

# Structural Changes and Cellular Localization of Resuscitation-Promoting Factor in Environmental Isolates of *Micrococcus luteus*

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**Abstract** Dormancy among nonsporulating actinobacteria is now a widely accepted phenomenon. In *Micrococcus luteus*, the resuscitation of dormant cells is caused by a small secreted protein (resuscitation-promoting factor, or Rpf) that is found in “spent culture medium.” Rpf is encoded by a single essential gene in *M. luteus*. Homologs of Rpf are widespread among the high G + C Gram-positive bacteria, including mycobacteria and streptomycetes, and most organisms make several functionally redundant proteins. *M. luteus* Rpf comprises a lysozyme-like domain that is necessary and sufficient for activity connected through a short linker region to a LysM motif, which is present in a number of cell-wall-associated enzymes. Muralytic activity is responsible for resuscitation. In this report, we characterized a number of environmental isolates of *M. luteus*, including several recovered from amber. There was substantial variation in the predicted *rpf*

gene product. While the lysozyme-like and LysM domains showed little variation, the linker region was elongated from ten amino acid residues in the laboratory strains to as many as 120 residues in one isolate. The genes encoding these Rpf proteins have been characterized, and a possible role for the Rpf linker in environmental adaptation is proposed. The environmental isolates show enhanced resistance to lysozyme as compared with the laboratory strains and this correlates with increased peptidoglycan acetylation. In strains that make a protein with an elongated linker, Rpf was bound to the cell wall, rather than being released to the growth medium, as occurs in reference strains. This *rpf* gene was introduced into a lysozyme-sensitive reference strain. Both *rpf* genes were expressed in transformants which showed a slight but statistically significant increase in lysozyme resistance.

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## Introduction

We think of living bacteria as colony formers, but dormant bacteria are unable to form colonies when plated on a suitable solid medium unless they are first resuscitated, a poorly understood process that ultimately leads to the restoration of colony-forming ability. Dormancy may be considered as a response to environmental stress, and this is exemplified by the spore-forming bacteria. However, dormancy among nonsporulating bacteria has recently been gaining attention. In the course of our research on dormancy, we have isolated several strains of *Micrococcus luteus* that vary in the structure of a protein involved in resuscitation, called the resuscitation-promoting factor (Rpf).

Rpf was discovered following the work of Kaprelyants et al. [14], who had shown that starved cultures of *M. luteus*

could be resuscitated from a dormant state by “spent culture medium” of the same organism. In later publications, the active component (Rpf) was isolated and characterized from the spent culture medium [20–23]. Similar Rpf are widespread among the high G + C Gram-positive bacteria. In *Mycobacterium tuberculosis*, five Rpf-like proteins play an important role in breaking dormancy [20,22]. Rpf is encoded by a single essential gene in *M. luteus*. Its protein product has a highly conserved approximately 70-residue lysozyme-like domain connected through a short (ten residue) linker region to a LysM domain that is believed to facilitate binding to peptidoglycan and is found in a large number of cell-wall-associated proteins [3].

We are interested in dormancy of *M. luteus* isolated from amber [10,11]. Although it cannot be stated that the age of the amber also corresponds to the age and dormant period of the isolates, it can be argued that these “environmental” isolates have acquired long-term survival ability under oligotrophic conditions including a capacity to withstand the harsh chemicals present in amber. The Rpf protein, associated with resuscitation from dormancy, was significantly modified in these amber isolates [10]. In previous investigations, Matsuda et al. [19] had noted a slight variation (mostly single-nucleotide polymorphisms) between the *rpf* genes of different laboratory strains, whereas Greenblatt et al. indicated that the linker region was elongated in environmental strains isolated from amber [10]. The amber strains also varied from reference strains in their resistance to lysozyme and to  $\alpha$ -terpineol. It was not clear if these properties were distinct reactions to stress or an integral part of their dormancy program [16].

In this report, we examine in detail the structure of Rpf from several amber and environmental isolates and show that it is expressed and secreted. Roles for the Rpf linker in binding to the cell envelope and in environmental adaptation are proposed. To test whether Rpf with an extended linker contributes directly to the enhanced lysozyme resistance of the environmental strains, we inserted the gene from one such variant into a laboratory strain of *M. luteus*. Although this resulted in a small increase in lysozyme resistance, the main factor responsible for this phenotype was the extent of acetylation of the cell envelope peptidoglycan.

## Materials and Methods

### Organisms

Environmental isolates from amber (AMB #4, AMB #27, and AMB #29) have been previously described [10]; additional isolates from almond tree resin and from a purposely contaminated plate onto which we poured liquid nitrogen are designated as (ALM) and (LIQN), respectively.

Several additional isolates were derived from human skin and one from a cat's paw. Two reference strains of *M. luteus* were employed: the classical Fleming strain 2665, NCIMB 13267 (FLR), and the equivalent strain from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, no. 20030 (GCR). LYSV is a colony that was isolated from a zone of lysis in a lawn of the GCR strain that formed around a filter paper disk containing  $\sim 0.5\mu\text{g}$  lysozyme. *E. coli* strains TG1 and HSM174 (DE3) were used for cloning and for protein expression, respectively.

### Media

Organisms were generally grown at 30°C in conical flasks on an orbital shaker using either LB medium [10] or nutrient broth E (NBE—LabM) in the seed cultures for the lysozyme sensitivity studies. Resistance to lysozyme was demonstrated using cells grown on either succinate medium, containing 1% (w/v) succinic acid and 0.3% yeast extract (Difco), adjusted to pH7.4 with NaOH or medium 1 containing 10 mM-Hepes, 50 mM-KCl, 5 mM-MgSO<sub>4</sub>, pH7.3 [1].

### Microscopy

Routine examination was performed under oil illumination usually following Gram staining. For localization of Rpf, bacteria were exposed to anti-Rpf antibody (polyclonal rabbit antibodies that had been affinity-purified as described previously [23]) in Eppendorf tubes for 1 h and mounted in GVA mounting solution (Zymed 00/8000) on glass slides. The undiluted antibody had a concentration of 0.4 mg per milliliter and was used at a dilution of 1:100 in the microscopic examinations. The first antibody was followed by a secondary goat antirabbit fluorescent-labeled antibody, after which samples were washed three times with phosphate-buffered saline (PBS). The bacteria were examined using a Zeiss model 410 fluorescent microscope at 1,000 times magnification.

### Lysozyme Sensitivity

#### (a) Early logarithmic phase

In order to determine the sensitivity of different isolates of *M. luteus* to a range of lysozyme concentrations in the early log phase, 3-mm disks of sterile filter paper impregnated with 8  $\mu\text{l}$  of freshly prepared lysozyme in water (0.4–0.0025 mg/ml) were placed onto a freshly inoculated bacterial lawn ( $10^7$  cfu from an overnight culture in NBE). The plates were incubated at 37°C for 24 h and then examined for the presence of growth inhibition zones around the filter paper disks. Zone diameters were measured and recorded.

### (b) Late logarithmic phase

For the determination of the sensitivity of the different isolates of *M. luteus* to lysozyme in the late logarithmic phase, isolates were grown in succinate medium at 30°C as described [1]. Fresh cultures were inoculated (10% of the total volume) with samples from 24-h cultures. Cells 20-h postinoculation were centrifuged at 5,000×*g* for 10 min and resuspended in medium 1. The suspension was incubated with lysozyme (50 µg/ml of culture) for 30 min at 37°C. After incubation, the OD<sub>595</sub> was determined; cells were stained using Gram's stain and observed microscopically.

### Deacetylation and Measurement of Released Acetate

Bacterial peptidoglycan was deacetylated as described by Brumfitt et al. [6] by exposure to 0.2 M NaOH at 37°C for 30 min and then washed and resuspended in PBS. The cells were subsequently tested for lysozyme resistance as previously described. The measurement of released acetate was performed using the Megazyme Acetic Acid kit (K-ACETRM; Megazyme International Ireland Limited 2006). Small starter cultures from single colonies of the various isolates were inoculated into 45-ml LB medium and incubated overnight at 30°C. After determining the OD<sub>595</sub>, the cells were harvested by centrifugation and resuspended in 2 ml distilled H<sub>2</sub>O. To 1 ml of the slurry in an Eppendorf tube was added 0.25 ml of either H<sub>2</sub>O or 1 M NaOH, and tubes were incubated at 37°C for 30 min. Thereafter, tubes were centrifuged at 11,000 rpm, and the supernatants were analyzed by the Megazyme procedure. The method employs several coupled enzymatic reactions, the final readout of which is the change in A<sub>340</sub> with the oxidation of NADH. The slurry without NaOH serves to adjust for acetic acid in the medium trapped interstitially and released from the cell, while a blank cuvette corrects for downward drift (spontaneous oxidation) in NADH absorption.

### Western Blotting

Protein separation was performed by electrophoresis in 12% polyacrylamide gels in the presence of sodium dodecyl sulfate [17]. Rpf in suitably concentrated samples (100 ng/ml) of culture supernatant was detected by Western blotting using polyclonal rabbit antibodies that had been affinity-purified as described previously [23]. The supernatant of the environmental strains was concentrated fourfold before being applied to the membrane. In dot blots of cells, a suspension of 10<sup>8</sup> was applied to the membrane. The antibodies were used at a dilution of 1:3,000. The secondary antibody was conjugated with alkaline phosphatase.

### ELISA

The procedure was essentially as described by Mukamolova et al. [22]. Culture supernatant (5–200 µl) was added to plastic 96-well plates (Costar) at 37°C for 45 min. Unbound antigen was removed by washing three times with PBS containing Tween (PBS-T; PBS containing 0.05% Tween-80). Affinity-purified rabbit antibodies raised against Rpf, specific towards native Rpf and recombinant forms (1:1,000), was added, and the plates were incubated at 37°C for 1 h. After three washes with PBS-T, the secondary antibody (goat antirabbit), alkaline phosphatase conjugate (Sigma, 1:5,000), was added. After washing three times with PBS-T, phosphatase substrate (*p*-nitrophenyl phosphate) was added, and the plates were incubated at room temperature for 30 min. Standardization of the assay was performed using recombinant Rpf in the culture medium. Staining intensity was determined by scanning (405 nm) plates in a Labsystem optical reader.

### PCR Amplification of the *rpf* Gene

DNA was isolated from *M. luteus* using a standard phenol protocol [28]. Two primers, UpRpfFL (5'-ccggccagtagcgtg-catttcac-3') and LowRpfFL (5'-gcgggcctctcgtgtgga-3'), were used for polymerase chain reaction (PCR) amplification (4 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 56°C, 1 min at 72°C) with Taq polymerase. When used with the FLR strain, these primers give a 1,042-bp product encompassing the complete *rpf* coding sequence (672 nt) together with additional upstream and downstream sequences (370 nt). The sizes of the PCR products were estimated by agarose gel electrophoresis. DNA sequencing was undertaken by the sequencing units of either the Hebrew University or IBERS, Aberystwyth University.

### Influence of Anti-Rpf Antibodies on Bacterial Growth

*M. luteus* was grown at 30°C in conical flasks on an orbital shaker using LB medium overnight, diluted to an OD<sub>595</sub>=1.9 with LB medium and pipetted into plastic 96-well plates (Costar) containing anti-Rpf antibodies (1:100). Plates were incubated on an orbital shaker (300 rpm) at 30°C, and growth was monitored by scanning the OD<sub>595</sub> in a Labsystem optical reader. Experimental details are given in Mukamolova et al. [20]. In that reference, preimmune antibodies were shown to have no effect in this system.

### Cloning

The plasmid pMind was kindly provided by Brian Robertson of Imperial College, London [5]. LB medium was used throughout with addition of 10 µg kanamycin

(Km) per milliliter to the cultures of the transformed *M. luteus*, which were incubated at 30°C, while the transformed *E. coli* was maintained in LB medium containing 50 µg/km per milliliter and incubated at 37°C. Gene expression from pMind-based plasmids was induced by the addition of tetracycline (20 ng/ml).

The vector pET-19b was used for the initial cloning using the primers:

Upper: **GCCCATATGGCCACCGTGGACACCTG**  
Lower: **GGGGATCCGGTCAGGCGTCTCAGG**

The bold font specifies the restriction sites for *NdeI* and *BamHI*

For cloning in the vector pMind, the primers

Upper: **GGATCCGACCCGACCAAGGAGAAG**  
GACGAC,  
Lower: **ACTAGTTGACGGGCACCAGGCACGAG**  
were used

The bold font specifies restriction sites for *BamHI* and *SpeI*.

The PCR products were initially cloned in pGEM-T (Promega). Thereafter, pGEM *rpf<sub>LYSV</sub>*, pGEM *rpf<sub>AMB #4</sub>*, and the two vectors, pET-19b and pMind, were digested with the restriction enzymes *NdeI* + *BamHI* or *BamHI* + *SpeI*, respectively, and the fragments were ligated into their corresponding vectors. The ligated products were transformed into *E. coli* strain TG1, and selected colonies were examined by PCR, and the sequences of the cloned genes were confirmed. pET-19b *rpf<sub>LYSV</sub>* and pET-19b *rpf<sub>AMB #4</sub>* were transformed into *E. coli* strain HSM174 (DE3) by electroporation (Bio-Rad). Transformation of *M. luteus* GCR with pMind *rpf<sub>LYSV</sub>* and pMind was performed with the TransformAid Kit (Fermentas).

#### Isolation and Purification of Recombinant Proteins

The recombinant Rpf was expressed in *E. coli* HSM174 (DE3). The protein containing a His10-tag at the N terminus was isolated by sonicating the bacteria previously induced with 1 mM IPTG. The bacteria were harvested by centrifugation and frozen in binding buffer (BB—5 mM imidazole pH 8/0.5 M NaCl/20 mM Tris-HCl). After thawing, RNAase and DNAase were added followed by addition of urea to a final concentration of 8 M. Cells were sonicated two to three times for 30 s. After centrifugation, a Ni<sup>2+</sup> chelation column (*V*=2 ml; Ni<sup>2+</sup>-coordinated iminodiacetic acid immobilized on Sepharose 6B) was loaded with the supernatant. The Biological LP system (Bio-Rad) was utilized for elution and refolding of the protein on the column: the first of a series of washing steps was with BB. The second, a refolding step, was with BB plus urea declining linearly from 8 M to zero. The (third) elution step

was with a linear gradient from 5 to 500 mM imidazole. Protein eluted at about 250 mM imidazole (a total volume of 0.3 ml was collected (monitored by A<sub>280</sub>)). Lastly, the Rpf-containing fraction was dialyzed against 50 mM Tris, pH 7.2, and 50 mM NaCl (final protein concentration 100–200 µg/ml). The protein was stored at +4°C for up to 1 week without significant loss of activity.

#### Muramidase Activity

To determine the muramidase activity of the recombinant Rpf, 4-β-D-*N,N',N''*-triacetylchitotrioside ((NAG)<sub>3</sub>-MUF; Sigma, Germany) was used as a substrate. A final concentration of 6 µM MUF was incubated at Rpf concentrations from 1 to 20 µg/ml in the presence of 5 mM MgSO<sub>4</sub> in 50 mM citric acid–Na citrate buffer, pH 6.0. After incubation at 37°C for 3 h, reactions were terminated by adding 2 µl 10 M NaOH. Fluorescence intensity was detected with a RF-5301PC fluorimeter (“SHIMADZU,” Japan) at an excitation wavelength of 360 nm and a readout of 450 nm.

#### Influence of Recombinant Variant Rpf on the Lag Phase of Growth

Cells of *M. luteus* were grown for 3 days in rich medium (LabM) followed by centrifugation and washing three times with LabM. The same medium was inoculated with these starved washed cells in a dilution of 1:10,000 in 96-well plates and incubated at 30°C in a MULTISCAN Analyzer. Lag phase was calculated as the time when the OD<sub>595</sub> increased by 15% from the initial level.

## Results

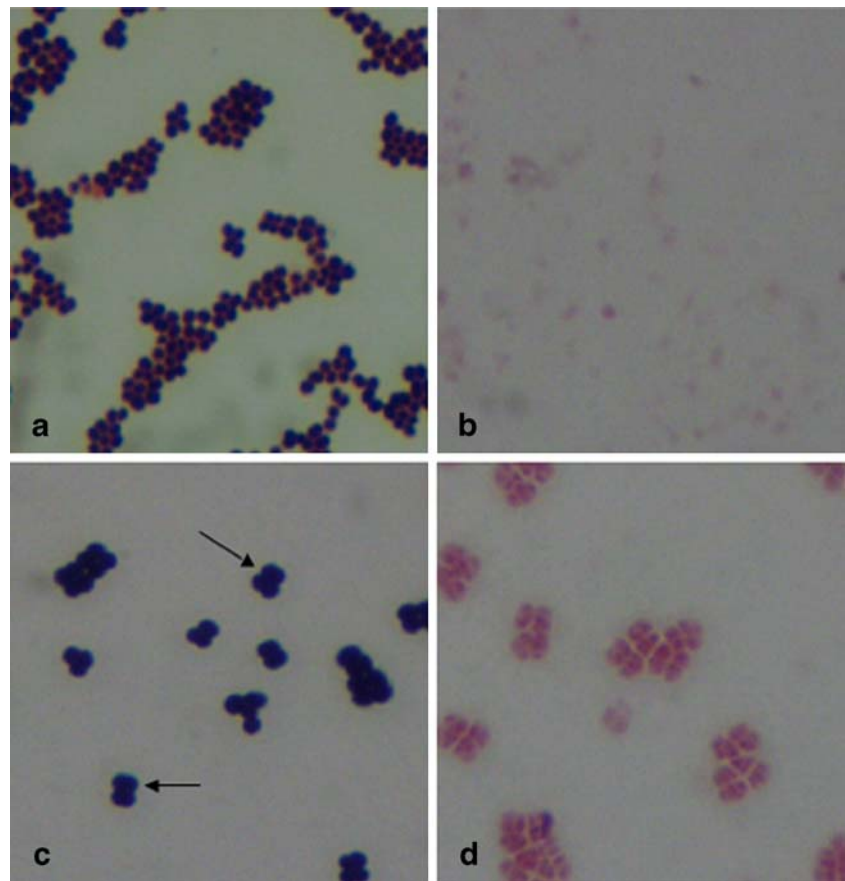
#### Phenotypic Characterization

All the *M. luteus* strains had similar colonial morphology. They formed bright yellow colonies on LB medium, and they were indistinguishable by light reflection from colonial surfaces. Light microscopy of Gram-stained preparations of the various isolates revealed that they were very similar. Most of the strains formed tetrads characteristic of *M. luteus*, but in the FLR strain the tetrads tended to coalesce to form many-celled clusters (Fig. 1a, c), and the individual cells appeared smaller. Electron microscopy was not especially informative in terms of cell wall thickness.

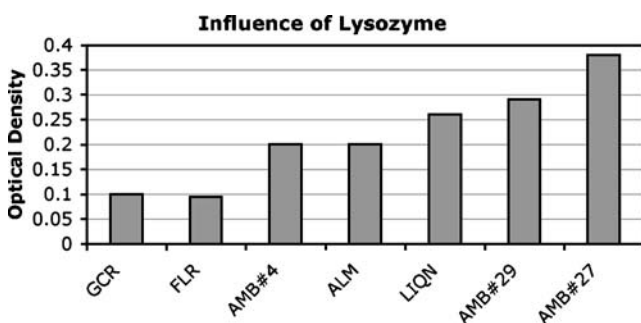
#### Lysozyme Sensitivity

In order to prepare DNA from the various isolates, standard lysozyme treatment was employed using cells grown in LB.

**Figure 1** **a** Gram stain of FLR. Note the occasional tetrads, but more frequent appearance of clusters of bacteria. **c** Gram stain of AMB #27—the tetrads are more frequent. **b** Gram stain of FLR after a half-hour exposure to lysozyme (50 mg/ml); it is difficult to discern cell outlines. **d** Gram stain of AMB #27 after lysozyme, cell outlines still remain

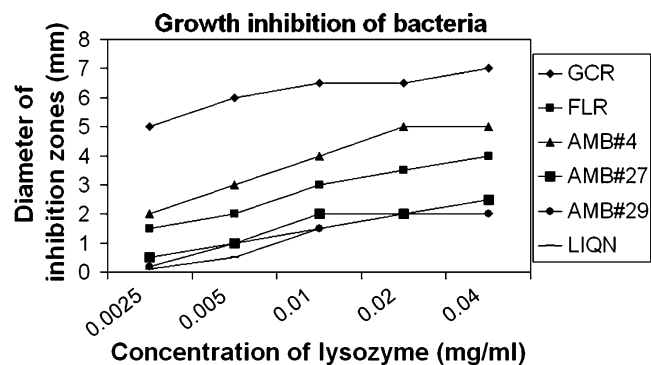


The two reference strains (FLR and GCR) were extremely sensitive and lysed rapidly, as expected, when exposed to lysozyme in late log phase, whereas the environmental isolates were much less sensitive (Figs. 1b–d, 2). A somewhat different picture was observed when lysozyme sensitivity was assayed using a disk diffusion agar method (Fig. 3) where cells are in exponential growth. AMB #4 was intermediate in sensitivity to the two reference strains—GCR and FLR, while the three environmental isolates, AMB #27, AMB #29, and LIQN were more resistant. The observed differences in lysozyme sensitivity suggest that



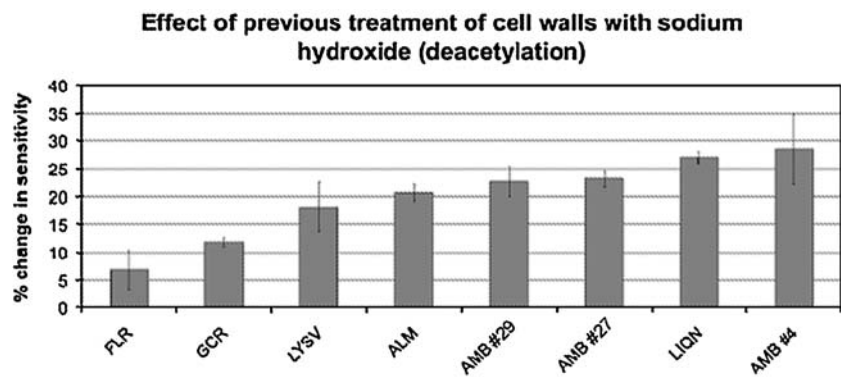
**Figure 2** OD of bacterial suspensions after exposure to lysozyme. Note that, although all show a reduction in OD, the environment isolates remain at higher ODs than the reference strains

cell wall architecture is different at different stages of growth and also that it varies from one strain to another. After incubating the plates for a few days, colonies appeared in the zones of clearance of the two reference strains, GCR and FLR (data not shown). This phenomenon has been observed previously [6,18]. One representative colony (denoted LYSV) derived from the GCR strain was further investigated in parallel with the various environmental isolates.



**Figure 3** Measurement of the clear zones around filter paper disks impregnated with lysozyme. In these newly seeded plates, AMB#4 is intermediate in sensitivity between the two reference strains, suggesting that sensitivity to lysozyme varies in different phases of growth

**Figure 4** Untreated and NaOH-treated cells were exposed to lysozyme. The OD value of the untreated cells minus the OD of the treated cells  $\times 100$  was percent change. NaOH deacetylation changed lysozyme sensitivity in all the populations, but the environmental strains showed greater change. The bars are standard deviation



According to previous reports, the lysozyme sensitivity of bacterial peptidoglycan is profoundly affected by *O*-acetylation [4,9,26]. We therefore determined whether deacetylation of peptidoglycan prepared from all strains rendered their wall material equally sensitive to lysozyme. The data in Fig. 4 show clearly that even the wall material from the reference strains become more sensitive to lysozyme when pre-exposed to alkali, but the difference between the pretreated and the alkali-treated walls was much greater with cell walls from the environmental isolates and the lysozyme-resistant variant. This suggests that differences in the degree of acetylation of the cell wall peptidoglycan are a major factor in explaining the increased resistance of the environmental isolates. Table 1 is a measure of the released acetate due to the exposure to alkali. It can be seen that the two reference strains released little if any acetate compared to the environmental isolates. Clearly, there is a correlation between the linker length, lysozyme resistance, and acetate released. The amber and environmental strains that are lysozyme resistant have substantially acetylated peptidoglycan and an elongated Rpf linker, whereas the reference strains that are lysozyme sensitive have weakly acetylated peptidoglycan and a shortened Rpf linker.

#### Rpf Variation

In order to further explore the relationship between Rpf linker length and the acetylation/lysozyme resistance of bacterial

peptidoglycan, the *rpf* genes were amplified and sequenced from three amber isolates (AMB #4, AMB #27, AMB #29), three skin isolates (CatPaw, Finger, Breast), three other environmental isolates (ALM, LIQN, & Air), and the GCR reference strain as well as the lysozyme-resistant variant apparently derived from it (LYSV). The deduced sequences of the Rpf proteins encoded by these genes are compared with the published sequence of Rpf from the Fleming strain (FLR) in Fig. 5a. The Rpf proteins of the two reference strains, FLR and GCR, were identical (as were the gene sequences), whereas, in most of the environmental and skin strains, the distance between the Rpf and LysM domains was increased by expansion of the low complexity linker region that separates them. This linker essentially comprises a series of five-residue repeats (consensus sequence [Q/E]AAA[E/A/D]); only three copies are present in the reference strains, whereas 16–21 copies are found in several of the environmental and skin isolates (Fig. 5b).

This pentapeptide repeat was also amplified in the LYSV strain. All six of the GCR strain variants isolated from the cleared zone around a lysozyme-impregnated disk gave PCR products of the same size with primers UpRpfFL and LowRpfFL, and the representative that was sequenced (LYSV) had 21 copies of the five-residue repeat in the linker between the Rpf and LysM domains. However, when similar experiments were carried out with the FLR strain yielding 30 independent variants, there was no evidence from PCR analyses that any of the strains had an expanded linker region.

**Table 1** The amount of released acetate after treatment with 0.2 M NaOH as measured by the Megazyme acetic acid assay

Isolate	Control $\mu\text{g}$ acetate/OD of bacteria	NaOH $\mu\text{g}$ acetate/OD of bacteria	Mean $\pm$ SD
FLR	3.53	2.48	Not detectable
GCR	1.55	2.47	0.92 $\pm$ 2.21
AMB #4	4.65	22.6	17.9 $\pm$ 1.31
AMB #27	3.01	21.7	18.7 $\pm$ 2.54
AMB #29	3.91	20.0	16.1 $\pm$ 1.50
LIQN	2.76	22.6	19.9 $\pm$ 4.6
LYSV	2.61	19.3	16.7 $\pm$ 4.29

**a**

FLR	MDTMTLFTTSATRSRRATASIVAGMTLAGAAAVGFSAPAQAATVDTWDRLAECESNGTWD	60
GCR	MDTMTLFTTSATRSRRATASIVAGMTLAGAAAVGFSAPAQAATVDTWDRLAECESNGTWD	60
CatPaw	MDTMTLFTTSATRSRRATASIVAGMTLAGAAAVGFSAPAQAATVDTWDRLAECESNGTWD	60
Breast	MDTMTLFTTSATRSRRATASIVAGMTLAGAAAVGFSAPAQAATVDTWDRLAECESNGTWD	60
AMB#4	--MTLFTTSATRSRRATASIVAGMTLAGAAAVGFSAPAQAATVDTWDRLAECESNGTWD	57
GCRLM	--MTLFTTSATRSRRATASIVAGMTLAGAAAVGFSAPAQAATVDTWDRLAECESNGTWD	57
Finger	-----LAGAAAVGFSAPAQAATVDTWDRLAECESNGTWD	34
Air	-----RATASIVAGMTLAGAAAVGFSAPAQAATVDTWDRLAECESNGTWD	45
ALM	-----AGAAAVGFSAPAQAATVDTWDRLAECESNGTWD	51
LIQN	-----TWDRLAECESNGTWD	15
AMB#27/29	-----TLAGAAAVGFSALQAATVDTWDRLAECESNGTWD	47

FLR	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	120
GCR	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	120
CatPaw	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	120
Breast	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	120
AMB#4	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	117
GCRLM	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	117
Finger	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	94
Air	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	105
ALM	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	111
LIQN	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	75
AMB#27/29	INTGNIFYGGVQFTLSSWQAVGGEGYPHQASKAEQIKRAEILQDLQGWAWPLCSQKLGL	107

FLR	TQADADAGDVDATEAAPVAVERTATVQRQSAADEAAAAEQAAA-----	162
GCR	TQADADAGDVDATEAAPVAVERTATVQRQSAADEAAAAEQAAA-----	162
CatPaw	TQADADAGDVDATEAAPVAVERTATVQRQSAADEAAAAEQAAA-----	162
Breast	TQADANAG-----AAAAEQAAA-----	136
AMB#4	TQADADAGDVDATEAAPVAVERTATVQRQSAADEAAAAEQAAAAEAAAAEAAAAE--	175
GCRLM	TQADADAGDVDA---APVAVERTATVQRQSAADETAADQAAAEQAAADQAAAEQAAADQA	174
Finger	TQADADAGDVDA---APVAVERTATVQRQSAADETAADQAAAEQAAADQAAAEQAAADQA	151
Air	TQADADAGDVDA---APVAVERTATVQRQSAADETAADQAAAEQAAADQAAAEQAAADQA	162
ALM	TQADADAGDVDATEAAPVAVERTATVQRQSAADEAAAAEQAAAEQAAADQAAAEERWAAE--	169
LIQN	TQADADAGDVDATEAAPVAVERTATVQRQSAADEAAAAEQAAAEQAAADQAAAEERWAAE--	133
AMB#27/29	TQADADAGDVDATEAAPVAVERTATVQRQSAADEAAAAEQAAAEQAAADQAAAEERWAAE--	165

FLR	-----	162
GCR	-----	162
CatPaw	-----	162
Breast	-----	136
AMB#4	-----QAAADQAAAEERWAAEQAAAEQAAADKAAAQ-----	207
GCRLM	AAERWAAKQAAAEQAAAEERWAAKQAAADQAAAEERWAAKQAAAEQAAADKAAAQ-----	227
Finger	-----AAERWAAKQAAADQAAAEERWAAKQAAAEQAAADKAAAQRAAAAEK	196
Air	AAERWAAKQAAADQAAAEERWAAKQAAADQAAAEERWAAKQAAAEQAAADKAAAQRAAAAEK	222
ALM	-----QAAADQAAAEERWAAEQAAADQAAAEERWAAEQAAAEQAAADKAAAQRAAAAE-	220
LIQN	-----QAAADQAAAEERWAAEQAAAEQAAADKAAAQRAAAAE-	169
AMB#27/29	-----QAAADQAAAEERWAAEQAAAEQAAADKAAAQLAAAAE-	201

**Figure 5 a** The translated amino acid sequences of Rpf from the reference strains and the environmental isolates. Note the large gap in the former where the linker insertion occurs. The insertion contains the motif: [Q/E]AAA[E/A/D]. **b** The aligned linkers. Note the

conversion in the first amino acid of the motif from Q or E to R and K as the linker lengthens. The motif enlarges to six amino acids near its terminus





## Rpf Expression

The observed changes in Rpf in the different isolates might variously affect protein expression and/or function. Western blotting of concentrated samples of culture supernatant demonstrated that the proteins were expressed in all the strains tested. As expected, in strains where the linker region was substantially elongated, the estimated size of the protein was increased, e.g., the deduced sizes of Rpf from AMB #4 and GCR were ~40 and ~30 kDa, respectively (Fig. 6a).

The observed differences in Rpf structure did not have any significant effect on the timing of Rpf expression during the bacterial growth cycle. Bacteria were grown to stationary phase overnight in NBE, and fresh cultures were inoculated the following morning. Samples were taken at three points during the growth cycle in batch culture, and equivalent volumes were assayed for the presence of Rpf using Western blotting (Fig. 6a). In the figure, the supernatant of GCR cultures was not concentrated, while those of AMB#4 were concentrated fourfold. Rpf expression starts in the early log phase, with a peak in the late log phase, while it is either reduced or is stable during the stationary phase. The amount of secreted Rpf in these samples was quantified by enzyme-linked immunosorbent assay (ELISA), and significant differences were found between different strains (Table 2, also see Fig. 6b). In Fig. 6b,  $1 \times 10^8$  cells were spotted on the membrane. The Rpf concentration in supernatant from the GCR strain reached a maximum of over  $7 \mu\text{g/ml}$  whereas in supernatant from the AMB #4 strain the concentration was reduced about fourfold ( $1.7 \mu\text{g/ml}$ ). The Rpf concentration in supernatant from the LYSV strain ( $0.115 \mu\text{g/ml}$ ) was more than 60-fold reduced compared with its putative parental strain, GCR.

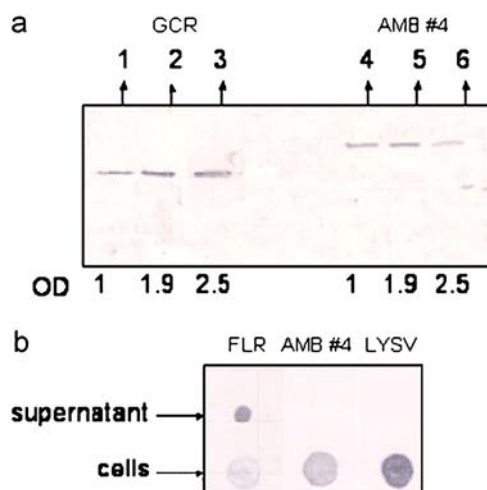
Since lower concentrations of Rpf were found in the culture supernatants of the environmental isolates, we surmised that the protein might be bound more tightly to the bacterial cell wall in these strains. Dot blots demonstrated that, unlike the reference strains (FLR and GCR) in which the majority of the Rpf was in the culture supernatant, Rpf in the environmental isolates essentially remained associated with (i.e., bound to) the bacterial cells (Table 2 and Fig. 6b). The ELISA values confirmed the lowered amount of the variant Rpf as well as its tendency to remain bound to the cell. The FLR strain produced  $3.9 \mu\text{g}$  Rpf per milliliter in total, 83% of which was released into the supernatant. At the same cell density, the LYSV strain produced only  $1.3 \mu\text{g}$  Rpf per milliliter, only 9% of which was released into the supernatant. Microscopic observation of fluorescent anti-Rpf antibody-labeled cells confirmed this conclusion; cells of the FLR and GCR strains were only very weakly fluorescent, whereas cells of the

AMB #4 and LYSV strains showed bright fluorescence (Figs. 7 and 10).

There is evidence that members of the Rpf protein family stimulate bacterial growth from an extracytoplasmic location [26]. This has been confirmed for several isolates by adding anti-Rpf antibodies to the liquid culture medium. Complete growth inhibition was observed when all isolates were inoculated into LB containing anti-Rpf antibodies at a 1:10 dilution (data not shown). Inhibition was transient, resulting in delayed bacterial growth when anti-Rpf antibodies were added at a 1:100 dilution. The LYSV strain, which grew more rapidly than the other two strains seemed to recover more rapidly from growth inhibition mediated by anti-Rpf antibodies than the other strains tested (Fig. 8).

## Detection of *rpf*<sub>LYSV</sub> and *rpf*<sub>GCR</sub> and the Expression of Recombinant *rpf*<sub>LYSV</sub> in the GCR Strain

The PCR products encompassing the *rpf* gene obtained from the GCR and LYSV strains had sizes of approximately 830 and 1,090 bp, respectively (data not shown). When *rpf*<sub>LYSV</sub> cloned in pMind was introduced into the GCR strain, Western blots confirmed that the recombinant protein was expressed (Fig. 9). In the figure,  $1 \times 10^8$  cells were applied to the membrane. Two bands were apparent, the stronger band corresponds to the chromosomally encoded Rpf<sub>GCR</sub>, while the weaker band corresponds to the plasmid-encoded Rpf<sub>LYSV</sub>. Comparison of the band intensities (Fig. 9) shows that about 25% of the total is accounted for by the



**Figure 6** a: Western blots of the supernatants of GCR and AMB #4, with increasing growth. The supernatants of GCR were not concentrated, while those of AMB #4 were concentrated fourfold. Note the increased molecular weight of the Rpf of AMB #4. The ODs correspond to bacterial growth of the logarithmic phase (1.0), late logarithmic (1.9), and stationary phase (2.5). b: Dot blots of the supernatant and lysed cells;  $1 \times 10^8$  cells were applied to the membrane. The reference strain is represented by FLR

**Table 2** Rpf concentrations ( $\mu\text{g/ml}$ ) in the supernatant during life cycles of AMB #4, GCR, and LYSV

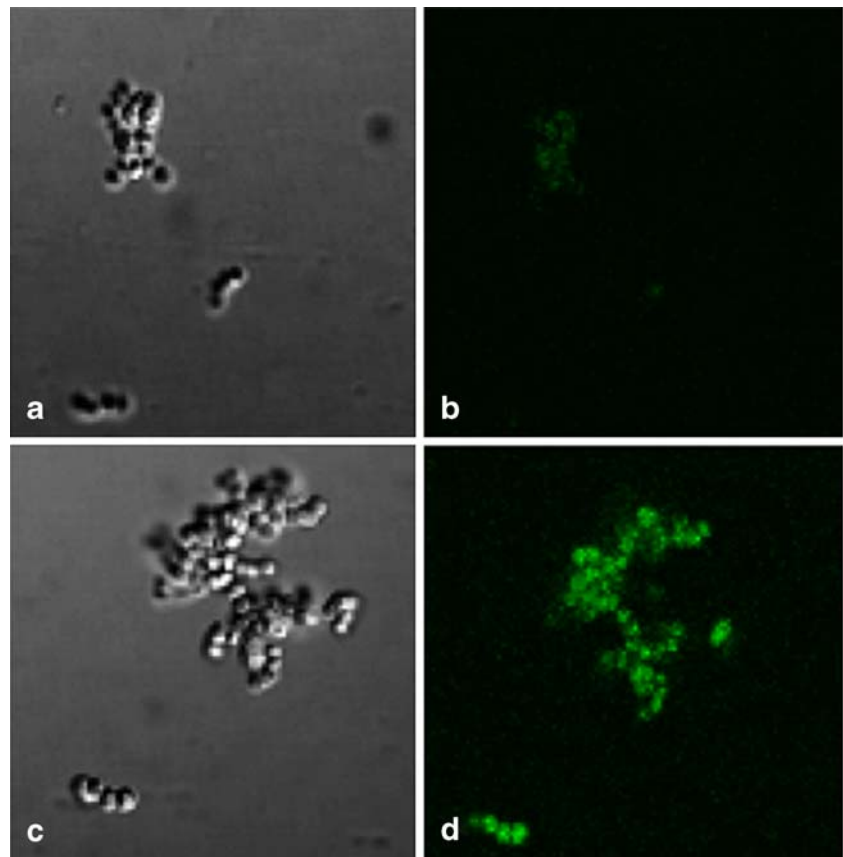
Isolate OD	AMB#4	GCR	LYSV
1	1.650	3	0.05
1.9	1.7	7	0.115
2.5	1.225	7.2	0

plasmid-encoded Rpf<sub>LYSV</sub> and 75% by the chromosomally encoded Rpf<sub>GCR</sub>.

#### Localization of Rpf<sub>LYSV</sub> by Immunofluorescence

The GCR(pMind) control, like the parental GCR strain, shows almost no detectable immunofluorescence. However, the GCR(pMind *rpf*<sub>LYSV</sub>) strain shows clear but nonuniform fluorescence (Fig. 10). Since the only difference between the fluorescent and nonfluorescent cells is the presence of Rpf<sub>LYSV</sub>, we conclude that the linker possessing the repetitive motif [Q/E] AAA[Q/E/D] is responsible for the change.

**Figure 7** Phase and fluorescent images of FLR and AMB #4 treated with affinity-purified antibody to Rpf and visualized by a second fluorescein-labeled antibody. **a** FLR, phase image; **b** FLR, fluorescent image; **c** AMB #4 phase image; **d** AMB #4, fluorescent image. The FLR is only dimly visible while AMB #4 is very bright

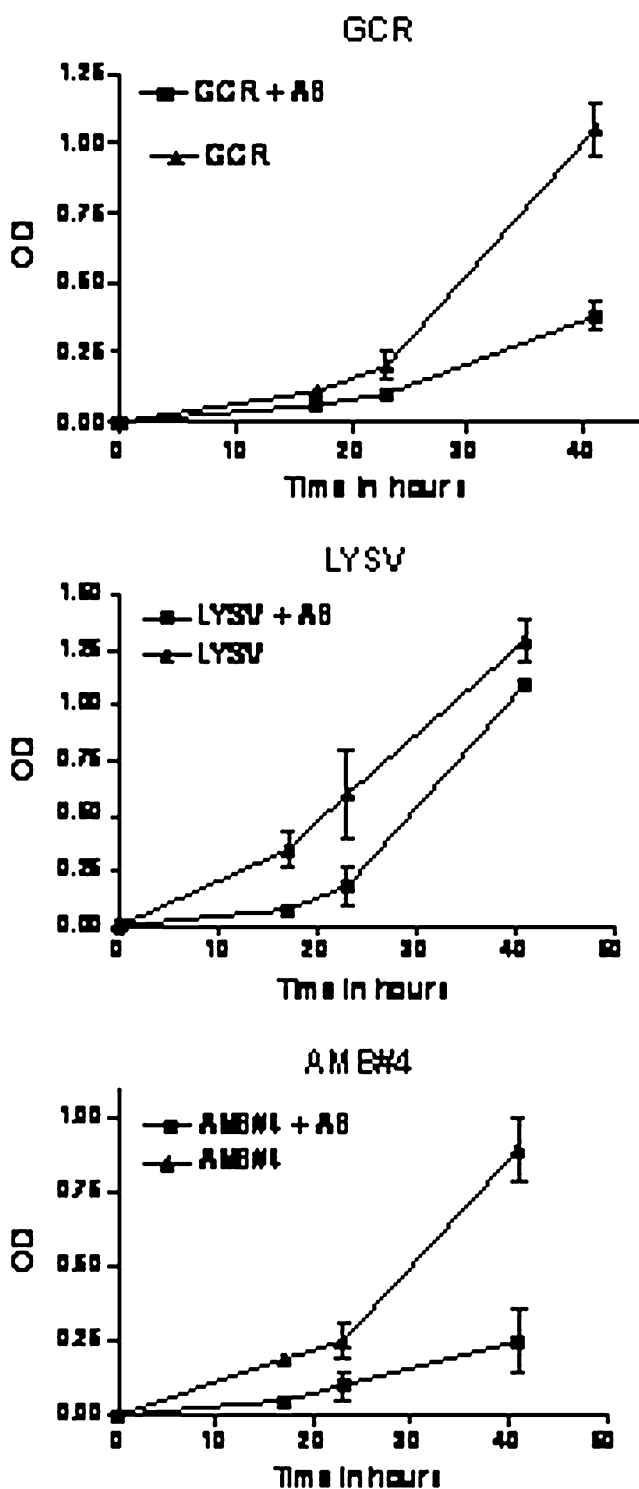


#### Lysozyme Sensitivity

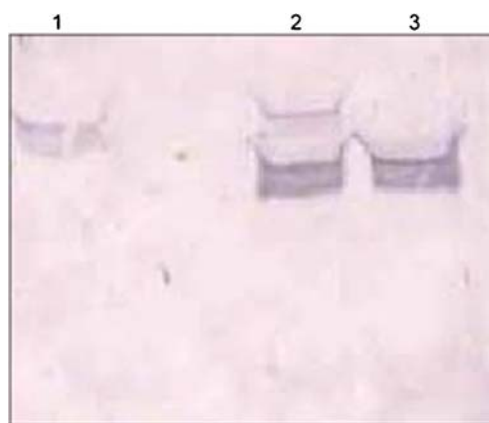
The GCR(pMind) and GCR(pMind *rpf*<sub>LYSV</sub>) strains were tested for lysozyme resistance. The GCR(pMind) strain presented a 21.5 mm ( $\pm 0.6$  mm) zone of growth inhibition whereas the GCR(pMind *rpf*<sub>LYSV</sub>) strain presented a 19.2 mm ( $\pm 0.4$  mm) inhibition zone. This difference was reproduced in six individual experiments, which consistently showed that the diameter of the lytic zone of the GCR (pMind *rpf*<sub>LYSV</sub>) strain was about 11% reduced as compared with that of the GCR strain.

#### Biological Activity

Figure 11 demonstrates that both the recombinant Rpf proteins (from the FLR and the AMB #4 strains) decrease the lag time before the inception of growth of *M. luteus* cells. When added at a concentration of 20 ng/ml, there is a 70% reduction with both proteins. However, the protein from the Fleming strain had a lower threshold, being minimally active at 5 ng/ml and more at 10 ng/ml, while the AMB #4 protein bearing the elongated linker is only active at the highest effective concentration. The lack of activity at high concentrations has been reported previously [22].



**Figure 8** Growth curves of GCR, AMB #4, and LYSV treated with 1:100 affinity-purified antibody to Rpf. The bars indicate standard deviation. Note the greater growth of LYSV and its more rapid recovery



**Figure 9** A Western blot showing (1) LYSV, (2) GCRpMind/rp/LYSV, and (3) GCRpMind. The double band in 2 represents the expressed recombinant and the constitutive protein;  $10^8$  cells were applied to the membrane

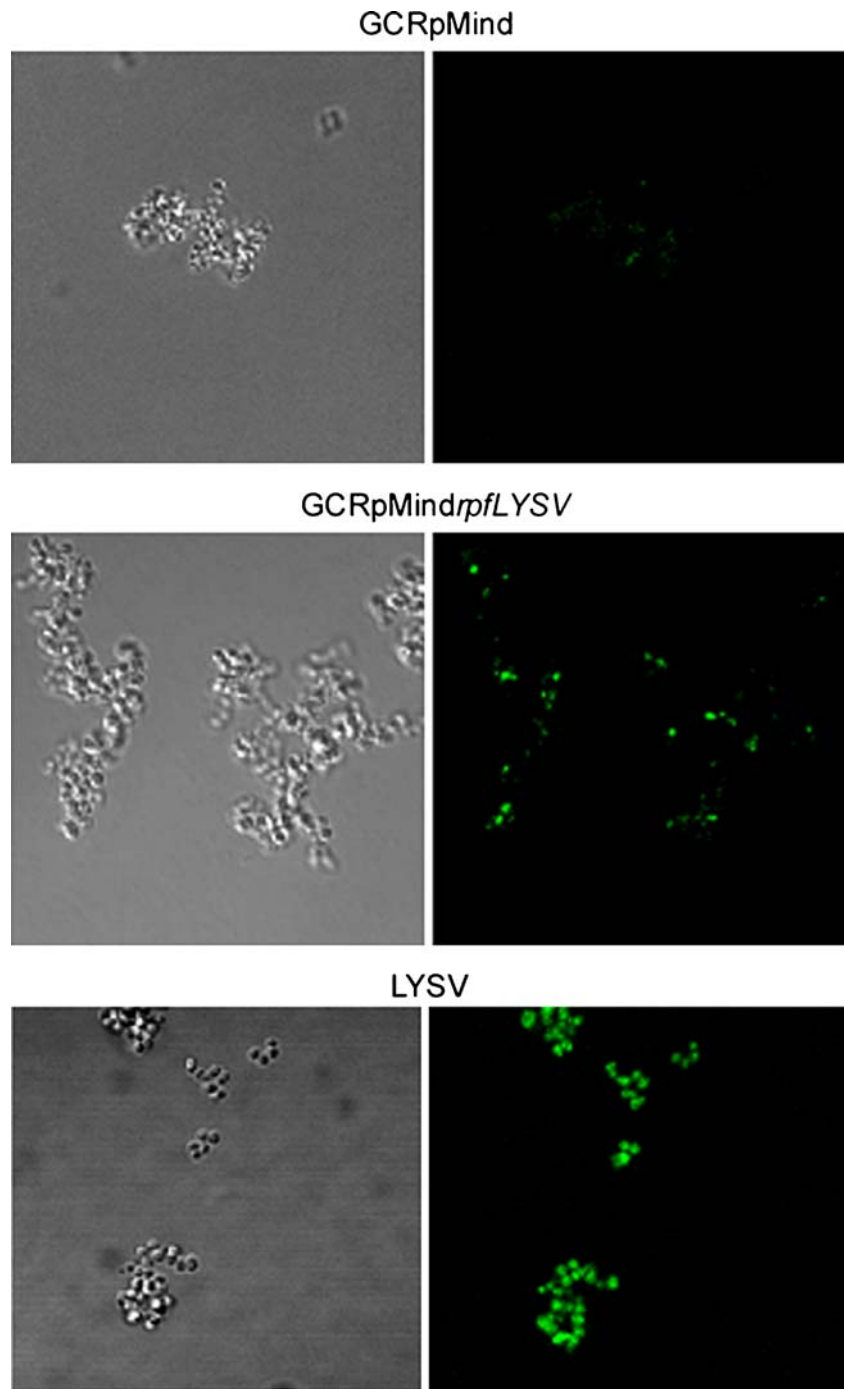
### Muramyltic Activity

The muramidase activities of the recombinant proteins were measured using a synthetic substrate, (NAG)<sub>3</sub>-MUF. All proteins examined had similar specific activities, which varied from 1.3 for the AMB #4 protein to 1.9 ( $\pm 0.4$ ) for the LYSV protein to 3.7 ( $\pm 2.4$ ) for the FLR protein (units of nanomole MUF per hour).

### Discussion

The exquisite sensitivity of *M. luteus* to lysozyme has been known for decades, and many investigators have taken advantage of this to produce protoplasts from these organisms for a variety of purposes [2,8,12,25,29]. It was therefore surprising that we experienced difficulty removing the peptidoglycan envelope with lysozyme during routine DNA extraction from several newly isolated environmental strains of this organism. Both the amber isolates, the skin isolates, and the other environmental isolates shared this characteristic, suggesting perhaps that the reference strains are not typical of the species as a whole. Environmental isolates presumably have to adapt to a wider range of different (stressful) conditions than “domesticated” laboratory strains. One such stress would be the secretion of lysozyme by the organisms on whose skin it grows, as well as other cell wall lytic enzymes secreted by competitors. It therefore seems plausible to suggest that the reference strains may have “relaxed” their cell wall structure during prolonged maintenance in the laboratory environment. Certainly, from our results, peptidoglycan acetylation makes an important contribution to the observed lysozyme tolerance of the environmental isolates. Whether or not activation of peptidoglycan *O*-acetyltransferase is responsible (as proposed by

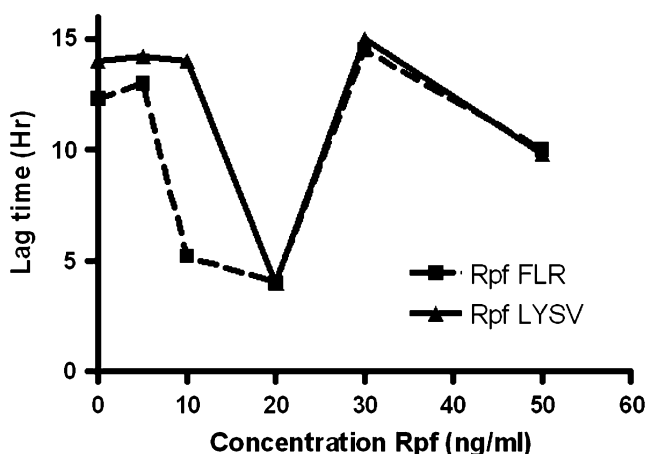
**Figure 10** Phase and fluorescent images, formaldehyde-fixed cells were treated first with a rabbit anti-Rpf antibody and then secondly with an antirabbit fluorescent antibody. The empty plasmid did not affect the faint fluorescence of GCR, while the plasmid containing the variant *rpf* conferred a spotty fluorescence. LYSV, similar to the environment strains, was brightly fluorescent (Fig. 7). The images were magnified  $\times 1,000$



Bera et al. [4] to explain the lysozyme resistance of virulent staphylococci) should be explored. Another characteristic that correlated with enhanced lysozyme tolerance was the length of the repetitive low complexity linker region in their Rpf proteins. Perhaps a longer linker is required for Rpf to exert its physiological function when peptidoglycan is acetylated? To test directly whether linker length might be related in some way to lysozyme tolerance, we isolated lysozyme-tolerant derivatives of both the FLR and the GCR strains. The results of these investigations were indetermi-

nate. Thirty independently isolated derivatives of the FLR strain had unchanged Rpf linker regions, whereas six independently isolated derivatives of the GCR strain had an extended Rpf linker region. Since the length of the linker in the six GCR isolates was apparently the same, we cannot rule out the possibility that these colonies were simply laboratory contaminants.

In the doctoral thesis of one of the authors [16], some additional phenotypic differences are described. When suspended over a cushion or 55% percoll as described by



**Figure 11** The influence of recombinant Rpf of FLR and LYSV on the lag time of *M. luteus* (FLR). Lag time was measured as the time required to be 15% greater than the initial OD

Peleg et al. [24], the AMB #4, AMB #27, and AMB #29 strains either remained above the cushion or dispersed within it, whereas the reference strains pelleted, indicating a difference in cell density. A second feature separating the three amber isolates from other strains was their resistance to  $\alpha$ -terpineol. This test was performed much like the lysozyme test described above by placing a filter paper disk impregnated with 3%  $\alpha$ -terpineol on a freshly seeded lawn of bacteria. The amber isolates grew up to the disk while LIQN, ALM, and the two reference strains, FLR and GCR, showed very clear zones of growth inhibition.

Sequencing of the *rpf* gene from a range of environmental isolates indicated that most of them had extended linker regions compared with those of the two reference strains. Western blotting demonstrated that each of the strains expressed and secreted Rpf and that the protein accumulated to the greatest extent during late log phase. The amber strains and the lysozyme-tolerant LYSV strain produced smaller quantities of Rpf, which had a reduced tendency to accumulate in the culture medium than the Rpf of the FLR strain. In spite of this, the growth of all strains tested was inhibited by anti-Rpf antibodies, consistent with previous results indicating that Rpf is essential for growth of *M. luteus* (although the LYSV strain appeared to be less sensitive than the other two strains tested—GCR and AMB #4). This may possibly be connected with differences in Rpf localization between the strains or the more rapid growth of LYSV.

Keep et al. [15] have suggested that the critical action of Rpf is in its muralytic activity and, indeed, the available evidence suggests that muralytic activity is responsible for the resuscitation and growth-promoting activities of Rpf [21]. This is consistent with the structural similarity between the Rpf proteins and lysozyme [7,15]. The repetitive motif [Q/E]AAA[E/A/D] is predicted to form an alpha helix. Such

repetitive structures commonly associated with lectins [30]. Perhaps this explains the greatly enhanced association of Rpf<sub>AMB #4</sub> and Rpf<sub>LYSV</sub> with the cell envelope. Our findings indicate that Rpf proteins possessing a short linker are readily secreted into to the growth medium, whereas those with a longer (and therefore more flexible) linker remain bound to the cell hinting, perhaps, at a possible function for the linker region in facilitating binding to the bacterial cell wall. Would it enhance the muralytic activity? We have demonstrated the muralytic activity of Rpf proteins with extended linker regions. A tighter association with the cell wall may make the Rpf more effective even though its bulk concentration is low. Indeed, Rpf concentrations in the supernatants of mycobacterial cultures are also very low and in these organisms Rpf proteins are predominantly localized on the cell surface [13,27]. Lysozyme resistance of the environmental isolates and selected variants presumably results from some alteration of peptidoglycan structure/organization (of which acetylation is one factor), and Rpf structure may adjust to that of the altered peptidoglycan. Using an antibody to block the surface activity of Rpf in mycobacteria, Mukamolova et al. [23] have shown growth inhibition, stating that “sequestration of these proteins at the cell surface might provide a means to limit or even prevent bacterial multiplication in vivo.” In the three strains of *M. luteus* exposed to anti-Rpf antibody, inhibition was observed but seemed greater for LYSV. However, this was in part due to its more rapid growth. In both LYSV and AMB#4, the linker is elongated and cell-bound, and there seems no difference between them and GCR where the Rpf is released.

Figure 5b illustrates a number of features of the linker structure in a variety of *M. luteus* isolates when viewed from the perspective of its length. With the exception of the Breast strain, all begin with QSAAD. Using the Air strain Rpf as standard, thereafter, each five-member repeat begins with Q or E until the eighth repeat where RW appears. This pattern continues until the 17th member of the series when K is the initial amino acid. All repeats are pentapeptides until number 21, where a hexapeptide repeat is introduced. What is the origin of the elongated linker region in the environmental strains? The draft version of the *M. luteus* genome sequence (courtesy of the Joint Genome Institute) provides no evidence of additional related sequences within *M. luteus*. Has it been introduced from another organism? In *Magnetococcus* sp MC-1, there is a protein (TPR repeat-containing protein, ACCESSION YP\_866069) of unknown function with 97 five-member repeats of the same motif [Q/E]AAA[E/A/D]. Returning to Fig. 5b, gaps or deletions of four, five, seven, and nine copies of the pentapeptide repeat are apparent, suggesting perhaps that there has been a gradual shortening of the linker under the relaxed conditions of repeated laboratory culture of the strains now in culture collections (FLR and GCR).

Our findings indicate that environmental isolates of *M. luteus* produce a variant Rpf with an elongated linker. We also have found that all of the strains with the elongated linker show increased resistance to lysozyme. This resistance is correlated with and probably due to acetylation of the peptidoglycan. Additionally, we have found that, in all forms with the enlarged Rpf, a great preponderance of the protein remains cell-wall-associated. The converse is seen in the GCR and FLR strains where the great majority of the Rpf is released to the culture medium. If, as suggested above, the [Q/E]AAA[E/A/D] pentapeptide repeats give lectin-like properties to these proteins, this could explain the tendency of the variant Rpf proteins to remain cell-associated [30].

The questions raised by these observations relate to (1) whether the *rpf* gene with the elongated linker is responsible for the cell-bound nature of its protein and (2) if this variant Rpf is somehow related to the greater lysozyme resistance. To answer these questions, we cloned the variant *rpf* gene which codes for the elongated linker into pMind and transformed the GCR strain with it. These transformed cells show several interesting characteristics. Firstly, both Rpfs are expressed. Secondly, the organism transformed with pMind*rpf*<sub>LYSV</sub> is fluorescent when treated with anti-Rpf antibody. Thirdly, there is a slight but significant increase in their resistance to lysozyme. Since the GCR (pMind) control, like the parental GCR strain, shows almost no detectable immunofluorescence and the GCR (pMind *rpf*<sub>LYSV</sub>) is fluorescent, it would seem that the presence of multiple repeats of the [Q/E]AAA[E/A/D] pentapeptide confers on the expressed Rpf an enhanced ability to adhere to the bacterial cell wall. It is therefore likely that the elongated linker does explain why Rpf<sub>LYSV</sub> is not released.

Does the enlarged Rpf confer increased resistance to lysozyme? We suspect that the elongated Rpf linker described herein is a consequence of the adaptation of the protein to changes in peptidoglycan structure, in particular acetylation. The variant Rpf in the GCR(pMind *rpf*<sub>LYSV</sub>) strain does measurably enhance the transformed cell's resistance to lysozyme. Moreover, this enhancement is proportional to its additional contribution to the Rpf content of the cell. By binding tightly to the peptidoglycan, it may compete with exogenously added lysozyme for active catalytic sites. This binding might also serve to stabilize the three-dimensional structure of the cell wall. Once a quantitative assay for muralytic activity has been developed, it may be possible to address this question.

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