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Proteins of the Rpf (Resuscitation Promoting Factor) Family Are Peptidoglycan Hydrolases

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Abstract—The secreted *Micrococcus luteus* protein, Rpf, is required for successful resuscitation of dormant "non-culturable" *M. luteus* cells and for growth stimulation in poor media. The biochemical mechanism of Rpf action remained unknown. Theoretical predictions of Rpf domain architecture and organization, together with a recent NMR analysis of the protein structure, indicate that the conserved Rpf domain has a lysozyme-like fold. In the present study, we found that both the secreted native protein and the recombinant protein lyse crude preparations of *M. luteus* cell walls. They also hydrolyze 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside, a synthetic substrate for peptidoglycan muramidases, with optimum activity at pH 6. The Rpf protein also has weak proteolytic activity against N-CBZ-Gly-Gly-Arg-β-naphthylamide, a substrate for trypsin-like enzymes. Rpf activity towards 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside was reduced when the glutamate residue at position 54, invariant for all Rpf family proteins and presumably involved in catalysis, was altered. The same amino acid substitution resulted in impaired resuscitation activity of Rpf. The data indicate that Rpf is a peptidoglycan-hydrolyzing enzyme, and strongly suggest that this specific activity is responsible for its growth promotion and resuscitation activity. A possible mechanism of Rpf-mediated resuscitation is discussed.

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Key words: proteins of the Rpf family, peptidoglycan hydrolases, "non-culturable" cells

Rpf (resuscitation promoting factor), a protein secreted by the Gram-positive bacterium *Micrococcus luteus*, stimulates the resuscitation of dormant *M. luteus* cells [1]. The mechanism of Rpf action remained unknown; however, the low concentrations at which Rpf is active allowed an analogy to be drawn with eukaryotic cytokines, and it became known as a growth factor. The encoding gene, *rpf*, was identified and sequenced, and the recombinant protein expressed in *Escherichia coli*. Rpf-like proteins are found in a number of Gram-positive bacteria. In particular, five Rpf-like proteins were identified in the genome of *Mycobacterium tuberculosis* [1]. It is interesting to note that *M. tuberculosis* belongs to the same group of Gram-positive bacteria as *M. luteus* (the actinobacteria, with (G+C)-rich DNA) and like the latter, it is capable of transformation into a dormant, metabolically inactive life form [2]. The N-terminal region of *M. luteus* Rpf, comprising 75 amino acid residues, is called the "conserved" region, to distinguish it from the "variable", C-terminal Rpf region, comprising 109 residues and including a LysM domain involved in binding of peptidoglycan from the cell wall. The conserved region has sequence similarity with *M. tuberculosis* proteins (up to 75% identical residues). However, apart from this conserved 75-residue domain, there is no similarity between the remaining segments of Rpf-like proteins of *M. tuberculosis*. At the present time, more than 30 genes from various microorganisms encode proteins containing a segment corresponding to the "conserved" Rpf region, which suggests that they should be classified into the special family denoted "Rpf-like proteins". The physiological role and biochemical mechanism of these proteins remain unknown. However, it was shown *in vitro* that

Abbreviations: MPN) most probable number (of resuscitated cells); $(NAG)_3$-MUF) 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside.

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Rpf-like proteins from *M. tuberculosis* are able to stimulate cell growth in old *Mycobacterium bovis* cultures, and antibodies against Rpf inhibit the growth of *M. tuberculosis* [3]. Evidence was obtained showing that *M. luteus* Rpf is essential for cell proliferation (*rpf* null-mutants could not be obtained unless a second functional copy of the *rpf* gene was present on an autonomous plasmid) [4]. However, deletion of the two *rpf*-like genes of *Corynebacterium glutamicum* did not affect bacterial growth, but impaired the initiation of bacterial growth after long periods of storage [5].

The inactivation of individual *rpf*-like genes in *M. tuberculosis* did not reveal substantial differences in the growth of this bacterium *in vivo* and *in vitro* [6]. However, simultaneous deletion of three out of five *rpf* genes resulted in a marked reduction of *M. tuberculosis* virulence *in vivo* [7]. Moreover, dormant forms of mutant strains obtained following extended incubation in stationary phase were not capable of the spontaneous resuscitation shown previously for wild type cells. These experiments confirmed the importance of the Rpf proteins for resuscitation of dormant mycobacteria, and indicated that these proteins may be involved in the mechanism of *M. tuberculosis* persistence during latent tuberculosis.

According to the tertiary Rpf structure predicted within the Ten Most Wanted program (http://www.doembi.ucla.edu/TMW/), the conserved Rpf domain has a lysozyme-like fold [8]. This suggested that Rpf could be an enzyme with lytic activity towards peptidoglycan [9]. Moreover, based on an analysis of the amino acid sequences of Rpf-like proteins it was suggested that they belong to the family of lytic transglycosylases ([10], PFAM database). Recent work by Cohen-Gonsaud et al. making use of high resolution NMR for a structural study of the conserved Rpf domain of *M. tuberculosis* RpfB has confirmed this theoretical prediction; however direct experimental proof of Rpf muralytic activity was not obtained [11].

In this work, an experimental investigation was made of the enzymatic activity of native (isolated from *M. luteus* culture medium) and recombinant Rpf as well as several altered forms obtained by site-directed mutagenesis. Experimental data are presented, indicating that Rpf has peptidoglycan hydrolase activity and a possible mechanism for the growth-promoting effect of Rpf on dormant or "non-culturable" bacterial cells is proposed.

MATERIALS AND METHODS

Native Rpf protein was isolated from *M. luteus* culture medium of organisms cultivated on a pre-dialyzed Nutrient Broth (NB) (Himedia, India) at 200 rpm and 30°C. To prepare the medium, the low molecular weight fraction, obtained after dialysis, was used. Cell suspensions (A_{600} = 1.2-1.5) were centrifuged at 3000*g* for 35 min. The supernatant was filtered through a bacterial filter (0.22 μm, Whatman, USA), applied to a column of DEAE-Sepharose FF (Sigma, Germany), washed with 40 mM phosphate buffer, pH 7.6, in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) and 80 mM NaCl, and eluted with a NaCl gradient (80-350 mM). The eluted fractions were concentrated by ultrafiltration and then fractionated by HPLC (Akvilon, Russia) on a Superdex 200 HR column.

Preparation of Rpf and its modified derivatives. Recombinant protein from *E. coli* strain LMG194, containing the Rpf-HisTag/pBAD/g111 plasmid, was prepared by cultivation of producer cells in RM medium containing (g/liter): casamino acids, 20; Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; (ml/liter): 1 M thiamine solution, 0.1; 1 M $MgCl_2$ solution, 1; pH 7.4, with addition of glucose up to a final concentration of 0.2% and ampicillin to 100 μg/ml at 37°C with shaking at 150 rpm. Induction was performed by adding 0.01% L-arabinose (DiaM, Russia) after the optical density of the cell culture at 600 nm had reached 0.5-0.7 unit. After incubation for 4 h, the cell suspension was centrifuged at 4000 rpm for 15 min. The cells were resuspended in a buffer containing 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, 10 μg/ml RNase, and 10 μg/ml DNase, and lysed using an ultrasonic disintegrator (Soniprep 150, Japan), three times for 30 sec. After centrifugation (12,500*g* for 30 min) the supernatant was diluted three times and applied to an affinity column containing Ni-NTA-agarose (Qiagen, Germany) equilibrated with SB buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0). The column was sequentially washed with 10 volumes of SB, 10 volumes of SB containing 10 mM imidazole (Sigma), and finally with 2 volumes of SB containing 20 mM imidazole. Elution was performed using 20 mM Tris-HCl buffer, pH 7.2, containing 100 mM histidine (Sigma). The eluate was dialyzed against 20 mM Tris-HCl buffer, pH 7.2, for 16-18 h at 4°C. The final protein preparation was diluted with an equal volume of glycerol and stored at −20°C until use. The protein amount was measured spectrophotometrically (A_{280}). The resultant recombinant Rpf preparations obtained were homogeneous after denaturing gel electrophoresis and colloidal Coomassie staining (represented by two forms with molecular weights of 27 and 31 kD). Both bands were detected by immunoblotting using specific antibodies against Rpf.

Using site-directed mutagenesis [12], mutant recombinant proteins with replacements E54Q, E54A, E54K, C53K, C114T and double replacements E54K/D48A and C53K/C114T were obtained. The recombinant proteins were prepared according to the same protocol as unmodified Rpf. A plasmid encoding a mutant protein containing only the variable segment of Rpf was obtained based on the pET-19b plasmid (Novagen). A DNA fragment corresponding to the Rpf

variable segment was amplified from the pET-19b–Rpf construct previously described [1]. Primer sequences were as follows: 5′-CAAGCATATGGTGGAGGGTG-GCTGGACC-3′; 5′-CTAGTTATTGCTCAGCGGTG-3′. The resulting fragment was cleaved with *Nde*I and *Bam*HI restriction endonucleases and cloned into the corresponding sites in pET-19b. The recombinant protein was obtained as described above for the unmodified protein.

Total cell wall preparation from *M. luteus*. *M. luteus* cells grown in NB medium at 30°C and 200 rpm until A_{600} ~1.5 were pelleted by centrifugation at 10,000 rpm for 15 min. The pellet was rinsed with 0.9% NaCl three times, then resuspended in 20 ml of 4% SDS solution and autoclaved for 20 min at 121°C. After that, the suspension was centrifuged at 12,500*g* for 15 min. The pellet was washed three times with 0.1% Triton X-100 solution to remove SDS, and then three times with 10 mM Tris-HCl buffer, pH 8.0. The suspension was finally dried on a rotary evaporator until completely dry and stored at –20°C.

Measurement of Rpf lytic activity towards total cell wall extract from *M. luteus*. The dried cell wall pellet treated with SDS was resuspended in 50 mM phosphate buffer, pH 7.0, and homogenized. The amount of pellet was optimized in such way that A_{600} of the suspension after homogenization was 0.22-0.25. The measurement was performed in a standard 96-well plate. Each well contained 300 μl cell wall suspension and the final concentration of Rpf was 1 μg/ml. Lytic activity was detected as decrease in absorbance of the cell wall suspension and registered using a Multiscan Ascent plate spectrophotometer (Thermo Labsystems, Finland) with built-in shaker and thermostat. The measurements were made at 5-min intervals over a 2-h period at 30°C with shaking at 900 rpm. The data were processed using Ascent Software, version 2.6, supplied together with the instrument.

Determination of muramidase activity of Rpf and its modified derivatives. To determine the muramidase activity of recombinant protein, 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside ($(NAG)_3$-MUF) (Sigma) was used as a substrate. The protein (final concentration 1-10 μg/ml) was incubated with the substrate (final concentration 8 μM) in 50 mM citrate buffer, pH 6.0, in the presence of 5 mM $MgSO_4$ for 3 h at 37°C. Fluorescence intensity was registered using an RF-5301PC fluorimeter (Shimadzu, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

Analysis of proteolytic (peptidase) Rpf activity. To determine peptidase activity of native Rpf preparations, N-CBZ-Gly-Gly-Arg-β-naphthylamide (Sigma) was used as a substrate. Native protein at a concentration of 5 μg/ml was incubated with the substrate (final concentration 55 μM) in 50 mM Tris-HCl buffer, pH 8.0, in the presence of 5 mM $MgSO_4$ for 25 h at 22°C. Fluorescence intensity of the reaction products was measured on the fluorimeter using excitation and emission wavelengths of 330 and 420 nm, respectively. One unit of enzymatic activity was defined as the value of the fluorescence intensity consequent upon substrate hydrolysis by trypsin at a concentration of 1 pg/ml at 37°C and under the above conditions.

Protein electrophoresis according to O'Farrell and Laemmli was performed using standard protocols.

Western blotting and ELISA. Affinity-purified rabbit antibodies against Rpf [3] were used for Western blot analysis and ELISA. These antibodies were specific towards native Rpf and all recombinant forms, including aggregated forms and degradation products (antibody dilution 1 : 3000). Anti-rabbit IgGs (Sigma) at a dilution of 1 : 5000 were also used. ELISA was performed according to the previously described protocol [4].

Protein sequencing. Analysis of C-terminal amino acid sequences was performed by the carboxypeptidase method followed by identification of dansyl chloride-modified amino acids [12]. N-Terminal sequences were analyzed according to the Edman technique.

Mass spectrometry. Proteins were identified after SDS-PAGE according to Laemmli. Gel treatment, trypsinolysis, and peptide extraction were performed using standard protocols [13, 14]. MALDI-TOF mass spectra were obtained on an Ultraflex (Bruker, Germany) instrument in positive ionization mode [15].

Determination of Rpf lytic activity towards total cell wall extract from *M. luteus* in polyacrylamide gel. The study was performed on a glass plate with a 15% polyacrylamide gel (without SDS) containing 0.1% total cell wall from *M. luteus*. Rpf solution (20 μl; 100 μg/ml) was added to the plate and incubated at 37°C for 18 h after complete absorption of the protein solution into the gel. Lytic activity was displayed as a clearing spot on the gel corresponding to the zone of Rpf application.

Production and resuscitation of "non-culturable" *M. smegmatis* cells. "Non-culturable" *M. smegmatis* cells (strain mc^2155) were prepared by growing bacteria in a modified Hartman's–de Bont medium containing 0.5% bovine serum albumin (Cohn-analog, Sigma) to stationary phase [16]. In some cases, cells transformed with plasmids encoding secreted Rpf and its mutant forms were used. An end-point dilution method, based on the preparation of a series of 10-fold dilutions of bacterial suspension in a fresh nutrient medium until a concentration of 1 cell per ml of medium was attained, was used to resuscitate "non-culturable" cells. Resuscitation was performed in 48-well Corning plates, containing 0.45 ml of Sauton's medium supplemented with 0.05% yeast extract in each well. Sauton's medium contains (per liter): 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$; 4 g L-asparagine, 60 ml glycerol; 0.05 g ferric ammonium citrate; 2 g $Na_3C_6H_5O_7$; 0.1 mg $ZnSO_4$. The wells also contained 50 μl of the corresponding serial dilutions of cell cultures and Rpf-producing cells, viz. *M. luteus* at the concentration of 10^5-

10^6 cells/ml. The plates were incubated for 6 days at 37°C with agitation (150 rpm). The most probable number (MPN) of resuscitated cells was estimated statistically using the standard tables [17], taking into account the number of wells with visible bacterial growth for each dilution (triplicate measurements). The MPN of resuscitated cells represents the average number of resuscitated cells per ml inoculum, taken from the most concentrated suspension.

RESULTS

Rpf forms secreted into the culture medium by *M. luteus*. Two polypeptides that interact with antibodies against the entire recombinant Rpf or its conserved domain are found in the culture supernatant during the cultivation of *M. luteus* in a liquid medium from early logarithmic and up to stationary phase. The larger polypeptide has molecular weight of about 25 kD and the smaller one of 12-15 kD (Fig. 1). Microsequencing of the N-terminus of the immunoreactive 25 kD protein from the culture medium has shown that the five N-terminal amino acid residues correspond to the predicted sequence of the secreted form of Rpf (ATVDT), based on analysis of the *rpf* gene sequence. Sequencing of the C-terminus also revealed concordance of the protein sequence with that predicted for Rpf and based on the *rpf* gene sequence.

Protein analysis using MALDI-TOF/MS has confirmed that the protein with a molecular weight of 25 kD (according to electrophoresis data) is the secreted form of Rpf, and that its actual molecular weight is 19 kD, which

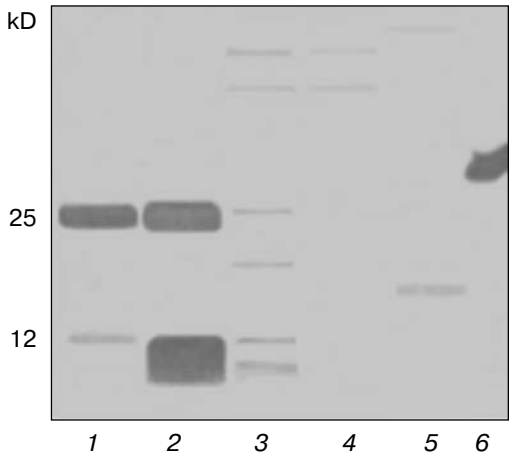

Fig. 1. Secretory Rpf forms in culture medium of *M. luteus*. Western blotting of supernatant aliquots taken from *M. luteus* culture at different growth stages: *1*) logarithmic phase (A_{600} = 0.8, 12 h); *2*) end of logarithmic phase (A_{600} = 6.3, 24 h); *3*) stationary phase (A_{600} = 7.5, 44 h); *4*) late stationary phase (A_{600} = 7.0, 68 h); *5*) pre-stained protein molecular weight markers for immunoblotting (Bio-Rad, USA); *6*) recombinant Rpf, 10 ng.

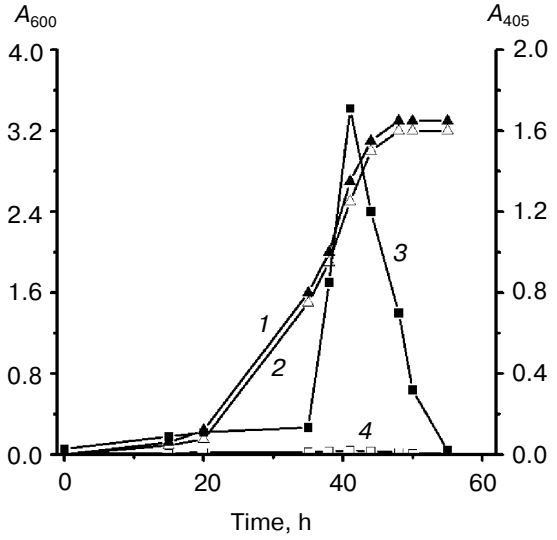

Fig. 2. Rpf content in *M. luteus* culture medium (wild type and mutant with inactivated chromosomal *rpf* gene) during growth on NB medium: *1*) growth curve for wild type; *2*) growth curve for mutant; *3*) dynamics of Rpf accumulation in culture medium (wild type); *4*) dynamics of Rpf accumulation in culture medium (mutant).

corresponds to the theoretical molecular weight of the predicted gene product (19,147 daltons). The polypeptide with a molecular weight 12 kD is probably a truncated form resulting from Rpf proteolysis. Overestimation of molecular weight following denaturing gel electrophoresis was observed for other Rpf-like proteins [3]. The concentration of both forms (with molecular weights of 25 and 12 kD) in the culture medium of *M. luteus* falls to a non-detectable level (less that 2 μg/liter culture) during the late stationary phase (Fig. 1). It is noteworthy that Rpf with a molecular weight of 25 kD represents the major protein secreted by *M. luteus* into the culture medium; its concentration reaches 0.5-1 mg/liter (depending on the culture density) as determined by ELISA. However, the concentration of the corresponding proteins in other bacterial producers of Rpf-like proteins, for instance, *M. bovis* [3] and *M. smegmatis* [16], is about a thousand times lower. Moreover, as was shown previously, Rpf concentrations effective for the stimulation of cell growth are within the range of tens of picograms to nanograms per ml of medium [3]. It would therefore appear that the high Rpf concentrations secreted by *M. luteus* are excessive in relation to its proposed function as a growth factor. We have compared Rpf levels in the culture supernatant in wild type cells and in cells where *rpf* gene was inactivated by allelic exchange, and Rpf was synthesized from a plasmid carrying the *rpf* gene (such cells would not be viable without the plasmid) [4]. As seen from Fig. 2, despite the similarity between the growth curves for two strains in the rich medium, the amount of Rpf secreted into the culture medium by the transformant with the inactivated *rpf* gene

was three orders of magnitude lower (comparable with the ELISA detection limit) than that secreted by wild type cells. This indicates that even very small amounts of secreted Rpf are sufficient to maintain growth of *M. luteus*. An increase in temperature during cultivation of *M. luteus* with an inactivated *rpf* gene resulted in aggregation and formation of cellular clumps.

Peptidoglycan hydrolase activity of Rpf. In view of the predicted structural similarity between Rpf and lysozyme-like proteins, the peptidoglycan hydrolase activity (typical for enzymes involved in the processing of bacterial cell wall) of Rpf was measured. As seen from Fig. 3, the application of Rpf to the polyacrylamide gel plate, containing *M. luteus* cell walls, resulted in the formation of a transparent zone, corresponding to cell wall hydrolysis. The recombinant protein containing only the variable Rpf region did not display such lytic activity. Similar lysis zones (but observed for much lower effective concentrations) were found for lysozyme (data not shown). Other protein preparations employed as controls that did not have cell wall degrading activity (e.g., bovine aldolase; Sigma) did not exhibit any lysis zones (data not shown).

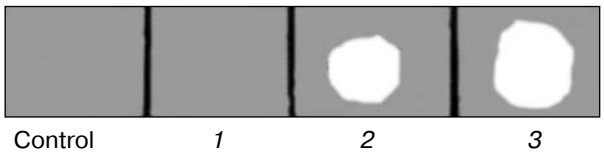

Fig. 3. Lysis zones in polyacrylamide gel containing total cell wall extract from *M. luteus* treated with SDS: *1*) recombinant proteins corresponding to variable Rpf region; *2*) recombinant Rpf; *3*) native Rpf.

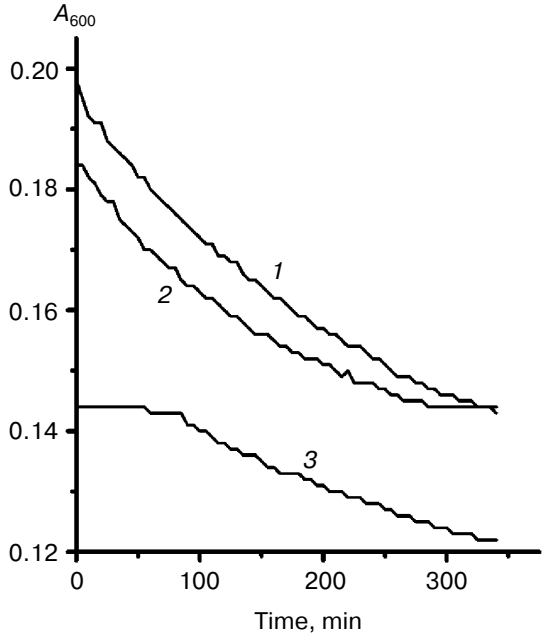

Fig. 4. Lytic activity of Rpf and its mutant form with glutamate replacement (E54A) towards total cell wall extract from *M. luteus*. Lytic activity was registered by changes in absorbance at 600 nm and 30°C. *1*) Recombinant Rpf; *2*) mutant Rpf (E54A); *3*) buffer (control).

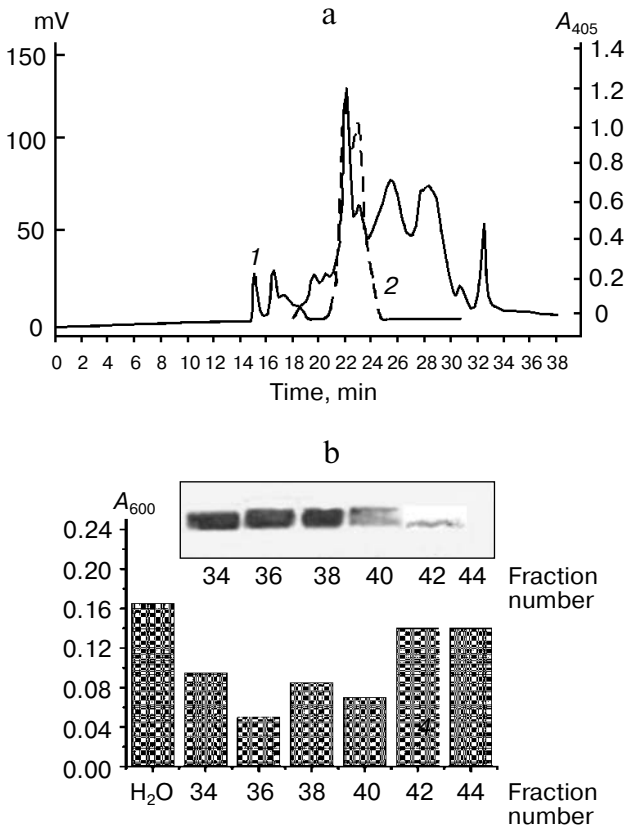

Fig. 5. Gel filtration of supernatant from *M. luteus* culture, immunoblotting, and lytic activity of the fractions obtained. *M. luteus* cells were grown on dialyzed NB medium until A_{600} reached 2.0. Supernatant taken from the culture was applied to a BioSep-SEC-2000 column (Phenomenex, USA) using an HPLC system, followed by analysis of obtained fractions by ELISA with Rpf-specific antibodies. a: *1*) HPLC elution profile (on left ordinate, optical detector voltage proportional to optical density at 280 nm); *2*) ELISA data. b) Immunoblotting of gel chromatography fractions with Rpf-specific antibodies and their lytic activity towards the total cell wall extract from *M. luteus*. Fractions 34-44 (in panel (b)) correspond to fractions eluted after 21-26 min (in panel (a)). For determination of lytic activity, equal volume of each fraction was added to the preparation of cell walls and incubated for 24 h at 37°C. Absorbances were measured at 600 nm.

The second approach to the determination of Rpf lytic activity was based on the registration of absorbance changes for *M. luteus* cell walls (prepared in the presence of SDS) upon incubation with recombinant Rpf. These experiments demonstrate a small but reproducible decrease in absorbance (A_{600}) of the cell wall suspension (Fig. 4). The effect was dependent on Rpf concentration.

Boiling of the Rpf preparation resulted in a loss of activity, indicating the enzymatic mechanism of Rpf action upon the cell walls. Native Rpf also displayed lytic activity towards the cell wall preparation. To confirm that the detected lytic activity of the native Rpf preparation was an intrinsic property of this protein, gel filtration experiments were performed using HPLC and Rpf isolated from culture supernatant. ELISA and immunoblotting revealed the presence of Rpf in those particular fractions that displayed lytic activity (Fig. 5).

The abovementioned results indicated that Rpf has lytic activity towards *M. luteus* cell wall preparations. However, these data did not provide any evidence concerning the precise nature of the Rpf substrate. To test the hypothesis that Rpf is a peptidoglycan hydrolase, we used the specific synthetic substrate for peptidoglycan hydrolases (muramidases), $(NAG)_3$-MUF, which is a model analog of bacterial cell wall polymers. It was found that recombinant Rpf hydrolyzes $(NAG)_3$-MUF with formation of the fluorescent product, MUF, thus indicating that Rpf has β-1-4-glycolytic activity (Fig. 6). The reaction was optimal at pH 6. Substrate hydrolysis was activated by addition of Mg or Ca salts (10 mM), but was strongly inhibited in the presence of Zn salts. The specific activity of different recombinant Rpf preparations was very variable, with a maximum specific activity of 21 ± 12 nmol MUF/h per mg of protein. Native Rpf was also able to hydrolyze this substrate with a specific activity of 7 ± 5 nmol MUF/h per mg of protein. For comparison, the specific activity of egg white lysozyme towards the same substrate was about 900 nmol MUF/h per mg of protein. Thus, cell wall lysis by Rpf, both in suspension and in polyacrylamide gel, as well as hydrolysis of a specific lysozyme substrate, indicated that Rpf has peptidoglycan hydrolase activity.

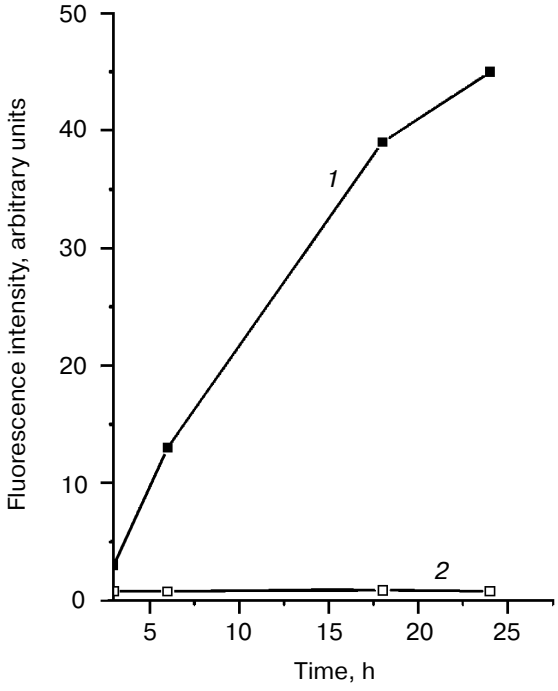

Fig. 7. Peptidase activity of native Rpf. Fluorescent substrate (N-CDZ-Gly-Gly-Arg-β-naphthylamide) was incubated with native Rpf in Tris-HCl buffer (pH 8.0): *1*) native Rpf; *2*) buffer (control). Complete substrate hydrolysis by trypsin corresponded to fluorescence of 850 arbitrary units.

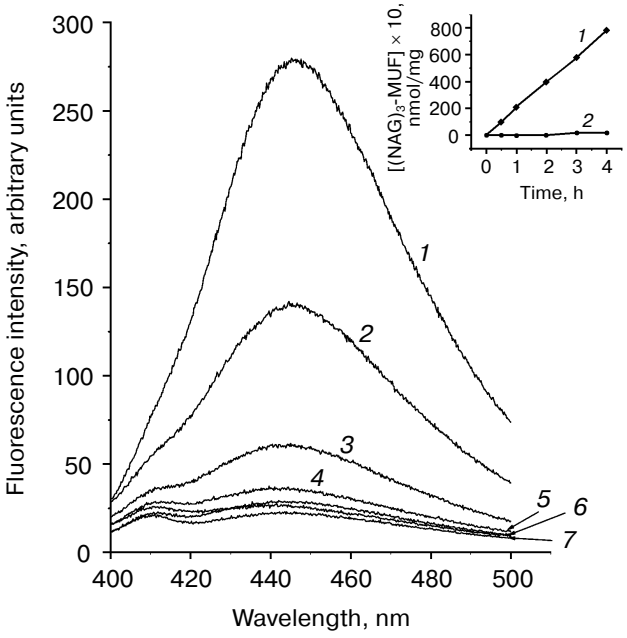

Fig. 6. Muramidase activity of recombinant Rpf proteins. Fluorescent substrate (4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside) was incubated with various recombinant proteins in citrate buffer (pH 6.0) during 3 h at 37°C: *1*) Rpf ; *2-6*) mutant Rpf forms: E54Q (*2*), E54A (*3*), E54K (*4*), C53K + C114T (*5*), E54K + D48A (*6*); *7*) buffer (control). The insert shows time dependence of hydrolysis of fluorescent substrate by recombinant Rpf (*1*); buffer (control) (*2*).

Peptidase activity of Rpf. We have noticed that during periods of storage native Rpf preparations in some cases underwent cleavage and formed products with various molecular weights. According to MALDI-TOF data, the two main peptides formed have molecular weights of 10,004 and 9245 daltons. Inspection of the Rpf sequence indicates that the cleavage site is located near residue Pro71 at the end of the conserved domain. Repeated purification of Rpf preparations did not prevent its self-degradation, which indicated that Rpf may possibly possess intrinsic proteolytic activity. Various substrates were screened and it was shown that native Rpf preparations hydrolyzed N-CBZ-Gly-Gly-Arg-β-naphthylamide, used for the assay of trypsin activity (Fig. 7). This reaction is optimal at pH 8. Recombinant Rpf preparations also displayed proteolytic activity. The specific activity of various preparations of native Rpf reached 28,800 ±

800 units/mg. The specific activity of recombinant protein preparation was ca. 16,000 ± 9000 units/mg.

Site-directed mutagenesis of Rpf. According to the theoretical model of Rpf tertiary structure (http://www.doe-mbi.ucla.edu/TMW/) and the published NMR data [11], there is a highly conserved glutamate residue in all Rpf homologs (Glu54 in Rpf), which corresponds to the catalytically active Glu35 in lysozyme and lytic transglycosylases Slt35 (E162) and Slt70 (E478) [18, 19]. Based on this similarity, we have investigated several mutant forms of Rpf, where different amino acid residues were replaced by site-directed mutagenesis. The effects of these replacements were studied using resuscitation of "non-culturable" cells of *M. smegmatis*, using the model developed by us previously [16]. The cells could be reactivated if they contained a plasmid encoding a secreted form of Rpf. In this work, we investigated the effect of amino acid replacement in the Rpf molecule on resuscitation of "non-culturable" cells. The results of these experiments are presented in the table. As a control, we used resuscitation of *M. smegmatis* upon co-cultivation with *M. luteus* cells (as was shown earlier, this approach allowed reactivation of wild type [16]). As seen in the table, the isosteric replacement, E54Q, merely resulted in a decrease in the extent of reactivation. Replacements E54A and especially E54K resulted in significantly more profound inhibition of Rpf resuscitation activity. Other replacements were also investigated, for instance, the two cysteine residues, which seem likely to be involved in the formation of a functionally significant intramolecular disulfide bond. Single replacements of Cys53 and Cys114 resulted in partial inhibition of activity, whereas the double replacement led to virtually complete Rpf inactivation.

In parallel, we also investigated the effect of these amino acid replacements in the Rpf molecule on its enzymatic activity towards the substrate $(NAG)_3$-MUF. As seen in Fig. 6, the isosteric replacement, E54Q, did not result in a complete loss of activity. However, replacements E54A and particularly E54K caused a dramatic inhibition of enzymatic activity. The maximal inhibition was seen in the mutant form with replacement of both cysteine residues. The rate of hydrolysis of *M. luteus* cell walls by the mutant Rpf containing E54A replacement was reduced compared with the unmodified protein (Fig. 4).

DISCUSSION

Rpf was first isolated as a protein factor mediating resuscitation of dormant *M. luteus* cells *in vitro*. The relatively high (unusual for cytokines) Rpf concentrations in the culture medium, together with the associated proteolytic activity we previously reported [9], the predicted lysozyme-like fold of the Rpf domain, the presence of a LysM domain (a characteristic module for protein attachment to the cell wall) at the C-terminus of Rpf, and weak similarity of the Rpf active site to that of lytic transglycosylases [10, 20] led us to search for specific enzymatic functions of the Rpf-like proteins, which would indicate an involvement in the processing of bacterial cell walls.

Reactivation of "non-culturable" *M. smegmatis* cells transformed by pAG plasmid carrying wild type and mutant *rpf* genes

Mutation	MPN			Resuscitation index
	AGX	AGR	AGX + *M. luteus*	
Empty vector	$<10^2$	$(1.8 \pm 1.2) \cdot 10^7$	$(3.9 \pm 1.9) \cdot 10^5$	$<5.5 \cdot 10^{-6}$
E54Q	$(4.3 \pm 1.7) \cdot 10^4$	$(2.3 \pm 1.1) \cdot 10^6$	$(4.7 \pm 2.1) \cdot 10^6$	$1.9 \cdot 10^{-2}$
E54A	$(1.1 \pm 0.9) \cdot 10^4$	$(1.8 \pm 1.2) \cdot 10^7$	$(3.0 \pm 2.0) \cdot 10^5$	$6.0 \cdot 10^{-4}$
E54K	$<10^2$	$(1.8 \pm 1.2) \cdot 10^7$	$(4.4 \pm 3.0) \cdot 10^6$	$<5.5 \cdot 10^{-6}$
E54K/D48A	$(2.0 \pm 1.5) \cdot 10^3$	$(1.8 \pm 1.2) \cdot 10^7$	$(3.0 \pm 2.5) \cdot 10^5$	$1.1 \cdot 10^{-4}$
C53K	$(2.9 \pm 2.1) \cdot 10^4$	$(2.3 \pm 1.1) \cdot 10^6$	$(3.0 \pm 0.4) \cdot 10^6$	$1.3 \cdot 10^{-2}$
C114T	$(4.0 \pm 3.5) \cdot 10^4$	$(2.3 \pm 1.1) \cdot 10^6$	$(2.8 \pm 1.6) \cdot 10^6$	$1.7 \cdot 10^{-2}$
C53K/C114T	$<10^2$	$(1.8 \pm 1.2) \cdot 10^7$	$(1.6 \pm 0.9) \cdot 10^5$	$<5.5 \cdot 10^{-6}$

Note: Reactivation of "non-culturable" cells secreting various mutant forms of Rpf (AGX) was measured by the end-point dilution method (see "Materials and Methods"). Reactivation of each strain upon co-cultivation with *M. luteus* (AGX + *M. luteus*) was used as a positive control. Resuscitation index was calculated as the ratio between the most probable number (MPN) of cells for AGX and AGR (wild type) in the same experiment.

Both native (i.e., isolated from *M. luteus* culture medium) and recombinant (isolated from *E. coli* producer strain LMG194, transformed with a corresponding plasmid) Rpf preparations exhibited activity towards the model peptidoglycan substrate and the cell wall extract. In the present investigation, this conclusion is confirmed by two experimental approaches: activity against crude cell wall preparations in solution and in polyacrylamide gel, and hydrolysis of a specific, artificial lysozyme substrate. Other experimental data also confirm this conclusion [12]. Moreover, there is a LysM motif at the C-terminus of Rpf, which is responsible for protein binding to peptidoglycan in the bacterial cell wall [21]. It indicates an ability of the Rpf molecule to bind to the cell wall, which is the obvious substrate for this protein.

Since the conserved domain is common for all Rpf-like proteins (up to 75% of identical residues), it can be predicted that all Rpf-like proteins are peptidoglycan hydrolase enzymes. At the present time peptidoglycan hydrolases are divided into five classes: muramidases (EC 3.2.1.17), N-acetyl-glucosaminidases (EC 3.2.1.52), lytic transglycosylases (EC 3.2.1.x), N-acetylmuramoyl amidases (EC 3.5.1.28), and peptidoglycan-D,D-endopeptidases (EC 3.4.99.17). According to current models, these enzymes form multienzyme complexes, which cooperatively remodel the bacterial cell wall [22].

The fact that Rpf hydrolyzes the synthetic muramidase substrate $(NAG)_3$-MUF indicates that Rpf possesses β-1-4-glycolytic activity, intrinsic for muramidases, N-acetyl-glucosaminidases, and lytic transglycosylases. Thus, Rpf-like proteins probably belong to one of the abovementioned classes of peptidoglycan hydrolases.

The relatively low proteolytic activity associated with Rpf, which was found previously [9] and studied in detail in the present work using a synthetic model substrate for trypsin-like proteinases, could be nonspecific and related to its peptidoglycan hydrolase activity. However, it may represent specific proteolytic activity towards an as yet unidentified substrate in the *M. luteus* cell wall. In this context, Rpf resembles some recently discovered enzymes with lysozyme-like activity that also exhibit peptidase activity. Such enzymes include P5 protein from phi-6 bacteriophage [23], shellfish lysozyme [24], and, perhaps, destabilase from the medicinal leech [25]. It is interesting that shellfish lysozyme displayed maximal lysozyme activity at pH 5.0, and isopeptidase activity at pH 7.0, since similar pH optima were found for the corresponding activities of Rpf. It has been proposed that the differences in pH optima indicate a spatial separation between the active sites responsible for the different activities [24].

The predicted tertiary structure for the Rpf domain indicates that it is lysozyme-like [8], as has recently been confirmed by NMR analysis [11]. However, in our experiments, replacement of Glu54 (a catalytically important amino acid in lysozyme-like proteins) with the structurally similar residue, glutamine, did not lead to the complete loss of activity (as has been documented in the case of lysozyme and lytic transglycosylases). At the same time, replacement of Glu54 by amino acids with less structural similarity resulted in a more pronounced decrease in activity (Fig. 6). This might suggest some difference between the catalytic mechanisms of lysozyme and Rpf; however, the involvement of Glu54 in catalysis cannot be excluded at present. As was previously suggested [11], the Rpf molecule is stabilized by a disulfide bond. The data obtained in this work indicate that the cysteine residues are possibly involved in catalysis or in the maintenance of structural integrity. It is significant that the effects of specific amino acid replacements on Rpf enzymatic activity in the reaction with the synthetic substrate correlate with their influence on biological activity. Indeed, the resuscitation efficiency of "non-culturable" *M. smegmatis* cells as influenced by the various modified Rpf proteins (table) varied in accordance with the activity data obtained using the synthetic substrate (Fig. 6), which directly confirms the leading role played by Rpf in the process of reactivation.

Despite the fact that the mechanism of resuscitation of dormant cells by Rpf still remains unknown, a hypothesis can be made based on the known enzymatic properties of this protein. It is known that during the activation of bacterial spores the first detected activities are so called lytic activities, specific for resuscitation and growth, which are realized by germination-specific lytic enzymes (GSLE) [26], in particular, N-acetyl-glucosaminidase, lytic transglycosylase, and amidase activities. Cooperative action of these enzymes results in decondensation of the spore cortex, a thickened cell wall found in bacterial spores. As a result of this process, spores become more sensitive to external and trophic stimuli (amino acids, ions, active oxygen forms, etc.), which in turn can induce their transformation into active life forms [27]. The presence of an abnormally thickened cell wall in "non-culturable" cells of *M. luteus* is reminiscent of the spore cortex, lending credence to a proposed role for these proteins in breaking dormancy by their modification of the bacterial cell envelope.

The cytokine-like mechanism of Rpf action suggested previously [1] implies the binding between Rpf and its hypothetical receptor on the cell surface, which triggers a biochemical cascade ultimately resulting in the resumption of cell division. However, despite all efforts, a receptor for Rpf has not yet been found on the cell surface of *M. luteus* (Mukamolova and Young, unpublished data). Moreover, the relatively high concentration of Rpf in the culture medium is not consonant with a cytokine-like mode of action. However, in other bacteria, such as *M. tuberculosis*, *M. smegmatis*, and *C. glutamicum*, the secreted levels of Rpf-like proteins are very low [3]. In this work, we have also found that very low amounts of Rpf are sufficient for biological activity (Fig. 2), which may indicate an additional function(s) for *M. luteus* Rpf.

In summary, we may conclude that the secreted Rpf protein and its structural homologs (Rpf-like proteins) are peptidoglycan hydrolase enzymes, presumably, lytic transglycosylases. The latter are involved in the processing or remodeling of bacterial cell walls *in vivo*, both during normal cell wall expansion as a result of vegetative growth and upon addition to "non-culturable" or dormant bacterial populations, when they act as reactivating agents.

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