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Comparative expression analysis of *rpf*-like genes of *Mycobacterium tuberculosis* H37Rv under different physiological stress and growth conditions

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Mycobacterium tuberculosis H37Rv possesses five resuscitation-promoting factors, RpfA–E, which are required for the resuscitation of dormancy in mycobacteria induced by prolonged incubation of the culture in stationary phase. This study explores the transcriptional profile of all the *rpf*-like genes of *M. tuberculosis* H37Rv in the exponential phase, stationary phase, non-culturable phase and Rpf-mediated resuscitation phase. The relative expression was also monitored under acid stress, nutrient starvation and low-oxygen (hypoxia) conditions by real-time quantitative PCR. We show differential relative expression of the *rpf* genes during different stages of growth and under stress. During early resuscitation, all *rpf* genes were expressed with maximal expression ratios for *rpfA* and *rpfD*. *rpfC* was consistently expressed during all stages of growth and nutrient starvation. Acid stress induced higher relative expression of *rpfD* and *rpfE* and hypoxia of *rpfC* and *rpfE*. These results therefore provide further evidence that the *rpf* genes perform distinct roles during cell growth and cell survival under different physiological stresses, and are consistent with the *rpf*-like genes being differentially regulated.

INTRODUCTION

Persistence and reactivation play significant roles in the pathogenesis of *Mycobacterium tuberculosis*. Only a proportion of persons infected with *M. tuberculosis* develop active tuberculosis, while the majority of them harbour the bacilli for an extended period of time in the form of latent infection without producing clinical symptoms. Approximately one-third of the world's population is infected with latent *M. tuberculosis*, and run the risk of reactivation, causing active disease (WHO, 2010; Gedde-Dahl, 1952). Hence, an understanding of mycobacterial persistence and reactivation is crucial for effective control of the disease.

The translation from replicating to a dormant state and then to reactivation remains an enigma. In recent years, a class of protein has been identified that act like growth factors for dormant mycobacteria. The protein known as resuscitation-promoting factor was originally identified in *Micrococcus luteus* as a ~16–17 kDa protein that is secreted by actively growing bacteria. The protein in picomolar concentrations is able to resuscitate dormant *Micrococcus luteus* or mycobacteria generated by prolonged incubation in spent culture medium (Mukamolova *et al.*, 1998a, b; Shleeva *et al.*, 2002). *M. tuberculosis* contains five Rpf homologues which share sequence as well as functional homology with Rpf of *Micrococcus luteus* in their capacity to resuscitate dormant *Mycobacterium bovis* BCG or *M. tuberculosis in vitro* (Mukamolova *et al.*, 2002). The five *rpf*-like genes of *M. tuberculosis* H37Rv are distributed throughout the chromosome and are designated *rpfA* (Rv0867c), *rpfB* (Rv1009), *rpfC* (Rv1884c), *rpfD* (Rv2389c) and *rpfE* (Rv2450c) (http://genolist.pasteur.fr/tuberculist/). The presence of five *rpf*-like genes in *M. tuberculosis* is intriguing, as all five genes are expressed *in vitro* and *in vivo* in the lungs of mice acutely infected with *M. tuberculosis* (Tufariello *et al.*, 2004; Mukamolova *et al.*, 2002; Downing *et al.*, 2004). Transcripts for all the *rpf*-like genes are detectable at the earliest time points, although relative expression has been shown to differ at later time points during growth in culture (Tufariello *et al.*, 2004; Mukamolova *et al.*, 2002). Strains harbouring deletions of individual *rpf*-like genes from *M. tuberculosis* do not show significant alteration of growth *in vitro* and *in vivo* (Tufariello *et al.*, 2004; Downing *et al.*, 2004). Earlier studies have confirmed that *rpfA–E* are collectively dispensable for growth of *M. tuberculosis in vitro* and *in vivo* and suggest a functional hierarchy within this multigene family under different conditions (Kana *et al.*, 2008). Deletion of individual *rpfB–E* genes has been shown to be accompanied by upregulation of some or all of the remaining *rpf*-like genes (Downing *et al.*, 2004), although later studies with multiple *rpf* mutants have clearly shown

Abbreviations: NC, non-culturable; NCC non-culturable cells.

A set of supplementary methods, describing the protocol for cDNA synthesis, a supplementary figure, showing amplification efficiency for each primer set, and a supplementary table, showing the data used to calculate the normalized ratios, are available with the online version of this paper.

that *rpf*-like genes do not compensate for the loss of other genes by transcriptional upregulation; instead they suggest that *rpf*-like genes are likely to be regulated by other distinct mechanisms (Downing *et al.*, 2005; Kana *et al.*, 2008). In this respect, it is worth noting that *rpfA* has been shown to be regulated by cAMP receptor protein (Rickman *et al.*, 2005), while *rpfC* is positively regulated by the sigma factor SigD (Raman *et al.*, 2004). RpfB and RpfE have been shown to interact with mycobacterial endopeptidase RipA (Rpf-interacting protein), resulting in peptidoglycan hydrolysis. RipA does not interact with RpfA, C and D, adding to the complexity of Rpf function and regulation (Hett *et al.*, 2007).

In this study, we have explored by real-time PCR the relative expression of all five *rpf*-like genes of *M. tuberculosis* H37Rv during different growth stages, ranging from exponential phase to the non-culturable (NC) state and subsequent Rpf-mediated resuscitation. The relative expression profiling was further extended to three different physiological stress conditions; namely, nutrient starvation, hypoxia and acid stress.

METHODS

Bacterial strains and culture conditions. *M. tuberculosis* H37Rv was grown in Sauton's medium containing albumin–dextrose–catalase (ADC; Becton Dickinson) and Tween 80 (0.05 %, v/v) without shaking at 37 °C. For the resuscitation of NC cells of *M. tuberculosis*, filter-sterilized supernatant of actively growing *M. tuberculosis* culture (OD_{600} 0.5) was used. Methylene blue (1.5 μg ml^{-1}) was used as a redox indicator in hypoxia experiments.

Formation of non-culturable cells (NCC) and their resuscitation. The NC state was achieved as described by Shleeva *et al.* (2003). Briefly, NCC of *M. tuberculosis* were obtained by prolonged incubation of culture in Sauton's medium containing Tween 80 (0.05 %, v/v) and ADC for 6 months at 37 °C (Shleeva *et al.*, 2003; Salina *et al.*, 2006). Samples were taken at regular intervals and passed through a 23-gauge syringe needle before plating to make the culture homogeneous. Plating was done on MB7H10 agar medium containing oleic acid–albumin–dextrose–catalase (OADC; Becton Dickinson). After 6 months of incubation, *M. tuberculosis* became NC, as c.f.u. levels were less than 5 cells ml^{-1}. Resuscitation of NCC was induced by cell-free culture supernatant of exponential phase (OD_{600} 0.5) *M. tuberculosis* H37Rv culture, as described by Shleeva *et al.* (2003). The cells were removed by centrifugation at 6000 ***g*** for 10 min and the supernatant was filter-sterilized with a 0.22 μm pore-size filter (Whatman) at 4 °C and used within 20 min.

RNA isolation from different growth stages. An exponential-phase starter culture of *M. tuberculosis* H37Rv was used to inoculate the working culture at an approximate density of 10^6 c.f.u. ml^{-1} (based on the estimate that OD_{600} $1 = 5 \times 10^8$ c.f.u. ml^{-1}) in Sauton's medium containing ADC and Tween 80 at 37 °C without shaking. RNA extraction was done at different growth stages such as early exponential phase (OD_{600} 0.2, 5-day-old culture), exponential phase (OD_{600} 0.6, 3-week-old culture), stationary phase (OD_{600} 2.5, 3-month-old culture), NC state (OD_{600} 4.5, 6–month-old culture) and resuscitation phase (OD_{600} 0.2, 3 days after resuscitation). From the various growth phases described above, 30 ml culture was harvested in triplicate from three independent experiments. RNA isolation was done according to the kit protocol (RNeasy Mini kit, Qiagen) and quantified by Qubit fluorometer (Invitrogen).

Acid stress. *M. tuberculosis* H37Rv culture was grown in 100 ml Sauton's medium (pH 7.2) containing Tween 80 (0.05 %, v/v) at 37 °C without shaking. When the OD_{600} reached 0.6, cells were divided into three 30 ml aliquots, centrifuged at 6000 ***g*** for 10 min at room temperature, resuspended in 50 ml Sauton's medium (pH 4.5, pH 5.5 or pH 7.2) containing Tween 80 (0.05 %, v/v) and kept at 37 °C without shaking. After 24 h of acid shock, 30 ml culture from each flask was pelleted. RNA isolated from the culture grown in Sauton's medium at pH 7.2 was used as a control or positive calibrator for relative expression analysis. RNA isolation was done according to the kit protocol (RNeasy Mini kit, Qiagen) and quantified by Qubit fluorometer (Invitrogen). RNA was isolated from three independent experiments.

Nutrient starvation. An exponentially growing *M. tuberculosis* H37Rv culture (OD_{600} 0.6) in Sauton's medium with Tween 80 (0.05 %, v/v) was centrifuged at 6000 ***g*** for 10 min at room temperature. Cells were washed twice with PBS with Tween 80 (0.05 %, v/v) pH 7.2 and resuspended in 100 ml PBS-Tween (pH 7.2). The culture was incubated at 37 °C without shaking. Cells were harvested at 0, 24 and 96 h. Cells harvested at 0 h served as a control (Betts *et al.*, 2002). RNA isolation was done by using the RNeasy Mini kit (Qiagen) and quantified by Qubit fluorometer (Invitrogen). The experiment was repeated three times with three independent experimental set-ups.

Hypoxia conditions. An aerobic exponentially growing culture of *M. tuberculosis* H37Rv was diluted to OD_{600} 0.005 in MB7H9 medium containing ADC, Tween 80 (0.05 %, v/v) and the redox dye methylene blue (1.5 μg ml^{-1}). The culture was transferred to sealed rubber-capped tubes (50 % headspace ratio) and kept standing at 37 °C. Anaerobiosis was monitored by decolorization of the redox indicator methylene blue (Boon *et al.*, 2001). Complete decolorization of the dye was obtained after 25 days incubation at 37 °C. For control samples, tubes were not sealed and were kept at 37 °C with periodic exposure to air by shaking for the same period of time. The cells were pelleted on the 26th day in triplicate, and RNA was isolated by using the RNeasy Mini kit (Qiagen) and quantified by Qubit fluorometer (Invitrogen).

cDNA synthesis. Each RNA sample was treated with RNase-free DNase I (Fermentas) and heat-inactivated according to the manufacturer's instructions. RNA integrity was checked by formaldehyde agarose gel electrophoresis. After staining with ethidium bromide, three sharp bands of 23S, 16S and 5S rRNA were seen. DNA contamination was checked by (no reverse transcription) PCR for each RNA sample in a gradient cycler (Mastercycler gradient, Eppendorf) using a 16S rRNA primer. No amplification was seen after 30 cycles of amplification. PCR cycling parameters were: initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, extension at 72 °C for 30 s for 30 cycles, followed by a final extension of 10 min at 70 °C. PCR was performed in a 20 μl reaction mix containing 1.25 U DreamTaq DNA polymerase (Fermentas), 16S rRNA primers (0.2 μM each), RNA template (100 ng), $MgCl_2$ (final concentration 2.0 mM) and dNTPs (final concentration 0.2 mM). PCR products were electrophoresed in a 2.5 % agarose gel and visualized by ethidium bromide staining.

A 300–400 ng RNA sample was used for cDNA synthesis using random hexamer primers with the RevertAid First Strand H Minus cDNA synthesis kit (Fermentas). A detailed protocol for cDNA synthesis is available with the online version of this paper. Real-time quantitative PCR was performed in a LightCycler 480II instrument (Roche) using a LightCycler 480 SYBR Green I Master kit (Roche).

Primers were designed using DNASTAR primer design software and purchased from Sigma–Aldrich. All primer sets were optimized for annealing temperature and concentration to ensure that only a single product of the correct size would be amplified. The optimum annealing temperature and primer concentration were found to be 68 °C and 0.5 μM.

PCR efficiency and real-time PCR. Amplification efficiency for each primer set was calculated by serially diluting exponential-phase cDNA template. Efficiency values were calculated by the software in LightCycler 480II and were in the optimum range. Efficiency values for each primer set are given in Table 1 and Supplementary Fig. S1, and showed high linearity in the investigated range of 0.1–100 ng cDNA.

For LightCycler reactions, a master mix of the following components was prepared: 7.0 μl PCR-grade water, 1.0 μl (0.5 μM) forward primer, 1.0 μl (0.5 μM) reverse primer, 10 μl 2× Master Mix, 1.0 μl cDNA (50–100 ng). A multiwell plate was sealed with sealing foil, centrifuged at 1500 ***g*** for 1 min and loaded into the LightCycler 480II instrument. Amplification was performed in triplicate wells for each sample analysed; a control reaction with no template (water) was run with all reactions. In each set of reactions, 16S rRNA was used as a reference gene for normalization of cDNA amount. Real-time PCR analysis was performed using the following optimized assay conditions: denaturation program (95 °C for 10 min); amplification and quantification program repeated for 45 cycles (95 °C for 10 s, 68 °C for 20 s, 72 °C for 30 s with a single fluorescence measurement); melting curve program (95 °C for 10 s, 70 °C for 1 min with continuous fluorescence measurement at 97 °C); and finally a cooling step at 40 °C for 30 s.

Relative quantification analysis was done using an efficiency-calibrated model (Pfaffl., 2001), and the normalized ratio was calculated as:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}}}{(E_{\text{reference}})^{\Delta \text{CP}_{\text{reference}}}}$$

Where CP=crossing point, $\Delta \text{CP}_{\text{target}} = \text{CP}_{\text{control}} - \text{CP}_{\text{sample}}$, $\Delta \text{CP}_{\text{reference}} = \text{CP}_{\text{control}} - \text{CP}_{\text{sample}}$, E_{target}=amplification efficiency of target gene, and $E_{\text{reference}}$=amplification efficiency of reference gene.

Melting curve analysis was performed after each run in a LightCycler 480II instrument to confirm the specificity of the primers.

Statistical analysis. CP values for each run were used to calculate mean CP with SD and SE. The normalized ratio was calculated using the efficiency-calibrated model (see Supplementary Table S1). The relative expression ratio was calculated from triplicate normalized ratios for each gene with SD (Table 2). Student's *t* test was performed to determine the significance of the relative expression ratios. $P<0.05$ was considered significant (*), $P<0.01$ was considered highly significant (**) and $P>0.05$ was considered not significant.

RESULTS

rpf-like genes are differentially expressed during growth of the cell

The relative expression of all five *rpf* genes (*rpfA–E*) was followed in exponential, stationary, NC and resuscitation phases. All five *rpf*s (*rpfA–E*) were expressed in the early exponential phase (Tufariello *et al.*, 2004; this study), and hence RNA from this stage was taken as a positive calibrator for quantification of relative expression in different growth stages (Fig. 1). *rpfB*, *rpfC* and *rpfE* had a higher expression ratio in exponential phase; the relative expression of *rpfB* and *rpfE* was reduced in stationary phase. The expression ratio of *rpfD* was lower in exponential phase but higher in the stationary and NC phases. However, only *rpfC* was found to be upregulated consistently in the exponential to stationary and NC phases. The relative expression of *rpfA* remained lower in exponential and NC phase but higher in resuscitation phase. Thus, from the data it appears that *rpfC* and *rpfD* have a role in stationary phase adaptation and persistence, while *rpfB* and *rpfE* may be required in exponential phase. Interestingly, in resuscitation phase, the relative expression

Table 1. Primer sequences with amplicon size and melting temperature

Amplification efficiency for each primer set was calculated using a LightCycler 480II instrument by serially diluting exponential-phase cDNA template in the investigated range of 0.1–100 ng cDNA with high linearity.

Gene	Forward and reverse primer sequences (5′–3′)	Amplicon size	T_m*	Amplification efficiency
rpfA	ggtgtgcggccgcgggttatcg	179 bp	69.57 °C	2.042
	ccagcggtgcgggcaggtcgttag		71.25 °C	
rpfB	cgacgctaagcaggtgtggacgac	221 bp	67.84 °C	1.909
	cactcagcagccccgcgacattgg		69.55 °C	
rpfC	gtcacggcatccatgtcgctctcc	154 bp	67.84 °C	2.015
	cccaggtggccggcttgaact		65.68 °C	
rpfD	gccgcgagtccccagcaacagat	144 bp	67.77 °C	2.152
	ggccgcgaggaacgtcaggatg		67.70 °C	
rpfE	ccagccggtatcgccaatg	269 bp	60.98 °C	1.855
	ccaccggactcgcactg		60.00 °C	
16S rRNA	tcccgggccttgtacaca	75 bp	67.70 °C	1.935
	ccactggcttcgggtgtta		66.00 °C	

*Melting temperature.

Table 2. Relative expression of all five *rpf*-like genes of *M. tuberculosis* H37Rv during different growth stages and under physiological stress conditions

The mean expression ratio and SD were calculated from the normalized ratio of three independent experiments. A.S., acid stress; N.S., nutrient starvation.

Sample	Genes	Mean expression ratio	SD
Exponential phase	*rpfA*	0.516610415	0.063507482
	rpfB	2.100984083	0.20351674
	rpfC	5.153812825	0.416394402
	rpfD	0.06102806	0.015142959
	rpfE	2.945745991	0.803414735
Stationary phase	*rpfA*	1.11898035	0.09392843
	rpfB	0.596770509	0.06906787
	rpfC	4.782993608	1.013816931
	rpfD	9.408208285	2.240292524
	rpfE	0.254524841	0.03918638
NC phase	*rpfA*	0.186841133	0.044517313
	rpfB	0.143705398	0.017466593
	rpfC	7.47137892	1.540350093
	rpfD	1.645698175	0.173943021
	rpfE	0.309567954	0.134784157
Resuscitation phase	*rpfA*	147.88404	11.75758243
	rpfB	8.137067142	0.619541303
	rpfC	4.356160476	0.961283965
	rpfD	117.187855	11.81078374
	rpfE	16.40931644	2.205469802
A.S. 4.5	*rpfA*	0.18969541	0.027988916
	rpfB	0.219301832	0.063692007
	rpfC	0.096401007	0.014285684
	rpfD	7.023885745	1.087480935
	rpfE	15.74334861	2.899078774
A.S. 5.5	*rpfA*	0.128864525	0.052478946
	rpfB	0.136357764	0.052055954
	rpfC	0.097237528	0.006761298
	rpfD	3.741203353	1.195968654
	rpfE	52.96238918	17.83526707
N.S. 24 h	*rpfA*	26.15805962	7.022619657
	rpfB	19.97932126	3.041090912
	rpfC	21.65962658	4.007682004
	rpfD	46.87336873	16.7076694
	rpfE	1.486512097	0.356684446
N.S. 96 h	*rpfA*	0.075462404	0.023846414
	rpfB	0.067609707	0.030488955
	rpfC	12.08445555	2.679209677
	rpfD	2.65486	0.963807116
	rpfE	0.2550596	0.126099371
Hypoxia	*rpfA*	0.544439636	0.244295824
	rpfB	0.89992703	0.176465309
	rpfC	11.35487408	4.30441611
	rpfD	0.186790824	0.03958978
	rpfE	22.06182767	2.390987321

of all five *rpf* genes was enhanced, with the maximal expression ratio of *rpfA* and *rpfD* on the third day of resuscitation (Fig. 1, Table 2). On the 10th day of resuscitation, the relative expression of all five *rpf* genes was almost identical to that observed in exponential phase (data not shown).

Relative expression of *rpf*s under stress conditions

The expression of all five *rpf*-like genes of *M. tuberculosis* H37Rv was analysed in mildly acidic [acid stress (A.S.) pH 5.5] and highly acidic (A.S. pH 4.5) conditions compared

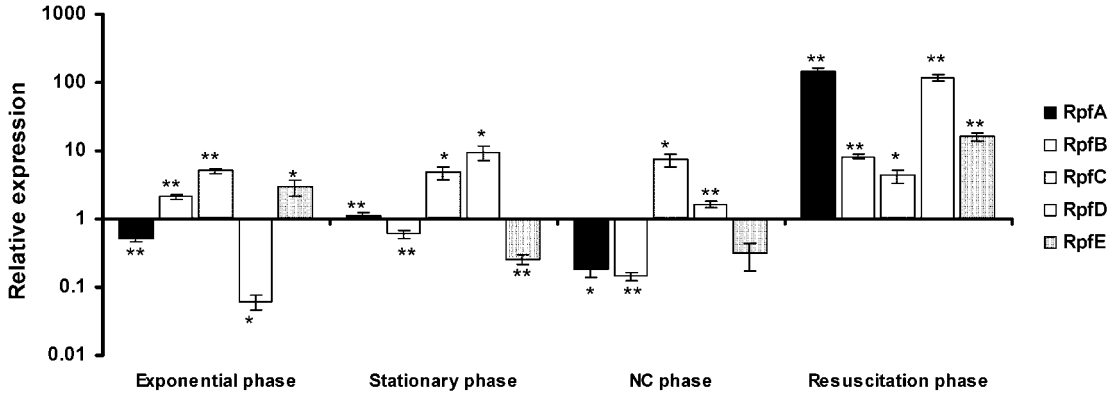

Fig. 1. Relative expression analysis of all five *rpf* genes (*rpfA–E*) was followed in exponential, stationary, NC and resuscitation phases by real-time PCR. *M. tuberculosis* H37Rv was grown in Sauton's medium containing ADC and Tween 80 at 37 °C without shaking. RNA extraction was done at different growth stages as described in Methods. Early exponential-phase RNA was used as a positive calibrator and 16S rRNA was used as a reference gene for normalization. Data represent mean relative expression values of three independent experiments with SD values (Table 2). $P<0.05$ was considered significant (*), $P<0.01$ was considered highly significant (**) and $P>0.05$ was considered not significant.

with the control grown at normal pH, as described in Methods. RNA of the control culture was used as a positive calibrator for real-time quantitative PCR (qRT-PCR). In mildly acidic (pH 5.5) and highly acidic conditions (pH 4.5), the relative expression of *rpfE* and *rpfD* was increased but the expression ratios of *rpfA*, *rpfB* and *rpfC* were decreased. In highly acidic conditions, *rpfE* had the maximum expression ratio among all *rpf*s (Fig. 2, Table 2).

The relative expression of all five *rpf* genes was observed under hypoxia conditions generated as described in Methods. A control culture was used as a positive calibrator for relative expression analysis. After 25 days of culture incubation, complete decolorization of the redox dye methylene blue was observed. At this time point, the *rpfE* and *rpfC* expression ratios were increased, with a maximum expression ratio of *rpfE*. The relative expression of *rpfA*, *rpfB* and *rpfD* was lower compared with the control (Fig. 3, Table 2). We analysed the relative expression of all five *rpf*-like genes during 24 and 96 h of nutrient starvation (N.S.), using 0 h RNA as a positive calibrator and control. It was observed that during 24 h of nutrient stress, the relative expression of all five *rpf* genes increased with respect to the control. During prolonged incubation of the culture for 96 h, *rpfC* and *rpfD* had increased expression ratios but the relative expression of *rpfA*, *rpfB* and *rpfE* was decreased (Fig. 4, Table 2).

DISCUSSION

In this paper, we have analysed the expression of the five *rpf*-like genes of *M. tuberculosis* H37Rv under different

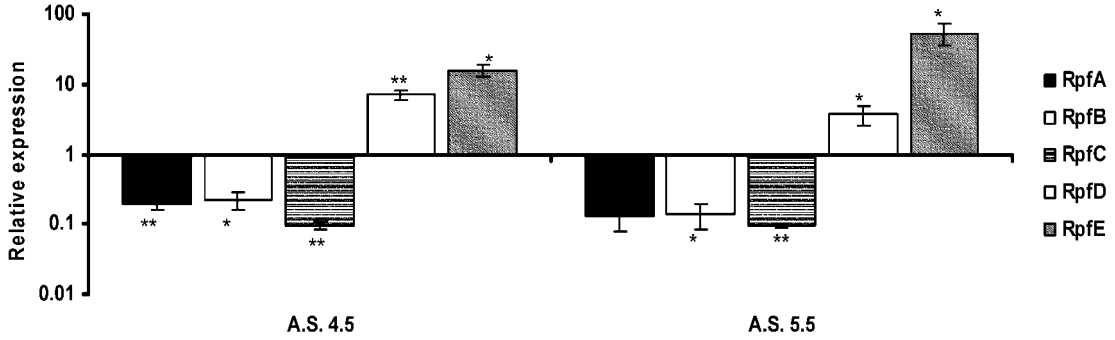

Fig. 2. Relative expression of all five *rpf*-like genes of *M. tuberculosis* H37Rv was analysed under mildly acidic (pH 5.5, A.S. 5.5) and highly acidic (pH 4.5, A.S. 4.5) conditions. Acid stress was generated as described in Methods. RNA isolated from the culture grown in Sauton's medium, pH 7.2, was used as a control or positive calibrator for relative expression analysis. 16S rRNA was used as a reference gene for normalization. Data represent mean relative expression values of three independent experiments with SD values (Table 2). $P<0.05$ was considered significant (*), $P<0.01$ was considered highly significant (**) and $P>0.05$ was considered not significant.

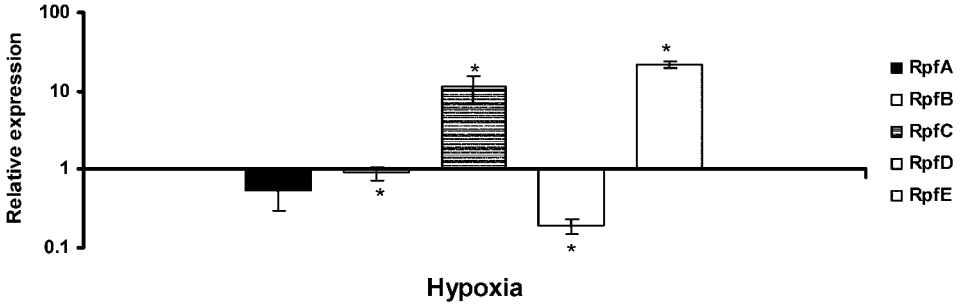

Fig. 3. Relative expression analysis of all five *rpf*-like genes of *M. tuberculosis* H37Rv under hypoxia. Hypoxia was generated as described in Methods. RNA isolated from the culture grown aerobically in MB7H9 medium containing ADC and Tween 80 (0.05 %. v/v) was used as a control or positive calibrator for relative expression analysis. 16S rRNA was used as a reference gene for normalization. Data represent mean relative expression values of triplicate experiments with SD values (Table 2). $P<0.05$ was considered significant (*), $P<0.01$ was considered highly significant (**) and $P>0.05$ was considered not significant.

growth and physiological stress conditions. It was found that all *rpf* genes were expressed in early resuscitation phase with maximum relative expression for *rpfA* and *rpfD*. Only *rpfC* was observed to show consistently increased relative expression during all stages of growth and nutrient starvation. It was also observed that acid stress induced higher relative expression of *rpfD* and *rpfE* and hypoxia of *rpfC* and *rpfE*. The earlier results on expression of the five *rpf* (*A–E*) genes in axenic culture from early exponential to stationary phase by semiquantitative RT-PCR revealed the expression of all five genes at early time points but differences at later stages of growth (Mukamolova *et al.*, 2002; Downing *et al.*, 2004; Tufariello *et al.*, 2004). The studies showed that *rpfE* was expressed maximally in the early exponential phase of growth, with expression falling markedly and remaining low throughout (Tufariello *et al.*, 2004). Three of the other *rpf*-like genes, *rpfA rpfB* and *rpfD*, had higher expression in exponential phase but reduced expression in stationary phase. Only *rpfC* (rv1884c) was expressed at an undiminished level throughout when compared with early exponential phase (Tufariello *et al.*, 2004). In extended stationary phase (4-month-old culture) of *M. tuberculosis*, expression of all five *rpf*-like genes can be detected (Tufariello *et al.*, 2004), while no expression is observed in stationary phase and 5-month-old cultures of *M. bovis* BCG (Mukamolova *et al.*, 2002). Differential expression of the five *rpf*-like genes in *M. tuberculosis* appears to be related to differential regulation and this is supported by functional and mutational analysis of *rpf* genes.

RpfB has been shown to interact with a putative mycobacterial endopeptidase, designated Rpf-interacting protein A (RipA) (Hett *et al.*, 2007). The two proteins colocalize in the septa of actively dividing cells, suggesting a role for the RipA–RpfB complex in peptidoglycan hydrolysis during cell division. RipA also interacts with RpfE but not with RpfA, RpfC and RpfD, suggesting that these Rpfs may act via a distinct mechanism and/or on a different

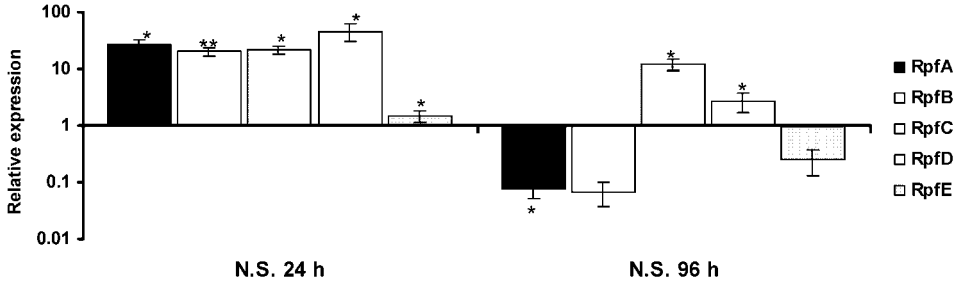

Fig. 4. Relative expression of all five *rpf*-like genes during 24 and 96 h of nutrient starvation (N.S.) analysed by real-time PCR. Nutrient stress was generated as described in Methods. Cells harvested at 0 h served as control or positive calibrator for relative expression analysis. 16S rRNA was used as a reference gene for normalization. Data represent mean relative expression values of three independent experiments with SD values (Table 2). $P<0.05$ was considered significant (*), $P<0.01$ was considered highly significant (**) and $P>0.05$ was considered not significant.

substrate or pathway (Hett *et al.*, 2007, 2008). Similarly, colony formation is delayed in *M. tuberculosis* with mutated *rpfB*, and colonies are much smaller than those of the wild type (Tufariello *et al.*, 2004). The higher expression of *rpfB* and *rpfE* observed in exponential phase in our study may thus be related to cell division and growth. RpfC is by far the most abundant resuscitation-promoting factor in *M. tuberculosis* (Mukamolova *et al.*, 2002; Tufariello *et al.*, 2004). We also found a consistently higher expression ratio of *rpfC* from exponential to stationary to NC phase. *rpfC* appears to be directly regulated by the sigma factor SigD, and *in vitro* transcription of *rpfC* has been found to be completely dependent on SigD (Raman *et al.*, 2004). The expression of SigD has been linked to the stringent response, with an increased expression in response to starvation that is partly Rel-dependent (Raman *et al.*, 2004). We also observed enhanced *rpfC* expression during nutrient starvation (Fig. 3). Hence, *rpfC* may have a role in stationary-phase adaptation and persistence.

Expression of the *rpf*-like genes during resuscitation of NCC to active growth by *M. tuberculosis* culture supernatant was also examined. Samples were taken on days 3 and 10 of resuscitation. Day 3 was chosen as changes in the composition of the cell population have been reported to occur as early as 3 days after inoculation of NCC in resuscitation medium (Shleeva *et al.*, 2003). The progression of the culture from the NC phase to the resuscitation phase on day 3 resulted in an increase in expression of all five *rpf* genes. The expression of all the *rpf*s in early resuscitation phase is anticipated, as it is a recovery phase from the NC state to active growth. The higher expression ratio of all the *rpf* genes in early resuscitation phase correlates with mutational studies, which clearly suggest that deletion of three or more genes results in impairment of resuscitation from an NC state (Kana *et al.*, 2008; Downing *et al.*, 2005). Deletion of any one of the five *rpf*-like genes does not affect the growth and ability to resuscitate spontaneously from an NC state *in vitro* (Downing *et al.*, 2005). Similarly single-deletion mutants have been found to be phenotypically similar to wild-type *M. tuberculosis* H37Rv *in vivo* (Downing *et al.*, 2005). However, the multiple mutant strains KDT8 (Δ*rpfA* Δ*rpfB* Δ*rpfC*) and KDT9 (Δ*rpfA* Δ*rpfC* Δ*rpfD*), which lack three of the five *rpf*-like genes, are significantly but differentially attenuated in a mouse infection model. These mutants are also unable to resuscitate spontaneously *in vitro* (Downing *et al.*, 2005). In another study, quadruple mutants (Δ*rpfACBD* and Δ*rpfACBE*) of *M. tuberculosis* were found to be significantly attenuated in a murine infection model, being severely impaired in growth and persistence (Kana *et al.*, 2008). We observed higher relative expression of *rpfA* and *rpfD* during early resuscitation. *rpfA* has been shown to be subject to regulation by the cAMP receptor protein (Rickman *et al.*, 2005), and is reported to be the most potent resuscitation-promoting factor that exhibits activity at femtomolar concentrations (Mukamolova *et al.*, 2002). Among all five Rpf-like proteins of *M. tuberculosis*, RpfD is considered the most efficient in terms of resuscitation-promoting activity (Cohen-Gonsaud *et al.*, 2005).

Phagosomes containing mycobacteria begin to acidify rapidly after phagocytosis and the pH drops rapidly to 5.5 or lower. The acidification signal is used by mycobacteria to induce the expression of genes needed to alter phagosomal maturation (Hackam *et al.*, 1998; Oh & Straubinger, 1996; Sturgill-Koszycki *et al.*, 1994). The induction of *rpfE* under mildly acidic conditions has been reported previously (Fisher *et al.*, 2002). The loss of *rpfE* from the triple mutant Δ*rpfACB* has been shown to have pronounced effect on persistence of tubercle bacilli in the lungs of mice (Kana *et al.*, 2008). We observed that in highly acidic conditions the expression ratios of *rpfE* and *rpfD* were highest among all *rpf*s, which suggests a role for both *rpfE* and *rpfD* in acid-induced stress conditions.

M. tuberculosis is generally regarded as a strictly aerobic bacillus, although it can survive for a long period of time under microaerophilic conditions (Wayne & Hayes, 1996). An *in vitro* model to study this persistence state is the Wayne dormancy model, in which the bacteria down-regulate their metabolism due to reduced access to oxygen (Wayne & Hayes, 1996). *rpfE* has been shown to have a role in persistence, and *rpfC* is regulated by the transcriptional regulator SigD required for stationary phase adaptation and persistence (Raman *et al.*, 2004). Our results suggest that both *rpfC* and *rpfE* have a role in cell survival during hypoxia-induced persistence, and that *rpfD* and *rpfE* have a role in adaptation of *M. tuberculosis* in acid-induced stress conditions, indicating that *rpfE* is important for the survival of *M. tuberculosis* H37Rv under stress conditions induced by low oxygen and pH.

M. tuberculosis H37Rv arrests growth, decreases its respiration and becomes resistant to front-line drugs such as isoniazid, rifampicin and metronidazole under nutrient-starved conditions (Betts *et al.*, 2002). It has been shown that the respiration rate of a nutrient-starved culture declines rapidly over the first 96 h of starvation (Betts *et al.*, 2002). We have observed that during 96 h of nutrient stress *rpfC* and *rpfD* had higher relative expression, and the expression ratio of *rpfA*, *rpfB* and *rpfE* was decreased. As discussed above, *rpfC* is under the control of the putative sigma factor SigD, which shows enhanced expression during nutrient starvation (Betts *et al.*, 2002). Starvation-induced expression of *sigD* is blunted in a *relA* mutant strain (Dahl *et al.*, 2003), suggesting that *sigD* is a part of the RelA regulon required for the stringent response for survival of *M. tuberculosis* during nutrient starvation. It has been reported that expression of *relA* was induced under nutrient starvation (Betts *et al.*, 2002), suggesting the increased expression of *sigD* together with increased expression of *rpfC* in nutrient starvation.

Conclusion

These studies are consistent with *rpfA* and *rpfD* playing important roles in reducing the lag phase during resuscitation, as they have maximum expression ratios in the initial phase of resuscitation (3 days after resuscitation). Only *rpfC* has a higher expression ratio from exponential phase to the NC state compared with early exponential phase, suggesting that *rpfC* has a consistently increased relative expression among all *rpf*s in *M. tuberculosis*. The results suggest that acid stress induces expression of *rpfD* and *rpfE* and that hypoxia induces expression of *rpfC* and *rpfE*. Nutrient starvation induces all the *rpf* genes in the early phase (24 h), but later after 96 h, *rpfC* and *rpfD* have higher relative expression. The five *rpf* genes are differentially expressed under different growth stages and stress conditions, suggesting that they have distinct roles during cell growth and cell survival under stress conditions.

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