

ABSTRACT

Johnathon Steven Nolen

Examination of resuscitation-promoting factors in potential fish pathogens:

Mycobacterium shottsii and *Mycobacterium pseudoshottsii*

(Under the Direction of DR. RUSSELL KARLS)

Defining mechanisms of gene regulation is critical for understanding how pathogenic mycobacteria survive in diverse environments and for identifying novel drug targets. For the human pathogen *Mycobacterium tuberculosis*, resuscitation factors are believed to play an important role in primary infections as well as for reactivation of latent infections. The main goal of the project is to identify the inter-species conservation and function of an important resuscitation-promoting factor (Rpf) from recently identified potential fish pathogens *Mycobacterium shottsii* and *Mycobacterium pseudoshottsii*. A significant impediment in mycobacterial research is the slow growth of these microbes *in vitro*, necessitating very long incubations that sometimes take months for appreciable growth. This research investigates if RpfB proteins accelerate growth *in vitro* and if Rpf proteins from one *Mycobacterium* species impact the growth of others. As proteins from this class are secreted into culture media, culture filtrates from aged bacterial cultures are tested for growth stimulation of low-density mycobacterial cultures. Recently, RpfB from *M. tuberculosis* H37Rv was over-expressed in *E. coli* strain CH3λDE3 and purified using Ni⁺²-NTA chromatography. Further tests for growth stimulation are in progress. This research is beneficial in that Rpf proteins could potentially be used as a diagnostic marker for *M. tuberculosis* infection and may be especially helpful for diagnosing latent infections. Also, use of purified Rpf proteins may help accelerate culture of *M. tuberculosis* in sputum, thereby aiding in more-rapid diagnosis and treatment.

INDEX WORDS: Mycobacteria, *Mycobacterium tuberculosis*, resuscitation-promoting factor (Rpf), *Mycobacterium shottsii*, *Mycobacterium pseudoshottsii*, RpfB, culture filtrates, aged bacterial cultures, Ni⁺²-chromatography

EXAMINATION OF RESUSCITATION-PROMOTING FACTORS IN POTENTIAL FISH
PATHOGENS: *MYCOBACTERIUM SHOTTSII* AND *MYCOBACTERIUM PSEUDOSHOTTSII*

by

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A Thesis Submitted to the Honors Council of the University of Georgia

In Partial Fulfillment of the Requirements for the Degree

BACHELOR OF SCIENCE

in MICROBIOLOGY

with HONORS

Athens, Georgia

2010

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DEDICATION

I dedicate this thesis to my family, friends, and faith. I thank my family for all the support they have given me, in doing a fine job in raising all three of their children, and in setting perfect examples of how God can use our lives. I want to thank my closest friend, Mary Elizabeth Golden, for her support and her everlasting friendship through my high school and college careers. Most of all, I want to thank my Lord and Savior, Jesus Christ. He has tremendously blessed me with a great family and the opportunity to continue my education in order to further His kingdom.

ACKNOWLEDGEMENTS

I am very grateful for Dr. Russell Karls accepting me into his lab my senior year when I had no prior microbiology lab experience. I am also very grateful to Dr. Tuhina Gupta for allowing me to work with her on this project and for her mentorship. I have learned a lot about research laboratory policies and techniques. It was a valuable experience that I will always remember. I wish to thank Dr. Frederick Quinn for his valuable input with my project. In addition, I thank Dr. Vincent Starai for his insight and guidance with protein purification. I am extremely grateful for the kindness that was shown towards me by all lab members throughout my time in the lab.

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CHAPTER 1 INTRODUCTION

Mycobacterium tuberculosis is a bacterium that usually attacks the lungs and may cause symptoms of tuberculosis: chronic cough, blood-tinged sputum, fever, night sweats, and weight loss. Presently, no vaccine has been shown to reproducibly protect against tuberculosis. Although certain drugs are available to treat *M. tuberculosis* infections, most are only effective against actively-dividing bacteria. It is thought that some *M. tuberculosis* bacteria enter into a dormant state in response to stress signals sensed inside a human host and are thus protected from antibiotics.

A resuscitation-promoting factor (Rpf) was first discovered in *Micrococcus luteus* (Kell, et al.). This protein was found to be required for growth stimulation of dormant cells and growth enhancement in poor media (media containing low levels of nutrients). Rpf proteins have been found to stimulate growth of high G+C Gram-positive organisms, which includes *Mycobacterium bovis* (BCG), *Mycobacterium kansasii*, *Mycobacterium avium*, *Mycobacterium smegmatis*, and *Mycobacterium tuberculosis* (Kell, et al.).

To pursue the role of resuscitation-promoting factors, Zhu and colleagues tested the effects of Rpfs on mycobacteria cultured for longer than one month (Zhu, et al.). Without Rpfs, the mycobacteria were viable, but not actively replicating. The results showed that addition of Rpfs shortened the lag phase and enhanced the growth of *M.*

tuberculosis. As *M. tuberculosis* infections often result in latent infections, Rpf proteins may be important in helping the bacteria emerge from a latent state (Young, et al.). Since Rpf proteins have been found to enhance the growth of such organisms, many experiments have been performed to identify the mechanism by which these proteins work.

It has been hypothesized that Rpf proteins have peptidoglycan hydrolysis activity, meaning that the presence of Rpf proteins may aid during cell division by breaking down the peptidoglycan layer of the bacteria (Telkov, et al.). Telkov and colleagues tested this hypothesis by applying Rpf proteins to a polyacrylamide gel containing *M. luteus* cell walls. They observed the formation of transparent zones when Rpf proteins were present, and no transparent zones when Rpf proteins were absent. This suggested cell wall hydrolysis activity (Henderson, et al.). The activity of peptidoglycan hydrolysis either alters the mechanical properties of the cell wall to facilitate cellular division, or it releases lysis products that may function as anti-dormancy signals (Kaprelyants, et al.).

Rpf proteins have also been found in *M. tuberculosis*. There are five *rpf*-like genes distributed throughout the chromosome in *M. tuberculosis* strain H37Rv. These genes are *Rv0867c* (*rpfA*), *Rv1009* (*rpfB*), *Rv1884c* (*rpfC*), *Rv2389c* (*rpfD*), and *Rv2450c* (*rpfE*). Each encodes a protein with a highly-conserved RPF domain consisting of approximately seventy amino acids (Turapov, et al.). Experiments have been performed to test the significance of this Rpf domain. A *M. tuberculosis* mutant with a deletion in the Rpf domain of RpfB exhibited a defect in persistence following infection of mice and impaired reactivation from late-stage growth *in vitro* (Mukamolova, et al.). Similar *in vitro*

reactivation defects were observed in a *M. tuberculosis* mutant deleted for the entire *rpfB* gene. This implies that the Rpf domain plays a key role in the activities of bacteria containing Rpfs.

The main purpose of this project was to isolate Rpf proteins to develop them into a diagnostic test for *M. tuberculosis* infection. The *Mycobacterium* species used in this project were *M. shottsii* and *M. pseudoshottsii*. Because they share the same genus, these species have similarities to *M. tuberculosis*. The advantages of using these species are i) they are slow-growing species, which should magnify a resuscitation phenotype and ii) they are not respiratory pathogens like *M. tuberculosis*, enabling them to be studied outside of a high-containment laboratory. The results of this project could be beneficial because *M. tuberculosis* also grows very slowly and clinical diagnosis may be accelerated if addition of Rpf proteins accelerates the growth of *M. tuberculosis* in clinical specimens. In addition, if Rpf proteins can be purified and used to generate antibodies, the antibodies could potentially be used to detect the presence of *M. tuberculosis*. This would be especially helpful for diagnosing people with latent *M. tuberculosis* infection.

CHAPTER 2 METHODS AND MATERIALS

Studies examining resuscitation properties of mycobacterial spent media

M. shottsii was grown in approximately 50 ml Middlebrook 7H9 broth supplemented with 0.5% glycerol, 10% albumin-dextrose- catalase (ADC, BD/Difco, Inc.), and 0.25% Tween 80 (7H9tgADC) at 25 °C. When cultures reached a density of 1 at 600 nanometers (OD₆₀₀), the cells were pelleted by centrifugation (2056 g for 15 minutes). Culture supernatant was then collected and filtered through 0.22µm Nalgene filters (127-0020). An aliquot (100 µl) of late-stationary phase (six-month old) *M. shottsii* culture was inoculated into culture bottles containing either 50 ml of fresh 7H9tgADC medium or 50 ml total volumes of fresh medium containing 25% or 50% of the OD₆₀₀ = 1 *M. shottsii* spent medium. Growth at 25°C was monitored by measuring the optical density at 600 nm over 29 days. The spent medium was only inoculated in *M. shottsii* because there was insufficient spent medium to test for both *M. shottsii* and *M. pseudoshottsii*.

This same method was used to prepare spent medium from *M. marinum* and from *M. tuberculosis*. Spent media from *M. marinum* and *M. tuberculosis* strain Erdman was inoculated in subcultures of 6-month aged *M. shottsii* bacteria and 6-

month aged *M. pseudoshottsii*. Growth was monitored by measuring the absorbance at 600 nm over time.

Screening *E. coli* CH3λDE3 pSLH2 transformants for production of RpfB

Plasmid pSLH2, which encodes for *M. tuberculosis* H37Rv RpfB with an amino-terminal multiple histidine tag was kindly provided by Michael Young (University of Aberystwyth). The 6-His-RpfB gene is under control of an IPTG-inducible promoter. This plasmid was used to transform *E. coli* strain CH3λDE3 kindly provided by Dr. Vincent Starai (Dept. of Microbiology, University of Georgia). Individual transformant clones were checked for production of RpfB after induction with 1 mM IPTG and incubation for two or four hours with shaking (220 rpm) at 37°C. Soluble fractions were prepared and analyzed by SDS-PAGE. The results showed that induction of certain clones (11 and 12) yielded increased production of a protein product of the expected size for RpfB (figure 5). This suggested that either would be suitable for purification of RpfB protein.

Preparation of charged nickel-NTA columns

Four-ml nickel-NTA columns (Novagen, Inc.) were poured and resin allowed to settle (2-ml total bed volume). The columns were washed at least 5 times with sterile water at 4°C. Charge buffer (obtained from a His-Bind Purification Kit

(Novagen, Inc.) was prepared by mixing 4 ml of 8X charge buffer with 28 ml of autoclaved water. The column was then filled with 1X charge buffer. The column was then vortexed and allowed to settle for 5 minutes before draining the liquid. This procedure was repeated until all of the 1X charge buffer was run through the column. The columns were then washed with 3 volumes of binding buffer (5 mM imidazole, 300 mM NaCl, 50 mM Tris HCl pH 8.0, 10% (v/v) glycerol, and 1 tablet of Roche protease inhibitor cocktail). The column was now ready for use.

Purification of recombinant *M. tuberculosis* RpfB

A two-liter culture of *E. coli* strain CH3λDE3 transformed with plasmid pSLH2 (clone 11) was grown until O.D. 600 nm reached 0.5 - 0.6 at which point the culture was divided into two and induced with IPTG. The two cultures were kept in shakers at 220 rpm set at different temperatures: 25°C and 37°C. After 4 hours the cells were harvested by centrifugation (4000 g, 20 min). The pellets were stored at -80°C until used. Later, the cell pellets were resuspended in 30 ml cold phosphate-buffered-saline (PBS) pH 7.4 with protease inhibitors tablet (Roche). Without vortexing, the pellets were mixed on an orbital mixer (BD Adams™ Nutator) at 4°C. The cell suspensions were then passed twice through a French press (11,000 psi).

The cell lysates were centrifuged (14,000 x g, 45 min, 4°C). The soluble and insoluble fractions were collected and all fractions were stored on ice. The soluble fractions from 25°C and 37°C cultures were further used for RpfB purification using Ni-NTA columns.

Based on colony screening of *E. coli* CH3λDE3 pSLH2 transformants, clone 11 was selected for purification of RpfB. The clone was cultured at 37°C in duplicate flasks containing 1 L Luria broth containing carbenicillin [100 µg/ml]. When the culture reached an OD₆₀₀ = 0.5- 0.6, each culture was induced with 1 mM IPTG. One flask was transferred to a 25°C shaker, the other to a 37°C shaker. After 4 hours, the cultures were harvested by centrifugation (4000 x g, 30 min, 4°C). The cell pellets were stored at -80°C. Later, the cell pellets were suspended in approximately 30 ml of ice cold PBS, pH 7.4 containing protease inhibitor tablets (Roche). Without vortexing, the pellets were mixed on an orbital mixer (BD Adams™ Nutator) at 4 °C (in a cold room) until they were dissolved. From this point onward, all experiments were done in a cold room except for the SDS page gels. The cells were lysed by two passes through a French Press. The cell lysates were centrifuged (14,000 x g, 45 min, 4°C). The soluble and insoluble fractions were collected and all fractions were stored on ice. The soluble fractions from 25°C and 37°C cultures were further used for RpfB purification using Ni-NTA columns. The soluble fractions were then

incubated with 2-ml of charged Ni-NTA resin at 4°C for 18 hours and the mixture poured into a column. The flow through was collected from each column into labeled 50-ml tubes and then stored on ice. Ten column volumes of wash buffer containing 55 mM imidazole was added to each column. The first 500 µl eluted from the column with wash buffer was collected and stored on ice for subsequent SDS-PAGE analysis. The remaining wash buffer eluate was discarded. Next, three column volumes (a total of 6 ml) of elution buffer containing 550 mM imidazole was added. Six 1-ml fractions were collected in 1.5 ml tubes and stored on ice. Samples from each fraction were analyzed by SDS-PAGE. Fractions 2, 3, 4 and 5 contained a strong band of the expected size for RpfB were pooled and dialyzed against 1000 ml of PBS, pH 7.4, +10% glycerol+ 5 mM EDTA in Pierce 3,500 Molecular Weight Dialysis Cassettes . After 2 hours, the dialysis cassettes were placed into a beaker containing fresh PBS+10% glycerol for 2 hours, and then in a final beaker containing PBS+10% glycerol overnight. Samples were then removed from the dialysis cassettes. From the original 25°C culture, 2.5 ml was recovered. From the original 37°C culture, 3.0 ml was recovered.

Testing recombinant *M. tuberculosis* RpfB for resuscitation of *M. shottsii* and *M. pseudoshottsii*

The purified and dialyzed recombinant 6-His-RpfB protein preparations from 25°C and 37°C cultures were tested for growth stimulation of late stationary phase (12- month old) *M. shottsii* and *M. pseudoshottsii* cultures. In triplicate 10-ml screw-capped glass tubes, was added 6 ml 7H9tgADC medium supplemented with or without the following amounts of recombinant RpfB: 0 µg, 95 µg, 190 µg or 380 µg. Each tube was inoculated with 10 µl of 12 months old culture of *M. shottsii* or *M. pseudoshottsii*. Absorbance at 600 nm was monitored over time.

CHAPTER 3 RESULTS AND DISCUSSION

Mycobacterium shottsii and *M. pseudoshottsii* culture supernatants have resuscitation qualities

It is known that *M. tuberculosis* excretes resuscitation factors into culture medium and that the spent medium collected from actively-replicating cultures can be used to resuscitate growth of stationary phase *M. tuberculosis* cells that have been subcultured into fresh medium (Mukamolova, et al.). We examined if marine mycobacteria: *M. shottsii* and *M. pseudoshottsii* exhibit similar properties. To test if *M. shottsii* and *M. pseudoshottsii* secrete products into culture medium that aid in resuscitation of aged *M. shottsii* and *M. pseudoshottsii* bacteria, aged *M. shottsii* and *M. pseudoshottsii* cells were generated by culture in Middlebrook 7H9tgADS broth for 6 to 12 months. For culture supernatants from actively-dividing cells, *M. shottsii* and *M. pseudoshottsii* were cultured in 7H9tgADS to an optical density at 600 nm = 1.0 and the cells removed by centrifugation and filtration. Aged *M. shottsii* or *M. pseudoshottsii* aliquots were then subcultured into fresh medium containing 0, 25 or 50% of the OD₆₀₀ = 1.0 spent medium and monitored for growth over time. The results indicated that something is present in the spent medium that resuscitates aged *Mycobacterium shottsii* and *Mycobacterium pseudoshottsii* (figure 1).

When no spent media is added, there is little to no growth. As the amount of spent media present was increased, so did growth of the organism. The O.D.₆₀₀ at day 15 was 0.2 for *M. shottsii* cultured in 50% spent medium, whereas no visible growth was detected for *M. shottsii* cultured without spent medium (figure 1).

Aged *M. shottsii* bacteria can be resuscitated by supernatant from OD₆₀₀ = 1.0 culture of *M. pseudoshottsii*

Evidence of interspecies resuscitation by culture supernatants was observed. Growth (as measured by culture optical density) of *M. shottsii* began earlier when spent medium was present. The more spent medium, the earlier cultures grew and the higher the O.D.₆₀₀. Spent medium from *M. marinum* stimulated growth of both *M. shottsii* and *M. pseudoshottsii* (figure 2). For example, an O.D.₆₀₀ of 0.2 is reached by both *M. shottsii* and *M. pseudoshottsii* around day six with 50% spent medium, where *M. shottsii* and *M. pseudoshottsii* with 0% or 25% spent never reach an O.D.₆₀₀ of 0.2. Similar growth patterns were also demonstrated when *M. shottsii* and *M. pseudoshottsii* were inoculated with varying amounts of *M. tuberculosis* spent medium (figure 3). An O.D.₆₀₀ = 0.2 was reached around day 27 for *M. shottsii* cultured with 50% *M. tuberculosis* spent medium, while *M. shottsii* cultured with 25% *M. tuberculosis* spent medium reached O.D.₆₀₀ = 0.2 around day 33. *M. shottsii*

without spent medium did not reach an $\text{O.D.}_{600} = 0.2$ by the time this experiment was stopped. *M. pseudoshottsii* cultured with either 25% or 50% *M. tuberculosis* spent medium reached an $\text{O.D.}_{600} = 0.2$ at around 14 days. There was a peak for *M. pseudoshottsii* growth at around 27 days, and then the O.D._{600} began to decline.

The above studies indicate that *M. shottsii* can be resuscitated by culture supernatants from several *Mycobacterium* species. We hypothesize that the resuscitation is due to the presence of RpfB in the culture supernatants. Therefore, to test this hypothesis, the RpfB protein from *M. tuberculosis* was expressed in *E. coli* with an amino terminal hexahistidine tag using plasmid pSLH2 (Kell, et al.).

Testing recombinant *M. tuberculosis* RpfB for resuscitation of *M. shottsii* and *M. pseudoshottsii*

Recombinant *M. tuberculosis* RpfB was purified from *E. coli* CH3λDE3/pSLH2 as described (chapter 2, figure 4). The recombinant *M. tuberculosis* RpfB was then tested for resuscitation of aged *M. shottsii* and *M. pseudoshottsii* bacteria by adding a different amounts of RpfB into fresh 7H9tgADS medium inoculated with either 6- 12 months old *M. shottsii* or 6- 12 months old *M. pseudoshottsii* culture. Growth was monitored over time. Growth promotion was expected to be similar to results previously described when spent culture media were tested. However, little or no

growth was observed in the cultures (data not shown). Based on gel analysis of the purification of the RpfB protein, it is evident that additional proteins had eluted from the nickel column along with RpfB. The major protein contaminant likely was not removed by subsequent dialysis using 3,500 Molecular Weight Cutoff Dialysis Cassettes. This suggests the possibility that a contaminating molecule in the RpfB preparation is interfering with RpfB activity. Alternatively, the resuscitation results seen with the spent medium studies could be due other proteins or factors in the culture supernatants. A third possibility is that Rpf proteins work in concert to promote resuscitation. If this is the case, it would predict that additional Rpf proteins could be purified and tested in combinations to determine what is necessary to promote resuscitation.

CHAPTER 4

SUMMARY AND FUTURE PLANS

Because growth of *M. tuberculosis* is very slow, it takes a long time by sputum culture to diagnose patients with the disease, especially latent *M. tuberculosis*. Resuscitation promoting factors may be useful as growth stimulants to accelerate growth of *M. tuberculosis* in clinical specimens, which would accelerate the diagnosis of infection and may also be useful in accelerating antibiotic susceptibility testing of the cultured strain. This would provide useful information for treatments that should be effective in treating infections with this strain of *M. tuberculosis*. From our studies growth of aged *M. shottsii* and *M. pseudoshottsii* bacilli was accelerated by the addition of spent media from different *Mycobacterium* species, suggesting something in the spent medium aids cell division. This could be a variety of enzymes or proteins, but efforts in purifying Rpf proteins should be pursued to examine their effect on growth of these organisms. If growth promotion is found to be associated with Rpf proteins, then antibodies to these proteins could be generated to begin to develop a diagnostic test.

Future efforts will test what substances in the spent medium are responsible for growth acceleration. Such efforts include fractionating the spent media to detect the growth-accelerating factors. Also, it will be necessary to perform another round of protein purification of recombinant *M. tuberculosis* RpfB to improve the purity

of the protein preparation. Beneficial experiments in the future include studying growth promoting effects of purified RpfB on *M. shottsii*, *M. pseudoshottsii* and *Mycobacterium avium* subspecies *paratuberculosis* (MAP). In addition, western blotting with RpfB protein as a diagnostic marker may be possible if rabbits latently infected with *M. tuberculosis* produce antibodies that recognize this protein. If so, then rabbits could be cleared of active disease using antibiotic therapy. Serum from these animals could then be tested for antibodies that recognize RpfB. Finally, expression of *rpf* genes could be studied using real time RT-PCR of the *rpfA-E* homologs in *M.shottsii*, *M. pseudoshottsii* and MAP.

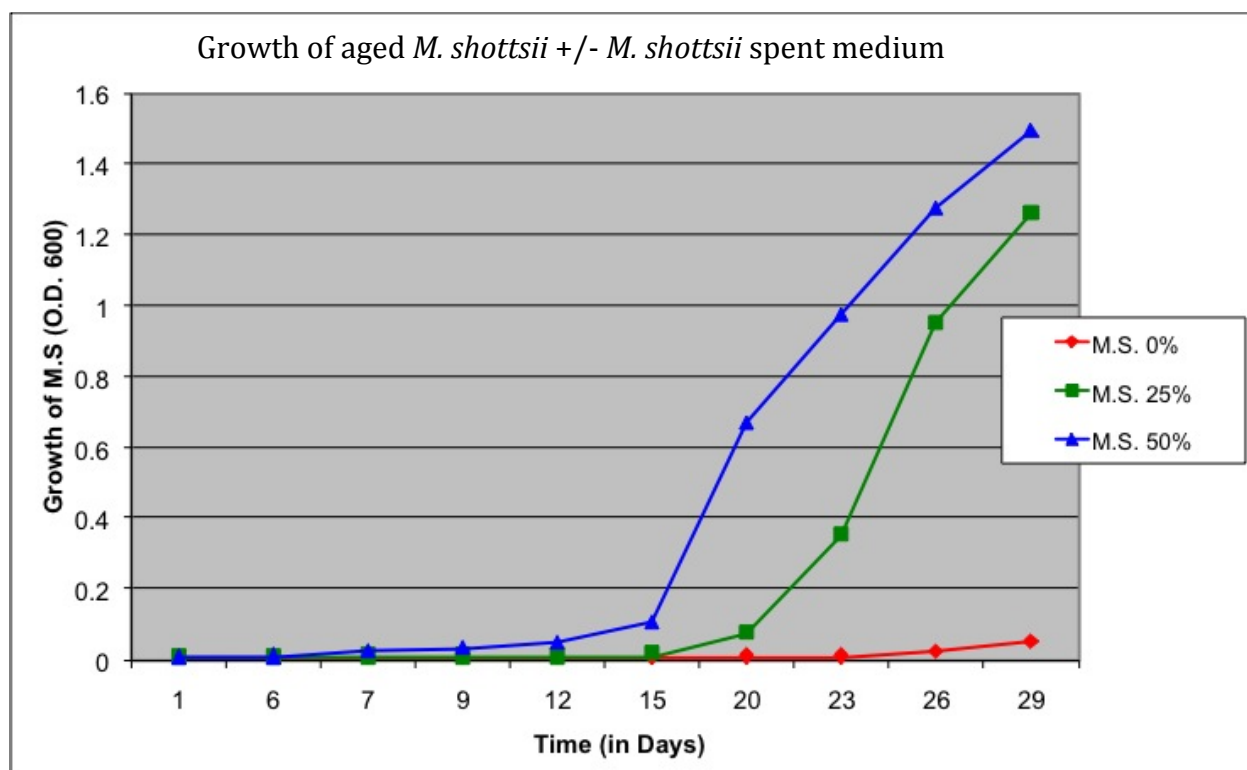
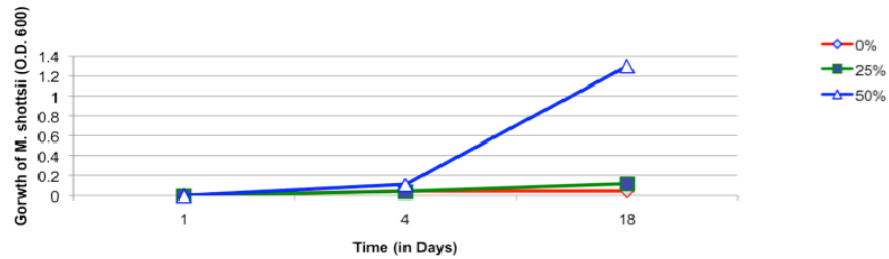


Figure 1: Spent medium from *M. shottsii* promotes growth of aged *M. shottsii*

M. shottsii was cultured in Middle Brook 7H9 broth supplemented with 0.5% glycerol, 0.25% Tween-80 and 10% (v/v) albumin-dextrose-catalase (7H9gtADC) at 25°C until the culture reached an optical density at 600 nm = 1.0. The spent media was collected and filtered through 0.22 µm Nalgene filters after removal of the cells by centrifugation (200 x g, 30 min, 25°C). An aliquot (100 µl) of late stationary phase (six-month old) *M. shottsii* culture was transferred to either 50 ml fresh 7H9gtADC medium or medium supplemented with 25% or 50% of the OD₆₀₀ = 1 *M. shottsii* spent medium. Growth at 25°C was monitored by measuring the optical density at 600 nm.

A. Growth of aged *M. shottsii* +/- *M. marinum* spent medium



B. Growth of aged *M. pseudoshottsii* +/- *M. marinum* spent medium

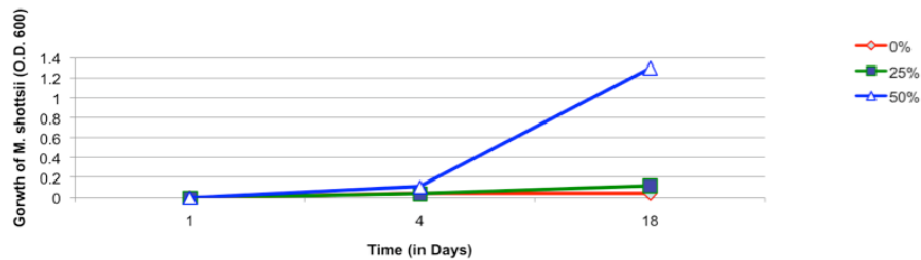
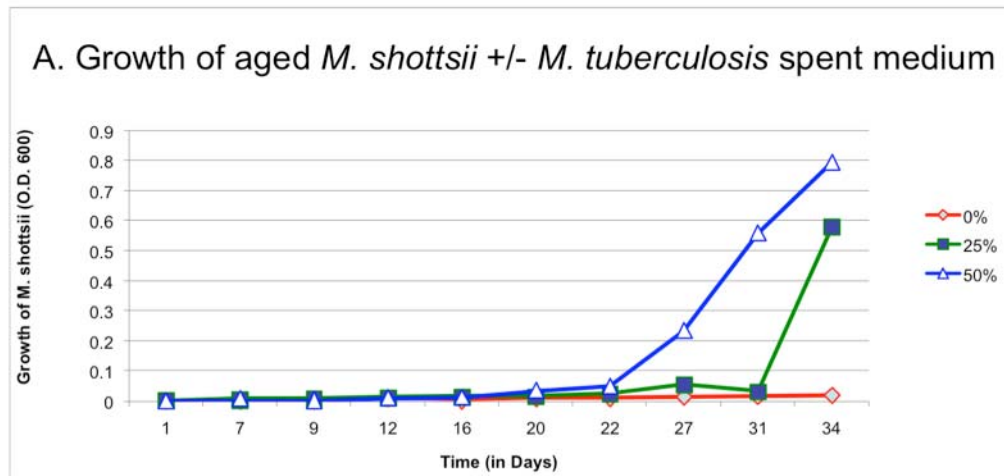


Figure 2: Spent medium from *M. marinum* promotes growth of aged *M. shottsii* and *M. pseudoshottsii*

Using similar methodology as described in Figure 1, spent medium was obtained from a $OD_{600} = 1.0$ culture of *M. marinum* and tested for growth promotion of *M. shottsii* (A) and *M. pseudoshottsii* (B) bacilli obtained from 6-month old cultures. The percentage of spent medium is indicated.



B. Growth of aged *M. pseudoshottsii* +/- *M. tuberculosis* spent medium

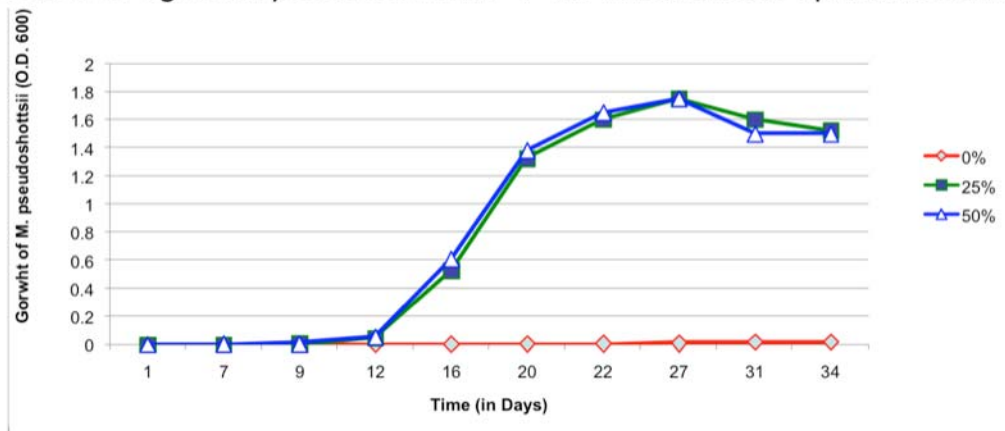


Figure 3: Spent medium from *M. tuberculosis* promotes growth of aged *M. shottsii* and *M. pseudoshottsii*

M. tuberculosis strain Erdman was cultured in 7H9gtADC broth at 37°C until absorbance at 600 nm reached 1.0. The spent media was collected and filtered through 0.22 µm membranes after removal of the cells by centrifugation (200 x g, 30 min, 25°C). An aliquot (10 µl) of late stationary phase (6-month old) culture of *M. shottsii* or *M. pseudoshottsii* culture was transferred to 10 ml fresh 7H9gtADC medium supplemented with either 0%, 25% or 50% *M. tuberculosis* spent media and cultured at 25°C. Absorbance at 600 nm was monitored over time for resuscitation of *M. shottsii* (A) and *M. pseudoshottsii* (B).

Culture CH3λDE3/pSLH2 in LB + carbenicillin to OD₆₀₀=0.5 at 25°C and 37°C → Induce for 4 hr with 1 mM IPTG → Collect cells and freeze pellets → Suspend pellets in PBS + protease inhibitors → Lyse cells by French Press → Charge NTA resin with nickel → Wash resin with binding buffer → Mix resin with CH3λDE3/pSLH2 lysate for 18 hr to allow His6-RpfB to bind resin → pour into a column → wash column with binding buffer → wash column with wash buffer → elute RpfB with elution buffer → analyze fractions for RpfB → pool RpfB fractions → dialyze against PBS + 10 % glycerol + 5 mM EDTA → dialyze against PBS + 10% glycerol

Figure 4: Outline of Protein Purification of 6-His-RpfB from CH3λDE3/pSLH2

1. Protein MW standards
2. Clone 1 uninduced
3. Clone 1 induced for 2 hrs
4. Clone 1 induced for 4 hrs
5. Clone 11 uninduced
6. Clone 11 induced for 2 hrs
7. Clone 11 induced for 4 hrs
8. Clone 12 uninduced
9. Clone 12 induced for 2 hrs
10. Clone 12 induced for 4 hrs

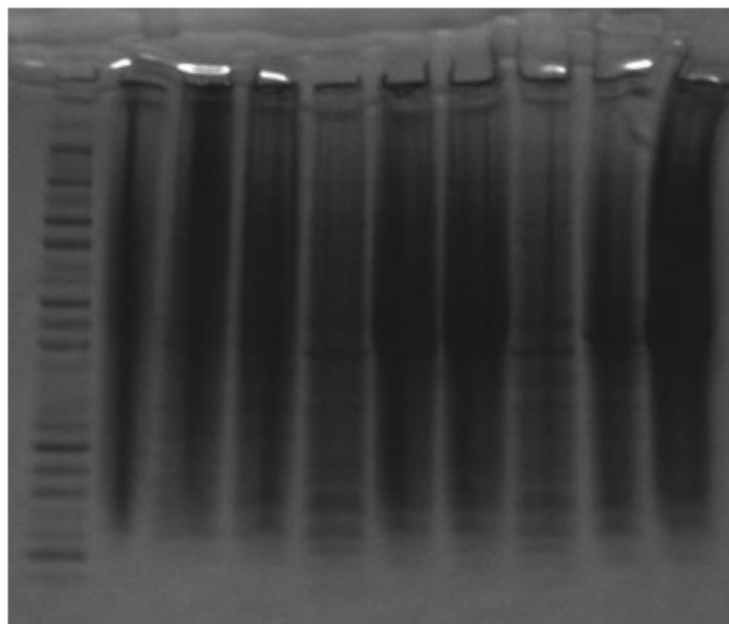


Figure 5: Screening *E. coli* CH3λDE3/pSLH2 transformants for production of RpfB

Plasmid pSLH2 encoding *M. tuberculosis* H37Rv RpfB with an N-terminal 6-His tag under control of an IPTG-inducible promoter was used to transform *E. coli* strain C43λDE3. Individual transformant clones were checked for production of RpfB after induction with 1 mM IPTG for the times indicated. Soluble fractions were prepared and analyzed by SDS-PAGE. The results showed that induction of clones 11 and 12 yielded increased production of a protein product of the expected size for RpfB.

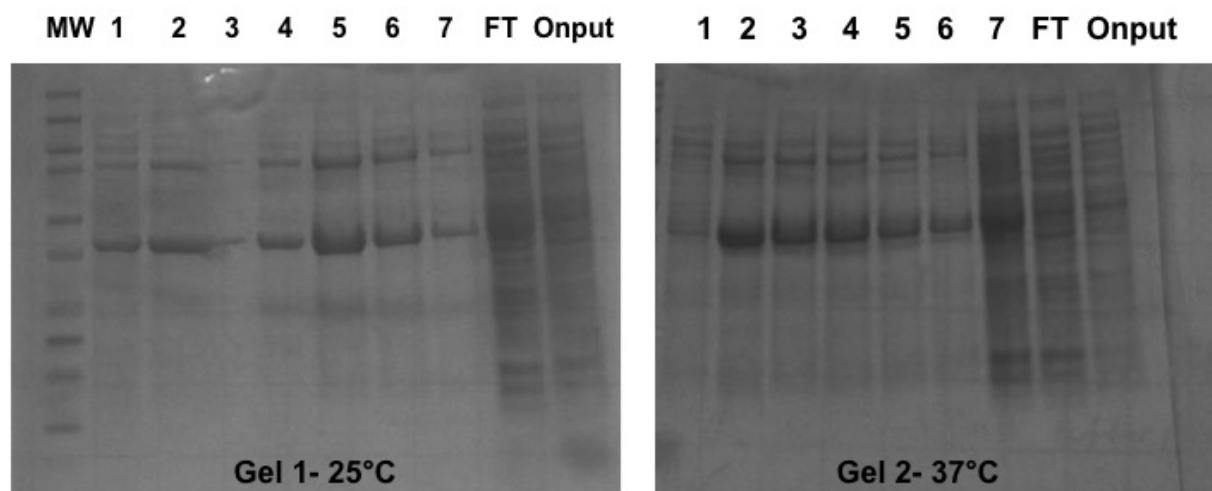


Figure 6: Purification of recombinant RpfB from *E. coli* clone 11 grown at 25°C and 37°C

Luria broth containing carbenicillin [100 µg/ml]. When the culture reached an $OD_{600} = 0.5$, each culture was induced with 1mM IPTG. One flask was transferred to a 25°C shaker, the other to a 37°C shaker. After 4 hours, the cultures were harvested by centrifugation (4000 x g, 30 min, 4°C). The cell pellets were stored at -80°C. Later, the cell pellets were suspended in cold PBS, pH7.5 containing protease inhibitors. The cells were lysed by French Press. The cell lysates were centrifuged (14,000 x g, 45 min, 4°C). The soluble and insoluble fractions were collected. The soluble fraction was then incubated with a Ni-NTA resin at 4°C for 18 hours and the mixture poured into a column. The resin was washed and eluted with 50 mM and 550 mM imidazole, respectively. Fractions were collected and analyzed on SDS-PAGE gel. Loaded from right to left are the column onput, the flowthrough (FT), and sequential aliquots obtained with 550 mM imidazole elution. The leftmost lane contains protein molecular weight standards (MW).

WORKS CITED

Keep, Nicholas H.; Ward, John M.; Cohen-Gonsaud, Martin; Henderson, Brian.
Trends in Microbiology, Jun2006, Vol. 14 Issue 6, p271-276, 6p; DOI:
10.1016/j.tim.2006.04.003; (AN 21262768).

Mukamolova, G.V., A.S. Kaprelyants, D.I. Young, M. Young, D.B. Kell, A bacterial cytokine. Proc. Acad. Sci. USA 95 (1998) 8916-8921.

Mukamolova, G.V., A.S. Kaprelyants, D.B. Kell, M. Young, Adoption of the transiently non-culturable state- a bacterial survival strategy?. Adv. Microb. Physiol. 47 (2003) 65-129)

Mukamolova, G. V., O. A. Turapov, et al. (2002). "gene of." Molecular Microbiology **46**: 611-621.

Telkov, M.V., G. R. Demina, S. A. Voloshin, E. G. Salina, T. V. Dudik, T. N. Stekhanova, G. V. Mukamolova, K. A. Kazaryan, A. V. Goncharenko, M. Young, and A. S. Kaprelyants, Proteins of the Rpf (Resuscitation Promoting Factor) Family Are Peptidoglycan Hydrolases, ISSN 00062979, Biochemistry (Moscow), 2006, Vol. 71, No. 4, pp. 414-422. © Pleiades Publishing, Inc., 2006. 414-422

Zhu, W., B.B. Plikaytis, and T.M. Shinnick, Resuscitation factors from mycobacteria: homologs of *Micrococcus luteus* proteins. Tuberculosis (Edinb) 83 (2003) 261-269).