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 muriolytic activity of Rpf and they have been identified as peptideglycan 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 Rpfs 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 RipA, C and D and could serve as prospective target along with Rpfs 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 Rpfs 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 Rpfs may act via distinct mechanism and or on different substrate or pathway.

To investigate additional potential interacting partner of Rpfs 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
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 mc³155 was grown in M7H9 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 (DH1) 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 DH1M and kanamycin resistant E. coli DH1M [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 Sau3A enzyme and DNA fragments ranging between 0.2 kb to 2 kb were eluted from the gel, treated with CIAP and ligated with BamHI linearized pUT18c vector. The 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 OD₆₅₀ was recorded. Cells were then permeabilized by 35 µl of tolune 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 tolune. For enzymatic reaction, aliquots (0.1 ml (0.5 ml) of permeabilized cells were added to buffer PM2 (70 mM Na₂HPO₄, 12H₂O, 30 mM NaH₂PO₄·H₂O, 1 mM MgSO₄, 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. OD₅₇₀ was recorded. The enzymatic activity A (milliter unit) was calculated according to the following equation, A=100 × (OD₅₇₀ of the culture/time × V × OD₆₅₀). 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 pT7Z57R/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 Ni²⁺. 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-pMF | 5'CGGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pMR | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pMAF | 5'AAAACCTGCAGCGCGGAGACGACGAC3' |
| pKT25-pMAR | 5'CGGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pBF | 5'CGGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pBR | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pCF | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pCR | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pDF | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pDR | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pEF | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pKT25-pER | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pUT18C-sucAF | 5'GGGAATTCGCCACCGTGGACACCTG3' |
| pUT18C-sucAR | 5'GGGAATTCGCCACCGTGGACACCTG3' |

Table 2: Primers used in this study.

For cloning in pUB vectors

| MF | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| MR | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| SucA | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| SucAR | 5'GGGATCCAGCGCGGAGACGACGAC3' |

For real-time PCR

| pA | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pB | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pC | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pD | 5'GGGATCCAGCGCGGAGACGACGAC3' |
| pE | 5'GGGATCCAGCGCGGAGACGACGAC3' |

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 complementation (M-PFC) was used to study in vivo protein-protein interaction between RpfS from *M. tuberculosis* with SucA protein in mycobacterial host [16]. sucA gene was cloned in pUA400 and rpf (A-E) genes were cloned in pUA8300 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-^Hyg^ 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 OD$_{600}$ nm of 0.8. Cells were diluted in fresh 7H9 medium, and ~10$^6$ 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::pRpfM and pUT18c::M. tuberculosis library were plated on LB agar containing ampicillin, kanamycin and indicator X-gal for blue colonies representing cya genes in M-PFC vectors. Three blue 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 corresponding cya/ genes in M-PFC vectors. All three inserts were found in frame with T18 fragment of pUT18c vector. The clone containing in frame fusion of rpfS and sucA genes 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* Rp, 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 Rpfs from *M. tuberculosis***

Interaction of rpfs with full-length sucA gene was studied. All five rpfs (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 rpfs 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 Rpfs in vivo**

In *vivo* protein-protein interaction between Rv1248c (SucA) and Rp (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 rpfs (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 µg/ml) (Figure 3a). Results confirmed the interaction of SucA with Rpfs 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 330 nm and emission wavelength of 590 nm (Figure 3b).

**Expression analysis of rpfs and sucA gene in different growth stages**

Transcriptional analysis of rpfs and sucA was done in exponential, stationary, NC and resuscitation phase of *M. tuberculosis* H37Rv by real
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 (Rpfs), a family of extracellular bacterial proteins from M. luteus and M. tuberculosis has been shown to stimulate growth of dormant mycobacteria. Rpfs is present as a single, essential gene in M. luteus and five homologues (rpfA-E) in M. tuberculosis. Experimental evidence suggests that Rpfs 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 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 Rpfs, we made an attempt to identify additional interacting partners of Rpfs 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 partners was defined by nucleotide sequencing as sucA (r1248c) gene encoding α-ketoglutarate carboxylase (kgd) 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 Rpf A of M. tuberculosis, which interacted with both Rpf B and E and not with other Rpfs from M. tuberculosis. The selective interaction might be related to differential substrate specificity.

Interaction of SucA with Rpfs 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 A-E 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-ketoglutarate dehydrogenase 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 kgd (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 Succinyl 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 RipA 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 muramyl 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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