AN ABSTRACT OF THE THESIS OF

Rachel Tennant for the degree of Master of Science in Microbiology presented on March 4, 2005.

Title: *Mycobacterium avium* genes upregulated upon infection of *Acanthamoeba castellanii* demonstrate a common response to the intracellular environment.

Abstract approved: Redacted for privacy

Luiz E. Bermudez

*Mycobacterium avium* is a ubiquitous environmental organism found in both water and soil. It can cause disease in patients with previous pulmonary conditions, as well as immunosuppressed patients, with the most prevalent being AIDS patients. Studies have indicated that passage through amoeba, a common environmental protozoa, increases virulence of *M. avium*. Using an *M. avium* GFP-promoter library in *M. smegmatis*, and real-time PCR, this study seeks to examine genes expressed during amoeba infection.

First, *M. avium* and *M. smegmatis* survivability in *Acanthamoeba castellanii* were determined. It was found that both species were able to infect and multiply within amoeba for at least 4 days.

After screening the GFP-promoter library using *A. castellanii*, it was determined that 20 genes were expressed at various timepoints. These included
genes involved in metabolic pathways, protein transcription and translation, and macromolecule degradation. Eight of these genes were also found to be upregulated in macrophage infection.

Finally, real-time PCR was used to confirm expression of five chosen genes. Genes were found to be upregulated by at least two-fold.

Through these studies we have determined that the *M. avium* GFP-promoter library is an effective tool for studying gene upregulation. We have also determined at least some of the genes used by *M. avium* during amoeba infection, as well as the ones that are shared in macrophage infection.
Mycobacterium avium Genes Upregulated Upon Infection of Acanthamoeba castellani
Demonstrate a Common Response to the Intracellular Environment

by
Rachel C. Tennant

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Redacted for privacy

Major Professor, representing Microbiology

Redacted for privacy

Chair of the Department of Microbiology

Redacted for privacy

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Rachel C. Tennant, Author
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Mycobacterium avium Genes Upregulated Upon Infection of Acanthamoeba castellanii Demonstrate a Common Response to the Intracellular Environment

Introduction

Mycobacteria are a group of gram-positive acid-fast organisms capable of inhabiting a wide range of environments. Some of these organisms are pathogens, having the ability to infect humans and animals. Until recently, environmental mycobacteria were not considered to be of great importance. However, emerging evidence has shown that pathogenic environmental mycobacteria are being implicated in a variety of diseases, especially in immunosuppressed individuals.

Environmental mycobacterial diseases

Mycobacteria are widespread in the environment, and there are several different species causing a variety of diseases. These range from gastrointestinal infections to skin and soft tissue infections to disseminated infection.

Waterborne mycobacterial species causing disease can include Mycobacterium marinum and Mycobacterium chelonae, which are associated with skin granulomas acquired via waterborne exposure (swimming pools and fish tanks), and Mycobacterium kansasii, which is associated with pulmonary disease. There is also an increasing link between Mycobacterium avium ssp. paratuberculosis and Crohn’s disease, which is a chronic inflammatory condition in the intestinal tract. However, the environmental mycobacteria that draws the most interest is Mycobacterium avium complex or MAC. MAC will be the focus of this study.
\textit{Mycobacterium avium} complex is composed of \textit{M. avium ssp. avium} and \textit{Mycobacterium intercellulare}. This complex is responsible for most cases of non-tuberculosis mycobacterial infections in developed countries (Horsburgh, 1996). MAC infections can occur in children, the elderly, patients who are immunosuppressed, and patients with pre-existing pulmonary disease, including people with conditions such as silicosis, pneumoconiosis, emphysema, bronchiectasis and cystic fibrosis (Falkinham, 2002). It is also often found in co-infections with \textit{M. tuberculosis} and \textit{Pneumocystis carinii} (Raju and Schluger, 2000). \textit{M. avium} is the most common species isolated from AIDS patients (Guthertz et al., 1989) and can cause bacteremia and disseminated multiorgan bacterial disease, as well as pulmonary infections in these individuals (Crowle et al., 1992).

The immune response to \textit{Mycobacterium avium}

Entry of \textit{M. avium} into the body is thought to occur through the bronchial or intestinal mucosa. The bacteria are then phagocytosed by host macrophages through complement receptors and C-3 mediated phagocytosis (Bermudez et al., 1991). Complement receptor mediated phagocytosis bypasses the oxidative response, but the cells may still be activated. \textit{M. avium} can also prevent fusion of the phagosome and lysosome (Sturgill-Koszycki et al., 1994). From this point, \textit{M. avium} multiplies until the macrophage, increasing apoptosis of those cells, and exiting bacteria infect surrounding macrophages.

To help understand the mechanisms responsible for the ability of \textit{M. avium} to survive and thrive in macrophages, studies have been undertaken to identify the genes
that are upregulated during *M. avium* infection of macrophages. Genes involved in several different functions have been found to play important roles in macrophage infection. Genes such as *nirB* (anaerobiosis), *dppD* (hypothetical ABC-transporter), and *lprC* (esterase) have all been found to be upregulated during macrophage infection, and during infection of mice (Danelishvili et al., 2004; Hou et al., 2002). Hou et al., in 2002, also showed that genes for such pathways as mycobactin biosynthesis, the tricarboxylic acid cycle, and nitrate extrusion were upregulated. In addition, he found several genes that are potentially involved directly in adherance and entry (*mce*) and antigenic variation (genes encoding for PPE proteins). This broad range of gene types upregulated during macrophage infection provides insight into the complex pathway by which *M. avium* infects the human host.

**Mycobacteria and the environment**

Mycobacteria are found in many environmental reservoirs. These can include natural and municipal water, soils, aerosol, protozoans, animals, and humans. Some type of waterborne exposure is thought to be the main source of *M. avium* in AIDS patients. DNA fingerprints of isolates obtained from the blood of patients matched isolates from the patients drinking water collected from the buildings where the individuals lived (von Reyn et al., 1994). *M. avium* has been recovered from biofilms within water distribution systems (Norton et al., 2004), and it has been shown to have varying degrees of chlorine susceptibility, with many strains showing a resistance of more than 500 times that of *Escherichia coli* (Falkinham et al., 2001). *M. avium* has been shown to be resistant to
chloramines, chlorine dioxide, and ozone (Taylor et al., 2000). Unfortunately, these are the major agents of disinfection used by most municipal water treatment plants.

Other residents of water systems are amoeba. These free-living protozoa (such as *Acanthamoeba* and *Nagleria*) have been isolated worldwide from freshwater ponds and lakes, drinking water systems, hot springs and spas, swimming pools, and sewage (Schuster and Visvesvara, 2004). Amoeba feed by phagocytosis and have small vacuoles for food digestion and water elimination. In many ways amoeba have been found to be very similar to human macrophages.

In 1987, Lock et al. examined the phagocytic capacity of *Acanthamoeba castellanii* as compared to that of human polymorphonuclear leukocytes (PMNLs). In this study, it was found that PMNLs were less able than amoeba to ingest a variety of bacteria. Acanthamoeba use carbohydrate binding proteins for phagocytosis (Brown et al., 1975), which are also used by macrophages. *A. castellanii* has also been shown to have a “respiratory burst” upon phagocytosis (Davies and Edwards, 1991), which again is a common mechanism in phagocytes. Both have been found to exhibit cyanide-insensitive O₂ uptake and increased O₂ consumption during phagocytosis (Davies and Edwards, 1991). The ability to infect amoeba might have prepared *M. avium* to move into the human host.

Another known advantage for bacteria inhabiting within amoeba is that of resistance to bactericidal agents. Miltner et al. showed that *M. avium* grown within *A. castellanii* was less susceptible to rifabutin, azithromycin, and clarithromycin. Amoeba are resistant to water treatment with ozone, monochloramine, copper-silver ionization,
and chlorine dioxide (Thomas et al., 2004). This would mean that amoeba are potentially very important to bacteria in providing protection from standard water treatments.

At least one bacteria is known to use amoeba as a reservoir. *Legionella pneumophila* is the etiologic agent of Legionnaire’s disease. This disease can manifest itself in a lethal pneumonia. The association of *Legionella pneumophilia* with amoeba is well known. When cultivated together, replication of *Legionella pneumophilia* occurs, even though the bacteria could not survive in the medium alone (Holden et al., 1984). It has also been found that coculture with *Acanthamoeba castellanii* enhances invasion (Cirillo et al., 1997).

Studies of *Legionella pneumophila* genes involved in entry and survival of *Legionella pneumophila* into amoeba and macrophage show much overlap. For example, the gene *rtxA* (repeats in structural toxin) in *Legionella pneumophila* has been shown to play a similar role in adherence and entry in both amoeba and macrophages (Cirillo et al., 2002). Another gene, *mip* (macrophage infectivity potentiator), has been shown to impair *Legionella pneumophila*’s ability to infect both macrophages and protozoa (Cianciotto and Fields, 1992). Other genes such as *pmi* (protozoa and macrophage infectivity), *icm* (intracellular multiplication) and *dot* (defect in organelle trafficking) play important roles in infectivity of protozoa and human macrophages (Hagele et al., 2000). Due to the similarity of genes for adherence and entry for both protozoa and human macrophages, it would seem that *Legionella pneumophila* may be using amoeba as a “primer” for macrophage invasion.
An important question in the study of *M. avium* is whether it might also be using amoeba to prepare for macrophage invasion. The benefits of the ability to use amoeba have been shown in both resistance to adverse environments and resistance to antimicrobials. Could amoeba also be providing a practice ground to improve invasion into host macrophages?

Species such as *M. avium*, *Mycobacterium fortuitum*, and *M. marinum* all invade and replicate within amoeba (Cirillo et al., 1997). *M. avium* cultured in amoeba have both enhanced entry into epithelial cells as well as increased virulence in macrophage and mouse models (Cirillo et al., 1997). This would seem to indicate, along with the protection provided by the amoeba from bactericidal agents, that there is a distinct advantage provided to *M. avium* by amoeba.
Mycobacterium avium genes upregulated upon infection of Acanthamoeba castellanii demonstrate a common response to the intracellular environment.

Rachel C. Tennant and Luiz E. Bermudez

To be submitted to Veterinary Microbiology
Abstract

Using a previously created *Mycobacterium avium* genomic library with GFP-promoter trap in *Mycobacterium smegmatis*, we screened for genes which are upregulated after passage through *A. castellanii*. Clones exhibiting a 2.5-fold or greater increase in GFP expression, out of a total of 10,000 clones, were selected for further examination. Upregulation was confirmed in subsequent experiments. A total of 20 clones showed an increase in expression after 24 and 48 h. Homologues were determined, and genes were found to encode for a variety of functions, including metabolic pathways, protein transcription and translation, and macromolecule degradation. Eight out of the 20 genes were found to be the same as those upregulated in macrophage infection. Five genes were selected to confirm upregulation in *M. avium* upon amoeba infection, using real time PCR. These genes were found to be upregulated at least 2.5-fold in *M. avium*.

These results indicate that the GFP promoter library in *M. smegmatis* is a valid system for studying gene upregulation in *M. avium*, and that many *M. avium* genes are commonly upregulated following macrophage and amoeba infection.

Introduction

*Mycobacterium avium* is an emerging opportunistic pathogen. It is known to cause infections in both humans and animals, being especially prevalent in immunocompromised individuals, such as HIV-infected patients (Inderlied et al., 1993). *M. avium* infections are also found in patients with pre-disposing lung conditions, like emphysema, bronchiectasis, and cystic fibrosis (Kilby et al., 1992; Teirstein et al., 1990). Other entities that are associated with increased risk include deficiency in interleukin-12
receptor (Altare et al., 1998), deficiency of interferon-gamma receptors and immunosuppression due to transplantation (Dorman et al., 2004; Simpson et al., 1982). In both AIDS patients and those with other immunodeficiencies, infections are disseminated.

*M. avium* is a ubiquitous organism, often found in soil and water sources. Studies of *M. avium* in water systems report a range of 10 to 700,000 CFU/l, with higher numbers in the distribution system than downstream (Falkinham et al., 2001). Chlorine, the main agent of bacterial control in water systems, has been shown to have varying effects on *M. avium*, with some strains being less susceptible than others (Falkinham, 2003). This characteristic may allow slow-growing *M. avium* strains to remain in drinking water systems from weeks to months.

Free-living amoeba are common inhabitants of water, including treated drinking water. One proposed theory is that, like *Legionella pneumophila*, *M. avium* may use amoeba as an intermediate host. It has been shown that *M. avium* will grow in co-culture with amoeba, often found within the outer walls of double-walled cysts of amoeba (Steinert et al., 1998). It has also been found in non-cystic amoeba in the environment (unpublished observation). Prior interaction between *M. avium* and amoeba was shown to enhance both entry and intracellular replication of *M. avium* in macrophages, and to be associated with a more virulent phenotype of *M. avium* in the beige mouse model (Cirillo et al., 1997).

Studies involving *Legionella* have indicated that genes which play a role in macrophage invasion and replication are often the same genes as those found with roles
in amoeba invasion and replication. The gene htrA, however, is necessary for intracellular replication in macrophages, but is not required for survival in amoebas (Pedersen et al., 2001). In contrast, genes such as rtxA, which is involved in adherence and entry of macrophages and amoeba (Cirillo et al., 2002), as well as icmT, which is necessary for pore-formation mediated cytolysis (Molmeret et al., 2002), which allows the bacteria to exit the macrophage or amoeba.

Although studies have been carried out to determine M. avium genes upregulated upon infection of macrophages, no information exists regarding genes required during life within amoeba. Therefore, we seek to undertake experiments with the goal of identifying M. avium genes upregulated during infection of amoeba.

Methods and Materials

**Bacteria, amoeba, and growth conditions.** M. smegmatis mc² 155 was kindly provided by Dr. William Jacobs, Jr. (Albert Einstein School of Medicine, NY). M. smegmatis was cultured on 7H10 agar, supplemented with oleic acid, albumin, dextrose, and catalase (OADC, Difco Laboratories, Detroit MI) at 37°C. The strain was used for survival studies, as well as for the construction of the M. avium GFP-promoter library (Danelishvili et al., 2004). M. avium 104 strain was isolated from blood the of an AIDS patient. It was cultured in 7H9 broth supplemented with OADC at 37°C. Acanthamoeba castellanii was obtained from ATCC (ATCC number 30234). It was maintained in 712 PYG media, in the dark, at room temperature.

*Mycobacterium smegmatis* and *Mycobacterium avium* ability to survive within *A. castellanii.* Due to the fact that the M. avium promoter GFP library was constructed in
M. smegmatis, survivability of M. smegmatis in A. castellanii was determined. We also defined the ability of M. avium to survive in amoeba and the time points for RNA extraction of intracellular M. avium in A. castellanii. A. castellanii was seeded in a 12-well flat bottom tissue culture plates at 1.0 x 10^8 cells, in 712 PYG media, at room temperature, and allowed to adhere overnight. Preparation of a disperse inoculum was carried out as previously described (Cirillo et al., 1997). Established monolayers were infected with disperse 10^7 bacteria (M. smegmatis mc^2 155 or M. avium) and incubated at either 30°C or 37°C for 2 h. A. castellanii monolayer was then washed 3 times with Hanks Balanced Salt Solution (HBSS) to remove extracellular bacteria, a method previously demonstrated to efficiently remove uningested bacteria (Cirillo et al., 1997). A. castellanii monolayer then was lysed with 0.5% SDS for 30 min (concentration that has no effect on mycobacteria viability) and passed through a 26-gauge needle to ensure complete lysis of the amoeba at 0, 24, 48, 72 and 96 h time points. The lysate was plated onto 7H11 agar plates and grown for 10 days at 37°C to determine the number of colonies of intracellular M. smegmatis or intracellular M. avium.

M. avium promoter GFP library. Library construction has been reported previously (Danelishvili et al., 2004). Briefly, M. avium genomic DNA was extracted and digested with Sau3A. Fragments ranging from 300-1000bp were selected using gel electrophoresis and cloned into plasmid pEMC1 (Danelishvili et al., 2004). Plasmids were expanded in E. coli, purified, and then transformed into M. smegmatis. Positive clones were selected using kanamycin 50 µg/ml on 7H10 agar. The pools containing 5
clones each were stored per well of a 96-well plate. A total of 10,000 clones, representing approximately 2.5-times the size of the *M. avium* genome, were used.

**Infection of *A. castellanii* with library.** Amoebas in 712 PYG medium were seeded in a 96-well flat-bottom tissue culture plate at $1.0 \times 10^5$ cells per well, and allowed to adhere overnight at room temperature in the dark. Resultant monolayers were infected with $10^6$ bacteria (MOI 10) and allowed to incubate at $30^\circ$C for 2 h. To remove extracellular bacteria, amoeba were washed 3 times with HBSS and media was replaced (Cirillo et al., 1997). GFP expression was measured prior to infection to establish a baseline and at 4, 24, and 48 h after infection, using a Cytoflourimeter II (Biosearch, Bedford MA). Wells exhibiting at least a 2-fold increase in the level of GFP over baseline were lysed with 0.25% SDS and plated onto 7H11 agar plates with 50 µg/ml kanamycin. Twenty individual clones per well were selected from plates and cultured in 7H9 Middlebrook broth containing 50 µg/ml kanamycin. They were used to infect fresh amoeba following the same procedure described above, with expression of GFP being measured at the same time points of 4, 24, and 48 h. Individual clones exhibiting at least a 2.5-fold increase in GFP expression over baseline were selected for sequencing. The selected clones were propagated in 7H9 broth with 50 µg/ml kanamycin, and plasmids were extracted using the Stratagene Plasmid Extraction Kit, as recommended by the manufacturer (Stratagene, San Diego CA). Plasmids were then transformed, using electroporation, into *E. coli*. The *E. coli* was grown in LB broth in presence of 50 µg/ml kanamycin. Plasmids were again extracted and sent for sequencing with primers specific for the GFP and kanamycin sequences in the pEMC1 plasmid.
Sequencing and Analysis. Sequencing was performed by the Center for Gene Research and Biotechnology, Oregon State University. Sequence analysis was performed using the BLAST service provided by the National Center for Biotechnology Information (www.ncbi.nih.gov). To determine *M. avium* gene function we searched for either *M. tuberculosis* or *M. avium* ssp. *paratuberculosis* homologues to the genes upregulated in our study. This was done using Tuberculist (http://genolist.pasteur.fr/TuberculList/) and BLAST.

RNA extraction and isolation. *M. avium* grown in 7H9 broth was used as a control, and intracellular *M. avium* from infected amoebas was obtained for experimental RNA. Amoebas were infected as described above, using *M. avium* strain 104. At 24 h, amoeba were lysed with .5% SDS for 30 min and passed through a 23-gauge needle to insure complete breakage of amoebas. Resulting lysate was centrifuged at 400x rpm for 5 min to removed lysed amoeba from suspension. Supernatant was centrifuged at 3200x rpm, and resulting pellet was used for obtaining RNA. RNA was extracted using a series of phenol/chloroform extractions as previously described (Danelishvili et al., 2004). RNA was cleaned using an RNA clean kit and treated with DNAse I prior to real-time PCR. Integrity of RNA was confirmed OD_{260/280nm} absorption and gel electrophoresis. Total RNA was reverse transcribed with Superscript II Plus RNase H- Reverse Transcriptase (Invitrogen, Carlsbad CA) per manufacturer's instructions.

Real Time PCR. SYBR green technology was used to perform real time PCR. Run protocol for the LightCycler was as follows: cycle 1, denaturation step, 95°C for 10 min; cycle 2, amplification and quantation, repeated 40 times, 95°C for 30s, 63°C for 30s,
and 72°C for 2 min with one fluorescence measurement. Threshold cycle (Ct) was quantified as described in User Bulletin #2 for ABI PRIMS 7700 sequence detection system (ABI). Threshold cycle is defined as the fractional cycle number at which the fluorescence reaches 10x the standard deviation of the baseline. iCycler I software (BIORAD, Hercules CA) was used for quantitative analysis, and a relative quantification was used in which the expression levels of *M. avium* target genes were compared to a standard curve generated using several dilutions of a known quantity of amplicons. As previously described (Danelishvili et al., 2004), an amplification-based strategy was used to determine fold change in gene expression. For each gene amplification, before calculating fold change the Ct values were normalized to the Ct of the 16sRNA. The following formula was used:

\[
\text{fold change} = 2^{-\Delta(\Delta\text{Ct})}
\]

where

\[
(\Delta\text{Ct}) = \text{Ct, target} - \text{Ct, 16sRNA},
\]

\[
\Delta(\Delta\text{Ct}) = \Delta\text{Ct, expressed} - \Delta\text{Ct, control}
\]

Standard deviations were calculated, and samples were compared to values for gene MA2610c, which was used as a negative control because it is not expressed upon *M. avium* infection of amoeba.

Results

*M. smegmatis* and *M. avium* ability to survive within *A. castellanii*. To analyze *M. smegmatis* and *M. avium* ability to survive in amoeba, we determined CFU/ml at 4, 24, 48, 72, and 96 h after inoculation, at temperatures of 30°C and 37°C. As demonstrated in Figure 1, both *M. smegmatis* and *M. avium* showed ability to survive
Figure 1. *M. smegmatis* and *M. avium* growth in *A. castellanii*, both at 30°C and 37°C. Data points represent the average of 4 experiments ± SD.
within amoeba at temperatures of 30°C and 37°C. However, at 37°C, a significant number of amoeba were noted to become detached from the bottoms of the wells. In addition, at 37°C, amoeba is not very effective and capable of destroying intracellular bacteria. For these reasons, we chose 30°C for the temperature of incubation of *A. castellanii* infected with the *M. smegmatis* GFP-promoter library and for infection of *A. castellanii* with *M. avium*.

*M. avium* gene expression determined by GFP library. Over 10,000 clones of the *M. avium* promoter library were screened for increased GFP expression. Twenty clones were identified as having a 2.5-fold or greater increase in expression of GFP (Table 1). Five genes showed upregulation only at the 24 h time point. Nine genes showed upregulation only at the 48 h time point. Six genes showed upregulation at both 24 and 48 h time points. There was no significant increase in GFP expression at the 4 h time point over baseline. The genes identified belong to a variety of classes including transcription regulation, protein translation, metabolic pathways, energy metabolism, degradation of macromolecules, DNA replication, membrane proteins, and genes with unknown function. One gene of unknown function was found to be in a region that shared very little homology with other closely related bacteria such as *M. tuberculosis* or *M. avium ssp. paratuberculosis*. It also had no homology with other bacteria. This region was found to occupy 50kb of the *M. avium* genome, and to have a G+C content of 69%, which is similar to the G+C content of *M. avium*. 
Table 1. *M. avium* promoters upregulated during infection of *A. castellanii*.

<table>
<thead>
<tr>
<th>Clone</th>
<th><em>M. avium</em> gene homologue in M. tb (Rv) or MAP</th>
<th>% Similarity</th>
<th>Gene function</th>
<th>Fold change in GFP</th>
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<tr>
<td>1D12</td>
<td>Rv0688</td>
<td>82</td>
<td>Ferridoxin reductase</td>
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</tr>
<tr>
<td>2C2</td>
<td>Rv0252/<em>nirB</em></td>
<td>84</td>
<td>Nitrate Reductase flavoprotein. Contains nitrite and sulfite reductases iron-sulfur/sidroheme-binding site</td>
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<td>2H5</td>
<td><em>M. avium</em> Gene</td>
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<td>Integral membrane protein</td>
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</tr>
<tr>
<td>13F8</td>
<td>MAP2001c</td>
<td>93</td>
<td>FAD-binding monooxygenase motif</td>
<td>2.5</td>
</tr>
<tr>
<td>14A4</td>
<td>Rv1685c</td>
<td>81</td>
<td>Hypothetical protein, possible transcriptional regulator</td>
<td>2.9</td>
</tr>
<tr>
<td>18C2</td>
<td>Rv0358</td>
<td>65</td>
<td>Conserved hypothetical protein</td>
<td>2.6</td>
</tr>
<tr>
<td>18G5</td>
<td>MAPfadE25</td>
<td>90</td>
<td>Acetyl CoA dehydrogenase</td>
<td>2.5</td>
</tr>
<tr>
<td>19C2</td>
<td>Rv1497/<em>lipL</em></td>
<td>77</td>
<td>Esterase</td>
<td>3.2</td>
</tr>
<tr>
<td>20F6</td>
<td>Rv0002/<em>dnaN</em></td>
<td>88</td>
<td>DNA polymerase III</td>
<td>2.5</td>
</tr>
<tr>
<td>22A5</td>
<td>Rv2839c/<em>infB</em></td>
<td>89</td>
<td>Initiator factor If-2</td>
<td>2.8</td>
</tr>
<tr>
<td>22E3</td>
<td>Rv3795/<em>embB</em></td>
<td>74</td>
<td>Arabinosyl transferase</td>
<td>3.6</td>
</tr>
</tbody>
</table>
Analysis of *M. avium* gene expression in amoeba by quantitative real time PCR.

Five genes were chosen for analysis by real time PCR (Table 2). This allowed us to confirm upregulation of genes identified in the *M. smegmatis* system in *M. avium*, and would validate the use of the *M. smegmatis* library. The following *M. avium* genes were chosen for analysis: Ma0359 (membrane protein), Ma-*lipL* (esterase), Ma-*embB* (cell wall biosynthesis), Ma-*lprC* (lipoprotein), and Ma-*nirB* (nitrate reductase flavoprotein). These represent a variety of classes found to be upregulated in the GFP-promoter library. The gene Ma2610c did not show upregulation in amoeba using the promoter library. This gene served as a negative control.

Real time PCR efficiency was determined using a dilution series of cDNA template with a fixed concentration of primers. Slopes calculated by the LightCycler software were used with the following formula to calculate efficiency: \( E = 10^{(-1/\text{slope})} \). These calculations indicated both a high real time efficiency with a high linearity.

Because expression of 16s RNA is constant independent of conditions, target genes from both control and experimental groups were normalized to the expression level of the 16sRNA gene. All 5 genes showed significant induction upon *M. avium* infection of amoeba, while no upregulation was observed with Ma2610e, the negative control, confirming that genes detected using the GFP promoter library were expressed by *M. avium* (Figure 2).

Discussion

*M. avium* is an organism commonly encountered in the environment, mainly in soil and water sources (Falkinham et al., 2001; Inderlied et al., 1993). As an
Table 2. Sense and antisense primers for real time PCR

<table>
<thead>
<tr>
<th>Clone</th>
<th>Target</th>
<th>Primers</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12C6</td>
<td>Ma0359</td>
<td>5'-CGGCTGGCTGGTGTTCTGTTGCTGCTGTTG-3'</td>
<td>567</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-ACGCCGGAGAAGTCGAAAC-3'</td>
<td></td>
</tr>
<tr>
<td>19C2</td>
<td>Ma-lipL</td>
<td>5'-GCTGGAGCTGGCCGCA-3'</td>
<td>1477</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CGGCCAGCTACAGGTGTTCGTCGTC-3'</td>
<td></td>
</tr>
<tr>
<td>22E3</td>
<td>Ma-embB</td>
<td>5'-GCGGTGATCTCTCCTGGGCCTGCTGCTG-3'</td>
<td>1219</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-AGTGCAACCAGGGCTCGCGTACA-3'</td>
<td></td>
</tr>
<tr>
<td>7B7</td>
<td>Ma-lpcR</td>
<td>5'-CTGACGGGCTGTTCCTCCGCTCGTCTG-3'</td>
<td>489</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTAGGAATTGGTCGCCAGCGTCA-3'</td>
<td></td>
</tr>
<tr>
<td>2C2</td>
<td>Ma-nirB</td>
<td>5'-CTCAAAGGATGGATTCCAGGACGTGCTGAGGC-3'</td>
<td>1250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GGGTCGCCCGGCAGCTCGCTG-3'</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Upregulation of *M. avium* genes upon infection of *A. castellanii*, as determined by real-time PCR. The data represents the average of 2 independent experiments ± SD.
environmental microbe, *M. avium* shares the environment with other bacteria, as well as protozoa. Recently, *M. avium* has been shown to infect and survive in Acanthamoeba (Cirillo et al., 1997), and there is evidence suggestive that it is associated with the upregulation of genes associated with virulent phenotype of the bacterium. To seek a better understanding of the impact of the intracellular environment in Acanthamoeba on *M. avium*, we examine how amoeba infection impacts the regulation of *M. avium* genes.

Genes found to be upregulated in this study span a wide variety of classes. These include metabolic pathways, transcriptional regulators, DNA replication, energy metabolism, protein translation and modification, and degradation of macromolecules. Several of the genes seen to be upregulated upon Acanthamoeba infection have an unknown function. Several of the genes only show upregulation after 24 h of infection but not at the 4 h time point. This can be explained by an initial period of adaptation following uptake, in which many bacteria stop multiplying. It is very likely that this period of adaptation lasts less than 24 h. Among the genes upregulated is a promoter found to have no homology to bacterial sequences stored in the database. An examination of the region found a 50kb section of the *M. avium* genome that does not have significant homology to *M. tuberculosis* or *M. avium ssp. paratuberculosis*. The region has a G+C content of 69%, which suggests that the sequence either belongs to mycobacteria or has been acquired from another organism with high G+C content in the genome. This region, therefore, might be an evolutionary adaptation that helped *M. avium* become able to infect macrophages, due to the similarity between macrophage and
amoeba vacuoles (Brown et al., 1975; Cirillo et al., 1997; Davies and Edwards, 1991; Lock et al., 1987).

Eight of the genes found in this study were the same as those identified in infection of macrophages by *M. avium* (Danelishvili et al., 2004; Hou et al., 2002). These included genes involved in protein translation, energy metabolism, and genes of unknown function. Studies with *Legionella pneumophila* suggested that Legionella expresses many of the same virulence genes when within macrophages and amoeba (Cirillo et al., 2002; Molmeret et al., 2002). In contrast, some virulence genes are expressed exclusively in one environment. For example, the *htrA* is a necessary gene for full virulence in macrophages but is not needed in amoeba (Pedersen et al., 2001). This was the case of the *M. avium* gene in the 50Kb exclusive region, which is only expressed in amoeba but not in macrophages. This data seems to indicate that *M. avium* uses some mechanisms for survival in the intracellular environment that are common for amoeba and macrophage.

The *nirB* gene has been shown to be over-expressed in Mycobacteria by both Hou and Danelishvili. This gene is part of the denitrification process, encoding for a nitrogen reductase. Genes in this category, such as *nirK* and *nirV*, have been shown to regulate in virulence in Brucella in mice (Baek et al., 2004).

The *lprC* is another gene in common between macrophage and amoeba infection. This gene encodes for a lipoprotein, which may have a role in bacteria binding to host proteins, and in envelope remodeling (Danelishvili et al., 2004). Also upregulated were a ferredoxin reductase (Ma0688) and several dehydrogenases (*serA2* and MAPfadE25).
homologue). Ferredoxin reductase is an enzyme that catalyzes the oxidation and reduction of ferredoxin or adrenodoxin in the presence of NADP. Iron starvation response in *Pseudomonas aeruginosa* has been shown to lead to a cascade that not only activates ferredoxin, ferredoxin reductase and several dehydrogenases, but also activates genes encoding virulence factors such as TonB and exotoxinA (Ochsner et al., 2002).

Using an *M. avium* GFP promoter library, we have identified 20 genes upregulated during infection of amoeba. Five of those genes were then confirmed by real-time PCR. This again validates the use of an *M. smegmatis* for the study of upregulation of *M. avium* genes. Many genes expressed have unknown function, but future studies involving inactivation of these genes may provide information on their roles in the pathogenesis of the infection.
References


Discussion

Until recently, studies of genes upregulated in bacteria and after passage through intermediate hosts have been limited to those of *Legionella pneumophila* and amoeba. In this study, we hoped to determine if gene upregulation occurred in *M. avium* after passage through an intermediate host.

A gene region of great interest that was found in this study is that of the 2H5 promoter insert. It occurs within a 50 kb region, having a G+C content of 69% and having little homology to *M. avium*’s closest relatives (Figure 3). Its G+C content is 69%. Evolutionarily, it is important that *M. avium* may have acquired the ability to reside within amoeba in the environment. This trait may have later provided *M. avium* with an advantage in infecting human macrophages, due to the similarity between amoeba and human macrophages.

Several genes that were determined to be upregulated in this study have been found to have important functions in other bacteria. Because iron is essential for growth in many bacterial species, regulation of iron is extremely important. Ferredoxin reductase has been shown to be one of a cascade of genes expressed during iron-starvation of *Pseudomonas aeruginosa* (Ochsner et al., 2002). Several dehydrogenases, iron-binding proteins, and virulence factors are expressed as well. Expression of ferredoxin reductase and other genes may provide a sensing mechanism for the bacteria, enabling them to turn on virulence genes when it is most advantageous to do so. For
**Mycobacterium tuberculosis**

Rv3770A  Rv3771c  serT  hisC2  Rv3773c  echA21  lipE  Rv3776

Rv3770B  argU

**Mycobacterium avium**

hisC2  50kb region with 2H5 promoter insert  Rv3773c

Figure 3. *M. avium* region with clone 2H5 promoter insert as compared to *M. tuberculosis*.
bacteria that have adapted to living intracellularly, sensing and responding to environmental stimuli is vital. Also, several types of iron have been found to be stimulatory to growth of *M. avium* (Kimsell et al., 1995).

The *nir* class of genes is a group of genes that has received much attention. The *nirA* and *nirB* have been found to be expressed in three different studies of macrophage infection by *M. avium* (Danelishvili et al., 2004; Dubnau and Smith, 2003; Hou et al., 2002). They have also been linked to virulence in *Shigella flexneri* (Jin et al., 2002). In Brucella, another intracellular pathogen, inactivation of *nirK* resulted in a decrease of *in vivo* growth. While the role of this gene is unclear, it is postulated that nitrate reductase genes may confer some advantage by providing the bacteria with a mechanism to combat toxic NO produced by macrophages during infection (Baek et al., 2004).

One of the first problems I ran into early on was when I started using the GFP-promoter library. I found duplicate clones in different wells of the same plate. After looking at many steps in the process of inoculating and washing the amoeba and bacteria, it was determined that the aseptic technique could be improved during the wash step. This required changing tips more frequently and being more careful not to aerosolize the wash solution by expelling it more slowly and away from the plates.

After solving this problem, I discovered that I was sequencing several of the same clones from a single well. In an effort to make the work more efficient, I introduced a screening process for the clones. After determining a 2.5-fold increase in fluorescence in the individual clones, the plasmids were extracted, propagated in *E. coli*, and then digested with *EcoRI* and *XbaI*. This digest excised the insert from the plasmid. After gel
electrophoresis, multiple plasmids with the same size inserts were not sequenced. This allowed me to screen out duplicate clones from the same well. (Figure 4) This also sorted out any plasmids that were expressing without an insert or promoter being present. (Figure 5)

RNA extractions from intracellular bacteria can be difficult. After several fruitless attempts to extract RNA from M. avium that had been incubated with amoeba, I discovered a few problems with my techniques. The first was that I had underestimated the strength of the amoeba cell wall. Lysing with 0.25% SDS for 30 minutes was insufficient to cause breakage in the amoeba. Most of the amoeba were discovered to be intact by light microscopy. After increasing this to 0.50% SDS, and passing the amoeba through a 26-gauge needle, lysis was confirmed. The second problem was that a very large volume of infected amoeba are necessary to recover sufficient bacteria for RNA extraction. This problem was solved by increasing the amount of amoeba, as well as the amount of innoculum.

After RNA extraction, the importance of DNAse treatment, as well as clean-up of RNA, became obvious. First, RNA was DNAse treated, then used for real-time PCR. It was noted that some samples were taking in excess of 35 cycles to reach threshold (Figure 6). RNA was then DNAse treated, cleaned with an RNA clean-up kit, and used for no-RT control PCR reactions with 16s primers (Figure 7). When the no-RT controls were run on agarose gels, it was noted that there was DNA contamination before DNAse treatment and clean-up. After clean-up, Ct (threshold cycle) decreased, and valid results were obtained (Figure 8).
Figure 4. Plasmid inserts from pEMC1 plasmids. Lane A – 1kb marker, Lane B-D – plasmids after digestion with EcoRI and XbaI, note that lanes B and D have the same size plasmid therefore are the same clone.
Figure 5. Plasmids digested with EcoRI and XbaI. Note there are no plasmid inserts present.
Figure 6. Real-time PCR before clean-up and DNAse treatment of RNA.
Figure 7. No-RT controls with 16s primers before and after RNA DNAse treatment and clean-up. Lane A – marker, Lane B – control RNA before treatment, Lane C – experimental RNA before treatment, Lane D – control RNA after treatment, Lane E – experimental RNA after treatment.
Figure 8. Real-time PCR after RNA treatment with DNAse and clean-up.
Several useful conclusions, as well as technique improvements, were gained from this study. Most importantly, we have provided insight into the genes which regulate the mechanism that \textit{M. avium} uses to infect amoeba, as well as found some commonalities with the system used to infect macrophages. Future research may provide more insight into whether amoeba provide an important reservoir for \textit{M. avium} in the environment and whether this reservoir plays an important role in virulence for this bacteria.
References


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