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Muralytic activity of *Micrococcus luteus* Rpf and its relationship to physiological activity in promoting bacterial growth and resuscitation

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Summary

The culturability of several actinobacteria is controlled by resuscitation-promoting factors (Rpfs). These are proteins containing a *c.* 70-residue domain that adopts a lysozyme-like fold. The invariant catalytic glutamate residue found in lysozyme and various bacterial lytic transglycosylases is also conserved in the Rpf proteins. Rpf from *Micrococcus luteus*, the founder member of this protein family, is indeed a muralytic enzyme, as revealed by its activity in zymograms containing *M. luteus* cell walls and its ability to (i) cause lysis of *Escherichia coli* when expressed and secreted into the periplasm; (ii) release fluorescent material from fluorescamine-labelled cell walls of *M. luteus*; and (iii) hydrolyse the artificial lysozyme substrate, 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside. Rpf activity was reduced but not completely abolished when the invariant glutamate residue was altered. Moreover, none of the other acidic residues in the Rpf domain was absolutely required for muralytic activity. Replacement of one or both of the cysteine residues that probably form a disulphide bridge within Rpf impaired but did not completely abolish muralytic activity. The muralytic activities of the Rpf mutants were correlated with their abilities to stimulate bacterial culturability and resuscitation, consistent with the view that the biological activity of Rpf results directly or indirectly from its ability to cleave bonds in bacterial peptidoglycan.

Introduction

Some bacteria such as *Bacillus* and *Clostridium* spp. can form dormant endospores, which represent an intermediate state between life and death. They show no obvious signs of vitality, but they nevertheless retain the ability to germinate and to resume growth and division when they detect specific (usually chemical) signals in their environment. Although the actinobacteria do not make endospores, several of them can enter a state in which they have much reduced metabolic activity and lose culturability. They require resuscitation in a nutrient-poor liquid medium before they can resume active growth on an agar-solidified medium. The adoption of this 'non-culturable' state may represent a survival strategy that helps these organisms to persist during nutrient-limited conditions unfavourable for growth (Mukamolova *et al.*, 2003). Although the mechanisms by which 'non-culturable' cells are produced remain unknown, their very existence has important implications for microbial population ecology and for the control of infectious disease (Kell *et al.*, 1998). For example, some (at least) of the persistent cells of *Mycobacterium tuberculosis* responsible for reactivation tuberculosis (TB) in humans may be in a 'non-culturable' state (Mukamolova *et al.*, 2003). Evidence to support this comes from experiments with a chronic mouse model of TB, in which the viable count of *M. tuberculosis* 10 months post-infection was two orders of magnitude higher when measured in a liquid medium that permits resuscitation (MPN) than when measured by conventional plating (cfu) on a solid medium that does not (Dhillon *et al.*, 2004). The presence of *M. tuberculosis* DNA in culture-negative and superficially normal human lung tissue during latent infection is also consistent with this view (Hernández-Pando *et al.*, 2000).

'Non-culturable' forms of *Micrococcus luteus* can be resuscitated using the supernatant from late logarithmic phase laboratory cultures (Kaprelyants *et al.*, 1994). The active component is a secreted protein called Rpf – resuscitation-promoting factor (Mukamolova *et al.*, 1998). Rpf homologues are widespread throughout the actinobacteria (Kell and Young, 2000) and biological activity (resusci-

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tation or growth promotion) has been demonstrated for several representatives, including all five proteins found in *M. tuberculosis* and its close relatives (Mukamolova *et al*., 2002a; Zhu *et al*., 2003). The gene encoding Rpf could not be inactivated in *M. luteus* except in the presence of a second functional copy (Mukamolova *et al*., 2002b), whereas the individual *rpf*-like genes of *M. tuberculosis* were dispensable for growth *in vitro* and *in vivo* (Downing *et al*., 2004; Tufariello *et al*., 2004). Triple mutants of *M. tuberculosis* showed significantly attenuated virulence in mice and were unable to resuscitate spontaneously from the 'non-culturable' state *in vitro* (Downing *et al*., 2005). A *Corynebacterium glutamicum* mutant lacking both of its *rpf*-like genes grew as well as did the wild type, but regrowth after a period of storage was significantly impaired (Hartmann *et al*., 2004).

The extreme potency of Rpf (activity at picomolar concentrations), and the strict requirement for Rpf to permit growth under certain defined conditions, suggested that this protein should be regarded as a bacterial growth factor (Mukamolova *et al*., 1998). A mode of action based on specific enzymatic activity was also considered, and this was supported by the prediction that Rpf has a lysozyme-like fold (Finan, 2003; Kazarian *et al*., 2003; Cohen-Gonsaud *et al*., 2004; http://predictioncenter2.gc.ucdavis.edu/). However, attempts to mimic Rpf activity by exogenous addition of traces of lysozyme were unsuccessful (G.V. Mukamolova, unpublished). In this paper we demonstrate that Rpf possesses muralytic activity. A modelling approach was employed to generate a predicted structure for the Rpf fold and this was used to inform site-directed mutagenesis experiments to identify residues that are important for muralytic and physiological activity. The results are consistent with the view that the muralytic activity of Rpf is probably responsible for its observed activity both in resuscitating dormant cells and in stimulating growth when bacteria are inoculated at low density into a minimal medium or held in a prolonged stationary phase. One possible interpretation is that Rpf remodels the cell wall throughout bacterial growth and that its activity is especially important during the transition from a 'non-culturable' to an actively growing state.

While this work was in progress, the solution structure of the Rpf-like domain of *M. tuberculosis* RpfB (Rv1009) was published, confirming the lysozyme-like fold and also providing some data showing the importance of a conserved glutamate residue for growth stimulatory activity (Cohen-Gonsaud *et al*., 2005).

Results

Structure prediction of Rpf

In 2002, *M. luteus* Rpf was included in the 'Ten Most Wanted' list (TMW), the initiative to predict by a community-wide effort the structures of the 10 most wanted proteins of unknown structure, suggested by biologists on the basis of their biological importance (Abbott, 2001). Predictions for the 10 selected proteins were collected and analysed, and the results were presented at the world structure predictors' community meeting, CASP5, in December 2002 (Tramontano, 2003). There were 54 predictions for Rpf (target TMW0001) resulting in a consensus prediction of a lysozyme-like fold for the Rpf domain. One of us (A.G.M.) participated in both CASP5 and TMW and communicated to the others this consensus prediction together with the 3-D co-ordinates of a detailed model of the Rpf domain structure.

The Rpf domain was predicted to have a minimal lysozyme fold, common to all known members of the lysozyme super-family in the SCOP database (Murzin *et al*., 1995). The selected model of the Rpf domain was constructed on the basis of not one, but several structures from different lysozyme families, aiming to provide a structural explanation of every conserved feature in the multiple sequence alignment of the Rpf domain family. In particular, the conserved glutamate residue (E54) aligned with the catalytically essential invariant glutamate residue, suggesting that the Rpf domain might possess an enzymatic activity.

The recent determination of the *M. tuberculosis* RpfB domain (PDB entry 1xsf; Cohen-Gonsaud *et al*., 2005), which has a 50% sequence identity to the *M. luteus* Rpf domain, allowed a direct comparison of the predicted and experimental structures (Fig. 1). The predicted and observed folds of the Rpf domains are very similar indeed. Moreover, 59 of 78 (> 75%) of the modelled residues appear aligned correctly and can be superimposed with an r.m.s. deviation of 2.0 Å. The correctly aligned residues include many of those modified by mutagenesis in this study.

Rpf is a muralytic enzyme

According to the predicted structure of *M. luteus* Rpf, and the recently solved solution structure of the Rpf domain of *M. tuberculosis* RpfB (Cohen-Gonsaud *et al*., 2005), the Rpf domain has a lysozyme-like fold and the Rpf proteins may therefore show muralytic activity. This was initially investigated using zymography. Native, secreted Rpf was partially purified from culture supernatants and tested following electrophoresis through polyacrylamide gels containing either cell wall fragments or autoclaved whole cells as substrate. A clearance band was observed corresponding to a 25 kDa protein (Fig. 2B) that reacted with anti-Rpf antibodies. At least two additional supernatant proteins substantially larger than Rpf also had muralytic activity, but anti-Rpf antibodies did not detect them (Fig. 2A). Recombinant Rpf (expressed from pET19b with

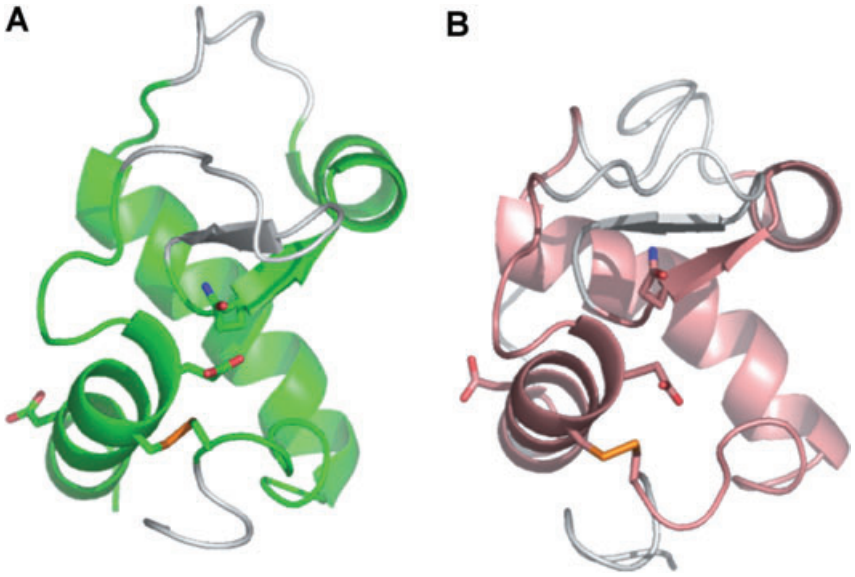

Fig. 1. Structure of the Rpf domain. The theoretical model of the *M. luteus* Rpf domain guiding this study (A) is compared with the recent experimental structure of the *M. tuberculosis* RpfB domain (B). Coloured regions have both similar backbone conformation and correctly predicted sequence alignment. Depicted with sticks are the side-chains of selected conserved residues targeted by site-directed mutagenesis in A (first number) and their counterparts in B (second number): D48/286, C53/291, E54/292, Q72/310 and C114/355.

a polyhistidine tag at the N-terminus) isolated from *Escherichia coli* usually gave negative results in this assay for muralytic activity. Removal of the polyhistidine tag or repositioning it at the C-terminus or varying the assay conditions did not improve activity. This was finally achieved by Rpf expression and secretion into the *E. coli* periplasm, providing an environment conducive to disulphide bridge formation. The protein thus produced yielded a distinct clearance band of the expected size in zymograms (Fig. 2C). Sometimes, multiple clearance bands were observed, probably representing different forms of Rpf. Nucleic acid strongly inhibited the observed muralytic activity, in accordance with previous observations on muralytic enzymes from *E. coli* (Kusser and Schwarz, 1980). The recombinant protein was less active than the native protein and both lost activity after storage at 4°C for 1 week.

Hyper-expression of Rpf in the E. coli *periplasm causes bacterial lysis*

When the *rpf* gene is expressed without its signal sequence from the T7 promoter in pET19b, it accumulates internally as inclusion bodies with no evidence of bacterial lysis (Mukamolova *et al*., 1998; 2002b). However, arabinose-induced expression and secretion of Rpf in *E. coli* from the recombinant pBAD/gIIIb vector resulted in substantial lysis of the bacteria (Fig. 3). Lysis was confirmed microscopically and has been described previously for muralytic enzymes expressed in *E. coli* (Fischer *et al*., 1993; Ehlert *et al*., 1995). Lysis was observed in LB and other rich media but it was much reduced in the SOB medium (containing 8.5 mM Mg^{2+} and a reduced salt concentration) that was employed for routine protein expression. Lysis was correlated with the accumulation of Rpf and was not observed in strains harbouring either the expression vector alone or a derivative expressing calmodulin (Fig. 3). *M. luteus* Rpf contains an N-terminal Rpf domain and a C-terminal LysM domain (Bateman and Bycroft, 2000) separated by a linker region (Mukamolova *et al*., 1998). It was previously reported that a truncated form of Rpf comprising only the Rpf domain retained biological activity (Mukamolova *et al*., 2002b). As expected, when expressed separately, the LysM domain showed no evidence of muralytic activity on zymograms (data not shown) nor was there any lysis of the *E. coli* expression host (Fig. 3). We were unable to express sufficient quantities of the Rpf domain alone in a recombinant pBAD/gIIIb vector to test its activity.

Hydrolysis of M. luteus *cell walls labelled with fluorescamine*

A semi-quantitative estimate of the muralytic activity of Rpf was obtained using *M. luteus* cell walls labelled with fluorescamine as previously described (Mintz *et al*., 1975). Under the assay conditions employed here (see *Experimental procedures*), 10 μg purified recombinant Rpf hydrolysed 16.9 ± 1.3% of the fluorescamine-labelled wall material per hour. The observed activity was well above background, which was 0.27 ± 0.1% for buffer only and 0.54 ± 0.02% for a purified extract from *E. coli* harbouring the empty expression vector, pBAD/gIIIb. The activity of purified recombinant Rpf was between five- and six-fold lower than that of an equivalent amount of freshly prepared hen egg white lysozyme. Similar results were obtained using three different batches of recombinant Rpf. The observed activity was completely abolished by boiling

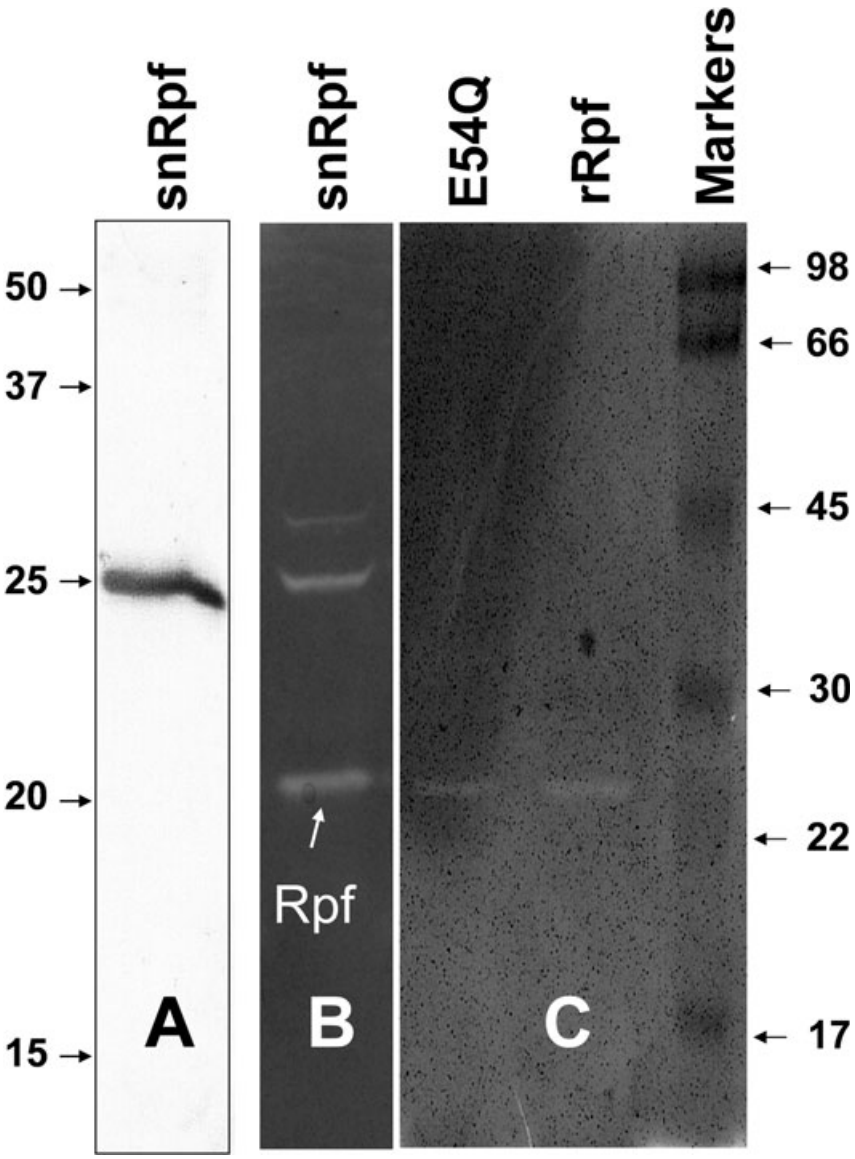

Fig. 2. Rpf zymograms. Rpf partially purified from concentrated *M. luteus* culture supernatant (A and B) or recombinant Rpf and the E54Q mutant (C) were separated by SDS-PAGE (12.5% gels). The zymogram gels (B and C) contained 0.16% w/v *M. luteus* cell walls. The Western blot (A) was incubated with polyclonal rabbit anti-Rpf antibodies.

or by precipitation using 70% ethanol. These experiments confirmed that Rpf has muralytic activity. Attempts to employ this assay using crude extracts of *E. coli* cells producing Rpf were not successful, owing to comparatively high endogenous activity.

Rpf has lysozyme-like activity

In view of its structural similarity to the c-type lysozymes, and (noting *a posteriori*) the fact that the Rpf-like domain of *M. tuberculosis* RpfB (Rv1009) can bind N,N′,N″-triacetylchitotriose (tri-NAG) (Cohen-Gonsaud *et al*., 2005), we determined whether recombinant Rpf hydrolyses the synthetic, artificial lysozyme substrate, 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside (MUF tri-NAG). Activity with this substrate was significant and optimal at pH 6 (Table 1). Different batches of recombinant protein showed substantial variation in specific activity. This was probably because different preparations contained different proportions of the protein in the form of aggregates (as revealed by gel filtration), which are completely inactive (data not shown). The activity of Rpf in this assay (nmoles MUF released per minute per milligram protein) is approximately 50-fold less than that of hen egg white lysozyme.

Measurement of the residual activity of altered Rpf proteins

To identify residues that are important for biological activity, 16 different mutated forms of *rpf* were generated and the corresponding proteins produced, as indicated in the

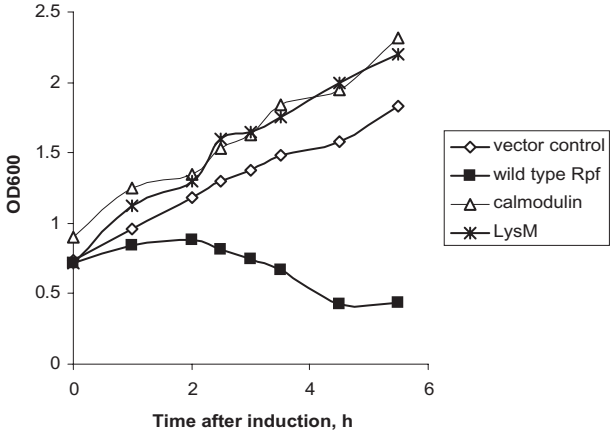

Fig. 3. Lysis of *E. coli* cells during Rpf expression. *E. coli* TOP 10 strains were grown in LB medium to OD_{600} of 0.7 and then induced with 0.01% arabinose. The culture density was monitored with time thereafter.

Experimental procedures. As zymography provides only a qualitative measure of muralytic activity, the purified recombinant proteins were assayed *in vitro* using fluorescamine-labelled cell walls of *M. luteus* (Fig. 4A). Some of these proteins were also assayed using MUF tri-NAG (Table 1). The extent of *E. coli* lysis during arabinose-induced expression of these proteins in LB is also indicated in Fig. 4A. The correlation coefficient between *M. luteus* cell wall hydrolysis and *E. coli* lysis during protein expression was 0.81 (see Fig. 4B).

The biological activity of the various recombinant proteins *in vivo* was determined by expressing them from plasmids that replicate autonomously in *M. luteus* and *Mycobacterium smegmatis*. The observed phenotypes therefore result from the combined expression of the altered form of Rpf from the ectopic, plasmid-encoded gene and wild-type protein(s) from the endogenous chromosomal gene(s). Rpf function was assayed in *M. luteus* in two different ways. Firstly, Rpf stimulates the growth of this organism when inoculated into poor media. The growth enhancement associated with the presence of additional ectopic copies of *rpf* (as compared with the control strain harbouring the empty vector plasmid) is shown in Fig. 5A. The predominant effect of increased Rpf availability is a reduction of the lag phase either when it is endogenously produced as in this first assay, or exogenously added, as shown previously (Mukamolova *et al*., 1998; 2002a). There is also an increase in colony size when organisms are plated on agar-solidified medium (data not shown) as was previously observed when Rpf was expressed in *My. smegmatis* (Mukamolova *et al*., 2002a). The results of these experiments were summarized in Table 2 by recording the number of generations of growth that occurred in the 96 h period following the inoculation of each culture.

The effects of expression of the mutant proteins on the culturability of *M. luteus* on an agar-solidified minimal medium formed the basis of the second assay (some representative data are shown in Fig. 5C). The results for all the mutants are presented in Table 2 as the ratio of the cfu obtained when bacteria were plated on a poor and on a rich medium having previously grown to stationary phase in a rich medium (effects on culturability are most pronounced under these conditions).

In a third assay we examined the ability of selected proteins to resuscitate 'non-culturable' cells of the heterologous organism, *My. smegmatis*, the genome of which encodes four Rpf-like proteins (http://www.tigr.org/tigr-scripts/CMR2/GenomeTabs.spl?database=gms). When grown to stationary phase under carefully controlled conditions, this organism loses the ability to form colonies or grow when re-inoculated into liquid medium. However, cells expressing and secreting *M. luteus* Rpf acquire the ability to resuscitate spontaneously when re-inoculated in liquid medium, thus providing a convenient assay for the resuscitation activity of the Rpf mutants. The effectiveness of resuscitation was estimated using the MPN method, essentially as described previously (Shleeva *et al*., 2004) – see *Experimental procedures*. These data are also summarized in Table 2.

The invariant glutamate residue

Rpf has a lysozyme-like fold, shows muralytic activity *in vitro*, and like all other members of the Rpf protein family, it shares an absolutely conserved glutamate residue whose counterparts in lysozyme (E35) and in two lytic transglycosylases, Slt35 (E162) and Slt70 (E478) are involved in catalysis (Grutter *et al*., 1983; Thunnissen *et al*., 1995; van Asselt *et al*., 1999a,b). We therefore concentrated initially on the effect of replacing the corresponding residue of Rpf. The isosteric E54Q substitution retained activity in zymograms (Fig. 2C) although there was some attenuation of muralytic activity (Fig. 4A, Table 1). It is noteworthy that activity was by no means

Table 1. MUF tri-NAG hydrolysis by Rpf and Rpf mutants.

Protein[a]	Specific activity[b]
Rpf (wild type)	20.6 ± 11.6
E54Q	13.5 ± 4.5
E54A	6.6 ± 4
C53K + C114T	1.2 ± 0.3
E54K + D48A	< 0.5
Lysozyme	934

a. All measurements were made using secreted recombinant proteins isolated from *E. coli*.
b. MUF released (nmoles per milligram per hour). Values are given together with the standard deviation; at least three independent batches of protein were assayed in duplicate.

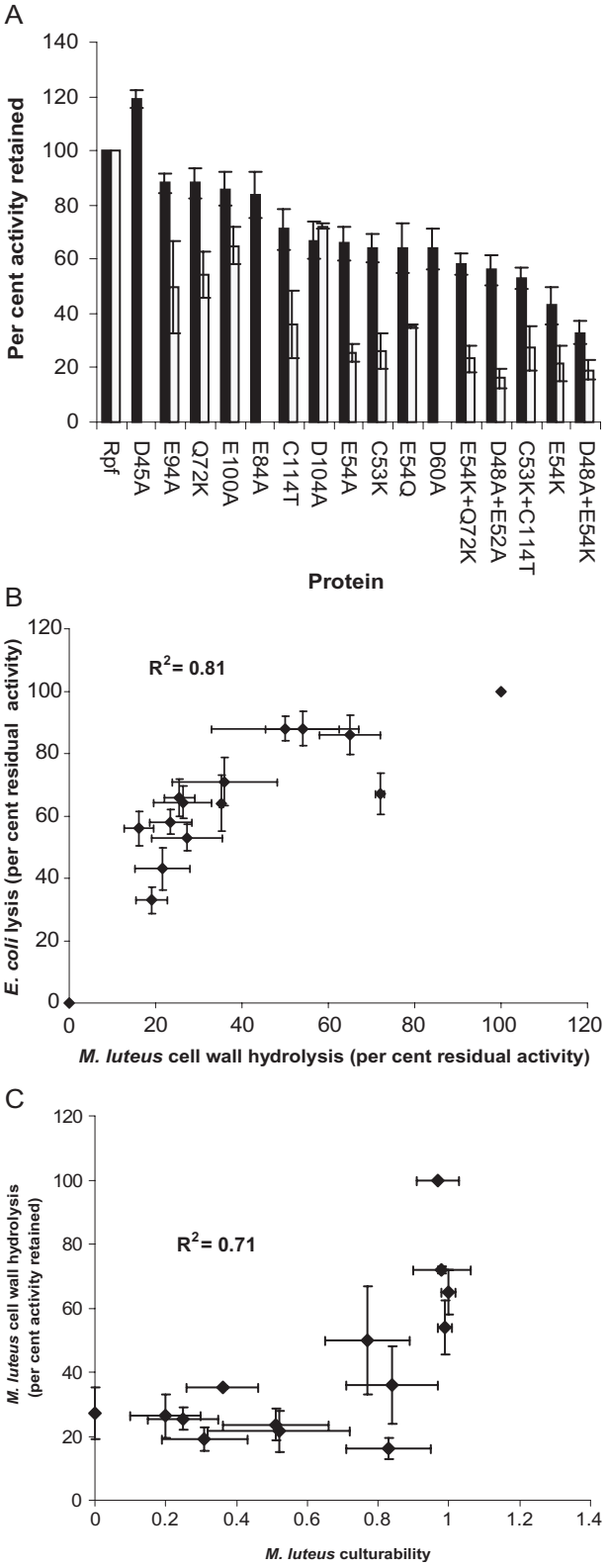

Fig. 4. Muralytic activity of the mutant Rpf proteins. Lysis of *E. coli* during protein expression, measured as described in *Experimental procedures*, is shown as filled columns in A. Muralytic activity was determined using *M. luteus* cell walls labelled with fluorescamine as described in *Experimental procedures*. Ten micrograms of each protein was used per assay. All samples were assayed in triplicate and the average values for two independent experiments with different protein preparations are shown as open columns in A (assays were not performed on the D45A, D60A and E84A mutants). For both assays, the residual activity is expressed as a percentage of that observed with the wild-type protein. The relationship between *M. luteus* cell wall hydrolysis and *E. coli* lysis during protein expression is shown in B. The relationship between the culturability of *M. luteus* strains expressing the mutant Rpf proteins (data from Table 2) and the muralytic activity of the purified proteins is shown in C. The error bars in B and C represent one standard deviation.

completely abolished as is the case for the corresponding substitution in lysozyme or the *E. coli* lytic transglycosylase, Slt70 (Grutter *et al*., 1983; Thunnissen *et al*., 1994). The *M. luteus* strain expressing the E54Q mutant showed weak but significant growth stimulation in lactate minimal medium (LMM) medium (Fig. 5A). This experiment was repeated on four separate occasions and growth of the strain expressing E54Q was always slightly better than that of the empty vector control with an average lag phase reduction of 17 ± 7 h. However, culturability on LMM plates was reduced compared with that of either the wild-type strain harbouring the empty vector (data not shown) or the strain hyper-expressing wild-type Rpf (Table 2 and Fig. 5C). The E54Q mutant showed a 2 log impairment in its ability to promote the resuscitation of *My. smegmatis* (Table 2). These results indicate that although the physiological function of the E54Q mutant was impaired, it was not completely abolished. Similar attenuation of both muralytic and physiological activity was observed with the E54A mutant (Fig. 4A and Table 2). However, this mutant showed further impairment in its ability to hydrolyse MUF tri-NAG and resuscitation of *My. smegmatis* fell by another 2 logs (Tables 1 and 2). The E54K mutant had more pronounced attenuation of muralytic activity (Fig. 4A), and resuscitation of 'non-culturable' cells of *My. smegmatis* was completely abolished (Table 2), but physiological activity in *M. luteus* was not significantly different from that observed with the other substitutions tested (Table 2). The various measurements of muralytic and physiological activity are not perfectly correlated with each other (e.g. Fig. 4C; see also *Discussion*). Moreover, the data show that the invariant glutamate residue is important, though not absolutely necessary for Rpf function; the extent to which function is impaired in the various assays depends on the amino acid residue that replaces it.

Replacement of other residues

According to the modelling and the structural data, residue Q72 would be accessible within the active site cleft

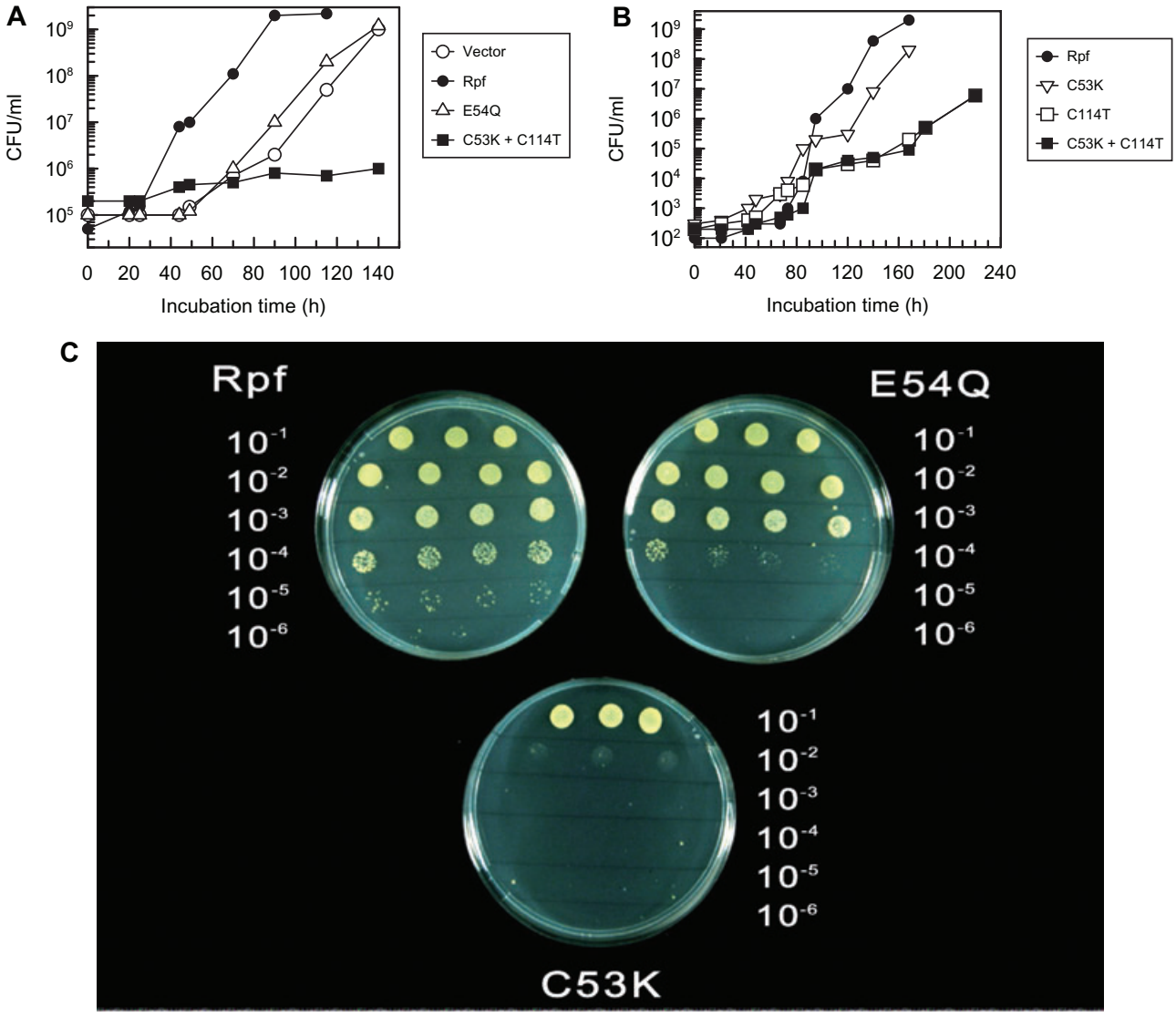

Fig. 5. Growth of *M. luteus* strains expressing Rpf mutants. LMM cultures (20 ml) were inoculated with *c.* 10^5 cells ml^{-1} (A) or 10^2 cells ml^{-1} (B) from a 48 h pre-culture in NBE in 100 ml flasks. Cultures were incubated at 30°C with shaking (200 r.p.m. min^{-1}) and growth was monitored at intervals by plating serial dilutions in triplicate on NBE agar. In C, horizontal rows represent replicate 10 µl samples from serial dilutions (10^{-1}–10^{-6}) of an overnight NBE culture spotted on LMM agar and incubated for 120 h at 30°C.

and might potentially be involved in the interaction of Rpf with its substrate. The Q72K replacement reduced muralytic activity against *M. luteus* cell walls by about 50% (Fig. 4A). However, there was only a slight effect on *E. coli* lysis (Fig. 4A) and both *M. luteus* growth and *My. smegmatis* resuscitation were unimpaired (Table 2). Moreover, the muralytic and physiological activities of a protein bearing both Q72K and E54K alterations were not significantly more attenuated than those of the E54K mutant (Fig. 4A, Table 2). These data suggest that Q72 does not have an important role in Rpf function.

As substitution of the presumptive catalytic glutamate residue did not completely abolish activity, we also examined the effect of substitutions of the other eight acidic residues in the Rpf domain. Residue D48 is highly conserved among the Rpf-like proteins and we examined the effect of substituting alanine for this residue in combination with E54K and E52A. The D48A + E54K mutant showed a hydrolytic activity (Fig. 4A) and a physiological activity (Table 2) similar to that of the E54K protein, although it had no activity against MUF tri-NAG (Table 1). The D48A + E52A protein was unstable *in vitro*. This probably accounts for its low muralytic activity, as *M. luteus* cells expressing the D48A + E52A protein lysed rapidly once they reached stationary phase in batch culture (data not shown). The culturability and recovery of this strain from stationary phase was comparable to that of the strain hyper-expressing wild-type Rpf. A mutant harbouring

Table 2. Physiological activity of the Rpf mutants in *M. luteus* and *My. smegmatis*.

Rpf mutant	Growth of *M. luteus* in LMM[a] (number of generations)	*M. luteus* culturability[b]	*My. smegmatis* resuscitation index[c]
Empty vector	5.46 ± 1.82	0.95 ± 0.04	$< 5.5 \times 10^{-6}$
Rpf wild type	13.55 ± 1.74	0.97 ± 0.06	1
E54Q	6.77 ± 1.49	0.36 ± 0.1	1.9×10^{-2}
E54A	7.26 ± 2.04	0.25 ± 0.1	6.0×10^{-4}
E54K	6.94 ± 1.8	0.52 ± 0.2	$< 5.5 \times 10^{-6}$
Q72K	13.18 ± 0.81	0.99 ± 0.02	1
E54K + Q72K	6.53 ± 1.77	0.51 ± 0.15	$< 5.5 \times 10^{-6}$
E54K + D48A	5.83 ± 1.8	0.31 ± 0.12	1.1×10^{-4}
D48A + E52A	11.67 ± 1.39	0.83 ± 0.12	5.0×10^{-2}
E94A	11.38 ± 3.8	0.77 ± 0.12	ND
E100A	11.02 ± 0.7	1.00 ± 0.02	1.7×10^{-4}
D104A	13.75 ± 1.85	0.98 ± 0.08	ND
D45A	9.05 ± 2.61	1.00 ± 0.2	ND
D60A	12.2 ± 1.8	0.97 ± 0.12	ND
E84A	13.93 ± 2.04	0.86 ± 0.13	ND
C53K	8.46 ± 0.97	0.20 ± 0.1	1.3×10^{-2}
C114T	4.87 ± 1.88	0.84 ± 0.13	1.7×10^{-2}
C53K + C114T	3.8 ± 1.29	$< 10^{-3}$	$< 5.5 \times 10^{-6}$

a. Growth of *M. luteus* in LMM using as inoculum cells from a 48 h pre-culture in NBE. Bacteria were diluted to give an initial density of *c.* 10^2 cfu ml^{-1} in some experiments and *c.* 10^5 cfu ml^{-1} in others. The number of cfu was determined at zero time and 96 h post-inoculation by plating on NBE agar. The number of generations is given by $\log_2 cfu_{96}/cfu_0$; these values summarize the results of six independent experiments – a typical example is shown in Fig. 5.
b. Cells grown to stationary phase in NBE for 48 h were plated on LMM and NBE. The values given are cfu_{LMM}/cfu_{NBE} (average of four independent experiments).
c. MPN of the strain expressing the Rpf mutant/MPN of the strain expressing wild-type Rpf (determined in the same experiment).
ND, not determined.

D48A alone was not constructed, because the physiological behaviour of the D48A + E54K mutant was similar to that of the E54K mutant and that of the D48A + E52A mutant was similar to that of the wild-type protein. Muralytic activity appeared to be retained based on the extensive lysis of *M. luteus* expressing D48A + E52A in stationary phase, even though it could not be assayed reliably *in vitro*.

The remaining acidic residues in Rpf were also substituted with alanine. The E94A mutant was also unstable (cf. D48A + E52A mutant), but in this case sufficient protein was obtained to show that it had substantial muralytic activity (Fig. 4A) and *M. luteus* cells expressing this protein lysed when they entered stationary phase in batch culture. Expression of the E94A mutant in *M. luteus* also caused a slight reduction in culturability (Table 2). None of the other substitutions, viz. D45A, D60A, E84A, E100A and D104A, had a significant effect on muralytic activity, though expression of the E84A and D45A proteins in *M. luteus* did slightly impair culturability and recovery from stationary phase respectively (Table 2).

The Rpf proteins contain two highly conserved cysteine residues predicted to form a disulphide bridge. These were mutated to conform to the residues found at equivalent positions in the Rpf-like domain of a mycobacteriophage tape measure protein (Pedulla *et al.*, 2003). The combined C53K + C114T replacement substantially reduced muralytic activity (Table 1, Fig. 4A). This protein showed no resuscitation activity, and it had a highly deleterious effect on *M. luteus* culturability and resumption of growth after extended stationary phase (Table 2). The inhibitory effects of expression of this protein on the growth of *M. luteus* cells on a minimal medium are shown in Fig. 5. The C53K or C114T alterations alone had similar negative effects on muralytic activity as were observed following the replacement of both cysteine residues. However, the same was not true for physiological activity, where replacement of either residue alone had a significant but lesser effect than replacement of both residues (Table 2). The predominant effect on culturability was associated with the C53K replacement (Table 2), whereas the predominant effect on growth rate was associated with the C114T replacement (Fig. 5B). Finally, both the C53K and the C114T mutants showed reduced but nevertheless significant activity in the *My. smegmatis* resuscitation assay (Table 2).

Discussion

The first indication that Rpf might be a muralytic enzyme was provided by its weak sequence similarity to the c-type lysozymes (Finan, 2003; Kazarian *et al.*, 2003). Structural modelling of the Rpf domain, as presented here and also by others reinforced this idea (Cohen-Gonsaud *et al.*, 2004). This was also suggested by the frequent occurrence in the Rpf-like proteins of domains such as LysM (Bateman and Bycroft, 2000), which are characteristic of cell wall-associated proteins (Ravagnani *et al.*, 2005). The

recently published structure of the Rpf-like domain of *M. tuberculosis* RpfB (Rv1009), showing that it does indeed adopt a lysozyme-like fold, has added further support (Cohen-Gonsaud *et al*., 2005). These authors showed that the Rpf-like domain of *M. tuberculosis* RpfB bound the oligosaccharide, tri-NAG, but that this domain had no measurable lysozyme-like activity (Cohen-Gonsaud *et al*., 2005).

In this paper we have shown that both native *M. luteus* Rpf and a recombinant his-tagged form expressed and secreted into the *E. coli* periplasm possess muralytic activity, using zymography as well as an assay based on fluorescamine-labelled *M. luteus* cell walls. Moreover, Rpf may be able to catalyse similar reactions to those catalysed by lysozyme, as it hydrolyses MUF tri-NAG, an artificial lysozyme substrate. The predicted and recently published structure of the corresponding domain of *M. tuberculosis* RpfB suggests that these proteins belong to the family of lytic transglycosylases (Finan, 2003; Cohen-Gonsaud *et al*., 2004; 2005; Ravagnani *et al*., 2005). Like lysozyme (which is purely a hydrolase), the lytic transglycosylases cleave the β-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine residues within peptidoglycan (Holtje *et al*., 1975). They are thought to play an important role in the synthesis and recycling of peptidoglycan and the production of 1,6 anhydromuramic acid, which does not occur during lysozyme-mediated catalysis, allows energy conservation that is vital for the synthesis of new glycan stands. Moreover, in lytic transglycosylases and in goose-type lysozymes a single acidic residue (often glutamate) within the active centre mediates catalysis (Weaver *et al*., 1995). The corresponding residue lying in the proposed active centre of the Rpf-like proteins is an absolutely conserved glutamate, which might therefore be predicted to play an essential role in cell wall hydrolysis. Although replacement of the corresponding glutamate residue in the Rpf-like domain of RpfC (Rv1884c) caused a loss of the growth stimulatory activity seen with the wild-type protein, the effect of this replacement on the resuscitation-promoting activity of RpfC was not reported – nor indeed was it shown that this protein has muralytic activity (Cohen-Gonsaud *et al*., 2005).

Using *M. luteus* Rpf, we have shown here that replacement of the presumptive catalytic glutamate residue by either glutamine or alanine attenuated, but did not completely abolish, both hydrolytic and physiological (resuscitation and growth stimulatory) activities. A more drastic change, in the E54K mutant, resulted in further attenuation of hydrolytic activity and complete loss of resuscitation promoting activity. These data indicate that E54 plays an important role in both hydrolytic and physiological activities. However, the retention of residual activity in the E54Q and E54A mutants is not consistent with an essential role for this glutamate residue in catalysis, as is considered to be the case for lysozyme. Replacement of the corresponding glutamate residue in two *E. coli* soluble lytic transglycosylases, Slt35 and Slt70, resulted in complete loss of activity (Thunnissen *et al*., 1994; van Asselt *et al*., 1999a). Owing to the substantial redundancy of muralytic enzymes in *E. coli*, the precise physiological function of these enzymes remains unclear (Heidrich *et al*., 2002). In other cases of presumed lytic transglycosylase activity where a precise physiological function can be ascribed (e.g. type IV secretion apparatus, phage tail protein and plasmid conjugation apparatus) the picture is less clear-cut. Replacement of the corresponding glutamate residue sometimes resulted in only partial impairment of function (Moak and Molineux, 2000; Hoppner *et al*., 2004), whereas in others, complete inactivation was reported (Mushegian *et al*., 1996; Bayer *et al*., 2001). However, none of these reports provides data showing impairment of both biochemical and physiological function as reported here. This was however, shown for an *N*-acetyl glucosaminidase (formerly thought to be a muramidase) of *Salmonella typhimurium*, in which the corresponding glutamate residue was replaced (Nambu *et al*., 1999).

Direct comparison of the activities of the altered Rpf proteins with that of the wild-type protein revealed that attenuation of hydrolytic activity does correlate, albeit imperfectly, with impairment of physiological function. The correlation coefficient between *M. luteus* cell wall hydrolysis and culturability was 0.69 and that between *M. luteus* cell wall hydrolysis and growth following an extended stationary phase was 0.71 (see also Fig. 4B). Although the correlation is far from perfect, and there was substantial variability between replicates, we nevertheless contend that the correlation is biologically significant. There is no reason to suppose that the different physiological assays we employed would all respond to the same extent to impairment of the muralytic activity of Rpf; some may be much more sensitive than others. Moreover, the physiological assays were complicated by the fact that activity was measured in a genetic background in which wild-type Rpf is present, the mutant protein being expressed from an autonomous plasmid-encoded gene. A more rigorous test would have necessitated the production of multiple *M. luteus* strains in which the chromosomal copy of *rpf* was replaced with a single copy of each mutated derivative. Given the rudimentary state of the genetic tools currently available for this organism, this would be a Herculean task. Moreover, the available evidence indicates that *rpf* is an essential gene in *M. luteus* (Mukamolova *et al*., 2002b) and its replacement with mutated genes encoding products with severely impaired activity would probably be lethal. We have very recently constructed a strain of *M. luteus* in which *rpf* expression is controlled by a tetracy-

cline-dependent corynebacterial promoter, which should make it possible to undertake more stringent future analysis of the relationship between the biochemical and physiological activities of the altered Rpf proteins (G.V. Mukamolova and M. Young, unpublished; Blokpoel *et al*., 2005).

Given the observed correlation between the biochemistry and the physiology and in the absence of clear evidence to the contrary, we conclude that the observed muralytic activity is responsible, at least in part, for the growth stimulatory and resuscitation-promoting effects of the protein. However, we recognize that certain features of this protein are particularly important for its physiological activity, according to the available evidence. For example, the replacement of one or both cysteine residues had similar effects on muralytic activity (Fig. 4A), whereas the loss of physiological activity occasioned by the replacement of both cysteine residues was very pronounced and more subtle effects were seen following replacement of either one alone (Table 2, Fig. 5). These residues might be involved in the regulation of Rpf activity and not just in stabilization of Rpf secondary structure. The Rpf-like domain in the 'Barnyard' mycobacteriophage tape measure proteins lacks both cysteine residues, but is otherwise very similar to *M. luteus* Rpf (Pedulla *et al*., 2003) – indeed we introduced the C53K and C114T changes into *M. luteus* Rpf to make it conform more closely to this particular protein domain. Nevertheless, we cannot rule out the direct involvement of both cysteine residues in catalysis as was shown for GTP cyclohydrolase I (Auerbach *et al*., 2000). In this enzyme, the thiol groups of two cysteine residues are involved in the chelation of Zn^{2+} in the active centre of the enzyme. It is perhaps significant in this context that the addition of mercaptoethanol or DTT in renaturation buffer did not abolish Rpf activity in zymograms, suggesting that disulphide bridge formation is not essential for the muralytic activity of Rpf.

Although we do not know precisely how Rpf stimulates the growth and resuscitation of 'non-culturable' bacteria, an important corollary exits to the hypothesis that the loss of physiological function is a consequence of the loss of muralytic activity. The dormant cells that accumulate during extended stationary phase in *M. luteus* (and depend on Rpf for their resuscitation) should have altered cell wall architecture, as compared with that of actively growing cells. By analogy with other bacteria, a higher degree of cross-linking would be predicted during stationary phase (Pisabarro *et al*., 1985; Atrih *et al*., 1999; Signoretto *et al*., 2000; 2002), as well as other possible modifications (Payie *et al*., 1995). It has been shown that dormant *M. luteus* cells have a significantly thickened cell wall (Mukamolova *et al*., 1999), but detailed biochemical analyses have not yet been reported. Perhaps, like bacterial endospores, these dormant cells contain a chemically modified form of peptidoglycan (Atrih and Foster, 2001a,b; Chirakkal *et al*., 2002). Limited hydrolysis of this material may be required to permit renewed cell wall expansion as these dormant cells resume growth. Rpf is also required when cells resume growth under nutrient-poor conditions and these too are associated with enhanced cross-linking of the peptidoglycan in other organisms (Signoretto *et al*., 2000; 2002). Moreover, this is consistent with the observed production of Rpf in the lag phase before *M. luteus* cells start to multiply (Mukamolova *et al*., 2002b).

Lytic transglycosylases may be important for the transport of macromolecules across the bacterial cell wall [reviewed by Koraimann (2003)]. We may hypothesize that Rpf could facilitate the penetration of some compounds required for the resuscitation or growth of the bacteria. Indeed, there are compounds in the culture supernatant with resuscitation/growth stimulation activity, consistent with this idea (Sun and Zhang, 1999; Zhang *et al*., 2001). It is also possible that resuscitation and growth stimulation are indirect manifestations of the hydrolytic activity of Rpf. The very low concentrations of Rpf required for activity (Mukamolova *et al*., 1998; 2002a) would be consistent with the possible release of small quantities of muropeptides that could act as signalling molecules. Muropeptides have strong modulatory effects on the immune system in host–parasite interactions (Rosenthal *et al*., 1987; Dziarski, 2003; Girardin *et al*., 2003) and they have also been implicated in the control of cell wall expansion and β-lactam resistance in Gram-negative, though not in Gram-positive bacteria (Jacobs *et al*., 1994; 1997; Korsak *et al*., 2005). The attenuation of virulence in triple Rpf knockout mutants of *M. tuberculosis* could have as its underlying basis a failure to release immuno-modulatory muropetides that may play a role in the development of active tuberculosis.

Characterization of the precise muralytic activity of Rpf, the products of its action on bacterial peptidoglycan and their possible influence on bacterial growth and resuscitation are challenges for the future.

Experimental procedures

Model of the Rpf domain structure

The Rpf structure predictions by the participants of the TMW experiment are available from the TMW online archive at the new URL (http://predictioncenter2.gc.ucdavis.edu/) and can be accessed from the Data Sharing Page via anonymous login. The three-dimensional model of the Rpf domain, guiding this work, was constructed by using a knowledge-based approach that performed successfully in the CASP4 and CASP5 prediction experiments (Murzin and Bateman, 2001). The atomic co-ordinates of this model and a short description of its construction are available via web link

http://predictioncenter2.gc.ucdavis.edu/submit/predictions/T0001G044a1.

Organisms and media

Micrococcus luteus NCIMB 13267 (Fleming strain 2665) was grown in Nutrient Broth E (NBE, LabM), LMM and succinate minimal medium (SMM) as described previously (Mukamolova *et al*., 1998). *My. smegmatis* was grown in NBE or Middlebrook medium (Difco). *E. coli* strains XL2 Blue and TOP 10 were grown in LB or SOB medium (Sambrook *et al*., 1989). *E. coli* strain LMG194 was grown in RM, which contained following components (per litre): Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; casamino acids, 20 g; glucose, 2 g. Thiamine and $MgCl_2$ were added to a final concentration of 1 mM.

Micrococcus luteus *cell wall preparation*

Micrococcus luteus cells grown to stationary phase in 1 l LB medium overnight were centrifuged at 10 000 *g*, washed with deionized water, resuspended in 200 ml 5% (w/v) SDS and boiled for 20 min. Following centrifugation, the pellet was resuspended in 100 ml 4% (w/v) SDS and boiled again for 20 min. The pellet was then thoroughly washed six times with 100 ml hot (65°C) water to remove the SDS. It was finally washed with acetone, air-dried, weighed and stored at –20°C. Before use, cell walls were suspended in deionized water and passed through a 23-gauge syringe needle to disrupt large clumps. For some experiments the cell wall preparation was dispersed and further disrupted using a Biospec mini bead beater before use.

Activity determination by zymography

Zymograms were made as described by Huard *et al*. (2003). Protein samples were subjected to electrophoresis through 12.5% (w/v) SDS-PAGE in gels containing cell walls or cell wall fragments [0.16–0.2% (w/v)]. Following SDS-PAGE, the gels were rinsed in 100 ml deionized water with gentle shaking for 20 min, and then in 100 ml of renaturation buffer for 30 min. Finally, they were transferred to 100 ml fresh renaturation buffer and incubated at 30°C overnight. To improve contrast, gels were stained with 0.1% methylene blue in 0.01% KOH and then de-stained as described previously (Potvin *et al*., 1988). Renaturation in 25 mM Tris/HCl buffer, pH 6.0, containing 0.2% of Triton X-100 gave the best results. A distinct clearance band with native Rpf was obtained when gels were incubated at 30°C or lower. Activity was significantly reduced in a phosphate/succinate buffer at the same pH and the presence of Mg^{2+} or Ca^{2+} (various concentrations up to 10 mM) did not improve activity.

Activity assay using fluorescamine-labelled cell walls

Cell wall preparations were labelled with fluorescamine as described by Mintz *et al*. (1975). Samples (10 μg) of freshly isolated Rpf protein were mixed with 50 μg fluorescamine-labelled cell walls in reaction buffer (50 mM Tris/HCl, pH 6.0; 5 mM $CaCl_2$). The reaction mixture (final volume 200 μl) was incubated at 30°C for 4 h. The reaction was terminated by adding 200 μl 4 M LiCl followed by centrifugation at 30 000 *g* for 40 min to remove insoluble cell wall material. After dilution to a final volume of 2 ml using reaction buffer, solubilized cell wall material in the supernatant was quantified by measuring the fluorescence (excitation at 390 nm, emission at 475 nm) with a Shimadzu RF-5301PC spectrofluorimeter. A control in which LiCl was added at the onset of the reaction was included for each sample to measure the extent of any non-specific degradation. The reaction was essentially linear over the first 4 h. All measurements were made using triplicate samples and the experiments were repeated twice with two different protein preparations. The results in the text and in Fig. 4 are expressed as per cent hydrolysis (fluorescence relative to an equivalent sample digested to completion with an excess of hen egg white lysozyme). All assays were carried out using pBAD-derived proteins (see below).

Activity assay using a fluorogenic substrate

The ability of wild type and mutated forms of Rpf to hydrolyse the fluorogenic substrate, MUF tri-NAG (Sigma) was determined as follows. The reaction mixture (400 μl) contained 1.5–1.9 μg freshly prepared recombinant protein in 50 mM Na-citrate buffer pH 6 containing 5 mM $MgSO_4$ and 8 μM MUF tri-NAG. After incubation at 37°C for 3 h, reactions were terminated by adding 2 μl 10 M NaOH. Fluorescence, measured with a Shimadzu RF-5301PC spectrofluorimeter using an excitation wavelength of 360 nm, increased linearly over a 4 h reaction period and there was no measurable activity in the 'minus enzyme' control. All assays were carried out using pBAD-derived proteins (see below) and 4-methyl umbelliferone was employed as a standard.

Western blotting

Rpf was detected by Western blotting using polyclonal rabbit antibodies that had been affinity purified as described previously (Mukamolova *et al*., 2002a). The antibodies were used at a dilution of 1:1000.

Production of recombinant Rpf

N-terminally his-tagged recombinant Rpf was obtained and purified as described previously (Mukamolova *et al*., 1998). To improve activity, Rpf was also expressed and secreted into the *E. coli* periplasm using the pBAD/gIII system (Invitrogen). The *rpf* gene was amplified from *M. luteus* genomic DNA using primers ML10F 5′-AGAATTC***CCATGG***CCACCGTG GACACC-3′ and ML10R 5′-GCCGTC***GTCGAC***CTGCG GCAGGACGAG-3′ (restriction sites included for cloning purposes are in bold italics) and the following thermal cycler program: 95°C for 5 min, then 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min and finally 72°C for 7 min. The polymerase chain reaction (PCR) product was trimmed with NcoI and SalI and cloned into the corresponding sites of pBAD/gIIIb to produce a version of the *rpf* gene with its native N-terminal secretory peptide and a hexa-histidine tag on the C-terminus. The sequence was verified and the

recombinant pBAD/gIIIb derivative was transformed into *E. coli* TOP 10 and *E. coli* LMG194. To make recombinant protein, cells were grown in SOB medium (for TOP 10) or in RM medium (for LMG194) to an OD_{600} of ~0.5 and then *rpf* expression was induced with 0.01% arabinose. Cells were harvested by centrifugation after 4 h and used for protein purification as indicated by Invitrogen. As insufficient protein was obtained from the osmotic shock liquid bacteria were sonicated to extract proteins. Note that some of the observed variability might result from the secretion of different proportions of protein in different experiments. The LysM domain of Rpf was amplified from *M. luteus* genomic DNA, using primers ML11LysM_F 5′-TGCTCGCAGA***CCATGG***GCCTGA CCCAGGCT′-3 and ML10R. After trimming with NcoI and SalI it was cloned into the equivalent sites in pBAD/gIIIb.

Purification of recombinant proteins

Proteins were purified from 500 ml cultures of *E. coli* strains, 4 h post-induction. Cell pellets were resuspended in 10 ml binding buffer (BB – 50 mM NaH_2PO_4, pH 8.0 and 300 mM NaCl), containing DNAase and RNAase both at concentrations of 10 μg ml^{-1}. After sonication, the crude extract was centrifuged at 20 000 *g* for 30 min to remove cell debris and the supernatant applied to a Ni-NTA column (Quiagen), equilibrated with BB. The column was washed with 20 vol BB followed by 20 vol BB containing 10 mM imidazole. Rpf proteins were recovered by elution with 20 ml elution buffer (20 mM Tris/HCl, pH 7.2; 100 mM histidine). Samples were desalted using Sephadex G20 columns according to the manufacturer's instructions. In some experiments proteins were further purified using a MonoQ column (Pharmacia) as described previously (Mukamolova *et al*., 1998).

Site-directed mutagenesis

The Gene Taylor kit (Invitrogen) was employed according to the manufacturer's instructions for site-directed mutagenesis of the *rpf* gene. The template was a pMTL20 derivative containing a 1.3 kb SmaI fragment of *M. luteus* genomic DNA encoding *rpf* (Chambers *et al*., 1988; Mukamolova *et al*., 1998). The following PCR program was employed: 2 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C and 4 min at 68°C and finally 10 min at 68°C. Double the recommended amount of high fidelity Platinum polymerase (Invitrogen) was employed and 10% (w/v) DMSO was present in the PCR reactions. Following PCR, the DNA was transformed into *E. coli* strain DH5αTR1 and all mutations were confirmed by sequencing. The mutations made and the primers used are summarized in Table S1. PCR products were made from the mutated pMTL20 derivatives using the ML10F/R primers and they were cloned as NcoI-SalI fragments into the corresponding sites of plasmid pBAD/gIIIb for protein expression, as described above.

To determine the biological activity of the modified proteins, the genes encoding them were cloned as 1.3 kb SmaI fragments into plasmid pAGH (linearized with XbaI and blunt-ended using T4 polymerase). This plasmid replicates autonomously in *M. luteus* and *My. smegmatis* (Mukamolova *et al*., 2002a,b). The orientation of the insert was determined using diagnostic restriction enzymes and derivatives with *rpf* under control of P_{ami} (as well as its own promoter) were transformed into *M. luteus* and *My. smegmatis* as described previously (Mukamolova *et al*., 2002a,b). The presence of the correctly mutated *rpf* in each strain was reconfirmed by sequencing the PCR products obtained using primers AmiF (5′-CCTGGGCACCTCACCGATCGA-3′) and AmiR (5′-CCGCTGAATATCGGGGAGCTC-3′) with boiled cells as a template.

Escherichia coli *lysis during Rpf expression*

All strains, including a control containing the pBAD/gIIIb vector, were grown in LB medium to an OD_{600} of 0.5–0.7 and protein expression induced by adding arabinose to a final concentration of 0.02%. The culture density (OD_{600}) was then monitored at approximately hourly intervals. Examples of the curves obtained are given in Fig. 3. The culture density 5 h post-induction was expressed as a percentage of the maximum value attained (normally between 2 and 3 h following induction). During expression of wild-type Rpf the culture typically loses about 60% of its maximum OD_{600}. This is represented as the maximum (i.e. 100%) value in Fig. 4 and the values for the Rpf mutants are expressed as a percentage of this. In cases where there was no lysis a value of zero is recorded.

Preparation of crude cell extract and culture supernatant from M. luteus

Micrococcus luteus cells were grown in 10 ml NBE to OD_{600} of 1.0, washed twice with deionized water, resuspended in 0.5 ml sample buffer for SDS-PAGE and disrupted using a Biospec mini bead beater for 3×30 s. After centrifugation, the cell extract was boiled and applied to a 12.5% (w/v) polyacrylamide gel. To prepare concentrated culture supernatant, 2 ml samples of the supernatant from log-phase cultures OD_{600} of 1.5–2.0 was desalted on Sephadex G20 columns and then precipitated by adding 4 vol ice-cold ethanol. The protein pellet was reconstituted in 20 μl sample buffer and applied to a polyacrylamide gel, as above. Bands corresponding to Rpf were identified by immunoblotting with either anti-Rpf or anti-His tag antibodies as described previously (Mukamolova *et al*., 2002a).

Micrococcus luteus *growth assay*

Micrococcus luteus cells grown to stationary phase in NBE for 48 h were inoculated into 20 ml LMM, containing 50 μg hygromycin ml^{-1}. The initial cell concentration was either 10^5 or 10^2 cells ml^{-1}, depending on the experiment. Cultures were incubated at 30°C with shaking (200 r.p.m.) and sampled periodically to measure cfu by plating on NBE agar. To determine culturability, samples were plated on both NBE and LMM agar plates and the values given in Table 2 represent cfu_{LMM}/cfu_{NBE}. For the comparisons in Fig. 5C, 10 μl samples of serially diluted bacterial suspensions (10^{-1}–10^{-6}) were plated on LMM agar and incubated at 30°C for 120 h.

Mycobacterium smegmatis *resuscitation assay*

Plasmids containing mutated *rpf* genes were introduced into *My. smegmatis* as previously described (Shleeva *et al*., 2004). 'Non-culturable' cells of *My. smegmatis* strain mc^2155 were obtained after growth in modified Hartman's–de Bont medium supplemented with 0.5% BSA (Cohn – analogue, Sigma) until stationary phase as described by Shleeva *et al*. (2004). Culture samples were plated on NBE agar to check that they had attained a 'non-culturable' state. Resuscitation and MPN assays were performed in 48-well plastic plates (Corning) containing 0.45 ml Sauton's medium supplemented with 0.05% yeast extract (LabM). Serially diluted samples of *My. smegmatis* cultures (50 μl) were added to each well and plates were incubated at 37°C with agitation at 150 r.p.m. for 5 days. Wells with visible bacterial growth were counted as positive and MPN values were calculated using standard statistical methods (de Man, 1975). To ensure that the bacteria were 'non-culturable' and not simply dead, controls were performed in each experiment. Bacteria were resuscitated as above except that 10^5–10^6 *M. luteus* cells (i.e. organisms actively producing and secreting Rpf) were added to the wells at the time of inoculation. The *M. luteus* cells were taken from an exponentially growing NBE culture and diluted 100-fold in Sauton's medium (they do not interfere with the measurement of *My. smegmatis* resuscitation as they do not grow in Sauton's medium).

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References

Abbott, A. (2001) Computer modellers seek out 'Ten Most Wanted' proteins. *Nature* **409:** 4.

van Asselt, E.J., Dijkstra, A.J., Kalk, K.H., Takacs, B., Keck, W., and Dijkstra, B.W. (1999a) Crystal structure of *Escherichia coli* lytic transglycosylase Slt35 reveals a lysozyme-like catalytic domain with an EF-hand. *Structure Fold Des* **7:** 1167–1180.

van Asselt, E.J., Thunnissen, A.M., and Dijkstra, B.W. (1999b) High resolution crystal structures of the *Escherichia coli* lytic transglycosylase Slt70 and its complex with a peptidoglycan fragment. *J Mol Biol* **291:** 877–898.

Atrih, A., and Foster, S.J. (2001a) Analysis of the role of bacterial endospore cortex structure in resistance properties and demonstration of its conservation amongst species. *J Appl Microbiol* **91:** 364–372.

Atrih, A., and Foster, S.J. (2001b) *In vivo* roles of the germination-specific lytic enzymes of *Bacillus subtilis* 168. *Microbiology* **147:** 2925–2932.

Atrih, A., Bacher, G., Allmaier, G., Williamson, M.P., and Foster, S.J. (1999) Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. *J Bacteriol* **181:** 3956–3966.

Auerbach, G., Herrmann, A., Bracher, A., Bader, G., Gutlich, M., Fischer, M., *et al.* (2000) Zinc plays a key role in human and bacterial GTP cyclohydrolase I. *Proc Natl Acad Sci USA* **97:** 13567–13572.

Bateman, A., and Bycroft, M. (2000) The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD). *J Mol Biol* **299:** 1113–1119.

Bayer, M., Iberer, R., Bischof, K., Rassi, E., Stabentheiner, E., Zellnig, G., and Koraimann, G. (2001) Functional and mutational analysis of p19, a DNA transfer protein with muramidase activity. *J Bacteriol* **183:** 3176–3183.

Blokpoel, M.C.J., Murphy, H.N., O'Toole, R., Wiles, S., Runn, E.S.C., Stewart, G.R., Young, D.B., and Robertson, B.D. (2005) Tetracycline-inducible gene regulation in mycobacteria. *Nucl Acids Res* **33:** e22.

Chambers, S.P., Prior, S.E., Barstow, D.A., and Minton, N.P. (1988) The pMTL *nic*-cloning vectors. 1. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* **68:** 139–149.

Chirakkal, H., O'Rourke, M., Atrih, A., Foster, S.J., and Moir, A. (2002) Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination. *Microbiology* **148:** 2383–2392.

Cohen-Gonsaud, M., Keep, N.H., Davies, A.P., Ward, J., Henderson, B., and Labesse, G. (2004) Resuscitation-promoting factors possess a lysozyme-like domain. *Trends Biochem Sci* **29:** 7–10.

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

Dhillon, J., Lowrie, D.B., and Mitchison, D.A. (2004) *Mycobacterium tuberculosis* from chronic murine infections that grows in liquid but not on solid medium. *BMC Infect Dis* **4:** 51.

Downing, K.J., Betts, J.C., Young, D.I., McAdam, R.A., Kelly, F., Young, M., and Mizrahi, V. (2004) Global expression profiling of strains harbouring null mutations reveals that the five *rpf*-like genes of *Mycobacterium tuberculosis* show functional redundancy. *Tuberculosis* **84:** 167–179.

Downing, K.J., Mischenko, V.V., Shleeva, M.O., Young, D.I., Young, M., Kaprelyants, A.S., *et al.* (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.

Dziarski, R. (2003) Recognition of bacterial peptidoglycan by the innate immune system. *Cell Mol Life Sci* **60:** 1793–1804.

Ehlert, K., Höltje, J.V., and Templin, M.F. (1995) Cloning and expression of a murein hydrolase lipoprotein from *Escherichia coli*. *Mol Microbiol* **16:** 761–768.

Finan, C.L. (2003) *Autocrine growth factors in streptomycetes*. PhD Thesis. Aberystwyth: University of Wales.

Fischer, B., Perry, B., Phillips, G., Sumner, I., and Goodenough, P. (1993) Physiological consequence of expression

of soluble and active hen egg white lysozyme in *Escherichia coli*. *Appl Microbiol Biotechnol* **39:** 537–540.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., *et al.* (2003) Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* **300:** 1584–1587.

Grutter, M.G., Weaver, L.H., and Matthews, B.W. (1983) Goose lysozyme structure: an evolutionary link between hen and bacteriophage lysozymes? *Nature* **303:** 828–831.

Hartmann, M., Barsch, A., Niehaus, K., Puhler, A., Tauch, A., and Kalinowski, J. (2004) The glucosylated cell surface protein, Rpf2, containing a resuscitation-promoting factor motif, is involved in intercellular communication of *Corynebacterium glutamicum*. *Arch Microbiol* **182:** 299–312.

Heidrich, C., Ursinus, A., Berger, J., Schwarz, H., and Holtje, J.V. (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.

Hernández-Pando, R., Jeyanathan, M., Mengistu, G., Aguilar, D., Orozco, H., Harboe, M., *et al.* (2000) Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection. *Lancet* **356:** 2133–2138.

Holtje, J.V., Mirelman, D., Sharon, N., and Schwarz, U. (1975) Novel type of murein transglycosylase in *Escherichia coli*. *J Bacteriol* **124:** 1067–1076.

Hoppner, C., Liu, Z., Domke, N., Binns, A.N., and Baron, C. (2004) VirB1 orthologs from *Brucella suis* and pKM101 complement defects of the lytic transglycosylase required for efficient type IV secretion from *Agrobacterium tumefaciens*. *J Bacteriol* **186:** 1415–1422.

Huard, C., Miranda, G., Wessner, F., Bolotin, A., Hansen, J., Foster, S.J., and Chapot-Chartier, M.P. (2003) Characterization of AcmB, an *N*-acetylglucosaminidase autolysin from *Lactococcus lactis*. *Microbiology* **149:** 695–705.

Jacobs, C., Huang, L.J., Bartowsky, E., Normark, S., and Park, J.T. (1994) Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction. *EMBO J* **13:** 4684–4694.

Jacobs, C., Frere, J.M., and Normark, S. (1997) Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in Gram-negative bacteria. *Cell* **88:** 823–832.

Kaprelyants, A.S., Mukamolova, G.V., and Kell, D.B. (1994) Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell-free spent medium at high dilution. *FEMS Microbiol Lett* **115:** 347–352.

Kazarian, K.A., Yeremeev, V.V., Kondratieva, T.K., Telkov, M.V., Kaprelyants, A.S., and Apt, A.S. (2003) *Proteins of Rpf Family as Novel TB Vaccine Candidates.* First International Conference on TB Vaccines for the World, Montreal, Canada.

Kell, D.B., and Young, M. (2000) Bacterial dormancy and culturability: the role of autocrine growth factors. *Curr Opin Microbiol* **3:** 238–243.

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.L., and Barer, M.R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Ant van Leeuwenhoek* **73:** 169–187.

Koraimann, G. (2003) Lytic transglycosylases in macromolecular transport systems of Gram-negative bacteria. *Cell Mol Life Sci* **60:** 2371–2388.

Korsak, D., Liebscher, S., and Vollmer, W. (2005) Susceptibility to antibiotics and beta-lactamase induction in murein hydrolase mutants of *Escherichia coli*. *Antimicrob Agents Chemother* **49:** 1404–1409.

Kusser, W., and Schwarz, U. (1980) *Escherichia coli* murein transglycosylase. Purification by affinity chromatography and interaction with polynucleotides. *Eur J Biochem* **103:** 277–281.

de Man, J.C. (1975) The probability of most probable numbers. *Eur J Appl Microbiol* **1:** 67–78.

Mintz, G., Herbold, D.R., and Glaser, L. (1975) A fluorescent assay for bacterial cell wall lytic enzymes. *Anal Biochem* **66:** 272–278.

Moak, M., and Molineux, I.J. (2000) Role of the Gp16 lytic transglycosylase motif in bacteriophage T7 virions at the initiation of infection. *Mol Microbiol* **37:** 345–355.

Mukamolova, G.V., Kaprelyants, A.S., Young, D.I., Young, M., and Kell, D.B. (1998) A bacterial cytokine. *Proc Natl Acad Sci USA* **95:** 8916–8921.

Mukamolova, G.V., Kormer, S.S., Kell, D.B., and Kaprelyants, A.S. (1999) Stimulation of the multiplication of *Micrococcus luteus* by an autocrine growth factor. *Arch Microbiol* **172:** 9–14.

Mukamolova, G.V., Turapov, O.A., Young, D.I., Kaprelyants, A.S., Kell, D.B., and Young, M. (2002a) A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Mol Microbiol* **46:** 623–635.

Mukamolova, G.V., Turapov, O.A., Kazaryan, K., Telkov, M., Kaprelyants, A.S., Kell, D.B., and Young, M. (2002b) The *rpf* gene of *Micrococcus luteus* encodes an essential secreted growth factor. *Mol Microbiol* **46:** 611–621.

Mukamolova, G.V., Kaprelyants, A.S., Kell, D.B., and Young, M. (2003) Adoption of the transiently non-culturable state – a bacterial survival strategy? *Adv Microb Physiol* **47:** 65–129.

Murzin, A.G., and Bateman, A. (2001) CASP2 knowledge-based approach to distant homology recognition and fold prediction in CASP4. *Proteins* (Suppl. 5)**:** 76–85.

Murzin, A.G., Brenner, S.E., Hubbard, T., and Chothia, C. (1995) SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J Mol Biol* **247:** 536–540.

Mushegian, A.R., Fullner, K.J., Koonin, E.V., and Nester, E.W. (1996) A family of lysozyme-like virulence factors in bacterial pathogens of plants and animals. *Proc Natl Acad Sci USA* **93:** 7321–7326.

Nambu, T., Minamino, T., Macnab, R.M., and Kutsukake, K. (1999) Peptidoglycan-hydrolyzing activity of the FlgJ protein, essential for flagellar rod formation in *Salmonella typhimurium*. *J Bacteriol* **181:** 1555–1561.

Payie, K.G., Rather, P.N., and Clarke, A.J. (1995) Contribution of gentamicin 2′-*N*-acetyltransferase to the O acetylation of peptidoglycan in *Providencia stuartii*. *J Bacteriol* **177:** 4303–4310.

Pedulla, M.L., Ford, M.E., Houtz, J.M., Karthikeyan, T., Wadsworth, C., Lewis, J.A., *et al.* (2003) Origins of highly mosaic mycobacteriophage genomes. *Cell* **113:** 171–182.

Pisabarro, A.G., de Pedro, M.A., and Vazquez, D. (1985) Structural modifications in the peptidoglycan of *Escheri-*

chia coli associated with changes in the state of growth of the culture. *J Bacteriol* **161:** 238–242.

Potvin, C., Leclerc, D., Tremblay, G., Asselin, A., and Bellemare, G. (1988) Cloning, sequencing and expression of a *Bacillus* bacteriolytic enzyme in *Escherichia coli*. *Mol Gen Genet* **214:** 241–248.

Ravagnani, A., Finan, C.L., and Young, M. (2005) A novel firmicute protein family related to the actinobacterial resuscitation-promoting factors by non-orthologous domain displacement. *BMC Genomics* **6:** 39.

Rosenthal, R.S., Nogami, W., Cookson, B.T., Goldman, W.E., and Folkening, W.J. (1987) Major fragment of soluble peptidoglycan released from growing *Bordetella pertussis* is tracheal cytotoxin. *Infect Immun* **55:** 2117–2120.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Shleeva, M., Mukamolova, G.V., Young, M., Williams, H.D., and Kaprelyants, A.S. (2004) Formation of non-culturable cells of *Mycobacterium smegmatis* in stationary phase in response to growth under sub-optimal conditions and their Rpf-mediated resuscitation. *Microbiology* **150:** 1687–1697.

Signoretto, C., Lleo, M.M., Tafi, M.C., and Canepari, P. (2000) Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. *Appl Environ Microbiol* **66:** 1953–1959.

Signoretto, C., del Mar Lleo, M., and Canepari, P. (2002) Modification of the peptidoglycan of *Escherichia coli* in the viable but nonculturable state. *Curr Microbiol* **44:** 125–131.

Sun, Z., and Zhang, Y. (1999) Spent culture supernatant of *Mycobacterium tuberculosis* H37Ra improves viability of aged cultures of this strain and allows small inocula to initiate growth. *J Bacteriol* **181:** 7626–7628.

Thunnissen, A.M.W.H., Dijkstra, A.J., Kalk, K.H., Rozeboom, H.J., Engel, H., Keck, W., and Dijkstra, B.W. (1994) Doughnut-shaped structure of a bacterial muramidase revealed by X-ray crystallography. *Nature* **367:** 750–754.

Thunnissen, A.M., Isaacs, N.W., and Dijkstra, B.W. (1995) The catalytic domain of a bacterial lytic transglycosylase defines a novel class of lysozymes. *Proteins* **22:** 245–258.

Tramontano, A. (2003) Of men and machines. *Nat Struct Biol* **10:** 87–90.

Tufariello, J.M., Jacobs, W.R., Jr, and Chan, J. (2004) Individual *Mycobacterium tuberculosis* resuscitation-promoting factor homologues are dispensable for growth *in vitro* and *in vivo*. *Infect Immun* **72:** 515–526.

Weaver, L.H., Grutter, M.G., and Matthews, B.W. (1995) The refined structures of goose lysozyme and its complex with a bound trisaccharide show that the 'goose-type' lysozymes lack a catalytic aspartate residue. *J Mol Biol* **245:** 54–68.

Zhang, Y., Yang, Y., Woods, A., Cotter, R.J., and Sun, Z. (2001) Resuscitation of dormant *Mycobacterium tuberculosis* by phospholipids or specific peptides. *Biochem Biophys Res Commun* **284:** 542–547.

Zhu, W., Plikaytis, B.B., and Shinnick, T.M. (2003) Resuscitation factors from mycobacteria: homologs of *Micrococcus luteus* proteins. *Tuberculosis (Edinb)* **83:** 261–269.

Supplementary material

The following supplementary material is available for this article online:

Table S1. Mutagenic primers.

This material is available as part of the online article from http://www.blackwell-synergy.com

