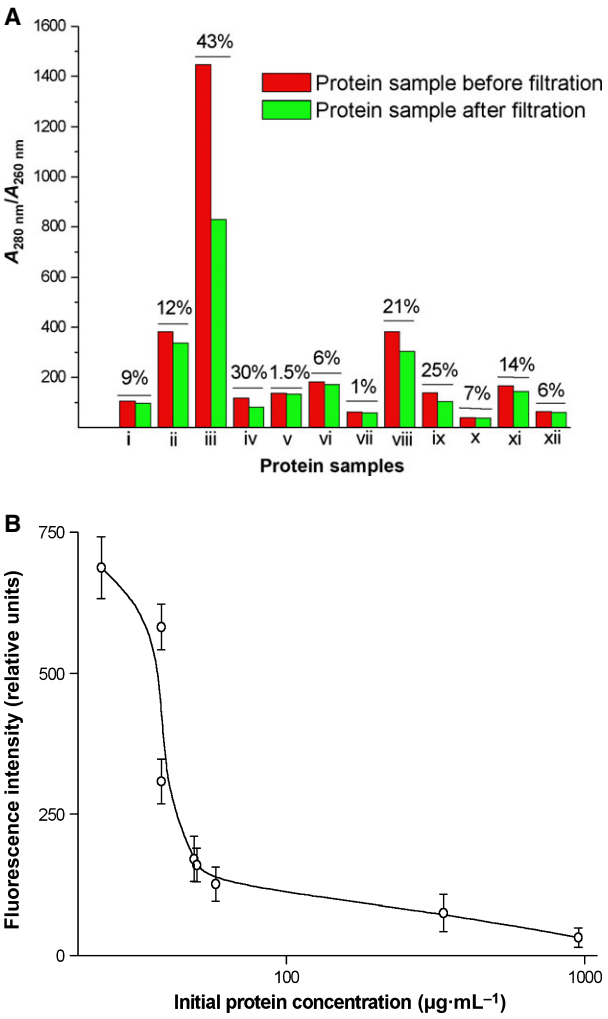

Fig. 3. Expression of the RpfB protein at high concentrations leads to its aggregation and consequent loss of its activity. (A) Concentrated RpfB samples contain large amounts of aggregates. The bars represent different batches (12) of different concentrations of recombinant RpfB. The proteins were filtered through 0.2-μm filters, and protein concentrations before and after filtration were compared. It is clear that the more concentrated samples contained significant amounts of large aggregates. The numbers above the bars indicate protein loss (% of the initial concentration). (B) Concentrated RpfB is less active. Experiments were conducted on an artificial Rpf substrate, MUF-3-NAG [12, 13], and were similar to those depicted in Fig. 2. Equal aliquots of the proteins (15 μg·mL^{-1}) were added to the MUF-3-NAG degradation assay. The initial protein concentration is shown on the *x*-axis.

The same analysis of supernatants obtained from PG that was not exposed to the enzymes indicated no detectable PG hydrolysis products. This confirmed the hypothesis that the release of muropeptides in the supernatant results from the enzymatic action of the two proteins.

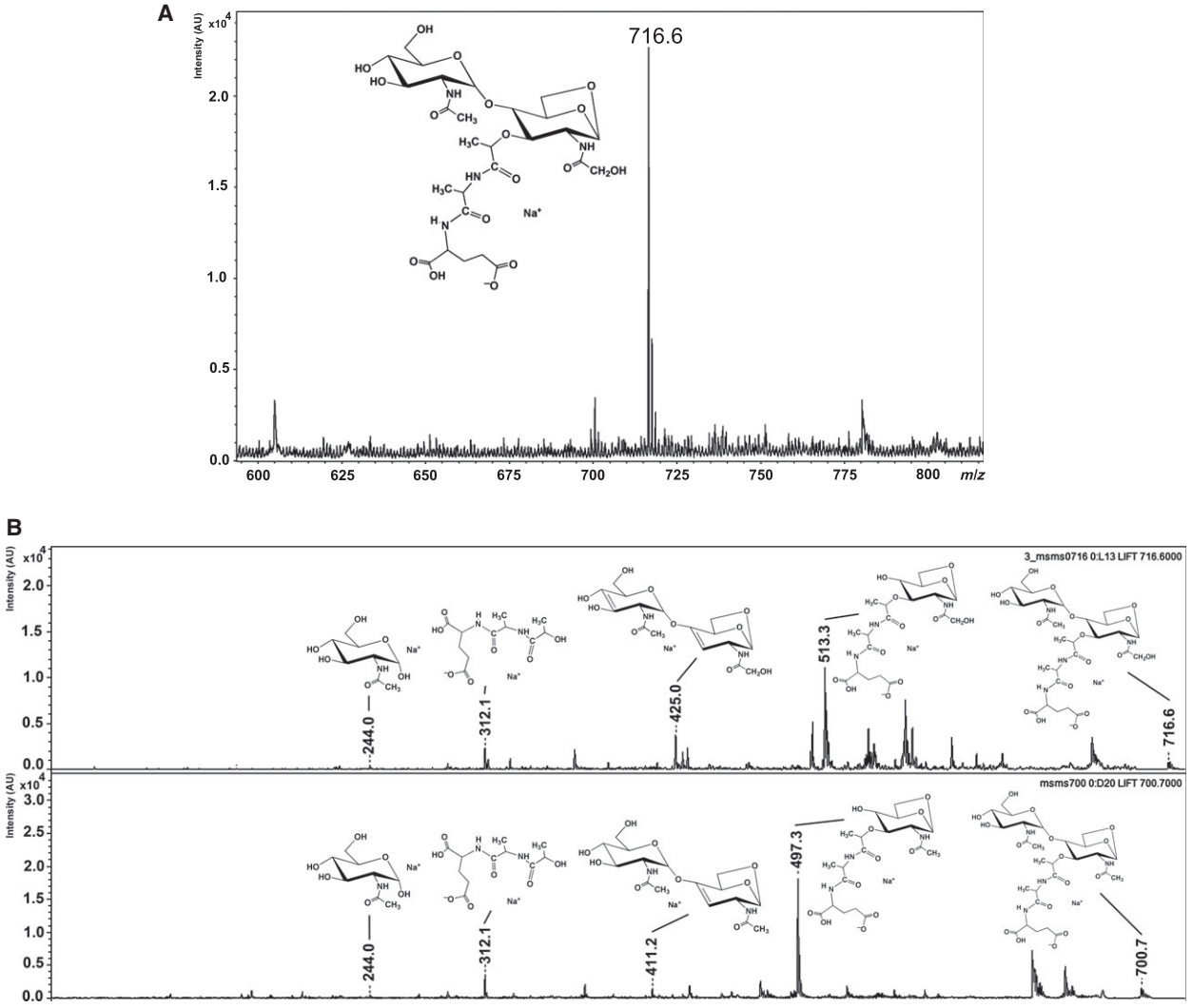

Fig. 4. (A) MS spectra (MALDI) of the supernatant filtered through a 3-kDa filter, obtained after mycobacterial PG (*Mycobacterium smegmatis*) hydrolysis in the presence of $RpfB_c$ + $RipA_c$ (both 10 $\mu g \cdot mL^{-1}$). (B) Fragmentation spectra of the MS peaks, *m*/*z* 716.6 and *m*/*z* 700.7. Top: fragmentation of the peak (*m*/*z* 716.6) resulting from PG digestion by both $RpfB_c$ and $RipA_c$ [*N*-acetylglucosaminyl-β(1→4)-*N*-glycolyl-1,6-anhydromuramyl-L-alanyl-D-isoglutamate]. Bottom: fragmentation of the peak for the synthetic analogue anhydroGMDP (*m*/*z* 700.7). The fragmentation pattern of the identified product matches the fragmentation pattern of the synthesized product. In both cases, fragments with the following *m*/*z* ratios could be detected: GlcNAc −244; dipeptide −312; disaccharide fragments −411 and 425; and monosaccharide-dipeptide −513.3 and 497.3. The observed mass differences in the initial compounds may be explained by the fact that the natural PG from mycobacteria consists of a large number of glycolyl-modified fragments of muramic acid.

Resuscitation activity of $RpfB_c$ and $RipA_c$

We tested the resuscitation activity of both $RpfB_c$ and $RipA_c$, and evaluated their combined effect on bacterial resuscitation. It is worth noting that the effect of RpfB on bacterial resuscitation has previously been studied [32]; however, no evidence has yet been reported on the ability of RipA to resuscitate bacteria. For these experiments we used nonculturable dormant *M. smegmatis* cells that were obtained after prolonged cultivation in K^+-deficient medium [32]. In these experiments, the effectiveness of resuscitation was judged by the appearance of platable cells [estimated by colony-forming unit (CFU) counting] during the incubation of nonculturable cells in fresh liquid medium. Incubation of *M. smegmatis* nonculturable cells in the liquid medium without any additions resulted in the appearance of some platable cells, but only after 160 h of reactivation. The addition of either individual protein ($RpfB_c$ or $RipA_c$) or a mixture of the two to the resuscitation medium significantly stimulated

resuscitation and the growth of the initially dormant bacteria ($RipA_c$ was more effective than $RpfB_c$; $P < 0.05$) (Fig. 5A). In this experiment, the individual enzymes were added at 10 ng·mL^{-1}, a concentration that was experimentally determined from a dose–response curve obtained with the following concentrations: 1, 5, 10, 20 and 50 ng·mL^{-1} (Fig. 5B).

Enhanced resuscitation was observed after 160 h of incubation in the presence of both proteins in comparison with arithmetical addition of the effect (CFU value on the ordinate axis) of each protein ($P < 0.01$). Thus, the initially observed synergy in PG degradation experiments (Fig. 2) was also observed for resuscitation (Fig. 5A). It is worth noting that this result also provides the first experimental evidence of RipA participation in resuscitation.

Synergy of $RpfB_c$ and $RipA_c$ relies on the joint reaction product

To determine whether resuscitation was directly stimulated by the proteins or by the products of their action, we first tested a 3-kDa-filtered enzyme–PG hydrolysate mixture. This sample (100 μg·mL^{-1}) showed a resuscitation activity similar to that obtained for the isolated proteins (data not shown), denoting the existence of the physiologically active low molecular weight compound in the mixture. Next, we tested a synthetic analogue of the muropeptide that was discovered in the digestion mixture.

As in the resuscitation experiments (Fig. 5), we found that addition of synthetic anhydroGMDP to the dormant culture resulted in the multiplication initially of nonculturable cells (Fig. 6). Remarkably, the concentration dependency of resuscitation in the presence of anhydroGMDP revealed a bell-shaped trend, with a wide range of resuscitation activity from 9 to 225 ng·mL^{-1}, and maximal activity in the range of ~ 40–100 ng·mL^{-1} (Fig. 6, inset). However, the maximal number of resuscitated cells in the presence of a particular muropeptide concentration varied from one experiment to another. For example, the resuscitation effect shown in Fig. 6 at 225 ng·mL^{-1} of anhydroGMDP is close to the maximum possible CFU recovered for the dormant culture after resuscitation, according to the inserted graph plot.

It is noteworthy that application of a set of other muropeptides or their fragments, including the monosaccharide-dipeptide (MurNAc-L-Ala-D-isoGlu), amino acids [Ala, Glu(n)], GlcNAc, the disaccharide (GlcNAc-MurNAc), and the dipeptide (L-Ala-D-Glu), did not result in any stimulatory activity in our nonculturable mycobacteria resuscitation assay at the same

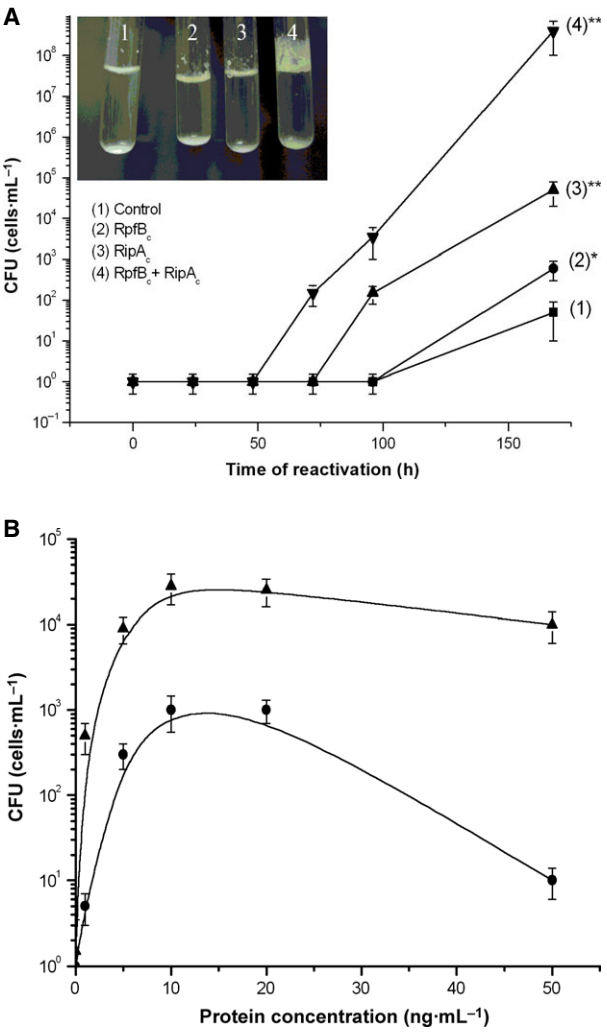

Fig. 5. Resuscitation of the dormant *Mycobacterium smegmatis* nonculturable cells by $RpfB_c$ and $RipA_c$, alone and in combination. Nonculturable cells (initial $D_{600\ nm} = 0.3$) were inoculated in the resuscitation medium, and supplemented with $RpfB_c$ and $RipA_c$, alone and in combination, and agitated at 37 °C. (A) Periodically, samples were removed to count CFUs. ■, control; ●, $RpfB_c$, 10 ng·mL^{-1}, $*P < 0.05$ versus control at 160 h; ▲, $RipA_c$, 10 ng·mL^{-1}, $**P < 0.01$ versus RpfB at 160 h; ▼, $RpfB_c$/$RipA_c$ 10/10 ng·mL^{-1}, $**P < 0.01$ versus $RpfB_c$ + $RipA_c$ at 160 h. Inset: test tubes with the reactivated *M. smegmatis* culture after 160 h of resuscitation. One of five typical experiments is shown. (B) Concentration dependency of the resuscitation effect after 160 h of incubation: ▲, $RipA_c$; ●, $RpfB_c$. The error bars represent the standard error of the mean (three repeats for each time point or protein concentration). Typical results from one independent experiment are shown.

(equimolar) range of concentrations as for anhydroGMDP (data not shown). This finding suggests that the precise chemical nature of the muropeptides generated by the combined action of both $RpfB_c$ and

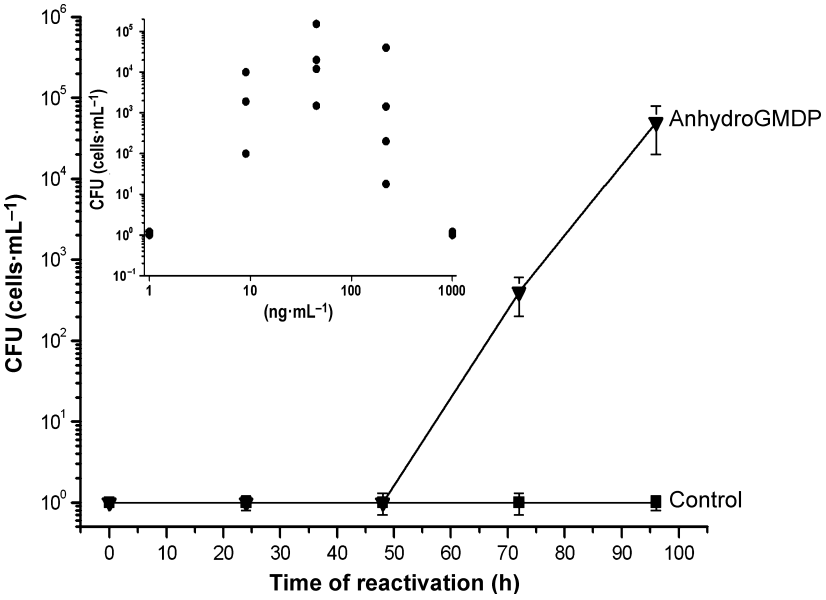

Fig. 6. AnhydroGMDP stimulates the resuscitation of *Mycobacterium smegmatis* nonculturable cells. The resuscitation procedure was performed similarly to the experiment shown in Fig. 5. The concentration of anhydroGMDP was 225 ng·mL^{-1}. The error bars represent the standard error of the mean (three repeats for each time point). A typical result of one independent experiment is shown. Inset: concentration dependency. CFU number (relative error < 30%) at the end of resuscitation (96 hs) versus anhydroGMDP concentration is shown. Each dot represents the result of one independent experiment.

$RipA_c$ is an important molecular determinant in the process of resuscitation of the dormant mycobacteria.

Discussion

Modulation of PG biosynthesis and composition is crucial for normal cellular growth and division, as well as the maintenance of dormancy or resuscitation in mycobacteria. In *M. tuberculosis*, Rpf-mediated PG cleavage seems to be important for resuscitation [3], although this has not yet been verified. The cell division hydrolase RipA was shown to colocalize with RpfB at the bacterial septa [16]; however, the role of RipA in resuscitation from dormancy has not yet been established. In this study, we determined the RpfB/RipA-mediated process of PG degradation in relation to resuscitation from dormancy. We provide experimental evidence that RipA is actually involved in the process of dormant mycobacterial cell resuscitation, aside from its known role in cell division. Additionally, we observed a correlation between RpfB and RipA synergy in PG degradation and in the resuscitation of the dormant mycobacteria.

Analysis of the chemical nature of the products formed as a result of PG hydrolysis in the presence of both enzymes provided several clues to better understand the molecular mechanism of this reaction. In spite of the existence of several structural studies on RpfB [10,11,14], information on its mechanism is still lacking. The RpfB catalytic domain shows structural similarity to lysozyme, an enzyme whose mechanism is well studied [14,33–35]. In hen egg white lysozyme, two residues (Glu35 and Asp52) are critical for enzyme activity: Glu35 acts as a proton donor to the glycosidic bond, and Asp52 acts as a nucleophile [34,35]. In contrast to hen egg white lysozyme, RpfB does not have a residue equivalent to Asp52 from c-type lysozymes; the lone extra acidic residue in the substrate-binding cleft of Rpf is Asp312, which, in turn, exists only in RpfB. Moreover, this aspartate is spatially distant, and is not conserved in the *M. tuberculosis* homologues (data not shown). Consistent with these findings, we show that RpfB acts through a mechanism resembling that of lytic transglycosylases rather than that of lysozymes. Indeed, we have identified the product of the joint action of RpfB and RipA: anhydroGMDP. Similarly to lytic transglycosylases [36], RpfB cleaves the β-1,4-glycosidic bond between MurNAc and GlcNAc, and forms a 1,6-anhydro ring at the MurNAc residue (Fig. 4).

We synthesized this product of RpfB–RipA synergistic action, and tested its activity in resuscitation experiments. Interestingly, we observed that this muropeptide was able to stimulate the resuscitation of nonculturable cells, similarly to RpfB or RipA (Figs 5 and 6). Remarkably, the concentration dependence of its resuscitation effect appeared to be bell-shaped, like the concentration curve of the Rpfs' stimulatory activity *in vitro* [37] and the resuscitation activity of large PG fragments towards nonculturable *M. smegmatis* cells [29]. The nature of this dependence remains unclear, although it could be theoretically explained by the probable inhibition of downstream processes in the reactivation pathways, owing to the presence of excess substrate.

The resuscitation effects observed after anhydroGMDP addition to the dormant cell cultures dem-

onstrate that the synergy exerted by RpfB and RipA in PG hydrolysis is caused by the forming reaction product of the particular chemical nature. Indeed, anhydroGMDP, and not the other muropeptide fragments, directly induced resuscitation (Fig. 6). Muropeptides have been shown to act as germinants for *Bacillus subtilis* spores through activation of the PrkC Ser/Thr kinase [38–40]. This mechanism proceeds through the recognition of muropeptides by the extracellular sensing PASTA domains of PrkC [39,40]. Our results showed resuscitation activity for the disaccharide-oligopeptide, and not for the monosaccharide-oligopeptide (MurNAc-L-alanyl-D-iso-glutamate), and are in accord with the study of Lee *et al.* [41], in which a set of synthetic muropeptides were tested as germinants for *B. subtilis* spores. However, the resuscitation effect of anhydroGMDP in the current study is in apparent contrast to a previous finding [42] showing that several muropeptides with maximal affinity for the PASTA domains of PknB (the mycobacterial homologue of PrkC) had only limited resuscitation effects. This may be attributable to the observed concentration-dependent mode of anhydroGMDP-induced resuscitation (Fig. 6); this finding restricts the concentration range in which resuscitation activity can actually be observed [42].

It is well known that Gram-negative bacteria can efficiently recycle the resulting muropeptides during their life cycle [43]. In contrast, it has been assumed that Gram-positive bacteria do not recycle PG [44], although this concept is currently being challenged for *M. tuberculosis* [45,46]. Therefore, we cannot exclude a possible role of muropeptides as building blocks that are meant to be incorporated in mycobacterial PG during its biosynthesis and reactivation.

Experimental procedures

PG preparation

The procedure is based on *Mycobacteria protocols* [47]. *M. smegmatis* mc^2155 was grown in Sauton's medium (pH 7.0) with agitation (200 r.p.m.) at 37 °C until the culture reached the stationary phase (30–48 h) [32]. Cells were collected by centrifugation (4000 ***g***, for 30 min), washed three times with 0.9% NaCl, and resuspended in breaking buffer [2% w/v Triton X-100 in PBS (0.1 M KH_2PO_4, 0.01 M NaCl, pH 7.4)]. The bacterial suspension was sonicated on ice for 10 cycles (each cycle −1 min). Broken cells were centrifuged at 8000 ***g*** for 15 min, and left overnight in the breaking buffer to extract the remaining soluble material. The insoluble fraction containing cell wall material was centrifuged at 8000 ***g*** and 4 °C. To remove the associated proteins, the pelleted cell wall material was extracted three times with 2% SDS in PBS at 95 °C for 1 h. The treated pellet was successively washed with water, 80% acetone in water, and acetone, and then lyophilized to yield the purified cell walls (rich in mycolic acids, arabinogalactan, and PG).

Mycolic acids were removed by saponification – refluxing with 2% KOH in methanol/toluene solution (1 : 1) for 48 h. The insoluble residue was collected by centrifugation (10 000 ***g*** for 5 min). The pellet was washed repeatedly with excess methanol before lyophilization. To remove arabinogalactan from the arabinogalactan–PG complex, the cell wall sample was incubated in the presence of 0.1 M H_2SO_4 at 37 °C for 5 days. The PG obtained was washed four or five times with water, and lyophilized. The final PG samples were kept at 4 °C.

PG labelling and hydrolysis

The purified PG fraction (2 $mg \cdot mL^{-1}$) was dissolved in 100 mM freshly prepared sodium carbonate buffer (pH 8.5; adjusted with concentrated HCl). FITC labelling was carried out in the dark for 16 h at room temperature with stirring (Sigma, St. Louis, MO, USA; final concentration 1 $mg \cdot mL^{-1}$). Labelled PG was collected by centrifugation (13 000 ***g***, for 10 min), and thoroughly washed until the supernatant lacked any unbound dye. The labelled material was stored at −20 °C in 50 mM NaH_2PO_4 (pH 6.0). To assess the hydrolytic activity of the proteins, the labelled cell wall PG was incubated with $RpfB_{280–362}$ and $RipA_{332–472}$, alone and in combination, for 96 h at 37 °C. The reactions were stopped by heating at 80 °C for 10–20 min. The fluorescence intensity of the collected supernatant was measured at an excitation wavelength of 492 nm and an emission wavelength of 515 nm [17,48].

Recombinant protein isolation and purification

$RpfB_{280–362}$ was purified from 200 mL of an *E. coli* producer strain (kanamycin-resistant), according to a previously published protocol [49]. To ensure maximal resuscitation activity, the initial concentration of the protein was no more than 100–200 $\mu g \cdot mL^{-1}$, and IPTG induction was performed for no longer than 2 h. $RpfB_{280–362}$ was dialysed against 50 mM citric acid/sodium citrate buffer (pH 6.0).

$RipA_{332–472}$ was obtained by use of an *E. coli* producing strain (ampicillin-resistant), and was maintained in the same conditions as $RpfB_{280–362}$ [15,48]. The concentration of the protein was 400–600 $\mu g \cdot mL^{-1}$. The proteins were maintained at 4 °C for 1 month without loss of activity.

MALDI-TOF MS analysis

Sample preparation

The sample (1.5 μL) was mixed with 0.5 μL of 2,5-dihydroxybenzoic acid (Aldrich, Schnelldorf, Germany;

10 mg·mL^{-1} solution in 20% acetonitrile, 0.5% trifluoroacetic acid) on the MALDI target plate. The mixture was air-dried.

Mass spectra

Mass spectra were recorded on the Ultraflextreme BRUKER MALDI-TOF/TOF spectrometer (Germany) equipped with an Nd UV laser in positive reflectron ionization mode. The measurement accuracy was 70 p.p.m. For fragmentation spectra, the tandem mode was used with optimized laser power (100 shots, 100 Hz).

Muropeptide synthesis

AnhydroGMDP (*N*-acetylglucosaminyl-β(1-4)-*N*-glycolyl-1, 6-anhydromuramyl-L-alanyl-D-isoglutamate) was synthesized from GMDP (*N*-acetylglucosaminyl-β(1–4)-*N*-acetylmuramyl-alanyl-D-isoglutamate) according to previously published methods [50–53].

N-acetylglucosaminyl-β(1–4)-MurNAc

This was isolated from the *Micrococcus luteus* biomass according to Hoshino's protocol [51]; the yield was ~ 1% of the disaccharide (~ 1 g from 100 g of wet biomass).

Trifluoracetate-L-alanyl-D-isoglutamate

N-tert-butoxycarbonyl-L-alanyl-D-glutamate benzyl ester was synthesized according to a previously published protocol [52]. The solution of 1.6 g (3.6 mmol) of the protected dipeptide in 5 mL of trifluoroacetic acid was incubated for 30 min at 20 °C. After evaporation, 1.65 g of trifluoracetate dipeptide (trifluoracetate-L-alanyl-D-isoglutamate) as amorphous powder was obtained.

N-acetylglucosaminyl-β(1–4)-*N*-acetylmuramyl-alanyl-D-isoglutamate

N-acetylglucosaminyl-β(1–4)-MurNAc [496.5 mg (1 mmol)] was dissolved in 3 mL of dimethylformamide. *N*,*N*′-disuccinimide carbonate (256 mg) and 120 μL of *N*-methylmorpholine were added to the mixture. The mixture was incubated for 6 h at 20 °C.

Trifluoracetate-L-alanyl-D-isoglutamate (498.15 mg) and 460 μL of *N*-methylmorpholine in 10 mL of 2-picoline were added to the mixture.

The reaction mixture was incubated at 20 °C for 24 h. After evaporation, the residue was dissolved in 2 mL of H_2O, and applied to a Sephadex DEAE A-25 chromatographic column (1.5 × 50 cm) in 0.15 M acetic acid. After lyophilization, 518 mg (73%) of the disaccharide-dipeptide was obtained (97% purity).

AnhydroGMDP

The 1,6-anhydrodisaccharide dipeptide was synthesized according to Chun's method [53]. A saturated solution of *N*-acetylglucosaminyl-β(1–4)-*N*-acetylmuramyl-alanyl-D-isoglutamate in absolute acetonitrile was mixed with 20 mol% of $HClO_4{\cdot}SiO_2$. The temperature was raised to 80 °C to form the anhydro product. To purify the product after evaporation, the residue was dissolved in 2 mL of H_2O, applied to a C18 chromatographic column, and eluted in a gradient of H_2O to 20% CH_3OH acetic acid (yield of ~ 80%). The fragmentation spectrum is shown in Fig. 4.

To prepare the $HClO_4{\cdot}SiO_2$ catalyst, 1.25 g (12.5 mol) of 70% $HClO_4$ was added to a silica gel suspension (23.75 g, mesh-400) in diethyl ether. The mixture was concentrated, and the residue was heated to 100 °C for 72 h under vacuum to obtain $HClO_4{\cdot}SiO_2$ (0.5 nmol·g^{-1}) as a free-flowing powder.

Formation of nonculturable mycobacteria cells

M. smegmatis (strain mc^{2}155) was initially grown for 24–30 h at 37 °C on an orbital shaker (250 r.p.m.) in 20 mL of 2 × rich broth E medium (Himedia, India) with 0.05% (v/v) Tween-80. The bacteria were then inoculated into modified Hartman–de Bont medium [32]. Bacteria were grown in Hartman–de Bont medium with 0.5% BSA (fraction V, Cohn Analog; Sigma) at 37 °C with agitation for 72 h until NC was achieved.

Resuscitation procedure

The resuscitation of nonculturable *M. smegmatis* cells was accomplished with reactivation medium – twice-diluted Sauton's medium [29] supplemented with 0.6% glycerol, 0.025% yeast extract (LabM), and 0.05% Tween-80.

M. smegmatis nonculturable cells obtained as described above were washed once in 20–30 mL of the reactivation medium containing 0.05% Tween-80, and then resuspended in 20 mL of reactivation medium in a 150-mL flask to give an initial $D_{600\ nm}$ of 0.1–0.3. Aliquots of $RpfB_c$, $RipA_c$, 3-kDa filtrates of digested PG or synthetic anhydroGMDP were added to the individual flasks. The cells were incubated at 37 °C for 5–11 days with agitation at 100–120 r.p.m., and the cultures were sampled periodically for plating on NBE agar.

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Author contributions

VDN - Planned experiments, Performed experiments, Analyzed data, Contributed reagents or other essential material, Wrote the paper; GRD - Planned experiments, Performed experiments; MOS - Planned experiments, Performed experiments; SVG - Contributed reagents or other essential material; AR - Contributed reagents or other essential material; RB - Contributed reagents or other essential material, Wrote the paper; ASK - Planned experiments, Analyzed data.

References

1 Kaprelyants AS & Kell DB (1993) Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. *Appl Environ Microbiol* **59**, 3187–3196.

2 Kana BD, Gordhan BG, Downing KJ, Sung N, Vostroktunova G, Machowski EE, Tsenova L, Young M, Kaprelyants A, Kaplan G *et al.* (2008) The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*. *Mol Microbiol* **67**, 672–684.

3 Kana BD & Mizrahi V (2010) Resuscitation-promoting factors as lytic enzymes for bacterial growth and signaling. *FEMS Immunol Med Microbiol* **58**, 39–50.

4 Lillebaek T, Dirksen A, Baess I, Strunge B, Thomsen VØ & Andersen AB (2002) Molecular evidence of endogenous reactivation of *Mycobacterium tuberculosis* after 33 years of latent infection. *J Infect Dis* **185**, 401–404.

5 Wu X, Yang Y, Han Y, Zhang J, Liang Y, Li H, Li B & Wang L (2008) Effect of recombinant Rv1009 protein on promoting the growth of *Mycobacterium tuberculosis*. *J Appl Microbiol* **105**, 1121–1127.

6 Mukamolova GV, Turapov O, Malkin J, Woltmann G & Barer MR (2010) Resuscitation-promoting factors reveal an occult population of tubercle Bacilli in Sputum. *Am J Respir Crit Care Med* **181**, 174–180.

7 Downing KJ, Mischenko VV, Shleeva MO, Young DI, Young M, Kaprelyants AS, Apt AS & Mizrahi V (2005) Mutants of *Mycobacterium tuberculosis* lacking three of the five rpf-like genes are defective for growth in vivo and for resuscitation in vitro. *Infect Immun* **73**, 3038–3043.

8 Tufariello JM, Mi K, Xu J, Manabe YC, Kesavan AK, Drumm J, Tanaka K, Jacobs WR Jr & Chan J (2006) Deletion of the *Mycobacterium tuberculosis* resuscitation-promoting factor Rv1009 gene results in delayed reactivation from chronic tuberculosis. *Infect Immun* **74**, 2985–2995.

9 Biketov S, Potapov V, Ganina E, Downing K, Kana BD & Kaprelyants A (2007) The role of resuscitation promoting factors in pathogenesis and reactivation of *Mycobacterium tuberculosis* during intra-peritoneal infection in mice. *BMC* **17**, 146.

10 Cohen-Gonsaud M, Barthe P, Bagnéris C, Henderson B, Ward J, Roumestand C & Keep NH (2005) The structure of a resuscitation-promoting factor domain from *Mycobacterium tuberculosis* shows homology to lysozymes. *Nat Struct Mol Biol* **12**, 270–273.

11 Ruggiero A, Tizzano B, Pedone E, Pedone C, Wilmanns M & Berisio R (2009) Crystal structure of the resuscitation-promoting factor (DeltaDUF)RpfB from *M. tuberculosis*. *J Mol Biol* **385**, 153–162.

12 Mukamolova GV, Murzin AG, Salina EG, Demina GR, Kell DB, Kaprelyants AS & Young M (2006) Muralytic activity of *Micrococcus luteus* Rpf and its relationship to physiological activity in promoting bacterial growth and resuscitation. *Mol Microbiol* **59**, 84–98.

13 Telkov MV, Demina GR, Voloshin SA, Salina EG, Dudik TV, Stekhanova TN, Mukamolova GV, Kazaryan KA, Goncharenko AV, Young M *et al.* (2006) Proteins of the Rpf (resuscitation promoting factor) family are peptidoglycanhydrolases. *Biochemistry (Mosc)* **71**, 414–422.

14 Ruggiero A, Marchant J, Squeglia F, Makarov V, De Simone A & Berisio R (2013) Molecular determinants of inactivation of the resuscitation promoting factor B from *Mycobacterium tuberculosis*. *J Biomol Struct Dyn* **31**, 195–205.

15 Ruggiero A, Squeglia F, Esposito C, Marasco D, Pedone E, Pedone C & Berisio R (2010) Expression, purification, crystallization and preliminary X-ray crystallographic analysis of the resuscitation promoting factor interacting protein RipA from *M. tuberculosis*. *Protein Pept Lett* **17**, 70–73.

16 Hett EC, Chao MC, Steyn AJ, Fortune SM, Deng LL & Rubin EJ (2007) A partner for the resuscitation-promoting factors of *Mycobacterium tuberculosis*. *Mol Microbiol* **66**, 658–668.

17 Hett EC, Chao MC, Deng LL & Rubin EJ (2008) A mycobacterial enzyme essential for cell division synergizes with resuscitation-promoting factor. *PLoS Pathog* **4**, e1000001.

18 Squeglia F, Ruggiero A, Romano M, Vitagliano L & Berisio R (2014) Mutational and structural study of RipA, a key enzyme in *Mycobacterium tuberculosis* cell division: evidence for the L-to-D inversion of configuration of the catalytic cysteine. *Acta Crystallogr D Biol Crystallogr* **70**, 2295–2300.

19 Brennan PJ & Nikaido H (1995) The envelope of mycobacteria. *Annu Rev Biochem* **64**, 29–63.
20 Lavollay M, Arthur M, Fourgeaud M, Dubost L, Marie A, Veziris N, Blanot D, Gutmann L & Mainardi JL (2008) The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L D-transpeptidation. *J Bacteriol* **190**, 4360–4366.
21 Vollmer W & Höltje JV (2004) The architecture of the murein (peptidoglycan) in gram-negative bacteria: vertical scaffold or horizontal layer(s)? *J Bacteriol* **186**, 5978–5987.
22 Brennan PJ & Crick DC (2007) The cell-wall core of *Mycobacterium tuberculosis* in the context of drug discovery. *Curr Top Med Chem* **7**, 475–488.
23 Gupta R, Lavollay M, Mainardi JL, Arthur M, Bishai WR & Lamichhane G (2010) The *Mycobacterium tuberculosis* protein LdtMt2 is a nonclassical transpeptidase required for virulence and resistance to amoxicillin. *Nat Med* **16**, 466–469.
24 Correale S, Ruggiero A, Capparelli R, Pedone E & Berisio R (2013) Structures of free and inhibited forms of the L, D-transpeptidase LdtMt1 from *Mycobacterium tuberculosis*. *Acta Crystallogr D Biol Crystallogr* **69**, 1697–1706.
25 Priyadarshini R, Popham DL & Young KD (2006) Daughter cell separation by penicillin-binding proteins and peptidoglycan amidases in *Escherichia coli*. *J Bacteriol* **188**, 5345–5355.
26 Priyadarshini R, de Pedro MA & Young KD (2007) Role of peptidoglycan amidases in the development and morphology of the division septum in *Escherichia coli*. *J Bacteriol* **189**, 5334–5347.
27 Heidrich C, Ursinus A, Berger J, Schwarz H & Höltje JV (2002) Effects of multiple deletions of murein hydrolases on viability, septum cleavage, and sensitivity to large toxic molecules in *Escherichia coli*. *J Bacteriol* **184**, 6093–6099.
28 Cohen-Gonsaud M, Keep NH, Davies AP, Ward J, Henderson B & Labesse G (2004) Resuscitation-promoting factors possess a lysozyme-like domain. *Trends Biochem Sci* **29**, 7–10.
29 Nikitushkin VD, Demina GR, Shleeva MO & Kaprelyants AS (2013) Peptidoglycan fragments stimulate resuscitation of "non-culturable" mycobacteria. *Antonie Van Leeuwenhoek* **103**, 37–46.
30 Squeglia F, Romano M, Ruggiero A, Vitagliano L, De Simone A & Berisio R (2013) Carbohydrate recognition by RpfB from *Mycobacterium tuberculosis* unveiled by crystallographic and molecular dynamics analyses. *Biophys J* **104**, 2530–2539.
31 Chao MC, Kieser KJ, Minami S, Mavrici D, Aldridge BB, Fortune SM, Alber T & Rubin EJ (2013) Protein complexes and proteolytic activation of the cell wall hydrolase RipA regulate septal resolution in mycobacteria. *PLoS Pathog* **9**, e1003197.
32 Shleeva M, Mukamolova GV, Young M, Williams HD & Kaprelyants AS (2004) Formation of 'non-culturable' cells of Mycobacterium smegmatis in stationary phase in response to growth under suboptimal conditions and their Rpf-mediated resuscitation. *Microbiology* **150**, 1687–1697.
33 Matsushima Y, Miyazaki T & Kasai S (1968) Chemical evidence for the Phillips model of the action of hen egg white lysozyme. *Nature* **219**, 265.
34 Vocadlo DJ, Davies GJ, Laine R & Withers SG (2001) Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate. *Nature* **412**, 835–838.
35 Phillips DC (1967) The hen egg white lysozyme molecule. *Proc Natl Acad Sci USA* **57**, 483–495.
36 Höltje JV, Mirelman D, Sharon N & Schwarz U (1975) Novel type of murein transglycosylase in *Escherichia coli*. *J Bacteriol* **124**, 1067–1076.
37 Mukamolova GV, Turapov OA, Young DI, Kaprelyants AS, Kell DB & Young M (2002) A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Mol Microbiol* **46**, 623–635.
38 Shah IM, Laaberki MH, Popham DL & Dworkin J (2008) A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell* **135**, 486–496.
39 Squeglia F, Marchetti R, Ruggiero A, Lanzetta R, Marasco D, Dworkin J, Petoukhov M, Molinaro A, Berisio R & Silipo A (2011) Chemical basis of peptidoglycan discrimination by PrkC, a key kinase involved in bacterial resuscitation from dormancy. *J Am Chem Soc* **133**, 20676–20679.
40 Ruggiero A, De Simone P, Smaldone G, Squeglia F & Berisio R (2012) Bacterial cell division regulation by Ser/Thr kinases: a structural perspective. *Curr Protein Pept Sci* **13**, 756–766.
41 Lee M, Hesek D, Shah IM, Oliver AG, Dworkin J & Mobashery S (2010) Synthetic peptidoglycan motifs for germination of bacterial spores. *ChemBioChem* **11**, 2525–2529.
42 Mir M, Asong J, Li X, Cardot J, Boons GJ & Husson RN (2011) The extracytoplasmic domain of the *Mycobacterium tuberculosis* Ser/Thr kinase PknB binds specific muropeptides and is required for PknB localization. *PLoS Pathog* **7**, e1002182.
43 Park JT & Uehara T (2008) How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan). *Microbiol Mol Biol Rev* **72**, 211–227.
44 Reith J & Mayer C (2011) Peptidoglycanturnover and recycling in Gram-positive bacteria. *Appl Microbiol Biotechnol* **92**, 1–11.
45 Höltje JV, Kopp U, Ursinus A & Wiedemann B (1994) The negative regulator of beta-lactamase induction

AmpD is a N-acetyl-anhydromuramyl-L-alanine amidase. *FEMS Microbiol Lett* **122**, 159–164.

46 Prigozhin DM, Mavrici D, Huizar JP, Vansell HJ & Alber T (2013) Structural and biochemical analyses of *Mycobacterium tuberculosis* N-acetylmuramyl-L-alanine amidase Rv3717 point to a role in peptidoglycanfragment recycling. *J Biol Chem* **288**, 31549–31555.

47 Parish T & Stocker N (1998) Methods in molecular Biology. In Mycobacteria protocols, pp. 91–107. Humana Press, Totowa, NJ.

48 Ruggiero A, Marasco D, Squeglia F, Soldini S, Pedone E, Pedone C & Berisio R (2010) Structure and functional regulation of RipA, a mycobacterial enzyme essential for daughter cell separation. *Structure* **18**, 1184–1190.

49 Ruggiero A, Squeglia F, Pirone L, Correale S & Berisio R (2011) Expression, purification, crystallization and preliminary X-ray crystallographic analysis of a major fragment of the resuscitation-promoting factor RpfB from *Mycobacterium tuberculosis*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **67**, 164–168.

50 Rostovtseva L, Andronova T, Malkova V, Sorokina I & Ivaniv V (1981) Synthesis and antitumor activity of glycopeptides containing N-Acetylglucosaminil-(ß 1-4)-N-acetylmuramyl disaccharide unit. *Bioorg Khim* **12**, 1843–1858. In Russian.

51 Hoshino O, Zehavi U, Sînaÿ P & Jeanloz RW (1972) The isolation and structure identification of a discaccharide containing manno-muramic acid from Micrococcus lysodeikticus cell wall. *J Biol Chem* **247**, 381–390.

52 Zemlyakov A (1998) Synthesis of γ-benzyl tert-butoxycarbonyl-L-alanyl-D-glutamate and its derivatives. *Chem Nat Compd* **34**, 78–79.

53 Chun Y, Yan S, Li X, Ding N, Zhang W, Wang P, Li M & Li Y (2011) Synthesis of 1,6-anhydro sugars catalyzed by silica supported perchloric acid. *Tetrahedron Lett* **52**, 6196–6198.