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Contribution of the Rv1985c transcriptional regulator to Mycobacterium tuberculosis intracellular adaptation.

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CONTRIBUTION OF THE Rv1985c TRANSCRIPTIONAL REGULATOR TO *MYCOBACTERIUM TUBERCULOSIS* INTRACELLULAR ADAPTATION

Nicholas B. Wantland
B.S., University of Louisville, 2006

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Thesis Approved on

July 29, 2011

by the following Thesis Committee

Dr. James E. Graham (Chairman)

Dr. David A. Scott

Dr. Donald R. Demuth

DEDICATION

I would like to dedicate this work to my parents, without their continued support and encouragement, none of this would have been possible.

ACKNOWLEDEGMENTS

My deepest gratitude goes out to Dr. James E. Graham for his support and guidance during this project. He has always been a valuable source of information and provided me with anything that I needed. Without his direction, I doubt this project would have been successful. I am very fortunate to be a member of his lab.

A great amount of thanks goes out to Dr. Mohammad A. Alzohairy for his work creating the recombinant Rv1985c strains, as well as his assistance with this project.

I want to extend my appreciation to my committee members Dr. Donald R. Demuth and Dr. David A. Scott for their time and valuable insight over the past two years.

I want to thank Terry P. Manning and the McLeish Laboratory for the donor cells used in all the macrophage experiments.

Finally, I want to thank the Microbiology & Immunology Department and Oral Biology Department for making the past two years an enjoyable experience and creating a wonderful place to learn.

ABSTRACT

CONTRIBUTION OF Rv1985c TO *MYCOBACTERIUM TUBERCULOSIS* INTRACELLULAR ADAPTATION

Nicholas B. Wantland

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Mycobacterium tuberculosis causes the often-fatal infectious disease Tuberculosis, and infects approximately one third of the world's population. Gaining a better understanding of how these bacteria regulate gene expression for intracellular survival will likely aid in developing ways to reduce disease. Studies of bacterial RNA expression in experimentally infected primary human macrophages indicated open reading frame Rv1985c, encoding a putative transcriptional regulator, may contribute to intracellular survival. Comparison of a strain constitutively expressing Rv1985c and the isogenic wild-type parent by array hybridization identified genes potentially regulated by Rv1985c. These included a group encoding factors likely relevant to cell wall remodeling,

particularly modification of mycolic acids. Interestingly, of the 22 upregulated genes identified by significance analysis of microarray, S.A.M., among the highest expressed were some of those previously described as part of the DosR/DevR dormancy regulon. We verified that *Rv1985c* mRNA levels rose dramatically in the first 24 hours following phagocytosis, and confirmed a lack of increased *dosR/devR* transcription in the constitutively expressing Rv1985c strain. Finally we showed that a Rv1985c knock out mutant while able to grow normally in laboratory broth had reduced ability to colonize cultured human macrophages, and showed reduced cytopathic effects. Restoring *Rv1985c* reversed this attenuation of the mutant.

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CHAPTER I

INTRODUCTION

M. tuberculosis:

Mycobacterium tuberculosis is the causal agent of the often-fatal infectious disease Tuberculosis. Even though a vast amount of information has been gathered on *M. tuberculosis,* fist isolated and characterized by Koch in 1882, it remains one of the largest and most widespread infectious diseases on earth. It is a non-motile, rod shaped, predominantly intracellular pathogen with an approximately 18-20 hour doubling time. The species is characterized as acid-fast bacteria due to its ability resist decolorization by acid-alcohol after staining with aniline dye. This characteristic is due to the high concentration of lipids embedded in its cell wall, including the wax-like mycolic acids. While there are numerous *M. tuberculosis* strains the best characterized is H37Rv. Its genome consists of 3,924 open reading frames (ORFs) and a 65% G + C content (Cole et al., 1998). These bacteria primarily infect the host

respiratory tract and initially infect resident alveolar macrophages.

Infection & Transmission:

The main portal of entry for *M. tuberculosis* is through the respiratory tract. Infection by *M. tuberculosis* usually begins with the inhalation of the bacteria in the form of airborne droplets. They must then travel through the respiratory tract to the lung where they are phagocytosed by host alveolar macrophages (Russell *et al.*, 2001). If falling on the epithelium of the upper airway the bacteria are swallowed and destroyed in the G.I. tract. If the macrophages have been previously activated they can eliminate the bacteria but if not the bacteria persist and replicate inside these host cells. Next a granuloma or tubercle is formed by the recruitment of mononuclear cells from the surrounding blood vessels. At this stage an effective T-cell response contains the bacteria in a microscopic granule and is known as the latent stage where there are no outward signs of disease, and there is no transmission of bacteria from the host to others (Russell *et al.*, 2001) During this stage the bacteria are thought to be less active or in a state of non-replicating persistence

(NPR). Due to changes in the host, HIV, old age, malnutrition, or other circumstances that alter the host's immune system, the infection can again become active. The bacteria then escape from growing granulomas that merge with airways, liquefy, and spill out into the bronchial passages. This leads to a cough that expels bacteria from the lung into the external environment spreading the bacteria to a new host (Russell *et al.*, 2001)

Epidemiology:

According to the World Health Organization, approximately one third of the world's population is infected with the *M. tuberculosis* and in 2009 it accounted for 1.7 million deaths worldwide. The highest rates of new infections are found in Africa, South-East Asia, and the East Mediterranean.

Recent globally increasing numbers of HIV infected persons has also led to an increase in cases of active tuberculosis. A person who is infected with HIV and *M. tuberculosis* is many more times likely to have active disease then someone without a HIV infection, as the virus targets CD4+ T-cells essential for the control of *M. tuberculosis* infection.

Gene Regulation:

During the course of infection *M. tuberculosis* encounters a wide range of environments all of which exert different pressures and stresses on the bacterial cell. Inside the host bacteria must be able to resist the actions of reactive nitrogen and oxygen species as well as lowered pH and potentially toxic free fatty acids. They must also withstand the antimicrobial action of alveolar surfactant as well as hypoxic conditions when inside a granuloma. Outside of the host, during transmission *M. tuberculosis* must then face other pressures such as UV light, low temperature, desiccation, and starvation (Manganelli *et al.*, 2004). The bacteria must have means to regulate expression of its large genome to survive in these diverse environments. Approximately 5% of H37Rv's genome, 190 genes, is predicted transcriptional regulators that facilitate this process.

Wayne Model Shiftdown:

Wayne *et al.*, 1996, showed that bacteria when abruptly transferred from vigorously aerated cultures to anaerobic conditions quickly died but when gradually shifted to these conditions showed increased tolerance and survival. These cells where said to be in a nonreplicating persistent

state. Upon aeration the bacteria began growing again. This effect was later described as being controlled by the DevR/DosR regulon.

DevR/DosR and Hypoxic Response:

The DevR/DosR regulon is known to be activated when the bacterial cell encounters hypoxic conditions as well as nitric oxide and carbon monoxide species (Park *et al*, 2003). This regulon consists of approximately 48 genes, with a core group of 4-6 genes that are found across all *Mycobacteria* (Gerasimova *et al.*, 2011), which arrest growth and put the cell in a dormant/drug tolerant state (Chauhan *et al.*, 2011).

Hypothesis:

Previous studies with experimentally infected Thp-1 cell line macrophages indicated open reading frame *Rv1985c*, which encodes a putative lysR transcriptional regulator (Zhou *et al.*, 2010), contributes to the survival of the bacteria after phagocytosis. Genes that are under the control of this transcriptional regulator will contribute the survival of the bacteria during phagocytosis host by macrophages.

CHAPTER II

MATERIALS AND METHODS

Mycobacterium Strains and Media:

M. tuberculosis wild type strain, H37Rv (ATCC #25618), was obtained from the American Type Culture collection. Dr. Mohammad A. Alzohairy created the *Rv1985c* over expressing mutant strain by expressing *Rv1985c* constitutively from the *M. bovis* hsp60 promoter. Alzohairy also created the *ΔRv1985c* knock-out mutant strain, as well as the complemented *ΔRv1985c* strain. All bacteria were grown from frozen stocks in 7H9 Middlebrook media enriched with oleic acid, albumin, dextrose, and catalase (OADC) 1% Tween-80 at 37°C with shaking at 200 rpm to mid-log density for all experiments.

RNA Purification:

RNA purification first began by growing *M. tuberculosis* strains to mid-log phase (0.2 OD at 600nm) as described. For larger RNA preparations, 20ml of mid-log culture was spun down in multiple 1.5mL micro-centrifuge tubes in a refrigerated centrifuge, -4°C, at maximum speed

for 10 minutes. Making sure to keep the bacteria on ice the supernatant was removed and discarded. The bacterial pellets were lysed with RTL buffer with a small amount of fresh dithiothreitol, DTT, powder added. The lysed bacteria were subsequently added to bead beater tubes containing zirconia-silica beads along with 5uL of phenol as a disinfectant. The tubes were sealed with parafilm and heated @70°C for 1 minute; then placed into an ice bath and allowed to cool. Next the tubes were placed in a Forma FP120 bead beater to mechanically lyse the bacteria. The samples were agitated for four twenty second cycles at a maximum speed of six. Samples were then placed in an ice bath for 5 minutes in between bead beater cycles to prevent over heating. The bead beater tubes containing the samples were centrifuged at low speed for one minute to settle the zirconia-silica beads to the bottom of the tube allowing for easy removal of the RNA containing liquid. The liquid was removed and placed in a 2mL centrifuge tube containing and equal volume of 100% ethanol. The samples were purified using Qiagen RNAeasy kit following manufacturer's instructions. The purified RNA now in nuclease free water was precipitated overnight at -20°C using 2x volume 5M LiCl.

DNase Treatment:

Centrifuging the sample containing LiCl precipitated RNA at maximum speed for 10 minutes to form a RNA pellet. The RNA pellet was washed twice with 70% ethanol and allowed to air dry. Then the pellet was resuspended in 200uL cold H_2O. To the resuspended RNA 20uL of 10x DNase buffer was added along with 10uL Turbo DNase (Invitrogen). The sample was heated at 37°C for thirty minutes. To neutralize the reaction 20uL of EDTA was added and the sample heated at 75°C for 5 minutes. Following DNase treatment the sample was distributed in 5ug aliquots and precipitated with 2x volume 95% ethanol and 0.75x volume NH_4OAc. The samples were stored as precipitates at -20°C.

cDNA synthesis:

Approximately 2.5-5ug of precipitated DNA free RNA in a nuclease free micro-centrifuge tube was centrifuged and washed twice with 70% ethanol. The ethanol was then removed and the pellet allowed to air dry or 3 minutes at 37°C. To the RNA 1uL of random decamers primers were added along with 1uL of 10mM 4 dNTP mix and 13uL of H_2O. This mixture was heated at 65°C for five minutes and then allowed to incubate on ice for one minute. Contents of the tube were collected by centrifugation. Next 4uL of a 5x first strand DNA buffer (Invitrogen) was added along with 1uL 0.1M DTT,

1uL RNAse-out (Invitrogen), and 1uL superscript III (Invitrogen). The contents of the tube was mixed by pipetting the solution up and down. After that, the solution was allowed to incubate at room temperature for five minutes before being placed in a heat block at 50°C for 90 minutes. Finally, heating at 70°C for five minutes inactivated the reaction. The newly created cDNA was stored at -20°C for future use in RT-qPCR.

Array Hybridization:

Approximately 25ug of DNA free RNA was spun down for 10 minutes at maximum speed in a 1.5mL centrifuge tube. The RNA pellet was washed twice with 70% ethanol and allowed to dry for 3 minutes. 55uL of nuclease free water with 0.1mM EDTA was used to dissolve the pellet. Next 5uL decamers, 5 uL of 10mM dATP, dGTP, dTTP mix, 5uL 0.1mM dCTP, and 6.25uL p33 labeled dCTP was added to the dissolved RNA and the contents was heated at 65°C for 5 minutes; then allowed to incubate on ice for one minute. The content of the tube was collected by centrifugation; 20uL of 5x first strand buffer was added followed by 5ul of DTT o.1M, 5uL RNAse out, and 5uL superscript III. The reaction was allowed to incubate at room temperature for 5 minutes followed by incubation at 50°C for 90 minutes.

Heating the mixture at 70°C for 5 minutes inactivated the reaction.

Next a Sephadex G50 column was prepared by putting glass wool in the bottom of a syringe and then filling the syringe with G50 making sure there are no bubbles in the column. Next a few drops of 0.1M NaCl was added to the column to make sure it is functioning properly. The radio labeled cDNA is then run through the column and drops are collected into micro-centrifuge tubes. Approximately 3 drops are collected per tube and around 15 tubes are necessary to completely run the sample. The radioactivity of the tubes is then measured. If the cDNA synthesis worked properly you will have one large peak of radioactive tubes. The three tubes with the highest radioactivity were combined and NaOH was added so that the final concentration of the solution 0.4M NaOH. Probes were heated to 55°C for 15 minutes to hydrolyze RNA template. The probe was then added to a glass tube containing the nylon array and hybridization solution consisting of 0.5% SDS, 6x SSC, 5x Denhard's solution, and 100ug/mL salmon DNA. The array was allowed to hybridize overnight in a rotating incubator at a temperature of 65°C.

Following the overnight incubation the array is then washed with a mixture of 0.1XSSC & SDS. After washing the

array was then placed on blotting paper and wrapped in plastic wrap. The array is exposed to a phosphorimaging plate, Fuji Film, for 24 hours before it is read by a phosphorimager and analyzed using Array Vision Software.

Quantitative real-time PCR analysis (RT-qPCR):

Primers were selected for *sigA, sigE, Rv1985c, Rv0195, fdxA, hspX, devRS & devR* by Primer 3 software (Table 1) and tested for equal efficiency relative to standard *sigA* primers using dilutions of genomic DNA template. Samples were analyzed utilizing a DNA Opticon 2 Monitor (MJ Research) and SYBR Green (Finnzymes) for RT-qPCR.

Culturing of Macrophages:

Peripheral blood mononuclear cells, PBMCs, were obtained from Terry Manning of the Mcleish Laboratory. They were centrifuged at 1,600 RPM for five minutes at room temperature in a 50mL conical centrifuge tube to pellet the cells. The supernatant was removed and the cells were gently washed with 20mL of phosphate buffered saline, PBS. Again the cells were centrifuged and washed with 20 mL of PBS. Next the PBMCs were under laid with 1mL of heat inactivated fetal bovine serum, FBS, and left to sit for five minutes undisturbed as to allow any clumps to settle to the bottom of the tube. The top layer containing the PBMCs was removed, making sure to not disturb the serum

Gene	Sequences
Rv1985c	Forward-5' AGT CAC GCC CCG AAA CTG -3'
	Reverse-5' TTC TCG GGG AAC ATG CCC CAT C -3
Rv0195	Forward-5' AGT CAC GCC CCG AAA CTG -3'
	Reverse-5' CTC TGG CTG CGA TAC AAC AA -3'
sigE	Forward-5' ACG ATG CCG AAG ACC TGA -3'
	Reverse-5' TGG TGG TGA TGC GGT GTA -3'
devR	Forward-5' CAT CAA GGG AAT GGA GTT GG -3'
	Reverse-5' AAC CGC GAC ACG TAG TTC TT -3'
devRS	Forward-5' ATC GAA TTC TCG CCG ACC GAA TGT TCC TA -3'
	Reverse-5' ATC GGT ACC CGT AGT TGG GAG AGC GTG TG -3'
fdxA	Forward-5' CGT GGA TGT GAT GGA CAA GT -3'
	Reverse-5' GCA GGA CTT GGT GGA AAA AG -3'
hspX	Forward-5' CGA CAA GGA CGT CGA CAT TA -3'
	Reverse-5' CCG GAT CTG AAT GTG CTT TT -3'

Table 1. Table of primers used for RT-qPCR measurements.

layer, and placed in a new sterile centrifuge tube. The cells were once again centrifuged and the supernatant was removed. The PBMCs are then brought up in 15ml of RPMI 1640 containing, additional L-glutamine, 0.5% nonessential amino acids, 1% Hepes, and 5% heat inactivated FBS. The concentration of the cells was measured using trypan blue and a hemocytmeter and adjusted to $2x10^6$ cells/mL by adding additional media. The cells were then placed in a Teflon bowl and allowed to sit undisturbed in a 37°C incubator for 5 days. After the five-day incubation the cells and media were removed and placed in 50mL conical centrifuge tube. Prewarmed PBS with 2% FBS is used to help wash the cells out of the Teflon bowl. A cell scraper was also used to help remove any adherent cells. The cells were spun down in a centrifuge at 1,600 RMP for 5 minutes. The supernatant was then removed and the cells were washed with 20mL of PBS containing 2% FBS. Then the cells were brought up in the same RPMI media as before to a density of $5x10^6$/mL. One mL of media containing cells was added to each well of a chamber slide, for microscopic analysis, and 4mL for each well in a six well plate, for RNA time course experiments. Allowing the cells to adhere for two days before removing non-adherent cells and washing with RPMI

media. Next the media was replaced and the cells were ready for infection with *M. tuberculosis*.

Cell Culture Infection Model:

Bacteria were grown as shaking cultures as described above from frozen stocks. Before the infection the bacteria were placed in a 15mL centrifuge tube along with a small amount of zirconia-silica beads and vortexed gently to disrupt clumps. The bacteria were then centrifuged at 300rmp with a 4in rotor (Beckman) for 3 minutes to pellet any undisrupted clumps of bacteria leaving a single cell suspension. The bacteria were opsonized by incubation with an equal volume of non-inactivated FBS for 20 minutes before dilutions were made in RPMI with 2% FBS. Infections were carried out with a one to one MOI.

Kinyoun Staining Procedure:

Infection media was first removed followed by a wash with phosphate buffered saline. Each well of the chamber slide was then fixed with 0.5ml of a 3% formaldehyde solution and allowed to set for thirty minutes. After the thirty minute fixation the formaldehyde solution was removed and replaced with 0.5 ml of cold methanol that had been placed in a -80°C freezer for at least thirty minutes before use. The methanol was then allowed to sit on the slides for fifteen minutes. After the fifteen-minute soak

the methanol was removed and the slides were allowed to air-dry before the upper portion of the chamber slide was removed. At this point the slide was heated at 75°C for thirty minutes to ensure the monolayer was "baked on" to the slide. The slide was then ready to be stained and must be done so as soon as possible to achieve the best staining results. If it had been a long period of time between the slide fixation and staining the slides were re-soaked in cold methanol and heat fixed before continuing with the staining.

The slides were then saturated with Kinyoun Carbol Fuchsin stain consisting of 1g basic fuchsin, 2g phenol crystals, 5ml 95% ethanol, & 25mL of H20 for 20 minutes to stain the bacteria. The slides were washed with 95% ethanol, DIFCO's TB Quick Stain Reagent B, and H_20 until the wash water ran clear. Next the slides were stained with 1% w/v methylene blue for 20 min to stain the macrophage monolayer. The slides were rinsed with water to remove excess stain and allowed to air dry before being viewed under the microscope.

Harvesting of *M. tuberculosis* from macrophage monolayers:

The six well plates that contained the infected macrophage monolayers were removed from the incubator and placed on ice. The media was removed and the monolayers

washed twice with prewarmed PBS before being lysed with 1% Triton X-100. The contents of the well, which contained bacteria, Triton X-100 and macrophage cell debris was collected in a 1.5mL micro-centrifuge and spun at very low speed in a refrigerated centrifuge, 0°C, for 10 minutes. This step pellets the macrophage debris while still leaving the bacteria in suspension. The supernatant was removed to a new centrifuge tube that was then spun at maximum speed in the same centrifuge for 10 minutes to pellet the bacteria. The liquid on top of the bacterial pellet was discarded and the bacteria lysed as described previously in the RNA purification procedure.

Biohazards and laboratory safety:

M. tuberculosis is a Class 3 biohazardous agent. All experiments with live *M. tuberculosis* were performed in the certified BSL3 facility of Dr. James E. Graham in the Department of Microbiology and Immunology at the University of Louisville. Nicholas B. Wantland was trained in the relevant safety practices.

CHAPTER III

RESULTS

Improved RNA Purification:

Obtaining pure stable RNA is essential for achieving proper results when performing array hybridizations as well as with RT-qPCR. I tried various methods to determine which would yield the highest quality RNA. After numerous trials the best method involved lysis with RLT buffer (Qiagen RNeasy mini kit) with DTT added, followed by "bead beating" with silica–zirconium beads and the use of silica column binding. The captured RNA was then precipitated overnight with LiCl at -20°C before undergoing a DNAse treatment and second precipitation with ethanol–ammonium acetate (Figure 1).

Microscopic Analysis of *Rv1985c*:

Separate macrophage monolayer cultures (Figure 2) were infected with H37Rv, Δ*Rv1985c*, & Δ*Rv1985c* + *Rv1985c* strains at a 1:1 MOI and examined at 24, 48, 72, and 96 hours post infection. While all macrophage cultures were equally inoculated amounts the Δ*Rv1985c* mutant showed a significant decrease in its uptake and early survival (Figure 3)

indicating *Rv1985c* plays an important role in the early infection process. Plating of the inocula media and washes showed very few colonies, suggesting more than 90% of the inocula bacteria were phagocytosed and destroyed. Factors contributing to survival are therefore likely needed during this initial infection in this model.

There was also a substantial loss in the macrophage monolayer over the course of 96 hours in the wild type and the complemented Δ*Rv1985c* (Figure 4). This loss in monolayer can be attributed to the amount of bacteria that infected and survived in the macrophages during the first 18 hours of infection as well as the subsequent growth of the bacteria as seen by the average bacteria per cell (Figure 5). *Rv1985c* showed a contribution to both aspects of colonization.

RT-qPCR Analysis of *Rv1985c* during macrophage infection:

Cultured primary human macrophages were infected with H37Rv and total RNA was collected at time points 0, 4, 24 hours post infection. cDNAs prepared from the isolated RNA was then analyzed by RT-PCR as described in Methods. The results showed an increase in *Rv1985c* mRNA levels after the first 24 hours post infection by approximately 15 fold over its expression in bacteria growing at mid-log in broth (Figure 6). This identifies *Rv1985c* as being expressed

early after phagocytosis by resting primary human macrophages and suggests it may play a role in adaptation to this dynamic environment.

Array Hybridization *Rv1985c*:

During the course of infection *M. tuberculosis* encounters a wide range of environments and external stimuli. In response to these stimuli the bacterial transcriptome must be able to adapt and respond to ensure bacterial survival. Through previous work, done by Dr. Mohammad Alzohairy, the transcriptional regulator *Rv1985c* was identified as belonging to a regulatory cascade initiated by the CpsA. Transcriptional regulator CpsA has been shown previously to be important for the pathogenesis of *M. tuberculosis* and, *Rv1985c* is one of only two transcriptional regulators absent from Bacille Calmette Guerin, BCG. I therefore attempted to identify RNAs whose expression was controlled by this transcriptional regulator.

The constitutively *Rv1985c* expressing strain (*Rv1985c+*) was first analyzed to determine its increase in *Rv1985c* mRNA levels. RT-qPCR indicated that the *Rv1985c* constitutively expressing strain showed an approximately two-fold increase in its *Rv1985c* mRNA level over that H37Rv wild type strain (Figure 7). This small increase was

however significant as determined by Rest software (Pfaffl *et al.*, 2002). The constitutively expressing strain was then grown to mid-log density and the RNA collected and purified. Approximately 25ug of DNAse treated RNA was the converted to radiolabeled cDNA and used for array analysis as described in the Methods section. The *Rv1985c* strain array hybridization was performed in triplicate and compared to three existing H37Rv mid-log broth arrays.

To compare different arrays (Figure 8) with different overall levels of hybridization, we determined the mean intensity for the 16 genomic DNA spots on each array (at corners and on mid-line). The individual spot intensity values for each array were then normalized by a ratio of its genomic spots to the average intensity for all genomic spots on all of the arrays compared. Analysis with BRB Array Tools, S.A.M., also applies a median normalization, where an algorithm chooses a reference array among all compared whose own median hybridization intensity signal is median among those compared. Individual gene intensities on each array are then normalized by subtracting the median difference among all features relative to the reference array. There were 30 ORFs determined to be significantly (Table 2) different between H37Rv and *Rv1985c+* by significance analysis of microarray, S.A.M., with a 90th

percentile confidence and the false discovery rate among the 30 significant ORFs set to 0.10; with 22 ORFs having increased expression and eight with decreased.

Of the 22 ORFs whose hybridization increased interestingly the largest increases were for *Rv1738*, *fdxA*, *Rv3131*, & *hspx/acr*. All of these have been previously described as being part of the DosR/DevR dormancy regulon (Voskuil 2003). Alpha-crystallin protein, Acr, encoded by *acr* has been shown to be expressed highly during hypoxic conditions, stationary growth phase as well as in nitric oxide enriched environments (Vasudeva-Rao *et al.*, 2008). A member of the small heat shock protein family, Acr forms high-molecular weight aggregates as well as having chaperone activity in vitro (Park *et al.*, 2003). *fdxA, which encodes ferredoxin A,* is involved in an alternate pathway for electron transport (Voskuil *et al.*, 2003). *Rv1738* & *Rv3131* encode conserved hypothetical proteins with no known function. Also noteworthy was the increased mRNA levels of *desA2* (acyl-desaturase), *desA3* (linoleoyl-CoA desaturase), and *fbpA* (mycolyl-transferase, mediating permeability) as well as a decrease *fabB* (malonyl CoA-acyl carrier protein), *fbpC2* (mycolyl-transferase), and *acpM* (meromycolate extension acyl carrier, involved in mycolic acid synthesis). We verified the increased fdxA and

hspX/acr mRNA levels by RT-qPCR and confirmed the lack of increased *dosR/devR* transcription in the constitutively expressing *Rv1985c* strain (Figure 7).

Figure 1.

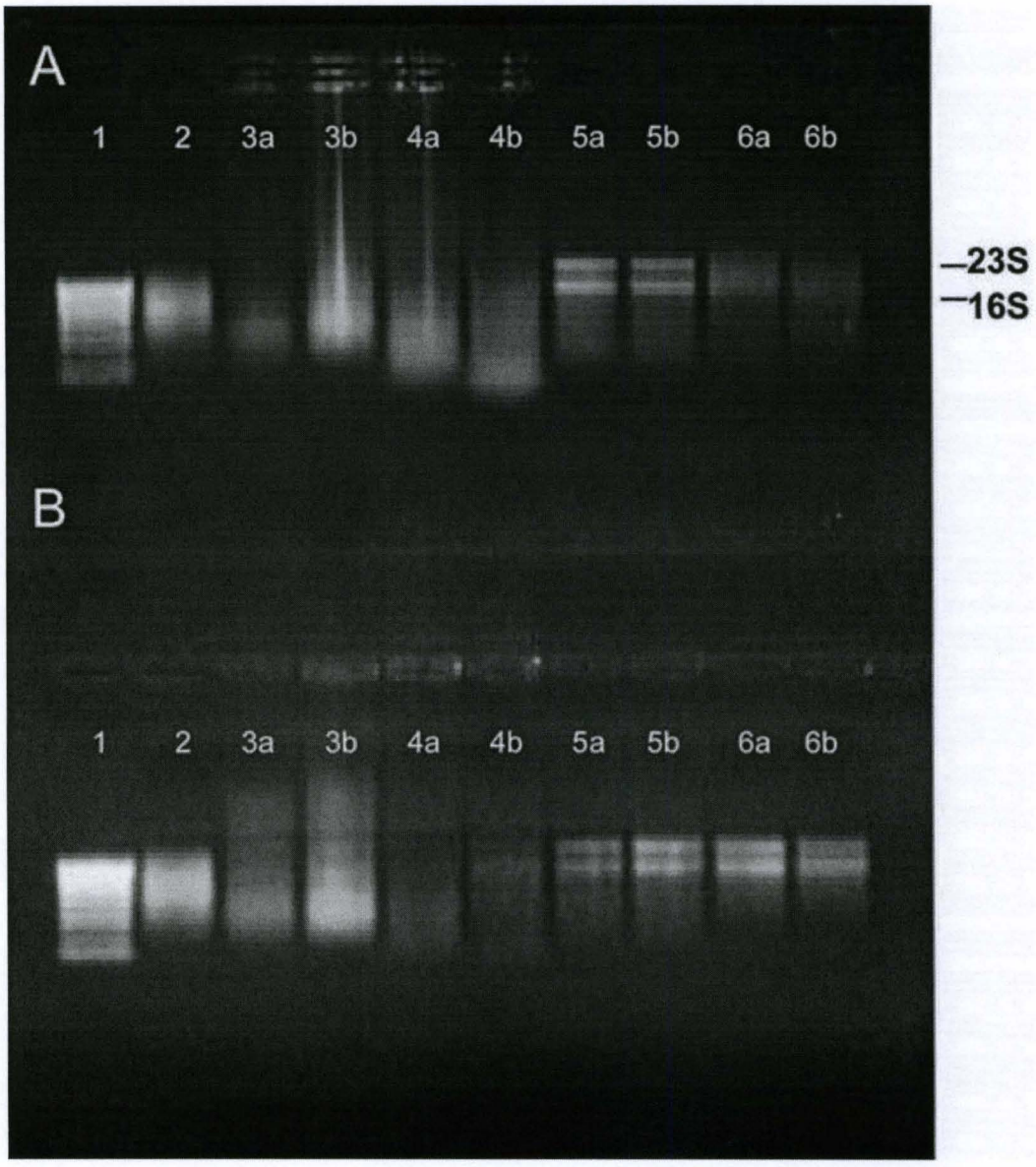
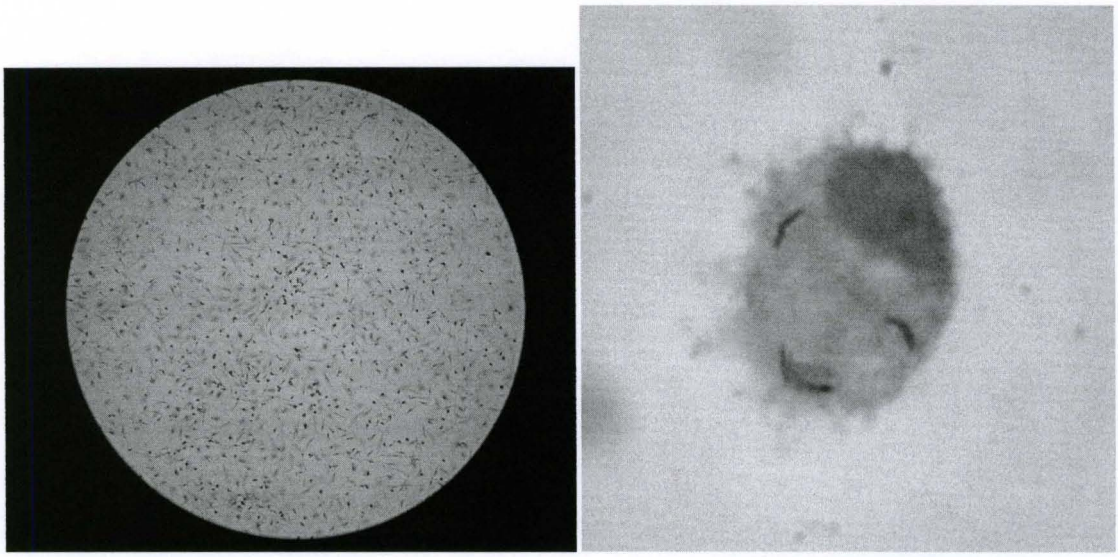


Figure 1. The figure shows RNA samples run on a nondenaturing agarose gel. Panel A lane one depicts a standard DNA ladder, lane 2 a bacteriophage MS2 RNA standard, lanes 3a & 3b duplicate phenol-chloroform extractions, lanes 4a & 4b phenol-chloroform extractions with DNase treatment, lanes 5a & 5b purification only by silica column binding, lanes 6a & 6b silica column binding with DNase treatment. Panel B shows parallel samples as listed above but after an additional precipitation by ethanol-ammonium acetate. RNA used for research described is shown in lanes 6a and 6b.

Figure 2.

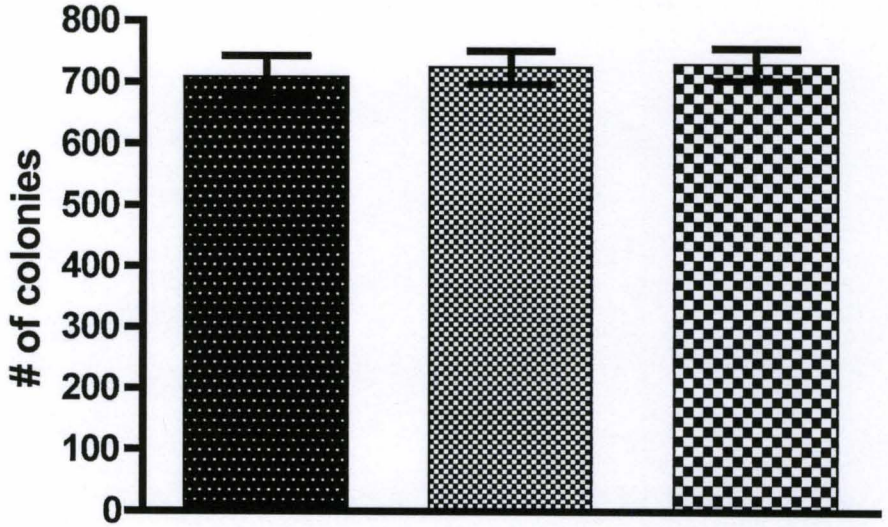


A **B**

Figure 2. Human PBMC infection model. "Panel A" shows a representative primary human macrophage monolayer prior to infection. "Panel B" shows a cold Kinyoun acid-fast staining of a single H37Rv-infected macrophage at 48 hours post infection.

Figure 3.

A


800
700
600
500
400
300
200
100
0
# of colonies

B

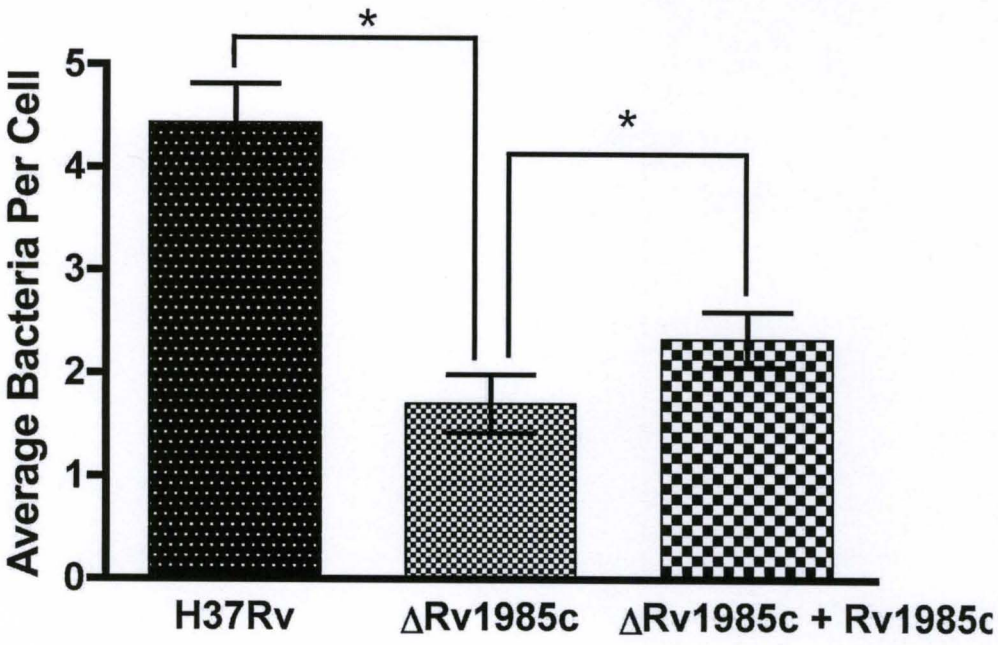

*
*
5
4
3
2
1
0
Average Bacteria Per Cell
H37Rv
ΔRv1985c
ΔRv1985c + Rv1985c

Figure 3. *Rv1985c* contributes to uptake and early intracellular survival. A) Shows similar numbers of mutant and isogenic H37Rv that were used as inoculum in the PBMC infection model described in Methods. Error bars show standard errors of mean plate counts from four independent dilutions of each inoculum. B) Δ*Rv1985c* showed a significant reduction in uptake up and survival at 18 hours in primary human macrophages $p < 0.005$. The complemented strain showed incomplete restoration in intracellular survival at 18 hours. Error bars show standard errors of mean bacterial counts.

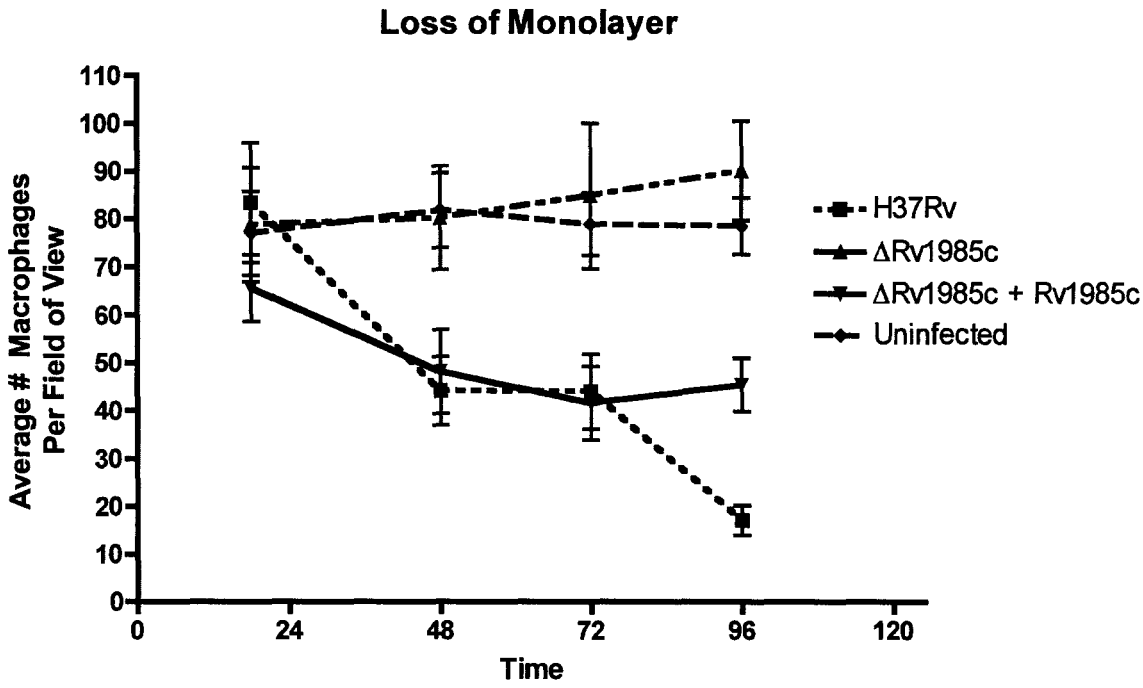


Figure 4. Cells infected by the *Rv1985c* mutant show better survival in the infection model. The reduced ability of the *Rv1985c* mutant to colonize cells and subsequent decreased growth rate resulted in improved survival of macrophage monolayers in the infection model. Restoring *Rv1985c* reversed this attenuation of the mutant. Error bars show standard errors of mean cell counts.

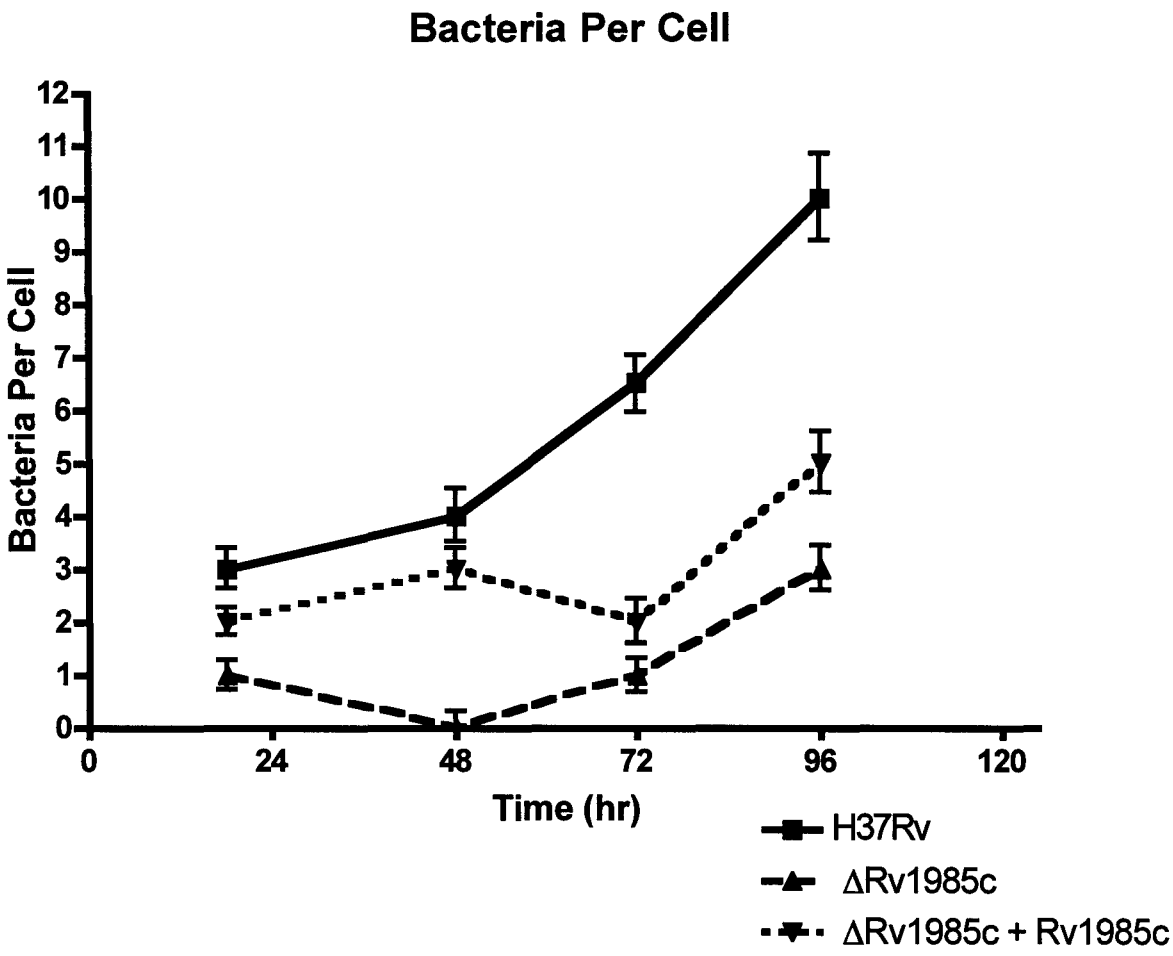


Figure 5. *Rv1985c* contributes to intracellular growth. The Figure shows the average bacteria per cell as determined by acid-fast staining and microscopy at the indicated time points. Error bars show standard errors of mean bacterial counts.

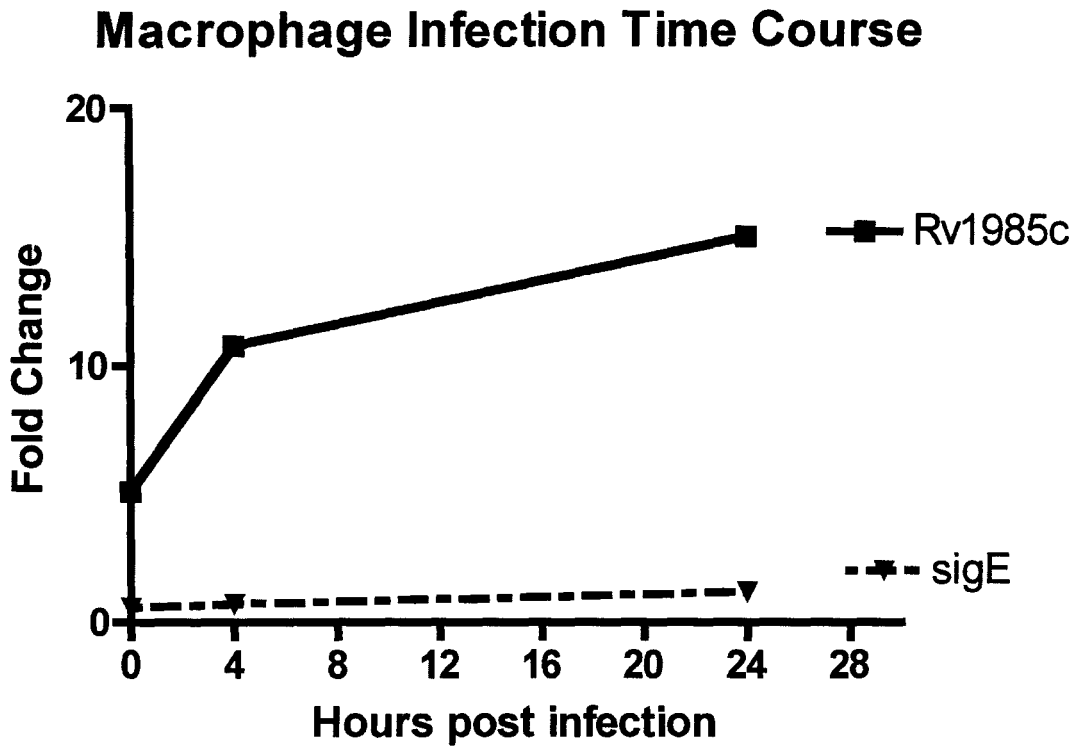


Figure 6. *Rv1985c* mRNA levels increase on phagocytosis. The Figure shows the results of RT-qPCR analysis of *M. tuberculosis* mRNA levels during the early time course of primary human macrophage infection. Changes in mRNA levels were determined by normalizing to *sigA* mRNA levels, and are shown relative to those in mid-log phase broth cultures. Changes in mRNA levels other than for *sigE* were determined to be significant by the REST software tool ($p < 0.05$) (Pfaffle, *et al.*, 2005)

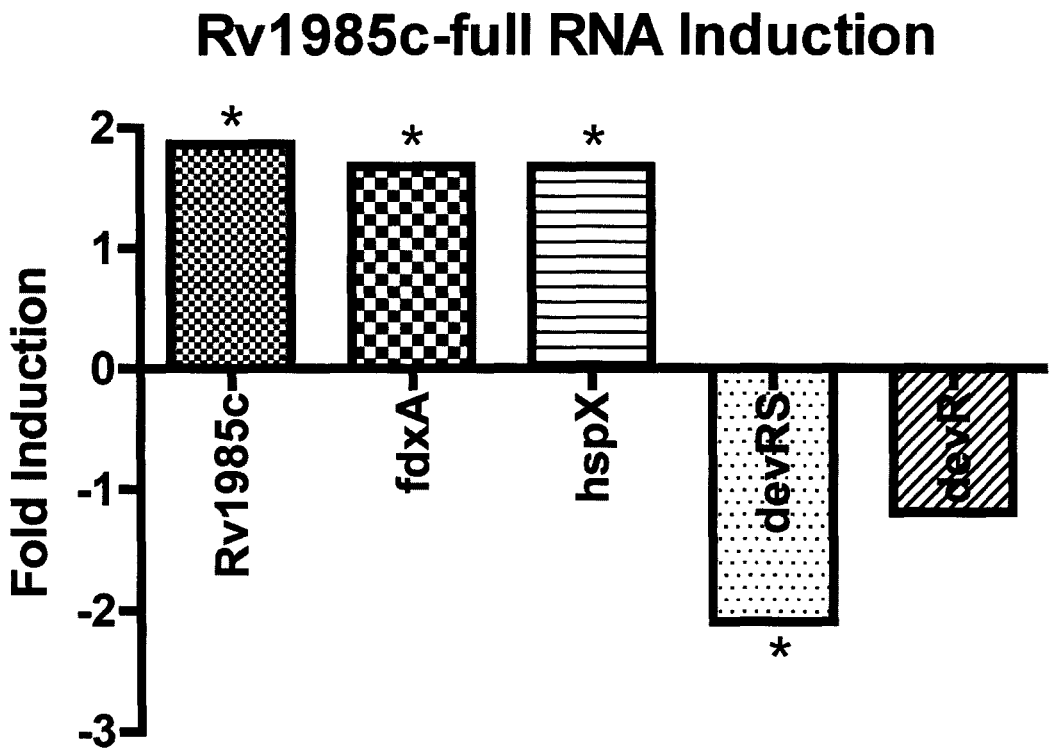


Figure 7. Changes in mRNA levels with constitutively expressed *Rv1985c*. The figure shows changes in relative mRNA steady-state levels for the indicated mRNAs relative to *sigA*. mRNAs levels were measured by RT-qPCR as described in Methods. Changes except for *devR* were determined to be statistically significant with a p-value of at least 0.005, by REST software (Pfaffl *et al.*, 2002).

Figure 8.

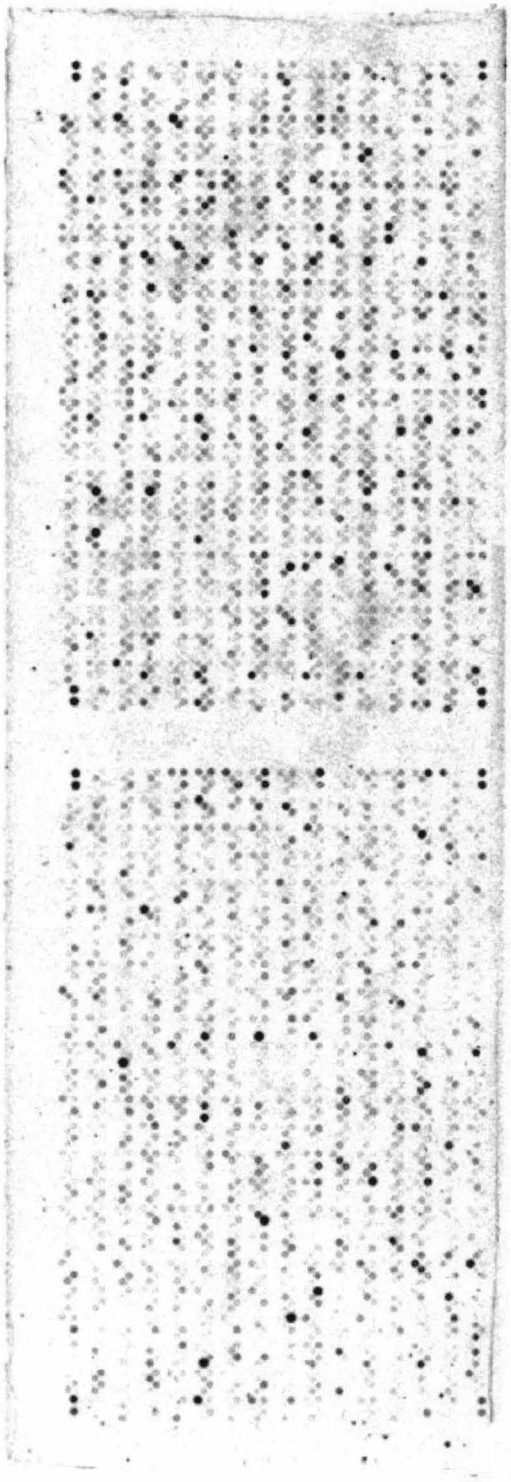


Figure 8. Representative array hybridization image. The array shown is hybridized with a 1st-strand cDNA probe prepared from H37Rv constitutively expressing *Rv1985c* grown to mid log in Middlebrook 7H9 OADC as described in Methods.

mRNA	annotation	H37Rv	+ *Rv1985c*	fold-change
*Rv1738**	conserved hypothetical protein	10	164	16
*fdxA**	ferredoxin	7	91	13
*Rv3131**	conserved hypothetical protein	10	87	9
*hspX/acr**	heat shock protein	8	68	8
Rv0872c	PE_PGRS-15 glycine rich protein	6	50	8
Rv1059	conserved hypothetical protein	6	44	7
Rv3031	conserved hypothetical protein	14	93	7
hrp1	putative inosine phosphate dehydrogenase	10	64	6
Rv0109	PE_PGRS-1 glycine rich protein	10	61	6
bfrB	bacterioferritin	26	144	6
*Rv0079**	hypothetical protein	8	46	5
Rv0829	is 6110' transposase	33	170	5
ltp3	lipid carrier protein	16	81	5
Rv3489	hypothetical protein	45	219	5
rplC	ribosomal protein L3	53	242	5
Rv0047c	conserved hypothetical protein	9	40	5
nusE	transcription elongation factor	86	377	4
whiB1	transcriptional regulatory protein	38	165	4
desA3	linoleoyl-coadesaturase	9	37	4
fbpA	mycolyl transferase	107	435	4
desA2	acyldesaturase	21	82	4
Rv0195	transcriptional regulatory protein	15	54	4
Rv0232	transcriptional regulatory protein	24	6	- 4
Rv2407	conserved hypothetical protein	26	6	- 4
fbpC2	mycolyl transferase	33	7	- 5
fabD	malonylcoa-acyl carrier protein	32	6	- 5
groES	10k Da chaperonin	135	23	- 6
acpM	meromycolate extension acyl carrier	261	35	- 8
Rv3388	pe-pgrs family protien	243	25	- 10
lppQ	conserved lipoprotein	84	6	- 14

Table 2: List of mRNAs whose levels were significantly changed by constitutive *Rv1985c* expression. We used S.A.M. (significance analysis of microarrays) with 90% confidence and a false discovery rate set to 0.1 to identify differentially expressed genes. Asterisk indicates members of the previously described DosR/DevR regulon (Voskuil el, 2009).

CHAPTER IV

DISSCUSSION

Desaturases and Mycolic Acid Modification:

Mycolic acids are beta-hydroxy fatty acids with a long alpha-alkyl side chain (60- 80C) found in the cell wall of *M. tuberculosis* (Takayama *et al.*, 2005). Mycolic acids can help the bacteria resist chemical and oxidative damage and help the bacteria survive inside host macrophages (Todar, 2011). There are only three acyl-desaturases in the H37Rv genome DesA1, DesA2, and DesA3. Acyl-desaturase, *desA2,* works outside the cell by introducing double bonds to mycolic acid precursors altering side chains. Interestingly desA2 requires a ferredoxin to transfer electrons from NADH or NADPH for its bond forming activity. While no specific ferredoxin has been identified in this process FdxA has been shown to be a potential candidate (Chang, 2009). We also saw an increase in *bfrB* mRNA levels, whose encoded product a bacterioferritin, may provide iron necessary for FdxA synthesis and activity.

DesA3 (linoleoyl-CoA desaturase) is involved in the production of a major component of membrane phospholipids and triglycerides, oleic acid. It is also considered one

of approximately 200 genes necessary for survival of *M. tuberculosis* inside a granuloma (Chang, 2009). Its role in this process, like that of *desA2,* is centered around modification of the cell membrane and wall. Also, it is worthy to note that desaturases, DesA2 and DesA3 are short-lived enzymes with relatively unstable mRNA and under constant transcriptional control (Chang, 2009).

Finally, the decrease in expression of mRNA of *fabB* (malonyl CoA-acyl carrier protein) and *acpM* (meromycolate extension acyl carrier), which are essential in the synthesis of mycolic acids (Barry *et al.*, 1998) coupled with the increase in *desA2* transcription suggests a switch from mycolic acid synthesis to the remolding of existing mycolic acids.

***fbpA* and *fbpC2*:**

FbpA and FbpC2 are two of three mycolyl-transferase paralogs long considered the *M. tuberculosis* "antigen 85 complex". Their role consists of transfer of mycolates to arabinogalactans on the exterior of the cell wall required for maintaining the integrity of the bacterial cell envelope (Puech *et al.*, 2002). By constitutively expressing *Rv1985c,* the steady state mRNA level of *fbpA* mRNA goes up while the steady state level of *fpbC2* goes down. According to Puech *et al,* 2002 a decrease in the

expression of *fpbC2* can lead to increased cell permeability. Modifications to the cell wall can lead to increased permeability for new nutrients or decreased to avoid host factors. Paralogs may perform slightly different activities but are particularly useful for fine tuning regulated expression from alternate promoters under conditions of environmental signals.

Alpha-crystallin:

Alpha-crystallin has been shown to be required for *M. tuberculosis* to grow optimally within macrophages. Also, during bacterial stress Acr localizes to the cell wall and membrane (Quinn *et al.*, 2002). It may also act on other proteins to aid in maintaining stability (Quinn *et al.*, 2002). The location and activity suggests a protective role of alpha-crystallin not only for the cell as a whole but potentially for mycolic acid modification going on outside the cell.

Rv1985c may bind dnaA:

In a late 2010 paper Zhou *et al.* determined the crystal structure of Rv1985c protein and showed that it can bind to DNA and prevent helix opening at oriC in vitro; thus preventing replication. In vivo they speculate that *Rv1985c* may have a dual role, and act as a marker of physiological circumstance by activating dnaA during times

of good nutrition to accelerate growth or conversely inhibit chromosomal replication during non-favorable circumstances (Zhou *et al.*, 2010). I did not see any reduced growth with constitutive *Rv1985c* expression. Further experiments will be needed to confirm this proposed activity and determine if it requires additional factors not present at mid-log phase in laboratory cultures

Increased *Rv1985c* expression in response to Phagocytosis:

Rv1985c is highly expressed during the first 24 hours following phagocytosis by primary human macrophages. This timing of expression coincides with a drastic change in the environment when there would need to be a change in the mycolic acids on the outside of the cell as well as increasing the cells ability to deal with a more stressful environment. The specific mechanisms suggested by the RNA expression and mutant studies described will need to be characterized by directed experiments.

Future Experiments:

Future experiments will include the verification of the increase in expression of desA2 & desA3 as well as the decrease in expression of fabB and acpM by RT-qPCR. Also I will attempt to demonstrate the modification of mycolic acid by solvent extraction and mass spectronomy and by a neutral-red cytochemical reaction. Finally, chromatin

immunoprecipitation assay, ChIP, of *Rv1985c* will be utilized to confirm the binding sites of *Rv1985c* to the DNA operator regions and determine direct and indirect roles in transcriptional regulation during intracellular adaptation

Conclusion:

The aforementioned studies show a contribution of *Rv1985c* to the intracellular adaptation of *M. tuberculosis.* They indicate modifications to mycolic acids and the cell membrane of the bacteria that may contribute to the ability to survive and grow in primary human macrophages.

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