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# The *rpf* gene of *Micrococcus luteus* encodes an essential secreted growth factor

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## Summary

***Micrococcus luteus* secretes a small protein called Rpf, which has autocrine and paracrine signalling functions and is required for the resuscitation of dormant cells. Originally isolated from the supernatant of actively growing cultures, Rpf was also detected on the surface of actively growing bacteria. Most molecules may be sequestered non-productively at the cell surface, as a truncated form of the protein, encompassing only the 'Rpf domain' is fully active. The C-terminal LysM module, which probably mediates binding to the cell envelope, is not required for biological activity. Rpf was essential for growth of *M. luteus*. Washed cells, inoculated at low density into a minimal medium, could not grow in its absence. Moreover, the incorporation of anti-Rpf antibodies into the culture medium at the time of inoculation also prevented bacterial growth. We were unable to inactivate *rpf* using a disrupted form of the gene, in which most of the coding sequence was replaced with a selectable thiostrepton resistance marker. Gene disruption was possible in the presence of a second, functional, plasmid-located copy of *rpf*, but not in the presence of a *rpf* derivative whose protein product lacked the secretory signal sequence. As far as we are aware, Rpf is the first example of a truly secreted protein that is essential for bacterial growth. If the Rpf-like proteins elaborated by *Mycobacterium tuberculosis* and other mycobacteria prove similarly essential, interference with their proper functioning may offer novel opportunities for protecting against, and treating, tuberculosis and other mycobacterial disease.**

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## Introduction

*Micrococcus luteus* is a member of the high G + C cohort of Gram-positive bacteria that may be isolated from human skin, soil and water. After prolonged incubation in stationary phase in laboratory culture, the non-sporulating *M. luteus* assumes a dormant state, with the formation of morphologically differentiated structures that do not resemble spores and are not immediately culturable (Kaprelyants and Kell, 1993a; Kaprelyants *et al.*, 1993; 1994; Mukamolova *et al.*, 1995a,b; 1998a; Votyakova *et al.*, 1994). Adoption of this state of low metabolic activity (Kaprelyants and Kell, 1993a,b) in natural environments would permit survival for extended periods when conditions are not conducive for growth.

Under laboratory conditions, viable cells of *M. luteus* promoted the resuscitation of 'non-culturable' cells (Votyakova *et al.*, 1994). Activity resided in a small protein, the *r*esuscitation-*p*romoting *f*actor (Rpf), that is produced by viable cells and is found in the supernatant of actively growing cultures (Mukamolova *et al.*, 1998b; 1999). The predicted product of the *rpf* gene, which encodes Rpf, has a secretory signal sequence at its N-terminus. When a recombinant form lacking the signal sequence was added to dormant cells they were resuscitated. These features suggested that following secretion, Rpf functions from an extra-cytoplasmic location by interaction with a component of the bacterial cell envelope. A requirement for exogenously added Rpf was also conferred on what were previously normally growing cells by extensive washing, and inoculation at low cell density in a minimal medium (Mukamolova *et al.*, 1998b). Therefore, in addition to its function in resuscitating 'non-culturable' cells, Rpf has a more general role as a bacterial growth factor or cytokine (Mukamolova *et al.*, 1998b). In this paper we explore the possibility that Rpf production and secretion are in fact essential for growth of *M. luteus*.

## Results

### *A single* rpf*-like gene in* M. luteus

The *rpf* gene of *M. luteus* (acc. no. Z96935), whose product was isolated and characterized previously (Mukamolova *et al.*, 1998b), appears to form a monocistronic operon (Fig. 1A). The upstream (incomplete) open

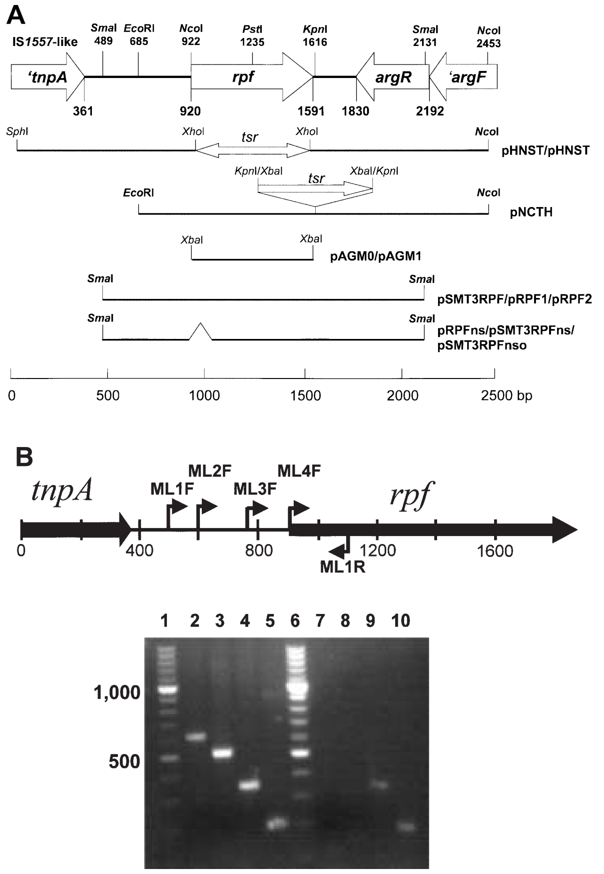

**Fig. 1.** A. The chromosomal context of *rpf* in *M. luteus*.
B. The localization of the *rpf* promoter using RT-PCR. PCR (lanes 2–5) and RT-PCR (lanes 7–10) products obtained with primers ML1F + ML1R (lanes 2 and 7), ML2F + ML1R (lanes 3 and 8), ML3F + ML1R (lanes 4 and 9), ML4F + ML1R (lanes 5 and 10). Lanes 1 and 6 contain a 100 bp ladder.

reading frame (ORF) lies on the same strand as *rpf* and encodes a protein with substantial similarity to the IS*1557* transposase of *Mycobacterium tuberculosis* (65% identity over 60 residues with Rv3798 and Rv1313c). Hybridization probes containing this region detected at least five different restriction fragments in genomic DNA (data not shown), consistent with the presence of multiple copies of an IS*1557*-like element in *M. luteus* NCIMB 13267. Immediately downstream from *rpf* is a stem–loop structure that may correspond to a transcription terminator. The downstream ORFs encoding ArgR and ArgF are convergently transcribed with *rpf*. The former contains PFAM motif 01316 (ArgR repressor DNA-binding domain) and shows greatest similarity to ArgR from various streptomycetes, mycobacteria and corynebacteria (44% identity over 93 residues with ArgR of *Streptomyces clavuligerus*). The latter partial ORF contains PFAM motif 00185 (ornithine carbamoyltransferase, Asp/Orn binding domain) and it shows similarity to ArgF from a wide variety of organisms (62% identity over 86 residues with ArgF of *Corynebacterium glutamicum*).

Using mRNA purified from exponentially growing bacteria, reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out using a single reverse primer (ML1R) and four different forward primers (ML1F-ML4F) from the 559 bp intergenic region between *rpf* and *tnpA* (Fig. 1A). Products were only obtained with the two *rpf*-proximal primers (Fig. 1B), indicating that *rpf* is transcribed from a promoter lying between co-ordinates 594 and 754 in Fig. 1A, and confirming that *rpf* is transcribed independently of the upstream ORF.

Southern hybridization experiments had previously suggested that *M. luteus* may contain a second *rpf*-like gene (Mukamolova *et al*., 1998b). However, the additional hybridization signal previously observed was in fact associated with a cryptic plasmid, pMLU1, harboured by *M. luteus* NCIMB 13267. This was detected by vector sequences in the probe that was used in the earlier work. It was not seen when a PCR product was used as probe (data not shown). The characteristics of pMLU1 (accession no. AJ439695), which closely resembles plasmid pMBCP isolated from *Ralstonia pickettii* (96% sequence identity with AF144733), will be described elsewhere. The available evidence therefore indicates that in contrast to most other organisms that contain *rpf*-like genes (Kell and Young, 2000), *M. luteus* is unusual in that it contains a single gene, lying in a monocistronic operon.

### *Rpf production during the growth cycle in batch culture*

To monitor Rpf production during the bacterial growth cycle, mRNA was isolated at various times following inoculation of stationary phase cells at high density into (rich) NBE medium. Within 30 min of inoculation, transcripts (normalized per cell) were just detectable by RT-PCR (using primers ML1R and ML4F – see Fig. 1). The detection limit was <1 pg cDNA per $10^8$ cells, which was roughly equivalent to 10 mRNA molecules per cell (assuming both mRNA extraction and conversion of mRNA to cDNA were essentially complete). Within 1 h of inoculation, transcripts were abundant and they remained so throughout the lag phase (Fig. 2A shows the results of a representative experiment). Transcript abundance per cell declined substantially while the bacteria were still in early mid-log phase and transcripts were not detected in late stationary phase (48 h post inoculation). Enzyme-linked immunosorbent assays (ELISAs) in which Rpf accumulation in culture supernatant was monitored under similar conditions revealed that Rpf became detectable only 5 h post inoculation, by which time almost two doublings had occurred (Fig. 2B). Rpf accumulated in the culture medium during early mid-exponential phase and

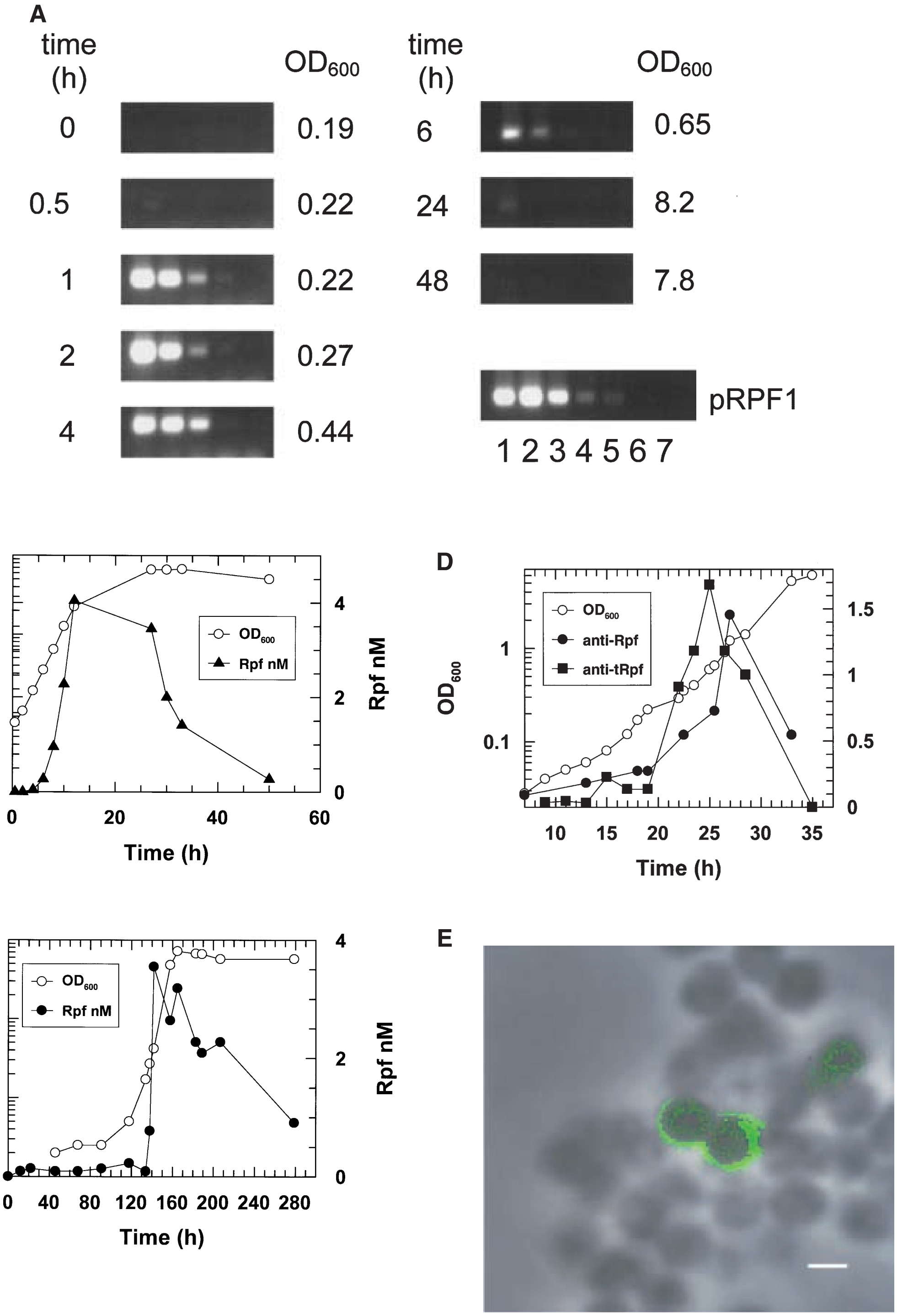

**Fig. 2.** Kinetics of *rpf* expression and Rpf accumulation during bacterial growth.

A. Relative amounts of *rpf* mRNA following inoculation of NBE with ~ $10^8$ cfu ml$^{-1}$ were estimated by RT-PCR, using serially diluted cDNA samples isolated from a standard number of cells at the times stated (the corresponding culture densities are also given). The band intensities of the PCR product obtained using serial 10-fold dilutions of pRPF1 as template are also shown (lane 5 contains 0.94 pg pRPF1).

B–D. The results of ELISAs of Rpf in the supernatants of NBE (B and D) and LMM (C) cultures various times after inoculation with cells. These were previously grown in: B, NBE for 20 h ($OD_{600}$ = 4.6, $10^7$ cells ml$^{-1}$ inoculated); C, LMM for three days ($OD_{600}$ = 5.0, 4.$10^6$ cells ml$^{-1}$ inoculated); D, NBE for 24 h ($10^4$ cells ml$^{-1}$ inoculated). Anti-Rpf antibodies were used in B and C.

E. Confocal microscopy of bacteria incubated with anti-Rpf antibodies, followed by a FITC-labelled secondary antibody was used to visualize Rpf on the bacterial cell envelope.

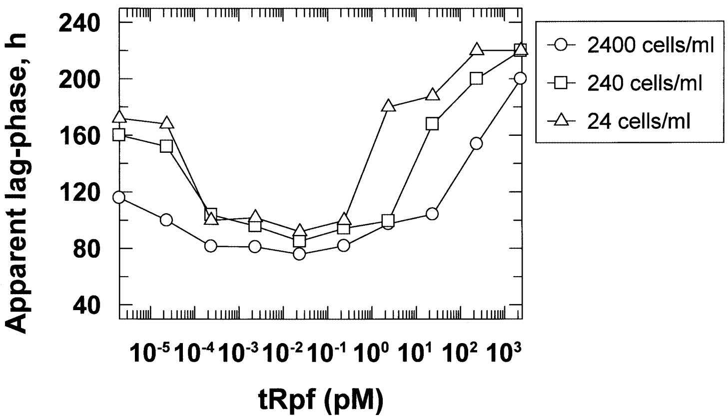

**Fig. 3.** The lysM module at the C-terminus of Rpf is not required for biological activity. Serially diluted samples of the truncated form of Rpf (residues $A_{42}$-$L_{118}$ with an N-terminal his-tag) were added to LMM that had been inoculated at low cell density with *M. luteus*. The time to detectable turbidity (apparent lag phase) was determined (see *Experimental procedures*). The values on the ordinate are control values (apparent lag phase in the absence of tRpf).

declined thereafter, reaching a value close to zero 50 h post inoculation. There was a clear delay between the onset of *rpf* transcription and the appearance of Rpf in the culture supernatant. Similar results to those shown in Fig. 2B were obtained after inoculation of bacteria in LMM (Fig. 2C); growth was evident several hours before Rpf was detectable in the culture supernatant. Moreover, the pattern of *rpf* transcription was very similar to that shown in Fig. 2A (data not shown). Finally, bacteria were inoculated into NBE at a lower initial density in order to investigate in greater detail the relationship between Rpf appearance and bacterial growth. A younger inoculum was used for this experiment to ensure that the growth rate was constant over the period of measurement (Fig. 2D). During the early stages very little Rpf was detected in the culture supernatant, but there was massive release of Rpf between 19 and 25 h (Fig. 2D). In this particular experiment, Rpf accumulation was monitored using two different antibodies. Although the curves were qualitatively similar, the protein detected by the anti-truncated Rpf antibodies appeared sooner than that detected by the anti-Rpf antibodies. One possible explanation is that different conformational forms of Rpf may be present in the culture supernatant, which are differentially detected by the two antibody preparations.

Examination of bacteria by confocal microscopy after labelling with anti-Rpf antibodies (see *Experimental procedures*) revealed the probable reason for the delay between *rpf* expression and Rpf accumulation in the culture supernatant. Rpf was present on the bacterial cell surface (Fig. 2E). The organisms shown in the Figure were from mid-exponential phase, and there was considerable heterogeneity with respect to the intensity of their fluorescence. In other samples taken at a much earlier stage during the growth cycle in batch culture (1 h post inoculation), a more substantial fraction of the cells (~ 30%) were labelled (data not shown).

### *A truncated form of Rpf is fully active*

The LysM module present at the C-terminus of Rpf probably promotes its association with the peptidoglycan of the cell envelope (Bateman and Bycroft, 2000). To determine whether the LysM module is necessary for biological activity, a truncated version of Rpf was produced corresponding to residues $A_{42}$-$L_{118}$ with an N-terminal his-tag (see *Experimental procedures*). Intact Rpf, is usually active at pM concentrations (Mukamolova *et al*., 1998b), whereas the truncated form was active at fM concentrations (Fig. 3). This experiment indicates that bacterial growth is stimulated by the ~ 70 residue 'Rpf domain' shared by all Rpf-like proteins (Kell and Young, 2000) and that the LysM module of Rpf is not required for biological activity.

### *Rpf is required for bacterial growth*

Previous work (Mukamolova *et al*., 1998b) suggested that unless bacteria have Rpf molecules located at their cell surface, they are unable to grow. This hypothesis was tested using anti-Rpf antibodies. In contrast to preimmune serum (and the IgG fraction purified from it) immune serum inhibited bacterial growth. Further experiments were carried out using affinity-purified antibodies. Complete growth inhibition was observed when bacteria were inoculated into a minimal medium (LMM) containing anti-Rpf antibodies at a 1 : 10 000 dilution and this inhibition was abolished if recombinant Rpf was added simultaneously (Fig. 4). As there is no evidence from Western blotting experiments that our antibodies react with any other *M. luteus* proteins (data not shown), their inhibitory effect on bacterial growth is most probably accounted for by sequestration of Rpf molecules located at or near the bacterial cell surface. These data strongly support the hypothesis that Rpf is required for bacterial growth. The antibody-mediated inhibition of bacterial growth in liquid

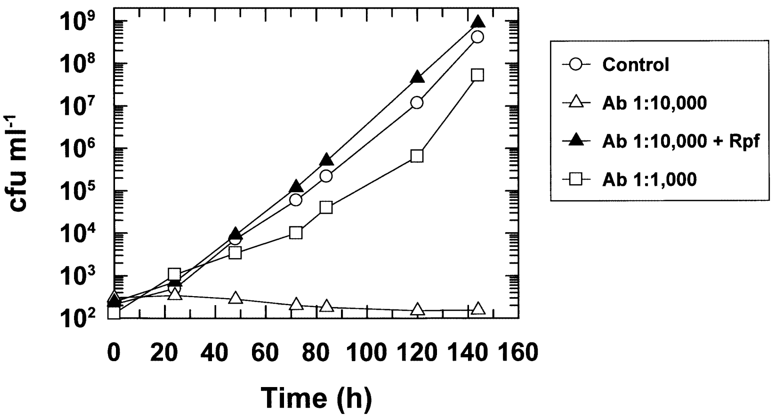

**Fig. 4.** Anti-Rpf antibodies inhibit bacterial growth. *Micrococcus luteus* was inoculated at low cell density in LMM containing anti-Rpf antibodies and Rpf (200 pM) as indicated, and samples were taken with time for determination of cfu.

medium was dependent on the antibody concentration. In the experiment shown in Fig. 4, for example, there was no inhibition when the antibody preparation was used at a 1 : 1000 dilution. The reason for a lack of inhibition at elevated antibody concentrations has not yet been established. As might be expected, the optimal antibody concentration for growth inhibition was dependent on the size of the inoculum, with higher concentrations being required to inhibit larger inocula (data not shown). Finally, the antibodies were without any inhibitory effect when added to actively growing cells (e.g. cultures at $OD_{600} = 1.0$) and they did not inhibit colony formation on agar-solidified medium (data not shown).

### Rpf *is an essential gene in* M. luteus

Genetic methods (see *Experimental procedures*) were used to determine whether *rpf* is an essential gene in *M. luteus*. A thiostrepton resistance (Tsr$^R$) cassette was successfully inserted into the *Kpn*I site that lies just downstream from the 3′ end of *rpf*, using the insert from plasmid pNCTH (see Fig. 1A). A total of 58 transformants was obtained and all 12 tested by Southern hybridization had the expected structure.

Many attempts were made to inactivate *rpf* using a DNA fragment in which the majority of the *rpf* coding sequence had been replaced by the Tsr$^R$ cassette (*rpf*::*tsr* inserts from plasmids pHNST and pHNSTo). In some experiments, recombinant Rpf was added at several different concentrations (100 pM, 10 pM, 1 pM) coincident with plating on selective medium whereas in others, the transformed bacteria were incorporated into a soft agar overlay containing 50% (v/v) sterile culture supernatant. In another series of experiments, Rpf or sterile culture supernatant was provided during the phenotypic expression period before plating on selective medium. None of these treatments permitted the recovery of bacteria in which *rpf* had been inactivated. A total of only 14 transformants was obtained and all of them retained an intact copy of *rpf* (as revealed by PCR and Southern hybridization). Six of them were Hyg$^R$ and they contained pHNST (a low-level contaminant in the DNA preparation used for all of the transformation experiments – see *Experimental procedures*). The *rpf*::*tsr* insert from plasmid pHNST contains part of an IS*1557*-like element (see Fig. 1). Some (or all) of the eight remaining transformants may have arisen from recombination with copies of this element located elsewhere in the bacterial chromosome, but this was not further investigated. Finally, we also attempted to obtain a *rpf* null mutant by cultivation of transformed cells in liquid medium. After a 1 h recovery period in NBE, post transformation, samples were serially diluted in tubes containing a rich (NBE) or poor (LMM) medium supplemented with thiostrepton and either recombinant Rpf or culture supernatant. In total, 14 different experiments were carried out and bacteria from 91 different tubes at the lowest dilutions where growth had occurred were screened by PCR using primers MLF5 + MLR5. All of the tube cultures gave a 728 bp PCR product corresponding to the wild-type gene. Two of them gave, in addition, a 1215 bp product corresponding to the inactivated gene (*rpf*::*tsr*). We were unable to subculture these two clones in liquid or on solid media, whereas the remainder were culturable (77 of them were also Hyg$^R$, indicating that they contained pHNST – see above).

The results of all of these experiments suggested that it is not possible to disrupt *M. luteus rpf*, consistent with the hypothesis that it is an essential gene.

This conclusion was reinforced by experiments using strains carrying a second, plasmid-located copy of *rpf*. A 1375 bp *Sma*I fragment containing the entire *rpf* gene together with its promoter (see Fig. 1A and B) was incorporated into plasmid pSMT3 (Hyg$^R$) (Garbe *et al*., 1994) in both orientations, generating plasmids pSMT3RPF and pSMT3FPR. In the former, *rpf* expression is assured from $P_{Rpf}$ as well as the strong $P_{hsp60}$ promoter. In the latter, *rpf* would be expressed from its own promoter but the strong $P_{hsp60}$ promoter would potentially produce a counter-transcript of *rpf*. These plasmids were introduced into *M. luteus* together with the vector alone (pSMT3) and the resulting $Hyg^R$ strains were transformed with the *rpf*::*tsr* insert isolated from plasmid pHNST. The strain harbouring plasmid pSMT3RPF yielded 28 thiostrepton-resistant transformants and in 95% of those tested by Southern hybridization, the chromosomal copy of *rpf* had been inactivated. One of these transformants, denoted MA1, was retained. The strains harbouring plasmid pSMT3 and pSMT3FPR yielded only three $Tsr^R$ transformants each. In all cases, the chromosomally located *rpf* gene was still intact.

In plasmid pAGM0, the *rpf* gene together with its presumed ribosome binding site, but lacking its promoter, was placed under the control $P_{ami}$ from *My. smegmatis* (Parish *et al*., 1997). Plasmids pAGM0 and pAGH (vector), were introduced into *M. luteus* and the resulting strains (MA2 and MA3 respectively) were then transformed with the *rpf*::*tsr* insert from plasmid pHNST. Only five transformants were obtained when strain MA3 harbouring plasmid pAGH was used as recipient and all of them retained an intact *rpf* gene. A total of 310 $Ts^R$ transformants were obtained with strain MA2 harbouring pAGM0 (*rpf* expressed from $P_{ami}$). The majority (75%) of these transformants contained the inactivated *rpf*::*tsr* gene as a result of homologous recombination. One of these strains, denoted MA14, was retained. Its growth in either rich (NBE) or minimal (LMM) medium was not dependent on added acetamide, consistent with a previous report (Parish *et al*., 1997) indicating that there is an appreciable basal level of expression from $P_{ami}$ in the absence of inducer (acetamide). Expression of the plasmid-encoded copy of *rpf* in strain MA14 was verified by RT-PCR and Rpf was detected in the culture supernatant by Western blotting (data not shown). We have attempted to cure plasmid pAGM0 by repeated subculturing of strain MA14. When subcultured in the presence of thiostrepton, colonies arose that contained pAGM0 integrated by single cross-over homologous recombination into the *rpf* region (pAGM0 has limited homology – 69 bp upstream and 46 bp downstream – with the corresponding *rpf*::*tsr* region of the bacterial chromosome). This was deduced by Southern hybridization. These colonies retained $Ts^R$ and were weakly $Hyg^R$. When strain MA14 was subcultured in the absence of thiostrepton, colonies were recovered which arose by double cross-over homologous recombination. They contained the intact *rpf* gene and were sensitive to both thiostrepton and hygromycin. These experiments provided strong evidence that *rpf* is an essential gene in *M. luteus*.

Finally, a 1255 bp *Sma*I fragment containing a modified *rpf* gene lacking codons 2–41 (signal sequence), but retaining the *rpf* promoter, was incorporated into plasmid pSMT3 ($Hyg^R$) (Garbe *et al*., 1994) in both orientations, generating plasmids pSMT3RPFns and pSMT3RPFnso (see Fig. 1A). These plasmids were established in *M. luteus* and the resulting strains were then transformed with the *rpf*::*tsr* insert from plasmid pHNST. In six separate experiments (three with each strain), a total of only 56 $Ts^R$ transformants was obtained. A PCR analysis revealed that the chromosomal copy of *rpf* was intact in all of them. These results, compared with those previously obtained for the strain harbouring pSMT3RPF (see above), establish that Rpf must be secreted in order to assume its essential biological function.

## Discussion

Gram-positive bacteria secrete a substantial repertoire of proteins into their environment. Bioinformatic analysis suggests that *Bacillus subtilis* exports about 300 proteins (Tjalsma *et al*., 2000), and a recent proteomic survey suggests that the actual number may be even greater (Antelmann *et al*., 2001). More than a third of exported proteins remain anchored at the external membrane surface (Tjalsma *et al*., 2000). Examples include components of the ABC family of bacterial transporters, some at least of the penicillin-binding proteins and the spore germinant receptors. Most of these proteins are not essential for viability, although there are exceptions such as PrsA, which is a post-translocation molecular chaperone (Vitikainen *et al*., 2001). Signal peptidases remove the signal sequences from many exported proteins (von Heijne, 1990; Tjalsma *et al*., 2000). Some of these, including *M. luteus* Rpf, have one or more LysM modules that promote their subsequent association with the bacterial cell wall (Bateman and Bycroft, 2000). Prominent among these are the autolysins, involved in cell wall expansion (Shockman *et al*., 1996; Smith *et al*., 1996).

Collectively, the penicillin-binding proteins and the autolysins are probably essential for bacterial growth (Shockman *et al*., 1996) but they show functional redundancy; individual genes can be inactivated without impairing viability (Margot *et al*., 1994; 1998; Blackman *et al*., 1998; Smith *et al*., 2000). Moreover, the existence of stable L-forms of many organisms, including *B. subtilis* (Allan, 1991; Allan *et al*., 1993), indicates that the cell wall might even be regarded as dispensable under certain very specialized growth conditions. Other secreted proteins include hydrolases responsible for the utilization of polymeric substrates, which are non-essential except in the absence of alternative substrates. Some secreted proteins are cleaved to generate signalling peptides that control the development of specialized physiological states (Kaiser and Losick, 1993; Lazazzera and Grossman, 1998). Examples include competence for genetic transformation in *Streptococcus pneumoniae* (Havarstein *et al*., 1995), endospore formation in *B. subtilis* (Perego, 1997; Perego and Brannigan, 2001) and conjugation in *Enterococcus faecalis* (Clewell, 1993; Havarstein *et al*., 1995; Perego, 1997; Lazazzera and Grossman, 1998; Perego and Brannigan, 2001). These too are non-essential functions, except under highly specialized conditions.

As will be evident from the above, whether or not a particular gene is essential may depend on environmental circumstances and, in many cases therefore, essentiality has to be defined operationally. Nevertheless, there is a cohort of genes whose products are essential for growth under all known conditions, such as those concerned with processes like replication, transcription and translation. As far as we are aware, *rpf* of *M. luteus* represents the first example of an essential gene that falls into this cohort, whose product is truly secreted. The experiments reported here showed that *rpf* could not be disrupted in *M. luteus* unless a second functional copy of the gene, whose product was secreted, was also present. Essentiality was also indicated by the Rpf-reversible growth inhibition caused by the addition of anti-Rpf antibodies to the culture medium of *M. luteus* (Fig. 4). Other organisms related to *M. luteus* (e.g. mycobacteria, streptomycetes and corynebacteria) contain multiple *rpf*-like genes. They may show functional redundancy; like the autolysins and possibly also the penicillin-binding proteins, they may have been recruited to fulfil distinct but overlapping cellular functions. Indeed, the five *rpf*-like genes of *My. tuberculosis* all have similar biological activity to that of Rpf (Mukamolova *et al*., 2002), which invites speculation that they may be essential collectively, but dispensable individually.

We were unable to construct a *rpf* null mutant, even when the growth medium was supplemented with recombinant Rpf. This was not unexpected when recombinants were selected on solid medium. We have been unable to demonstrate Rpf activity when cells depleted of Rpf (e.g. dormant cells) are plated (Kaprelyants *et al*., 1994). Rpf activity is only demonstrable in liquid medium. Therefore, we also attempted to recover a *rpf* null mutant by continuously maintaining transformed bacteria in liquid medium supplemented with recombinant Rpf. These experiments were also unsuccessful, but there may be many possible explanations for this. For example, biologically active recombinant Rpf may not persist for long enough when added to the culture medium. Alternatively, the natural cycle of Rpf production, secretion, sequestration on the cell wall, release, and finally degradation, may be vital for bacterial growth and difficult to mimic in culture using an exogenous source of protein. These experiments were therefore indeterminate; the possibility that a *rpf* null mutant could be obtained in a Rpf-supplemented environment cannot be excluded on the basis of the evidence currently available. Dormant cells of *M. luteus* essentially represent phenocopies of a *rpf* null mutant. They have *temporarily* lost the ability to make Rpf and can be resuscitated (i.e. persuaded to recommence endogenous production) by exogenous supplementation.

To investigate whether the LysM module at the C-terminus of Rpf is necessary for biological activity, a truncated derivative was produced, essentially comprising only the ~70-residue conserved domain (Kell and Young, 2000). Full-length Rpf is active at pM concentrations (Mukamolova *et al*., 1998b). The truncated form of *M. luteus* Rpf was active at fM concentrations. Evidently, the LysM-mediated interaction with (presumably) peptidoglycan (Bateman and Bycroft, 2000) is not required for biological activity. The great majority of Rpf molecules on the cell surface (see Fig. 2E) may be sequestered in non-productive interactions with the cell envelope peptidoglycan. They may form a reservoir of molecules that are released into the medium during the later stages of the growth cycle in batch culture (see Fig. 2B–D). LysM module-mediated interaction with the peptidoglycan could potentially regulate Rpf activity; bound molecules might be protected from proteolysis. Rpf activity therefore results from interaction of the highly conserved Rpf domain with a surface-located molecule, the nature of which is currently unknown.

The precise location of *rpf* between, and downstream from, genes encoding an IS*1557*-like transposase on one side and genes concerned with arginine biosynthesis on the other is unlikely to have any important biological significance. On the basis of the currently available genome sequence information, no other organisms have *rpf*-like genes in a similar genetic context (see, for example, Fig. 1B in Mukamolova *et al*., 2002).

The kinetics of *rpf* expression showed some interesting features. Firstly, transcript abundance did not simply reflect the period of active bacterial growth, but seemed to ‘anticipate’ it. Transcripts were detectable 30 min after stationary phase cells had been inoculated at high density into NBE; they were abundant 1 h post inoculation (Fig. 2A), at which time bacterial growth had not yet commenced. During the following 3 h, transcripts remained abundant, as the bacteria underwent the transition from lag phase to active growth. Transcript abundance had declined substantially after 6 h, at which time the bacteria were still growing actively. Second, there appeared to be a delay between inoculation of bacteria and the appearance of Rpf in the culture medium (Fig. 2B–D). This may simply be a reflection of the sensitivity (detection limit) of the ELISA. However, assuming that the transcripts observed during the lag phase were translated, many of the resulting protein molecules may have remained cell-associated (Fig. 2E). Perhaps the later release of Rpf into the culture medium represents a signal anticipating the onset of a period of less active growth in preparation for stationary phase? Evidence in support of this hypothesis comes from the observed inhibition of bacterial growth when high concentrations of Rpf-like proteins are incorporated into the bacterial growth medium (see Fig. 2 in Mukamolova *et al*., 2002).

Finally, we do not know why high concentrations of anti-Rpf antibodies did not inhibit bacterial growth when they were incorporated into the culture medium (Fig. 4). Perhaps complete inhibition occurs when antibody and antigen concentrations are approximately equivalent. High antibody concentrations might effectively present Rpf to the molecule with which it interacts on the bacterial cell envelope. Monoclonal antibodies will be required in order to explore these suggestions. In conclusion, however, the present work shows that the *rpf* gene of *M. luteus* encodes a secreted protein that is essential for cell multiplication.

## Experimental procedures

### *Organisms and media*

*Micrococcus luteus* NCIMB 13267 ('Fleming strain 2665') was grown at 30°C in conical flasks on an orbital shaker using either lactate minimal medium, LMM (Kaprelyants and Kell, 1993a), succinate minimal medium, SMM (Mukamolova *et al*., 1999) or Nutrient Broth E, NBE (LabM). *Escherichia coli* strain XL-2 Blue was used for cloning DNA fragments.

### *DNA manipulations and RT-PCR*

Standard methods were used for DNA manipulations, PCR and Southern hybridization (Southern, 1975; Sambrook *et al*., 1989). The DNA segments flanking the *rpf* gene were isolated by inverse PCR and sequenced commercially and they have been deposited in the EMBL database under accession number Z96935 (updated). For reverse transcriptase PCR experiments, RNA was prepared from culture samples containing $2 \times 10^8$ bacteria using the RNeasy Mini Kit (Qiagen) and DNA was removed using the DNA free™ kit (Ambion). Reverse transcription (25 μl) reactions contained 2 μg RNA, 1 μg of the reverse primer, ML1R (5′-TTCATGTCCCAGGTGCCGTT-3′, nt 1104–1085 in Fig. 1), 40 U RNAsin ribonuclease inhibitor (Promega) and 30 U AMV reverse transcriptase (Promega). Reactions 1 h were performed at 60°C and terminated by incubation at 75°C for 5 min. Control reactions, lacking AMV reverse transcriptase were performed simultaneously. For PCR reactions, samples (1–4 μl, or serial 10-fold dilutions, thereof) of the RT reaction products were used as template in the presence of the reverse primer, ML1R, and one of the forward primers. These were: ML1F (5′-GGTCGAACGCGACTCAGGTC-3′, nt 491–510 in Fig. 1); ML2F (5′-CAAATCCGGCCAGTAGCGTG-3′, nt 594–613 in Fig. 1); ML3F (5′-TCGAACCGTGATCTGACCGA-3′, nt 754–773 in Fig. 1); ML4F (5′-GAGGACTCGCCATGGACACC-3′, nt 909–928 in Fig. 1). After denaturation for 5 min at 94°C, samples were subjected to 30 cycles of: 30 s at 94°C, 30 s at 55°C, 60 s at 72°C, followed by 7 min at 72°C. No PCR product was produced in any of the control reactions in which reverse transcriptase had been omitted from the previous step.

### *Plasmid constructions*

In the following constructions, extensive use was made of cassettes encoding genes that confer thiostrepton resistance (Kieser *et al*., 2000) and hygromycin resistance (Blondelet-Rouault *et al*., 1997), both of which are selectable in *M. luteus*. Plasmid pRPF1 is pMTL20 (Chambers *et al*., 1988) containing the 1375 bp *Sma*I fragment of *M. luteus* DNA that encodes *rpf* (Fig. 1A). Plasmid pRPF2 was similarly constructed, starting from a derivative of pMTL20 that had been cleaved with *Mlu*I + *Sph*I, blunt-ended with T4 polymerase and re-ligated, to remove the *Nco*I and *Pst*I sites from the polylinker. A 943 bp segment upstream from *rpf* was amplified from *M. luteus* genomic DNA using primers MLF1 (5′-CGCGCaTGCACCGGGC-3′, nt 24–39 in Fig. 1, *Sph*I site underlined was introduced) and MLR1 (5′-CACGGctCGAGCGGTG-3′, nt 968–952 in Fig. 1, *Xho*I site underlined was introduced), trimmed with *Sph*I + *Xho*I and cloned in pMTL20. Similarly, an 894 bp segment downstream from *rpf* was amplified using primers MLF2 (5′-TCGGCCtcGAGCTCGT-3′, nt 1560–1575 in Fig. 1, *Xho*I site underlined was introduced) and MLR2 (5′-aCCATGGGCCAGGAGG-3′, nt 2451–2440 in Fig. 1, *Nco*I site underlined) trimmed with *Xho*I + *Nco*I and cloned in pMTL20. The two fragments were verified by sequencing, ligated together at their *Xho*I sites and cloned in *Sph*I + *Nco*I-digested pMTL6000 (N. P. Minton, personal communication). In a subsequent step, the thiostrepton resistance cassette (~ 1.1 kbp *Xho*I fragment) was inserted in both orientations into the *Xho*I site. Finally, the hygromycin resistance cassette (~ 2.3 kbp *Bam*HI fragment) was cloned into the *Bam*HI site in the plasmid backbone, generating plasmids pHNST and pHNSTo. These plasmids differ with respect to the orientation of *tsr* within *rpf* (Fig. 1).

For control experiments, a 1770 bp *Eco*RI-*Nco*I fragment of *M. luteus* DNA, containing *rpf*, *argR* and part of *argF* was cloned in pMTL20. The resulting plasmid was linearized with *Kpn*I, end-filled with T4 polymerase and the thiostrepton resistance cassette (~ 1.1 kbp end-filled *Xba*I fragment) inserted. Finally, the hygromycin-resistance cassette (~ 2.3 kbp *Hin*dIII fragment) was inserted at the *Hin*dIII site in the plasmid backbone generating plasmid pNCTH.

A second, plasmid-encoded copy of *rpf* was introduced into *M. luteus* under the control of either $P_{ami}$ (Parish *et al*., 1997) or $P_{hsp60}$ (Garbe *et al*., 1994). For the former, a 728 bp fragment, containing *rpf* with its presumed ribosome-binding site, was amplified from pRPF1. The product obtained with primers MLF5 (5′-TGCCTCtagaGCCGATCAGCGAGGA-3′, nt 889–913 in Fig. 1, *Xba*I site introduced is underlined) and MLR5 (5′-gtcTctaGaGGGCCGGTCAGGCGTC-3′, nt 1616–1591 in Fig. 1, *Xba*I site introduced is underlined) was trimmed with *Xba*I, and cloned into pMTL20. After sequence verification, the fragment was subcloned into the *Xba*I site of plasmid pAGH – this is plasmid pAGAN91 (T. Parish, personal communication) containing the hygromycin-resistance cassette in the *Bam*HI site at position 8281 – to generate pAGM0, in which *rpf* is transcribed from $P_{ami}$. In the related plasmid, pAGM1, the insert is in the opposite orientation and $P_{ami}$ generates a counter-transcript of *rpf*. To place *rpf* under control of $P_{hsp60}$, the 1375 bp *Sma*I fragment containing *rpf* and its promoter was isolated from pRPF1 and inserted into the *Eco*RV site of pSMT3 (Garbe *et al*., 1994) to generate pSMT3RPF. The related plasmid, pSMT3FPR, contains the 1375 bp *Sma*I fragment in the opposite orientation.

Finally, a modified form of *rpf*, whose product lacks the secretory signal sequence, was constructed as follows. The standard M13 reverse and forward sequencing primers were used in conjunction with primers JUN1 (5′-GCCATGGACAC CATGGCCACCGTGGACACC-3′, fusion of nt 917–931 to nt 1043–1057 in Fig. 1) and JUN2 (complement of JUN1), respectively, to amplify two DNA segments from plasmid pRPF1. The two DNA segments were then used as template and fused in a mega-PCR reaction with primers G4 (5′-GACCCGACCAAGGAGAAG-3′ nt 858–875 in Fig. 1) and G9 (5′-CGAGCTCCTGGCCGACGT-3′ nt 1574–1557 in Fig. 1) to generate a 606 bp fragment, which was confirmed by sequencing. The PCR product was then digested with *Nco*I + *Pst*I to release a 194 bp fragment, in which the initiation codon of *rpf* is fused to the codon corresponding to the first (alanine) residue of the secreted form of Rpf. This fragment was used to replace the corresponding 314 bp *Nco*I-*Pst*I fragment of pRPF2, to generate pRPFns. The 1255 bp *Sma*I fragment of pRPFns was then cloned into the *Sma*I site of pSMT3 in both orientations, to give plasmids pSMT3RPFns and pSMT3RPFnso.

### *Transformation of* M. luteus

Previously published methods (Kloos, 1969; Shiota and Nakayama, 1989) were adapted as follows for transforming *M. luteus* NCIMB 13267. Bacteria were grown in 300 ml conical flasks, containing 50 ml of LMM, to an $OD_{600} = 2.0$–2.5. Flasks were incubated on ice for 20 min, cells were harvested by centrifugation and washed with 50 mM Tris/HCl buffer, pH 7.5. The pellet was resuspended in 2 ml of the same buffer, containing 50 mM $CaCl_2$ and 0.5% Na glutamate. Linear DNA fragments (10 μg) were denatured at 90°C for 20 min and quickly chilled on ice; plasmid DNA (1 μg) was used without denaturation. Cells (0.5 ml) were mixed with DNA (50 μl samples) and incubated at 30°C with shaking for 30 min. After adding 1 ml of NBE, samples were further incubated without antibiotic selection for 10–16 h before plating on NBE agar containing the appropriate selective antibiotic (50 μg thiostrepton $ml^{-1}$, 200 μg hygromycin $ml^{-1}$ or 50 μg kanamycin $ml^{-1}$). Plates were incubated for up to 7 days at 30°C.

Using the above method, we obtained low frequency transformation with several replicative plasmids, which was adequate for our current purpose. Plasmid pSMT3 (Garbe *et al*., 1994) yielded ~ $10^3$ transformants per μg DNA. Surprisingly, pMTL20TSR also replicated autonomously in *M. luteus* ($10^2$ transformants per μg DNA). This is a pUC-like cloning vector (Chambers *et al*., 1988), lacking a conventional Gram-positive replicon, into which a selectable thiostrepton resistance ($Tsr^R$) cassette (Kieser *et al*., 2000) was incorporated at the *Hin*dIII site. A similarly marked derivative of the p15a-based plasmid, pMTL6000 (N. P. Minton, personal communication) did not appear to replicate autonomously, but derivatives containing *M. luteus* chromosomal DNA fragments were also able to persist (data not shown). Restriction fragments, rather than intact plasmids, were therefore used for introducing DNA into the *M. luteus* chromosome. As transformation frequencies were low, DNA fragments containing a selectable *tsr* gene were always isolated from vectors marked with a selectable *hyg* gene. In some experiments, this permitted phenotypic differentiation of transformants arising by double cross-over recombination with the bacterial chromosome, from those arising by establishment of intact plasmid molecules that almost invariably contaminated gel-purified restriction fragments.

For *rpf* disruption experiments, a mixture of equal amounts of pHNST and pHNSTo was digested with *Bam*HI and *Sca*I. The 2970 bp fragment, containing the insert shown in Fig. 1, in which most of the *rpf* coding sequence has been replaced by the thiostrepton resistance cassette, was gel-purified, heat-denatured (Oh and Chater, 1997) and used for transformation. In control experiments, plasmid pNCTH was digested with *Eco*RI and *Xho*I to release a 2870 bp fragment containing the insert shown in Fig. 1, in which there is a thiostrepton resistance cassette at the *Kpn*I site just downstream from *rpf*. This fragment was also gel-purified and heat-denatured (Oh and Chater, 1997) before transformation.

### *ELISA methods*

Culture supernatant (5–200 μl) was added to plastic 96-well plates (Costar), which were incubated at 37°C for 1 h. The wells were then washed three times with PBS-T (phosphate-buffered saline containing 0.05% Tween-80). The primary antibody was either rabbit anti-Rpf (1 : 10 000) or sheep anti-truncated Rpf (1 : 1000), (see Mukamolova *et al*. 2002). It was added and incubation was at 37°C for 45 min. After washing three times with PBS-T, the secondary antibody was added. This was either anti-rabbit, alkaline phosphatase conjugate (Sigma, 1 : 5000) or anti-sheep, alkaline phosphatase conjugate (Sigma 1 : 20 000). After washing three times again with PBS-T the wells incubated with phophatase substrate (*p*-nitrophenyl phosphate tablet set, Sigma) for 30 min at room temperature. Staining intensity was determined by scanning (405 nm) plates in a Labsystem optical reader. The assay was calibrated using different concentrations of recombinant Rpf protein in the relevant culture medium. The detection limits for the anti-Rpf and the anti-tRpf antibodies were 50 pM Rpf and 30 pM tRpf respectively.

### *Confocal microscopy*

*Micrococcus luteus* was grown in NBE ($OD_{600} = 0.3$), washed with TBS (50 mM Tris/HCl, 150 mM NaCl, pH 7.5) and labelled, without fixation, for 3 h at 30°C with anti-Rpf antibodies (Mukamolova *et al*. 2002) (1 : 100 in 100 ml TBS containing 0.2% BSA). After three washes with TBS, FITC-labelled goat anti-rabbit IgG (Sigma F-1262) was used as secondary antibody (1 : 100 in 100 ml TBS containing 0.2% BSA; 1 h incubation at 30°C). Bacteria were examined with a BioRad MRC1024ES confocal microscope with excitation at 488 nm (100 mW argon laser). Some bacteria probably lost their surface-located Rpf during the 4 h preparation period and visible bleaching occurred during microscopic examination of samples.

### *Cell viability by plating*

Serial dilutions were prepared in LMM, plated in triplicate on agar-solidified NBE (LabM) and incubated for 3 days at 30°C. Cell suspensions were passed 10 times through a 23-gauge needle to break up loose aggregates before dilution.

### *Production of truncated Rpf*

A truncated form of Rpf was obtained as described by (Mukamolova *et al*., 2002).

### *Rpf activity assay*

Growth of *M. luteus* was monitored in a Bioscreen C growth analyser (Labsystems, Finland) using a 600 nm filter. The freshly prepared recombinant protein was diluted 1 : 100 in LMM and sterilised by filtration (0.22 µm, Gelman). After breaking up aggregates (see above), late log cultures ($OD_{600} = 3.5–4.0$) were serially diluted using growth medium. Samples (5 µl) of each dilution (five to ten replicates) were added to wells containing medium (200 µl), together with serially diluted protein. Incubation was at 30°C with continuous shaking and measurements were taken hourly for 200 h.

### *Influence of anti-Rpf antibodies on bacterial growth*

*Micrococcus luteus* ($10^2$ colony-forming units, cfu) was inoculated into 20 ml LMM in a conical flask. Anti-Rpf antibodies, as well as immune and preimmune rabbit serum (and the IgG fraction purified from it) were as described by (Mukamolova *et al*., 2002). They were added, together with Rpf (200 pM), as appropriate, and the bacteria were incubated at 30°C on an orbital shaker. Growth was monitored by measuring cfu.

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