

Defining New Drug Targets Through Protein-Protein Interaction: Interaction of Resuscitation Promoting Factors with SucA of TCA Cycle in *M. tuberculosis* H37Rv

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Abstract

Upon infection with *Mycobacterium tuberculosis*, only a small percentage causes active infection while the rest goes into latent infection. These latent bacilli can reactivate under immunocompromised conditions and cause active disease. Little is known about the mechanism by which the mycobacteria reactivate. A family of extracellular bacterial proteins, known as resuscitation promoting factors (Rpf) from *Micrococcus luteus* and *M. tuberculosis* has been shown to stimulate growth of dormant mycobacteria as well as reactivation of chronic tuberculosis in mice. Rpf is present as a single, essential gene in *M. luteus* and five homologues (rpfA-E) in *M. tuberculosis*. The ability to stimulate culturability and resuscitation appears to be related to muralytic activity of Rpf and they have been identified as peptidoglycan glycosidases. Rpf B has earlier been shown to interact and synergize with Rpf-interacting protein A (RipA), an endopeptidase to cleave bonds in bacterial peptidoglycan suggesting distinct role of Rpf B in cell wall hydrolysis. In order to further understand the role of other Rpf in resuscitation of dormant mycobacteria, we used an *E. coli* two hybrid system to identify SucA of TCA cycle as an interacting partner of Rpf from *M. tuberculosis* (Rpf A, C and D) and *M. luteus* (RpfM). The *in vivo* protein-protein interaction was confirmed by M-PFC system in mycobacterial host, *in vitro* by FRET analysis. An enhanced expression of SucA and Rpf genes was observed during resuscitation phase. We hypothesize that during transition from nonculturable to resuscitation phase mycobacteria cleaves its hard breaking cell wall by endopeptidase RipA interacting with Rpf B and increases its metabolic energy generation by evoking TCA cycle, interacting with Rpf A, C and D and could serve as prospective target along with Rpf and RipA for development of new anti-tuberculosis drugs preventing reactivation of dormant bacilli.

Keywords: *M. tuberculosis*; Rpf; RipA; SucA; Protein-protein interaction; Resuscitation; Drug targets

Introduction

Pathogenic, slow growing mycobacteria can persist for a long period of time in the host by passing into a dormant or non-replicating persistent state and reactivate under opportune moment [1,2]. However, the mechanism of reactivation remains unclear, sufficient evidence has gathered towards involvement of resuscitation promoting factors (Rpf) of *Micrococcus luteus* and *Mycobacterium tuberculosis* in resuscitation of dormant mycobacteria [3,4]. *M. luteus* codes for a single rpf gene while *M. tuberculosis* genome contains five rpf homologues rpfA-E. All these proteins are either extracellular or membrane bound and share a 70-amino acid residues segment, known as Rpf domain, necessary and sufficient for biological activity [5-7]. All the five Rpf are individually able to resuscitate the dormant mycobacteria and individual deletion mutants of rpf gene do not display any growth defect *in vitro* or attenuation *in vivo* [8]. Although individual rpf-like genes appear non-essential for growth *in vitro*, a strain of *M. tuberculosis* lacking rpfB gene was shown to delayed reactivation in a mouse dormancy model [9] and two strains of *M. tuberculosis* in which three rpf genes were deleted were both attenuated in mice and did not reactivate in an *in vitro* reactivation assay [10]. Recently we have shown that all the five genes are differentially expressed under different conditions of growth and physiological stress conditions, which suggest that these proteins may be differentially regulated to cope up with different stress conditions encountered in the host [11].

The structural study of Rpf domain shows their homology to lysozyme and functional study suggests their role in cleavage of bacterial peptidoglycan during reactivation of dormant bacilli. Evidence came from the demonstration of protein-protein interaction of RpfB with a putative mycobacterial endopeptidase, designated as Rpf-interacting protein A (RipA) [12,13]. The two proteins co-localize in the septa of dividing cells suggesting a role for the RipA-RpfB complex in

peptidoglycan hydrolysis during cell division. RipA also interact with RpfE but not with the RpfA, RpfC and RpfD, suggesting that these Rpf may act via distinct mechanism and or on different substrate or pathway.

To investigate additional potential interacting partner of Rpf we screened the whole genomic library of *M. tuberculosis* H37Rv using bacterial two-hybrid system (Daniel Ladant, Pasteur Institute). The study led us to identify Rv1248c annotated as sucA gene in TCA cycle of *M. tuberculosis*. SucA interacted with Rpf A, C and D of *M. tuberculosis* and Rpf of *M. luteus* (RpfM). The protein-protein interaction was confirmed *in vivo* by M-PFC system in mycobacterial host and *in vitro* by FRET analysis using purified recombinant RpfM and SucA protein.

Materials and Methods

Strains and culture conditions

The bacterial strains of bacterial two-hybrid system were provided by Daniel Ladant and M-PFC system was obtained from UAB Foundation, USA. The bacterial strains used in the study are described in Table 1. *E. coli* cells were propagated in Luria Bertani (LB) medium and LB agar plate. *M. tuberculosis* H37Rv was cultivated at 37°C in

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Strains/plasmids	Description	Reference
<i>E. coli</i> DHM1	NaI ^r , cya-854, nonreverting adenylate cyclase defeceint Cya strain	Pasteur Institute
pKT25	Km ^r , encodes T25 fragment of B. pertussis adenylate cyclase enzyme	Pasteur Institute
pKT25zip	Km ^r , derivative of pKT25 having genetically fused leucine Zipper of GCN4 (I) in frame to T25 fragment	Pasteur Institute
pUT18c	Amp ^r , encodes T18 fragment of adenylate cyclase enzyme	Pasteur Institute
pUT18czip	Amp ^r , derivative of pUT18c having genetically fused leucine Zipper of GCN4 in frame to T18 fragment	Pasteur Institute
pKT25- <i>rpfM</i>	Km ^r , derivative of pKT25 encoding <i>M. luteus rpf</i> gene without secretory signal in frame to T25 fragment at BamH1/Kpn1	This study
pKT25- <i>rpfA</i>	Km ^r , derivative of pKT25 encoding <i>rpfA</i> gene without secretory signal in frame to T25 fragment at Pst1/BamH1	This study
pKT25- <i>rpfB</i>	Km ^r , derivative of pKT25 encoding <i>rpfB</i> gene without secretory signal in frame to T25 fragment at BamH1	This study
pKT25- <i>rpfC</i>	Km ^r , derivative of pKT25 encoding <i>rpfC</i> gene without secretory signal in frame to T25 fragment at BamH1	This study
pKT25- <i>rpfD</i>	Km ^r , derivative of pKT25 encoding <i>rpfD</i> gene without secretory signal in frame to T25 fragment at BamH1	This study
pKT25- <i>rpfE</i>	Km ^r , derivative of pKT25 encoding <i>rpfE</i> gene without secretory signal in frame to T25 fragment at BamH1	This study
pKT25- <i>inhA</i>	Km ^r , derivative of pKT25 encoding <i>inhA</i> gene without secretory signal in frame to T25 fragment at BamH1	This study

Table 1: Strains and plasmids used in this study.

Middlebrook 7H9 medium (Difco) supplemented with 0.2% glycerol, 0.05% tween-80 (Sigma) and 10% (v/v) albumin-dextrose-catalase (ADC; BD, USA). *M. smegmatis* mc²155 was grown in MB7H9 medium and plated on MB7H11 agar medium. For mycobacteria, kanamycin (25 µg/ml), hygromycin (50 µg/ml) and trimethoprim (40 µg/ml to 50 µg/ml) were used. For *E. coli*, kanamycin (50 µg/ml), ampicillin (100 µg/ml) nalidixic acid (50 µg/ml), hygromycin (150 µg/ml) and X-gal (40 µg/ml) were used.

Bacterial two-hybrid system

The bacterial adenylate cyclase-based two hybrid (BATCH) system was used as a primary *in vivo* screen to detect functional interactions between two proteins [14]. BATCH consists of two vectors, pKT25 coding for T25 fragment and pUT18c coding for T18 fragment of *Bordetella pertussis* adenylate cyclase enzyme. The test is based on the functional complementation between two fragments of the catalytic domain of the adenylate cyclase (AC) to reconstitute a cAMP signaling cascade in *E. coli*. The catalytic domain consists of two complementary fragments, T25 and T18, both fragments are necessary to reconstitute a fully active enzyme. The detection of protein-protein interaction with the BATCH system requires co-expression of proteins of interest as fusions with the T25 and T18 fragments in an *E. coli cya*⁻ strain (DHM1) and detection of the resulting *cya*⁺ phenotype on X-gal containing media [14]. Initially *rpfM* gene (549 bp; without signal sequences) of *M. luteus* was cloned in frame with sequence encoding T25 fragment in pKT25 vector using primers described in Table 2. The recombinant plasmid pKT25::*rpfM* was transformed into *E. coli cya*⁻ strain DHM1 and kanamycin resistant *E. coli* DHM1 [pKT25::*rpfM*] transformants were selected. The transformants were confirmed for the presence of insert by restriction digestion and nucleotide sequencing. *M. tuberculosis* H37Rv genomic library was prepared in pUT18c vector. The genomic DNA was digested with *Sau*3A enzyme and DNA fragments ranging between 0.2 kb to 2 kb were eluted from the gel, treated with CIAP and ligated with *Bam*H1 linearized pUT18c vector. The efficiency of genomic library was checked by transforming an aliquot of library in *E. coli* DH5a and isolating plasmids from 50 individual clones. Clones were digested with *Xba*I and *Eco*R1 to release the insert. More than 80% clones had insert of varying sizes. The pUT18c plasmid having library of *M. tuberculosis* genomic fragments was transformed into *E. coli* DHM1 [pKT25::*rpfM*] and co-transformants were selected on LB agar plate containing ampicillin, kanamycin and indicator X-gal for blue colonies at 30°C after 72 h. Three rounds of screening were done. The blue colonies were assayed for β-galactosidase activity [14]. The presence of insert in blue transformants was confirmed by restriction analysis and identity by nucleotide sequencing. The primers used in

cloning of *rpf* genes of *M. luteus* and *M. tuberculosis* in pKT25 vector are described in Table 2.

BATCH complementation assay

Efficiency of interaction between different hybrid clones was quantified by measurement of β-galactosidase activity in liquid culture [15]. For β-galactosidase activity measurement, bacteria were grown in the presence of 0.5 mM IPTG and appropriate antibiotic at 30°C for 14 h to 16 h. Cells were diluted 1:5 in M63 medium and optical density OD₆₀₀ was recorded. Cells were then permeabilized by 35 µl of toluene and 35 µl of 0.1% SDS in 2.5 ml of bacterial suspension. The tubes were vortexed for 10 sec and incubated at 37°C for 30 to 40 min for evaporation of toluene. For enzymatic reaction, aliquots (0.1 ml to 0.5 ml) of permeabilized cells were added to buffer PM2 (70 mM Na₂HPO₄·12H₂O, 30 mM NaHPO₄·H₂O, 1 mM MgSO₄, and 0.2 mM MnSO₄, pH-7.0) containing 100 mM β-mercaptoethanol to a final volume of 1 ml. The tubes were incubated at 28°C in a water bath for 5 min. The reaction was started by adding 0.25 ml of 0.4% O-nitrophenol-β-galactosidase (ONPG) in PM2 buffer (without β-mercaptoethanol). The reaction was stopped by adding 0.5 ml of 1 M Na₂CO₃ solution. OD₄₂₀ was recorded. The enzymatic activity A (miller unit) was calculated according to the following equation, A=100 × (OD₄₂₀ of the culture/time × V × OD₆₀₀). Where V represents the volume of culture.

Cloning, expression and purification of SucA protein of *M. tuberculosis* H37Rv and *M. luteus* Rpf protein

PCR amplification of *sucA* (Rv1248c, 3645 bp) was done from *M. tuberculosis* H37Rv genomic DNA and *M. luteus rpf* gene (549 bp; without signal sequences) from *M. luteus* genome. Amplified products were cloned in pTZ57R/T cloning vector (Fermentas). Presence of insert was confirmed by restriction digestion and nucleotide sequencing. Fragments were excised from the vector by restriction digestion and cloned in pET41a expression vector. Right oriented clones were transformed in expression host *E. coli* BL21 (DE3). Optimum expression and solubility of both the proteins were standardized with IPTG concentration and temperature. Recombinant proteins were purified by affinity chromatography using His tag Ni⁺⁺- NTA column (Qiagen).

Labeling of RpfM and SucA protein by alexa flour protein labeling kit

Fluorescence resonance energy transfer (FRET) is a technique used to measure the interaction between two molecules labeled with two different fluorophores (the donor and the acceptor) by the transfer of energy from the excited donor to the acceptor. FRET analysis was used to study physical protein-protein interaction by labeling

For cloning in bacterial two-hybrid plasmids	
pKT25- <i>rpf</i> MF	5'CGGGATCCCGCCACCGTGGACACCTG3'
pKT25- <i>rpf</i> MR	5'GGGGTACCTCAGGCCTGCGGCAGGAC3'
pKT25- <i>rpf</i> AF	5'AAAACTGCAGGCATGGCCGCTCAGGCGAC3'
pKT25- <i>rpf</i> AR	5'CGGGATCCTCAGCCGATGACGTACGG3'
pKT25- <i>rpf</i> BF	5'CGGGATCCCGCATGCTGCAAAACGGTGACG3'
pKT25- <i>rpf</i> BR	5'CGGGATCCTCAGCGCGCCGACCCGCTC3'
pKT25- <i>rpf</i> CF	5'CGGGATCCGGGTCCAGCCCGAACTG3'
pKT25- <i>rpf</i> CR	5'CGGGATCCTCAGCGCGGAATACTTGC3'
pKT25- <i>rpf</i> DF	5'CGGGATCCGAAAGCCGACGACATCGATTG3'
pKT25- <i>rpf</i> DR	5'CGGGATCCTCAATCGTCCCTGCTCCCG3'
pKT25- <i>rpf</i> EF	5'AAAACTGCAGGCGACGACGCGGGCTTGAC3'
pKT25- <i>rpf</i> ER	5'CGGGATCCTCAGCGCGGCGGCGCC3'
pKT25- <i>inh</i> AF	5'CGGGATCCCATGACAGGACTGCTGGACGGC3'
pKT25- <i>inh</i> AR	5'CGGGATCCTAGAGCAATTGGGTGTGCG3'
pUT18C- <i>suc</i> AF	5'CGGAATTCGAGATGTACCGCAAGTC3'
pUT18C- <i>suc</i> AR	5'CCCAAGCTTTCAGCCGAACGCCTCGT3'
For cloning in pet41a expression vector	
MLRF	5'CGGAATTCGCCACCGTGGACACCTG3'
MLRR	5'CCCAAGCTTTCAGGCCTGCGGCAGGAC3'
SucAF	5'CGGAATTCGAGATGTACCGCAAGTC3'
SucAR	5'CGGAATTCCTCAGCCGAACGCCTCGT3'
For cloning in pUAB vectors	
F300A	5'GGATCCATGAGTGGACGCCACCGT3'
R300A	5'AAGCTTTCAGCCGATGACGTACGG3'
F300B	5'GGATCCATGTTGCGCCTGGTAGTC3'
R300B	5'AAGCTTTCAGCGCGCACCCGCTC3'
F300C	5'GGATCCGTGCATCCTTTCGCGGCC3'
R300C	5'AAGCTTTCAGCGCGGAATACTTGC3'
F300D	5'GGATCCATGACACCGGGTTTGCTTAC3'
R300D	5'AAGCTTTCATCGTCCCTGCTCCC3'
F300E	5'GGATCCTTGAAGAACGCCCGTACG3'
R300E	5'AAGCTTTCAGCCGCGGCGGCC3'
FRip400	5'GAATTCGATGAGACGGAATCGCCGT3'
RRip400	5'AAGCTTCTAGTACTCGATGTATCGGAC3'
FSuc400	5'GAATTCGATGTACCGCAAGTTCGCG3'
RSuc400	5'CCCAAGCTTTCAGCCGAACGCCTCGTC3'
For real-time PCR	
<i>rpf</i> A	5'GGTGTGCGGCCGCGGTTATCG3' 5'CCAGCGGTGCGGGCAGGTCTGTAG3'
<i>rpf</i> B	5'CGACGCTAAGCAGGTGTGGACGAC3' 5'CACTCAGCAGCCCCGCGACATTGG3'
<i>rpf</i> C	5'GTCACGGCATCCATGCTGCTCTCC3' 5'CCCAGGTGGCCGGCTTGAAGT3'
<i>rpf</i> D	5'GCCGCGAGTCCCCAGCAACAGAT3' 5'GGCCGCGAGGAACGTCAGGATG3'
<i>rpf</i> E	5'CCAGCCGGTATCGCCAATG3' 5'CCACCGGACTCGACTG3'
16S rRNA	5'TCCCGGGCCTTGACACA3' 5'CCACTGGCTTCGGGTGTTA3'
<i>rv</i> 1248c	5'AGGGACCCGACCACTTCTG3' 5'GGTCTCCGATGCCGTCTCA3'

Table 2: Primers used in this study.

the proteins with alexa flour dye (Molecular probe). Purified SucA protein was labeled with alexa flour 488 dye (Absorbance_{max}=495 and Emission_{max}=519) and purified Rpf protein (*M. luteus*) with alexa

flour 546 dye (Absorbance_{max}=556 and Emission_{max}=573). Labeling of proteins was done according to manufacturer's protocol. The fluorescence was recorded in Fluorometer (Perkin Elmer).

Cloning of *sucA* and *rpf* genes in M-PFC vectors

Mycobacterial protein fragment complementation (M-PFC) was used to study *in vivo* protein-protein interaction between Rpf from *M. tuberculosis* with SucA protein in mycobacterial host [16]. *sucA* gene was cloned in pUAB400 and *rpf* (A-E) genes were cloned in pUAB300 at *EcoRI/HindIII* and *BamHI/HindIII* restriction sites respectively. Two plasmids were co-transformed in *M. smegmatis* and selected on MB7H11 medium containing 0.5% glucose, kanamycin (25 µg/ml) and hygromycin (50 µg/ml). Km^r-Hyg^r colonies were screened for growth in presence of trimethoprim. Appropriate positive and empty vector control were used.

Resazurin assay

M. smegmatis clones containing interacting plasmids were cultured in Middlebrook 7H9 medium containing hygromycin and kanamycin to an OD₆₀₀ nm of 0.8. Cells were diluted in fresh 7H9 medium, and ~10⁶ cells were added to clear-bottom 96-well micro-titer plates. Outer perimeter wells were filled with sterile water to prevent dehydration. TRIM was dissolved in dimethyl sulfoxide, and 2-fold serial dilutions of the drug were made in 0.1 ml of 7H9 in microtiter plates. Wells containing drug only and no *M. smegmatis* cells were the autofluorescence controls. Additional controls consisted of wells containing cells and medium only and empty vectors serve as negative control. Plates were incubated for 12 h at 37°C, after which 30 µl of resazurin (Sigma) solution was added to the wells containing cells only, further incubated, and observed for the appearance of a pink color. The result was recorded after 6 h as fluorescence intensity was measured in a fluorometer; Synergy plate reader (Biotek) in bottom-reading mode with excitation at 530 nm and emission at 590 nm.

Transcriptional analysis of *rpfs* and *sucA* genes in different growth stages

Transcriptional analysis of *rpfs* and *sucA* gene was performed in exponential, stationary, nonculturable and resuscitation phase of *M. tuberculosis* H37Rv by real time-PCR. RNA was isolated from different growth phases and real time-PCR was performed as described earlier [11].

Results

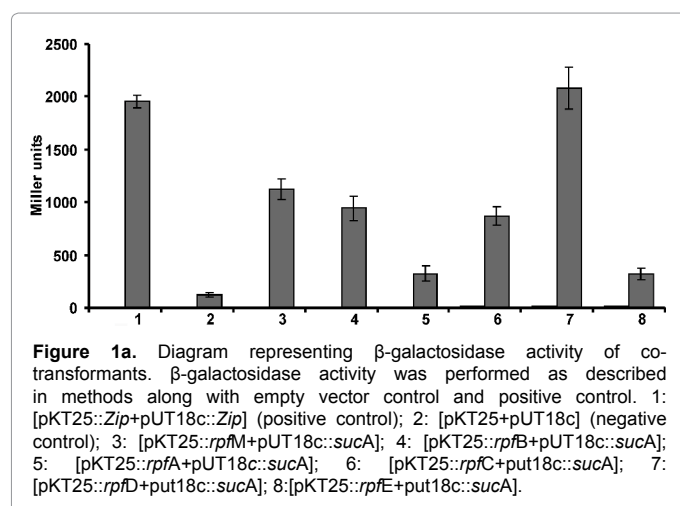
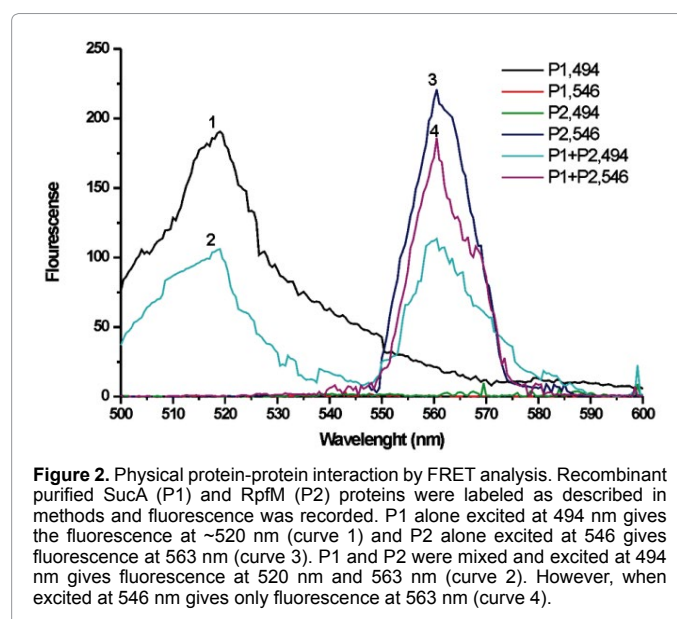
Identification of interacting partner of Rpf by bacterial two-hybrid system

The bacterial two-hybrid system was used as primary *in vivo* screen to detect functional interactions between two proteins. Screening of interacting partner of RpfM was done against random library of *M. tuberculosis* H37Rv. Co-transformants containing pKT25::*rpf*M and pUT18c::*M. tuberculosis* library were plated on LB agar containing ampicillin, kanamycin and indicator X-gal for blue colonies representing *cya*⁺ colonies were selected after 72 h of incubation at 30°C. Approximately 1 × 10⁶ clones were screened. Three blue clones were found and were assayed for β-galactosidase activity to confirm true positive interactions. The identity of the insert was made by nucleotide sequencing using pUT18c specific primers and sequences were blast with *M. tuberculosis* H37Rv genome in NCBI. The three clones contained inserts, which belonged to Rv0266c, Rv0111 and Rv1248c genes of *M. tuberculosis*. All three inserts were found in frame with T18 fragment of pUT18c vector. The clone containing in frame fusion of *rv*1248c gene showed highest β-galactosidase activity (Figure

1a) and was analyzed in detail. The interaction between RpfM and SucA was confirmed using full-length gene sequence of *sucA*.

In vitro protein-protein interaction

Physical protein-protein interaction was seen by FRET analysis [17] of the two proteins (SucA and RpfM) labeled with different alexa flour protein labeling dye (Molecular probe). Results suggested that when purified protein SucA, labeled with 488 dye (P1) was excited at 494 nm it gave sharp fluorescence at ~520 nm (Figure 2; P1) and purified *M. luteus* Rpf, labeled with 546 dye was excited at 546 nm, gave sharp peak of fluorescence at ~563 nm (Figure 2; P2). SucA and RpfM produced no signals when excited at 546 nm and 494 nm respectively. However, when both the labeled proteins were mixed in equal concentration and excited at 494 nm, the fluorescence peak at 520 nm was quenched and a sharp peak appeared at 563 nm, which indicates the transfer of energy from P1 to P2 due to close proximity of both the proteins. Quenching of fluorescence of donor (SucA) and increase in fluorescence of acceptor (RpfM) molecule suggest protein-protein interaction between SucA and RpfM (Figure 2).



Interaction of SucA with Rpf from *M. tuberculosis*

Interaction of *rpf*s with full-length *sucA* gene was studied. All five *rpf*s (A-E) were cloned in frame in pKT25 vector and co-transformed with pUT18c::*sucA* in *E. coli* DHM1 strain. The primers used for cloning of *rpf* genes and *sucA* gene in pKT25 and pUT18c vector are described in Table 1. Results suggest that SucA interacted with Rpf A, C and D which was confirmed by β -galactosidase activity (Figures 1a and 1b). No interaction was seen with empty vector or *inhA* gene cloned in pKT25 vector.

M-PFC and resazurin assay confirm the interaction of SucA and Rpf in vivo

In vivo protein-protein interaction between Rv1248c (SucA) and Rpf (A-E) was assayed in mycobacterial host using M-PFC system [17]. The system is based on fusion of protein of interest with domains of murine dihydrofolate reductase (mDHFR) cloned in two plasmids. Functional reconstitution of the two-mDHFR domains can occur in mycobacteria, thereby allowing selecting for mycobacterial resistance against trimethoprim (TRIM). *sucA* gene was cloned in pUAB400 and *rpf* (A-E) was cloned in pUAB300 vectors. Two plasmids were co-transformed in *M. smegmatis* and selected on MB7H11-Km-Hyg plate containing 0.5% glucose. The positive interaction resulted in growth in presence of trimethoprim (50 μ g/ml) (Figure 3a). Results confirmed the interaction of SucA with Rpf A, C and D. The intensity of interactions was assayed by resazurin reduction assay. Samples were processed in 96-well plates according to Methods. A change from non-fluorescent blue to fluorescent pink indicates reduction of resazurin and is indicative of protein-protein interaction. The change in color intensity was measured using a microplate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm (Figure 3b). TRIM concentration used ranged from 256 μ g/ml to 2 μ g/ml. Samples were analyzed in duplicate, and all wells contained equal numbers of *M. smegmatis* cells (10^6 colony-forming units).

Expression analysis of *rpf*s and *sucA* gene in different growth stages

Transcriptional analysis of *rpf*s and *sucA* was done in exponential, stationary, NC and resuscitation phase of *M. tuberculosis* H37Rv by real





Figure 3a. *In vivo* interaction of *rv1248c* (*sucA*) and five *rpf*s (*rpfA-E*) were assayed by M-PFC system in mycobacterial host. *sucA* was cloned in pUAB400 and *rpf* (A-E) was cloned in pUAB300. Two plasmids were co-transformed in *M. smegmatis* and selected on MB7H11-Km-Hyg plate containing 0.5% glucose and Trimethoprim (50 µg/ml). 1: [pUAB100::pUAB200] (positive control); 2: [pUAB300::pUAB400] (negative control); 3: [pUAB300*rpfD*::pUAB400*sucA*]; 4: [pUAB300*rpfC*::pUAB400*sucA*]; 5: [pUAB300*rpfE*::pUAB400*sucA*]; 6: [pUAB300*rpfA*::pUAB400*sucA*]; 7: [pUAB300*rpfB*::pUAB400*sucA*]; 8: [pUAB300*rpfB*::pUAB400*sucA*].

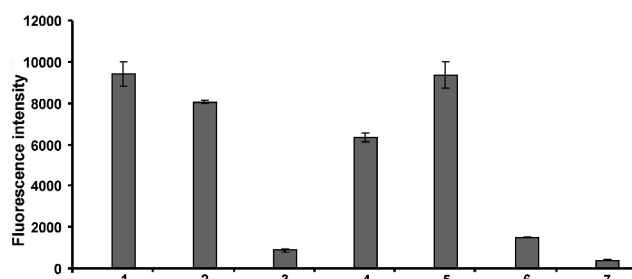


Figure 3b. Intensity of interaction was assayed by resazurin reduction assay with increasing concentration gradient of Trimethoprim. The change in color intensity was measured by using a microplate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm. TRIM concentration was used in the range from 256 µg/ml to 2 µg/ml. Samples were analyzed in triplicate, and all wells contained equal numbers of *M. smegmatis* cells. 1: [pUAB100::pUAB200] (positive control); 2: [pUAB300::pUAB400] (negative control); 3: [pUAB300*rpfD*::pUAB400*sucA*]; 4: [pUAB300*rpfC*::pUAB400*sucA*]; 5: [pUAB300*rpfE*::pUAB400*sucA*]; 6: [pUAB300*rpfA*::pUAB400*sucA*]; 7: [pUAB300*rpfB*::pUAB400*sucA*]; 8: [pUAB300*rpfB*::pUAB400*sucA*].

time-PCR. The results were expressed as relative expression in different growth phases using early log phase RNA as an internal calibrator and 16S rRNA as a reference gene. Transition of non-culturable state to resuscitation phase increases the expression of *sucA* and *rpf* (A-E) genes, which suggest their simultaneous increase in expression at transcriptional level in the resuscitation, phase (Figure 4).

Discussion

Resuscitation promoting factors (Rpf), a family of extracellular bacterial proteins from *M. luteus* and *M. tuberculosis* has been shown to stimulate growth of dormant mycobacteria. *Rpf* is present as a single, essential gene in *M. luteus* and five homologues (*rpfA-E*) in *M. tuberculosis*. Experimental evidence suggests that Rpf play a distinct role in bacterial resuscitation and re-growth as well as reactivation of chronic tuberculosis in mice. Resuscitation from dormant stage requires remodeling of cell wall and influx of energy by activation of

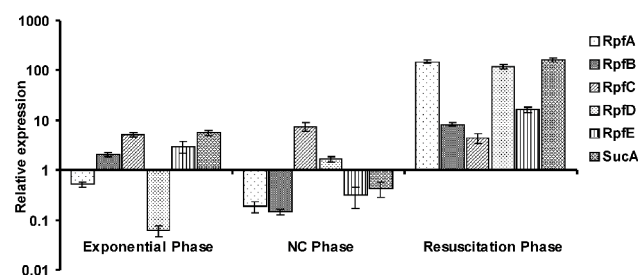


Figure 4. Relative expression analysis of all the five-*rpf* genes (*rpfA-E*) was followed in exponential, nonculturable (NC) and resuscitation phase by real-time PCR. *M. tuberculosis* H37Rv was grown in Sauton's medium containing ADC and tween-80 at 37°C without shaking. RNA extraction was done at different growth stages as described in methods. Early log phase RNA was used as a positive calibrator and 16S rRNA was used as a reference gene for normalization. Data represents mean relative expression value of three independent experiments with SD.

metabolism. Some interesting data has emerged on interaction of Rpf B and Rpf E with cell division protein, named Rpf-interacting protein A (Rip A) in *M. tuberculosis* using a yeast two-hybrid screen [12]. The interaction was confirmed by *in vitro* and *in vivo* co-precipitation assays. Both RpfB and RipA are peptidoglycan hydrolase, capable of digesting cell wall material and co-localize to the septa of growing mycobacteria and thus may play a role in the late stages of mycobacterial cell division, possibly during resuscitation or regrowth from a stressed state [13].

In order to extend the understanding of the mechanism of resuscitation by Rpf, we made an attempt to identify additional interacting partners of Rpf in the genome of *M. tuberculosis* H37Rv by using bacterial two-hybrid system (Daniel Ladant, Pasteur Institute) and Rpf from *M. luteus* as bait. The positive interaction was visualized by appearance of blue colony in presence of X-gal and quantitatively by determination of β -galactosidase activity. The identity of interacting partner was defined by nucleotide sequencing as *sucA* (*rv1248c*) gene encoding α -ketoglutarate decarboxylase (*kdg*) of TCA cycle. *SucA* was found to interact with RpfA, C and D of *M. tuberculosis* along with Rpf from *M. luteus*. This selective interaction has also been observed with RipA protein of *M. tuberculosis*, which interacted with Rpf B and E and not with other Rpf from *M. tuberculosis*. The selective interaction might be related to differential substrate specificity.

Interaction of *SucA* with Rpf from *M. tuberculosis* and *M. luteus* was confirmed in both *E. coli* and mycobacterial host. The physical interaction between RpfM and *SucA* was demonstrated by FRET analysis. FRET is widely used for co-localization and functional interaction studies and reliably used to measure intermolecular distances on a nanometer scale [17]. Rpf from *M. luteus* was used in FRET analysis because of the ease of expression and purification of recombinant protein. Expression of *M. tuberculosis* *rpf* genes in T7 expression vector did not yield significant expression. It is to be noted that Rpf proteins are active in picomolar concentration. It thus appears that low production may be an intrinsic property of protein.

M. tuberculosis lacks detectable α -ketodehydrogenase activity and drives a variant TCA cycle by operating separate oxidative and reductive half cycle of TCA leading to α -ketoglutarate and glutamate via the oxidative branch and succinate via the reductive branch. Both branches are linked by *kdg* (*SucA*) and succinic semialdehyde dehydrogenase to produce succinate from α -ketoglutarate via succinic semialdehyde (SSA) [18]. By pass pathway from α -ketoglutarate to succinate via SSA is not uncommon among microbes and represents an adaptation of their metabolism in diverse environment especially

anaerobic or microaerophilic milieu [18]. One such example is *E. coli*, which operates a complete TCA cycle aerobically and switches under anaerobic conditions to a branched pathway lacking KDH or in *Helicobacter pylori*, which thrive under microaerophilic conditions, operates a branched TCA pathway [19-22]. The knockout of *sucA* gene in *E. coli* cause activation of pentose phosphate (PP) pathway and the glyoxylate shunt while fluxes through the glycolysis and the TCA cycle was downregulated [23]. The mutation delayed the colony formation; glucose uptake rate was lower as compared to parent strain [24]. Although *rv1248c* was identified as an essential gene [24], mutants of *M. tuberculosis* deleted in *rv1248c* could be generated [25]. The mutants grew like wild type in medium containing both carbohydrates (dextrose and glycerol) and fatty acids (Tween 80) under a CO₂ enriched atmosphere but a marked defect in growth was observed when mutant was grown in medium containing carbohydrates as the sole carbon source. The growth was fully inhibited by the presence of 3-nitro-propionate whereas the growth of wild type remains unaffected suggesting the role of *kgd* in growth on carbohydrates as the sole carbon source.

Recent report suggests the existence of two possible ways of TCA cycle in *M. tuberculosis*. In anaerobic condition, where sole carbon source are fatty acids an anaerobic type α -ketoglutarate dehydrogenase drives the synthesis of succinyl-CoA and in aerobic condition, with sole carbon source carbohydrates, variant TCA cycle through *kgd* operates the formation of Succinate by Succinic semialdehyde [25]. Thus, it appears that mycobacteria during transition from dormant to resuscitation phase switch over to normal TCA cycle by activation of *kgd* (*rv1248c*) through variant TCA cycle. Ours is the first report on interaction of Rpf with SucA protein of *M. tuberculosis*. Does this interaction result in resumption of TCA cycle remains to be seen? In *M. tuberculosis*, expression of *sucA* gene has been demonstrated during both logarithmic growth, stationary phase, in human macrophages and was enhanced in resuscitation phase [26]. It could be possible that interaction of SucA with Rpf might be resulting in switch from anaerobic to aerobic pathway.

The resuscitation of dormant bacilli by resuscitation promoting factors (Rpf) has opened a new understanding on remodeling of cell wall of dormant bacilli by RpfB protein with lysozyme like activity binding to RipB an endopeptidase and cause peptidoglycan hydrolysis, thereby aiding in mycobacterial reactivation of growth [27]. Undisputed role of Rpfs and RipA in resuscitation from dormancy makes them attractive targets for development of new drugs preventing resuscitation of dormant *M. tuberculosis*.

A novel class of 2-nitro-phenylthiocyanates (NPT) compounds that inhibit muralytic activity of Rpfs were reported. These compounds suppressed resuscitation of dormant cells of *M. smegmatis* and delayed resuscitation of dormant *M. tuberculosis*. However, at similar concentration, no inhibition of the growth of active mycobacteria was observed [28]. *rv1248c*, identified in this study as interacting partner of Rpfs is essential or required for normal growth of *M. tuberculosis* [24] and is the first gene shown to encode a Kgd. Kgd is lacking in humans and may represent a potential target for chemotherapy of tuberculosis.

Author Summary

Reactivation of dormant mycobacteria into active infectious state must hydrolyze the rigid peptidoglycan layer and encompass flow of energy through resumptions of normal metabolic pathways. Recently, interaction of resuscitation promoting factors (RpfB and RpfE) with an endopeptidase RipA suggests the role of protein-protein interaction in cleavage of peptidoglycan layer of mycobacteria. In this paper, we

have demonstrated the interaction of RpfA, C, D and *M. luteus* Rpf with SucA (*Rv1248c*) of TCA cycle. We confirmed the interaction of SucA with Rpfs *in vivo* in mycobacterial host and *in vitro* by FRET analysis. The enhanced co-expression of *rpf* and *rv1248c* genes also supports their role during resuscitation. We hypothesize that the interaction of SucA with Rpfs results in activation of normal TCA cycle for the generation of energy efflux. It could be possible that the protein-protein interaction of Rpfs with SucA and RipA results in switch from dormant to active growing phase.

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