AN ABSTRACT OF THE DISSERTATION OF

MELANIE J. HARRIFF for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on June 8, 2007.

Title: Mechanisms for the Interaction of Environmental Mycobacteria with Host Cells

Abstract approved:

Luiz E. Bermudez Michael L. Kent

Environmental mycobacteria are important opportunistic pathogens for many hosts, including humans, cattle, and fish. Two well-studied species are *Mycobacterium avium* subsp. *avium*, a significant cause of disseminated bacterial disease in patients with AIDS, and *Mycobacterium avium* subsp. *paratuberculosis*, the cause of Johne's disease in cattle. Many other species that are considerable sources of infections in fish, such as *Mycobacterium chelonae* and *Mycobacterium marinum*, also have zoonotic potential. To gain knowledge about the invasion of epithelial cells by environmental mycobacteria, selected genes and proteins involved in the uptake of *M. avium* by HEp-2 cells were analyzed by a variety of methods. Two transcriptional regulators (MAV_3679 and MAV_5138) were identified as being involved in invasion. A mycobacterial protein (CipA) with an amino acid sequence suggestive of an ability to be a part of the scaffolding complex that forms during cell signaling

leading to actin polymerization was found to putatively interact with host cell Cdc42. Fusion of CipA to GFP, expressed in *Mycobacterium smegmatis*, revealed that CipA localizes to a structure on the surface of bacteria approaching HEp-2 cells. To establish whether species of environmental mycobacteria isolated from different hosts use similar mechanisms to *M. avium* for interaction with the mucosa, and for survival in macrophages, assays to determine invasion and replication were performed in different cell types, and a custom DNA microarray containing probes for known mycobacterial virulence determinants was developed. Bacteria cultured from macrophages indicated differences between the ability of *M. avium* and other environmental species to replicate in human, mouse, and carp cell lines. Hybridization of genomic DNA isolated from EM species against the sequenced MAC104 strain of *M. avium* showed that there are genes and regions of the chromosome absent from a subset of those species that may be important in determining host specificity and the in differences in virulence observed *in vitro* and *in vivo*. Finally, the intestinal epithelium was determined to be the primary route of infection for mycobacteria in zebrafish, similar to *M. avium* infection in humans. *M. avium* does not infect zebrafish, but the comparative virulence and genomics of some species isolated from fish suggest that they could be used as a surrogate model to continue progress in understanding the interaction between environmental mycobacteria and epithelial cells.

Mechanisms for the Interaction of Environmental Mycobacteria with Host Cells

by

Melanie J. Harriff

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented June 8, 2007

Commencement June 2008

Doctor of Philosophy dissertation of Melanie J. Harriff presented on June 8, 2007.

APPROVED:

Co-Major Professor, representing Molecular and Cellular Biology

---

Co-Major Professor, representing Molecular and Cellular Biology

---

Director of the Molecular and Cellular Biology Program

Dean of the Graduate School

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

Melanie J. Harriff, Author

ACKNOWLEDGEMENTS

Thank you to Mike Kent and Luiz Bermudez for providing me the opportunity to complete my degree at Oregon State University, and for their many contributions to my professional and personal development. Thank you also to the members of my committee, Chris Bayne, John Fowler, and Doug Barofsky, who have freely offered their time and advice.

A special thanks to those with whom I have worked in the laboratory. Without their friendship, professional collaboration, and contribution to my projects, I wouldn't be here today. To name a few: Julie Early, Lia Danelishvili, Martin Wu, Marta Alonso-Hearn, Heather Duchow, Sanjai Tripathi, and Mike McNamara from the Bermudez lab; and Chris Whipps, Virginia Watral, Janell Bishop-Stewart, and Jen Ramsey from the Kent lab.

For Tracey, Duke, and Satchel, whose endless love, friendship, and loyalty have been a light in each day, and who have sustained me throughout, I have immeasurable gratitude, appreciation, and love. It has been a joy to go through this experience with you at my side.

I would like to acknowledge the encouragement of friends who have passed through my life during this chapter, and to thank my family for their willingness to examine ideas beyond their own experience.

Finally, thanks to the Molecular and Cellular Biology Program and the Department of Biomedical Sciences for funding and support over the past few years. I appreciate being given the opportunity to represent the faculty, staff, and students of

these programs, and also am grateful for the contribution that they have made in my graduate career. I am indebted to Denny Weber for her assistance and support while I have been here, and much appreciate the efforts of Gail, Rosa, and Sheri in the MCB office.

CONTRIBUTION OF AUTHORS

Chapter 2: Martin Wu collected the RNA and performed the original microarray hybridization comparing MAC109 and MAC109:Δ*fadD2*; Cara Wilder, as a rotation student under my supervision, made the strains over-expressing MAV_3679 and MAV_5138 and performed invasion assays with those strains; and Michael McNamara made the MAV_4671:GFP construct. Chapter 3: Martin Wu performed the macrophage invasion assays for the six strains of *M. salmoniphilum*. Drs. Luiz Bermudez and Mike Kent were advisors on all manuscripts.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF TABLES

DEDICATION

This dissertation is dedicated to my grandmother, Carol F. Smith.

Chapter 1. Introduction

The genus *Mycobacterium*

The genus *Mycobacterium* consists of aerobic, rod-shaped, non-motile members of the Actinomycetes. Over 100 species of mycobacteria are recognized, and although they can be divided into fast- or slow-growing groups according to their replication in laboratory medium, the many species have a number of characteristics in common. The genomes of mycobacterial species are high in G+C content and, due to the presence of mycolic acids and mycolates, the cell wall is thick and waxy compared to other bacteria. As a result of these compounds and the resulting thick cell wall, mycobacteria are not typically classified by staining as Gram-positive or Gram-negative, but instead are acid-fast. In addition, mycobacteria are very hardy, surviving in harsh environments, and are naturally resistant to many antibiotics.

Mycobacteria are widespread in their environment and diverse in pathogenicity to humans and animals. Whereas many species, such as *Mycobacterium smegmatis,* are strictly saprophytic and not known to cause disease in humans or animals, many others are pathogens. Among the latter group are the familiar *Mycobacterium tuberculosis*, the cause of human tuberculosis, and *Mycobacterium bovis* and *Mycobacterium microti*, obligate pathogens of cattle and mice causing tuberculosis-like disease. Still other members of the genus *Mycobacterium* are opportunistic pathogens for a variety of hosts. The *Mycobacterium avium* Complex (MAC) includes a number of species known to cause disease in cattle, birds, and pigs, and importantly in humans with immunosuppressive conditions such as AIDS. *Mycobacterium marinum* and *Mycobacterium chelonae*

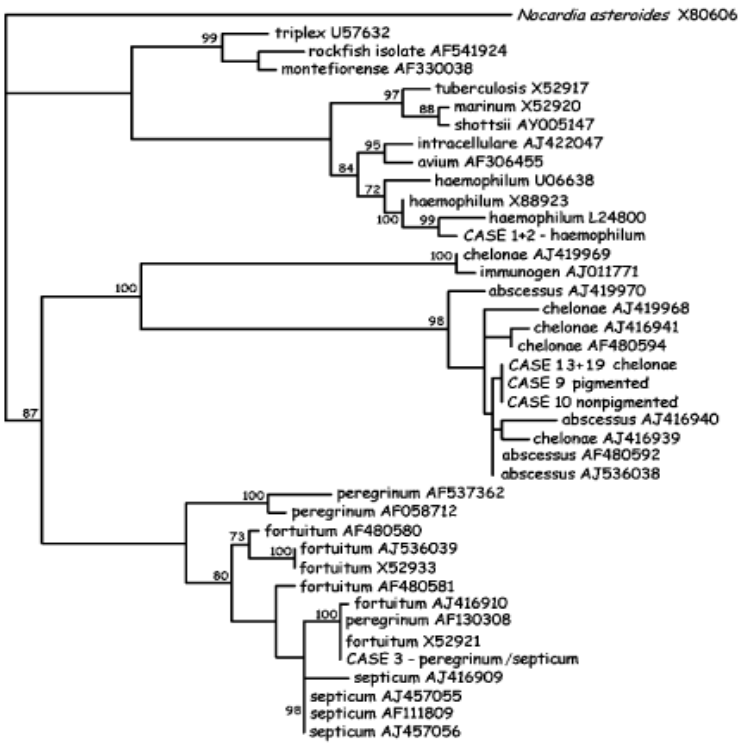

Figure 1.1 A phylogenetic tree showing the relationship between a subset of mycobacterial species, including many of those used in this study, based on molecular methods. (Kent *et al.*, 2004; Whipps *et al.*, 2007).

are among many other opportunistic species commonly isolated from fish and other cold-blooded vertebrates, and also cause infection in humans. A phylogenetic tree shows the relationship between many of these pathogenic species of mycobacteria (Figure 1.1).

Mycobacterial infection in humans

With the exception of *Mycobacterium ulcerans*, the cause of the Buruli ulcer, pathogenic mycobacteria survive intracellularly, inhabiting a number of cell types including macrophages, dendritic cells, and epithelial cells. The means by which each species enters these host cells varies depending on the host and mycobacterial

species. *M. tuberculosis* enters the lung cavity when airborne droplets discharged from an infected person are inhaled. Once in the alveolar spaces of the lung, *M. tuberculosis* invades both alveolar epithelial cells and macrophages. Within the alveolar macrophages, the bacterium is able to replicate and survive by preventing phagolysosomal fusion and subsequent killing. The immune response to *M. tuberculosis* leads to the formation of granulomas in the lung tissue.

Other mycobacteria enter host cells after being ingested. In humans and animal species, some members of the MAC invade epithelial cells lining the wall of the intestine. In humans, *M. avium* subsp. *avium* enters enterocytes, and translocates across the epithelial wall. It then infects macrophages in the sub-mucosal space and disseminates to other macrophages throughout the body (Figure 1.2) (Sangari *et al.,* 1999; Sangari *et al.,* 2001). In contrast, the closely related *Mycobacterium avium* subsp. *paratuberculosis* is believed to enter the intestinal lining of cattle through M cells, specialized immune cells found in Peyer's patches (Momotani *et al.,* 1998; Sigur-Dardottir *et al.,* 2001), although a more recent study suggests that this species can also cross epithelial cells that are not a part of the Peyer's patches (Sigur-Dardottir *et al.,* 2005). From there, the bacteria are similarly able to translocate to macrophages and disseminate.

In addition to gaining access to the intracellular environment via the epithelial tissues of the lungs and intestine, many mycobacteria are thought to enter cells directly from sites of tissue damage. *M. chelonae* is often isolated from the visceral tissues of fish (Decostere *et al.*, 2004); however, it has also been implicated in eye infections in humans following laser surgery (Brown-Elliot and Wallace, 2004).

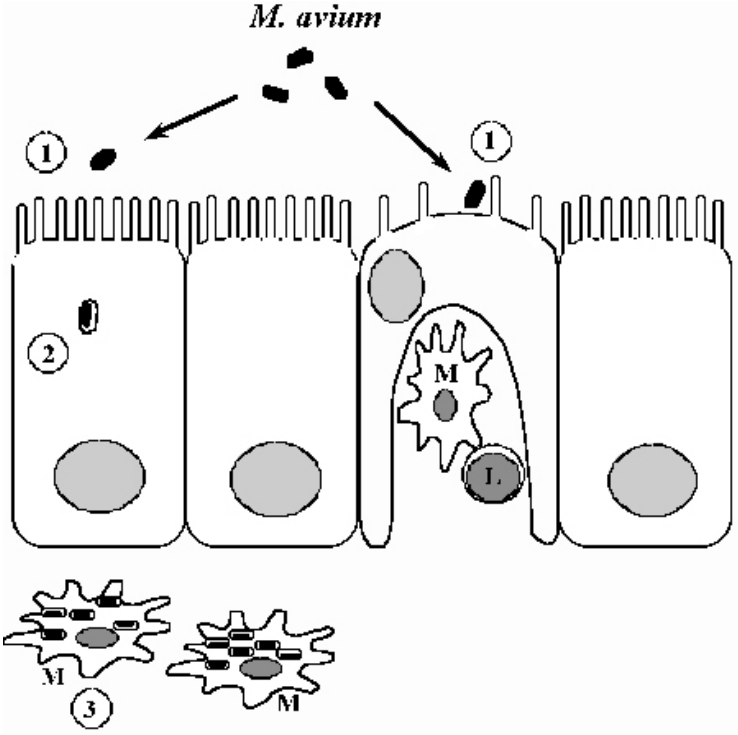

Figure 1.2 A model from Sangari *et al.* (1999) showing (1) the interaction of *M. avium* with the intestinal epithelium. (2) The bacterium is subsequently translocated to the basolateral surface, where it is (3) taken up by macrophages and disseminated. A later study showed that uptake was primarily by enterocytes (2001).

Whereas *M. marinum* is commonly known for granuloma-forming infections of fish (Decostere *et al*., 2004), fish handlers have been infected with this bacterium, familiarly known as "fish-tank tuberculosis" or "fish-fancier's finger." When there is a preexisting lesion on the skin, *M. marinum* is able to colonize human skin where a granuloma-like nodule forms at the site of infection. Unlike the dissemination of infection seen in fish, these infections of human skin typically do not spread beyond the local site of infection, likely due to the host temperature (Petrini, 2006).

Mycobacterial infection in fish

M. chelonae, M. marinum, and *Mycobacterium fortuitum* are the species most frequently implicated in fish mycobacteriosis (Chinabut, 1999). Many other species

have been isolated from outbreaks in fish facilities and in the wild, however, including *Mycobacterium peregrinum* (Kent *et al.*, 2004), *Mycobacterium haemophilum* (Kent *et al.*, 2004; Whipps *et al.,* 2007), and *Mycobacterium triplex* (Herbst *et al.,* 2001; Whipps *et al.*, 2003; Poort *et al.,* 2006) (See Appendix I), all of which have zoonotic potential. Although some mycobacteria cause chronic infections, with few signs of disease and little mortality, other species can cause severe outbreaks, with high levels of mortality (Kent *et al.*, 2004). A new species, *Mycobacterium shottsii*, is very prevalent, causing mycobacteriosis in almost half the striped bass from Chesapeake Bay (Rhodes *et al.,* 2004). Signs of fish mycobacteriosis include skin ulceration, emaciation and granulomatous lesions throughout the viscera (Decostere *et al.*, 2004) (Figure 1.3). In both diagnostic and experimental cases, mycobacteria are seen in the lumen and lining of the intestine, throughout visceral organs, within the swim bladder and the cells that line it, and in many other tissues (Figure 1.4). Despite the importance of mycobacteriosis in zebrafish facilities, the natural route of infection for mycobacteria was only recently experimentally determined in this thesis to be primarily the intestine (Harriff *et al.*, 2007) (See Chapter 4), and little is known about the molecular mechanisms by which

Figure 1.3 A lesion on the skin of a striped bass (www.vims.org), and granulomas in the visceral organs of a European tench (www.was.org) infected with mycobacteria.

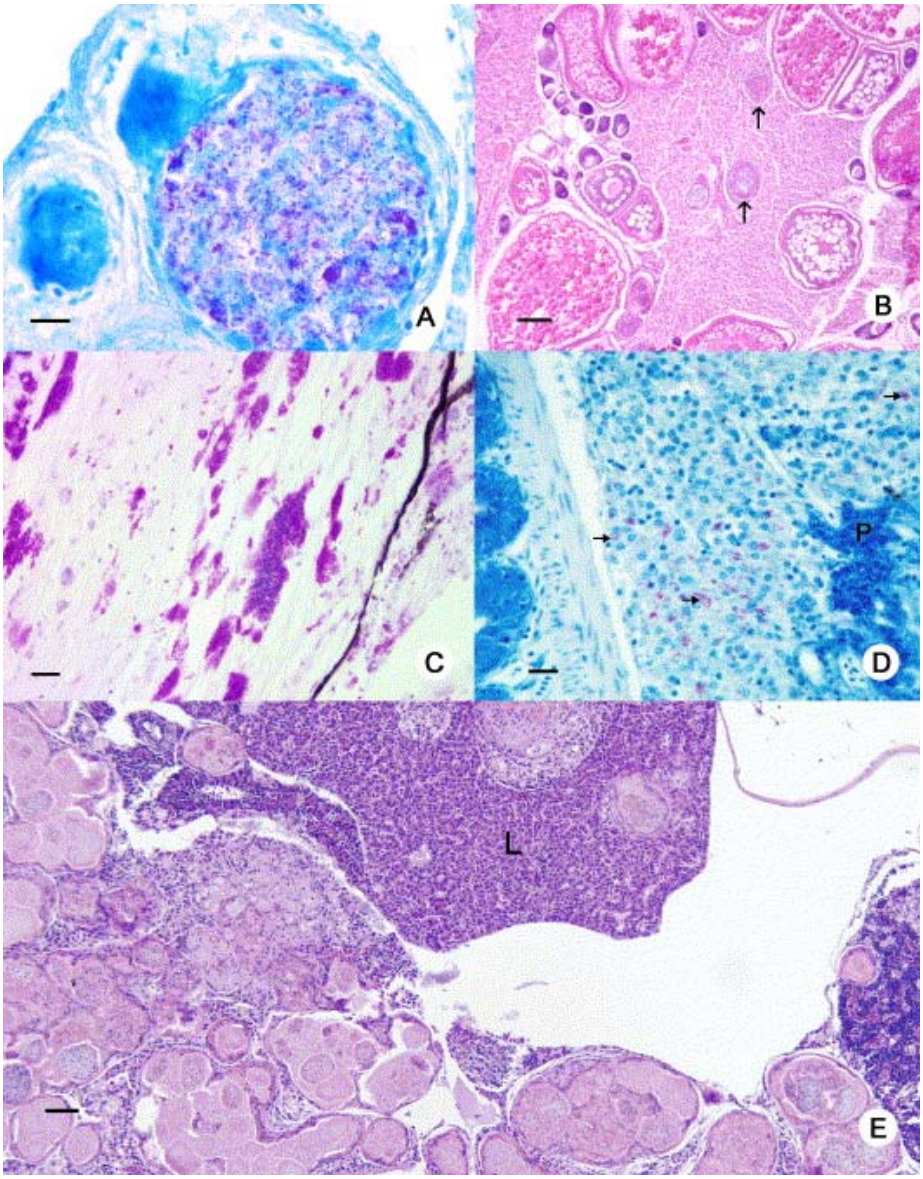

Figure 1.4 Mycobacteria in zebrafish tissues. A) and B) Typical granulomas containing acid fast bacilli in their centers; C) Acid-fast bacilli in the spinal cord not associated with infection; D) Diffuse infection in pancreatic tissue (P) near the intestinal epithelium; and E) disseminated infection with multiple granulomas in liver tissue (Kent *et al.*, 2004).

these organisms cause disease in fish. An additional question asked in this thesis was how the mechanisms by which fish pathogens infect the epithelial cells of the fish intesting compare to those of *M. avium*.

Invasion of intestinal epithelial cells by prokaryotic pathogens

In general, many intracellular pathogens first invade intestinal epithelial cells. In addition to *Mycobacterium*, members of *Salmonella*, *Listeria*, *Yersinia*, and *Shigella* are enteroinvasive pathogens that gain entry to the host by infecting the cells lining the intestinal epithelium. The uptake of these bacteria by the epithelial cells is an active process by the bacteria in which they exploit the machinery of these normally non-phagocytic host cells in various ways.

Because of the relative ease in genetically manipulating these other intracellular pathogens, much more is known about their molecular mechanisms of invasion than for *Mycobacterium*. There are two main mechanisms by which bacteria are known to enter epithelial cells (Figure 1.5). In the first, termed the "zipper" mechanism of entry in a number of reviews (Alonso and Garcia-del Portillo, 2004; Cossart and Sansonetti, 2004), the pathogen expresses a protein on its surface that interacts with a surface receptor of the host cell. This interaction starts signaling cascades leading to cytoskeletal rearrangement in the host cell, and ultimately the formation of a vacuole around the bacterium. Bacteria that employ the second, or "trigger" mechanism of entry (Alonso and Garcia-del Portillo, 2004; Cossart and Sansonetti, 2004), have secretion systems that inject effectors into the host cell. These effectors bring about cytoskeletal rearrangement and vacuole formation by directly interacting with and affecting the host cell proteins in the cytoskeleton signaling cascades.

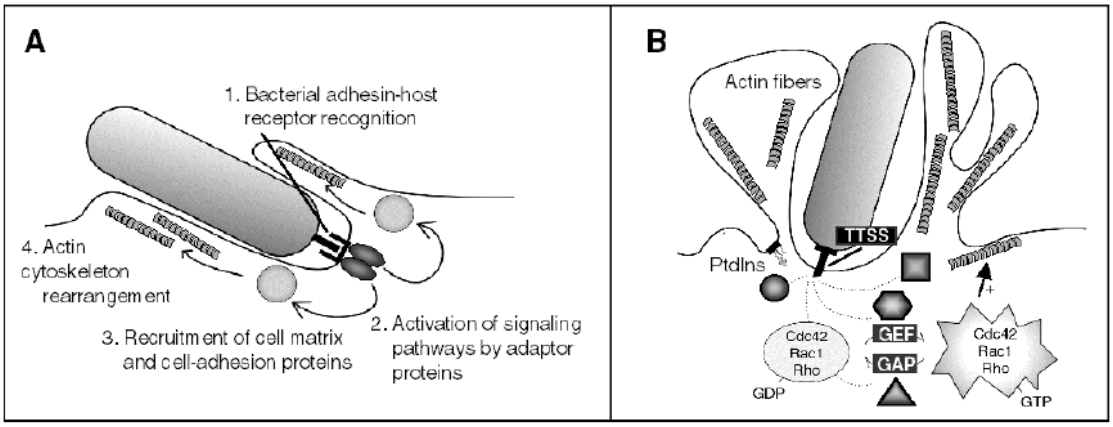

Figure 1.5 A drawing showing the two mechanisms by which intracellular pathogens are taken up by epithelial cells. A) The zipper mechanism, in which the bacterium interacts with host cell molecules on the exterior of the cell, leading to actin cytoskeleton rearrangement. B) The trigger mechanism, in which the bacterium secretes effectors into the host cell that effect actin cytoskeleton rearrangement (Alonso and Garcia-del Portillo, 2004).

Yersinia sp., which include the species causing the plague, are an example of a pathogen that enters epithelial cells by the zipper mechanism. This bacterium expresses an outer membrane protein, invasin, which binds to integrin receptors on host cells. Activation of receptors upon bacterial binding leads to tyrosine phosphorylation (Rosenshine *et al.,* 2003), activation of the small GTPase, Rac, the Arp2/3 complex, and possibly N-WASp (McGee *et al.,* 2001), and ultimately actin polymerization. The resulting cytoskeleton rearrangement eventually leads to engulfing of the bacterium in a vacuole.

The well-studied Type III Secretion Systems (TTSS) of *Salmonella* sp. and other intracellular pathogens provide an example of the trigger mechanism of host cell invasion. Zhou and Galan (2001) review this process in *Salmonella*. Briefly, *Salmonella* sp. express proteins, encoded on the Salmonella pathogenicity island, SPI-1, that form a needle-like structure called the injectosome that spans the plasma

membrane and extends into the extracellular space. Upon contact with the mucosal membrane and host epithelial cells, this system is activated such that the injectosome forms a translocon pore in the host cell membrane through which effector proteins are secreted directly into the host cell. It is not known how the initial changes in actin nucleation begin, but it is thought that one of the translocon pore proteins, SipC, may play a role. Proteins secreted through the translocon pore, including SopE and SopE2, interact with the small GTPases, Cdc42 and Rac, activating them and the resulting cascades lead to further actin polymerization. Other secreted effectors including SopB, which stimulates actin rearrangements, and SipA, which stabilizes actin filaments, play a role in the process. The end result is substantial actin nucleation and formation of filopodia and lamellipodia that form a pocket around the bacterium. *Salmonella* sp. not only activate the uptake process, but regulate it as well. After the pocket has formed around the bacterium, additional secreted effectors, like SptP, reverse the effects of the first effectors, leading to actin depolymerization and closing of the pocket around the bacterium (Zhou and Galan, 2001).

Although these pathogens have all developed strategies that allow them to invade the cells lining the intestinal epithelium, the mechanisms by which they do so are varied. Additionally, the resulting disease pathology is diverse, both among the different groups of bacteria, as well as in different hosts. This leads to complications in studying these mechanisms in an *in vivo* model. Many of these processes have been elucidated in cell culture and studies in a mouse model are of questionable medical relevance because the effect on the intestinal mucosa is not the same in mice and humans. Despite these difficulties, a large amount is known about the abilities of

these pathogens to take advantage of host cell processes to invade the intestinal mucosa.

M. avium invasion of intestinal epithelial cells

In contrast to other intracellular pathogens of humans, little is known about *M. avium* invasion of intestinal epithelial cells. *M. avium* generally disseminates throughout the body to cause disease only in patients with immune suppression. Indeed, MAC-related infections are the most common bacterial infection in patient with AIDS. Less commonly, *M. avium* can cause infections of the lung in otherwise healthy people. Little is known how the bacteria are able to cross the intestinal mucosa in these patients, however. An early study by Mapother and Songer (1984) showed that, as with other intracellular pathogens, phagocytosis of *M. avium* by human intestinal epithelial cells requires an active process by the bacterium, involving the regulation of the host cell cytoskeleton (Figure 1.6). They showed that this process is unique to the pathogenic mycobacteria, as saprophytes such as *M. smegmatis* are not efficiently phagocytosed by the same cells (Mapother and Songer, 1984). In a later study, using protein kinase inhibitors, Bermudez and Young (1994) showed that *M. avium* could still bind the HT-29 intestinal mucosal cells and HEp-2 laryngeal cells, but was not phagocytosed, indicating that the bacterium was likely binding to a receptor on the host cell surface.

In attempts to determine what these host cell receptors might be, Schorey *et al.* (1996) found that *M. avium* expresses fibronectin attachment proteins (FAPs), indicating that fibronectin and integrins in the extra-cellular matrix could be involved in the invasion process. Labo *et al.* (1998) showed that the *M. avium* genome has

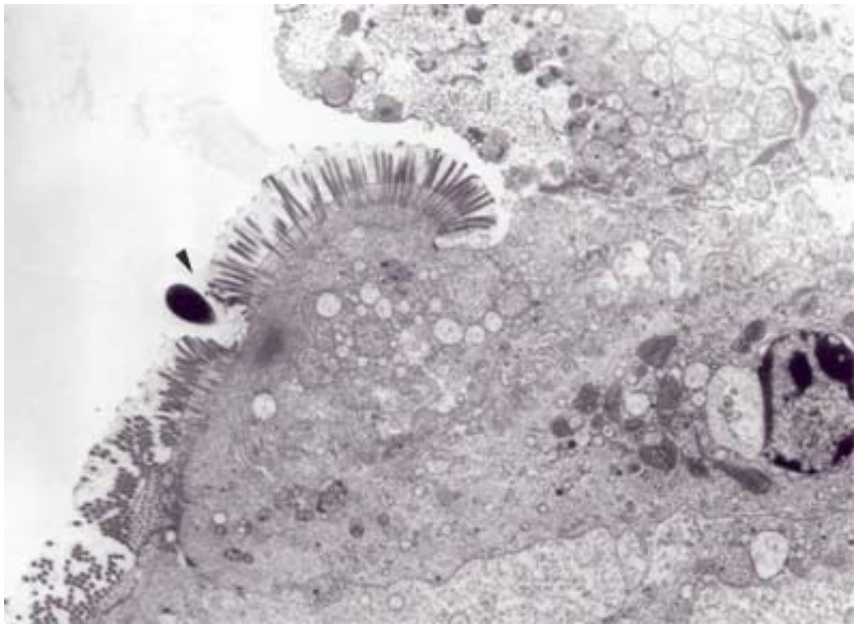

Figure 1.6 Electron micrograph of *Mycobacterium avium* invading epithelial cells of the mouse intestine. Membrane ruffling, effacing of the microvili, and actin filaments are visible, indicating that entry is an active process involving cytoskeletal rearrangement (Luiz E. Bermudez).

genes that are similar to the invasin genes of *Listeria,* further suggesting a means of entry more like the zipper mechanism. Later studies, however, concluded that *M. avium*, unlike other mycobacteria and intracellular pathogens, preferentially enters enterocytes, as opposed to the M cells found in Peyer's patches (2001). Because fibronectin and integrins are expressed only on M cells in the lumen, it is unlikely that FAPs or invasins play an important role in *M. avium* invasion of the intestinal epithelium. No further studies have found additional host cell-binding proteins that could be involved in the invasion of epithelial cells.

Sequencing of the genomes of *M. avium* and other mycobacteria has revealed that mycobacteria do not have type III secretion systems. If *M. avium* uses a trigger mechanism of entry into epithelial cells, it must do so by a novel secretion apparatus. Although the secretion apparatus is not apparent, more recent evidence suggests that

M. avium is able to induce actin polymerization and cytoskeletal rearrangement using mechanisms similar to those of *Salmonella*. In one study, Sangari *et al.* (2000) used pharmacological inhibitors of phosphoinositol-3-kinase (PI-3K) and the small GTPases, Rho, Rac, and Cdc42, to show that PI-3K and Rho were a part of the pathways involved in bacterial uptake into HT-29 cells, while Rac and Cdc42 did not play a role. An *M. avium* invasion mutant analyzed in a recent study suggested that the Cdc42/Arp2/3/N-WASp actin polymerization cascade does play a role in invasion, however (Dam *et al.*, 2006). In this study, interruption of the *M. avium* *fad*D2 gene led to decreased invasion of HEp-2 cells. Further analysis showed that N-WASp was not phosphorylated and did not bind to the Arp2/3 complex upon invasion with the mutant strain. Incubating the mutant strain with supernatant from the wild-type bacteria restored the invasion phenotype to near wild-type levels, suggesting that mycobacteria secrete proteins involved invasion of epithelial cells (Dam *et al.*, 2006).

Although not discussed in the chapters of this thesis, we were unable to detect proteins in the supernatant of the wild-type *M. avium* that were not present in the *fadD2* mutant in normal laboratory infection conditions. It is not yet clear if this process is triggered by binding of the bacterium to specific receptors on the host cell, or if *M. avium* has its own secretion system analogous to that of the TTSS of *Salmonella* and other intracellular pathogens. An aim of this thesis was to identify whether there are *M. avium* proteins that are on the surface or are secreted with roles in invasion.

Gene regulation and expression by intracellular pathogens upon epithelial cell invasion

The expression of many of the genes involved in invasion of epithelial cells is often controlled by a cascade of transcriptional regulators which are in turn regulated by a two-component or other regulatory system in response to an environmental stimulus. In *Salmonella*, the *hilA* gene appears to be central to controlling the expression of genes in the SPI-1 pathogenicity island, which encodes the TTSS and secreted effectors responsible for inducing the cytoskeletal changes seen in epithelial cell uptake of the bacterium. The *hilA* gene is both positively and negatively regulated by a number of factors that recognize environmental stimuli (Johnston *et al.*, 1996; Altier *et al.*, 2000; Fahlen *et al.*, 2000; Wilson *et al*., 2001; Baxter *et al.*, 2003), including the *fadD* gene (Lucas *et al.,* 2000) (Figure 1.7).

No one central regulatory gene has been found for regulation of mycobacterial entry of epithelial cells. Studies have identified a few regulatory genes important to invasion of epithelial cells. As previously mentioned, our laboratory identified the *M. avium fadD2* gene as being involved in regulation of the activation of the host cell Cdc42 signaling pathway leading to actin polymerization (Dam *et al.,* 2006). Another genome-wide screen identified a gene, *fadE20*, which may be involved in epithelial cell invasion (Miltner *et al.,* 2005). More is known about the regulation of genes important to macrophage invasion and survival. Screens in *M. tuberculosis* and *M. avium* have identified a number of genes important to entry and persistance in macrophages, including the two-component response regulators and sensor kinases, *mtrA/B* (Fol *et al*., 2006), *regX3/senX3* (Parish *et al.,* 2003a; Parish *et al.,* 2003b),

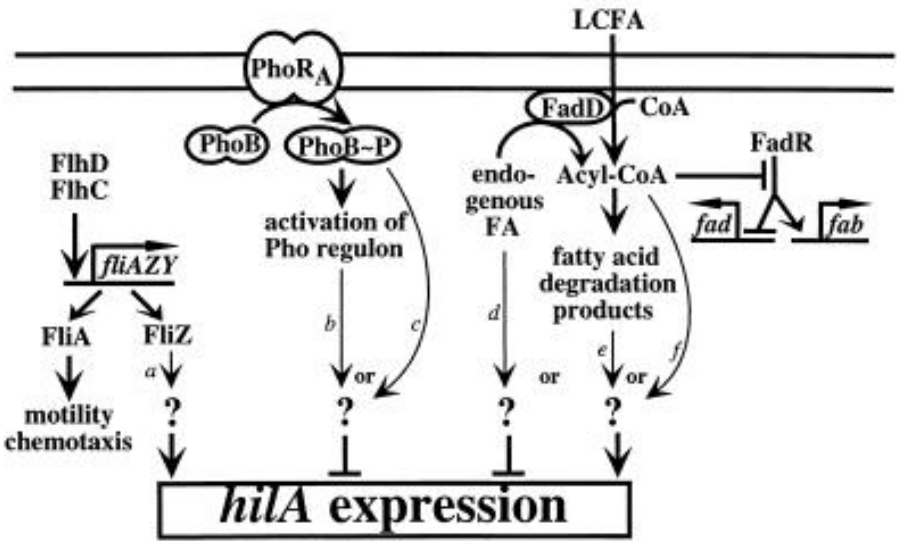

Figure 1.7 Proposed model of regulation of the *Salmonella* pathogenicity island 1 (SPI-1) by *hilA* and other upstream regulators. From Lucas *et al.* (2000)

and *phoP/R* (Perez *et al.,* 2001). Very little is known, however, about either the environmental stimuli that these genes are responding to or the mechanisms by which they induce downstream gene expression. It is also unknown whether any of these regulators also play a role in epithelial cell invasion, a question examined in this thesis.

Zebrafish as a host model for mycobacteriosis

Zebrafish have long been important models in developmental biology research. Recently, this organism has also been recognized for its advantages as a model host for pathogens, including *Salmonella typhimurium* (van der Sar *et al.,* 2003), *Streptococcus pyrogenes* (Neely *et al.,* 2002; Brenot *et al.*, 2004), and *M. marinum* (Davis *et al.*, 2000; Prouty *et al.,* 2003). Zebrafish have the potential to be significant tools to study pathogens of mammals, as they have advantages over other commonly

used models. Unlike familiar invertebrate models, such as the *Drosophila* and *C. elegans,* zebrafish have a fully developed innate and adaptive immune system (van der Sar *et al.,* 2004). Additionally, both forward and reverse (Wienholds *et al.,* 2003) genetic screens, and real-time visualization (Davis *et al.,* 2000; van der Sar *et al.,* 2003) are possible, giving the zebrafish advantages over other vertebrate infection models. Zebrafish are not without disadvantages, however, as there are still limited tools for immune system analyses, few cell lines, and a lower incubation temperature than mammalian cells (van der Sar *et al.,* 2004).

Prouty *et al.* (2003) and Pozos and Ramakrishnan (2004) have overcome the potential problem of the incubation temperature of *M. tuberculosis* compared to zebrafish by developing *M. marinum* infection of zebrafish as a surrogate model for *M. tuberculosis* infections. Using bacteria expressing fluorescent markers, the Ramakrishnan group has been able to track the movement of *M. marinum* in the macrophages of translucent zebrafish embryos (Davis *et al.*, 2000; Volkman *et al.,* 2004; Swaim *et al.,* 2006), gaining more insight into the formation of and persistence of mycobacteria within graulomas.

Concluding Remarks

Briefly, the aims of this thesis involved analyzing the following:

- The interaction of environmental mycobacteria (specifically *M. avium*) with the host intestinal epithelium.
- Comparative genomics and virulence of environmental mycobacteria isolated from humans and fish.
- Mycobacterial infection of zebrafish.

The objective of the aims in this thesis was to gain more understanding of the interaction of environmental mycobacteria, particularly species causing disease in humans and fish, with the intestinal cell epithelium and macrophages. *M. avium* is a species we know more about, so the first aim involved further elucidating the role of the *fadD2* gene and other *M. avium* proteins that interact with host cell Cdc42 in the invasion process. Based on literature suggesting a role for *fadD* in regulation of intestinal cell invasion in *Salmonella*, gene expression in the *M. avium fadD2* mutant deficient in epithelial cell invasion was further analyzed. Transcriptional regulators under the control of the *fadD2* gene were examined for their ability to influence the regulation of genes involved in invasion of epithelial cells. Additionally, *M. avium* proteins that putatively interact with host cell Cdc42 were identified and investigated for their role in invasion.

Many other species of environmental mycobacteria are important to fish health, and a number of species infecting fish also have zoonotic potential. To examine the mechanisms of invasion for some of these species, assays were performed to screen for invasion and growth in macrophages from humans, mice, and carp. Additionally, genomic DNA from these species was hybridized against a custom DNA microarray containing probes representing known virulence genes of mycobacteria.

Many of these environmental mycobacteria cause infections in zebrafish, an important model for developmental research and infectious diseases. *In vivo* infection experiments to identify the route of mycobacterial infection in zebrafish were

conducted, and *M. avium* was tested in this system to determine if zebrafish may be a model organism to study the interaction of *M. avium* with the intestinal epithelium.

References

Alonso, A., and F. Garcia-del Portillo. 2004. Hijacking of eukaryotic functions by intracellular bacterial pathogens. Int Microbiol **7:**181-191.

Altier, C., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer. 2000. Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. Mol Microbiol **35:**635-646.

Baxter, M. A., T. F. Fahlen, R. L. Wilson, and B. D. Jones. 2003. HilE interacts with HilD and negatively regulates *hilA* transcription and expression of the *Salmonella enterica* serovar *Typhimurium* invasive phenotype. Infect Immun **71:**1295-1305.

Bermudez, L. E., and L. S. Young. 1994. Factors affecting invasion of HT-29 and HEp-2 epithelial cells by organisms of the *Mycobacterium avium* complex. Infect Immun **62:**2021-2026.

Brenot, A., K. Y. King, B. Janowiak, O. Griffith, and M. G. Caparon. 2004. Contribution of glutathione peroxidase to the virulence of *Streptococcus pyogenes*. Infect Immun **72:**408-413.

Brown-Elliott, B. A., and R. J. Wallace, Jr. 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin Microbiol Rev **15:**716-746.

Chinabut, S. 1999. Mycobacteriosis and Nocardiosis. CAB International, New York.

Cossart, P., and P. J. Sansonetti. 2004. Bacterial invasion: the paradigms of enteroinvasive pathogens. Science **304:**242-248.

Dam, T., L. Danelishvili, M. Wu, and L. E. Bermudez. 2006. The *fadD2* Gene Is Required for Efficient *Mycobacterium avium* Invasion of Mucosal Epithelial Cells. J Infect Dis **193:**1135-1142.

Davis, J. M., H. Clay, J. L. Lewis, N. Ghori, P. Herbomel, and L. Ramakrishnan. 2002. Real-time visualization of *mycobacterium*-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity **17:**693-702.

Decostere, A., K. Hermans, and F. Haesebrouck. 2004. Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. Vet Microbiol **99:**159-166.

Fahlen, T. F., N. Mathur, and B. D. Jones. 2000. Identification and characterization of mutants with increased expression of *hilA*, the invasion gene transcriptional activator of *Salmonella typhimurium*. FEMS Immunol Med Microbiol **28:**25-35.

Fol, M., A. Chauhan, N. K. Nair, E. Maloney, M. Moomey, C. Jagannath, M. V. Madiraju, and M. Rajagopalan. 2006. Modulation of *Mycobacterium tuberculosis* proliferation by MtrA, an essential two-component response regulator. Mol Microbiol **60:**643-657.

Harriff, M. J., L. E. Bermudez, and M. L. Kent. 2007. Experimental Exposure of zebrafish (*Danio rerio* Hamilton) with *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: A potential model for environmental mycobacterial infection. J Fish Dis. **29:** 1-13.

Herbst, L.H., S.F. Costa, L.M. Weiss, L.K. Johnson, J. Bartell, R. Davis, M. Walsh & M. Levi. 2001. Granulomatous skin lesions in moray eels caused by a novel *Mycobacterium* species related to *Mycobacterium triplex*. Infection and Immunity **69,** 4639-46.

Johnston, C., D. A. Pegues, C. J. Hueck, A. Lee, and S. I. Miller. 1996. Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. Mol Microbiol **22:**715-727.

Kent, M. L., C. M. Whipps, J. L. Matthews, D. Florio, V. Watral, J. K. Bishop-Stewart, M. Poort, and L. Bermudez. 2004. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. Comp Biochem Physiol C Toxicol Pharmacol **138:**383-390.

Labo, M., L. Gusberti, E. D. Rossi, P. Speziale, and G. Riccardi. 1998. Determination of a 15437 bp nucleotide sequence around the *inhA* gene of *Mycobacterium avium* and similarity analysis of the products of putative ORFs. Microbiology **144 (Pt 3):**807-814.

Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee. 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar *typhimurium*. J Bacteriol **182:**1872-1882.

Mapother, M. E., and J. G. Songer. 1984. In vitro interaction of *Mycobacterium avium* with intestinal epithelial cells. Infect Immun **45:**67-73.

McGee, K., M. Zettl, M. Way, and M. Fallman. 2001. A role for N-WASP in invasin-promoted internalisation. FEBS Lett **509:**59-65.

Miltner, E., K. Daroogheh, P. K. Mehta, S. L. Cirillo, J. D. Cirillo, and L. E. Bermudez. 2005. Identification of *Mycobacterium avium* genes that affect invasion of the intestinal epithelium. Infect Immun **73:**4214-4221.

Momotani, E., D. L. Whipple, A. B. Thiermann, and N. F. Cheville. 1988. Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. Vet Pathol **25:**131-137.

Neely, M. N., J. D. Pfeifer, and M. Caparon. 2002. *Streptococcus*-zebrafish model of bacterial pathogenesis. Infect Immun **70:**3904-3914.

Parish, T., D. A. Smith, S. Kendall, N. Casali, G. J. Bancroft, and N. G. Stoker. 2003. Deletion of two-component regulatory systems increases the virulence of *Mycobacterium tuberculosis*. Infect Immun **71:**1134-1140.

Parish, T., D. A. Smith, G. Roberts, J. Betts, and N. G. Stoker. 2003. The *senX3-regX3* two-component regulatory system of *Mycobacterium tuberculosis* is required for virulence. Microbiology **149:**1423-1435.

Perez, E., S. Samper, Y. Bordas, C. Guilhot, B. Gicquel, and C. Martin. 2001. An essential role for *phoP* in *Mycobacterium tuberculosis* virulence. Mol Microbiol **41:**179-187.

Petrini, B. 2006. *Mycobacterium marinum*: ubiquitous agent of waterborne granulomatous skin infections. Eur J Clin Microbiol Infect Dis **25:**609-613.

Poort, M. J., C. M. Whipps, V. G. Watral, W. F. Font, and M. L. Kent. 2006. Molecular characterization of a *Mycobacterium* species in non-native poeciliids in Hawaii using DNA sequences. J Fish Dis **29:**181-185.

Pozos, T. C., and L. Ramakrishnan. 2004. New models for the study of *Mycobacterium*-host interactions. Current opinion in immunology **16:**499-505.

Prouty, M. G., N. E. Correa, L. P. Barker, P. Jagadeeswaran, and K. E. Klose. 2003. Zebrafish-*Mycobacterium* marinum model for mycobacterial pathogenesis. FEMS Microbiol Lett **225:**177-182.

Rhodes M.W., H. Kator, I. Kaattari, D. Gauthier, W. Vogelbein & C.A. Ottinger. 2004. Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay. Diseases of Aquatic Organisms **61,** 41-51.

Rosenshine, I., V. Duronio, and B. B. Finlay. 1992. Tyrosine protein kinase inhibitors block invasin-promoted bacterial uptake by epithelial cells. Infect Immun **60:**2211-2217.

Sangari, F. J., J. Goodman, and L. E. Bermudez. 2000. *Mycobacterium avium* enters intestinal epithelial cells through the apical membrane, but not by the basolateral surface, activates small GTPase Rho and, once within epithelial cells, expresses an invasive phenotype. Cell Microbiol **2:**561-568.

Sangari, F. J., J. Goodman, M. Petrofsky, P. Kolonoski, and L. E. Bermudez. 2001. *Mycobacterium avium* invades the intestinal mucosa primarily by interacting with enterocytes. Infect Immun **69:**1515-1520.

Sangari, F. J., A. Parker, and L. E. Bermudez. 1999. *Mycobacterium avium* interaction with macrophages and intestinal epithelial cells. Front Biosci **4:**D582-588.

Schorey, J. S., M. A. Holsti, T. L. Ratliff, P. M. Allen, and E. J. Brown. 1996. Characterization of the fibronectin-attachment protein of *Mycobacterium avium* reveals a fibronectin-binding motif conserved among mycobacteria. Mol Microbiol **21:**321-329.

Sigur-Dardottir, O. G., C. M. Press, and O. Evensen. 2001. Uptake of *Mycobacterium avium* subsp. *paratuberculosis* through the distal small intestinal mucosa in goats: an ultrastructural study. Vet Pathol **38:**184-189.

Sigur-Dardottir, O. G., A. M. Bakke-McKellep, B. Djonne, and O. Evensen. 2005. *Mycobacterium avium* subsp. *paratuberculosis* enters the small intestinal mucosa of goat kids in areas with and without Peyer's patches as demonstrated with the everted sleeve method. Comp Immunol Microbiol Infect Dis **28:**223-230.

Swaim, L. E., L. E. Connolly, H. E. Volkman, O. Humbert, D. E. Born, and L. Ramakrishnan. 2006. *Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. Infect Immun **74:**6108-6117.

van der Sar, A. M., B. J. Appelmelk, C. M. Vandenbroucke-Grauls, and W. Bitter. 2004. A star with stripes: zebrafish as an infection model. Trends Microbiol **12:**451-457.

van der Sar, A. M., R. J. Musters, F. J. van Eeden, B. J. Appelmelk, C. M. Vandenbroucke-Grauls, and W. Bitter. 2003. Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections. Cell Microbiol **5:**601-611.

Volkman, H. E., H. Clay, D. Beery, J. C. Chang, D. R. Sherman, and L. Ramakrishnan. 2004. Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. PLoS biology **2:**e367.

Whipps, C. M., S. T. Dougan, and M. L. Kent. 2007. *Mycobacterium haemophilum* infections of zebrafish (*Danio rerio*) in research facilities. FEMS Microbiol Lett **270:**21-26.

Whipps, C. M., V. G. Watral, and M. L. Kent. 2003. Characterization of a *Mycobacterium* sp. in rockfish, *Sebastes alutus* (Gilbert) and *Sebastes reedi* (Westrheim & Tsuyuki), using rDNA sequences. J Fish Dis **26:**241-245.

Wienholds, E., F. van Eeden, M. Kosters, J. Mudde, R. H. Plasterk, and E. Cuppen. 2003. Efficient target-selected mutagenesis in zebrafish. Genome research **13:**2700-2707.

Wilson, R. L., S. J. Libby, A. M. Freet, J. D. Boddicker, T. F. Fahlen, and B. D. Jones. 2001. Fis, a DNA nucleoid-associated protein, is involved in *Salmonella typhimurium* SPI-1 invasion gene expression. Mol Microbiol **39:**79-88.

Zhou, D., and J. Galan. 2001. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect **3:**1293-1298.

Chapter 2

Mycobacterium avium genes MAV_5138 and MAV_3679 are transcriptional regulators that play a role in invasion of epithelial cells, in part by their regulation of CipA, a putative surface protein interacting with host cell signaling pathways

Melanie J. Harriff[1,3], Martin Wu[1], Cara Wilder[2], Michael McNamara[1,3], Michael L. Kent[1,2,3], and Luiz E. Bermudez[1,2,3]

Journal of Bacteriology: *In preparation*

[1]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis OR, 97331
[2]Department of Microbiology, Oregon State University, Corvallis OR, 97331
[3]Molecular and Cellular Biology Program, Center for Gene Research and Biotechnology, Oregon State University, Corvallis, Oregon, USA

Abstract

The *Mycobacterium avium* complex (MAC) is an important group of opportunistic pathogens for birds, cattle, swine, and immune-suppressed humans. Although invasion of epithelial cells lining the intestine is the chief point of entry for these organisms, little is known about the mechanisms by which members of the MAC are taken up by these cells. Studies with *M. avium* have shown that cytoskeletal rearrangement via activation of the small G-protein Cdc42 is involved, and that this activation is regulated in part by the *M. avium fadD2* gene. We show that the *fadD2* gene regulates a number of genes upon exposure to HEp-2 cells, including transcriptional regulators, membrane proteins, and secreted proteins. Over-expression of two of these repressor regulators (MAV_5138 and MAV_3679) led to increased invasion of HEp-2 cells, as well as altered expression of other genes. The protein product of one of the regulated genes, named CipA, was shown to putatively interact with Cdc42. This protein has domains that resemble the PXXP motif of human Piccolo proteins, which bind SH3 domains in proteins involved in the scaffold complex formed during cytoskeletal rearrangement. Although CipA was not detected in the cytoplasm of HEp-2 cells exposed to *M. avium*, the recombinant protein was shown to be expressed on the surface of *Mycobacterium smegmatis* incubated with HEp-2 cells. These results suggest that members of the MAC have a novel mechanism for activating cytoskeletal rearrangement, prompting uptake by host epithelial cells, and that this mechanism is regulated in part by *fadD2*, MAV_5138, and MAV_3679.

Introduction

Environmentally encountered organisms of the *Mycobacterium avium* complex (MAC) are known for being pathogens of birds and swine, and are a common cause of opportunistic infection in patients with AIDS. In non-AIDS patients, MAC can be isolated as the etiologic agent of lung infections. In AIDS patients, *M. avium* enters the intestinal mucosa primarily through the epithelial lining of the small intestine (Damsker and Bottone, 1985). After translocation of the bacterium across the epithelium cell, the bacterium is taken up by submucosal macrophages and disseminates.

Understanding of the invasion of host mucosal epithelial cells by MAC has been slow due to difficulties in genetic manipulation of these organisms. It is known, however, that other intracellular pathogens impact host signaling pathways triggering cytoskeletal rearrangement as a means to achieve invasion of non-phagocytic epithelial cells. For example, *Cryptosporidium parvum* has been shown to affect actin polymerization in host cells during invasion (Elliot *et al.,* 2001), and *Bordatella* (Masuda *et al.,* 2002) and *Salmonella* (Stender *et al.,* 2000) can modify small GTPases such as Rac and Cdc42 through activities similar to eukaryotic guanine nucleotide exchange factors and GTPase-activating proteins.

A transposon mutant library of MAC serovar 109 (MAC109) was screened in our laboratory for clones with impaired ability to enter human laryngeal epithelial (HEp-2) cells. A number of genes were found to be important for invasion of these cells, including the *fad*D2 gene (Dam *et al.,* 2006). This gene encodes a fatty acyl CoA synthetase involved in fatty acid degradation. In *Salmonella*, the *fad*D gene has

been established as a regulator of invasion through *hil*A expression (Lucas *et al.,* 2000).

Further analysis of the Δ*fad*D2 mutant strain of MAC109 (MAC109: Δ*fad*D2) showed that this strain did not activate the Cdc42 pathway leading to cytoskeletal reorganization. Previous studies have shown that Cdc42 activates N-WASp indirectly through phosphorylation, and that N-WASp subsequently binds and activates the Arp2/3 complex, leading to actin polymerization (Rohatgi *et al.,* 1999). Studies in our laboratory indicated that invasion by the Δ*fad*D2 mutant did not result in N-WASp phosphorylation or binding to and activation of the Arp2/3 complex. The Δ*fad*D2 mutant invasion efficiency could be restored by addition of supernatant from HEp-2 cells infected with the wild-type MAC109 strain (Dam *et al.,* 2006), suggesting the presence of secreted proteins and secretory systems associated with this mechanism of invasion.

Very little is known about surface proteins or secretory systems of mycobacteria involved in epithelial cell invasion. In *Mycobacterium tuberculosis* and *Mycobacterium avium* subsp. *paratuberculosis*, a number of secreted or surface proteins have been shown to be involved in macrophage or epithelial cell entry, including the mycobacterial cell entry (Mce) family of proteins (Gioffre *et al.*, 2005), the ESAT-6 family of proteins (Brodin *et al.,* 2004), a tyrosine phosphatase (PtpA) (Bach *et al.,* 2006), and the heparin-binding hemagglutinin protein (HbhA) (Reddy and Hayworth, 2002; Sechi *et al.,* 2006), but the mechanisms by which these proteins function in invasion are unknown. Kitaura and colleagues (2000) found five *M. avium* proteins that bind fibronectin, including Ag85 and Mpb51. Fibronectin is

expressed on the surface of M cells rather than enterocytes, and *M. avium* preferentially enters enterocytes (Sangari *et al.,* 2000), suggesting that these proteins are not primarily important for epithelial cell invasion. A recent study identifying secreted proteins of *M. tuberculosis* by proteomic methods indicated that a large portion of the secreted proteins were previously unknown, and that almost 40% of the proteins were secreted by a mechanism other than the general secretory pathway (Malen *et al.,* 2007), indicating there are also likely to be many surface and secreted proteins and systems by which these proteins are secreted by *M. avium* that are not yet identified.

In the present study, the role of the *fad*D2 gene in regulation of invasion was examined and putative surface or secreted proteins that could be responsible for the effect on the Cdc42 signaling pathway were identified. Our results suggest that *M. avium* invasion of epithelial cells is regulated in part by *fad*D2 and other downstream transcriptional regulators, and that the mechanism of invasion involves the activation of actin polymerization through interaction of a bacterial surface structure with the host cell membrane and Cdc42.

Materials and Methods

Cell Culture. Laryngeal cells (HEp-2 cells) were obtained from the American Type Culture Collection (ATCC, Catalog #CCL-23) and cultured in RPMI 1640 medium (Invitrogen, Carlsbad CA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island NY). Cells were cultured in 25 cm^2 or 75 cm^2 flasks (Corning).

Bacteria. *M. avium* serovar 109 (MAC109) and *M. avium* serovar 104 (MAC104) are virulent clinical isolates obtained from the blood of AIDS patients. *Mycobacterium smegmatis* mc^2 155 was a gift from Dr. William Jacobs Jr. (Albert Einstein School of Medicine, NY). All mycobacterial strains were cultured on 7H11 Middlebrook agar or in 7H9 Middlebrook broth (Difco Laboratories, Detroit MI) with 10% oleic acid, albumin, dextrose, and catalase (OADC, Hardy Diagnostics). The *fad*D2 transposon mutant (MAC109:Δ*fadD2*) was generated as described by Dam *et al.* (2006) and was grown on media supplemented with 400 μg/ml kanamycin. Other recombinant strains were generated as described below and cultured in broth or media supplemented with either 400 μg/ml or 50 μg/ml kanamycin. For infection, bacteria were grown at 37 °C to log phase in broth prior to inoculation. Luria-Bertani broth and agar (Difco Laboratories, Detroit MI) with indicated antibiotics were used to culture all *Esherichia coli* strains.

RNA extraction from mycobacteria. RNA was isolated and purified from mycobacterial strains as follows. Thirty ml mid-log culture of the wild-type and mutant were each divided into two parts, then centrifuged at 3000 g at 4°C. The pellets were washed with Hank's balanced salt solution (HBSS, Invitrogen, Carlsbad CA) and re-suspended in HBSS at a concentration of 3 X 10^8/ml. HEp-2 cells that were first washed with HBSS were exposed to the wild-type and recombinant MAC109 strains for 15 minutes at 37°C. Extracellular bacteria were then recovered from the flasks, and centrifuged at 3600 rpm at 4 °C, in addition to the bacteria resuspended in HBSS that were not exposed to cells. The pellets were suspended in 1 mL Trizol (Invitrogen, Carlsbad CA) and transferred to 2 mL screw cap tubes with

0.4 mL glass beads. The samples were shaken three times for 30 sec at maximum speed in a bead beater, and periodically inverted. RNA was extracted from the aqueous Trizol solution with Chloroform:isoamyl alcohol (24:1) and isopropanol and washed with 75% ethanol. Resuspended RNA was treated with TurboDNase (Ambion, Austin TX) for 30m at 37 °C, and precipitated with 100% ethanol and 3M sodium acetate (pH 5.2). Purity and quantity were analyzed by spectrophotometer.

Preparation of samples for microarray analysis. cDNA probe synthesis and array hybridization was performed using the Sigma Genosys Panorama cDNA Labeling and Hybridization kit (Sigma-Genosys, St. Louis MO) according to the manufacturer's protocol. Briefly, 3 μg of each RNA sample was combined with cDNA labeling primers and heated to 90°C for 2 minutes. After primer annealing, buffer, AMV Reverse Transcriptase, dNTPs and ^{32}P-dCTP (Amersham, Buckinghamshire UK) were added to each sample and incubated at 42°C for 3 hours. Unincorporated nucleotides were removed using the Sephadex G-25 gel-filtration spin column.

Hybridization and data analysis. The Panorama gene array blots, containing probes representing all known *M. tuberculosis* open reading frames, were pre-hybridized in the Hybridization Solution (2X SSPE) for 1 hour at 65°C. The labeled samples were denatured in 2X SSPE and incubated with the blots overnight at 65°C. The blots were washed three times at room temperature with washing solution, three times with washing solution pre-heated to 65 °C, then air-dried. Phosphorimaging of the blots was performed using an Amersham Storm 840 at 50 μM resolution. Data files were sent to Sigma-Genosys for quantification of gene expression signals. Fold-change in

expression of genes after exposure to HEp-2 cells as compared to incubation in HBSS was analyzed for the wild-type MAC109 and MAC109:Δ*fad*D2.

Real-time PCR. HEp-2 cells were exposed to wild-type MAC109 and the MAC109:Δ*fad*D2, or the MAC109:pMH6 or MAC109:pMH7 over-expression strains (see below). After no exposure or a 15-minute exposure, RNA was extracted from the bacteria as described above. cDNA was generated from the RNA using the SuperScript III First Strand Synthesis for RT-PCR Kit (Invitrogen, Carlsbad CA) per the manufacturer's instructions. Briefly, 3 μg of each RNA was combined with 50 ng/μl random hexamers and a 10 mM dNTP mix and incubated at 65 °C for 5 minutes. 10X RT Buffer, 25 mM $MgCl_2$, 0.1 M DTT, and 40 U RNaseOUT were added to the primed RNAs and incubated at room temperature for 2 minutes. 200 U SuperScript III Reverse Transcriptase was added to each sample, and incubated for 10 minutes at room temperature, followed by 50 minutes at 50 °C. The reactions were terminated for 15 minutes at 70 °C, and then treated with RNase H for 20 minutes at 37 °C. Selected genes were amplified by Real-time PCR in a BioRad iQ iCycler, using SybrGreen (BioRad, Hercules CA) and the primers listed in Table 2.1. Fold-change in gene expression after exposure to HEp-2 cells was determined by the following formula:

$$\Delta Ct_{exp} = Ct_{exp/gene} - Ct_{exp/16S}$$

$$\Delta Ct_{cont} = Ct_{cont/gene} - Ct_{cont/16S}$$

$$\Delta(\Delta Ct) = Ct_{exp} - Ct_{cont}$$

$$\text{Fold change} = 2^{-\Delta(\Delta Ct)}$$

where “exp” refers to RNA samples from bacteria after exposure to HEp-2 cells, and “cont” refers RNA samples from bacteria in HBSS.

Vector construction. Strains of MAC109 over-expressing the MAV_5138 and MAV_3679 transcriptional repressor regulator genes were constructed as follows: Using the primers indicated in Table 2.2, the gene sequences were amplified from MAC109 genomic DNA, and the amplicons were ligated into pLDG13, a *Mycobacterium/E. coli* shuttle vector containing the strong G13 constitutive promoter from *Mycobacterium marinum* (Danelishvili *et al.*, 2005), at the HinDIII and EcoRI restriction sites. After screening for insertion in *E. coli*, the recombinant plasmids, pLDG13:MAV_5138 and pLDG13:MAV_3679 (named pMH7 and pMH6, respectively) were transformed to competent MAC109, resulting in MAC109:pMH6 and MAC109:pMH7.

For the two-hybrid screen, primers indicated in Table 2.2 were used to amplify human *cdc*42 placental isoform from the recombinant pcDNA3.1 plasmid provided by the Guthrie Research Institute (Sayre, PA). This fragment was then cloned into the pBT plasmid (Stratagene, LaJolla, CA) in frame with the λcI repressor gene at the EcoRI restriction site. Transformants were plated on LB agar containing 34.5 μg/ml chloramphenicol and screened by digestion and PCR. MAC104 genomic DNA was partially digested with Sau3A, and cloned into the pTRG plasmid (Stratagene, LaJolla CA) at the BamHI restriction site. Transformants were plated on LB agar containing 12.5 μg/ml tetracycline. 70,000 colonies were selected from 10 ligations plated onto 100 plates and combined to create an *M. avium* library downstream of the RNAP-α gene fragment (pTRG:MAClib).

Table 2.1 Primer sequences used for Real-time PCR[a].

Gene[b]	Forward primer	Reverse primer
16S rDNA	GAGTAACACGTGGGCAATCTG	GTCTGGGCCGTATCTCA
CipA	CCTGCGTGACCCGGTCTC	GCCGGACGTCATTGATATTGA
MAV_5138	GGTGCGGCCGTCTACTTCCAG	GGTGGTGCGGATCTCCTCGTC
MAV_3679	CCTCCGAGTCGATCCGCAAAC	CGTCGGTGAAATAGCGCAGCA
MAV_4139	CAGCGAGATCCTGACGTTCCT	GCACCCAGCCCTCGATCA
MAV_1190	CCGAGTGGTTGACCCGCATGA	GCCTGGGCGATGTCGATGGTG
MAV_3436	GTTCGGCTGGGCGTGGAA	CTCAGCCCGATCAGCGTGGAG
MAV_4666	GCTGCGGCTGAAGTACCAG	AGACGGTGCGGTCGGAGTTGA
MAV_4360	GCCGGGGGACAGGGACGTTAC	CGAGTTGGCGCAGGAACGA

[a] All PCR reactions were performed with an annealing temperature of 60 °C, with an extension time of 30 s.
[b]The forward and reverse primers amplify fragments of the indicated genes ranging in size from 200 - 400 base pairs.

The *M. avium* MAV_4671 gene (termed *cipA* for "Cdc42 interacting protein") was amplified from MAC104 genomic DNA using the primers listed in Table 2.2. The PCR product was cloned into the pBT plasmid at the EcoRI restriction site. The human *cdc*42 placental isoform gene product was amplified as indicated above and cloned into the pTRG plasmid at the EcoRI restriction site.

To construct a CipA:CyaA fusion protein, the primers listed in Table 2.2 were used to amplify the DNA encoding amino acids 2-400 of the *Bordatella pertussis cyaA* gene from pACYA, a kind gift from Gregory V. Plano (University of Miami, FL). The *cyaA* PCR product was ligated into pLDG13 at the PstI restriction site and screened in *E. coli*, resulting in pMH4. The *cipA* PCR product was amplified from MAC104 using the primers listed in Table 2.2. pMH4 and the CipA PCR product were digested with EcoRI, ligated, and screened in *E. coli*, resulting in pMH5, a

plasmid containing an in-frame fusion of *cipA* and *cyaA* behind the strong G13 promoter. The completed pMH5 construct was transformed by electroporation to MAC104 competent cells, and the resulting colonies were screened by PCR to confirm the presence of the plasmid. Resulting transformants were called MAC104:pMH5.

A promoterless GFPmut2 gene (obtained from Rafael Valdivia and Stanley Falkow, Stanford University) was inserted in the HinDIII and EcoRI sites of the pMV261 vector containing the Hsp60 promoter, resulting in pMV261:GFP. *CipA* was inserted between the promoter and the GFPmut2 gene at BamHI and EcoRI sites, in frame with GFP. Colonies were screened in *E. coli*, and the resulting pMV261:CipA:GFP was transformed to competent *M. smegmatis* mc^2155. PCR was used to screen *M. smegmatis* expressing GFP for the presence of the CipA:GFP sequence.

HEp-2 cell invasion assays. Assays were performed as described previously by Sangari and colleagues (2000). Briefly, MAC109, MAC109:pMH6, and MAC109:pMH7 were adjusted to 10^8/mL by McFarland standards and verified by plating serial dilutions. For each strain, 100 µL of this inoculum was added to 4 wells of a 24-well culture plate containing HEp-2 cells. After 30 minutes or a 1-hour infection time, the supernatant was removed, and the wells were washed 3 times with HBSS to remove extracellular bacteria. Sterile water containing a 1:5 dilution of 0.025% sodium dodecyl sulfate (SDS) was added to the wells to lyse the cells. The lysate was diluted serially and plated onto 7H11 agar to determine the colony forming units per ml (CFU/ml). Assays were performed in replicate, and the resulting CFUs

Table 2.2 Bacterial strains constructed for this study.

Bacterial strain name	Vector Name	Bacterial host	Use	Parent vector	Gene insert	Forward primer sequence	Reverse primer sequence
MAC109: pMH6	pMH6	*M. avium*	Over-expression of MAV_3679	pLDG13[a]	MAV_3679	TTTAAGCTTGCACCC GGATGCACGACGCTG	TTTGAATTCCTACGACG ACGAGAGGGTCAGGGG
MAC109: pMH7	pMH7	*M. avium*	Over-expression of MAV_5138	pLDG13[a]	MAV_5138	TTTAAGCTTGTGAGG GCTGACGAAGAGCGT	TTTGAATTCTTATGCCGT TACCCAGATCGC
E. coli: pBT:*cdc42*	pBT:*cdc42*	*E. coli*	Bacteriomatch 2-hybrid screen	pBT	*cdc42*[b]	GTGGAATTCCACCAT GCAGACAATTAAGTG	GTGGAATTCAACGGGCC CTCTAGACTCGAG
E. coli: pBT:*cipA*	pBT:*cipA*	*E. coli*	Bacteriomatch 2-hybrid screen	pBT	*cipA*	GAATTCCAACGTGAC GAACCCACAAGGACC	GATGAATTCGAGGTTGG CGAAGCAGGGGTCGTT
E. coli: pTRG:*cdc42*	pTRG: *cdc42*	*E. coli*	Bacteriomatch 2-hybrid screen	pTRG	*cdc42*	GTGGAATTCCACCAT GCAGACAATTAAGTG	GTGGAATTCAACGGGCC CTCTAGACTCGAG
MAC104: pMH4	pMH4	*M. avium*	MAV_4671: *Bordetella pertussis cyaA* fusion protein	pLDG13[a]	*cyaA*[c]	TTTCTGCAGCAATCG CATGAGGCTGGTTAC	TTTCTGCAGCTAGCGTT CCATCGCGCCCAG
MAC104: pMH5	pMH5	*M. avium*	MAV_4671: *Bordetella pertussis cyaA* fusion protein	pMH4	*cipA*	GAATTCCAACGTGAC GAACCCACAAGGACC	GATGAATTCGAGGTTGG CGAAGCAGGGGTCGTT
M. smeg:GFP	pGFP	*M. smegmatis*	Fluorescence microscopy	pMV261	GFPmut2[d]	GCTGGATCCTTGCTC AGGTTGGTAGGGC	GAGAATTCCGAAGCAG GGGTCGTTGTTG
M. smeg: CipA: GFP	pGFP:CipA	*M. smegmatis*	Fluorescence microscopy	pGFP	*cipA*	TCTGAATTCAAGAAG GAGATATACATAT	CAGGTAACTTATTTATT TTGTATAGTTC

[a] Danelishvili *et al.* (2005)

[b] Amplified from the pcDNA3.1:*cdc42* plasmid, obtained from the Guthrie Research Institute, Sayre PA

[c] Amino acids 2-400, amplified from pACYA (Day *et al*. (2003))

[d] Obtained from Rafael Valdivia and Stanley Falkow, Stanford University

from all assays were analyzed compared to the inoculum to determine the percent invasion after 30 minutes and 1 hour for the three strains.

Two-hybrid screen for protein-protein interaction. The recombinant pBT:*cdc*42 and pTRG:MAClib were co-transformed into the Bacteriomatch Two-Hybrid System (Stratagene, LaJolla CA) reporter strain *E. coli*. Transformants were plated onto LB agar containing 400 μg/ml carbenicillin, 12.5 μg/ml tetracycline, 34.5 μg/ml chloramphenicol, and 50 μg/ml kanamycin. Colonies that grew after 30 hours at 30° C were picked from these plates and transferred to LB agar containing the same antibiotics except carbenicillin and 80 μg/ml X-Gal and 200 μM phenylethyl β-D-thio galactoside (an X-Gal inhibitor), prepared in dimethyl formamide. Colonies that became blue in color in the presence of X-Gal and the inhibitor after 17 hours at 30 °C were further analyzed to determine the *M. avium* sequence of the insert in the pTRG vector. Co-transformation of the pBT:*cipA* vector with pTRG:*cdc*42 vectors and pBT:*cipA* with the pTRG:MAClib, and screening of the resulting transformants was repeated as described above.

Protein extraction and Western blot analysis. MAC104 and MAC104:pMH5 were grown to log phase in 7H9 media without OADC. After centrifugation, the pellets were resuspended in HBSS and the resulting inocula were used to infect HEp-2 cells in 6-well tissue culture plates or 75 cm^2 flasks at an MOI of 100:1. All steps post-infection were completed at 4° C or on ice. At 30 min, 1 hr, and 2 hr time points, the extracellular bacteria were removed from the wells and pelleted by centrifugation. The infected HEp-2 cells were lysed in water containing a protease inhibitor cocktail (Sigma), and then centrifuged to remove the cellular debris and intracellular bacteria.

The contents of the HEp-2 cells after lysis and centrifugation were incubated with mouse monoclonal α-CyaA [Santa Cruz Biotech (SCBT), CA] and agarose-conjugate α-IgG beads (SCBT, CA) overnight at 4 °C. The beads were washed four times with PBS and resuspended in Laemmli sample loading buffer (BioRad, Hercules CA). After denaturation by boiling for 5 minutes, the protein samples were run on a 12% Tris-HCl protein gel (Bio-Rad, Hercules, CA) for 1 hr at 150 V. The proteins were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus with a constant current of 15 V for 1 hr.

Western blotting was performed using the Odyssey Western Blotting system by Li-Cor (Lincoln NE) per the manufacturer's instructions. Briefly, the membrane was blocked in a 1:1 dilution of TBS and Odyssey blocking buffer overnight at 4° C. The membrane was then incubated with mouse α-cyaA antibody (1:300 in TBS containing 0.1% Tween-20) (Santa Cruz Biotech, CA) for 3 hrs. Following washes with TBS-0.1% Tween-20, the membrane was incubated with anti-mouse IgG linked to AlexaFluor680 (1:2000 in TBS containing 0.1% Tween and 0.01% SDS) (Li-Cor, Lincoln NE). After additional washes the membrane was scanned using the Odyssey Infrared Imager (Li-Cor, Lincoln NE).

Assay for cAMP in infected HEp-2 cultures. cAMP was assayed in uninfected HEp-2 cells and at 30 min, 1 hr, 2 hr, and 4 hr after contact between the bacteria and HEp-2 cells using the Direct cAMP Enzyme Immunoassay Kit (Sigma, St. Louis MO) per manufacturer's instructions. Briefly, MAC104 and MAC104:pMH5 were used to infect the cells at an MOI of 100:1. At each time point, extracellular bacteria were removed from the wells, and the infected cells were washed with HBSS, and

then lysed in 0.1M HCl. After centrifugation of the lysate at 600 x g, the supernatants were acetylated and neutralized in a 96 well plate. Each sample was then incubated with a cAMP conjugate and cAMP antibody for 2 hours at room temperature and then washed. The plate was read at 405 nm after incubation with the p-NPP substrate for 1 hour and addition of the stop solution. The levels of cAMP in the samples were calculated based on the standard curve generated from serial dilutions of known cAMP concentrations.

Fluorescence microscopy and immunohistochemistry.

M. smegmatis strains containing either the pMV261:GFP or pMV261:CipA:GFP vector, expressing GFP or GFP fused to the 3' end of the CipA protein, and *M. avium* stained with fluorescein were resuspended in RPMI at 10^7/mL, estimated by McFarland's standards. 100 μL of this inoculum was added to a monolayer of HEp-2 cells in each chamber of an 8-chamber slide with 100 μL fresh RPMI or 100 μL fresh RPMI containing 10 μL of a 10 mg/mL solution of cytochalasin D. Following a 15 min, 30 min, or 1 hr incubation time, the media was removed, and the wells were incubated with a 4% paraformaldehyde solution for 1 hr at room temperature. After washing, monolayers were examined by fluorescence microscopy, or further prepared for immunohistochemistry as follows.

Cells were permeabilized with a 0.1% Triton X-100, 0.1% sodium citrate solution for 2 min on ice. After blocking overnight in 10% Bovine Serum Albumin (BSA), wells were incubated with rabbit α-Cdc42 (Santa Cruz Biotech, CA), diluted 1:1000 in 10% BSA for 1 hr at room temperature, and then anti-rabbit IgG-Texas Red conjugate (Santa Cruz Biotech, CA), diluted 1:200 in 10% BSA for 1 hr. Monolayers

were examined by fluorescence microscopy.

Bioinformatics. The *M. avium* 104 genome sequence is posted on The Institute for Genome Research (TIGR) website (www.tigr.org). Sequences obtained from the microarray and positive pTRG vectors in the bacterial 2-hybrid screen were analyzed by the Basic Local Alignment Search Tool (BLAST) to find similarity to the published MAC104 genome and for putative conserved domains. Once *M. avium* gene sequences were obtained, the protein-specific iterated BLAST (PSI-BLAST) and the SignalP 3.0 server (Bendtsen *et al.*, 2004) were utilized to further characterize the gene sequences.

Statistical Analysis. Statistical analysis was based on the mean of three experiments +/- the standard deviation. Student's t-test was used to compare values for each strain. P-values < 0.05 were considered significant.

Results

The *M. avium fad*D2 gene is a regulator of gene expression upon exposure to HEp-2 cells. In order to determine if the *M. avium fad*D2 gene functions as a gene regulator upon epithelial cell invasion, microarrays were performed comparing gene expression in the wild-type MAC109 strain to MAC109:Δ*fad*D2 strain. Seventy-eight *M. avium* genes that were up-regulated at least 2-fold in the MAC109 strain after a 15 minute exposure to HEp-2 cells, were not up-regulated in the Δ*fad*D2 mutant strain (with a cut-off of 1.5 fold change). These genes are included in 7 functional categories, and are listed in Table 2.3. A large proportion of genes identified are conserved hypothetical proteins, or hypothetical proteins with an

Table 2.3 Genes upregulated in MAC109 but not MAC109:Δ*fadD2* after a 15 minute exposure to Hep-2 cells.

***M. tuberculosis* array gene**	**MAV Homolog**	**MAC109 Fold-change**	**MAC109: Δ*fadD2* fold-change**	**Difference in fold-Change**	**Gene functional category**	**Predicted Function**
Rv0008c	MAV_0012	2.1	-1.2	3.3	3	possible membrane protein
Rv0043c	MAV_0060	2.1	-1.1	3.2	9	probable transcriptional regulator
Rv0047c	MAV_0068	2.2	-1.1	3.3	10	conserved hypothetical protein
Rv0058/*dna*B	MAV_0079	2	-1.6	3.6	2	Helicase
Rv0863	MAV_0092	2	-1.1	3.1	10	conserved hypothetical protein
Rv0420c	MAV_0112	4	-4.3	8.3	3	possible transmembrane protein
Rv3887c	MAV_0157	5.5	2.2	3.3	3	conserved transmembrane protein
Rv3788	MAV_0234	2.3	-1.9	4.2	16	hypothetical protein
Rv3772	MAV_0250	2.2	-1.1	3.3	7	probable histidinol-phosphate aminotransferase
Rv3680	MAV_0448	2.3	-1	3.3	3	probable anion-transporter ATPase
Rv3669	MAV_0460	3.5	1.5	2	3	probable conserved transmembrane protein
Rv3666c/*dpp*A	MAV_0464	2.5	-1.8	4.3	3	probable periplasmic dipeptide-binding lipoprotein
Rv3665c/*dpp*B	MAV_0465	2.2	-1	3.2	3	probable dipeptide-transport integral membrane protein ABC transporter
Rv3587c	MAV_0566	2.1	-1	3.1	3	probable conserved membrane protein
Rv3513c	MAV_0644	2	-1.3	3.3	1	probable fatty-acid-CoA-ligase, involved in lipid degradation
Rv2303c	MAV_0646	3.6	-1.7	5.3	0	possible macrotetrolide antibiotic resistance protein
Rv3503c/*fdx*D	MAV_0653	2.6	-1.2	3.8	7	probable ferredoxin
Rv3494c/*mce*4F	MAV_0953	2.6	-1	3.6	0	possibly involved in host cell invasion, similar to putative secreted proteins
Rv0867c/*rpf*A	MAV_0996	2.1	-1.8	3.9	3	possible resuscitation-promoting factor
Rv0924c/*nramp*	MAV_1044	2	-1.4	2.4	3	divalent cation-transport integral membrane protein
Rv0975c/*fad*E13	MAV_1091	2.1	-1.2	3.3	1	probable acyl-CoA dehydrogenase, involved in lipid degradation
Rv0985c/*msc*L	MAV_1098	2.3	-1.1	3.4	3	possible large-conductance ion mechanosensitive channel
Rv1211	MAV_1356	2	-1.4	3.4	10	conserved hypothetical protein
Rv1212c	MAV_1357	3.8	-1.3	5.1	7	putative glycolyl transferase involved in cellular metabolism
Rv1231c	MAV_1370	2.1	-1	3.1	3	probable membrane protein
Rv1349	MAV_1567	6.2	1.3	4.9	3	multidrug transmembrane ABC transporter
Rv2419c	MAV_1752	2	-1.1	3.1	7	probable phophoglycerate mutase

Table 2.3 (continued).

M. tuberculosis **array gene**	**MAV Homolog**	**MAC109 fold-change**	**MAC109: Δ*fadD2* fold-change**	**Difference in fold-Change**	**Gene functional category**	**Predicted Function**
Rv2383c/*mbt*B	MAV_2009	2.1	-1.3	3.4	1	phenyloxazoline synthase
Rv2112c	MAV_2046	2.2	-1.4	3.6	10	conserved hypothetical protein
Rv2330c/lpp*P*	MAV_2076	2	-1.3	3.3	3	probable lipoprotein
Rv2326c	MAV_2082	2	-1.2	3.2	3	possible transmembrane ATP-binding protein ABC transporter
Rv2257c	MAV_2183	3.4	-1.8	5.2	10	conserved hypothetical protein
Rv2225/*pan*B	MAV_2239	2.5	-1.2	3.7	7	probable 3-methyl-2-oxobutanoate hydroxymethyltransferase
Rv2223c	MAV_2243	2	-1.2	3.2	3	probable exported protease
Rv2131c/*cys*Q	MAV_2360	2.3	-1	3.3	7	possible monophosphatase
Rv3820c/*pap*A2	MAV_2723	2.2	-1.4	3.6	1	possible conserved polyketide synthase
Rv1902c/*nan*T	MAV_2799	3.4	1.3	2.1	3	probable sialic acid transport integral membrane protein
Rv1872c/*lld*D2	MAV_2843	2.1	-1.2	3.3	7	possible l-lactate dehydrogenase
Rv1866	MAV_2852	2	-2.1	4.1	1	conserved hypothetical protein, supposed involvement in lipid degradation
Rv1844c/*gnd*L	MAV_2871	2.1	-1.2	3.3	7	probable 6-phosphogluconate dehydrogenase
Rv1037c	MAV_2921	2	-1.6	3.6	3	putative esat6-like protein
Rv0219	MAV_2996	2	-1.3	3.2	3	probable conserved transmembrane protein
Rv1621c/*cyd*D	MAV_3166	2.1	-1.2	3.3	7	probable transmembrane ATP-binding protein, ABC transporter
Rv1481	MAV_3297	3.7	1	2.7	3	probable membrane protein
Rv1406/*fmt*	MAV_3372	2	-1.8	3.8	2	probable methionyl tRNA formyltransferase
Rv2572c/*asps*	MAV_3447	2.3	-1.2	3.5	2	possible aspartyl tRNA synthetase
Rv2582/ppi*B*	MAV_3462	2.1	-1	3.1	2	probable peptidyl-prolyl cis-trans isomerase B
Rv2604c/*sno*P	MAV_3482	2.6	-1.1	3.7	7	probable glutamine anidotransferase
Rv2622	MAV_3501	2.3	-1.8	4.1	7	possible methyltransferase
Rv2708c	MAV_3601	2.3	-1.4	3.7	10	conserved hypothetical protein
Rv2740	MAV_3633	2.2	-1	3.2	10	conserved hypothetical protein
Rv2788/*sir*R	MAV_3679	2.4	-1.2	3.6	9	probable transcriptional regulator
Rv3228	MAV_4184	3.3	1	2.3	10	conserved hypothetical protein
Rv3254	MAV_4218	2.7	-1.3	4	10	conserved hypothetical protein, similar to secreted protein of S. coelicolor
Rv3270/*ctp*E	MAV_4235	3	-1.1	4.1	3	probable metal cation-transporting P-type ATPasae C

Table 2.3 (continued).

***M. tuberculosis* array gene**	**MAV Homolog**	**MAC109 fold-change**	**MAC109: Δ*fadD2* fold-change**	**Difference in fold-change**	**Gene functional category**	**Predicted Function**
Rv3370c/*dna*E2	MAV_4335	2	-1.5	3.5	2	DNA polymerase III alpha-chain
Rv3371	MAV_4336	2.5	-1.1	3.2	10	conserved hypothetical protein
Rv3415c	MAV_4360	4.9	1.1	3.8	10	conserved hypothetical protein, transcription repressed at low pH
Rv1751	MAV_4364	2.2	-1.2	3.4	7	probable oxidoreductase
Rv3458c/*rps*D	MAV_4399	2.2	-1.6	3.8	2	probable 30S ribosomal protein S4
Rv3465/*rml*C	MAV_4407	2.7	-1.8	4.5	7	dTDP-4-Dehydroramnose 3,5-Epimerase
Rv0696	MAV_4475	2.6	-1.2	3.8	7	probable membrane sugar transferase
Rv0694/*lld*D1	MAV_4477	2.4	-1.4	3.8	7	possible l-lactate dehydrogenase
Rv0561c	MAV_4582	2.1	-1	3.1	7	possible oxidoreductase
Rv0483/*lpr*Q	MAV_4666	5.2	1.5	3.7	3	probable conserved lipoprotein
Rv0479c	MAV_4671	4.4	-1.2	5.6	3	probable conserved membrane protein, secreted protein in M. leprae
Rv0474	MAV_4676	2	-1.2	3.2	9	probable transcriptional regulator
Rv0459	MAV_4690	2.3	-1	3.3	10	conserved hypothetical protein
Rv0356c	MAV_4776	2.4	-1.4	3.8	10	conserved hypothetical protein
Rv0893c	MAV_4873	2	-1.2	3.2	10	conserved hypothetical protein
Rv0269c	MAV_4893	2.7	-1.1	3.8	10	conserved hypothetical protein
Rv0224c	MAV_4945	2.2	-1.4	3.6	7	possible methyltransferase
Rv0209	MAV_4965	2.2	-1.2	3.4	16	hypothetical protein
Rv3089/*fad*D13	MAV_5018	2.3	-1.3	3.6	1	probable chain-fatty-acid-CoA ligase, involved in lipid degradation
Rv0158	MAV_5138	3.8	1.3	2.5	9	probable tetR-like transcriptional regulatory protein
Rv0155/*pnt*AA	MAV_5142	3.4	1.2	2.2	7	probable NAD(P) transhydrogenase (subunit alpha)
Rv0121c	MAV_5188	2.1	-1.4	3.5	10	conserved hypothetical protein
Rv3907c/*pcn*A	MAV_5294	3.6	1.6	2	2	probable poly(A) polymerase, transcription repressed at low pH

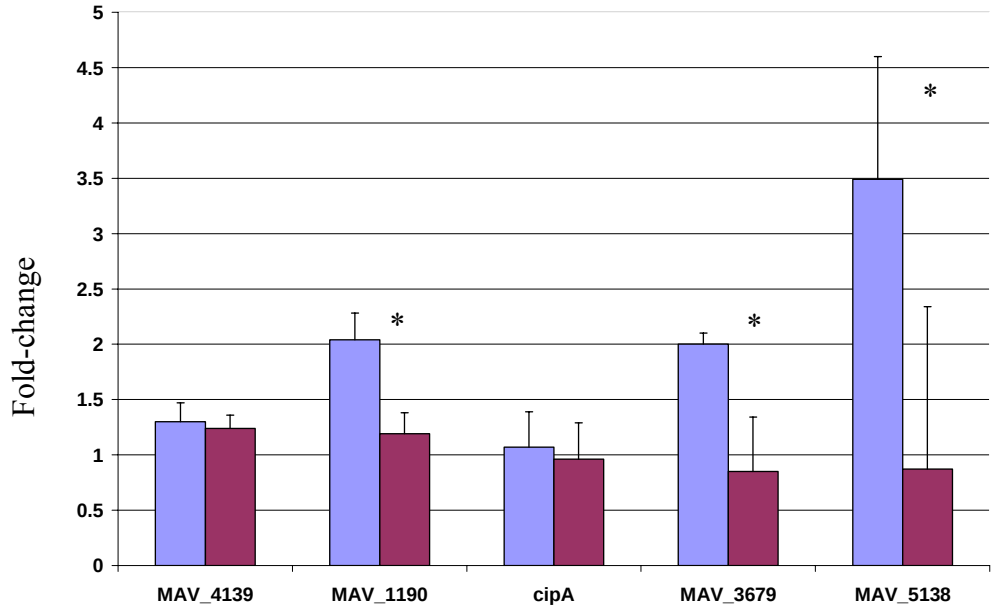

Figure 2.1 Real-time PCR results comparing the fold-change in gene expression upon 15 minute exposure to HEp-2 cells between the MAC109 and MAC109:Δ*fadD2* strains. The y-axis represents fold-change between broth grown bacteria and bacteria incubated for 15 minutes with HEp-2 cells, using the 16S rDNA as an internal control. * indicates significant differences between the two strains ($p < 0.05$). MAC109; MAC109:Δ*fadD2*

orthologue in *Mycobacterium bovis* (classes 10 and 16, respectively) with a total of 19 genes. Additionally, 21 genes are from functional class 3, cell wall and cell processes, and 19 genes are from function class 7, intermediary metabolism and respiration. The remaining proportion of genes belong to 4 functional classes; virulence, detoxification, adaptation (class 0, 2 genes), lipid metabolism (class 1, 6 genes), information pathways (class 2, 5 genes), and regulatory pathways (class 9, 3 genes).

Real-time PCR was performed to confirm the results of the microarray. Five genes were selected with varied responses to HEp-2 exposure based on the results of the microarray: MAV_5138, MAV_3679, and *cipA* were all up-regulated in the wild-

type (at least 2-fold increase over control), but not the mutant upon exposure to HEp-2 cells; MAV_4139 and MAV_1190 showed no up-regulation in either the wild-type or mutant. By real-time PCR analysis, the MAV_5138 and MAV_3679 were also shown to be up-regulated in the wild-type, but not the mutant after exposure to HEp-2 cells (Figure 2.1, $p < 0.05$). The *cipA* gene did not increase in expression upon exposure, but had higher expression compared to the *fad*D2 mutant under the same conditions (although this difference was not significant) (data not shown).

MAV_5138 and MAV_3679 are transcriptional regulators are involved in invasion of HEp-2 cells.

Based on their up-regulation in the wild-type *M. avium,* but not the *fad*D2 mutant strain upon exosure to HEp-2 cells, MAV_5138 and MAV_3679 were over-expressed in *M. avium* behind the G13 promoter. Strains over-expressing either transcriptional regulator had an increased percent invasion of HEp-2 cells compared to MAC109 (Figure 2.2). The MAC109:pMH7 strain (over-expressing MAV_5138) had significantly higher invasion after a 30 min incubation, while the MAC109:pMH6 strain (over-expressing MAV_3679) had significantly higher invasion after both a 30 min and 1 hr incubation ($p < 0.05$).

Real-time PCR was used to compare the expression of 7 genes in MAC109 to MAC109:pMH6 and MAC109:pMH7 grown in broth. Over-expression of the MAV_3679 repressor regulator led to up-regulation of all 7 genes in broth-grown bacteria (Figure 2.3A). Similarly, all 7 genes were up-regulated in broth-grown bacteria in the MAC109:pMH6 strain compared to the wild-type (Figure 2.3B).

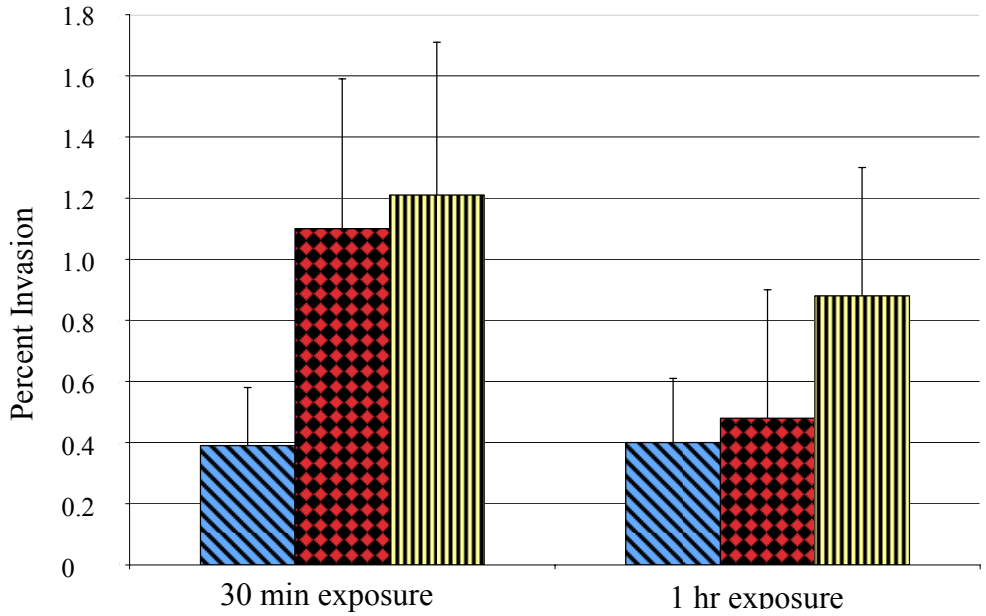

Figure 2.2 Percent invasion of HEp-2 cells by strains of *M. avium* over-expressing MAV_3679 or MAV_5138 after a 30 minute or 1 hour incubation time. Diagonal bars represent MAC109; checks represent MAC109:pMH6; and vertical bars represent MAC109:pMH7. Both recombinant strains have significantly higher invasion than MAC109 after 30 minutes, and MAC109:pMH7 also has a significantly higher invasion after 1 hour. * indicates significant differences from baseline ($p < 0.05$). MAC109; MAC109:pMH7; MAC109:pMH6.

***M. avium* expresses proteins that putatively interact with host cell Cdc42.** In a 2-hybrid screen, co-transformation of the pBT:*cdc*42 and pTRG:MAClib to the reporter strain *E. coli* resulted in the growth of 75 colonies on the selective media. Of these colonies, 15 became blue after growth on media containing X-Gal and an X-Gal inhibitor. After sequence analysis, three of these colonies contained an *M. avium* gene fragment in-frame with the RNAP-alpha gene fragment (Table 2.4). The C-terminal 25 amino acids of CipA were recovered from the target plasmid in the initial screen. Co-transformation of the full-length *cipA* gene in the pBT plasmid with pTRG:*cdc42* did not result in a positive interaction.

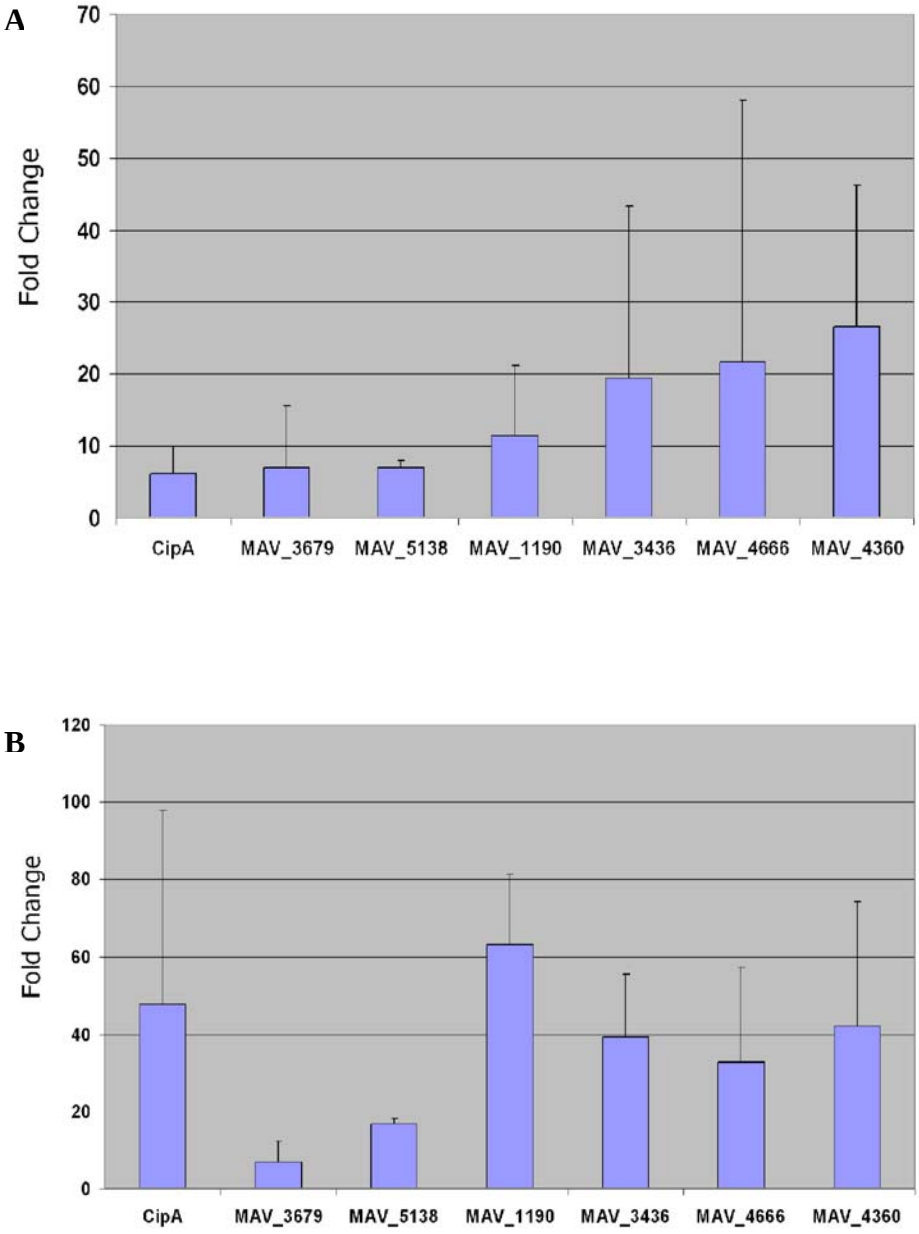

Figure 2.3 Fold-change in expression of genes in *M. avium* over-expressing A) MAV_3679, or B) MAV_5138, compared to wild-type MAC109 grown in culture medium. Results shown are the average and standard error of three experiments.

Table 2.4 Results of 2-hybrid screen for *M. avium* proteins interacting with human Cdc42 or full-length *M. avium* CipA.

Bait	*M. avium* gene	Putative function
Cdc42		
	CipA	Hypothetical protein
	MAV_1190	Patatin, putative esterase
	MAV_4139	Hypothetical protein
CipA		
	MAV_4952	Acyltransferase
	MAV_1043	Hypothetical protein, phage-related protein
	MAV_1300	Hypothetical protein
	MAV_3034	Oxidoreductase
	MAV_4319	Transposase, mutator family protein
	MAV_3595	Sigma factor, *mysA*
	MAV_0876	Transcriptional regulator, *iclR* family

The CipA amino acid sequence suggests it is a secreted or transmembrane protein with domains for interaction with eukaryotic proteins.

Prior to the recent annotation of *M. avium*, an amino acid sequence corresponding to the *cipA* gene was listed as a putative multicopper oxidase in the NCBI database. The coding sequence listed in this entry is shorter than annotated homologues in other mycobacterial species, and there is no sequence or biochemical evidence to support this putative function. The current annotation lists this protein as a hypothetical protein. There are very few homologues to CipA, and their predicted functions are diverse, including the following: a putative membrane protein in *M. tuberculosis* (Rv0479c) and other sequenced mycobacteria, a hypothetical protein in *M. avium* subsp. *paratuberculosis* (MAP3972c), and putative secreted proteins in *Mycobacterium leprae, Corynebacterium diptheriae, Rhodococcus* sp. RHA-1, and *Burkholderia cepacia*. According to the database, the first 100 amino acids are not found in any bacteria besides other mycobacterial species. A PSI-BLAST of this

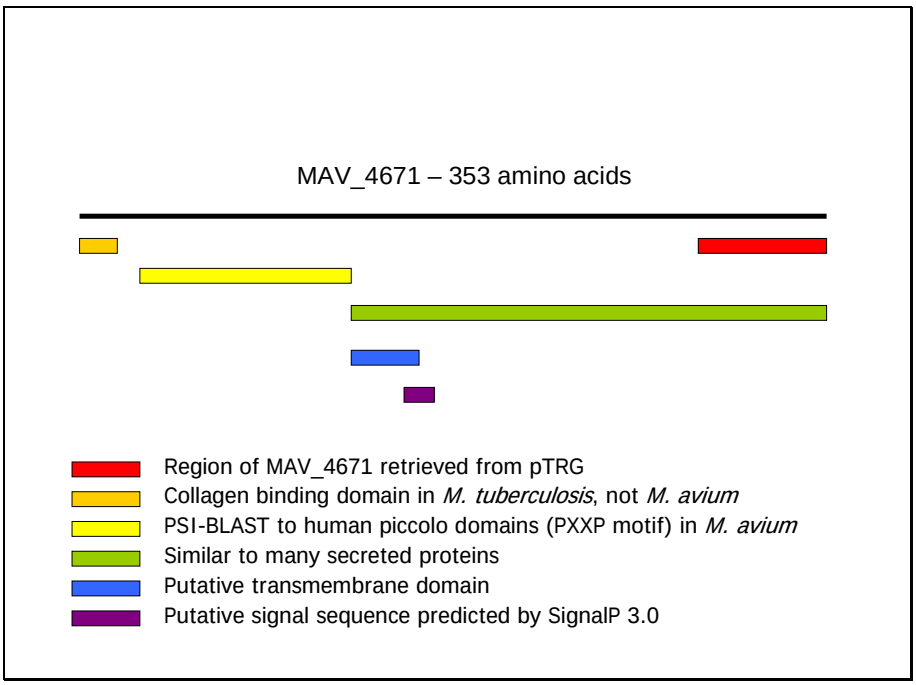

Figure 2.4 Domains/regions present in the CipA (MAV_4671) amino acid sequence.

sequence reveals that there are segments in the first 100 amino acids similar to the PXXP domains of the Piccolo domain proteins in human cells, which have been implicated to play a role in scaffolding proteins involved in actin dynamics (Fenster *et al.,* 2003). These domains are present in only *M. avium* and *M. avium* subsp. *paratuberculosis*, and none of the other characterized mycobacteria. The remaining 252 amino acids correspond to putative secreted proteins, and this region also contains a putative signal peptide, predicted by SignalP 3.0 (Bendtsen *et al.,* 2004). The region of this gene sequenced from the pTRG plasmid corresponds to the C-terminal 50 amino acids (Figure 2.4).

CipA is not secreted into the cytoplasm of HEp-2 cells.

To determine if CipA is a secreted protein, it was fused to amino acids 2-400 of the *B. pertussis* adenylate cyclase protein. Using antibodies against the *B. pertussis cyaA*

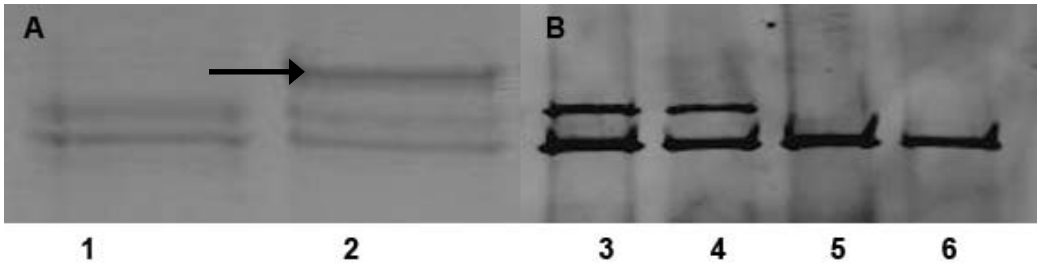

Figure 2.5 CipA expressed as a fusion protein with the *Bordatella pertussis* adenylate cyclase gene is not detected in the cytosolic or insoluble fractions of HEp-2 cells infected with *Mycobacterium avium*. Proteins were extracted from broth-grown *M. avium*, or from HEp-2 cells infected with strains of *M. avium*. An α-CyaA antibody and agarose-conjugate α-IgG beads were used to precipitate the CipA:CyaA fusion protein from the lysates. A) Protein gel stained with coomassie blue showing the expression of the 80 kDa CipA:CyaA fusion protein (arrow) in broth-grown *M. avium*. Lane 1: MAC104, Lane 2: MAC104:pMH5. B) Western blot showing proteins immunoprecipitated from HEp-2 cells infected with strains of *M. avium*. Lane 3: Soluble lysate fraction incubated with MAC104; Lane 4: Soluble lysate fraction incubated with MAC104:pMH5; Lane 5: Insoluble lysate fraction incubated with MAC104; Lane 6: Insoluble lysate fraction incubated with MAC104:pMH5.

tag, expression of the CipA:CyaA fusion protein was detected in *M. avium* by western blot (Figure 2.5A). After incubating HEp-2 cells with the MAC104:pMH5 strain, the fusion protein could not be detected in the cytoplasm or insoluble fraction of the lysed HEp-2 cells (Figure 2.5B). When in the cytoplasm of eukaryotic cells, the fragment of the adenylate cyclase protein leads to an increase in cAMP that can be assayed by ELISA. After incubation with MAC104:pMH5 for 1, 2, and 3 hours, there was no significant increase in cAMP levels compared to the wild-type MAC104 (Figure 2.6) There was an initial increase at 2 hours, but it was not significant, and the levels of cAMP in the cells incubated with the wild-type increased to nearly the same levels after 3 hours.

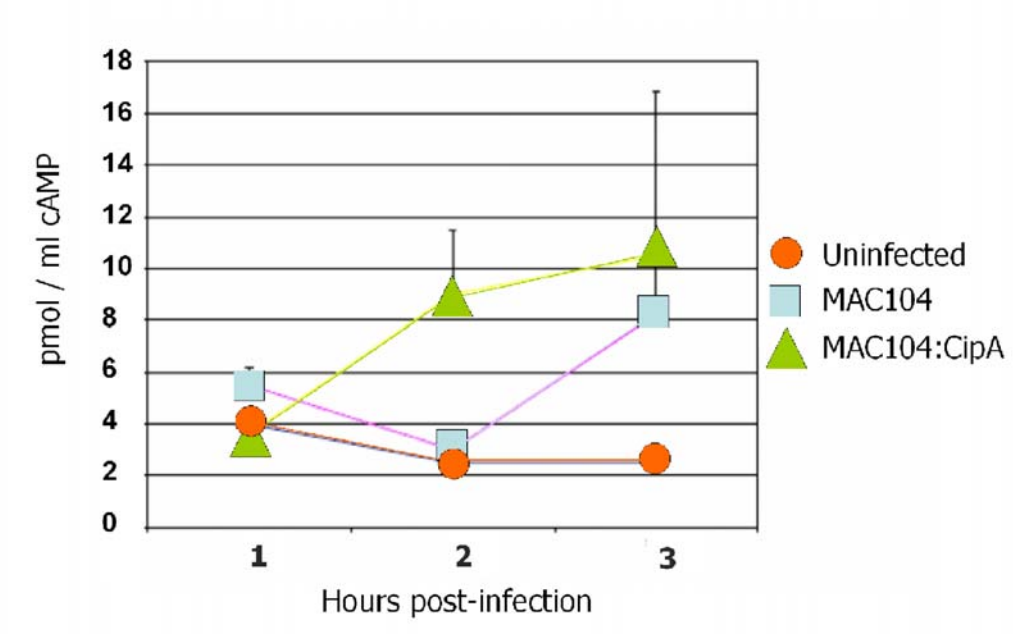

Figure 2.6 Levels of cAMP in infected HEp-2 cells after a 1, 2, or 3 hour incubation with wild-type MAC104, or MAC104 expressing CipA fused to *Bordatella pertussis* adenylate cyclase. Although there appears to be a difference between the wild-type and recombinant strains at 2 hours, this difference is not significant ($p < 0.05$) based on the results of three experiments. Additionally, the increase in cAMP levels seen in the wild-type after 3 hours suggests that the increase may be a result of the infection alone.

CipA localizes to the membrane of *M. smegmatis* when exposed to HEp-2 cells, with an associated accumulation of Cdc42 near *M. smegmatis* and *M. avium* at 15 and 30 minutes after exposure.

To determine if CipA is on the surface of bacteria, a strain of *M. smegmatis* expressing CipA fused to GFP was used to infect HEp-2 cells. Construction of recombinant strains of *M. avium* expressing this fluorescent fusion protein was unsuccessful. After an incubation time of 1 hour with the recombinant strains of *M. smegmatis*, a structure could be observed on the tip of bacteria near HEp-2 cells (Figure 2.7A). The HEp-2 cells had been treated with cytochalisin D to prevent

uptake of the bacteria. This structure was not present on wild-type *M. smegmatis* expressing GFP alone (Figure 2.7B).

To determine if Cdc42 was accumulating near the invading bacterium, immunohistochemistry was performed after infection of HEp-2 cells with the recombinant *M. smegmatis* strains, as well as fluroescein-labeled *M. avium.* After fixation, infected cells were incubated with antibodies against Cdc42 at 15 min, 30 min, and 1 hour after infection. Cdc42 could be observed in the cytoplasm of uninfected cells (Figure 2.7C), in cells infected with *M. smegmatis* containing pMV261:GFP or pMV261:CipA:GFP, and in cells infected with fluorescein-labeled *M. avium*. Cdc42 was observed to accumulate near mycobacteria at 15- and 30-min post-infection (Figure 2.7D and E), but not at 1 hr (Figure 2.7F).

MAV_4671 interacts with *M. avium* and HEp-2 cell proteins.

To identify putative *M. avium* proteins interacting with CipA, the *M. avium* protein library in the pTRG vector was screened with the CipA protein in the pBT vector, by co-transformation in the 2-hybrid reporter strain. In five separate co-transformations, 87 colonies grew on plates containing the selective antibiotic. Of these colonies, 23 were further analyzed after becoming blue in the second screen. Sequences retrieved from the pTRG vector indicated that 7 proteins putatively interact with CipA (Table 2.4), including MAV_3034, an oxidoreductase, and MAV_1300, a hypothetical protein with some loose similarity to intracellular transport proteins.

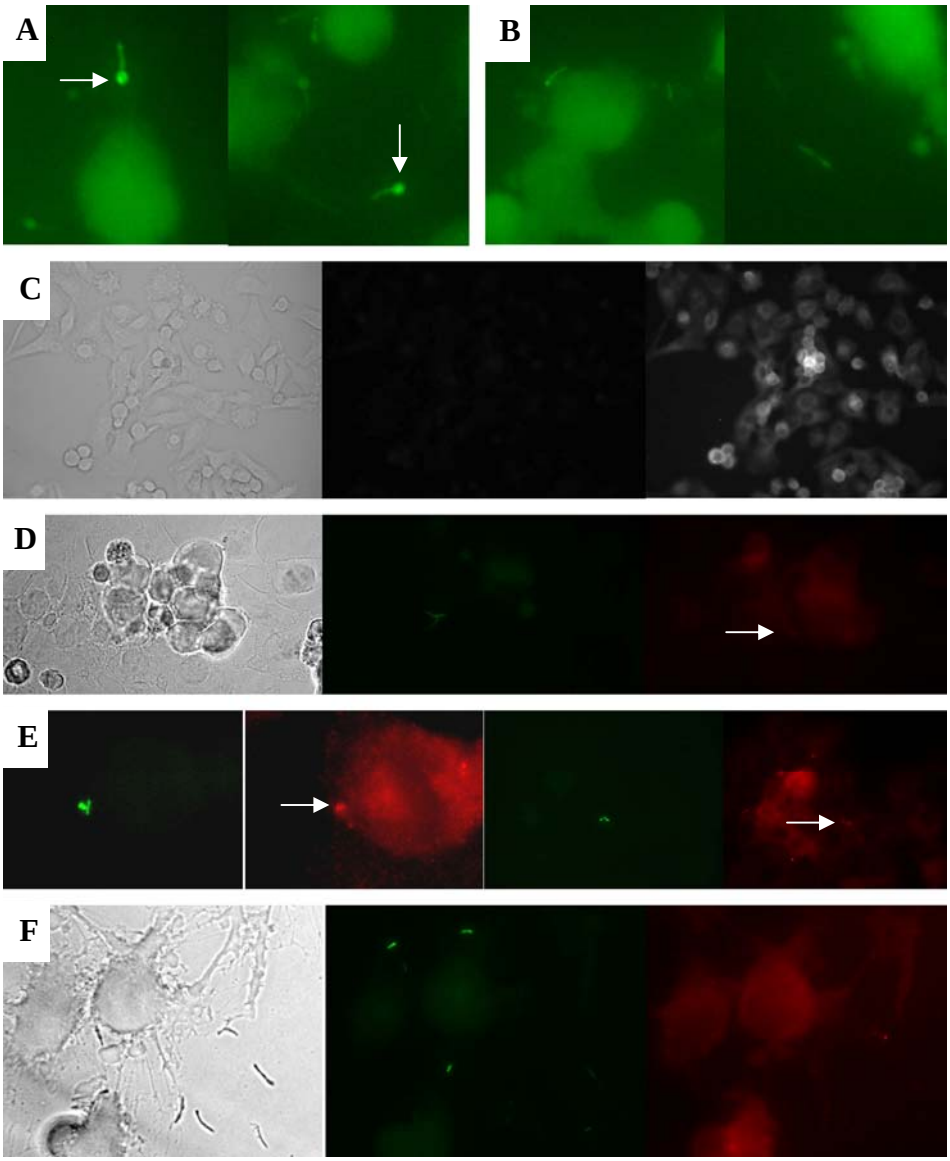

Figure 2.7 A-B) HEp-2 cells exposed to *M. smegmatis* expressing CipA fused to GFP, or GFP alone. A) Representative images of individual bacterial cells, expressing the fusion protein, in contact with cytochalasin D-treated HEp-2 cells. B) Representative images of individual bacterial cells expressing GFP only, in contact with cytochalasin D-treated HEp-2 cells. C-F) Uninfected cells or cells infected with either strain of *M. smegmatis*, or fluroescein-labeled *M. avium* were incubated with α-Cdc42 antibodies, and a Texas-red conjugated secondary. C) Uninfected cells; D) cells infected with *M. avium* for 15 min; E) cells infected with *M. avium* for 30 min; and F) cells infected with *M. smegmatis* for 1 hr.

Discussion

Mycobacterium avium is similar to other intracellular pathogens, such as *Salmonella* sp., *Yersinia* sp., and *Shigella* sp., in its ability to enter the cells of the intestinal epithelium by an active process involving cytoskeletal reorganization. There is little known, however, about the molecular mechanism of this process in *M. avium*. Unlike these other intracellular pathogens, *M. avium* does not have genes encoding proteins similar to surface invasins or internalins [as in the case of *Yersinia* (Isberg and Leong, 1988; Isberg and Leong, 1990) or *Listeria* (Cossart *et al.,* 2003)], or those that comprise type III secretion systems [as in the case of *Salmonella* (Zhou and Galan *et al.,* 2001) or *Shigella* (Sansonetti, 2001)], commonly involved in the secretion of proteins effecting cytoskeletal rearrangement and subsequent epithelial cell invasion. As a result, none of the pathways upstream or downstream of this triggering event are known either. It is plausible, however, to hypothesize that *M. avium* uses analogous mechanisms, as the end result is similar.

There is little doubt that regardless of the pathogen, genes involved in epithelial cell invasion are regulated in response to environmental cues such as pH, oxygen and osmolarity. A study by Bermudez and colleagues (1997) showed that exposure to low O_2 tension and hyperosmolarity led to increased invasion of epithelial cells by *M. avium*. Screens for *M. avium* or *Mycobacterium tuberculosis* genes involved in regulation of macrophage entry have identified a number of two-component response regulators, including *mtrA/B* (Fol *et al.,* 2006), *regX3/senX3* (Parish *et al.,* 2003a; Parish *et al.,* 2003b), and *phoP/R* (Perez *et al.,* 2001). Similar response regulators have not been identified for *M. avium* invasion of epithelial cells.

In *Salmonella*, however, Lucas *et al.* (2000) identified a number of genes that work independently to activate *hilA*, which in turn activates genes in the pathogenicity island SP-1, encoding the TTSS involved in epithelial cell invasion. The regulators identified by this group included two-component response regulators similar to those involved in mycobacterial invasion of macrophages, as well as *fadD*. The FadD protein is involved in the breakdown of endogenous and long-chain fatty acids, although the mechanism of regulation by this protein is yet unknown (Lucas *et al.,* 2000).

In two separate screens of mutant libraries of *M. avium*, mutations in the *fadD2* (Dam *et al.,* 2006) and *fadE20* (Miltner *et al.,* 2005) genes were associated with reduced invasion of epithelial cells. Further studies with the *fadD2* mutant strain revealed that it was deficient in the ability to activate the host cell Cdc42 signaling pathway, leading to actin polymerization via N-Wasp and the Arp2/3 complex (Dam *et al.,* 2006). This signaling pathway, from *fadD* in the bacterium to actin polymerization in the host, is involved in cell entry by other intracellular pathogens of both plants and animals (Barber *et al.,* 1997; Lucas *et al.,* 2000; Soto *et al.,* 2002). We hypothesized that the *M. avium fadD2* gene may also be involved in regulation of genes that effect epithelial cell invasion, and conducted experiments to determine the role of *fadD2* in this process.

In microarrays comparing the up-regulation of genes in a wild-type *M. avium* strain upon exposure to HEp-2 cells to the *fadD2* mutant strain, we identified many genes possibly regulated by *fadD2,* including putative secreted proteins, transport proteins, (trans)membrane proteins, and lipoproteins, based on similarity to other

bacterial proteins. The putative secreted proteins include an *esat*6-like protein, a probable exported protease, and MAV_4671 (CipA), a protein similar to both transmembrane and secreted proteins. One of the virulence, detoxification, adaptation genes identified on the array is also a putative secreted protein, *mce*4D, that is possibly involved in host cell invasion (Chitale *et al.,* 2001). The transport and transmembrane proteins include *dpp*A and *dppB,* components of an operon thought to be involved in transport of dipeptides across the membrane. An additional integral membrane protein is *mnt*H, an orthologue to many eukaryotic natural resistance-associated macrophage proteins (*nramp* proteins). A number of homologues to transcriptional regulators were also identified, including MAV_3679 and MAV_5138. Real-time PCR confirmed that MAV_3679 and MAV_5138 are regulated by *fadD2* upon invasion of epithelial cells, as they were not up-regulated in the *fadD2* mutant (Figure 2.1).

MAV_3679 encodes an ion-dependent repressor regulator, similar to *sirR* from *Staphylococcus epidermis*, *dtxR* from *Corynebacterium diptheriae, mntR* from *Staphylococcus aureus*, and both *ideR* and Rv2788 from *M. tuberculosis.* Many of these ion-dependent repressor regulators, which bind the operators of and regulate ABC transporters, have been shown to be involved in virulence (Ando *et al.,* 2003; Manabe *et al.,* 1999). Interestingly, constitutive expression of an ion-independent mutant of *dtxR* in both *S. aureus* and *M. tuberculosis* exhibited attenuation in mice (Ando *et al.,* 2003). In this study, constitutive over-expression of this gene behind a non-native strong promoter (G13) led to increased invasion of HEp-2 cells. Although it is possible that MAV_3679 is involved in the regulation of the *mntH* gene

(regulated by *mntR* in *S. aureus*) also identified in the microarray, the *M. tuberculosis* homolog of *mntH* is not involved in virulence (Domenech *et al.,* 2002), and the connection between expression of MAV_3679 and *M. avium mntH* was not further explored.

For its genome size, *M. avium* has a very large number of *tetR*-like transcriptional regulators, and more than almost all other sequenced bacteria (Ramos *et al.,* 2005). The family of *tetR* regulators has many members, and the MAV_5138 gene is most similar to a homolog in *M. tuberculosis* of the AcrR family member. Proteins in the AcrR family have not been shown to play a role in virulence, but other TetR-family repressor regulators, such as HapR of *Vibrio cholera* (Kovacikova and Skorupski, 2002) and TvrR of *Pseudomonas syringae* (Preiter *et al.,* 2005) have been implicated in virulence. In general, the TetR-family is important to regulation of genes in response to the environment. *M. avium* has more than twice as many *tetR*-like repressors than *M. tuberculosis*, so it is possible that there are as yet uncharacterized members in this large family involved in *M. avium* virulence. Similar to the *sirR*-like MAV_3679, over-expression of the MAV_5138 protein led to an increase in invasion of HEp-2 cells. *Pseudomonas syringae* is a plant pathogen that uses a TTSS to secrete effectors involved in virulence. Preiter and colleagues (2005) showed that *tvrR*, a *tetR*-like regulator, is involved in virulence of *P. syringae* in a TTSS-independent manner, lending support to the hypothesis that FadD2 and MAV_5138 are involved in virulence of *M. avium* through some uncharacterized secretion system.

In the case of both repressor regulators, it is unknown yet how these genes affect bacterial uptake. It is possible that the repressors are involved in down-regulation of genes expressed in soil or water when a host environment is sensed. It is also plausible that these genes would be involved in a transcriptional regulatory cascade leading to increased expression of genes important to virulence. Real-time PCR was used to analyze the expression of a number of hypothetical proteins in the MAV_3679 and MAV_5138 over-expression strains compared to wild-type (Figure 2.3). Increased expression of proteins shown to be up-regulated in the wild-type strain upon exposure to HEp-2 cells by microarray suggests that these regulators increase expression of genes with roles in the mechanism of pathogenesis. Interestingly, the real-time PCR results, and percent invasion with the these strain was highly variable. This result is not surprising, given that TetR-like repressors are thought to regulate their own expression (Ramos *et al.,* 2005). Also, unpublished studies in our laboratory with the GFP and RFP fluorescent reporters in *M. avium* suggest that the G13 promoter is not as strong or constitutive as it has been found to be in other species of mycobacteria (JV Early, M McNamara, personal communication), which could also lead to variable results.

In addition to analyzing bacterial genes that may be regulated by MAV_3679 or MAV_5138, and in turn, by FadD2, a bacterial 2-hybrid screen, with human Cdc42 as the bait, was used to explore this pathway from the host side. Fragments of three genes were retrieved from target plasmid after showing interaction with Cdc42: CipA, MAV_1190, and MAV_4139. These three genes were also up-regulated in the strains

of *M. avium* over-expressing both the MAV_3679 and MAV_5138 repressor regulators (although CipA up-regulation was not confirmed by Real-time PCR).

The MAV_1190 gene, corresponding to the Rv1063c/MAP1012c genes from *M. tuberculosis/M. avium* subsp. *paratuberculosis*, does not have a characterized function, but does have three conserved domains found on NCBI using the Conserved Domain Database (CDD) (Marchler-Bauer *et al.,* 2005): RssA (COG1752), Patatin-like phospholipase (pfam01734), and COG4667. A search for similar domain architectures revealed a number of other conserved domains including a serine/threonine-specific kinase subfamily (smart00220), a leucine-rich repeat family (cd00116), and ANK (cd00204) and ARM (cd00020), two families of domains implicated in mediating protein-protein interactions. Similar to the MAV_1190 gene, the MAV_4139 gene cannot be compared to other organisms to determine a putative function, but the amino acid sequence corresponds to a conserved domain in the CDD, COG5282. This domain represents an unknown conserved protein and has not been characterized further.

The CipA protein sequence also does not contain any domains collected in the conserved domain database, but does have similarity to domains by BLAST, as described in the results section (Figure 2.4). The piccolo domains in the first 100 amino acids of CipA, and the putative interaction with Cdc42, prompted us to analyze whether this protein is secreted or expressed on the surface of *M. avium* in such a way that it is a part of the protein scaffold complex that forms when Cdc42 is activated, and transmits that signal to downstream proteins, such as N-WASp. Using a construct expressing a CipA:CyaA fusion protein, the presence of this protein could

not be detected in the host cell cytoplasm by western blot, or increased levels of cAMP (Figures 2.5 and 2.6). In addition, we looked for cleavage of the protein into two fragments using tags at the N- and C-terminus, using shorter tag sequences (Day *et al.,* 2003), but could detect neither fragment in the cytoplasm by western blot (data not shown). It is possible the protein is present in the host cells, but at levels below an ability to detect it. Together, these results suggest, however, that the protein is not secreted into the cytoplasm of the host cells. A study by Cain and colleagues (2004) showed that the secreted effectors of *Salmonella* are also not secreted into the host cell cytoplasm, but localize to the cell membrane instead, from where they induce their effects on Cdc42 and downstream actin reorganization.

Because CipA was not detectable in the cytosol or the insoluble cell fractions, we hypothesized that this *M. avium* protein was expressed on the surface of the bacterium upon invasion of epithelial cells, forming part of a structure which interacts with or inserts into the host cell membrane. Morphological changes in the recombinant strain of *M. avium* expressing the CipA:CyaA fusion protein lent support to the localization of this protein to the bacterial membrane or cell wall (data not shown). Additionally, unpublished work in our laboratory has identified a protein in *M. avium* subsp. *paratuberculosis* that also putatively interacts with Cdc42, and could be a part of this structure. Although this protein, MAP3985c, is not homologous to CipA, they are located very near to each other in the chromosome (Figure 2.8). Both CipA and MAP3985c are in small operons containing a hydrolase. MAP3985c was identified based on its interaction with an oxidoreductase shown to be involved in *M. avium* subsp. *paratuberculosis* invasion of epithelial cells (Alonso-Hearn and

colleagues, submitted for publication). In this study, CipA was also shown to putatively interact with an oxidoreductase (Table 2.4).

There are many hypothetical proteins in this region, corresponding to putative transmembrane proteins, a Rho-kinase, and proteins with bacterial SH3 domains. Also present in this region are both characterized and uncharacterized transcriptional regulators, including *regX3/senX3* and MAV_4676, a regulator that was shown by microarray to be up-regulated upon exposure to HEp-2 cells, but not in the *fadD2* mutant strain, and the *hbhA* gene, encoding the surface heparin-binding hemagglutinin protein shown to adhere to epithelial cells (Reddy and Hayworth,

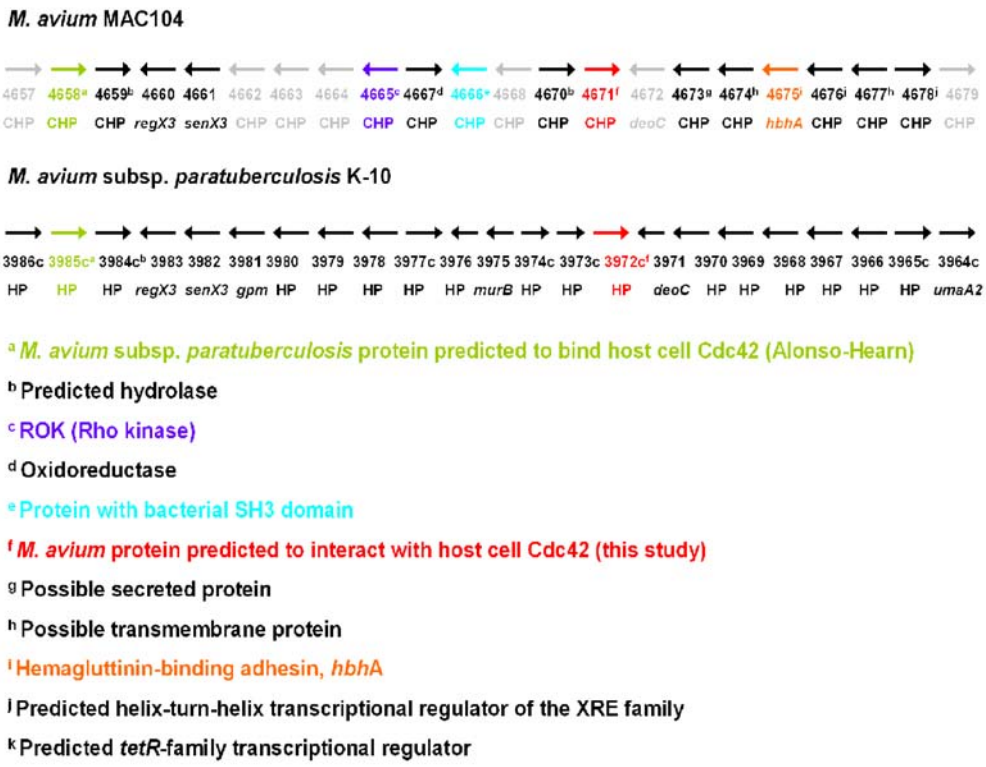

Figure 2.8 The *M. avium* (numbers represent MAV annotation) and *M. avium* subsp. *paratuberculosis* (numbers represent MAP annotation) chromosomal regions encompassing the CipA gene discussed in this study. CHP: Conserved hypothetical protein; HP: Hypothetical protein

2002; Sechi *et al.,* 2006). This region is currently being further analyzed for its role in the invasion of epithelial cells.

M. avium does not have a TTSS, but may have an analogous mechanism for getting proteins into the host cell where they can interact with host cell signaling pathways. Because the CipA protein has domains suggesting its binding to host cell proteins, but could not be shown to be secreted, we expressed this protein in *M. smegmatis,* fused to GFP, to see if it localized to the surface of the bacteria when epithelial cells were encountered (Figure 2.7). Interestingly, a structure was observed at the end of *M. smegmatis* near host cells. Additionally, at early time points, Cdc42 accumulated in areas of the cell near bacteria, suggesting that this structure may be involved in Cdc42 activation.

Our results lead us to hypothesize that the region of the genome including MAV_4671 is important to the invasion of epithelial cells by *M. avium*. Our model suggests that in the presence of host-specific environmental cues, various regulators including *fadD2* lead to activation of additional transcriptional regulators. These regulators, including MAV_3679 and MAV_5138, directly or indirectly activate the expression of proteins from this region, and likely other regions, that make up the components of a mechanism for altering host cell signaling. Observed protein-protein interactions and other data from our lab suggest that oxidoreductases may play a role in the regulation of this mechanism as well, perhaps acting as chaperones. As the bacterium comes into contact with the host cells, these proteins are inserted into the host cell membrane, where they form a complex with Cdc42 and other scaffolding proteins that are present leading to actin polymerization, and subsequent uptake of the

bacterium. Future work will address the identification and characterization of the proteins involved in this mechanism of epithelial cell invasion.

Acknowledgements

This work was supported by NIH Grant NCRR 5R24RR017386-02. Thank you to Denny Weber for editing this manuscript.

References

Ando, M., Y. C. Manabe, P. J. Converse, E. Miyazaki, R. Harrison, J. R. Murphy, and W. R. Bishai. 2003. Characterization of the role of the divalent metal ion-dependent transcriptional repressor *MntR* in the virulence of *Staphylococcus aureus*. Infect Immun **71:**2584-2590.

Bach, H., J. Sun, Z. Hmama, and Y. Av-Gay. 2006. *Mycobacterium avium* subsp. *paratuberculosis* PtpA is an endogenous tyrosine phosphatase secreted during infection. Infect Immun **74:** 6540-6.

Barber, C. E., J. L. Tang, J. X. Feng, M. Q. Pan, T. J. Wilson, H. Slater, J. M. Dow, P. Williams, and M. J. Daniels. 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. Mol Microbiol **24:**555-566.

Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak. 2004. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol **340:**783-795.

Bermudez, L. E., M. Petrofsky, and J. Goodman. 1997. Exposure to low oxygen tension and increased osmolarity enhance the ability of *Mycobacterium avium* to enter intestinal epithelial (HT-29) cells. Infect Immun **65:** 3768-3773.

Brodin P., I. Rosenkrands, P. Andersen, S. T. Cole, and R. Brosch. 2004. ESAT-6 proteins: protective antigens and virulence factors? Trends Microbiol **12**: 500-8.

Cain, R. J., R. D. Hayward, and V. Koronakis. 2004. The target cell plasma membrane is a critical interface for *Salmonella* cell entry effector-host interplay. Mol Microbiol **54:**887-904.

Chitale, S., S. Ehrt, I. Kawamura, T. Fujimura, N. Shimono, N. Anand, S. Lu, L. Cohen-Gould, and L. W. Riley. 2001. Recombinant *Mycobacterium tuberculosis* protein associated with mammalian cell entry. Cellular Microbiology **3:**247-254.

Cossart, P., J. Pizarro-Cerda, and M. Lecuit. 2003. Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. Trends Cell Biology **13:**23-31.

Dam, T., L. Danelishvili, M. Wu, and L. E. Bermudez. 2006. The *fadD2* Gene Is Required for Efficient *Mycobacterium avium* Invasion of Mucosal Epithelial Cells. J Infect Dis **193:**1135-1142.

Damsker, B., and E. J. Bottone. 1985. *Mycobacterium avium-Mycobacterium intracellulare* from the intestinal tracts of patients with the acquired immunodeficiency syndrome: concepts regarding acquisition and pathogenesis. J Infect Dis **151:**179-181.

Danelishvili, L., M. Wu, L. S. Young, and L. E. Bermudez. 2005. Genomic approach to identifying the putative target of and mechanisms of resistance to mefloquine in mycobacteria. Antimicrobial Agents and Chemotherapy **49:**3707-3714.

Day, J. B., F. Ferracci, and G. V. Plano. 2003. Translocation of YopE and YopN into eukaryotic cells by *Yersinia pestis yopN, tyeA, sycN, yscB* and *lcrG* deletion mutants measured using a phosphorylatable peptide tag and phosphospecific antibodies. Mol Microbiol **47:**807-823.

Domenech, P., A. S. Pym, M. Cellier, C. E. Barry, 3rd, and S. T. Cole. 2002. Inactivation of the *Mycobacterium tuberculosis* Nramp orthologue (*mntH*) does not affect virulence in a mouse model of tuberculosis. FEMS Microbiology Letters **207:**81-86.

Elliott, D. A., D. J. Coleman, M. A. Lane, R. C. May, L. M. Machesky, and D. P. Clark. 2001. *Cryptosporidium parvum* infection requires host cell actin polymerization. Infect Immun **69:**5940-5942.

Fenster, S. D., M. M. Kessels, B. Qualmann, W. J. Chung, J. Nash, E. D. Gundelfinger, and C. C. Garner. 2003. Interactions between Piccolo and the actin/dynamin-binding protein Abp1 link vesicle endocytosis to presynaptic active zones. The Journal of Biological Chemistry **278:**20268-20277.

Fol, M., A. Chauhan, N. K. Nair, E. Maloney, M. Moomey, C. Jagannath, M. V. Madiraju, and M. Rajagopalan. 2006. Modulation of *Mycobacterium tuberculosis* proliferation by MtrA, an essential two-component response regulator. Mol Microbiol **60:**643-657.

Gioffre, A., E. Infante, D. Aguilar, M.P. Santangelo, L. Klepp, A. Amadio, V. Meikle, I. Etchechoury, M.I. Romano, A. Cataldi, R. P. Hernandez, F. Bigi. 2005. Mutation in *mce* operons attenuates *Mycobacterium tuberculosis* virulence. Microbes Infect **7**: 325-34.

Isberg, R. R., and J. M. Leong. 1988. Cultured mammalian cells attach to the invasin protein of *Yersinia pseudotuberculosis*. Proc Nat Acad Sci USA **85:**6682-6686.

Isberg, R. R., and J. M. Leong. 1990. Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell **60:**861-871.

Kitaura, H., N. Ohara, M. Naito, K. Kobayashi, and T. Yamada. 2000. Fibronectin-binding poteins secreted by *Mycobacterium avium*. APMIS **108:** 558-64

Kovacikova, G., and K. Skorupski. 2002. Regulation of virulence gene expression in *Vibrio cholerae* by quorum sensing: HapR functions at the *aphA* promoter. Mol Microbiol **46:**1135-1147.

Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee. 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar *typhimurium*. J Bacteriol **182:**1872-1882.

Malen, H., F. S. Berven, K. E. Fladmark, and H. G. Wiker. 2007. Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. Proteomics **7**: 1702-18.

Manabe, Y. C., B. J. Saviola, L. Sun, J. R. Murphy, and W. R. Bishai. 1999. Attenuation of virulence in *Mycobacterium tuberculosis* expressing a constitutively active iron repressor. Proc Nat Acad Sci USA **96:**12844-12848.

Marchler-Bauer, A., J. B. Anderson, P. F. Cherukuri, C. DeWeese-Scott, L. Y. Geer, M. Gwadz, S. He, D. I. Hurwitz, J. D. Jackson, Z. Ke, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, G. H. Marchler, M. Mullokandov, B. A. Shoemaker, V. Simonyan, J. S. Song, P. A. Thiessen, R. A. Yamashita, J. J. Yin, D. Zhang, and S. H. Bryant. 2005. CDD: a Conserved Domain Database for protein classification. Nucleic Acids Res **33:**D192-196.

Masuda, M., M. Minami, H. Shime, T. Matsuzawa, and Y. Horiguchi. 2002. In vivo modifications of small GTPase Rac and Cdc42 by *Bordetella* dermonecrotic toxin. Infect Immun **70:**998-1001.

Miltner, E., K. Daroogheh, P. K. Mehta, S. L. Cirillo, J. D. Cirillo, and L. E. Bermudez. 2005. Identification of *Mycobacterium avium* genes that affect invasion of the intestinal epithelium. Infect Immun **73:**4214-4221.

Parish, T., D. A. Smith, S. Kendall, N. Casali, G. J. Bancroft, and N. G. Stoker. 2003. Deletion of two-component regulatory systems increases the virulence of *Mycobacterium tuberculosis*. Infect Immun **71:**1134-1140.

Parish, T., D. A. Smith, G. Roberts, J. Betts, and N. G. Stoker. 2003. The *senX3-regX3* two-component regulatory system of *Mycobacterium tuberculosis* is required for virulence. Microbiology **149:**1423-1435.

Perez, E., S. Samper, Y. Bordas, C. Guilhot, B. Gicquel, and C. Martin. 2001. An essential role for *phoP* in *Mycobacterium tuberculosis* virulence. Mol Microbiol **41:**179-187.

Preiter, K., D. M. Brooks, A. Penaloza-Vazquez, A. Sreedharan, C. L. Bender, and B. N. Kunkel. 2005. Novel virulence gene of *Pseudomonas syringae* pv. tomato strain DC3000. J Bacteriol **187:**7805-7814.

Ramos, J. L., M. Martinez-Bueno, A. J. Molina-Henares, W. Teran, K. Watanabe, X. Zhang, M. T. Gallegos, R. Brennan, and R. Tobes. 2005. The TetR family of transcriptional repressors. Microbiol Mol Biol Rev **69:**326-356.

Reddy, V. M. and D. A. Hayworth. 2002. Interaction of *Mycobacterium tuberculosis* with human respiratory epithelial cells (HEp-2). Tuberculosis (Edinb) **82:** 31-6.

Rohatgi, R., L. Ma, H. Miki, M. Lopez, T. Kirchhausen, T. Takenawa, and M. W. Kirschner. 1999. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. Cell **97:**221-231.

Sangari, F. J., J. Goodman, and L. E. Bermudez. 2000. *Mycobacterium avium* enters intestinal epithelial cells through the apical membrane, but not by the basolateral surface, activates small GTPase Rho and, once within epithelial cells, expresses an invasive phenotype. Cell Microbiol **2:**561-568.

Sansonetti, P. J. 2001. Microbes and microbial toxins: paradigms for microbial-mucosal interactions III. Shigellosis: from symptoms to molecular pathogenesis. American Journal of Physiology **280:** G319-323.

Sechi, L. A., N. Ahmed, G. E., Felis, I. Dupre, S. Cannas, G. Fadda, A. Bua, and S. Zanetti. 2006. Immunogenicity and cytoadherence of recombinant heparin-binding haemagglutinin (HbhA) of *Mycobacterium avium* subsp. *paratuberculosis*: Functional promiscuity or a role in virulence? Vaccine **24**: 236-43.

Soto, M. J., M. Fernandez-Pascual, J. Sanjuan, and J. Olivares. 2002. A *fadD* mutant of *Sinorhizobium meliloti* shows multicellular swarming migration and is impaired in nodulation efficiency on alfalfa roots. Mol Microbiol **43:**371-382.

Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold, and W. D. Hardt. 2000. Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Mol Microbiol **36:**1206-1221.

Zhou, D., and J. Galan. 2001. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes and infection / Institut Pasteur **3:**1293-1298.

Chapter 3

Species of environmental mycobacteria vary in their ability to grow in human, mouse, and carp macrophages, and differ regarding the presence of mycobacterial virulence genes observed by DNA microarray hybridization.

Melanie J. Harriff[1,2], Martin Wu[1], Michael L. Kent[1,2], Luiz E. Bermudez[1,2,§]

Applied and Environmental Microbiology: *Submitted*

[1] Department of Biomedical Sciences, College of Veterinary Medicine, 105 Magruder, Oregon State University, Corvallis, OR 97331
[2] Molecular and Cellular Biology Program, Center for Gene Research and Biotechnology, 3021 Agricultural and Life Sciences, Oregon State University, Corvallis, OR 97331

Abstract

There are many species of environmental mycobacteria (EM) that infect animals important to the economy and research that also have zoonotic potential. The genomes of very few of these species have been sequenced, and little is known about the molecular mechanisms by which most of these opportunistic pathogens cause disease. In this study, 18 isolates of environmental mycobacteria isolated from fish and humans (including strains of *Mycobacterium avium, Mycobacterium peregrinum, Mycobacterium chelonae,* and *Mycobacterium salmoniphilum*) were examined for their ability to grow in macrophage lines from humans, mice, and carp. Genomic DNA from 14 of these isolates was then hybridized against DNA from a reference *M. avium* strain on a custom microarray containing virulence genes of mycobacteria, and a selection of representative genes from metabolic pathways. The strains of EM had different abilities to grow within the three types of cell lines that grouped largely according to the host from which they were isolated. Genes identified as being putatively absent in some of the strains included those with response regulatory function, cell wall composition, and fatty acid metabolism, as well as a recently identified pathogenicity island important to macrophage uptake. Further understanding of the role these genes play in host specificity and pathogenicity will be important to gain insight into the zoonotic potential of certain environmental mycobacteria, as well their mechanisms of virulence.

Introduction

Environmental mycobacteria (EM) have long been recognized for their ability to cause opportunistic disease in humans as well as animals of economic and research significance, including cattle and fish. The influence of all members of this group on human and animal health is just emerging, however. Unlike the obligate pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae*, EM are cultured from the soil and water, and are extremely hardy in conditions that would not allow many other bacteria to survive. They are routinely isolated from many sources of water that have been treated for human consumption (Falkinham *et al*., 2001; Chang *et al.*, 2002), and once in the host, the great majority live within cells.

EM can cause a wide variety of disease pathology in humans and animals. In humans, the *Mycobacterium avium-intracellularae* complex (MAC) is the most common EM affecting patients with HIV, while *Mycobacterium ulcerans* is most common in non-HIV-positive individuals (Banks, 1994). Infection with MAC in otherwise healthy individuals is usually associated with pulmonary disease, while MAC causes disseminated disease in immune-suppressed individuals. Another member of the MAC, *Mycobacterium avium* subsp. *paratuberculosis*, is the cause of Johne's disease in ruminants, which is of particular economic concern to the cattle industry. *Mycobacterium marinum*, *Mycobacterium fortuitum*, and *Mycobacterium chelonae* cause dermal infections in humans. *M. chelonae* and *M. fortuitum* are associated with infection of injection or wound sites at hospitals (Brown-Elliot and Wallace, 2002), whereas infection with *M. marinum* is most commonly linked to contact with swimming pools and fish tanks (Decostere *et al*., 2004).

These three species of mycobacteria also cause disease in wild and cultured fishes (Chinabut, 1999). In addition, many other species causing infection in humans, such as *Mycobacterium triplex* (Whipps *et al*., 2003; Poort *et al*., 2006) (See Appendix I), *Mycobacterium peregrinum* (Kent *et al*., 2004), and *Mycobacterium haemophilum* (Kent *et al.,* 2004; Whipps *et al*., 2007), have been shown to lead to serious infections in fishes. Mycobacteriosis has a significant impact on groups using zebrafish (Astrofsky *et al*., 2000; Watral and Kent, 2007; Whipps *et al.,* 2007) or other fish, such as medaka (Sanders and Swaim, 2001), for research models, as the ability to obtain and maintain mycobacteria-free fish is necessary. Zebrafish have been recognized for over thirty years as an excellent model to study early vertebrate development (van der Sar *et al*., 2004). Many fish species, including zebrafish (Prouty *et al*., 2003; Pozos and Ramakrishnan, 2004) and goldfish (*Carassius auratus*) (Pattyn and Verdoolaeche-Van Loo, 1974; Talaat *et al*., 1998; Ruley *et al*., 2002), are now also being utilized as models for infectious diseases, including mycobacteria. For example, Ramakrishnan and colleagues (Prouty *et al*., 2003; Ramakrishnan, 2004) have proposed *M. marinum* as a surrogate model for *M. tuberculosis* because the two bacteria are genetically closely related.

Despite the importance of EM to the health of humans and many animals, very few have been studied in depth in the laboratory to determine the molecular and cellular basis for their ability to cause disease. Of the EM species mentioned, only *M. avium, M. avium* sp. *paratuberculosis, M. marinum* and *M. ulcerans* have had the genomes sequenced and annotated, and there are a limited number of published studies that have identified putative factors involved in virulence for these strains.

Microarray technology is being currently utilized for comparative studies of bacteria at the genomic DNA level, both within serovars of the same species and between species of the same genus. Porwollik *et al.* (2002, 2004, 2005) have done numerous experiments comparing the genomic DNA of serovars of *Salmonella enterica* with different virulence and hosts showing that there are often hundreds of genomic differences within serovars. Hamelin *et al.* (2006) used a microarray containing all known *Escherichia coli* virulence and antimicrobial resistance genes to survey the population of environmental *E. coli* isolates in the Great Lakes, specifically in areas with known pollution (Hamelin *et al*., 2007). Microarrays have also been used to compare the genomic DNA between strains of sequenced mycobacteria. Behr *et al.* (1999) first used a whole-genome library to compare the Bacille Calmette-Guérin (BCG) vaccine strain to *M. tuberculosis*. Their findings indicated at least 9 deletions in the BCG strains that may play a role in the difference in virulence between the two species. Similarly, microarrays have been used to compare genomic DNA between *M. tuberculosis* and *Mycobacterium microti,* a member of the same clade that has very low virulence in humans (Frota *et al*., 2004). For species of the MAC, including *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum*, and *Mycobacterium intracellularae*, multiple groups have used a whole genome microarray to analyze regions of deletion and polymorphisms that may explain some of the differences in pathogenicity and host specificity within this closely related group (Semret *et al*., 2004; Paustian *et al.,* 2005; Marsh *et al*., 2006).

For this study, the virulence and genomes of some of the unstudied and unsequenced EM were compared to a species that we know more about, *M. avium.*

Several strains of *M. avium*, *M. peregrinum*, *M. salmoniphilum* and *M. chelonae* were examined for their ability to invade and grow in human, mouse, and carp macrophage cell lines. Genomic DNA from a subset of these strains was then hybridized to a selective microarray containing probes for numerous genes thought to be involved in the pathogenicity of *M. avium* and *M. tuberculosis*. By comparing a number of EM species, and incorporating data from similar experiments with other EM species such as *M. marinum*, our knowledge about the molecular and cellular mechanisms of virulence used by this group of opportunistic pathogens will be expanded. Because there is a wide range of hosts for EM, it is interesting to explore whether there is genetic variation among these species that leads to different pathogenic potential.

Materials and Methods

Bacterial strains and culture. Eighteen strains of EM were analyzed in macrophage invasion assays and fourteen of these as well as *M. tuberculosis* H37Rv were hybridized against the DNA microarray. The strains were obtained from different sources and hosts and have been characterized previously, as listed in Table 3.1. The four serovars of *M. avium* and *M. tuberculosis* H37Rv were grown at 37 °C on Middlebrook 7H10 agar (Difco, Detroit MI) supplemented with oxalate acid-albumin-dextrose-catalase (OADC) (Difco, Detroit MI) for 7-10 days. Pure colonies were inoculated to 7H9 broth (Difco, Detroit MI) supplemented with 0.1% Tween-80 (Sigma) and OADC and grown to log phase in a shaking incubator prior to use in the assays. The MPATCC and MP101

Table 3.1 Species and Strains used in this study.

Species/ Strain	Reference name	Source	Temperature used for Growth on agar	Days Grown on 7H10 agar	Used in Macrophage Assays	Used in DNA-DNA Microarray Analysis
M. avium						
MAC104	Inderlied *et al.*[a]	Human Blood	37 °C	7-10	-	+
MAC100	Inderlied *et al.*[a]	Human Blood	37 °C	7-10	-	+
MAC101	Inderlied *et al.*[a]	Human Blood	37 °C	7-10	+	+
MAC109	Inderlied *et al.*[a]	Human Blood	37 °C	7-10	-	+
M. peregrinum						
MP101	Case 3[b]	Zebrafish Tissue	37 °C	5-7	+	+
MP102	This study	Tilapia Tissue	28.5 °C	5-7	+	+
MPATCC	ATCC 700686	Human Sputum	37 °C	5-7	-	+
M. chelonae						
MCH	Case 13[b]	Zebrafish Tissue	28.5 °C	3-5	+	+
MCJA	Case 9[b]	Zebrafish Tissue	28.5 °C	3-5	+	+
MCJ78	Case 10[b]	Zebrafish Tissue	28.5 °C	3-5	+	+
M. salmoniphilum						
MCTRASK	Whipps *et. al*[c]	Salmonid Tissue	28.5 °C	3-5	+	+
MCBAND	Whipps *et. al*[c]	Salmonid Tissue	28.5 °C	3-5	+	+
MCSABLE	Whipps *et. al*[c]	Salmonid Tissue	28.5 °C	3-5	+	+
MCMWF	Whipps *et. al*[c]	Salmonid Tissue	28.5 °C	3-5	+	+
MCSILETZ	Whipps *et. al*[c]	Salmonid Tissue	28.5 °C	3-5	+	-
MCER	Whipps *et. al*[c]	Salmonid Tissue	28.5 °C	3-5	+	-
MCAUS	Whipps *et. al*[c]	Salmonid Tissue	28.5 °C	3-5	+	-
M. tuberculosis						
H37Rv	ATCC 25618	Human Lung	37 °C	7-10	-	+

[a] Inderlied *et al.* (1987)
[b] Kent *et al.* (2004)
[c] Whipps *et al.* (2007)

strains of *M. peregrinum* were grown on similar media for 5-7 days prior to inoculation to broth, and the MP102 strain were grown at 30 °C. All *M. chelonae* and *M. salmoniphilum* strains were grown at 30 °C for 3-5 days prior to inoculation to broth. For macrophage assays, mycobacteria in log phase growth were washed and resuspended in Hank's balanced salt solution (HBSS). Inocula of approximately 3 x 10^7 were established using McFarland's concentration standards, followed by serial dilutions and plating on 7H10 to confirm the number of bacteria.

Cell lines and culture. Three cell lines were used in the macrophage assays. The human monocyte (U-937) (ATCC CRL-1593.2) and mouse peritoneal macrophage (RAW264.7) (ATCC TIB-71) cell lines were obtained from the ATCC, and the carp leukocyte cell line (CLC) was a gift from Jeffery Cirillo (University of Nebraska, Lincoln). U-937 cells were cultured in RPMI-1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis MO). RAW264.7 cells were cultured in DMEM (GIBCO) supplemented with 10% FBS. CLC cells were cultured in EMEM (ATCC) supplemented with 10% FBS, and 10 mM nonessential amino acids (Sigma, St. Louis MO) on collagen IV pre-coated flasks and plates. Cells were maintained in 25 cm^2 or 75 cm^2 flasks, and were transferred to 24-well culture plates, such that the monolayers were 80% confluent for the assays. The U-937 cells were treated with 500 ng/ml of Phorbol 12-Myristat 13-Acetate (PMA) (Sigma) overnight to promote maturation and adherence. U-937 and RAW264.7 cells were grown at 37 °C with 5% CO_2, while CLC cells were grown at 28.5 °C with 5% CO_2, until the time of the assays.

Macrophage assays. Assays were performed as described previously by Bermudez and Petrofsky (1997), except that infected 24-well plates were incubated at 37 °C and/or

28.5 °C, depending on the cell type and experiment. Briefly, the inoculum was added in duplicate wells for each time point to 24-well culture plates containing U-937, RAW264.7, or CLC cells at an MOI of 10. After a 1-hour infection time, the supernatant was removed, and the wells were washed 3 times with HBSS to remove extracellular bacteria. Sterile water containing a 1:5 dilution of 0.025% sodium dodecyl sulfate (SDS) was added to two of the monolayers to lyse the cells. The lysate was diluted serially and plated onto 7H10 agar to determine the colony forming units per ml (CFU/ml). Media was replenished in the remaining wells. After 2 and 4 days, the duplicate cell monolayers were lysed and plated as described above. Assays were performed in triplicate, and the resulting CFUs from all assays were analyzed to determine the uptake and growth of all indicated mycobacterial strains in the three cell types.

Microarray probe design and array assembly. Oligonucleotide probe sequences were generated from the sequences of 181 predicted ORFs of *M. avium* serovar 104 (MAC104) as described below. These 181 genes encode for proteins with predicted or characterized roles in virulence of mycobacteria, along with representative genes of various metabolic pathways. Additionally, the array probes included fifteen sequences from a recently identified pathogenicity island in *M. avium* (Danelishvili *et al.*, in press). MAC104 gene sequences were compared to *M. tuberculosis*, *M. smegmatis*, and other mycobacterial sequences when homologues were present. Regions that were most similar across multiple mycobacterial sequences for each gene were analyzed by ProbeSelect (Li and Stormo, 2001) againstthe entire *M. avium* genome. Parameters for probe design were specificity to the MAC104 gene, bias towards the 3' end of the selected gene sequences, minimal secondary structures, and a length of 49-51 nucleotides. As controls to use in

the establishment of a score for absence (see below), probe sequences were selected from 20 *M. tuberculosis* genes that could not be found in MAC104. Additionally, as negative controls, oligonucleotide probe sequences were designed for 5 ORFs of *Escherichia coli* K12. The probes designed for the array represent approximately 4% of the ORFs in the MAC104 genome. A complete list of the 224 oligonucleotide sequences can be found in Supplemental Table 3.1. Oligonucleotide probes were synthesized by Qiagen/Operon (Alameda CO). Lyophilized probes were resuspended in a buffer containing 3X SSC and 1.5M Betaine, and printed in quadruplicate on Corning UltraGap slides using a BioRobotics Microgrid II TAS robotic spotter. The assembled array had a total of 1120 features.

DNA Extraction, labeling, and hybridization. The strains indicated in Table 3.1 were grown in Middlebrook 7H9 supplemented with 10% OADC. After sufficient growth, the resulting bacterial pellet was resuspended in 10 mM Tris and 0.5 mM EDTA. Bacterial lysis was performed by overnight shaking with 20 mg/ml lysozyme (Sigma) at 37 °C, followed by addition of 1% SDS and 1 µg/µl proteinase K for 3 hours. After addition of 5M NaCl and CTAB, DNA was precipitated from the lysate twice by chloroform:isoamyl alcohol (24:1) (Sigma), twice by phenol:chloroform: isoamyl alcohol (25:24:1) (Sigma), then with cold isopropanol, and finally washed with 80% ethanol. The resulting DNA pellet was dried and resuspended in DNase-free water and treated with RNase (Roche). The quantity and purity of each DNA sample was evaluated by spectrophotometric analysis at 230, 260, and 280 nm.

DNA of the bacterial strains was fluorescently labeled by direct incorporation of fluorescent nucleotide analogs in a Klenow polymerase fill-in reaction. Each 60 µl

reaction was carried out with 4 µg DNA, partially digested with Sau3A1 (Roche), random primers (10 µg hexamers) (Invitrogen, Alameda CA), 6 µl 10X Klenow buffer (Roche), 1 mM Cy3- or Cy5-cCTP (Amersham), 10 µg dNTPs – dCTP, and 1000 U Klenow polymerase (Roche). After incubation at 37 °C for 5 hours, labeled DNA was purified using the Qiaquick PCR Purification Kit (Qiagen, Alameda CA).

Labeled DNAs (MAC104 as the reference and another strain as the test) were combined in a hybridization mixture containing the Cy3- and Cy5-labeled DNAs, a formamide-based hybridization buffer, and COT-1 DNA. The solution was applied to array slides that had been preincubated with the same hybridization mixture without DNAs, covered with a glass slip, and placed into a Boekel Scientific Hybridization Chamber for overnight incubation at 42 °C. The slides were then washed with 2X SSC (diluted from 20X SSC, Ambion), 0.2% SDS, 2X SSC, and finally 0.2X SSC. Arrays were scanned after 10 minutes in the dark using a ScanArray 4000 (Packard BioScience Company, Meriden CT).

Microarray data analysis for presence or absence of genes. Fluorescence intensities and background signals from each channel were determined using ScanArray 4000. Data was uploaded to QuantArray to quantify the intensities generated by the spot. Background fluorescence was subtracted from the fluorescence for each spot, and the data set was normalized using a Lowess transformation. To reduce the analysis of uninformative genes, spots for which the average signal intensity was less than

Table 3.2 Genes and primer sequences used for microarray verification.

Gene	Species used for primer sequence	Forward primer 5'-3'	Reverse primer 5'-3'
16S rDNA	Mycobacteria specific[a]	CGAACGGGTGAGTAACAC	TGCACACAGGCCACAAGG
MAV_4679	*M. avium*	TACAGCTGCGCGTACTTCGAG	TAGCGTTGCCGGCCGAAGTGC
	M. smegmatis	CCTCGACCCGACACAGACGTA	CATCCGGAAGAAGTCGTCGTA
MAV_1169	*M. avium*	ATGGAACAGCCGGTCGTCGAA	TCACGCCTCGAATCGATACCC
	M. smegmatis	CGATCAAGACCCGCGTGTTGG	TAGTGGGTCTCGGTGGCGTAG
MAV_0469	*M. avium*	CACGGCGGCCTTCTTCGACCT	ACAGCTTGTGCGCGGCGATCA
	M. smegmatis	ACAAGACCGTGATCGCCAAGT	GCCCGATGCCGAGACCACGAC
MAV_3729	*M. avium*	CCTGCAATTCACCGCGACCAC	CCCGGGCGGCCAGTTCGTCCT
	M. smegmatis	GACTGCAGTTCACGGGTACGA	CGGCGCGGTAGAACACGTAGG
MAV_0701	*M. avium*	ACGTCACCTCCGAAGTCGTAG	GTTCCTGACGGCGCGAGATTC
	M. smegmatis	GACGTCACCGCCGAAGTCGTA	GTGATGATGCCGGGTATGGAC
MAV_2510	*M. avium*	CAACTACCTGCCCACCGACCT	AAGTCCTCGAAATCGGCCATT
	M. smegmatis	AAATCGTCGAAATCGCTGATG	TTCTCGCAGGCCCGGATGAAC
MAV_0214	*M. avium*	AGCGAGCCGGCGTAGACGAAC	TTGTACCTGCTCGACGGGATG
	M. smegmatis	CCGCCCGCGTCACCCATCGAG	GCGGCTGCAAGACCTACAAGT
MAV_4209	*M. avium*	ATGGACACCATGAGGCAAAGG	TCACGGAGGTCCGGCCTTGTA
Mav_Inv1 (246-704)	*M. avium*	ACGTTCACGACCCGAGTTCAC	GCCGGTACTATGCGTCGATCC
Mav_Inv2 (421-872)	*M. avium*	GAGGGCCGGTCCAACGTGAAC	CATTTGAGCACTTGCCCGTAT
Mav_Inv3 (827-1296)	*M. avium*	TCCATTCAACCCGGCGCATTT	CATGATTCGGCGGTCGGGTTC
Mav_Inv4 (1322-1779)	*M. avium*	GCAGTCTCATCGGCGTTGATT	TCCATATTTCCGCCGAGCAAG
Mav_Inv5 (1737-2265)	*M. avium*	TCCGCAACACCAGAAATCGTG	TGGGTCAGTGCGGCGCAATGA

[a] Primers from Talaat *et. al* (1997)

the average intensity of the *E. coli* negative controls for that array were removed from the analysis.

The methods of Porwollik *et al.* (2002) were used to determine whether a gene was present or absent. Briefly, the ratio of the test strain to MAC104 for each spot was determined (Cy3/Cy5 or Cy5/Cy3, depending on the experiment); this ratio was $\log_2$ transformed, then normalized with respect to all ratios (such that the mean $\log_2 = 0$). The median value of the spots for each gene was calculated and genes with a median $\log_2$ of less than -1.05, based on 2-4 experiments, were scored as being absent. This cutoff was determined by calculating the average $\log_2$ value for all spots that should be present in both MAC104 and *M. tuberculosis* and subtracting two standard deviations.

PCR verification of microarray data. For a subset of genes with a median ratio of less than -1.05 in one or more strains tested, primers were designed to amplify regions encompassing the oligonucleotide sequence on the array. Primers were designed for each gene from both the MAC104 and *M. smegmatis* sequences. A range of annealing temperatures from 55 °C to 65 °C was used in situations were there was questionable or no amplification with an annealing temperature of 60 °C with either set of primers. Table 3.2 lists the genes and primers used to verify the presence or absence of these genes.

Statistical Analysis. For the macrophage assays, results shown are the average and standard deviation of three replicates. Differences in growth were considered significant by Student's t-test, $p < 0.05$.

Results

Environmental species of mycobacteria exhibit different patterns of growth in human, mouse, and carp cell lines. Human monocytes (U-937), mouse macrophages (RAW264.7), and carp leukocytes (CLC) were exposed to 1 strain of *M. avium*, 2 strains of *M. peregrinum,* 3 strains of *M. chelonae*, and 6 strains of *M. salmoniphilum* at 37 °C and/or 28.5 °C. Uptake after a 1 hour exposure, and growth within the cells after 2 and 4 days were assayed by counting the colony forming units (CFUs) collected from the lysed cells as described (Bermudez and Petrofsky, 1997). MAC101 grew 732% from the baseline to 2 days, and 1259% from 2 days to 4 days in U-937 cells incubated at 37 °C. This strain increased 1788% over 2 days from baseline and 324% from 2 days to 4 days in RAW264.7 cells grown at 37 °C. However, MAC101 decreased 155% after 2 days from the baseline, and decreased an additional 265% between 2 and 4 days in CLC cells grown at 28.5 °C (Table 3.3). MAC101 exhibited a similar pattern to MAC104 based on data from previous work (24). MAC101 was also assayed in U-937 and RAW264.7 cells at 28.5 °C, and CLC cells at 37 °C. In human and mouse cells at this lower temperature, increase in bacterial CFUs was observed for human cells (394% over baseline at 2 days, and 228% additional increase between 2 and 4 days), but growth was only observed after 2 days for mouse cells (324% between baseline and 2 days, with a subsequent decrease of 1778% between 2 and 4 days). In CLC cells at 37 °C, MAC101 did not exhibit growth after 2 days (-211%), but did grow between 2 and 4 days (437%) (Table 3.3).

Table 3.3 Growth of mycobacteria strains in human (U937), mouse (RAW264.7) and carp (CLC) cells.

	Time (days)			% change between days 2 and baseline	% change between days 4 and 2
Cell line and strain	**Baseline (0)**	**2**	**4**		
U937 (37 °C)					
MAC101	$3.85 \pm 1.06 \times 10^4$	$2.82 \pm 0.17 \times 10^5$	$3.55 \pm 0.64 \times 10^6$	732[a]	1259[b]
MP101	$4.65 \pm 0.07 \times 10^4$	$8.80 \pm 4.67 \times 10^5$	$3.15 \pm 0.5 \times 10^8$	1892	35795[b]
MP102	$1.98 \pm 0.06 \times 10^4$	$1.35 \pm 0.13 \times 10^5$	$1.51 \pm 1.75 \times 10^6$	682[a]	1119
MCH	$3.9 \pm 0.85 \times 10^4$	$1.77 \pm 0.33 \times 10^4$	$1.40 \pm 0.71 \times 10^4$	-220	-126
MCJA	$5.3 \pm 0.14 \times 10^4$	$2.9 \pm 0.28 \times 10^5$	$7.0 \pm 1.41 \times 10^5$	547[a]	241[b]
MCJ78	$5.5 \pm 0.71 \times 10^4$	$1.0 \pm 0.21 \times 10^5$	$5.0 \pm 1.13 \times 10^5$	182[a]	500[b]
MCBAND	$3.33 \pm 0.27 \times 10^4$	$4.93 \pm 0.33 \times 10^5$	$6.28 \pm 0.18 \times 10^5$	1480[a]	127[b]
MCTRASK	$6.25 \pm 0.45 \times 10^4$	$4.00 \pm 0.2 \times 10^5$	$6.88 \pm 0.27 \times 10^5$	640[a]	172[b]
MCSABLE	$3.3 \pm 2.7 \times 10^4$	$4.25 \pm 0.14 \times 10^5$	$6.55 \pm 0.17 \times 10^5$	1288[a]	154[b]
MCMWF	$5.95 \pm 0.09 \times 10^4$	$5.28 \pm 0.23 \times 10^5$	$6.98 \pm 0.28 \times 10^5$	887[a]	132[b]
MCAUS	$3.1 \pm 1.3 \times 10^3$	$3.1 \pm 0.15 \times 10^5$	$4.5 \pm 0.12 \times 10^5$	10000[a]	145[b]
MCSILETZ	$7.63 \pm 0.24 \times 10^4$	$9.38 \pm 0.18 \times 10^5$	$1.17 \pm 0.03 \times 10^6$	1737[a]	125[b]
MCER	$5.4 \pm 0.29 \times 10^4$	$3.1 \pm 0.11 \times 10^5$	$4.68 \pm 0.03 \times 10^6$	574[a]	1510[b]
U937 (28.5 °C)					
MAC101	$1.84 \pm 1.63 \times 10^4$	$7.25 \pm 3.46 \times 10^4$	$1.65 \pm 0.008 \times 10^5$	394	228
MP101	$2.35 \pm 0.78 \times 10^4$	$6.50 \pm 4.95 \times 10^4$	$7.64 \pm 1.22 \times 10^7$	277	117538[b]
MP102	$2.03 \pm 0.79 \times 10^4$	$1.09 \pm 0.33 \times 10^6$	$1.27 \pm 0.7 \times 10^6$	5369[a]	117
MCH	$2.88 \pm 0.08 \times 10^4$	$3.88 \pm 0.02 \times 10^5$	$5.60 \pm 0.35 \times 10^5$	1347	144
RAW264.7 (37 °C)					
MAC101	$3.30 \pm 1.56 \times 10^3$	$5.90 \pm 6.65 \times 10^4$	$1.91 \pm 0.24 \times 10^5$	1788	324
MP101	$1.00 \pm 0.14 \times 10^6$	$3.56 \pm 0.49 \times 10^6$	$1.40 \pm 0.14 \times 10^8$	356[a]	3933[b]
MP102	$1.65 \pm 0.07 \times 10^6$	$1.65 \pm 0.07 \times 10^5$	$7.0 \pm 0.7 \times 10^5$	-1000[a]	424[b]
MCH	$4.5 \pm 0.14 \times 10^3$	$4.08 \pm 1.17 \times 10^3$	$4.15 \pm 0.07 \times 10^3$	-110	102
MCJA	$4.75 \pm 0.35 \times 10^4$	$1.0 \pm 0.28 \times 10^5$	$1.01 \pm 0.05 \times 10^6$	211	1010[b]
MCJ78	$2.15 \pm 0.21 \times 10^4$	$5.85 \pm 0.21 \times 10^4$	$3.75 \pm 0.07 \times 10^5$	272[a]	641[b]
MCBAND	$4.6 \pm 0.2 \times 10^4$	$3.55 \pm 0.09 \times 10^5$	$7.93 \pm 0.15 \times 10^5$	772[a]	223[b]

[a] indicates significant change between baseline and 2 days, $p < 0.05$; [b] indicates significant change between 2 days and 4 days, $p < 0.05$

Table 3.3 (continued). Growth of mycobacteria strains in human (U937), mouse (RAW264.7) and carp (CLC) cells.

MCTRASK	$5.13 \pm 0.43 \times 10^4$	$1.01 \pm 0.04 \times 10^6$	$1.28 \pm 0.03 \times 10^6$	1969 [a]	127 [b]
MCSABLE	$5.4 \pm 2.27 \times 10^3$	$4.0 \pm 0.08 \times 10^5$	$7.0 \pm 0.2 \times 10^5$	7407 [a]	175 [b]
MCMWF	$6.33 \pm 0.27 \times 10^4$	$7.63 \pm 0.24 \times 10^5$	$8.38 \pm 0.24 \times 10^5$	1205 [a]	110
MCAUS	$3.3 \pm 0.29 \times 10^4$	$4.45 \pm 0.2 \times 10^5$	$5.68 \pm 0.27 \times 10^5$	1348 [a]	128 [b]
MCSILETZ	$3.88 \pm 0.15 \times 10^4$	$6.95 \pm 0.21 \times 10^5$	$9.3 \pm 0.24 \times 10^5$	1791 [a]	134 [b]
MCER	$7.3 \pm 0.35 \times 10^4$	$5.5 \pm 0.24 \times 10^5$	$7.8 \pm 0.12 \times 10^5$	753 [a]	142 [b]
RAW264.7 (28.5 °C)					
MAC101	$2.47 \pm 0.35 \times 10^7$	$8.0 \pm 0.27 \times 10^7$	$4.5 \pm 1.41 \times 10^6$	324 [a]	-1778
MP101	$7.00 \pm 1.41 \times 10^5$	$1.28 \pm 0.04 \times 10^6$	$1.29 \pm 1.21 \times 10^7$	183 [a]	1008
MP102	$2.44 \pm 0.78 \times 10^4$	$3.43 \pm 0.17 \times 10^5$	$1.92 \pm 0.44 \times 10^6$	1406 [a]	560 [b]
MCH	$3.95 \pm 0.21 \times 10^4$	$5.58 \pm 0.22 \times 10^5$	$8.13 \pm 0.24 \times 10^5$	1413 [a]	146 [b]
CLC (37 °C)					
MAC101	$4.0 \pm 0.3 \times 10^5$	$1.9 \pm 0.4 \times 10^5$	$8.3 \pm 0.3 \times 10^5$	-211	437 [b]
MP101	$5.60 \pm 0.30 \times 10^5$	$1.80 \pm 0.20 \times 10^6$	$4.60 \pm 0.40 \times 10^6$	321 [a]	256 [b]
MP102	$5.0 \pm 0.4 \times 10^5$	$2.0 \pm 0.2 \times 10^5$	$8.0 \pm 0.3 \times 10^4$	-250	-250 [b]
MCH	$6.3 \pm 0.3 \times 10^5$	$9.1 \pm 0.2 \times 10^4$	$5.6 \pm 0.3 \times 10^4$	-692 [a]	-163
CLC (28.5 °C)					
MAC101	$3.1 \pm 0.4 \times 10^5$	$2.0 \pm 0.3 \times 10^5$	$5.3 \pm 0.2 \times 10^5$	-155	265 [b]
MP101	$5.00 \pm 0.20 \times 10^5$	$1.00 \pm 0.30 \times 10^6$	$5.10 \pm 0.40 \times 10^6$	200 [a]	510 [b]
MP102	$3.0 \pm 0.3 \times 10^5$	$6.0 \pm 0.2 \times 10^5$	$8.0 \pm 0.3 \times 10^5$	200 [a]	133
MCH	$4.98 \pm 0.06 \times 10^4$	$7.08 \pm 0.15 \times 10^5$	$1.13 \pm 0.02 \times 10^6$	1422 [a]	1046 [b]
MCJA	$1.18 \pm 3.18 \times 10^4$	$2.80 \pm 0.14 \times 10^5$	$4.80 \pm 0.28 \times 10^5$	2373 [a]	171
MCJ78	$5.5 \pm 0.71 \times 10^4$	$3.0 \pm 0.71 \times 10^5$	$8.0 \pm 1.41 \times 10^5$	545 [a]	267 [b]
MCBAND	$3.28 \pm 0.23 \times 10^4$	$3.03 \pm 0.09 \times 10^5$	$6.13 \pm 0.15 \times 10^5$	924 [a]	202 [b]
MCTRASK	$6.80 \pm 0.29 \times 10^4$	$5.58 \pm 0.29 \times 10^5$	$7.8 \pm 0.34 \times 10^5$	821 [a]	140 [b]
MCSABLE	$4.55 \pm 0.27 \times 10^4$	$4.4 \pm 0.29 \times 10^5$	$6.93 \pm 0.33 \times 10^5$	967 [a]	158 [b]
MCMWF	$7.25 \pm 0.32 \times 10^4$	$6.15 \pm 0.4 \times 10^5$	$7.83 \pm 0.12 \times 10^5$	848	127 [b]
MCAUS	$2.78 \pm 0.27 \times 10^4$	$3.68 \pm 0.14 \times 10^5$	$5.85 \pm 0.14 \times 10^5$	1324 [a]	159 [b]
MCSILETZ	$5.5 \pm 0.24 \times 10^4$	$8.05 \pm 0.21 \times 10^5$	$1.03 \pm 0.03 \times 10^6$	1464 [a]	128 [b]
MCER	$8.2 \pm 0.35 \times 10^4$	$4.12 \pm 0.15 \times 10^5$	$7.8 \pm 0.18 \times 10^5$	502 [a]	189 [b]

[a] indicates significant change between baseline and 2 days, $p < 0.05$; [b] indicates significant change between 2 days and 4 days, $p < 0.05$

Two strains of *M. peregrinum* (MP101 and MP102) were tested in all three strains of cells at both temperatures. MP101 was able to infect and exhibit growth over 4 days in U-937 cells, RAW264.7 cells, and CLC cells at 37 °C and 28.5 °C (Table 3). MP102 was also able to replicate in different species of macrophages at 37 °C, but was not able to grow in the mouse macrophages at 28.5 °C between baseline and 2 days (-1000%), although it did show subsequent growth after 4 days (424%) (Table 3.3).

Of the 3 strains of *M. chelonae* tested, 2 were isolated from outbreaks in zebrafish colonies, and the other (MCH) was isolated from incidental infections of zebrafish. CFUs of the MCH strain of *M. chelonae* increased compared to baseline after 2 and 4 days in CLC cells at 28.5 °C, but did not grow in human or mouse cells at 37 °C or 28.5 °C (Table 3.3). The other strains isolated from zebrafish, MCJA and MCJ78, grew in all three cell types, regardless of the incubation temperature (Table 3.3).

Six isolates of *M. salmoniphilum* from salmonid fish grew in U-937 cells at 37 °C after 2 days, but exhibited little additional growth between 2 and 4 days, with the exception of the MCER strain (Table 3.3). In RAW264.7 cells incubated at 37 °C, there was again growth between baseline and 2 days, but little additional growth over the subsequent 2 days (Table 3.3). All six strains increased in CFUs in CLC cells at 28.5 °C over the course of the 4-day assay, with the trend of less growth between days 2 and 4 being consistent with the other cell types (Table 3.3).

Strains with comparable abilities to grow in macrophages also share genetic similarity. The genomic DNAs of a subset of the EM strains analyzed in macrophage assays (Table 3.3) were compared against genomic DNA from the reference strain, MAC104 by microarray analysis. Table 3.4 (*M. peregrinum* strains), Table 3.5

Table 3.4 Score[a] for genes predicted to be absent in *M. peregrinum* isolates.

	Strain		
Gene Number	MP101	MP102	ATCC MP
MAV1540	+	+	-1.14
MAV1705	+	+	-1.38
MAV2241	-1.13	+	+
MAV2479	-1.24	+	+
MAV3389 (*carB*)	+	+	-1.09
MAV4209 (*mtrA*)	+	+	-1.14
MAVinv1	-2.88	+*	-2.76
MAVinv2	-4.35	+	-1.97
MAVinv5	-3.46	-1.53	-2.00
MAVInv6	ND[b]	0.53	-3.35
MAVinv9	-3.66	+*	-1.88
MAVinv13	-2.63	+	-2.94
MAVinv14	-2.40	+	+
MAVinv15	-3.73	+	-1.15

[a] A negative value less than -1.05 indicates that the score was more than 2 standard deviation lower than the median score for a gene absent in *M. avium* as compared to *M. tuberculosis*.
[b] ND indicates that the score was not determined for that gene.
[*] Score is between 1 and 2 standard deviations below the median

(*M. chelonae* strains) and Table 3.6 (*M. salmoniphilum* strains) and Figure 3.1 summarize the genes that were found to be absent by microarray analysis. A summary of all results is available as Supplemental Table 3.1. The strains of *M. avium* are similar in their ability to grow in macrophages, and by microarray, there were only two genomic differences between MAC104 and the other three strains of *M. avium* tested. By the calculation described in the methods used to determine the presence or absence of a gene, MAV3729 (*mpt*53) was scored as absent in MAC100, and MAV2536 (*lpr*M) was scored as absent in MAC109. The *mpt*53 gene, however, was amplified from the genomic DNA of the MAC100 strain by PCR (Table 3.7).

M. peregrinum. The strain of *M. peregrinum* isolated from zebrafish replicated differently in macrophages than *M. avium*, and had more genomic differences than other strains of *M. avium* when compared to MAC104 (Table 3.4). Two genes were scored as absent from the MP101 strain (MAV2241 and MAV2479). No genes were absent from the MP102 strain, isolated from tilapia. The probes designed for the putative invasion island indicated that parts of the region are absent in the MP101 strain, but present in the MP102 strain. The MPATCC strain, isolated from human sputum, was also missing segments of the invasion island, as well as four other genes (MAV1540, MAV1705, MAV3389/*car*B, MAV4209/*mtr*A) (Table 3.4). Four regions of the invasion island were further analyzed by PCR, and could not be amplified in any of the three strains. The *mtr*A gene could be amplified by PCR from the DNA of the MPATCC strain (Table 3.7).

***M. chelonae* from zebrafish.** Although the MCH, MCJA, and MCJ78 strains were all isolated from zebrafish, the MCH strain had a different profile of growth in macrophages. Similarly, the MCH strain differs genomically from the other two strains. The genomic DNA of all three strains also had a different genomic profile from the strains of *M. peregrinum,* when compared to MAC104. The scores for presence or absence of genes are summarized in Table 3.5. There were five genes that were scored as absent in the MCH strain, including MAV4209/*mtr*A. Additionally, the invasion island is absent from this strain. None of the genes that were absent in the MCH strain are similar to those absent in MCJA or MCJ78. The MCJA strain had five genes that were absent, while the MCJ78 strain was missing seven genes. Of these, MAV1169/*kdp*E was common between both. In the case of four genes that were scored as absent in only one of the strains (MAV0182/*sod*A, MAV2510/*mmp*L5, MAV3608, and MAV4679/*pca*A),

Table 3.5 Score[a] for genes predicted to be absent in *M. chelonae* isolates.

Gene Name	Gene Number	Strain		
		MCH	MCJA	MCJ78
ppp	MAV0022	+*	+	+
	MAV0160	+	+	+
sodA	MAV0182	+	+*	-1.26
	MAV0663	+	-1.37	+*
eno	MAV1164	-1.30	+	+
kdpE	MAV1169	+	-2.05	-1.56
fadD6	MAV1351	+	+	+*
oppD	MAV1431	-1.24	+	+
glpD1	MAV2188	+	+	-1.54
mmpL5	MAV2510	+*	+*	-1.12
katG	MAV2753	+	+*	+
fbpB	MAV2816	+	+	-1.48
secA	MAV2894	+*	+	+
	MAV3608	+	-1.55	+*
whiB1	MAV4167	+	+	-1.35
mtrA	MAV4209	-1.13	+	+
regX3	MAV4660	+*	+	+
pcaA	MAV4679	+	+*	-1.35
pckA	MAV4963	-1.23	+	+
mmpL11	MAV4973	+	+	+*
mce1	MAV5015	+	-1.60	+
ephF	MAV5163	+	+*	+
	MAV5286	+*	+	+
	MAVinv1	-2.21	+*	+*
	MAVinv2	-2.24	-1.33	-1.43
	MAVinv3	-1.83	+*	ND[b]
	MAVinv4	-1.19	+*	+*
	MAVinv5	-2.73	+	+*
	MAVinv6	-2.46	-2.31	-3.004
	MAVinv7	-2.60	+*	+*
	MAVinv8	-1.60	+	ND[b]
	MAVinv9	-2.31	+	+
	MAVinv10	-2.52	+	+*
	MAVinv11	-1.20	ND[b]	+
	MAVinv12	-1.55	+	+*
	MAVinv13	-2.39	+	+*
	MAVinv14	-1.81	+	+
	MAVinv15	-1.30	+	-1.30

[a] A negative value less than -1.05 indicates that the score was more than 2 standard deviations lower than the median score for a gene absent in *M. avium* as compared to *M. tuberculosis*.

[b] ND indicates that the score was not determined for that gene.

[*] Score is between 1 and 2 standard deviations below the median

the score for the other strain was very near to the cutoff of -1.05 (Table 3.5). The invasion island was present in both MCJA and MCJ78 strains. PCR amplification from genomic DNA confirmed that MAV4209/*mtr*A and the invasion island were absent from MCH, and that the MAV1169/*kdp*E gene was absent in the MCJA and MCJ78 strains (Table 3.7).

***M. salmoniphilum* from salmonids.** The strains of *M. chelonae* isolated from salmonids were the most dissimilar group as compared to MAC104. All strains tested by macrophage assays had comparable growth in the three types of macrophages. The profile of genes that are present, absent, or putatively duplicated is very similar across the four strains tested by microarray. Table 3.6 summarizes the putatively absent genes in each strain; MCBAND had 21 genes that scored as absent, MCMWF had 25 genes, MCSABLE had 29 genes, and MCTRASK, 21 genes absent. Nine genes were absent in all four strains. Many others were absent in two or three of the strains. In these cases, the score was typically very close to the -1.05 cutoff in the other strain(s) (Table 3.6). Supplementary Table 3.1 shows a summary of these values while Figure 3.1 shows a visual representation of the presence and absence of all genes tested.

Amplification of a subset of genes by PCR had some correlation to the microarray results with these strains (Table 3.7). Although MAV_0469 and MAV_3729/*mpt53* scored as absent in four and three of the strains, respectively, both genes could be amplified from all four strains by PCR with primers designed from *M. smegmatis* sequence (but not with primers designed from *M. avium* sequence). Although MAV_0214/*fbp*A scored as absent in all four strains, it could be amplified by PCR

Table 3.6 Score[a] for genes predicted to be absent in *M. salmoniphilum* isolates.

Gene Name	Gene Number	Strain			
		MCBAND	MCMWF	MCSABLE	MCTRASK
Ppp	MAV0022	+	-1.28	+	+
	MAV0160	+*	-1.64	+*	+*
fbpA	MAV0214	-1.08	-1.17	-1.36	-1.77
embA	MAV0225	-2.23	-1.9	-3.66	-2.18
embC	MAV0229	+*	-1.88	-1.56	-1.06
	MAV0469	-1.53	-1.74	-2.1	-1.71
phoP	MAV0701	+	-1.21	+*	+*
phoT	MAV0767	+	-1.07	-1.55	+
Eno	MAV1164	+*	+*	-2.35	-1.22
kdpE	MAV1169	+	+	+	+
fadD6	MAV1351	+	+*	-1.61	-1.42
atpA	MAV1525	-1.61	-1.62	+*	-1.28
murA	MAV1531	-1.1	+*	-1.17	+
	MAV1540	-2.16	-1.34	-3.19	+*
fadA5	MAV1615	-1.05	+*	+*	+*
argS	MAV1724	-1.37	+*	-1.34	-1.19
mmpL12	MAV1761	+*	-1.09	-1.53	-1.06
sucA	MAV2273	+*	+*	+*	-1.4
	MAV2304	+*	+*	-1.28	+
lppM	MAV2323	-1.33	+*	+	+
katG	MAV2753	-2.22	-1.51	-3.81	-1.96
secA	MAV2894	-1.99	-1.63	-2.55	-2.71
secA2	MAV2894	+	-1.22	+	+
mgtC	MAV2903	+*	-1.16	+*	+*
uvrC	MAV3360	+*	+*	+	-1.07
carB	MAV3389	+	-1.37	+*	+*
Mpt53	MAV3729	-1.65	+*	-1.61	-1.19
pfkA	MAV3858	-1.48	+*	-1.76	-1.34
cstA	MAV3938	-1.72	-1.57	-1.72	+*
	MAV4168	-1.36	-1.9	-2.20	-1.24
mtrA	MAV4209	+	-1.5	-1.89	-1.14
dnaE2	MAV4335	+*	+*	-1.28	+*
secY	MAV4434	-1.37	+*	-1.39	-1.33
proC	MAV4650/51	-1.13	-1.98	-1.74	-1.28
regX3	MAV4660	+	+*	-1.05	+
udgA	MAV4823	-1.46	-1.7	-1.87	-1.63
pckA	MAV4963	+	+*	+	+
mmpL11	MAV4973	+*	-1.23	-1.08	+*
lprO	MAV5005	-1.28	+*	-1.94	+*
ephF	MAV5163	-1.66	-1.53	-2.74	+*
pepA	MAV5187	-1.61	-1.43	-1.92	-1.58
	MAV5286	+	-1.19	+	+

[a] A negative value less than -1.05 indicates that the score was more than 2 standard deviations lower than the median score for a gene absent in *M. avium* as compared to *M. tuberculosis*.
[b] ND indicates that the score was not determined for that gene.
[*] Score is between 1 and 2 standard deviations below the median

using primers designed from *M. smegmatis* from every strain except MCTRASK. MAV_0701/*pho*P was scored as absent in MCSABLE and MCTRASK, and also did not amplify from MCTRASK DNA by PCR. The *mtrA* gene, absent by microarray, could not be amplified from DNA of any of the four strains by PCR.

Discussion

Environmental mycobacteria (EM) have a significant ability to cause disease in humans and animals. Very little knowledge exists, however, about the mechanisms of disease for this group of pathogens, including those important to fish mycobacteriosis. To learn more about the virulence and genomics of some of these species with zoonotic potential, we used macrophage assays and a custom microarray to compare strains of different EM species to *M. avium*.

Many of the EM strains of *M. peregrinum* and *M. chelonae* were isolated from zebrafish and have been characterized previously (Kent *et al.,* 2004; Watral and Kent, 2007). The strains isolated from salmonid fishes were first classified as *M. salmoniphilum*, but the species was suppressed and its members assigned to *M. chelonae* (Ross, 1960; Arakawa and Fryer, 1984). However, the species has now been resurrected based on molecular analyses (Whipps, *et al.*, 2007). Because all the strains of *M. chelonae*, *M. salmoniphilum*, and the MP102 strain of *M. peregrinum* were unable to grow in culture at 37 °C, it was notable that many of these isolates were able to grow in human and mouse macrophages at the same temperature. Previous work with *M. marinum*, another fish pathogen with zoonotic potential, indicates that strains that are not able to grow in culture at 37 °C, are capable of growing in macrophages

Table 3.7 PCR confirmation of microarray results.

Gene Name	MAV Number	MAC104	MAC100	MAC101	MAC109	MP101	MP102	MPATCC	MCH	MCJA	MCJ78	MCBAND	MCTRASK	MCSABLE	MCMWF
16S		+	+	+	+	+	+	+	+	+	+	+	+	+	+
kdpE	MAV1169	+	+	+	+	+	+/-	+/-	+	-	-	+	+	+	+
pcaA	MAV4679	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Rv3661	MAV0469	+	+	+	+	+	+	+	+	+	+	+	+	+	+
mpt53	MAV3729	+	+	+	+	+	+/-	+	+	-	-	+	+	+	+
phoP	MAV0701	+	+	+	+	+	+	+	+	+	+	+	+	-	+/-
mmpL5	MAV2510	+	+	+	+	+	+	+	+	-	+/-	+	+	+	+
fbpA	MAV0214	+	+	+	+	+	+	+	+/-	+	+	+	-	+	+
mtrA	MAV4209	+	+	+	+	+	+	+	-	+	-	-	-	-	-
Inv1		+	+	+	+	-	-	+/-	-	-	-	+	+	+/-	-
Inv2		+	+	+	+	-	-	-	-	-	-	-	+	+/-	-
Inv3		+	+	+	+	+	+/-	-	+	-	-	+	+	+	+
Inv4		+	+	+	+	-	-	-	-	-	-	-	+	+/-	-
Inv5		+	+	+	+	-	-	-	-	-	-	+	+	+	-

+ indicates a single PCR products; +/- indicates multiple or faint PCR products; - indicates no PCR product

at that temperature (Kent *et al*., 2006). All strains of mycobacteria isolated from fish replicated in the carp cells, and many of those strains also grew in the human and mouse cells. In contrast, neither the MAC101 nor MAC104 strains (from humans) grew well in carp cells. This result does not seem to be a product of the optimum temperature for the cell type or bacterial strain, as the MAC101 strain was able to grow in human and mouse cells at 28.5 °C.

The sequenced species of mycobacteria have a wide range of alternate sigma factors, from four in *Mycobacterium leprae* to 28 in *M. smegmatis*. It is thought that many of the *M. tuberculosis* sigma factors are involved in growth in various conditions (Chen *et al,*. 2000; Rodrigue *et al*., 2006) and virulence (Smith, 2003; Manganelli *et al*., 2004). The proteins appear to function in regulatory networks with each other in response to various stimuli, but many of the downstream genes affected by the various sigma factors are yet unknown (Rodrigue *et al*., 2006). Studies have shown that the amino acid sequences of the *sig* genes in the avirulent BCG strain are slightly different from *M. tuberculosis* (Charlet *et al*., 2005) or that the genes are duplicated (Brosch *et al*., 2000; Manganelli *et al*., 2004), and that *Mycobacterium microti*, which has lower pathogenicity than *M. tuberculosis*, is missing the sigma factor, SigF (Geiman *et al*., 2004). SigJ does not appear to be involved in virulence, yet transcription is induced in human macrophages (Capelli *et al*., 2006). It is possible that the environmental strains of mycobacteria tested in this study have lost or gained multiple copies of some of these regulatory genes, leading to the ability to grow in the intracellular environment of a macrophage at 37 °C, even when they are incapable of growth in *in vitro* culture. Only *sig*E was analyzed by microarray in this study, and it was not determined to be absent or

Figure 3.1 Microarray hybridization results for all genes and strains examined. The dark vertical lines indicate the separation between the four species of mycobacteria tested. Boxes from left to right represent the individual strains as follows: (a) MAC100, (b) MAC101, (c) MAC109, (d) MP101, (e) MP102, (f) MPATCC, (g) MCH, (h) MCJA, (i) MCJ78, (j) MCBAND, (k) MCTRASK, (l) MCMWF, (m) MCSABLE. All genes used in the microarray are listed in Supplemental Table 1. The boxes in this figure from top to bottom represent this gene list as it ordered in S-Table 1. Genes that were 2 or more standard deviations below the median were considered to be putatively absent (score < -1.05), while genes that were 2 or more standard deviation above the median were considered to be putatively duplicated (score > 1) . A-G represent regions discussed further in the text. A) *fbpA*, *embA*, *embC*; B) *eno, kdpE*; C) MAV_1705, *argS, mmpL12*; D) *katG*; E) *mpt53*; F) *mtrA*; G) *M. avium* pathogenicity island (PI, regions 1-15)

- Indicates present gene;
- Indicates score between 1 and 2 standard deviations below the mean;
- Indicates between 2 and 3 standard deviations below the mean (putatively absent);
- Indicates more than 3 standard deviations below the mean (putatively absent);
- Indicates between 2 and 3 standard deviations above the mean;
- Indicates more than 3 standard deviations above the mean (putatively duplicated);
- Indicates no data was obtained for this gene.

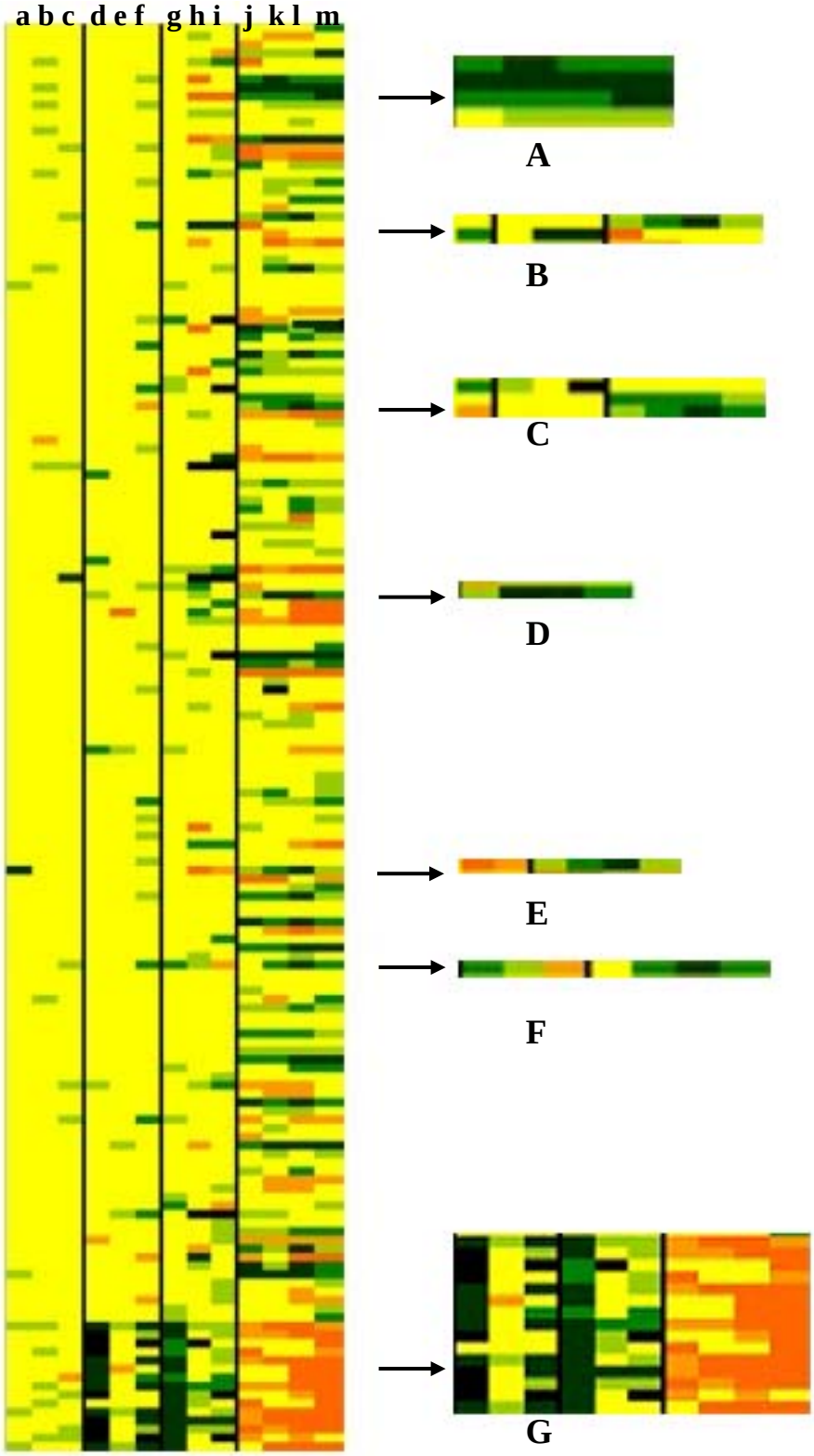

Figure 3.1 (continued)**.**

duplicated in any strain. Differences in sigma factors could be playing a role in the ability of these strains to survive and grow at the temperature, and possibly also in the differences in growth observed between strains. Future work will address this question.

The percent growth over 2 and 4 days in the three types of macrophages was not similar in mycobacteria isolated from the same hosts, or even in the same species, although there were some trends. The *M. salmoniphilum* strains isolated from salmonids and the MCJA and MCJ78 strains isolated from zebrafish showed growth over 2 and 4 days in all three macrophages lines. The MCH strain of *M. chelonae* isolated from zebrafish, however, was not able to grow in either mouse or human macrophages, or in carp cells incubated at 37 °C. When the human and mouse macrophages were incubated at a lower temperature, the MCH strain was able to grow. The MP101 strain of *M. peregrinum*, isolated from zebrafish, was able to grow in all cell types at either temperature, but the MP102 strain, isolated from tilapia, was not able to grow in mouse macrophages unless they were incubated at 28.5 °C. This strain was also unable to grow in carp cells incubated at 37 °C. As previously indicated, the *M. avium* strains tested were unable to multiply well in carp cells at either temperature.

The macrophage growth profile correlates well with other virulence data. Using intraperitoneal injection, Watral and Kent (2007) showed that the MCH strain of *M. chelonae* did not cause any significant disease or mortality in zebrafish, nor could it be cultured from the zebrafish weeks after exposure. Moreover, this isolate was obtained from a few fish as an incidental infection in a large research colony (Kent *et al*., 2004). In a different study in which zebrafish were exposed to mycobacteria by bath immersion or intubation, we showed that the MP101 strain of *M. peregrinum* (which did grow well

in mammalian macrophages) was consistently able to infect and grow in zebrafish tissues, although signs of disease and mortality were rare (Harriff *et al*., 2007) (See Chapter 4). In data not included in that study, the MP102 strain of *M. peregrinum* could neither be cultured from the zebrafish, nor did it cause any signs of disease. Similarly, we could not culture the *M. avium* MAC104 strain from zebrafish infected by bath immersion or intubation, and the infected fish showed no signs of disease (MJ Harriff, unpublished observations).

Genomic differences, based on microarray analyses, also correlated with phenotypic virulence data. A survey of the literature revealed over 120 genes predicted to play a role in virulence of different species of mycobacteria. Many of the genes selected for the microarray were identified by a number of genome- or proteome-wide screens designed to detect *M. avium* or *M. tuberculosis* genes or proteins upregulated in human macrophages (Braunstein *et al*., 2000; Dubnau *et al*., 2002; Hou *et al*., 2002; Danelishvili *et al.*, 2004). Over 50 of these gene products have no known or predicted function, including the open reading frames in a 3kb pathogenicity island (PI) identified in our lab to be important in virulence (Danelishvili *et al*., 2007). Little is known about how the genes encoded by the open reading frames in the PI may function in virulence, but it has been show to allow for infection of amoeba. This region is not present in *M. tuberculosis*, but is present in other members of the MAC. By microarray analysis, the island was completely absent in the MCH strain of *M. chelonae*, while portions of the island were also predicted to be absent in the ATCC and MP101 strains of *M. peregrinum*. Interestingly, the island is putatively duplicated in the *M. salmoniphilum* strains. This bacterium is a recognized pathogen of salmonids, based on both laboratory

transmission studies (Arakawa and Fryer, 1984) and field observations (Bruno *et al.,* 1998; Brocklebank *et al*., 2003). The *in vivo* and *in vitro* growth by these strains of mycobacteria suggests that the genes making up this pathogenicity island should be further studied for their role in the virulence of EM.

In addition to genes predicted to play a role in virulence, sequences representing selected genes of many of the metabolic pathways in mycobacteria were included. It is not known if differences or absence of metabolic pathways can play a role in host specificity or levels of pathogenicity. Differences in metabolic pathways are also important to differences in the basic phenotypic characteristics of species, which may play roles in environmental survival, host specificity, and pathogenicity. For example, *M. avium* subsp. *paratuberculosis* is very closely related to *M. avium* subsp. *avium* at the genomic level (Bannantine *et al*., 2003); however, multiple studies have revealed that each subspecies has regions and genes that are not present in the other (Semret *et al*., 2004; Paustian *et al*., 2005). Among some of the important findings, microarray analysis revealed that *M. avium* subsp. *paratuberculosis* has a truncation of an early gene in the mycobactin synthesis operon (Semret *et al*., 2004). Further study of this truncation could reveal the reasons for slow growth of *M. avium* subsp. *paratuberculosis,* as well as for differences in host and pathogenicity. Some of the species of EM tested in this study were putatively missing genes from metabolic pathways that may play a role in the ability to persist in certain environments.

Although many virulence genes identified in screens and included on this array have no putative function, others have been studied for their role in virulence. The *mtr*A gene was scored as absent in the MCH strain of *M. chelonae*, and three of the four strains

of *M. salmoniphilum* (with the fourth strain score being very near to the cutoff). This gene encodes the response regulator protein of a 2-component regulatory system that has been shown by Deretic and colleagues to be essential to growth in broth, and differentially expressed by virulent and avirulent mycobacteria (Zahrt and Deretic, 2000). While expression of this gene is constitutive and high in *M. tuberculosis*, it is induced to very high levels in the BCG vaccine strain upon entry into macrophages. A more recent study by Fol *et al*. (2006) showed that the regulation of the ratio of this protein product and its phosphorylated version are important to proliferation in macrophages. The protein product encoded by *mtrB* that phosphorylates MtrA is not essential (Zahrt and Deretic, 2000), suggesting that there are other kinases that play a role in modulation of this ratio. It is possible that in strains of EM without the MtrA protein, other response regulators have evolved to serve in the essential role for growth in the environment. When a host is encountered, however, the lack of this protein leads to an inability to replicate in the host cells. It is possible that lack of the *mtr*A gene is important to these differences in both *in vitro* and *in vivo* growth. Although the strains of *M. salmoniphilum* were able to grow in the human and mouse macrophages, the change in growth from 2 to 4 days was much reduced from the initial growth over 2 days (with the exception of one strain in human cells), suggesting that these strains are not able to persist as well in macrophages as strains with the *mtr*A gene.

The *mtr*A/B system was not the only 2-component regulatory system gene to be identified as being absent in certain strains of EM. The *kdp*E (MCJA and MCJ78) gene scored as absent in some of the strains, while the *pho*P and *reg*X3 also scored as absent (or nearly absent) in one to three of the *M. chelonae* or *M. salmoniphilum* strains. These

response regulatory genes have been shown by a number of groups to be important to growth and virulence of *M. tuberculosis* in animal models (Perez *et al*., 2001; Parish *et al.,* 2003; Sassetti and Rubin, 2003; Gonzalo *et al*., 2006), but there is still very little known about the mechanisms by which they do so.

Although microarrays have been a useful tool for comparing the genomes of different strains of sequenced mycobacteria, we determined that there are potential limitations to the use of this tool to compare unsequenced strains of EM. The cutoff score for absent genes was determined by hybridizing the DNA of *M. avium* and *M. tuberculosis* to oligonucleotides representing genes known to be present and absent in both strains. Even with a cutoff score of two standard deviations lower than the median absent score for *M. tuberculosis*, we were nevertheless able to amplify some genes by PCR that were identified as absent by the microarray. Other than 16S rDNA, ITS, and *hsp*60 DNA, there is very little sequence in the database for *M. peregrinum* and *M. chelonae*. This lack of sequence data makes it difficult to know what is the cause of the discrepancy between the microarray and PCR results. It is possible that, although present, the sequences of these genes are divergent enough from the MAC104 gene sequences that they do not hybridize to the oligonucleotide on the array. It is also possible that the oligonucleotide was selected from a region of the gene that is in fact absent, but the primers used for PCR are similar to regions of the genomic DNA that are still present. If this was the case, we would expect the amplified PCR fragments to have different sizes. As this was not the case, it is more likely that the former explanation accounts for the discrepancy. Further evidence for this explanation is that primers designed from *M. smegmatis* were required to amplify genes expected to be present from

some strains. On the other hand, there were many genes for which the test strain DNA so strongly hybridized to the array spot, compared to the MAC104 reference, to be considered putatively duplicated. Further optimization may improve this custom microarray as a tool for comparing the genomes and gene expression in unsequenced species of mycobacteria.

Given how little we know about the mechanisms of virulence for many of these species, this study has provided a useful set of data from which to base further investigation. Future study may involve an examination of the expression of these genes when the various strains of mycobacteria are exposed to cell types or an animal model, such as zebrafish or mice. Additionally, constructing recombinant strains expressing genes or regions that are putatively absent and looking for differences in the ability to grow in cell types or cause disease could provide insight as to the relative importance of those genes to virulence. As we isolate more species and strains of EM from hosts with which humans frequently come into contact, such as cattle, zebrafish, and other fishes, further understanding of how virulence is tied to the genomes of these organisms may provide a means by which we are able to determine the isolates with zoonotic potential. Finally, more knowledge about the mechanisms of virulence and pathogenic potential of these organisms may lead to advances in controlling disease in both humans and animals of economic and research importance, such as zebrafish.

Acknowledgements.

We would like to thank Scott Givan and Caprice Rosato of the Center for Gene Research and Biotechnology for support with microarray design, construction, and hybridizations. We would also like to thank Denny Weber for editing this manuscript.

This work was supported by NIH Grant NCRR 5R24RR017386-02.

References

Arakawa, C. K., and J. L. Fryer. 1984. Isolation and characterization of a new subspecies of *Mycobacterium chelonei* infectious for salmonid fish. Helgolander Meeresun **37:**329-342.

Astrofsky, K. M., M. D. Schrenzel, R. A. Bullis, R. M. Smolowitz, and J. G. Fox. 2000. Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities. Comp Med **50:**666-672.

Banks, J. 1994. Environmental mycobacteria, p. 265-276. *In* P. D. O. Davies (ed.), Clinical Tuberculosis. Chapman and Hall, London.

Bannantine, J. P., Q. Zhang, L. L. Li, and V. Kapur. 2003. Genomic homogeneity between *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* belies their divergent growth rates. BMC Microbiol **3:**10.

Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science **284:**1520-1523.

Bermudez, L. E., and M. Petrofsky. 1997. Regulation of the expression of *Mycobacterium avium* complex proteins differs according to the environment within host cells. Immunol Cell Biol **75:**35-40.

Braunstein, M., T. I. Griffin, J. I. Kriakov, S. T. Friedman, N. D. Grindley, and W. R. Jacobs, Jr. 2000. Identification of genes encoding exported *Mycobacterium tuberculosis* proteins using a Tn552'*phoA* in vitro transposition system. J Bacteriol **182:**2732-2740.

Brocklebank, J., S. Raverty, and J. Robinson. 2003. Mycobacteriosis in Atlantic salmon farmed in British Columbia. The Canadian veterinary journal **44:**486-489.

Brosch, R., S. V. Gordon, C. Buchrieser, A. S. Pym, T. Garnier, and S. T. Cole. 2000. Comparative genomics uncovers large tandem chromosomal duplications in *Mycobacterium bovis* BCG Pasteur. Yeast **17:**111-123.

Brown-Elliott, B. A., and R. J. Wallace, Jr. 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin Microbiol Rev **15:**716-746.

Bruno, D. W., J. Griffiths, C. G. Mitchell, B. P. Wood, Z. J. Fletcher, F. A. Drobniewski, and T. S. Hastings. 1998. Pathology attributed to *Mycobacterium chelonae* infection among farmed and laboratory-infected Atlantic salmon *Salmo salar*. Diseases of aquatic organisms **33:**101-109.

Cappelli, G., E. Volpe, M. Grassi, B. Liseo, V. Colizzi, and F. Mariani. 2006. Profiling of *Mycobacterium tuberculosis* gene expression during human macrophage infection: upregulation of the alternative sigma factor G, a group of transcriptional regulators, and proteins with unknown function. Res Microbiol **157:**445-455.

Chang, C. T., L. Y. Wang, C. Y. Liao, and S. P. Huang. 2002. Identification of nontuberculous mycobacteria existing in tap water by PCR-restriction fragment length polymorphism. Appl Environ Microbiol **68:**3159-3161.

Charlet, D., S. Mostowy, D. Alexander, L. Sit, H. G. Wiker, and M. A. Behr. 2005. Reduced expression of antigenic proteins MPB70 and MPB83 in *Mycobacterium bovis* BCG strains due to a start codon mutation in *sigK*. Mol Microbiol **56:**1302-1313.

Chen, P., R. E. Ruiz, Q. Li, R. F. Silver, and W. R. Bishai. 2000. Construction and characterization of a *Mycobacterium tuberculosis* mutant lacking the alternate sigma factor gene, *sigF*. Infect Immun **68:**5575-5580.

Chinabut, S. 1999. Mycobacteriosis and nocardiosis, p. 319-340. *In* W. B. Woo and P.T.K. (ed.), Fish Diseases and Disorders: Viral Bacterial and Fungal Infections, vol. 3. CAB Internation, New York.

Danelishvili, L., M. Wu, B. Stang, M. Harriff, S. Cirillo, J. Cirillo, R. Bildfell, B. Arbogast, L.E. Bermudez. 2007. Identification of *Mycobacterium avium* pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci USA *In press.*

Danelishvili, L., M. J. Poort, and L. E. Bermudez. 2004. Identification of *Mycobacterium avium* genes up-regulated in cultured macrophages and in mice. FEMS Microbiol Lett **239:**41-49.

Decostere, A., K. Hermans, and F. Haesebrouck. 2004. Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. Vet Microbiol **99:**159-166.

Dubnau, E., P. Fontan, R. Manganelli, S. Soares-Appel, and I. Smith. 2002. *Mycobacterium tuberculosis* genes induced during infection of human macrophages. Infect Immun **70:**2787-2795.

Falkinham, J. O., 3rd, C. D. Norton, and M. W. LeChevallier. 2001. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other Mycobacteria in drinking water distribution systems. Appl Environ Microbiol **67:**1225-1231.

Fol, M., A. Chauhan, N. K. Nair, E. Maloney, M. Moomey, C. Jagannath, M. V. Madiraju, and M. Rajagopalan. 2006. Modulation of *Mycobacterium tuberculosis* proliferation by MtrA, an essential two-component response regulator. Mol Microbiol **60:**643-657.

Frota, C. C., D. M. Hunt, R. S. Buxton, L. Rickman, J. Hinds, K. Kremer, D. van Soolingen, and M. J. Colston. 2004. Genome structure in the vole bacillus, *Mycobacterium microti*, a member of the *Mycobacterium tuberculosis* complex with a low virulence for humans. Microbiology **150:**1519-1527.

Geiman, D. E., D. Kaushal, C. Ko, S. Tyagi, Y. C. Manabe, B. G. Schroeder, R. D. Fleischmann, N. E. Morrison, P. J. Converse, P. Chen, and W. R. Bishai. 2004. Attenuation of late-stage disease in mice infected by the *Mycobacterium tuberculosis* mutant lacking the SigF alternate sigma factor and identification of SigF-dependent genes by microarray analysis. Infect Immun **72:**1733-1745.

Gonzalo Asensio, J., C. Maia, N. L. Ferrer, N. Barilone, F. Laval, C. Y. Soto, N. Winter, M. Daffe, B. Gicquel, C. Martin, and M. Jackson. 2006. The virulence-associated two-component PhoP-PhoR system controls the biosynthesis of polyketide-derived lipids in *Mycobacterium tuberculosis*. J Biol Chem **281:**1313-1316.

Hamelin, K., G. Bruant, A. El-Shaarawi, S. Hill, T. A. Edge, S. Bekal, J. M. Fairbrother, J. Harel, C. Maynard, L. Masson, and R. Brousseau. 2006. A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in *Escherichia coli* isolates from Great Lakes recreational waters. Appl Environ Microbiol **72:**4200-4206.

Hamelin, K., G. Bruant, A. El-Shaarawi, S. Hill, T. A. Edge, J. Fairbrother, J. Harel, C. Maynard, L. Masson, and R. Brousseau. 2007. Occurrence of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within the St. Clair River and Detroit River areas. Appl Environ Microbiol **73:**477-484.

Harriff, M. J., L. E. Bermudez, and M. L. Kent. 2007. Experimental exposure of zebrafish (*Danio rerio* Hamilton) to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: A potential model for environmental mycobacterial infection. J Fish Dis. **29:** 1-13.

Hou, J. Y., J. E. Graham, and J. E. Clark-Curtiss. 2002. *Mycobacterium avium* genes expressed during growth in human macrophages detected by selective capture of transcribed sequences (SCOTS). Infect Immun **70:**3714-3726.

Inderlied, C. B., L. S. Young, and J. K. Yamada. 1987. Determination of in vitro susceptibility of *Mycobacterium avium* complex isolates to antimycobacterial agents by various methods. Antimicrob Agents Chem. **31** 1697-1702.

Kent, M. L., V. Watral, M. Wu, and L. E. Bermudez. 2006. In vivo and in vitro growth of *Mycobacterium marinum* at homoeothermic temperatures. FEMS Microbiol Lett **257:**69-75.

Kent, M. L., C. M. Whipps, J. L. Matthews, D. Florio, V. Watral, J. K. Bishop-Stewart, M. Poort, and L. Bermudez. 2004. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. Comp Biochem Physiol C Toxicol Pharmacol **138:**383-390.

Li, F., and G. D. Stormo. 2001. Selection of optimal DNA oligos for gene expression arrays. Bioinformatics **17:**1067-1076.

Manganelli, R., R. Provvedi, S. Rodrigue, J. Beaucher, L. Gaudreau, and I. Smith. 2004. Sigma factors and global gene regulation in *Mycobacterium tuberculosis*. J Bacteriol **186:**895-902.

Marsh, I. B., J. P. Bannantine, M. L. Paustian, M. L. Tizard, V. Kapur, and R. J. Whittington. 2006. Genomic comparison of *Mycobacterium avium* subsp. *paratuberculosis* sheep and cattle strains by microarray hybridization. J Bacteriol **188:**2290-2293.

Parish, T., D. A. Smith, G. Roberts, J. Betts, and N. G. Stoker. 2003. The *senX3-regX3* two-component regulatory system of *Mycobacterium tuberculosis* is required for virulence. Microbiology **149:**1423-1435.

Pattyn, S. R., and G. Verdoolaeche-Van Loo. 1974. The result of inoculation of *Mycobacterium leprae* in the goldfish (*Carassius auratus*). Acta Zool Pathol Antverp **58:**109-116.

Paustian, M. L., V. Kapur, and J. P. Bannantine. 2005. Comparative genomic hybridizations reveal genetic regions within the *Mycobacterium avium* complex that are divergent from *Mycobacterium avium* subsp. *paratuberculosis* isolates. J Bacteriol **187:**2406-2415.

Perez, E., S. Samper, Y. Bordas, C. Guilhot, B. Gicquel, and C. Martin. 2001. An essential role for *phoP* in *Mycobacterium tuberculosis* virulence. Mol Microbiol **41:**179-187.

Porwollik, S., E. F. Boyd, C. Choy, P. Cheng, L. Florea, E. Proctor, and M. McClelland. 2004. Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. J Bacteriol **186:**5883-5898.

Porwollik, S., C. A. Santiviago, P. Cheng, L. Florea, and M. McClelland. 2005. Differences in gene content between *Salmonella enterica* serovar *enteritidis* isolates and comparison to closely related serovars *Gallinarum* and *Dublin.* J Bacteriol **187:**6545-6555.

Porwollik, S., R. M. Wong, and M. McClelland. 2002. Evolutionary genomics of *Salmonella*: gene acquisitions revealed by microarray analysis. Proc Natl Acad Sci U S A **99:**8956-8961.

Pozos, T. C., and L. Ramakrishnan. 2004. New models for the study of *Mycobacterium*-host interactions. Current opinion in immunology **16:**499-505.

Prouty, M. G., N. E. Correa, L. P. Barker, P. Jagadeeswaran, and K. E. Klose. 2003. Zebrafish-*Mycobacterium marinum* model for mycobacterial pathogenesis. FEMS Microbiol Lett **225:**177-182.

Ramakrishnan, L. 2004. Using *Mycobacterium marinum* and its hosts to study tuberculosis. Current Science **86:**82-92.

Rodrigue, S., R. Provvedi, P. E. Jacques, L. Gaudreau, and R. Manganelli. 2006. The sigma factors of *Mycobacterium tuberculosis*. FEMS Microbiol Rev **30:**926-941.

Ross, A. J. 1960. *Mycobacterium salmoniphilum* sp. nov. from salmonid fishes. Am. Rev. Respir. Dis. **81:**241-250.

Ruley, K. M., R. Reimschuessel, and M. Trucksis. 2002. Goldfish as an animal model system for mycobacterial infection. Methods Enzymol **358:**29-39.

Sanders, G. E., and L. E. Swaim. 2001. Atypical piscine mycobacteriosis in Japanese medaka (*Oryzias latipes*). Comp Med **51:**171-175.

Sassetti, C. M., and E. J. Rubin. 2003. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A **100:**12989-12994.

Semret, M., G. Zhai, S. Mostowy, C. Cleto, D. Alexander, G. Cangelosi, D. Cousins, D. M. Collins, D. van Soolingen, and M. A. Behr. 2004. Extensive genomic polymorphism within *Mycobacterium avium*. J Bacteriol **186:**6332-6334.

Smith, I. 2003. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clin Microbiol Rev **16:**463-496.

Talaat, A. M., R. Reimschuessel, and M. Trucksis. 1997. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. Vet Microbiol **58:**229-237.

Talaat, A. M., R. Reimschuessel, S. S. Wasserman, and M. Trucksis. 1998. Goldfish, *Carassius auratus*, a novel animal model for the study of *Mycobacterium marinum* pathogenesis. Infect Immun **66:**2938-2942.

van der Sar, A. M., B. J. Appelmelk, C. M. Vandenbroucke-Grauls, and W. Bitter. 2004. A star with stripes: zebrafish as an infection model. Trends in microbiology **12:**451-457.

Watral, V., and M. L. Kent. 2007. Pathogenesis of *Mycobacterium* spp. in zebrafish (*Danio rerio*) from research facilities. Comp Biochem Physiol C Toxicol Pharmacol **145:**55-60.

Whipps, C.M., W. R. Butler, F. Pourahmad, V. G. Watral, and M. L. Kent 2007b. Molecular systematics support the revival of *Mycobacterium salmoniphilum* (ex Ross 1960) sp. nov., nom. rev., a species closely related to *Mycobacterium chelonae*. Intl. J. Syst. Evol. Microbiol. *In press*.

Whipps, C. M., S. T. Dougan, and M. L. Kent. 2007. *Mycobacterium haemophilum* infections of zebrafish (*Danio rerio*) in research facilities. FEMS Microbiol Lett **270:**21-26.

Whipps, C. M., V. G. Watral, and M. L. Kent. 2003. Characterization of a *Mycobacterium* sp. in rockfish, *Sebastes alutus* (Gilbert) and *Sebastes reedi* (Westrheim & Tsuyuki), using rDNA sequences. J Fish Dis **26:**241-245.

Zahrt, T. C., and V. Deretic. 2000. An essential two-component signal transduction system in *Mycobacterium tuberculosis*. J Bacteriol **182:**3832-3838.

Supplemental Table 3.1 Scores predicting presence or absence for all genes and strains analyzed by custom microarray.

M. avium Gene Name	*M. avium* Gene Number	MAC100	MAC101	MAC109	MP101	MP102	MPATCC	MCH	MCJA	MCJ78	MCBAND	MCTRASK	MCSABLE	MCMWF
gyrA	MAV0006	0.072	0.426	0.126	0.121	-0.078	-0.012	0.593	-0.702	-0.215	0.535	1.438	1.18	0.663
rodA	MAV0021	0.012	-0.014	0.224	0.058	-0.184	-0.367	-0.098	-0.457	-0.147	1.263	0.57	0.649	0.002
ppp	MAV0022	0.053	0.4	0.346	0.237	0.003	0.073	-0.64	0.438	0.196	0.017	0.477	0.115	-1.276
	MAV0160	-0.002	-0.253	0.13	-0.317	0.058	0.551	0.696	0.734	1.083	-0.881	-0.874	-0.866	-1.644
sodA	MAV0182	0.063	-0.639	-0.451	0.366	0.744	-0.501	0.209	-0.89	-1.256	1.646	0.608	0.287	0.923
erp	MAV0207	-0.15	-0.342	-0.372	-0.012	-0.168	-0.144	-0.024	-0.486	-0.332	0.358	0.609	0.154	-0.05
fbpA	MAV0214	0.248	-0.432	-0.086	-0.319	-0.051	-0.592	-0.03	2.506	0.982	-1.077	-1.768	-1.361	-1.169
embA	MAV0225	0.113	-0.811	0.158	-0.402	-0.155	0.309	0.449	0.561	0.61	-2.228	-2.183	-3.659	-1.902
embC	MAV0229	0.038	-0.297	0.157	-0.13	-0.086	-0.426	-0.192	2.438	1.651	-1.006	-1.057	-1.556	-1.881
embB	MAV0229	-0.059	-0.7	-0.094	0.083	-0.277	-0.591	-0.116	0.322	0.432	-0.06	-0.566	-0.929	-0.977
proV	MAV0318	0.203	-0.271	0.035	-0.079	-0.097	-0.48	0.658	-0.962	-0.918	0.17	0.165	0.531	0.265
	MAV0385	-0.148	0.044	0.156	0.074	0.087	0.178	0.301	0.63	-0.066	0.054	-0.257	-0.732	-0.52
dppA	MAV0464	-0.217	-0.695	0.325	0.071	-0.079	0.83	0.066	0.015	0.45	0.266	0.906	0.839	0.348
	MAV0469	0.051	-0.42	0.098	0.345	-0.208	0.764	-0.361	2.984	1.127	-1.533	-1.708	-2.098	-1.741
	MAV0631	0.124	-0.245	-0.561	0.357	0.446	-0.68	-0.271	-0.46	-0.591	1.859	1.116	1.169	1.513
	MAV0633	-0.217	0.564	-0.165	0.383	0.839	0.452	-0.4	-0.294	-0.866	1.73	1.234	2.072	2.021
echA19	MAV0643	-0.508	0.436	0.371	-0.334	-0.094	0.53	0.416	0.442	0.65	-1.011	-0.301	-0.62	-0.872
	MAV0663	0.026	-0.988	0.408	0.193	0.003	0.296	0.199	-1.371	-0.751	0.403	0.033	0.496	0.916
phoP	MAV0701	0.301	-0.219	0.233	-0.248	-0.367	-0.854	-0.032	-0.206	-0.194	-0.307	-0.653	-0.711	-1.214
	MAV0750	-0.261	-0.446	-0.019	-0.313	0.01	0.222	-0.512	0.471	0.02	-0.008	-0.77	-0.308	0.143
phoT	MAV0767	0.043	0.238	-0.003	-0.089	-0.362	-0.117	-0.105	0.268	-0.299	-0.495	-0.321	-1.553	-1.07

Supplemental Table 3.1 (continued**).** Scores predicting presence or absence for all genes analyzed by custom microarray.

M. avium Gene Name	*M. avium* Gene Number	MAC100	MAC101	MAC109	MP101	MP102	MPATCC	MCH	MCJA	MCJ78	MCBAND	MCTRASK	MCSABLE	MCMWF
	MAV0996	-0.071	0.751	0.271	0.302	0.297	0.409	0.404	-0.305	-0.041	0.444	1.434	0.835	0.338
eno	MAV1164	0.105	-0.146	-0.647	0.096	0.278	-0.514	-1.301	0.049	0.811	-0.658	-1.218	-2.352	-0.997
kdpE	MAV1169	0.171	0.15	-0.061	0.071	0.016	-1.013	-0.312	-2.045	-1.575	1.917	0.034	0.735	0.987
kdpD	MAV1170	-0.017	-0.029	0.58	0.18	0.101	0.494	0.649	0.389	0.259	0.187	1.101	0.856	0.981
	MAV1210	0.141	0.52	0.078	0.751	0.567	0.468	-0.233	1.159	0.42	0.85	1.973	1.55	1.608
	MAV1285	-0.046	0.376	0.453	-0.622	-0.123	0.423	-0.478	0.196	0.029	0.084	0.712	0.227	-0.27
narG	MAV1303	0.132	0.205	-0.388	0.09	0.215	-0.01	0.769	-0.38	-0.481	-0.058	-0.75	-0.122	-0.514
fadD6	MAV1351	0.084	-0.687	-0.516	-0.014	-0.248	0.025	0.279	-0.234	-0.693	-0.116	-1.423	-1.61	-0.826
fadE6	MAV1351	0.058	0.096	0.261	-0.054	-0.274	0.382	0.192	-0.253	0.222	0.099	0.353	0.443	0.341
sigE	MAV1365	-0.59	-0.103	-0.344	-0.163	0.224	-0.301	-0.579	-0.151	0.146	0.607	-0.111	-0.434	-0.23
corA	MAV1379	-0.092	0.023	-0.154	-0.019	0.693	-0.032	0.148	0.145	0.404	0.188	0.215	0.805	0.442
lprC	MAV1424	-0.087	0.046	-0.132	0.634	0.271	0.454	-0.232	-0.37	0.05	0.487	0.154	0.14	0.627
oppA	MAV1429	0.098	0.084	-0.177	-0.017	-0.112	-0.174	0.137	-0.319	-0.324	1.288	0.594	1.548	1.405
oppD	MAV1431	0.186	-0.315	-0.149	-0.01	0.399	-0.92	-1.24	-0.302	ND	1.561	1.27	0.842	0.845
atpA	MAV1525	-0.304	0.031	0.284	-0.253	-0.095	0.1	0.373	1.605	0.586	-1.612	-1.279	-0.89	-1.624
mura	MAV1531	-0.187	-0.311	-0.29	-0.147	0.181	-0.043	0.491	0.983	0.153	-1.096	-0.357	-1.174	-0.606
	MAV1540	0.138	-0.058	0.051	-0.051	-0.1	-1.14	-0.462	0.146	0.359	0.308	0.105	0.064	0.469
	MAV1540	0.17	0.281	0.19	-0.152	-0.109	-0.422	0.202	0.085	-0.12	-2.165	-0.803	-3.189	-1.339
fadA4	MAV1544	0.021	0.302	0.198	0.076	0.445	-0.327	0.259	-0.472	-1.001	-0.905	-0.836	-0.399	0.035
fadA5	MAV1615	-0.005	0.009	0.123	0.195	-0.02	-0.086	-0.319	1.663	0.65	-1.067	-0.877	-0.986	-0.951

Supplemental Table 3.1 (continued**).** Scores predicting presence or absence for all genes analyzed by custom microarray.

M. avium **Gene Name**	***M. avium*** **Gene Number**	**MAC100**	**MAC101**	**MAC109**	**MP101**	**MP102**	**MPATCC**	**MCH**	**MCJA**	**MCJ78**	**MCBAND**	**MCTRASK**	**MCSABLE**	**MCMWF**
pdhA	MAV1675	-0.63	-0.445	-0.045	0.306	0.034	0.666	-0.569	0.378	0.062	0.111	1.162	0.693	-0.117
	MAV1705	-0.23	0.055	-0.468	0.473	0.224	-1.384	-0.89	0.615	ND	0.589	0.337	0.108	0.569
argS	MAV1724	0.057	0.45	-0.028	-0.123	-0.186	-0.23	0.168	0.438	-0.484	-1.372	-1.194	-1.341	-0.772
mmpL12	MAV1761	0.113	0.226	0.264	-0.048	-0.364	1.158	0.683	0.438	0.926	-0.896	-1.06	-1.533	-1.088
mmpl8	MAV1761	0.408	0.172	0.722	-0.098	-0.488	0.052	0.806	-0.664	-0.009	1.294	1.531	2.376	1.83
nirA	MAV1787	-0.22	0.022	0.395	0.448	0.231	0.827	0.293	0.563	0.683	-0.102	0.188	0.215	-0.601
mbtB	MAV2009	0.128	0.086	0.149	-0.005	0.006	0.292	0.501	-0.452	-0.39	-0.076	-0.512	-0.457	-0.039
mbtE	MAV2013	-0.116	1.13	0.205	0.044	-0.025	0.645	-0.021	0.46	0.054	-0.043	0.507	0.227	-0.03
rocE	MAV2087	0.072	-0.269	-0.364	0.138	0.416	-0.619	0.127	-0.176	-0.317	1.364	0.132	0.084	-0.295
glpD1	MAV2188	-0.041	-0.354	-0.08	-0.189	-0.112	-0.418	-0.172	0.127	-1.544	1.029	1.92	1.832	1.53
	MAV2241	-0.003	-0.527	-0.57	-0.114	-0.192	0.084	-0.52	ND	ND	-0.205	-0.415	-0.412	0.194
	MAV2241	-0.628	-0.156	0.588	-1.135	-0.188	-0.215	-0.478	0.583	-0.498	0.546	0.838	0.97	0.833
sucA	MAV2273	-0.012	0.177	0.142	-0.233	0.031	-0.107	-0.108	0.632	0.185	-0.72	-1.397	-0.607	-0.571
sucB	MAV2273	0.344	0.08	-0.122	0.291	0.065	-0.315	0.263	0.171	0.6	-0.033	-0.344	-0.282	0.4
	MAV2304	0.011	-0.151	-0.181	0.002	0.109	-0.047	0.627	-0.282	-0.186	-0.615	-0.487	-1.283	-0.766
lppM	MAV2323	-0.354	0.001	-0.077	-0.134	-0.061	0.11	0.918	0.695	-0.198	-1.331	-0.278	-1.044	-0.952
pbpB	MAV2330	-0.047	0.187	0.016	0.208	0.855	-0.202	0.424	-0.428	-0.349	0.506	-0.215	2.055	0.445
ppsA	MAV2371	0.029	0.279	-0.105	-0.115	-0.055	0.304	-0.326	0.902	-0.113	-0.619	-0.881	-0.812	0.339
	MAV2409	-0.282	0.155	0.185	-0.038	0.057	-0.028	0.088	-0.088	ND	0.415	-0.05	0.195	0.605
cobN	MAV2437	0.009	-0.404	-0.23	0.206	-0.352	-0.461	-0.349	-0.237	1.138	-0.344	-0.529	-0.588	-0.219

Supplemental Table 3.1 (continued)**.** Scores predicting presence or absence for all genes analyzed by custom microarray.

M. avium Gene Name	*M. avium* Gene Number	MAC100	MAC101	MAC109	MP101	MP102	MPATCC	MCH	MCJA	MCJ78	MCBAND	MCTRASK	MCSABLE	MCMWF
pks12	MAV2450	-0.16	-0.144	-0.093	-0.201	0.193	0.014	-0.015	-0.205	0.267	0.284	0.773	-0.109	-0.856
	MAV2479	0.03	0.598	0.192	-1.241	0.02	0.997	0.054	0.236	-0.011	-0.189	0.24	-0.015	0.802
mmpL5	MAV2510	-0.009	0.128	0.277	-0.257	-0.105	-0.521	-0.906	-0.775	-1.123	2.952	1.001	1.999	1.992
lprM	MAV2536	0.491	0.552	-2.05	0.274	0.041	0.701	0.023	ND	ND	-0.266	-0.455	-0.469	-0.578
err	MAV2716	0.161	0.171	-0.343	0.154	0.316	-0.645	-0.645	-1.019	-0.251	1.708	0.643	0.796	0.116
katG	MAV2753	0.05	-0.323	-0.255	-0.579	0.006	-0.433	0.061	-0.548	-0.034	-2.219	-1.958	-3.813	-1.51
fbpB	MAV2816	0.198	0.334	-0.088	0.561	0.716	0.149	0.99	-0.26	-1.479	0.507	0.175	1.581	1.691
bfrA	MAV2833	-0.032	0.39	0.153	0.822	1.95	0.73	0.481	-1.005	-0.3	1.197	0.951	2.778	2.649
ahpC	MAV2839	0.375	0.193	0.149	0.378	0.713	-0.276	0.709	-0.645	-0.73	1.323	1.511	2.989	2.228
lldD2	MAV2843	0.06	0.312	0.295	-0.17	-0.385	0.196	0.43	0.627	0.668	-0.407	0.396	-0.381	0.33
fadD33	MAV2874	-0.156	-0.299	0.068	0.114	0.225	0.598	0.051	-0.029	0.452	-0.016	1.116	0.369	0.072
secA2	MAV2894	-0.012	-0.286	-0.028	-0.245	0.163	-0.589	-0.236	0.646	-0.055	-0.257	-0.177	-0.354	-1.222
secA	MAV2894	0.044	-0.346	-0.458	-0.339	0.035	-0.316	-0.534	-0.434	ND	-1.986	-2.714	-2.552	-1.63
mgtC	MAV2903	-0.286	-0.335	0.102	0.05	-0.375	-0.467	-0.003	0.154	0.208	-1.014	-1.024	-0.927	-1.164
PPE	MAV2928	-0.025	-0.032	-0.457	0.0324	-0.232	-0.161	0.414	-0.645	0.186	2.35	2.435	2.918	2.631
PPE	MAV2928	-0.03	0.2	-0.202	0.124	0.06	0.919	0.847	-0.226	0.786	-0.331	-0.558	0.065	0.917
tlyA	MAV3077	0.036	-0.04	-0.302	-0.138	0.153	-0.97	-0.491	-0.352	-0.403	0.963	ND	-0.188	1.116
	MAV3105	-0.049	0.141	0.138	0.034	-0.068	0.031	0.145	0.084	-0.205	-0.235	0.08	0.314	-0.217
pks11	MAV3105	-0.338	0.694	-0.053	0.234	0.218	0.61	0.071	-0.547	-0.489	0.86	0.761	1.452	2.226
pks7	MAV3109	0.008	-0.002	-0.364	-0.137	-0.198	-0.344	0.142	0.767	0.488	-0.672	-0.17	-0.768	-0.274

Supplemental Table 3.1 (continued)**.** Scores predicting presence or absence for all genes analyzed by custom microarray.

M. avium **Gene Name**	***M. avium*** **Gene Number**	**MAC100**	**MAC101**	**MAC109**	**MP101**	**MP102**	**MPATCC**	**MCH**	**MCJA**	**MCJ78**	**MCBAND**	**MCTRASK**	**MCSABLE**	**MCMWF**
infC	MAV3127	-0.291	0.621	-0.015	-0.079	0.566	0.046	-0.376	0.368	0.188	-0.314	-0.894	-0.984	-0.028
chaA	MAV3179	-0.08	0.345	0.222	0.099	0.1	0.111	0.458	-0.11	0.319	0.024	0.073	-0.053	-0.28
	MAV3208	0.415	0.097	0.101	-0.148	0.397	0.663	0.259	-0.376	0.745	0.657	0.269	0.674	0.425
	MAV3209	-0.376	-0.145	0.343	-1.305	-0.606	-0.254	-0.645	0.053	-0.34	0.465	0.431	1.029	1.285
lipL	MAV3275	0.069	0.308	-0.184	0.47	0.02	0.492	0.748	-0.018	0.152	0.153	-0.067	0.005	0.67
can	MAV3303	-0.051	0.062	-0.093	0.336	0.333	0.148	0.081	-0.307	0.386	-0.347	-0.355	-0.438	-0.181
	MAV3321	0.096	-0.204	0.017	0.07	0.331	0.307	0.412	0.947	0.18	-0.383	-0.223	-0.177	-0.621
devB	MAV3331	0.234	0.085	0.081	-0.179	-0.022	0.132	0.066	0.084	-0.086	-0.109	-0.37	-0.195	-0.685
uvrc	MAV3360	0.141	0.448	0.167	-0.517	-0.087	0.382	-0.466	0.184	-0.3	-0.903	-1.069	-0.514	-0.853
carB	MAV3389	-0.17	-0.221	-0.326	-0.112	-0.337	-1.09	-0.4	-0.132	-0.062	-0.325	-0.792	-0.882	-1.374
	MAV3448	0.156	-0.501	-0.037	0.145	0.31	-0.459	0.55	-0.43	-0.52	0.663	0.383	0.341	0.424
secD	MAV3468	-0.065	0.055	0.279	0.134	0.492	-0.531	0.032	-0.263	0.33	-0.285	0.632	-0.453	-0.737
dut	MAV3589	-0.044	0.015	0.119	0.139	-0.061	0.045	0.029	1.901	0.593	-0.526	0.234	-0.15	0.602
ideR	MAV3604	0.329	-0.18	0.176	0.03	0.164	-0.564	-0.214	-0.259	-0.205	-0.287	-0.508	-0.275	0.001
	MAV3608	0.307	0.527	0.9	0.489	0.919	0.279	0.847	-1.547	-1.028	0.483	0.74	1.538	1.769
	MAV3611	0.154	-0.079	-0.022	0.732	0.092	0.008	0.584	-0.464	-0.342	-0.248	0.172	-0.141	-0.058
infB	MAV3693	0.148	-0.502	-0.122	0.005	0.437	-0.555	0.029	-0.41	-0.003	0.618	0.249	0.861	0.185
mpt53	MAV3729	-1.956	-0.086	0.163	0.025	-0.097	0.518	0.211	2.961	1.418	-1.651	-1.186	-1.612	-0.705
lepB	MAV3758	0.133	-0.516	0.136	0.512	0.609	0.261	-0.028	-0.099	0.338	3.002	1.637	0.849	1.412
ffh	MAV3770	0.019	0.864	0.752	0.243	-0.004	-0.262	0.217	-0.212	0.523	-0.51	-0.239	-0.594	-1.041

Supplemental Table 3.1 (continued)**.** Scores predicting presence or absence for all genes analyzed by custom microarray.

M. avium Gene Name	*M. avium* Gene Number	MAC100	MAC101	MAC109	MP101	MP102	MPATCC	MCH	MCJA	MCJ78	MCBAND	MCTRASK	MCSABLE	MCMWF
pfkA	MAV3858	-0.156	-0.219	-0.14	-0.322	-0.157	-0.998	0.034	0.915	0.158	-1.484	-1.345	-1.761	-0.976
	MAV3866	0.041	0.135	0.386	0.093	0.002	0.383	-0.393	0.012	-0.002	-0.503	0.199	-0.231	-0.258
fecB	MAV3910	-0.101	-0.409	-0.062	0.254	0.373	0.064	0.404	-0.208	-0.194	0.283	0.015	-0.227	0.016
csta	MAV3938	-0.252	-0.017	0.101	0.111	-0.227	0.049	0.497	0.456	-0.416	-1.722	-1.01	-1.721	-1.567
ftsX	MAV4002	-0.123	0.393	0.191	0.196	0.114	-0.054	0.982	0.045	-0.216	0.928	1.065	2.026	1.416
whiB1	MAV4167	0.103	0.486	0.221	-0.077	0.058	-0.327	0.748	-0.129	-1.35	-0.071	0.69	0.735	0.558
	MAV4168	-0.149	-0.261	0.096	-0.468	-0.343	-0.473	-0.386	-0.076	-0.139	-1.364	-1.235	-2.202	-1.905
	MAV4199	0.297	0.376	0.201	0.01	0.163	0.346	0.501	-0.535	-0.149	-0.5	-0.432	-0.711	0.184
mtrA	MAV4209	0.277	-0.257	-0.627	0.078	-0.515	-1.135	-1.134	-0.418	1.401	-0.466	-1.14	-1.89	-1.495
ctpC	MAV4235	0.01	0.104	-0.109	-0.215	-0.023	0.023	0.156	0.623	-0.068	-0.274	0.223	0.183	0.221
glpD2	MAV4278	0.178	0.263	-0.18	-0.118	-0.175	-0.478	-0.093	-0.113	-0.008	0.07	0.312	0.023	0.53
add	MAV4294	-0.034	0.423	0.176	-0.264	0.213	0.193	0.85	0.677	-0.308	-0.856	0.033	0.219	0.651
icd1	MAV4311	-0.206	-0.867	0.006	0.121	-0.212	0.393	-0.428	-0.156	0.3	0.392	1.213	0.57	-1.03
dnaE2	MAV4335	-0.046	0.028	-0.269	0.01	-0.336	0.521	-0.136	-0.091	0.14	-0.585	-0.55	-1.282	-0.594
guaB3	MAV4355	0.379	-0.244	-0.01	-0.071	0.339	0.017	-0.015	0.08	-0.239	0.641	0.127	0.461	0.324
mmsA	MAV4417	-0.064	0.627	-0.006	0.264	0.238	0.923	0.728	-0.062	0.198	0	0.363	0.697	0.196
secY	MAV4434	0.037	-0.299	-0.159	0.234	0.507	0.313	0.108	-0.149	0.301	-1.366	-1.334	-1.391	-0.74
	MAV4506	0.008	-0.036	-0.07	0.136	-0.647	0.466	0.285	0.293	0.073	-0.204	-0.075	-0.207	-0.326
	MAV4606	0.145	0.071	0.344	-0.091	-0.063	0.344	-0.18	0.476	0.466	-0.708	-0.646	-0.961	-0.952
proC	MAV4650	0.459	0.209	-0.049	0.103	-0.035	0.244	0.377	0.589	0.192	-1.135	-1.275	-1.736	-1.977

Supplemental Table 3.1 (continued)**.** Scores predicting presence or absence for all genes analyzed by custom microarray.

***M. avium* Gene Name**	***M. avium* Gene Number**	**MAC100**	**MAC101**	**MAC109**	**MP101**	**MP102**	**MPATCC**	**MCH**	**MCJA**	**MCJ78**	**MCBAND**	**MCTRASK**	**MCSABLE**	**MCMWF**
regX3	MAV4660	0.046	0.006	-0.078	0.135	0.019	0.529	-0.614	-0.115	-0.22	-0.229	-0.096	-1.054	-1.045
	MAV4676	-0.089	0.571	0.264	0.085	0.192	0.575	0.384	0.134	-0.841	0.117	0.346	0.694	0.935
pcaA	MAV4679	0.246	0.049	-0.649	-0.545	0.797	-0.308	0.509	-0.99	-1.354	2.344	1.083	1.343	0.938
aceA	MAV4682	0.007	0.613	-0.177	0.206	-0.125	0.847	0.216	-0.044	-0.047	0.866	1.781	1.23	0.339
hsp60	MAV4707	-0.224	-0.255	-0.03	-0.04	0.06	0.559	-0.202	-0.043	0.516	-1.971	-1.434	-1.905	-1.011
sodC	MAV4722	0.159	-0.046	-0.331	-0.043	-0.024	-0.356	0.631	0.617	0.161	-0.475	-0.581	-0.586	-0.255
	MAV4762	0.166	-0.302	-0.526	-0.028	-0.104	-1.024	-0.901	0.208	-0.454	1.679	0.826	1.577	1.107
	MAV4781	-0.268	-0.191	0.016	-0.242	-0.238	-0.036	-0.01	0.202	-0.557	-0.207	-0.672	-0.012	-0.012
rmlA	MAV4820	0.097	-0.349	-0.131	0.169	0.002	-0.093	0.083	-0.407	0.344	1.185	-0.519	-0.04	0.003
udgA	MAV4823	-0.152	-0.381	-0.099	-0.357	-0.752	-0.39	0.053	1.152	-0.202	-1.462	-1.628	-1.875	-1.697
nramp	MAV4859	0.083	0.335	0.454	-0.023	-0.163	-0.084	0.187	0.405	-0.044	-0.47	-0.734	-0.485	-0.671
	MAV4866	-0.119	-0.059	0.184	0.052	-0.33	-0.32	-0.283	-0.115	-0.278	-0.071	0.037	-0.232	-0.218
PPE	MAV4867	-0.045	0.342	-0.04	-0.069	-0.19	0.985	-0.125	0.363	0.339	-0.941	-0.45	-1.019	-0.149
fadD2	MAV4891	-0.001	-0.185	0.009	0.413	-0.416	0.117	-0.26	-0.224	-0.403	2.22	1.044	1.624	1.719
narK3	MAV4899	0.07	0.402	0.374	-0.031	0.229	0.578	0.014	-0.53	-0.824	0.671	1.205	1.394	0.448
nirB	MAV4904	0.034	0.071	-0.208	-0.045	0.061	0.829	-0.738	0.21	0.055	0.947	0.877	0.287	0.859
pckA	MAV4936	0.204	-0.414	-0.277	-0.226	-0.234	-0.037	-1.231	-0.089	1.693	0.875	0.286	0.061	-0.889
pckA	MAV4963	0.105	0.037	-0.117	-0.334	-0.608	-1.518	-1.001	ND	ND	-0.813	-0.75	-0.23	-0.364
	MAV4973	0.117	0.51	-0.349	-0.166	-0.122	0.448	0.754	0.034	-0.794	-0.06	0.212	0.14	-0.313
mmpL11	MAV4973	0.093	0.382	-0.392	-0.012	0.017	-0.235	0.847	0.634	-0.569	-0.846	-0.611	-1.084	-1.226

Supplemental Table 3.1 (continued)**.** Scores predicting presence or absence for all genes analyzed by custom microarray.

M. avium **Gene Name**	***M. avium*** **Gene Number**	**MAC100**	**MAC101**	**MAC109**	**MP101**	**MP102**	**MPATCC**	**MCH**	**MCJA**	**MCJ78**	**MCBAND**	**MCTRASK**	**MCSABLE**	**MCMWF**
lpqH	MAV5005	0.193	-0.435	0.234	1.05	0.37	0.34	0.638	0.381	-0.944	1.196	2.693	2.065	1.275
lprO	MAV5005	0.099	0.383	0.038	0.003	0.055	0.139	0.758	1.326	-0.053	-1.281	-0.799	-1.942	-0.633
mce1	MAV5015	0.58	0.771	-0.2	0.354	0.793	1.517	0.65	-1.604	-0.163	1.359	0.656	2.11	2.572
ephF	MAV5163	0.14	0.285	-0.186	0.019	0.09	-0.294	-0.325	-0.598	-0.268	-1.664	-0.547	-2.743	-1.535
pepA	MAV5187	-0.683	-0.184	-0.198	-0.096	-0.029	0.154	0.707	0.203	0.506	-1.613	-1.583	-1.922	-1.434
fusA2	MAV5189	0.34	0.586	0.077	-0.161	-0.212	0.644	0.23	-0.305	-0.613	0.156	0.525	0.221	-0.858
oxcA	MAV5191	0.45	-0.306	-0.074	0.093	-0.348	-0.342	0.031	0.186	-0.69	0.799	0.533	1.378	0.905
	MAV5243	0.143	0.198	0.072	-0.271	0.366	1.234	0.732	-0.462	-0.927	0.59	0.096	1.023	1.498
ctpA	MAV5246	-0.484	-0.189	0.384	-0.171	-0.189	0.115	-0.841	0.838	0.046	0.6	-0.524	-0.139	-0.853
	MAV5286	0.205	-0.387	-0.174	-0.056	0.031	-0.351	-1.04	-0.049	-0.093	0.274	-0.084	-0.158	-1.194
MAVInv1	MAVInv1	-0.556	-0.734	0.465	-2.875	-0.758	-2.762	-2.207	-0.79	-0.939	1.254	2.688	2.453	1.512
MAVInv2	MAVInv2	-0.034	-0.744	0.63	-4.35	0.158	-1.975	-2.224	-1.327	-1.428	1.206	2.343	3.476	3.284
MAVInv3	MAVInv3	-0.058	-0.334	-0.668	ND	-0.179	-0.293	-1.827	-0.564	ND	0.228	0.253	1.35	1.698
MAVInv4	MAVInv4	-0.401	-0.89	-0.442	-0.501	-0.111	-0.018	-1.883	-0.744	-0.373	2.947	0.895	0.716	1.813
MAVInv5	MAVInv5	-0.711	-0.09	-0.237	-3.457	-1.528	-2.001	-2.732	-0.291	-0.564	1.345	2.892	2.777	2.249
MAVInv6	MAVInv6	0.007	-0.36	0.135	ND	0.528	-3.348	-2.458	-2.307	-3.004	1.564	1.805	3.613	3.331
MAVInv7	MAVInv7	0.573	-0.246	-0.694	ND	-0.258	-0.556	-2.596	-0.583	-0.562	2.401	1.563	3.241	4.002
MAVInv8	MAVInv8	-0.084	-0.761	-0.776	ND	-0.423	ND	-1.605	0.3	ND	1.206	0.589	1.164	0.552
MAVInv9	MAVInv9	-0.578	-0.478	-0.29	-3.662	-0.525	-1.882	-2.305	0.109	-0.373	0.841	1.608	2.385	2.043
MAVInv10	MAVInv10	-0.303	-0.256	-0.023	ND	0.242	-0.918	-2.524	-0.406	-0.725	1.059	1.004	2.828	2.998
MAVInv11	MAVInv11	-0.461	-0.158	-0.463	ND	0.042	ND	-1.198	ND	0.286	0.716	0.332	0.823	1.658

Supplemental Table 3.1 (continued)**.** Scores predicting presence or absence for all genes analyzed by custom microarray.

M. avium **Gene Name**	***M. avium*** **Gene Number**	**MAC100**	**MAC101**	**MAC109**	**MP101**	**MP102**	**MPATCC**	**MCH**	**MCJA**	**MCJ78**	**MCBAND**	**MCTRASK**	**MCSABLE**	**MCMWF**
MAVInv12	MAVInv12	0.071	-0.753	-0.088	ND	-0.179	0.135	-1.552	0.317	-0.582	1.743	0.905	0.572	1.383
MAVInv13	MAVInv13	0.212	-0.06	0.387	-2.63	-0.235	-2.943	-2.387	-0.083	-0.952	1.186	1.613	1.961	1.712
MAVInv14	MAVInv14	0.64	-0.081	-0.478	-2.404	1.189	-0.378	-1.814	-0.268	-0.099	0.632	0.841	2.485	1.759
MAVInv15	MAVInv15	-0.444	-0.073	1.02	-3.728	0.145	-1.152	-1.303	-0.361	-1.314	0.422	0.51	1.686	1.914

Chapter 4

Experimental Exposure of zebrafish (*Danio rerio* Hamilton) with *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: A potential model for environmental mycobacterial infection

Melanie J. Harriff[2,3], Luiz E. Bermudez[2,3], and Michael L. Kent[1,2,3]

Journal of Fish Diseases: **29:** 1-13

[1] Center for Fish Disease Research, Department of Microbiology, 220 Nash, Oregon State University, Corvallis, OR 97331
[2] Department of Biomedical Sciences, College of Veterinary Medicine, 105 Magruder, Oregon State University, Corvallis, OR 97331
[3] Molecular and Cellular Biology Program, Center for Gene Research and Biotechnology, 3021 Agricultural and Life Sciences, Oregon State University, Corvallis, OR 97331

Abstract

The natural route by which fish become infected with mycobacteria is unknown. *Danio rerio* (Hamilton) were exposed by bath immersion and intubation to *Mycobacterium marinum* and *Mycobacterium peregrinum* isolates obtained from diseased zebrafish. Exposed fish were collected over the course of 8 weeks and examined for the presence of mycobacteriosis. Mycobacteria were consistently cultured from the intestines, and often from the livers and spleens of fish exposed by both methods. Mycobacteria were not observed in the gills. Histological analysis revealed that fish infected with *M. marinum* often developed granulomas accompanied by clinical signs of mycobacteriosis, while infection with *M. peregrinum* infrequently led to clinical signs of disease. Passage of the bacteria through environmental amoeba (*Acanthamoeba castellani*) was associated with increased growth of *M. peregrinum* over the course of 8 weeks, when compared to infection with the bacteria not passed through amoeba. The results provide evidence that zebrafish acquire mycobacteria primarily through the intestinal tract, resulting in mycobacterial dissemination.

Introduction

Mycobacteriosis is a frequently recognized disease of both captive and wild fishes (Chinabut, 1999). The infection is very common in aquarium fishes (Prearo *et al.,* 2004; Pate *et al.,* 2005), and is a major concern for zebrafish (*Danio rerio* Hamilton) handlers in research facilities (Astrofsky *et al.,* 2000; Kent *et al.,* 2004). Severity of infections can range from chronic, associated with low mortality, to more serious outbreaks in which the entire colonies had to be euthanized (Kent *et al.,* 2004). Typical manifestations of mycobacteriosis in fish include ulceration of the skin surface, general malaise and emaciation, and granulomatous lesions containing acid-fast bacteria through out the internal organs (Decostere *et al.,* 2004). *Mycobacterium chelonae, Mycobacterium fortuitum,* and *Mycobacterium marinum* are the species most frequently isolated from fish with mycobacteriosis (Chinabut, 1999); however, recent studies have revealed an increasing number of mycobacterial species that can be responsible for disease in fishes [Levi *et al.,* 2003; Whipps *et al.,* 2003; Rhodes *et al.,* 2004; Poort *et al.,* 2006 (See Appendix I)].

Zebrafish have been recognized for over thirty years as an excellent model to study early vertebrate development (van der Sar *et al.,* 2004). Many fish species, including zebrafish (Prouty *et al.,* 2003; Pozos and Ramakrishnan, 2004; Swaim *et al.,* 2006) and goldfish (*Carassius auratus*) (Pattyn and Verdoolaeche-Van Loo, 1974; Talaat *et al.,* 1998; Ruley *et al.,* 2002), have also been utilized as models for infectious diseases, including mycobacteria. For example, Ramakrishnan and colleagues (Prouty *et al.,* 2003; Ramakrishnan, 2004) have proposed *M. marinum* as a surrogate model for *Mycobacterium tuberculosis* because the two bacteria are genetically closely related. In

fish and amphibians, *M. marinum* produces granulomatous lesions similar to those observed in humans with tuberculosis (Davis *et al.,* 2002; Pozos *et al.,* 2004; Ramakrishnan, 2004).

There are many advantages to the zebrafish as a model for infectious diseases of mammals. They breed easily and can be kept in large numbers at relatively low cost, compared to mammalian models such as the mouse. Unlike the fruit fly (*Drosophila melanogaster*) and the nematode (*Caenorhabditis elegans*), zebrafish have an immune system more closely related to that of mammals, with fully developed innate and adaptive immune systems, including macrophages, T and B cells, and toll-like receptors, which respond to *M. marinum* infection (Jault *et al.,* 2004; Meijer *et al.,* 2004). Moreover, the zebrafish and human genomes contain many orthologous genes relating to immune function.

There are, however, some disadvantages to employing the zebrafish as a model organism. Compared with what is known about the mammalian immune system, relatively little is known about the details of zebrafish immunity, and reagents, such as antibodies against cell markers, have not yet been developed. In addition, no immortal zebrafish monocytic cell lines have been established. Finally, little is known about how zebrafish are infected naturally by pathogens.

Experimental infection of fish with mycobacteria (Pattyn and Verdoolaeche-Van Loo, 1974; Talaat *et al.,* 1997; Gauthier *et al.,* 2003), including zebrafish models (Prouty *et al.,* 2003; van der Sar *et al.,* 2004; Watral and Kent, 2007), has relied on intraperitoneal (IP) challenge or injection into the caudal vein of zebrafish embryos (Davis *et al.,* 2002). The only reference to experimental infection of adult fish other than by injection was by

Ross (Ross, 1970), in which mycobacteria were transmitted to guppies by feeding them infected salmon tissues. Zebrafish embryos, 5 hours post-fertilization, have been incubated with mycobacteria to achieve infection (Davis *et al.,* 2002). We are unaware of any scientific studies that show the route of transmission, although it is generally assumed that zebrafish become infected with mycobacteria following cannibalism of infected fish or by feeding on aquatic detritus (Belas *et al.,* 1995), or by vertical transmission, as has been shown in other fish species (Chinabut *et al.,* 1994).

The study of transmission and virulence of mycobacteriosis in zebrafish is important for two reasons: first, zebrafish are increasingly being used as models for mycobacterial infections; and second, mycobacterial outbreaks are of frequent concern in zebrafish research facilities. We investigated how zebrafish acquire mycobacteria, whether there is a dose-dependence, and if an intermediate host such as a protozoan that is commonly present in the water can enhance the ability of mycobacteria to infect fish. We successfully established infection in zebrafish with both *M. peregrinum* and *M. marinum*, using both bath and oral challenge, indicating that mycobacteria in water are primarily acquired by the fish intestinal tract.

Materials and Methods

Bacterial strains and growth conditions. *M. marinum* OSU214 strain was isolated from hybrid striped bass (*Morone saxatilis* X *Morone chrysops*) and previously characterized in our laboratory (Kent *et al.,* 2006b; Watral and Kent, 2007). *M. peregrinum* strain MP101, associated with a severe outbreak at a research facility, was obtained from zebrafish and previously described (Kent *et al.,* 2004). Prior to infection,

M. marinum was grown on Lowenstein-Jensen slants (Remel, Lenexa KS) at 30 °C for 10 days. Because the OSU214 strain does not grow well on Middlebrook 7H11 (Kent *et al.,* 2006b), blood agar plates were used for determining colony forming units (CFUs) after infection. The MP101 strain was grown on Middlebrook 7H11 supplemented with oleic acid-albumin-dextrose-catalase (OADC) (Hardy Diagnostics, Santa Maria CA) at 30 °C for 4 days prior to infection, and plated onto Middlebrook 7H11 with OADC and PACT (polymyxin E, amphotericin B, carbenicillin, and trimethropin) selective antibiotics (Remel, Lenexa KS) for CFU counts. After growth of the inoculum, both bacterial species were washed and resuspended in Hank's Balanced Salt Solution (HBSS) (Gibco, Grand Island NY), and inocula concentrations were adjusted by using McFarland's standards and verified by plating.

Fish. Zebrafish (*Danio rerio)*, approximately 12 weeks old, were obtained from the Zebrafish International Resource Center (ZIRC, University of Oregon, Eugene OR) and acclimated for 2 weeks at Oregon State University. Fish were anesthetized in a 0.02% solution of ethyl-3-aminobenzoate methanesulfonate salt (MS-222) (Argent, Redmond WA) prior to infection by IP injection or intubation, and moved to 10-gallon glass aquaria following infection. Water temperature was maintained at 28 °C, and ammonia and nitrate concentrations in the static aquaria were controlled by monitoring levels with test kits and periodic water changes. In addition, each aquarium was equipped with a biological filter made from a box type aquarium filter, which was filled with porous lava rock. The fish were fed daily, except on the day prior to infection or sacrifice. Fish were maintained under approval of the Oregon State University Institutional Animal Care and Use Committee #3114.

Mycobacterial infection of zebrafish. The experimental doses and number of fish infected and analyzed for all challenges is summarized in Table 4.1. Inocula were prepared as described above.

Intraperitoneal infection.

Twenty fish were injected intraperitoneally (IP) with 100 µl *M. marinum* or *M. peregrinum* suspension in HBSS containing 3 X 10^8 cfu/ml.

Bath immersion.

Twenty fish were immersed in 100 ml beakers containing either 2.2 X 10^7 cfu/ml *M. peregrinum* or 2.1 X 10^5 cfu/ml *M. marinum*, or a sham bath containing a 1:10 dilution of HBSS in sterile water (15 fish). After 2 hours immersion, fish were removed from the water containing bacteria and transferred to separate 10-gallon tanks with clean water.

Per-os infection.

M. marinum and *M. peregrinum* were suspended in HBSS at two concentrations. Fish were exposed by intubation to 50 µl of the suspension containing the following inocula: *M. marinum*, unknown concentration, estimated to be 3 X 10^7 cfu/fish (21 fish); *M. peregrinum,* 1.2 X 10^7 cfu/fish (27 fish) and 1.0 X 10^4 cfu/fish (24 fish); and *M. marinum*, 7.8 X 10^5 cfu/fish (39 fish), and 1.0 X 10^3 cfu/fish (26 fish) and sterile HBSS (15 fish). Plastic tubing was inserted over a 22-gaugue needle, attached to a syringe, and inserted into the esophagus of the fish. Visible expansion of the abdomen was observed upon inoculation.

Culture of bacteria from dead or euthanized fish.

At 1 week, 4 weeks, and 8 weeks post-infection, 3 – 5 fish from each group were euthanized and analyzed separately for the presence of bacteria. The intestine and a

combination of the liver and spleen were homogenized in cetylpyridine NaOH (Sigma, St. Louis MO) and allowed to incubate overnight. After this purification, the organ homogenates were plated on selective agar and examined for mycobacterial growth. For fish challenged by bath immersion with *M. peregrinum*, the gills were also homogenized and analyzed. Bacteria cultured from fish were identified by colony morphology, acid fast staining, and 16S rDNA sequence of representative samples as described by Kent *et al*. (2004). For identification of *M. marinum*, plates were left in the light at room temperature for 1-3 days to allow for development of yellow pigmentation. 16S rDNA sequence (924 base pairs) was obtained using the primers and methods described by Talaat *et al.* (1997).

Histology. At 1 week, 4 weeks, and 8 weeks post-infection, 2 - 3 fish were evaluated by histology for the occurrence of lesions consistent with mycobacteriosis (granulomas) and for the presence of bacteria. Fish were preserved in Dietrich's solution, decalcified for 48 hours with Cal-Ex (Fisher Scientific, Baltimore MD), and embedded in paraffin. Sagittal sections of each fish were cut and stained with hematoxylin and eosin (H&E) and Kinyoun's acid-fast stain. Nine fish infected by IP injection (all *M. marinum*) and four fish infected by bath exposure (2 *M. marinum* and 2 *M. peregrinum*) were processed the same as above, except that they were not decalcified prior to being embedded in paraffin.

Amoeba strain and growth conditions. *Acanthamoeba castellani* obtained from American Type Culture Collection (ATCC, Catalog No. 30234) was used for the experiment. Amoebae were cultured in 75 mm^2 tissue culture flasks (Corning, Corning NY) in F12 media (GIBCO, Grand Island NY) until 80% confluency was obtained. Amoebae were incubated in the presence of 3 X 10^8 bacteria for 3 days; the medium was

then removed and HBSS used to wash the cultures. After washing, amoebae were either left intact and resuspended in HBSS, or lysed with 0.5% Triton X-100 (Sigma, St. Louis MO) in sterile water. Bacteria from lysed amoebae were separated from the Triton X-100 and amoeba lysate by differential centrifugation (5 min at 1000 rpm, and then an additional 30 min spin of the resulting supernatant at 3500 rpm, all at 4 °C) and resuspended in HBSS. Bacterial from amoebae were plated for quantification prior to the fish infection.

Mycobacterial infection of zebrafish after passage through *A. castellani*. After passage through amoebae, *M. peregrinum* or *M. marinum* were used to infect zebrafish by intubation or bath immersion as described above. In one group for each strain of bacteria, the infected amoebae were lysed prior to fish infection as described above, while the infected amoebae were left intact for the other group. Based on prior evidence suggesting that 30% of bacteria will be phagocytosed by amoebae in 1 hour (L.E. Bermudez, personal communication), the number of bacteria removed from or within the amoebae and used to infect zebrafish was estimated to be 1×10^7, with actual concentration determined by plating. Each fish was exposed by intubation to 50 μl of the suspension containing the following inocula: *M. peregrinum*, from lysed amoebae, 1.49×10^7 cfu/fish; *M. peregrinum*, from whole amoebae, 2.04×10^7 cfu/fish; *M. marinum*, from lysed amoebae, 2.5×10^5 cfu/fish; and *M. marinum*, from whole amoebae, 1.7×10^2 cfu/fish. Fish were euthanized and processed as described above.

Statistical Analysis.

Statistical analysis was based on the mean CFUs from each type of organ +/- the standard deviation for no fewer than 3 fish at each time point. Statistical significance ($p<0.05$) was calculated between groups by ANOVA and within groups by Student's t-test.

Results

Background mycobacteriosis. Between 1 and 400 colonies were cultured from the intestines of 7 of 9 fish intubated with a sterile saline solution and 2 of 9 fish bathed in the sham control. The number of colonies exceeded 20 in only one of these 9 fish, and this fish was the only sample from which mycobacteria were cultured from the liver and spleen. Small subunit ribosomal RNA sequence data on these colonies (provided by C.M. Whipps, personal communication) identified *Mycobacterium fortuitum,* a species commonly isolated from fish without causing disease (Beran *et al.,* 2006). No visible external signs of disease were observed, and there was no evidence of mycobacterial infection (e.g., inflammation or granulomas) observed in the visceral organs of 6 fish from both control groups. *M. fortuitum* were not cultured from fish infected with *M. marinum* or *M. peregrinum*.

Challenge with *M. marinum* and *M. peregrinum* by intraperitoneal (IP) infection.

To ascertain if both strains could cause experimental infection, zebrafish were challenged with both strains by IP injection. Following challenge with *M. marinum*, most fish showed clinical evidence of mycobacteriosis and became moribund or died by 4 weeks. Histological findings were consistent with a mycobacterial infection. Only 2 of 6 fish

challenged with *M. peregrinum* by IP injection and analyzed by histology showed clinical signs of disease and/or granuloma formation (Table 4.1).

Challenge with *M. marinum* and *M. peregrinum* by bath immersion. The results of challenge by bath immersion are summarized in Table 4.1.

In bath exposure experiments with *M. marinum*, cumulative mortality was 10% after 8 weeks. Bacteria consistent with *M. marinum* [acid-fast, pigmented, slow-growing, matching 16S rRNA sequence (data not shown)] were isolated from all dead fish and from the intestines of 80% of the fish that were euthanized. Mycobacteria were also cultured from 50% of the liver/spleen homogenates. The bacterial concentration in the intestines increased 1 log between 4 and 8 weeks ($p<0.05$), but did not significantly change in the liver/spleen (Figure 4.1). Histological analysis revealed pathological changes consistent with mycobacteriosis in 50% (4/8) of the fish euthanized over 8 weeks. For 2 of these fish processed without decalcification, acid-fast bacilli were observed in various tissues, including the mesenteric tissue (Figure 4.2A).

Exposure by bath immersion with *M. peregrinum* resulted in 100% survival over 8 weeks. Bacteria consistent with *M. peregrinum* [acid-fast, white, fast-growing, matching 16S rRNA sequence (data not shown)] were isolated from the intestines of all infected fish. The bacterial load cultured from the intestines increased 2 logs between 1 and 4 weeks, and 1 log for the subsequent 4 weeks. *M. peregrinum* was not

Table 4.1 Summary of challenges with *M. marinum* and *M. peregrinum*

Infection Method	Species	Challenge Dose/fish	Sample Size	Mortality Rate (%)	Culture (%)			Granuloma Formation/ Inflammation (%)
					Intestine	Spleen/Liver	Gills	
IP Injection	*M. marinum*	3.0×10^7 (est.)	20	90	ND	ND	ND	100
	M. peregrinum	3.0×10^7	20	0	ND	ND	ND	33
Bath Immersion	*M. marinum*	[a]2.1×10^5	20	10	80	50	ND	50
	M. peregrinum	[a]2.2×10^7	20	0	100	50	0	25
	Control[c]	1:10 dil. HBSS	15	0	22	0	ND	0
Oral Intubation	*M. marinum*	3.0×10^7 (est.)	17	76	100	ND	ND	67
		7.7×10^5	39	5	70	50	ND	35
		1.0×10^3	26	4	75	33	ND	25
	M. peregrinum	1.2×10^7	27	7	100	75	ND	25
		1.0×10^4	24	0	100	42	ND	8
	Control[c]	50 ul HBSS	15	0	78	11	ND	0
Infection after Amoeba passage[b]	*M. marinum*	1.3×10^5	38	8	90	60	ND	25
	M. peregrinum	1.8×10^7	37	0	95	78	0	15

[a] Dose reflects the concentration of bacteria/ml

[b] Dose reflects the average of the bacteria from lysed and whole amoeba; percentages have been calculated for the combined groups

[c] Culture results for indicate percentages of fish with *M. fortuitum* growth. *M. fortuitum* was not cultured from non-control group

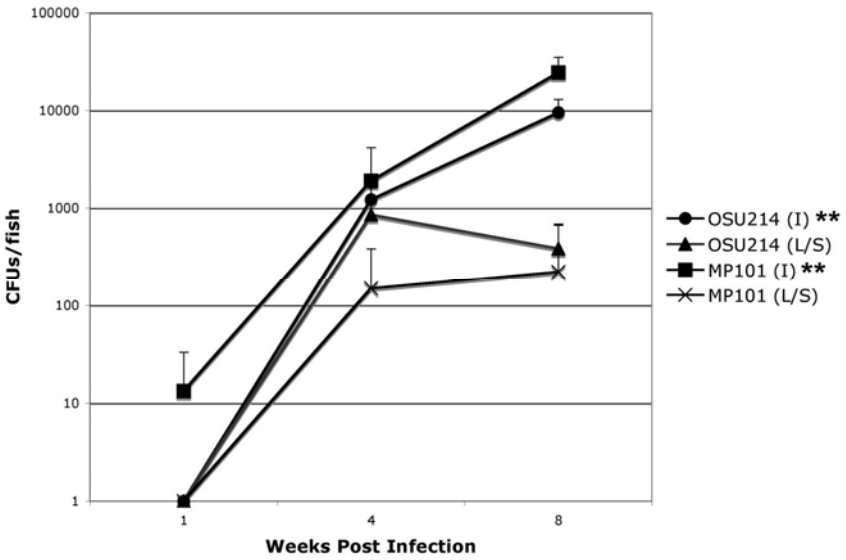

Figure 4.1 Colony forming units (CFUs) of bacteria cultured from the intestines, livers and spleens of zebrafish exposed to *Mycobacterium marinum* (OSU214) and *Mycobacterium peregrinum* (MP101) by bath immersion. Data shown are the average CFUs cultured from at least 3 fish at each time point from one representative experiment. I = Intestine, L/S = Liver/Spleen. * Indicates significant growth between 1 and 4 weeks ($p<0.05$); ** Indicates significant growth between 4 and 8 weeks ($p<0.05$).

recovered from the livers/spleens of the fish at 1 week post-infection (0/3 fish), but was isolated from 71% of the fish at 4 and 8 weeks (5/7) (Figure 4.1). Two fish analyzed by histology (without decalcification) had acid-fast bacilli in foci of chronic inflammation in multiple organs, including the kidney and testes (Figure 4.2B and 4.2C). Mycobacteria were not cultured from the gills, and histological analysis did not reveal the presence of lesions suggestive of mycobacterial infections in the gills of any fish samples.

Challenge with *M. marinum* and *M. peregrinum* by oral intubation. The results of challenge by oral intubation are summarized in Table 4.1.

Only 4 of 17 fish infected with *M. marinum* at an estimated dose of 3.0×10^7 cfu/fish survived beyond 5 weeks. The zebrafish showed clinical signs of

mycobacteriosis, including malaise, external skin ulceration, and diffuse erythema. Histological analysis revealed multiple granulomas in the visceral organs (including the kidney) of 10 of 15 fish (9 of which died prior to euthanization). In this experiment, the need to use media containing blood (e.g. 5% Sheep's Blood Agar) for the OSU214 strain of *M. marinum* had not yet been determined, and neither the original inoculum, nor CFU counts for fish sacrificed in this experiment were precisely determined by plate counts.

A second trial with two doses of *M. marinum* was then performed, in which all but 2 fish infected with *M. marinum* (7.8×10^5 cfu/fish) survived to the 8-week time point, with several showing numerous granulomas consistent with mycobacteriosis. At this dose, mycobacteria were cultured from both dead fish, from the intestines of 70% of euthanized fish and from the livers and spleens of 50% of the euthanized fish over the course of 8 weeks. CFUs recovered from the intestines were not significantly altered at any timepoint, although the number of bacteria did increase approximately 1 log between 4 and 8 weeks. In the liver/spleen samples, the CFUs increased significantly between 1 and 4 weeks and 4 and 8 weeks ($p<0.05$) (Figure 4.3). Granulomas consistent with mycobacteriosis were observed in the visceral organs and kidney of 35% (6/17) of the euthanized fish (Figure 4.2D). After challenge with a lower dose of *M. marinum* (1.0×10^3 cfu/fish), clinical signs of disease were not apparent and only 1 out of 26 fish died. Mycobacteria were not cultured from either the intestines or livers and spleens at the one-week time point (0/3 fish), but were

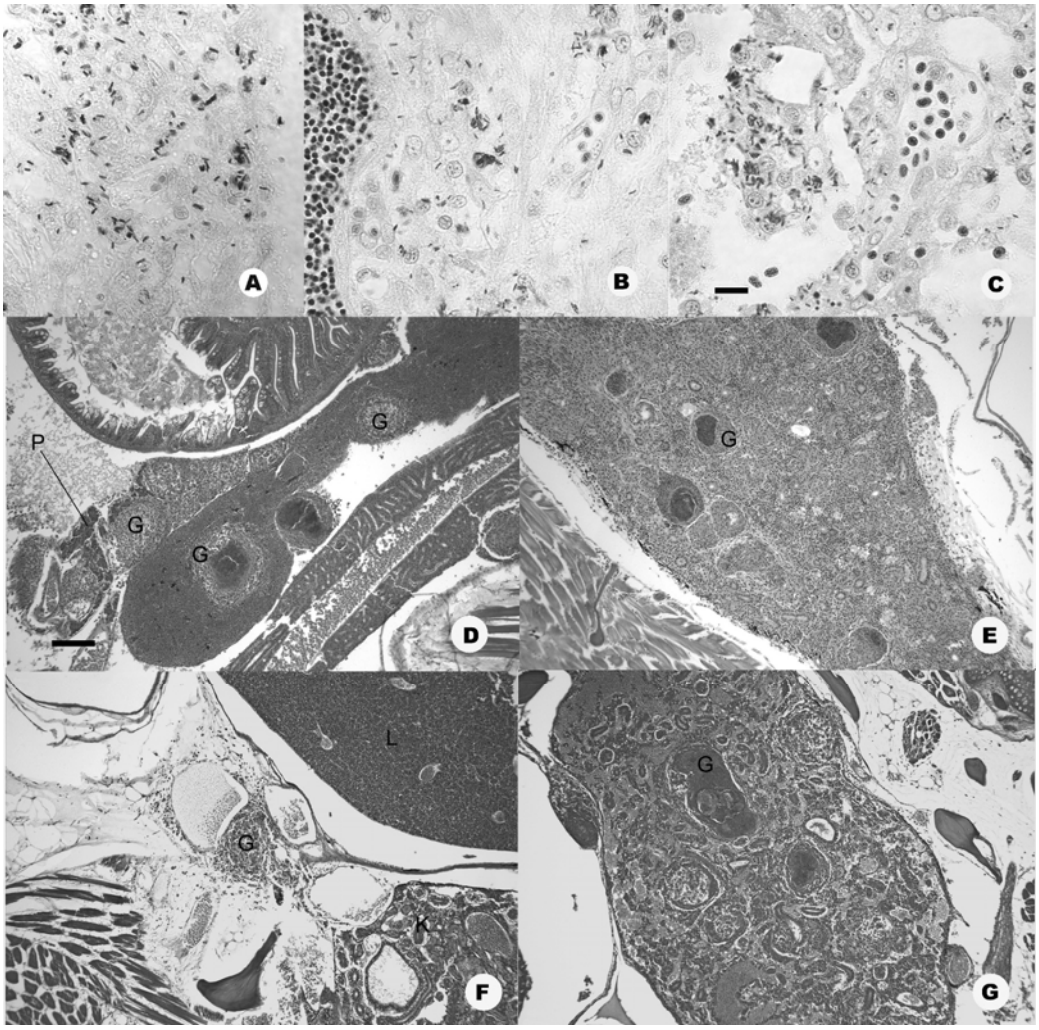

Figure 4.2 A-C: Acid-fast bacilli within zebrafish tissues from fish exposed to mycobacteria by bath immersion. Arrows = acid fast bacteria. Slides were prepared without decalcification. Bar = 10 µm. A) *M. marinum*, high dose, mesenteric tissue; B) *M. peregrinum*, high dose, testes; C) *M. peregrinum*, high dose, kidney. D-G: Granulomas (G) in tissues of zebrafish exposed to mycobacteria by intubation, with or without prior passage through amoeba. Hematoxylin & eosin, Bar = 40 µm. Images shown are representative of the fish that exhibited signs of mycobacteriosis. D) *M. marinum*, high dose, granulomas in the spleen; E) *M. peregrinum*, low dose, granulomas in the kidney; F) *M. marinum*, amoeba (lysed), granulomas in the tissue adjacent to the kidney (K), L = Liver; D) *M. marinum*, amoeba (whole), granulomas in the kidney.

recovered from the intestines of 100% of fish (9/9) and the livers/spleens of 42% of the fish euthanized afterwards. The number of CFUs in the organs increased approximately 0.5 log between 4 and 8 weeks ($p<0.05$) (Figure 4.3). Twenty-five percent (3/12) of the fish euthanized over 8 weeks had granulomas visible by histology.

In contrast to fish infected with the higher dose of *M. marinum*, those infected with the higher dose of *M. peregrinum* (1.2×10^7 cfu/fish) displayed no signs of mycobacteriosis. Despite the lack of signs of disease, mycobacteria were recovered from 100% of the intestines and 75% of the livers/spleens of the euthanized fish over 8 weeks. In both organ homogenates, the bacterial load increased by approximately 1 log between weeks 1 and 4, and decreased 0.5 log in the subsequent 4 weeks, although the difference

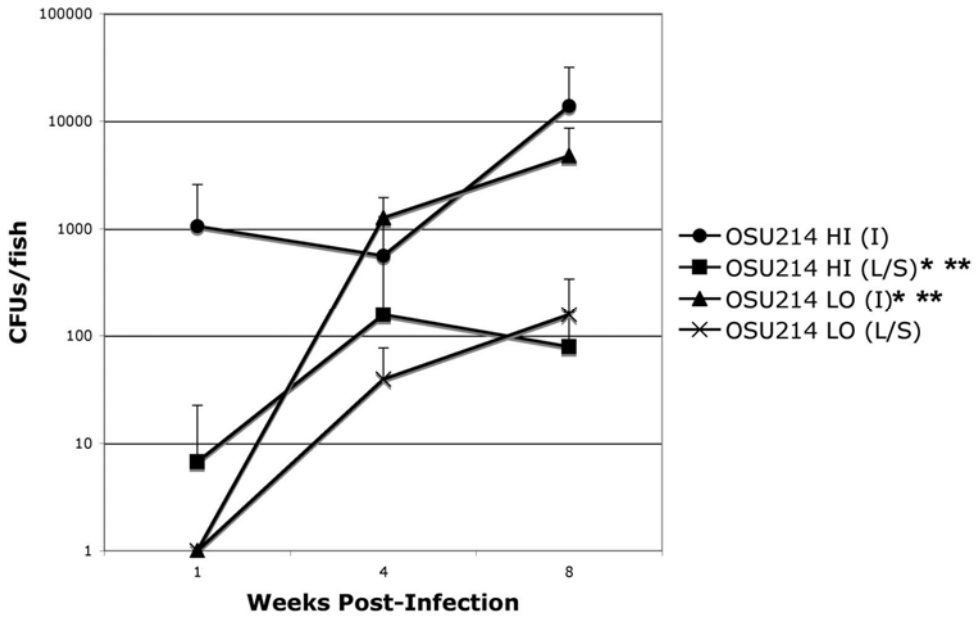

Figure 4.3 Colony forming units (CFUs) of bacteria cultured from the intestines, livers and spleens of zebrafish exposed to two doses of *Mycobacterium marinum* (OSU214) by intubation. Data shown are the average CFUs cultured from at least 3 fish at each time point from one representative experiment. I = Intestine, L/S = Liver/Spleen. * Indicates significant growth between 1 and 4 weeks ($p<0.05$); ** Indicates significant growth between 4 and 8 weeks ($p<0.05$).

was significant only in the intestines ($p<0.05$) (Figure 4.4). Granulomas were observed by H&E staining in 37.5% (3/8) of the fish at 1 and 4 weeks, but none of the fish had granulomas at 8 weeks (0/4 fish).

In the group infected with the low dose of *M. peregrinum*, there was no mortality and no signs of disease. Although granulomas were observed in only 1 of 12 fish (Figure 4.2E), colonies were consistently isolated from the organs of the fish. Mycobacteria grew from the intestines of 100% of the fish over 8 weeks, and from the livers and spleens of 67% of those fish (4/6) at 1 and 4 weeks. No bacteria grew from the livers and spleens at 8 weeks of infection (0/3 fish). In the intestines, there was an increase in CFUs of 1 log during the initial 4 weeks (although this change was insignificant) (Figure 4.4),

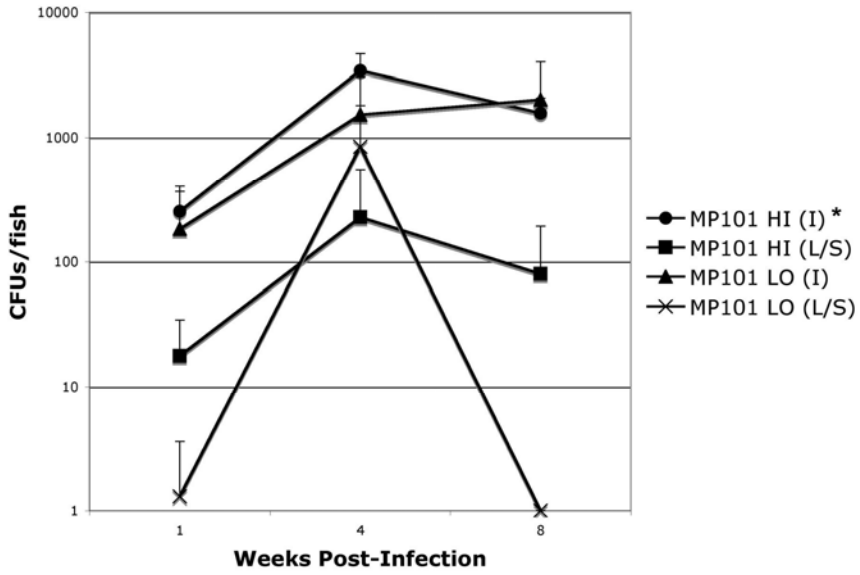

Figure 4.4 Colony forming units (CFUs) of bacteria cultured from the intestines, livers and spleens of zebrafish exposed to two doses of *Mycobacterium peregrinum* (MP101) by intubation. Data shown are the average CFUs cultured from at least 3 fish at each time point from one representative experiment. I = Intestine, L/S = Liver/Spleen. * Indicates significant growth between 1 and 4 weeks ($p<0.05$); ** Indicates significant growth between 4 and 8 weeks ($p<0.05$).

but no change was observed up to 8 weeks. In the livers and spleens, there was almost a 3-log increase between 1 and 4 weeks, but no mycobacteria were recovered at 8 weeks.

Effect of passage through *Acanthamoeba castellani* on mycobacterial infection of zebrafish. Both *M. peregrinum* and *M. marinum* were used to infect cultures of *A. castellani* for 3 days. Intracellular bacteria as well as bacteria obtained from lysed amoebae were subsequently used to infect zebrafish by intubation. The data from both groups were subsequently combined. Challenge with *M. marinum* contained within amoebae and from lysed amoebae resulted in infection of the zebrafish. Three fish became moribund prior to the end of the experiment in the group infected with *M. marinum* from lysed amoeba, and none died in the group infected with the bacteria inside whole amoebae. Although death of the three fish that died prior to the 1-week time point

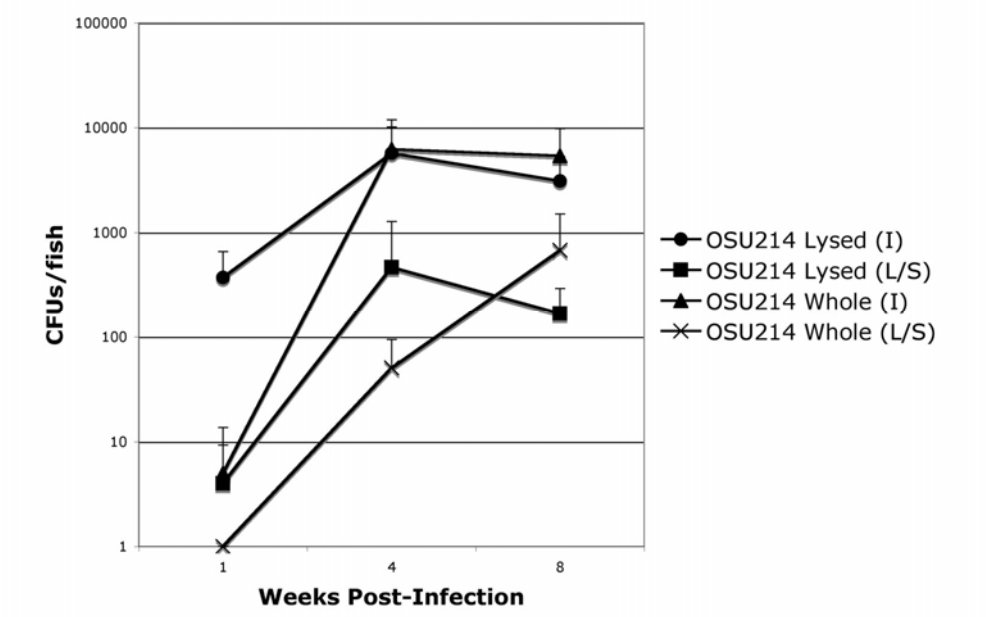

Figure 4.5 Colony forming units (CFUs) of bacteria cultured from the intestines, livers and spleens of zebrafish exposed to *Mycobacterium marinum* (OSU214) by intubation after passage through amoeba. Data shown are the average CFUs cultured from at least 3 fish at each time point from one representative experiment. I = Intestine, L/S = Liver/Spleen.

was likely from stress or handling, rather than mycobacteriosis, mycobacteria were nonetheless grown from the intestines of 2 of these fish. (The third fish was processed for histology). Of the fish euthanized over 8 weeks, bacteria were cultured from 90% of the intestines and from the livers and spleens of 60% of these fish. In each group, the number of bacteria increased from 1 to 3 logs between 1 and 4 weeks, but did not change between 4 and 8 weeks (Figure 4.5). There were no significant changes in the growth of the bacteria over the course of the 8 week trial, nor were there any statistical differences in the numbers of fish infected, when compared to the number of fish infected by bacteria without passage through amoebae. However, statistical analysis of the total number of

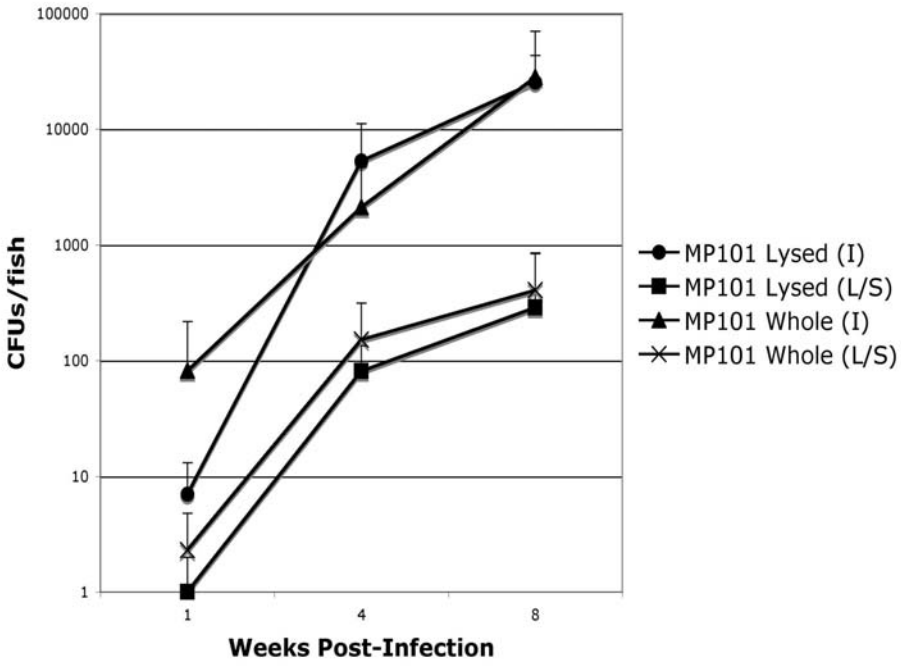

Figure 4.6 Colony forming units (CFUs) of bacteria cultured from the intestines, livers and spleens of zebrafish exposed to two doses of *Mycobacterium peregrinum* (MP101) by intubation after passage through amoebae. Data shown are the average CFUs cultured from at least 3 fish at each time point from one representative experiment. I = Intestine, L/S = Liver/Spleen.

bacteria isolated from fish infected with *M. marinum,* with and without prior passage through amoebae, suggests that passage through amoebae slightly enhances the ability of the bacteria to grow within zebrafish compared to infection without amoebae between 1 and 8 weeks ($p < 0.05$). Of the 12 fish analyzed by histology, 3 fish had granulomas and inflammation (Figure 4.2F and 4.2G).

As in the results of oral challenge with *M. peregrinum,* few fish (2/13) challenged with this species after passage through amoebae showed histological signs of mycobacteriosis, such as granulomas or inflammation. CFU counts, however, indicate that passage through amoebae does enhance the ability of *M. peregrinum* to grow within zebrafish between 1 and 8 weeks ($p < 0.05$). Similar to with *M. marinum*, however, the number of fish infected by bacteria with or without passage through amoebae was not significantly different. *M. peregrinum* was cultured from the intestines of 95% of the fish sacrificed over 8 weeks, and from the livers/spleens of 78% of these fish. Although not significantly, bacterial loads increased by at least 1.5 logs between 1 and 4 weeks, and again between 4 and 8 weeks (Figure 4.6).

Discussion

Zebrafish are becoming an important model for infectious diseases, including mycobacterial infections. Fish are commonly infected by mycobacteria, both in the natural environment, and in captivity, making them an even more relevant model. One of the present limitations, however, is that the natural route of infection by mycobacteria in fish has not been clearly demonstrated. Oral transmission through contaminated food is suspected to be a major source, as epizootics in hatchery-reared salmon in the 1950's and

1960's were associated with feeding unpasteurized fish (Ross, 1970; Belas *et al.,* 1995). Although there are many reports of experimental infection by intraperitoneal injection, controlled experiments investigating natural routes of exposure in adult fish are lacking. The study reported by Ross (1970), in which feeding infected salmon to guppies resulted in mycobacterial infection, is the only example from the literature of this type of study. Additionally, Ramakrishnan and colleagues have successfully transmitted mycobacteria to zebrafish embryos, 5 hours post-fertilization, by immersion (Davis *et al.,* 2002).

Although the route of mycobacterial infections in fish has not been addressed on an experimental level, other environmental mycobacteria are known to cause infection by the intestinal route. For example, an opportunistic human pathogen, *M. avium,* is able to invade epithelial cells of both the respiratory and intestinal tracts (Bermudez *et al.,* 1992; Sangari *et al.,* 1999; Sangari *et al.,* 2001; Yamazaki *et al.,* 2006). Although infection is likely to happen on a frequent basis, development of disease is more typical in immunosupressed individuals. Unlike many pathogenic bacteria, *M. avium* appears to interact preferentially with enterocytes, and not M cells found in Peyer's patches in mice (Sangari *et al.,* 2001). Little is known about receptors involved in the invasion of the enterocyte; however, it is thought that activation of host cell signaling pathways utilizing phosphorylation plays an important role (Sangari *et al.,* 2000; Dam *et al.,* 2006). Additionally, environmental factors of the intestinal lumen, such as low oxygen tension, increased osmolarity, and temperature impact the ability of *M. avium* to invade the intestinal mucosal cells (Bermudez *et al.,* 2000). Although zebrafish at the start of our study ranged in ages, it is known that adult zebrafish do not have Peyer's patches (Danilova and Steiner, 2002), indicating that mycobacterial infection in fish may also

occur by interaction with enterocytes, making zebrafish an acceptable model for *M. avium* and other mycobacteria.

In this study, zebrafish were challenged with two species of mycobacteria by IP injection, bath immersion, and oral intubation. Although there are some routes of infection that cannot be entirely ruled out by these experiments, the results show that zebrafish acquire mycobacteria primarily through the intestinal tract, similar to human acquisition of the environmental mycobacteria, such as *M. avium* (Sangari *et al.,* 1999). After immersion of the fish in water containing *M. peregrinum*, bacteria were not observed in the gills, either by histological analysis or by plating of gill homogenates. Both species of bacteria were, however, consistently cultured from the intestines of fish challenged by both immersion and intubation. In addition, our data suggest that the bacteria disseminate from the lining of the intestine to other visceral organs including the liver, spleen, and kidney. These results are consistent with observations from the ZIRC diagnostic service (http://zfin.org/zirc/documents/health. php), where mycobacteriosis in zebrafish from many research facilities is frequently diagnosed by histopathology. Several hundred cases of mycobacteriosis have been examined, with acid-fast bacilli observed in the intestinal or swim bladder epithelia, but never in the gill epithelium unless the fish exhibits a massive infection.

The control fish, whether intubated with or bathed in a sham HBSS solution, showed no histological signs of mycobacteriosis, but *M. fortuitum* was isolated in low numbers from the intestines of some fish. Whereas this species is recognized as a pathogen of fishes, it is a common aquatic bacterium and has been isolated from healthy fish and aquaria (Beran *et al.,* 2006). It is likely that these bacteria were normal flora of

the intestines of the fish. In fact, *M. fortuitum* has been observed in the lumen of healthy fish obtained for more recent experiments in our laboratory (Michael L. Kent, personal communication).

It should be noted that, in the present study, acid-fast bacteria were detected in fish exposed to *M. marinum* or *M. peregrinum* that had been processed without decalcification, but not in the other samples. This inability to detect acid-fast bacteria occurred even in fish with numerous granulomatous lesions, or in which large numbers of mycobacteria were cultured from the visceral organs. We subsequently demonstrated that the decalcification procedure we used, Cal-Ex 1 (Fisher Scientific, Baltimore, MD), (which contains 1.35 N HCl), completely inhibited the acid-fast staining of mycobacteria with a variety of acid-fast stains (Kent *et al.,* 2006a). In contrast, decalcification with 5% trichloroacetic acid or preservation in acidic fixatives such as Bouin's or Dietrich's did not affect these stains.

Mycobacterium marinum is a well-recognized pathogen of fishes, whereas *M. peregrinum* has only recently been associated with disease in fish (Kent *et al.,* 2004). Watral and Kent (2007), using intraperitoneal injections of zebrafish, evaluated the pathogenicity of the same strains of *M. marinum* and *M. peregrinum* that we examined here. Results from that study were similar to our present report in that *M. peregrinum* was capable of infecting fish, but only *M. marinum* caused significant mortalities.

Another consideration for environmental mycobacteria is the presence of potential reservoirs or intermediate hosts, such as amoebae. Previous studies have shown that passage through amoebae resulted in increased *M. avium* virulence in mice (Cirillo *et al.,* 1997; Cirillo *et al.,* 1999). In this study, while the *M. marinum* strain was able to cause

signs of disease and kill some fish, the amoebae only slightly enhanced the bacteria's ability to grow in the fish tissues. Although statistically valid, we cannot conclude that the amoebae enhanced infection with *M. marinum* because there were differences in the concentrations of the original inocula.The growth of the *M. peregrinum*, however, was enhanced after passage through amoebae, indicating that the role of amoebae in virulence enhancement may not be the same for all species. For both species, the bacterial counts from the fish infected after passage through amoebae were more variable than in samples infected with the bacteria alone. Although the net consequence of growth in amoebae is increased growth in the fish over 8 weeks, the effect must be initiated at the time of invasion. This suggests that the passage through amoebae leads the bacteria to enter the cells of the intestinal lining, possibly by different receptors and signaling pathways, thus changing their ability to grow within the cells. Free-living amoebae are found in the water of many fish culture facilities (De Jonckheere, 1979), and further studies on the role of these organisms as reservoirs for mycobacteria that infect fish should be undertaken.

We were able to obtain infection of zebrafish by intubation and bath immersion, although the doses of bacteria given to the fish in these experiments are likely much higher than any dose that would be encountered in the majority of natural environments. Nevertheless, there appeared to be a dose response for *M. marinum*. Interestingly, although signs of disease were more frequently observed at higher doses of *M. marinum*, CFU counts were lower than expected in the fish that were cultured at 8 weeks. These data suggest that it is possible for zebrafish to begin clearing the infection without a major inflammatory response, but further study is necessary. For *M. peregrinum*, the

dose seemed to have little relevance; similar levels of bacteria were cultured from fish organs, regardless of dose, and few fish developed clinical signs of disease at any dose. The only exception to this trend was the livers/spleens of fish infected with low dose of *M. peregrinum*. At all doses of either strain of bacteria, the bacteria were able to persist in the tissues of the fish for the duration of the 8-week experiment.

It is possible that when a fish is naturally exposed, bacteria could invade the cells of the intestinal lining and persist until a local or systemic immune suppressive event occurs, leading to an outbreak. Several types of stress may impair the fish's immune surveillance to a point where the bacteria are then able to grow to an overwhelming number. Stressors may include husbandry techniques, handling or crowding (Ramsay *et al.,* 2006), or the presence of concurrent infection with other pathogens. In fact, when we conducted a recent survey at a zebrafish facility, we diagnosed mycobacteriosis either by culture of the spleen and liver, or histology in many subclinical fish (C.M. Whipps, personal communication). There are several other examples of cryptic infections in naturally infected fish populations (Knibb *et al.,* 1993; Rhodes *et al.,* 2004; Kaattari *et al.,* 2005). In our experiment, based on development of clinical signs of infection, it is clear that the inflammatory response to the strain of *M. marinum* is greater than to the strain of *M. peregrinum*. A future direction in study will be to determine if different stressors, or levels of those stressors, are important in influencing the ability of species to induce an inflammatory response and cause disease in the fish.

We have demonstrated that mycobacterial infection in zebrafish (and likely in most other fish species) is acquired primarily through the intestinal tract. This

observation has implications for the control of infection, as well as for use of zebrafish as a model for mycobacterial diseases.

Acknowledgements.

This work was supported by NIH Grant NCRR 5R24RR017386-02.

We would like to thank the following colleagues at Oregon State University: Jerry Heidel and colleagues at the Veterinary Diagnostic Laboratory for assistance with acid-fast staining; Sheila Cleveland at the NIEHS Marine Freshwater Biomedical Science Center for assistance with histology; and Janell Bishop-Stewart and Virginia Watral at the Microbiology Department for assistance with fish infection and care.

References

Astrofsky, K. M., Schrenzel, M. D., Bullis, R. A., Smolowitz, R. M. & Fox, J. G. 2000. Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio)* facilities. Comp Med **50**, 666-72.

Belas, R., Fallon, P., and Hannaford, A. 1995. Potential application of molecular biology to the study of fish mycobacteriosis. Annu Rev Fish Dis **5**, 133-173.

Beran, V., Matlova, L., Dvorska, L., Svastova, P. & Pavlik, I. 2006. Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. J Fish Dis **29**, 383-93.

Bermudez, L. E., Petrofsky, M., Kolonoski, P. & Young, L. S. 1992. An animal model of *Mycobacterium avium* complex disseminated infection after colonization of the intestinal tract. J Infect Dis **165**, 75-9.

Bermudez, L. E., Wagner, D. & Sosnowska, D. 2000. Mechanisms of *Mycobacterium avium* pathogenesis. Arch Immunol Ther Exp (Warsz*)* **48**, 521-7.

Chinabut, S. 1999. Mycobacteriosis and Nocardiosis. Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections (P. T. K. W. a. D. W. Bruno), pp. 319-340. CAB International, New York.

Chinabut, S., Kanayati, Y., Pungkachonboon, T. 1994. Study of transovarian transmission of mycobacteria in *Betta splendens* Regan (L. M. Chou, Munro, A.D., Lam, T.J., Chen, T.W., Cheong, L.K.K., Ding, J.K., Hooi, K.K., Khoo, H.W., Phang, V.P.E., Shim, K.F., Tan, C.H.), Proc. Third Asian Fisheries Forum. Asian Fisheries Society, Manila, Phillipines.

Cirillo, J. D., Cirillo, S. L., Yan, L., Bermudez, L. E., Falkow, S. & Tompkins, L. S. 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. Infect Immun **67**, 4427-34.

Cirillo, J. D., Falkow, S., Tompkins, L. S. & Bermudez, L. E. 1997. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. Infect Immun **65**, 3759-67.

Dam, T., Danelishvili, L., Wu, M. & Bermudez, L. E. 2006. The fadD2 Gene Is Required for Efficient *Mycobacterium avium* Invasion of Mucosal Epithelial Cells. J Infect Dis **193**, 1135-42.

Danilova, N. & Steiner, L. A. 2002. B cells develop in the zebrafish pancreas. Proc Natl Acad Sci U S A **99**, 13711-6.

Davis, J. M., Clay, H., Lewis, J. L., Ghori, N., Herbomel, P. & Ramakrishnan, L. 2002. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity **17**, 693-702.

De Jonckheere, J. F. 1979. Occurrence of Naegleria and Acanthamoeba in aquaria. Appl Environ Microbiol **38**, 590-3.

Decostere, A., Hermans, K. & Haesebrouck, F. 2004. Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. Vet Microbiol **99**, 159-66.

Gauthier, D. T., Rhodes, M. W., Vogelbein, W. K., Kator, H. & Ottinger, C. A. 2003. Experimental mycobacteriosis in striped bass *Morone saxatilis*. Dis Aquat Organ **54**, 105-17.

Jault, C., Pichon, L. & Chluba, J. 2004. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. Mol Immunol **40**, 759-71.

Kaattari, I. M., Rhodes, M. W., Kator, H. & Kaattari, S. L. 2005. Comparative analysis of mycobacterial infections in wild striped bass *Morone saxatilis* from Chesapeake Bay. Dis Aquat Organ **67**, 125-32.

Kent, M. L., Heidel, J. R., Watral, W. G., Bishop-Stewart, J. K., Matthews, J. L. & Ostland, V. 2006. Some decalcification procedures inhibit acid fast staining of *Mycobacterium* spp. in tissue sections. Am. Fish. Soc./Fish Health Newslett. **34**, 15-17.

Kent, M. L., Watral, V., Wu, M. & Bermudez, L. E. 2006. In vivo and in vitro growth of *Mycobacterium marinum* at homoeothermic temperatures. FEMS Microbiol Lett **257**, 69-75.

Kent, M. L., Whipps, C. M., Matthews, J. L., Florio, D., Watral, V., Bishop-Stewart, J. K., Poort, M. & Bermudez, L. 2004. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. Comp Biochem Physiol C Toxicol Pharmacol **138**, 383-90.

Knibb, W., Colomi, A., Ankaoua, M., Lindell, D., Diamant, A. & Gordon, H. 1993. Detection and identification of a pathogenic marine mycobacterium from the European seabass *Dicentrachus labrax* using polymerase chain reaction and direct sequencing of 16S rDNA. Mol. Mar. Biol. Biotech. **2**, 225-232.

Levi, M. H., Bartell, J., Gandolfo, L., Smole, S. C., Costa, S. F., Weiss, L. M., Johnson, L. K., Osterhout, G. & Herbst, L. H. 2003. Characterization of *Mycobacterium montefiorense* sp. nov., a novel pathogenic Mycobacterium from moray eels that is related to *Mycobacterium triplex*. J Clin Microbiol **41**, 2147-52.

Meijer, A. H., Gabby Krens, S. F., Medina Rodriguez, I. A., He, S., Bitter, W., Ewa Snaar-Jagalska, B. & Spaink, H. P. 2004. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol Immunol **40**, 773-83.

Pate, M., Jencic, V., Zolnir-Dovc, M. & Ocepek, M. 2005. Detection of mycobacteria in aquarium fish in Slovenia by culture and molecular methods. Dis Aquat Organ **64**, 29-35.

Pattyn, S. R. & Verdoolaeche-Van Loo, G. 1974. The result of inoculation of *Mycobacterium leprae* in the goldfish (*Carassius auratus*). Acta Zool Pathol Antverp **58**, 109-16.

Poort, M. J., Whipps, C. M., Watral, V. G., Font, W. F. & Kent, M. L. 2006. Molecular characterization of a *Mycobacterium* species in non-native poeciliids in Hawaii using DNA sequences. J Fish Dis **29**, 181-5.

Pozos, T. C. & Ramakrishnan, L. 2004. New models for the study of Mycobacterium-host interactions. Curr Opin Immunol **16**, 499-505.

Prearo, M., Zanoni, R. G., Campo Dall'Orto, B., Pavoletti, E., Florio, D., Penati, V. & Ghittino, C. 2004. Mycobacterioses: emerging pathologies in aquarium fish. Vet Res Commun **28 (**Suppl 1), 315-7.

Prouty, M. G., Correa, N. E., Barker, L. P., Jagadeeswaran, P. & Klose, K. E. 2003. Zebrafish-*Mycobacterium marinum* model for mycobacterial pathogenesis. FEMS Microbiol Lett **225**, 177-82.

Ramakrishnan, L. 2004. Using *Mycobacterium marinum* and its hosts to study tuberculosis. Current Science **86**, 82-92.

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L. & Schreck, C. B. 2006. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquaculture **258**, 565-574.

Rhodes, M. W., Kator, H., Kaattari, I., Gauthier, D., Vogelbein, W. & Ottinger, C. A. 2004. Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay. Dis Aquat Organ **61**, 41-51.

Rhodes, M. W., Kator, H., Kotob, S., van Berkum, P., Kaattari, I., Vogelbein, W., Quinn, F., Floyd, M. M., Butler, W. R. & Ottinger, C. A. 2003. *Mycobacterium shottsii* sp. nov., a slowly growing species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). Int J Syst Evol Microbiol **53**, 421-4.

Ross, A. J. 1970. Mycobacteriosis among Pacific salmonid fishes. A symposium on diseases of fishes and shellfishes (S. F. Sniesko), pp. 279-283. American Fisheries Society, Washington D.C.

Ruley, K. M., Reimschuessel, R. & Trucksis, M. 2002. Goldfish as an animal model system for mycobacterial infection. Methods Enzymol **358**, 29-39.

Sangari, F. J., Goodman, J. & Bermudez, L. E. 2000. *Mycobacterium avium* enters intestinal epithelial cells through the apical membrane, but not by the basolateral surface, activates small GTPase Rho and, once within epithelial cells, expresses an invasive phenotype. Cell Microbiol **2**, 561-8.

Sangari, F. J., Goodman, J., Petrofsky, M., Kolonoski, P. & Bermudez, L. E. 2001. *Mycobacterium avium* invades the intestinal mucosa primarily by interacting with enterocytes. Infect Immun **69**, 1515-20.

Sangari, F. J., Parker, A. & Bermudez, L. E. 1999. *Mycobacterium avium* interaction with macrophages and intestinal epithelial cells. Front Biosci **4**, D582-8.

Swaim, L.E., Connolly, L.E., Volkman, H.E., Humbert, O. Born, D.E., & Ramakrishnan, L. 2006. *Mycobacterium marinum i*nfection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. Infect Immun **74**: 6108-6177.

Talaat A.M., Reimschuessel R. & Trucksis M. 1997. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. Veterinary Microbiology 58, 229–237.

Talaat, A. M., Reimschuessel, R., Wasserman, S. S. & Trucksis, M. 1998. Goldfish, *Carassius auratus*, a novel animal model for the study of *Mycobacterium marinum* pathogenesis. Infect Immun **66**, 2938-42.

van der Sar, A. M., Abdallah, A. M., Sparrius, M., Reinders, E., Vandenbroucke-Grauls, C. M. & Bitter, W. 2004. *Mycobacterium marinum* strains can be divided into two distinct types based on genetic diversity and virulence. Infect Immun **72**, 6306-12.

van der Sar, A. M., Appelmelk, B. J., Vandenbroucke-Grauls, C. M. & Bitter, W. 2004. A star with stripes: zebrafish as an infection model. Trends Microbiol **12**, 451-7.

Watral, V. & Kent, M. L. 2007. Pathogenesis of Mycobacterium spp. in zebrafish (*Danio rerio*) from research facilities. Comp Biochem Physiol C Toxicol Pharmacol 145, 55-60.

Whipps, C. M., Watral, V. G. & Kent, M. L. 2003. Characterization of a *Mycobacterium* sp. in rockfish, *Sebastes alutus* (Gilbert) and *Sebastes reedi* (Westrheim & Tsuyuki), using rDNA sequences. J Fish Dis **26**, 241-5.

Yamazaki, Y., Danelishvili, L., Wu, M., Hidaka, E., Katsuyama, T., Stang, B., Petrofsky, M., Bildfell, R. & Bermudez, L. E. 2006. The ability to form biofilm influences *Mycobacterium avium* invasion and translocation of bronchial epithelial cells. Cell Microbiol **8**, 806-14.

Zapata, A. & Amemiya, C. T. 2000. Phylogeny of lower vertebrates and their immunological structures. Curr Top Microbiol Immunol **248**, 67-107.

Chapter 5. Discussion

The general goal of this thesis was to study the interaction of environmental mycobacteria (EM) with host cells at the molecular level. Many species of EM are important health concerns to both humans and fish, including *Mycobacterium avium, Mycobacterium marinum,* and *Mycobacterium chelonae*. The *Mycobacterium avium* complex (MAC), an important concern for patients with AIDS, contains the species of EM that we know the most about; however, there is still little knowledge of the molecular mechanisms of host cell invasion. Species such as *M. marinum* and *M. chelonae* are a serious concern to those using fish as research models. Even less is known about these species that cause fish mycobacteriosis, including the natural route of infection. To elucidate more about the mechanisms of host cell invasion for all species of EM, a number of approaches were taken. First, experiments were performed with a mutant strain of *M. avium* deficient in epithelial cell invasion to identify genes and proteins involved in this mechanism. Second, strains of *M. avium* and EM isolated from fish were compared for their ability to invade and replicate in macrophages, and for the presence of known mycobacterial virulence determinants. Finally, the natural route of mycobacterial invasion in fish was determined experimentally, and the efficacy of zebrafish as a host model for *M. avium* was analyzed.

Aim 1: Interaction of *M. avium* with intestinal epithelial cells. The first aim involved understanding more about the role of FadD2 in the invasion of epithelial cells. Based on previous studies, we hypothesized that *M. avium* secretes molecules that interact with host cell signaling pathways to effect bacterial uptake, and that the secretion of these

molecules is regulated in part by FadD2. A microarray was utilized to obtain genome-wide information on the differences in gene expression in the wild-type versus the *fadD2* mutant strain when exposed to epithelial cells. Many genes with differences in expression were identified, including those encoding proteins that could potentially be involved in the secretion of molecules by *M. avium*. As most of the genes encoded hypothetical proteins with uncharacterized functions, a number of approaches were used to analyze these genes and proteins further.

Ideally, the first goals would have been to 1) construct knock-out mutants of a subset of these genes and observe changes in percent invasion of epithelial cells, and 2) analyze the differences in the secreted proteins between the wild-type and mutant strains, comparing these to the genes encoding secreted proteins identified by the microarray. As few groups have had success in targeting specific *M. avium* genes for knock out by allelic exchange, other methods were attempted. Allelic exchange via homologous recombination and gene interruption with an antibiotic resistance marker has been successful in *M. tuberculosis,* as well as *M. marinum*. For this reason, attempts were made to interrupt the *fadD2* gene in the MP101 strain of *M. peregrinum*, isolated from fish. This strain grows more rapidly than *M. avium*, and nothing was known about about its genetic tractability. If successful, the invasion phenotype of the knock-out strain would have been compared to that of the *M. avium* mutant, and if similar, *M. peregrinum* would have been used as a surrogate model to analyze the effect of deletion of selected genes on invasion. Unfortunately, although many colonies were screened, there were none in which a double crossover event had occurred. In parallel, a transposon mutant library of *M. avium* was screened for transposon insertion in specific genes using PCR.

No pools of mutants contained interrupted genes of interest, however. In addition, no differences were observed in the detectable proteins secreted by wild-type *M. avium* and the *fadD2* mutant strain in broth, or when exposed to epithelial cells, despite using multiple methods to concentrate the proteins.

Based on these results, alternate approaches were used to identify the pathways and proteins involved in the invasion process. First, a bacterial 2-hybrid assay was performed to identify putative secreted mycobacterial proteins that interact with host cell Cdc42. Second, a number of recombinant genes were transformed into *M. avium* such that their protein products would be over-expressed behind a constitutive promoter.

In the 2-hybrid screen, three proteins were observed to putatively interact with Cdc42. One of these proteins, encoded by MAV_4671, was of particular interest, as its expression upon exposure to epithelial cells was not up-regulated in the *fadD2* mutant strain. The amino acid sequence of this protein also suggested that it might be a transmembrane or secreted protein. All three genes were cloned behind a constitutive promoter, fused to a 6X-His tag. Unsuccessful attempts were made to observe the protein in either the broth supernatant or host cell cytoplasm, using antibodies to the 6X-His tag.

The focus was then turned to one of these proteins, MAV_4671, named CipA. The *cipA* gene was cloned behind the same promoter fused to a GSK tag. The GSK tag consists of a 20 amino acid sequence with a serine residue at position 9. In eukaryotic cell cytoplasm, this serine residue becomes phosphorylated, and antibodies have been developed against both the unmodified and phosphorylated tag sequences. Studies have shown that use of this tag to identify secreted prokaryotic proteins is superior to the *Bordatella pertussis* CyaA protein tag, used to assay increases in cytoplasmic cAMP.

Once again, the recombinant protein was not detectable by western blot. Finally, the *cipA* gene was cloned behind a stronger constitutive promoter, fused to the *cyaA* gene. Expression of the protein by *M. avium* was transiently detectable by western blot, however, the protein was never observed in the host cell cytoplasm, and levels of cAMP in the host cells did not increase significantly.

In the meantime, with the help of a rotation student, Cara Wilder, I was able to turn my efforts back to gene regulation on the bacterial side. Cara made recombinant strains of *M. avium* expressing two transcriptional repressor regulators (verified by real-time PCR to be upregulated during epithelial cell invasion) behind a strong promoter. Over-expression of these two genes (MAV_3679 and MAV_5138) led to an increase in bacterial uptake. Further experiments showed that over-expression of these regulators led to a change in expression of a number of other genes, including *cipA*.

In retrospect, considering my past and recent results, and those obtained recently by others in the laboratory, too much emphasis was placed on supporting the hypothesis that CipA was a secreted protein. Although the region of the genome that encodes this protein (Figure 2.8), and its amino acid sequence (Figure 2.4) are certainly of interest, broader thinking as to the function of this protein may have led to a different set of experiments, rather than repeated attempts at the same kind. An unexpected set of results from recent experiments supports this line of thinking. A new graduate student, Mike McNamara, was attempting to develop general plasmids for mycobacterial expression of proteins fused to GFP or RFP. To test his system using a variety of proteins, he cloned the *cipA* gene into his expression vectors. Although unable to detect fluorescence in *M. avium*, he was able to generate a strain of *Mycobacterium smegmatis* that expressed CipA

fused to GFP. Using this strain, I showed that, in *M. smegmatis*, CipA appears to be expressed on the surface of the bacterium when exposed to epithelial cells, and that Cdc42 appears to accumulate in the host cells near invading bacteria at early time points.

Additionally, Marta Alonso-Hearn, a postdoctoral researcher, identified a different *Mycobacterium avium* subsp. *paratuberculosis* protein that putatively interacts with Cdc42. The gene encoding this protein is very near to *cipA* in the genome, both genes are in small operons containing a putative hydrolase, and there are many other genes of interest in this region. Figure 2.8 in Chapter 2 outlines this region of the genome in both mycobacterial species. Dr. Alonso-Hearn identified her protein based on its lack of interaction with Cdc42 in an oxidoreductase mutant deficient in invasion of bovine epithelial cells. Interestingly, a second 2-hybrid screen indicated that CipA also has putative protein-protein interactions with a different oxidoreductase.

The combination of these results led us to propose a model for one of the mechanisms by which *M. avium* invades epithelial cells (Figure 5.1). The work described in Chapter 2 has laid the ground work to propose this model. Much remains to be done, however, to support the pathways and specific actions of proteins in the model. It is also likely that these mechanisms are only a part of the story. Host cell cytoskeleton rearrangement begins to take place before the bacteria comes into physical contact with the cell membrane, indicating that there are secreted proteins that have not yet been identified. Additionally, small G-proteins besides Cdc42 are involved in the uptake of bacteria by the host. Nonetheless, the proteins identified in this study are an important start in elucidating the molecular mechanisms by which *M. avium* invades epithelial cells.

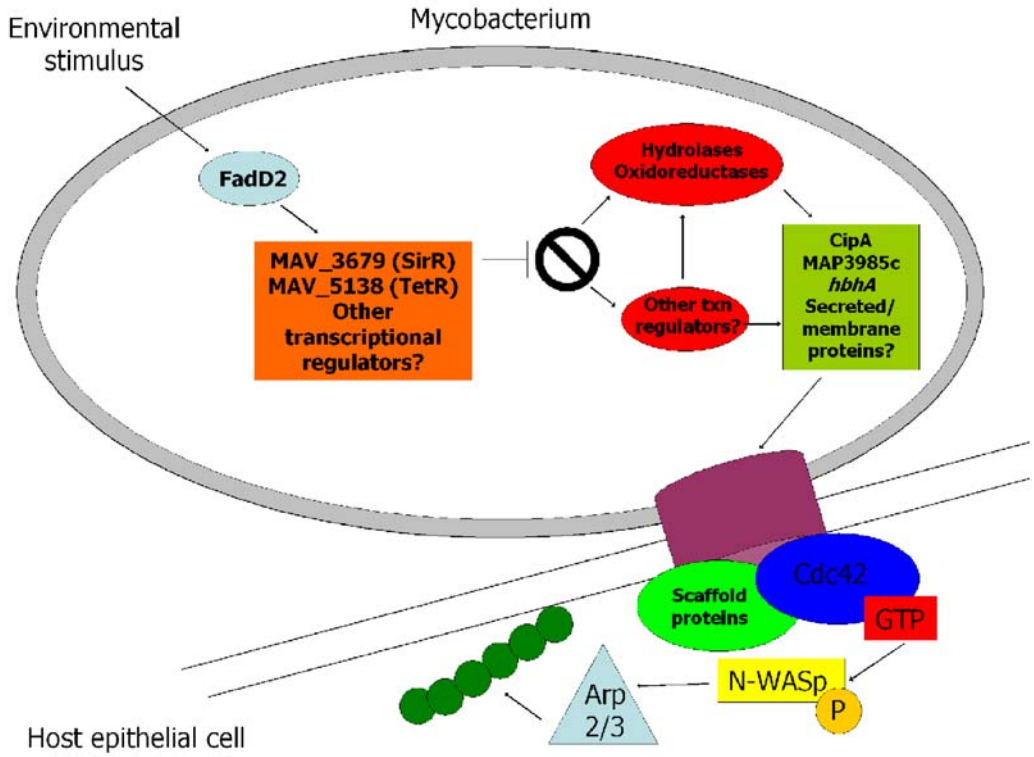

Figure 5.1 Proposed model of the molecular mechanisms of *Mycobacterium avium* invasion of epithelial cells regulated by FadD2.

Aim 2: Comparative analysis of the presence of virulence genes in species of EM isolated from fish. Members of the MAC are not the only pathogenic EM species. *Mycobacterium chelonae, Mycobacterium peregrinum,* and *Mycobacterium marinum* are only a few examples of EM with zoonotic potential that are very important in fish health. There are many reasons to be concerned about fish mycobacteriosis, among them being the importance of zebrafish as vertebrate models for research in developmental biology and infectious diseases. Increased understanding of the species of EM isolated from fish is important to those raising and using fish, for both diagnostic and preventative reasons. The second aim in this thesis involved analyzing the genomes of EM isolated from

outbreaks of mycobacteriosis and from chronic sub-clinical diagnostic cases in zebrafish, and correlating differences to the observed *in vivo* and *in vitro* virulence.

The original approach to this comparative study involved (1) conducting macrophage assays to identify the uptake and replication of these strains in human and mouse macrophages, and (2) using PCR to amplify a selected number of known virulence genes from the strains. At the time this work began, strains of *M. peregrinum*, *M. chelonae,* and *M. avium* were chosen based on the available isolates cultured from diagnostic cases. It was hypothesized that host specificity and virulence observed in the clinical cases would correlate to the ability of these strains to invade or replicate in particular macrophage cell lines, and that less virulent strains would also be lacking known mycobacterial virulence determinants.

In conducting the macrophage assays, it had to be considered that the fish isolates of mycobacteria did not grow in culture on plates at 37 °C, but this is the temperature at which the lines of mammalian macrophages are cultured. Interestingly, many of the fish isolates not only entered the human and mouse macrophages at this temperature, but also survived and replicated over the course of the 4-day assay. Based on some of these preliminary results, the isolates were used to infect the macrophages at a lower temperature (28.5 °C). In addition, lines of carp and catfish macrophages were also obtained. Carp are susceptible to mycobacteriosis, while catfish are naturally resistant. The catfish macrophages proved to be very difficult to culture, and CFU counts obtained were not consistent or reproducible, so this line was not pursued further. Although the cell lines were not as healthy at different temperatures, they survived for the 4-day assay,

and we were able to compare the various strains. Table 3.3 summarizes the results of the assays in all of these conditions.

In the meantime, the use of PCR to look at the presence of virulence genes in these strains was not productive. Multiple sets of primers and conditions were tried for each gene with mixed results using DNA from the isolates obtained from fish. Sequence data was not available for any of these species, making primer selection difficult, and although a positive PCR result was conclusive, a negative PCR result was not informative as to the presence or absence of this gene. To probe the DNA with a longer probe sequence, with the idea that there would be a better chance for hybridization, Southern blots were also attempted. These were also unsuccessful as to providing informative data. Probes for genes that had amplified by PCR in certain strains were not hybridizing the gDNA of those same strains in the Southern blot. More effort in optimization of the technique may have led to success. As the goal was to do a comparative study with many genes, it was determined that Southern blots were too labor intensive for each gene to continue.

Instead, a custom microarray was developed, using information gained from previous unsuccessful attempts to identify present and absent genes. Oligonucleotides generated for the array spots were developed for many more known virulence genes than would have been possible using PCR or Southern blot, allowing for a better analysis with which to correlate *in vivo* and *in vitro* virulence. The sequences for these probes were based on regions of genetic similarity among all sequenced strains of mycobacteria, increasing the chance that sequences of unknown mycobacteria would also be similar. Finally, the method used to analyze the results of hybridization was specifically

developed for determining the presence or absence of genes in unknown strains, based on known strains. Although the accuracy of these predictions did not prove to be perfect, based on subsequent PCR amplification, it was much more accurate than previous attempts.

The comparative analysis in Chapter 3 provides evidence that the pathogenicity of these species to the fish host, and possibly the zoonotic potential, correlates to the genomic composition of the organism. Although only a survey of mycobacterial genes were analyzed, important differences were observed in organisms with less virulence in diagnostic, *in vitro*, and *in vivo* settings. The pathogenicity island is an example of one of these differences that may prove to be useful in further development of diagnostic and preventative tools. This comparison of strains of EM will also be beneficial in the development of the zebrafish as a model for *M. avium* invasion of epithelial cells.

Future work should involve analyzing more of these species for the presence and expression of some of the genes identified in Chapter 2 of this thesis. Additionally, the way in which the environment and cellular response vary by host type and temperature, and the impact of this response on bacterial gene expression should be analyzed. Not presented in the chapters of this thesis was an unsuccessful attempt to use the custom microarray to look at the expression of present virulence genes in the different species. Many methods were used to extract RNA from intracellular mycobacteria in sufficient quantity and quality for use in a microarray. When fewer flasks of host cells were used, the quality of RNA improved due to the shorter time of processing, but the quantity decreased to inadequate levels for use on a microarray. If more flasks of host cells were used in order to increase the quantity of intracellular bacteria (and thus RNA), the quality

of the RNA, as well as the abililty to synchronize the timing among all the flasks decreased dramatically. Hybridization with one sample of high quality *M. avium* RNA isolated from intracellular bacteria resulted in disappointing hybridization to the microarray probes, and did not agree with Real-time PCR data conducted in parallel on the same samples. Given the opportunity to better optimize invasion percentages and the quality and quantity of extracted RNA, however, this tool could be a very effective way in which to compare the expression of virulence genes among strains of EM.

Technical difficulty and the need for optimization are not the only reasons why this tool was not pursued further. In the time after the macrophage assays and DNA microarrays were performed for this study, many new isolates from cases of fish mycobacteriosis were obtained. Data from *in vivo* infection experiments with many of these isolates (presented in Chapter 4 of this thesis, and in other work from the Kent laboratory cited in Chapter 3) suggest that the subset of strains should be reevaluated prior to undertaking a comparative gene expression analysis.

Aim 3: Identification of the route of infection in fish by mycobacteria. The means by which mycobacteria cause disease differs based on the bacterial species and host. Despite the importance and prevalence of mycobacteriosis in fish, the route of infection had not been experimentally determined. Almost all experimental studies with fish mycobacteriosis have involved infecting the fish by intraperitoneal (IP) injection. We hypothesized that 1) mycobacteria infect fish through the cells of the intestinal epithelium, similar to the route of *M. avium* infection in humans, 2) an assay for

experimental infection of fish using means other than IP injection could be developed and used, and 3) zebrafish could be used as a host model for *M. avium* infection.

To analyze this hypothesis, experiments were undertaken in which zebrafish were bathed in water containing various doses of mycobacteria. Originally, two strains of *M. peregrinum* isolated from outbreaks of mycobacteriosis in fish were used in infection experiments. Culturing colony forming units (CFUs) from the viscera of the fish without contamination by other gut bacteria and fungi proved to be very difficult, and the MP102 strain (see Chapter 3) was never isolated from the organs of the fish in these preliminary studies. Additionally, acid-fast bacteria were not observed in the tissues of fish preserved for histology, even when these fish came from the same groups in which many mycobacteria were cultured from the visceral organs. Further studies led to the discovery that decontamination of the organ homogenates with a cetylpiridine salt solution and subsequent plating on selective media successfully purified the mycobacteria from other bacteria and fungi. A study done by Kent and colleagues (cited in Chapter 4) also showed that the methods used in our lab to decalcify fish preserved for histology inhibited acid-fast staining. In the time since the experiments had begun, a new isolate of *M. marinum* had been isolated from fish that was proving to be highly virulent (based on IP injection studies). This strain was substituted in the experiments for the MP102 strain of *M. peregrinum* that had not led to infection, as it was proving to be a positive control in injection studies. Finally, with the help of Janell Bishop-Stewart in the Kent laboratory, a method was developed by which the zebrafish were infected by intubation, delivering the bacteria directly to the intestine.

With all of these considerations in place, studies presented in Chapter 4 show that, as with *M. avium* infection in humans, the intestine is the primary route of infection for fish mycobacteria. Even in fish infected by bath immersion, mycobacteria were not observed in the gills. On the other hand, mycobacteria have been observed in diagnostic cases in the swim bladder and its cell lining. It is probable that there are other minor routes of infection that could be explored.

Evidence from this work suggests that presence of the bacteria alone is not enough to cause disease. The doses used to infect the fish were much higher than would be encountered naturally. With these unnaturally high doses, there were still few cases in which the MP101 strain of *M. peregrinum* caused clinical signs of disease. Fish husbandry techniques and conditions (affecting fish stress) are likely to be important in development of disease, as is the presence of other hosts, such as amoebae. Although not significant, the numbers of bacteria cultured from the fish were higher for bacteria passaged through amoebae. As in all of the experiments, the CFUs were highly variable between individual fish, possibly leading to the lack of statistical significance. The organs of the fish were not weighed prior to homogenation because they were too small to register on the scale. Determining a method by which to weigh the organs and calculate CFUs/mg of tissue may aid in producing more consistent infection rates by these methods, as well as increase the statistical power in comparing groups.

Identification of the intestine as the primary route of infection for fish mycobacteria suggested that zebrafish could be developed as a model to study the interaction of *M. avium* with the intestinal epithelium. Specifically, pathways and protein functions, such as those proposed in Chapter 2 of this thesis, could be tested in zebrafish

as an alternative *in vivo* model to the mouse. Ideally, zebrafish would be infected with *M. avium* and mutant or recombinant strains that already exist. *M. avium* was not cultured from zebrafish after experimental infection by immersion or intubation, however, indicating the need to find a suitable surrogate species of mycobacteria. Infection of zebrafish with *M. marinum* is currently being used as a surrogate for the study of *Mycobacterium tuberculosis* granuloma formation and development. The work in Chapter 3 of this thesis should aid in selecting a species of EM for this use that not only has a similar genomic makeup to *M. avium*, but also has the ability to invade and grow in mammalian cells *in vitro.*

The scarcity of knowledge we have regarding the molecular mechanisms used by EM to invade host cells lent itself to the broad nature of the work presented in this thesis. This research has advanced what we know about specific proteins and pathways used by *M. avium* in epithelial cell invasion. Research tools, including a microarray and an *in vivo* host model, were developed for further study of these mechanisms in all EM important to human and fish health. Data regarding the ability of certain strains of mycobacteria to cause infection in fish also provides important information to fish handlers regarding the importance of proper husbandry techniques as a means to decrease mycobacteriosis in their facilities.

Bibliography

Alonso, A., and F. Garcia-del Portillo. 2004. Hijacking of eukaryotic functions by intracellular bacterial pathogens. Int Microbiol **7:**181-191.

Altier, C., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer. 2000. Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. Mol Microbiol **35:**635-646.

Ando, M., Y. C. Manabe, P. J. Converse, E. Miyazaki, R. Harrison, J. R. Murphy, and W. R. Bishai. 2003. Characterization of the role of the divalent metal ion-dependent transcriptional repressor MntR in the virulence of *Staphylococcus aureus*. Infect Immun **71:**2584-2590.

Arakawa, C. K., and J. L. Fryer. 1984. Isolation and characterization of a new subspecies of *Mycobacterium chelonei* infectious for salmonid fish. Helgolander Meeresun **37:**329-342.

Astrofsky, K. M., M. D. Schrenzel, R. A. Bullis, R. M. Smolowitz, and J. G. Fox. 2000. Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities. Comparative medicine **50:**666-672.

Bach, H., J. Sun, Z. Hmama, and Y. Av-Gay. 2006. *Mycobacterium avium* subsp. paratuberculosis PtpA is an endogenous tyrosine phosphatase secreted during infection. Infect Immun **74:**6540-6546.

Banks, J. 1994. Environmental mycobacteria, p. 265-276. *In* P. D. O. Davies (ed.), Clinical Tuberculosis. Chapman and Hall, London.

Bannantine, J. P., Q. Zhang, L. L. Li, and V. Kapur. 2003. Genomic homogeneity between *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* belies their divergent growth rates. BMC Microbiol **3:**10.

Barber, C. E., J. L. Tang, J. X. Feng, M. Q. Pan, T. J. Wilson, H. Slater, J. M. Dow, P. Williams, and M. J. Daniels. 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. Mol Microbiol **24:**555-566.

Baxter, M. A., T. F. Fahlen, R. L. Wilson, and B. D. Jones. 2003. HilE interacts with HilD and negatively regulates *hilA* transcription and expression of the *Salmonella enterica* serovar *typhimurium* invasive phenotype. Infect Immun **71:**1295-1305.

Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science **284:**1520-1523.

Belas, R., P. Fallon, and A. Hannaford. 1995. Potential application of molecular biology to the study of fish mycobacteriosis. Annu Rev Fish Dis **5:**133-173.

Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak. 2004. Improved prediction of signal peptides: SignalP 3.0. Journal of molecular biology **340:**783-795.

Beran, V., L. Matlova, L. Dvorska, P. Svastova, and I. Pavlik. 2006. Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. J Fish Dis **29:**383-393.

Bermudez, L. E., and M. Petrofsky. 1997. Regulation of the expression of *Mycobacterium avium* complex proteins differs according to the environment within host cells. Immunol Cell Biol **75:**35-40.

Bermudez, L. E., M. Petrofsky, and J. Goodman. 1997. Exposure to low oxygen tension and increased osmolarity enhance the ability of *Mycobacterium avium* to enter intestinal epithelial (HT-29) cells. Infect Immun **65:**3768-3773.

Bermudez, L. E., M. Petrofsky, P. Kolonoski, and L. S. Young. 1992. An animal model of *Mycobacterium avium* complex disseminated infection after colonization of the intestinal tract. J Infect Dis **165:**75-79.

Bermudez, L. E., D. Wagner, and D. Sosnowska. 2000. Mechanisms of *Mycobacterium avium* pathogenesis. Archivum immunologiae et therapiae experimentalis **48:**521-527.

Bermudez, L. E., and L. S. Young. 1994. Factors affecting invasion of HT-29 and HEp-2 epithelial cells by organisms of the *Mycobacterium avium* complex. Infect Immun **62:**2021-2026.

Braunstein, M., T. I. Griffin, J. I. Kriakov, S. T. Friedman, N. D. Grindley, and W. R. Jacobs, Jr. 2000. Identification of genes encoding exported *Mycobacterium tuberculosis* proteins using a Tn552'phoA in vitro transposition system. J Bacteriol **182:**2732-2740.

Brenot, A., K. Y. King, B. Janowiak, O. Griffith, and M. G. Caparon. 2004. Contribution of glutathione peroxidase to the virulence of *Streptococcus pyogenes*. Infect Immun **72:**408-413.

Brocklebank, J., S. Raverty, and J. Robinson. 2003. Mycobacteriosis in Atlantic salmon farmed in British Columbia. The Canadian veterinary journal **44:**486-489.

Brodin, P., I. Rosenkrands, P. Andersen, S. T. Cole, and R. Brosch. 2004. ESAT-6 proteins: protective antigens and virulence factors? Trends in microbiology **12:**500-508.

Brosch, R., S. V. Gordon, C. Buchrieser, A. S. Pym, T. Garnier, and S. T. Cole. 2000. Comparative genomics uncovers large tandem chromosomal duplications in *Mycobacterium bovis* BCG Pasteur. Yeast **17:**111-123.

Brown-Elliott, B. A., and R. J. Wallace, Jr. 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin Microbiol Rev **15:**716-746.

Bruno, D. W., J. Griffiths, C. G. Mitchell, B. P. Wood, Z. J. Fletcher, F. A. Drobniewski, and T. S. Hastings. 1998. Pathology attributed to *Mycobacterium chelonae* infection among farmed and laboratory-infected Atlantic salmon *Salmo salar.* Diseases of aquatic organisms **33:**101-109.

Cain, R. J., R. D. Hayward, and V. Koronakis. 2004. The target cell plasma membrane is a critical interface for *Salmonella* cell entry effector-host interplay. Mol Microbiol **54:**887-904.

Cappelli, G., E. Volpe, M. Grassi, B. Liseo, V. Colizzi, and F. Mariani. 2006. Profiling of *Mycobacterium tuberculosis* gene expression during human macrophage infection: upregulation of the alternative sigma factor G, a group of transcriptional regulators, and proteins with unknown function. Res Microbiol **157:**445-455.

Chang, C. T., L. Y. Wang, C. Y. Liao, and S. P. Huang. 2002. Identification of nontuberculous mycobacteria existing in tap water by PCR-restriction fragment length polymorphism. Appl Environ Microbiol **68:**3159-3161.

Charlet, D., S. Mostowy, D. Alexander, L. Sit, H. G. Wiker, and M. A. Behr. 2005. Reduced expression of antigenic proteins MPB70 and MPB83 in *Mycobacterium bovis* BCG strains due to a start codon mutation in *sigK*. Mol Microbiol **56:**1302-1313.

Chen, P., R. E. Ruiz, Q. Li, R. F. Silver, and W. R. Bishai. 2000. Construction and characterization of a *Mycobacterium tuberculosis* mutant lacking the alternate sigma factor gene, *sigF*. Infect Immun **68:**5575-5580.

Chinabut, S. 1999. Mycobacteriosis and nocardiosis, p. 319-340. *In* W. B. Woo and P.T.K. (ed.), Fish Diseases and Disorders: Viral Bacterial and Fungal Infections, vol. 3. CAB Internation, New York.

Chinabut, S., Y. Kanayati, and T. Pungkachonboon. 1994. Presented at the Proc. Third Asian Fisheries Forum, Manila, Phillipines.

Chitale, S., S. Ehrt, I. Kawamura, T. Fujimura, N. Shimono, N. Anand, S. Lu, L. Cohen-Gould, and L. W. Riley. 2001. Recombinant *Mycobacterium tuberculosis* protein associated with mammalian cell entry. Cell Microbiol **3:**247-254.

Cirillo, J. D., S. L. Cirillo, L. Yan, L. E. Bermudez, S. Falkow, and L. S. Tompkins. 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. Infect Immun **67:**4427-4434.

Cirillo, J. D., S. Falkow, L. S. Tompkins, and L. E. Bermudez. 1997. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. Infect Immun **65:**3759-3767.

Cossart, P., J. Pizarro-Cerda, and M. Lecuit. 2003. Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. Trends in cell biology **13:**23-31.

Cossart, P., and P. J. Sansonetti. 2004. Bacterial invasion: the paradigms of enteroinvasive pathogens. Science **304:**242-248.

Dam, T., L. Danelishvili, M. Wu, and L. E. Bermudez. 2006. The fadD2 Gene Is Required for Efficient *Mycobacterium avium* Invasion of Mucosal Epithelial Cells. J Infect Dis **193:**1135-1142.

Damsker, B., and E. J. Bottone. 1985. *Mycobacterium avium-Mycobacterium intracellulare* from the intestinal tracts of patients with the acquired immunodeficiency syndrome: concepts regarding acquisition and pathogenesis. J Infect Dis **151:**179-181.

Danelishvili, L., M. Wu, B. Stang, M. Harriff, S. Cirillo, J. Cirillo, R. Bildfell, B. Arbogast, L.E. Bermudez. 2007. Identification of *Mycobacterium avium* pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci USA *In press.*

Danelishvili, L., M. J. Poort, and L. E. Bermudez. 2004. Identification of *Mycobacterium avium* genes up-regulated in cultured macrophages and in mice. FEMS Microbiol Lett **239:**41-49.

Danelishvili, L., M. Wu, L. S. Young, and L. E. Bermudez. 2005. Genomic approach to identifying the putative target of and mechanisms of resistance to mefloquine in mycobacteria. Antimicrob Agents Chemother **49:**3707-3714.

Danilova, N., and L. A. Steiner. 2002. B cells develop in the zebrafish pancreas. Proc Natl Acad Sci U S A **99:**13711-13716.

Davis, J. M., H. Clay, J. L. Lewis, N. Ghori, P. Herbomel, and L. Ramakrishnan. 2002. Real-time visualization of *mycobacterium*-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity **17:**693-702.

Day, J. B., F. Ferracci, and G. V. Plano. 2003. Translocation of YopE and YopN into eukaryotic cells by *Yersinia pestis yopN, tyeA, sycN, yscB* and *lcrG* deletion mutants measured using a phosphorylatable peptide tag and phosphospecific antibodies. Mol Microbiol **47:**807-823.

de Jonckheere, J. F. 1979. Occurence of *Naegleria* and *Acanthamoeba* in aquaria. Appl Environ Microbiol **38:**590-593.

Decostere, A., K. Hermans, and F. Haesebrouck. 2004. Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. Vet Microbiol **99:**159-166.

Domenech, P., A. S. Pym, M. Cellier, C. E. Barry, 3rd, and S. T. Cole. 2002. Inactivation of the *Mycobacterium tuberculosis* Nramp orthologue (*mntH*) does not affect virulence in a mouse model of tuberculosis. FEMS Microbiol Lett **207:**81-86.

Dubnau, E., P. Fontan, R. Manganelli, S. Soares-Appel, and I. Smith. 2002. *Mycobacterium tuberculosis* genes induced during infection of human macrophages. Infect Immun **70:**2787-2795.

Elliott, D. A., D. J. Coleman, M. A. Lane, R. C. May, L. M. Machesky, and D. P. Clark. 2001. *Cryptosporidium parvum* infection requires host cell actin polymerization. Infect Immun **69:**5940-5942.

Fahlen, T. F., N. Mathur, and B. D. Jones. 2000. Identification and characterization of mutants with increased expression of *hilA*, the invasion gene transcriptional activator of *Salmonella typhimurium*. FEMS Immunol Med Microbiol **28:**25-35.

Falkinham, J. O., 3rd, C. D. Norton, and M. W. LeChevallier. 2001. Factors influencing numbers of *Mycobacterium avium, Mycobacterium intracellulare*, and other Mycobacteria in drinking water distribution systems. Appl Environ Microbiol **67:**1225-1231.

Fenster, S. D., M. M. Kessels, B. Qualmann, W. J. Chung, J. Nash, E. D. Gundelfinger, and C. C. Garner. 2003. Interactions between Piccolo and the actin/dynamin-binding protein Abp1 link vesicle endocytosis to presynaptic active zones. J Biol Chem **278:**20268-20277.

Fol, M., A. Chauhan, N. K. Nair, E. Maloney, M. Moomey, C. Jagannath, M. V. Madiraju, and M. Rajagopalan. 2006. Modulation of *Mycobacterium tuberculosis* proliferation by MtrA, an essential two-component response regulator. Mol Microbiol **60:**643-657.

Frota, C. C., D. M. Hunt, R. S. Buxton, L. Rickman, J. Hinds, K. Kremer, D. van Soolingen, and M. J. Colston. 2004. Genome structure in the vole bacillus, *Mycobacterium microti*, a member of the *Mycobacterium tuberculosis* complex with a low virulence for humans. Microbiology **150:**1519-1527.

Gauthier, D. T., M. W. Rhodes, W. K. Vogelbein, H. Kator, and C. A. Ottinger. 2003. Experimental mycobacteriosis in striped bass *Morone saxatilis*. Diseases of aquatic organisms **54:**105-117.

Geiman, D. E., D. Kaushal, C. Ko, S. Tyagi, Y. C. Manabe, B. G. Schroeder, R. D. Fleischmann, N. E. Morrison, P. J. Converse, P. Chen, and W. R. Bishai. 2004. Attenuation of late-stage disease in mice infected by the *Mycobacterium tuberculosis* mutant lacking the SigF alternate sigma factor and identification of SigF-dependent genes by microarray analysis. Infect Immun **72:**1733-1745.

Gioffre, A., E. Infante, D. Aguilar, M. P. Santangelo, L. Klepp, A. Amadio, V. Meikle, I. Etchechoury, M. I. Romano, A. Cataldi, R. P. Hernandez, and F. Bigi. 2005. Mutation in *mce* operons attenuates *Mycobacterium tuberculosis* virulence. Microbes Infect **7:**325-334.

Gonzalo Asensio, J., C. Maia, N. L. Ferrer, N. Barilone, F. Laval, C. Y. Soto, N. Winter, M. Daffe, B. Gicquel, C. Martin, and M. Jackson. 2006. The virulence-associated two-component PhoP-PhoR system controls the biosynthesis of polyketide-derived lipids in *Mycobacterium tuberculosis*. J Biol Chem **281:**1313-1316.

Hamelin, K., G. Bruant, A. El-Shaarawi, S. Hill, T. A. Edge, S. Bekal, J. M. Fairbrother, J. Harel, C. Maynard, L. Masson, and R. Brousseau. 2006. A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in *Escherichia coli* isolates from Great Lakes recreational waters. Appl Environ Microbiol **72:**4200-4206.

Hamelin, K., G. Bruant, A. El-Shaarawi, S. Hill, T. A. Edge, J. Fairbrother, J. Harel, C. Maynard, L. Masson, and R. Brousseau. 2007. Occurrence of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within the St. Clair River and Detroit River areas. Appl Environ Microbiol **73:**477-484.

Harriff, M., L. Bermudez, and M. L. Kent. 2007. Experimental exposure of zebrafish (*Danio rerio* Hamilton) to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: A potential model for environmental mycobacterial infection. J Fish Dis **29:** 1-13.

Herbst, L.H., S.F. Costa, L.M. Weiss, L.K. Johnson, J. Bartell, R. Davis, M. Walsh & M. Levi. 2001. Granulomatous skin lesions in moray eels caused by a novel *Mycobacterium* species related to *Mycobacterium triplex*. Infection and Immunity **69,** 4639-46.

Hou, J. Y., J. E. Graham, and J. E. Clark-Curtiss. 2002. *Mycobacterium avium* genes expressed during growth in human macrophages detected by selective capture of transcribed sequences (SCOTS). Infect Immun **70:**3714-3726.

Inderlied, C. B., L. S. Young, and J. K. Yamada. 1987. Determination of in vitro susceptibility of *Mycobacterium avium* complex isolates to antimycobacterial agents by various methods. Antimicrob Agents Chemother **31:**1697-1702.

Isberg, R. R., and J. M. Leong. 1988. Cultured mammalian cells attach to the invasin protein of *Yersinia pseudotuberculosis*. Proc Natl Acad Sci U S A **85:**6682-6686.

Isberg, R. R., and J. M. Leong. 1990. Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell **60:**861-871.

Jault, C., L. Pichon, and J. Chluba. 2004. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. Molecular immunology **40:**759-771.

Johnston, C., D. A. Pegues, C. J. Hueck, A. Lee, and S. I. Miller. 1996. Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. Mol Microbiol **22:**715-727.

Kaattari, I. M., M. W. Rhodes, H. Kator, and S. L. Kaattari. 2005. Comparative analysis of mycobacterial infections in wild striped bass *Morone saxatilis* from Chesapeake Bay. Diseases of aquatic organisms **67:**125-132.

Kent, M. L., J. R. Heidel, V. Watral, J. K. Bishop-Stewart, J. L. Matthews, and V. Ostland. 2006. Some decalcification procedures inhibit acid fast staining of *Mycobacterium* spp. in tissue sections. Am. Fish. Soc./Fish Health Newslett. **34:**15-17.

Kent, M. L., V. Watral, M. Wu, and L. E. Bermudez. 2006. In vivo and in vitro growth of *Mycobacterium marinum* at homoeothermic temperatures. FEMS Microbiol Lett **257:**69-75.

Kent, M. L., C. M. Whipps, J. L. Matthews, D. Florio, V. Watral, J. K. Bishop-Stewart, M. Poort, and L. Bermudez. 2004. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. Comp Biochem Physiol C Toxicol Pharmacol **138:**383-390.

Kitaura, H., N. Ohara, M. Naito, K. Kobayashi, and T. Yamada. 2000. Fibronectin-binding proteins secreted by *Mycobacterium avium*. Apmis **108:**558-564.

Knibb, W., A. Colomi, M. Ankaoua, D. Lindell, A. Diamant, and H. Gordon. 1993. Detection and identification of a pathogenic marine *mycobacterium* from the European seabass *Dicentrachus labrax* using polymerase chain reaction and direct sequencing of 16S rDNA. Mol. Mar. Biol. Biotech. **2:**225-232.

Kovacikova, G., and K. Skorupski. 2002. Regulation of virulence gene expression in *Vibrio cholerae* by quorum sensing: HapR functions at the *aphA* promoter. Mol Microbiol **46:**1135-1147.

Labo, M., L. Gusberti, E. D. Rossi, P. Speziale, and G. Riccardi. 1998. Determination of a 15437 bp nucleotide sequence around the *inhA* gene of *Mycobacterium avium* and similarity analysis of the products of putative ORFs. Microbiology **144 (Pt 3):**807-814.

Levi, M. H., J. Bartell, L. Gandolfo, S. C. Smole, S. F. Costa, L. M. Weiss, L. K. Johnson, G. Osterhout, and L. H. Herbst. 2003. Characterization of *Mycobacterium montefiorense* sp. nov., a novel pathogenic *Mycobacterium* from moray eels that is related to *Mycobacterium triplex*. J Clin Microbiol **41:**2147-2152.

Li, F., and G. D. Stormo. 2001. Selection of optimal DNA oligos for gene expression arrays. Bioinformatics **17:**1067-1076.

Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee. 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar *typhimurium*. J Bacteriol **182:**1872-1882.

Malen, H., F. S. Berven, K. E. Fladmark, and H. G. Wiker. 2007. Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. Proteomics **7:**1702-1718.

Manabe, Y. C., B. J. Saviola, L. Sun, J. R. Murphy, and W. R. Bishai. 1999. Attenuation of virulence in *Mycobacterium tuberculosis* expressing a constitutively active iron repressor. Proc Natl Acad Sci U S A **96:**12844-12848.

Manganelli, R., R. Provvedi, S. Rodrigue, J. Beaucher, L. Gaudreau, and I. Smith. 2004. Sigma factors and global gene regulation in *Mycobacterium tuberculosis*. J Bacteriol **186:**895-902.

Mapother, M. E., and J. G. Songer. 1984. In vitro interaction of *Mycobacterium avium* with intestinal epithelial cells. Infect Immun **45:**67-73.

Marchler-Bauer, A., J. B. Anderson, P. F. Cherukuri, C. DeWeese-Scott, L. Y. Geer, M. Gwadz, S. He, D. I. Hurwitz, J. D. Jackson, Z. Ke, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, G. H. Marchler, M. Mullokandov, B. A. Shoemaker, V. Simonyan, J. S. Song, P. A. Thiessen, R. A. Yamashita, J. J. Yin, D. Zhang, and S. H. Bryant. 2005. CDD: a Conserved Domain Database for protein classification. Nucleic acids research **33:**D192-196.

Marsh, I. B., J. P. Bannantine, M. L. Paustian, M. L. Tizard, V. Kapur, and R. J. Whittington. 2006. Genomic comparison of *Mycobacterium avium* subsp. *paratuberculosis* sheep and cattle strains by microarray hybridization. J Bacteriol **188:**2290-2293.

Masuda, M., M. Minami, H. Shime, T. Matsuzawa, and Y. Horiguchi. 2002. In vivo modifications of small GTPase Rac and Cdc42 by *Bordetella dermonecrotic* toxin. Infect Immun **70:**998-1001.
McGee, K., M. Zettl, M. Way, and M. Fallman. 2001. A role for N-WASP in invasin-promoted internalisation. FEBS Lett **509:**59-65.

Meijer, A. H., S. F. Gabby Krens, I. A. Medina Rodriguez, S. He, W. Bitter, B. Ewa Snaar-Jagalska, and H. P. Spaink. 2004. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Molecular immunology **40:**773-783.

Miltner, E., K. Daroogheh, P. K. Mehta, S. L. Cirillo, J. D. Cirillo, and L. E. Bermudez. 2005. Identification of *Mycobacterium avium* genes that affect invasion of the intestinal epithelium. Infect Immun **73:**4214-4221.

Momotani, E., D. L. Whipple, A. B. Thiermann, and N. F. Cheville. 1988. Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. Vet Pathol **25:**131-137.

Neely, M. N., J. D. Pfeifer, and M. Caparon. 2002. *Streptococcus*-zebrafish model of bacterial pathogenesis. Infect Immun **70:**3904-3914.

Parish, T., D. A. Smith, S. Kendall, N. Casali, G. J. Bancroft, and N. G. Stoker. 2003. Deletion of two-component regulatory systems increases the virulence of Mycobacterium tuberculosis. Infect Immun **71:**1134-1140.

Parish, T., D. A. Smith, G. Roberts, J. Betts, and N. G. Stoker. 2003. The *senX3-regX3* two-component regulatory system of *Mycobacterium tuberculosis* is required for virulence. Microbiology **149:**1423-1435.

Pate, M., V. Jencic, M. Zolnir-Dovc, and M. Ocepek. 2005. Detection of mycobacteria in aquarium fish in Slovenia by culture and molecular methods. Diseases of aquatic organisms **64:**29-35.

Pattyn, S. R., and G. Verdoolaeche-Van Loo. 1974. The result of inoculation of *Mycobacterium leprae* in the goldfish (*Carassius auratus*). Acta zoologica et pathologica Antverpiensia **58:**109-116.

Paustian, M. L., V. Kapur, and J. P. Bannantine. 2005. Comparative genomic hybridizations reveal genetic regions within the *Mycobacterium avium* complex that are divergent from *Mycobacterium avium* subsp. *paratuberculosis* isolates. J Bacteriol **187:**2406-2415.

Perez, E., S. Samper, Y. Bordas, C. Guilhot, B. Gicquel, and C. Martin. 2001. An essential role for *phoP* in *Mycobacterium tuberculosis* virulence. Mol Microbiol **41:**179-187.

Petrini, B. 2006. *Mycobacterium marinum*: ubiquitous agent of waterborne granulomatous skin infections. Eur J Clin Microbiol Infect Dis **25:**609-613.

Poort, M. J., C. M. Whipps, V. G. Watral, W. F. Font, and M. L. Kent. 2006. Molecular characterization of a *Mycobacterium* species in non-native poeciliids in Hawaii using DNA sequences. J Fish Dis **29:**181-185.

Porwollik, S., E. F. Boyd, C. Choy, P. Cheng, L. Florea, E. Proctor, and M. McClelland. 2004. Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. J Bacteriol **186:**5883-5898.

Porwollik, S., C. A. Santiviago, P. Cheng, L. Florea, and M. McClelland. 2005. Differences in gene content between *Salmonella enterica* serovar *Enteritidis* isolates and comparison to closely related serovars *Gallinarum* and *Dublin*. J Bacteriol **187:**6545-6555.

Porwollik, S., R. M. Wong, and M. McClelland. 2002. Evolutionary genomics of *Salmonella:* gene acquisitions revealed by microarray analysis. Proc Natl Acad Sci U S A **99:**8956-8961.

Pozos, T. C., and L. Ramakrishnan. 2004. New models for the study of *Mycobacterium*-host interactions. Current opinion in immunology **16:**499-505.

Prearo, M., R. G. Zanoni, B. Campo Dall'Orto, E. Pavoletti, D. Florio, V. Penati, and C. Ghittino. 2004. Mycobacterioses: emerging pathologies in aquarium fish. Veterinary research communications **28 Suppl 1:**315-317.

Preiter, K., D. M. Brooks, A. Penaloza-Vazquez, A. Sreedharan, C. L. Bender, and B. N. Kunkel. 2005. Novel virulence gene of *Pseudomonas syringae* pv. tomato strain DC3000. J Bacteriol **187:**7805-7814.

Prouty, M. G., N. E. Correa, L. P. Barker, P. Jagadeeswaran, and K. E. Klose. 2003. Zebrafish-*Mycobacterium marinum* model for mycobacterial pathogenesis. FEMS Microbiol Lett **225:**177-182.

Ramakrishnan, L. 2004. Using *Mycobacterium marinum* and its hosts to study tuberculosis. Current Science **86:**82-92.

Ramos, J. L., M. Martinez-Bueno, A. J. Molina-Henares, W. Teran, K. Watanabe, X. Zhang, M. T. Gallegos, R. Brennan, and R. Tobes. 2005. The TetR family of transcriptional repressors. Microbiol Mol Biol Rev **69:**326-356.

Ramsay, J. M., G. W. Feist, Z. M. Varga, M. Westerfield, M. L. Kent, and C. B. Schreck. 2006. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquaculture **258:**565-574.

Reddy, V. M., and D. A. Hayworth. 2002. Interaction of *Mycobacterium tuberculosis* with human respiratory epithelial cells (HEp-2). Tuberculosis (Edinburgh, Scotland) **82:**31-36.

Rhodes, M. W., H. Kator, I. Kaattari, D. Gauthier, W. Vogelbein, and C. A. Ottinger. 2004. Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay. Diseases of aquatic organisms **61:**41-51.

Rhodes, M. W., H. Kator, S. Kotob, P. van Berkum, I. Kaattari, W. Vogelbein, F. Quinn, M. M. Floyd, W. R. Butler, and C. A. Ottinger. 2003. *Mycobacterium shottsii* sp. nov., a slowly growing species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). Int J Syst Evol Microbiol **53:**421-424.

Rodrigue, S., R. Provvedi, P. E. Jacques, L. Gaudreau, and R. Manganelli. 2006. The sigma factors of *Mycobacterium tuberculosis*. FEMS Microbiol Rev **30:**926-941.

Rohatgi, R., L. Ma, H. Miki, M. Lopez, T. Kirchhausen, T. Takenawa, and M. W. Kirschner. 1999. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. Cell **97:**221-231.

Rosenshine, I., V. Duronio, and B. B. Finlay. 1992. Tyrosine protein kinase inhibitors block invasin-promoted bacterial uptake by epithelial cells. Infect Immun **60:**2211-2217.

Ross, A. J. 1970. Presented at the A symposium on diseases of fishes and shellfishes, Washington, DC.

Ross, A. J. 1960. *Mycobacterium salmoniphilum* sp. nov. from salmonid fishes. Am. Rev. Respir. Dis. **81:**241-250.

Ruley, K. M., R. Reimschuessel, and M. Trucksis. 2002. Goldfish as an animal model system for mycobacterial infection. Methods in enzymology **358:**29-39.

Sanders, G. E., and L. E. Swaim. 2001. Atypical piscine mycobacteriosis in Japanese medaka (*Oryzias latipes*). Comparative medicine **51:**171-175.

Sangari, F. J., J. Goodman, and L. E. Bermudez. 2000. *Mycobacterium avium* enters intestinal epithelial cells through the apical membrane, but not by the basolateral surface, activates small GTPase Rho and, once within epithelial cells, expresses an invasive phenotype. Cell Microbiol **2:**561-568.

Sangari, F. J., J. Goodman, M. Petrofsky, P. Kolonoski, and L. E. Bermudez. 2001. *Mycobacterium avium* invades the intestinal mucosa primarily by interacting with enterocytes. Infect Immun **69:**1515-1520.

Sangari, F. J., A. Parker, and L. E. Bermudez. 1999. *Mycobacterium avium* interaction with macrophages and intestinal epithelial cells. Front Biosci **4:**D582-588.

Sansonetti, P. J. 2001. Microbes and microbial toxins: paradigms for microbial-mucosal interactions III. Shigellosis: from symptoms to molecular pathogenesis. American journal of physiology **280:**G319-323.

Sassetti, C. M., and E. J. Rubin. 2003. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A **100:**12989-12994.

Schorey, J. S., M. A. Holsti, T. L. Ratliff, P. M. Allen, and E. J. Brown. 1996. Characterization of the fibronectin-attachment protein of *Mycobacterium avium* reveals a fibronectin-binding motif conserved among mycobacteria. Mol Microbiol **21:**321-329.

Sechi, L. A., N. Ahmed, G. E. Felis, I. Dupre, S. Cannas, G. Fadda, A. Bua, and S. Zanetti. 2006. Immunogenicity and cytoadherence of recombinant heparin binding haemagglutinin (HbhA) of *Mycobacterium avium* subsp. *paratuberculosis*: functional promiscuity or a role in virulence? Vaccine **24:**236-243.

Semret, M., G. Zhai, S. Mostowy, C. Cleto, D. Alexander, G. Cangelosi, D. Cousins, D. M. Collins, D. van Soolingen, and M. A. Behr. 2004. Extensive genomic polymorphism within *Mycobacterium avium*. J Bacteriol **186:**6332-6334.

Sigur-Dardottir, O. G., C. M. Press, and O. Evensen. 2001. Uptake of *Mycobacterium avium* subsp. *paratuberculosis* through the distal small intestinal mucosa in goats: an ultrastructural study. Vet Pathol **38:**184-189.

Sigur-Dardottir, O. G., A. M. Bakke-McKellep, B. Djonne, and O. Evensen. 2005. *Mycobacterium avium* subsp. *paratuberculosis* enters the small intestinal mucosa of goat kids in areas with and without Peyer's patches as demonstrated with the everted sleeve method. Comp Immunol Microbiol Infect Dis **28:**223-230.

Smith, I. 2003. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clin Microbiol Rev **16:**463-496.

Soto, M. J., M. Fernandez-Pascual, J. Sanjuan, and J. Olivares. 2002. A *fadD* mutant *of Sinorhizobium meliloti* shows multicellular swarming migration and is impaired in nodulation efficiency on alfalfa roots. Mol Microbiol **43:**371-382.

Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold, and W. D. Hardt. 2000. Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Mol Microbiol **36:**1206-1221.

Swaim, L. E., L. E. Connolly, H. E. Volkman, O. Humbert, D. E. Born, and L. Ramakrishnan. 2006. *Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. Infect Immun **74:**6108-6117.

Talaat, A. M., R. Reimschuessel, and M. Trucksis. 1997. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. Vet Microbiol **58:**229-237.

Talaat, A. M., R. Reimschuessel, S. S. Wasserman, and M. Trucksis. 1998. Goldfish, *Carassius auratus*, a novel animal model for the study of *Mycobacterium marinum* pathogenesis. Infect Immun **66:**2938-2942.

van der Sar, A. M., B. J. Appelmelk, C. M. Vandenbroucke-Grauls, and W. Bitter. 2004. A star with stripes: zebrafish as an infection model. Trends in microbiology **12:**451-457.

van der Sar, A. M., R. J. Musters, F. J. van Eeden, B. J. Appelmelk, C. M. Vandenbroucke-Grauls, and W. Bitter. 2003. Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections. Cell Microbiol **5:**601-611.

Volkman, H. E., H. Clay, D. Beery, J. C. Chang, D. R. Sherman, and L. Ramakrishnan. 2004. Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. PLoS biology **2:**e367.

Watral, V., and M. L. Kent. 2007. Pathogenesis of *Mycobacterium* spp. in zebrafish (*Danio rerio*) from research facilities. Comp Biochem Physiol C Toxicol Pharmacol **145:**55-60.

Whipps, C. M., W. R. Butler, F. Pourahmad, V. Watral, and M. L. Kent. 2007. Molecular systematics support the revival of *Mycobacterium salmoniphilum* (ex Ross, 1960) sp. nov., nom. rev., a species closely related to *Mycobacterium chelonae*. Intl. J. Syst. Evol. Microbiol. *In press*.

Whipps, C. M., S. T. Dougan, and M. L. Kent. 2007. *Mycobacterium haemophilum* infections of zebrafish (*Danio rerio*) in research facilities. FEMS Microbiol Lett **270:**21-26.

Whipps, C. M., V. G. Watral, and M. L. Kent. 2003. Characterization of a *Mycobacterium* sp. in rockfish, *Sebastes alutus* (Gilbert) and *Sebastes reedi* (Westrheim & Tsuyuki), using rDNA sequences. J Fish Dis **26:**241-245.

Wienholds, E., F. van Eeden, M. Kosters, J. Mudde, R. H. Plasterk, and E. Cuppen. 2003. Efficient target-selected mutagenesis in zebrafish. Genome research **13:**2700-2707.

Wilson, R. L., S. J. Libby, A. M. Freet, J. D. Boddicker, T. F. Fahlen, and B. D. Jones. 2001. Fis, a DNA nucleoid-associated protein, is involved in *Salmonella typhimurium* SPI-1 invasion gene expression. Mol Microbiol **39:**79-88.

Yamazaki, Y., L. Danelishvili, M. Wu, E. Hidaka, T. Katsuyama, B. Stang, M. Petrofsky, R. Bildfell, and L. E. Bermudez. 2006. The ability to form biofilm influences *Mycobacterium avium* invasion and translocation of bronchial epithelial cells. Cell Microbiol **8:**806-814.

Zahrt, T. C., and V. Deretic. 2000. An essential two-component signal transduction system in *Mycobacterium tuberculosis*. J Bacteriol **182:**3832-3838.

Zapata, A., and C. T. Amemiya. 2000. Phylogeny of lower vertebrates and their immunological structures. Current topics in microbiology and immunology **248:**67-107.

Zhou, D., and J. Galan. 2001. Salmonella entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect **3:**1293-1298.

Appendix I

Molecular characterization of a *Mycobacterium* species in non-native poeciliids in Hawaii using DNA sequences

Melanie J Poort[1,2,*], Christopher M Whipps[1], Virginia G Watral[1], William F Font[3], and Michael L Kent[1,2]

2006. Journal of Fish Diseases. **29**: 181-5.

[1]Center for Fish Disease Research, Department of Microbiology, 220 Nash, Oregon State University, Corvallis, OR 97331-3804, United States
[2] Department of Biomedical Sciences, College of Veterinary Medicine, 105 Magruder, Oregon State University, Corvallis, OR 97331, United States
[3]Department of Biological Sciences, Southeastern Louisiana University - 10736, Hammond, Louisiana 70402, United States

* M. Poort's publications following this one are authored under the name M.J. Harriff

Summary. Using molecular methods, C.W., V.W., and M. K. aided me in identifying a novel species of mycobacteria isolated from non-native poeciliid fish collected by W. F. in Hawaii. Like many of the other species of mycobacteria examined in my thesis work, *Mycobacterium triplex* has also been isolated from infections in humans. We were not able to culture this species of mycobacteria, however, and it was not used in further experiments.

Invasive or introduced fish species not only have the potential to displace native species from their ecological niches, but may introduce pathogens not normally encountered by native host species (Gozlan *et al.,* 2005). The shortfin molly (*Poecilia mexicana*), a species introduced to Hawaii, has been shown to be destructive to the native biota. Hoffman & Schubert (Hoffman and Schubert, 1984) reported that a parasitic copepod has been introduced to Hawaii through the establishment of infected shortfin mollies. Furthermore, other studies document the introduction of helminth parasites into native fish populations via poeciliid fish (Font and Tate, 1994; Font, 1997; Vincent and Font, 2003).

Poeciliid granulomatous disease (PGD) is common in poeciliid fishes, including shortfin mollies, in Hawaii. It is suspected to be caused by a *Mycobacterium* sp. as it is characterized by enlarged viscera with numerous granulomas in the kidney, a typical manifestation of mycobacterial infections in fish (Decostere *et al.,* 2004). Mycobacteriosis, caused by several species of *Mycobacterium*, has caused disease in numerous wild and captive fishes (Chinabut, 1999). Although not clearly documented, these outbreaks in wild fishes undoubtedly cause direct mortalities or reduced fitness due to increased predation. The purpose of this study was to determine if PGD is caused by a *Mycobacterium* sp., and characterize the bacterium, with the ultimate goal of determining if the bacterium is a threat to native freshwater fishes in Hawaii.

In this study, a total of 29 shortfin mollies (*Poecilia mexicana)* and three green swordtails (*Xiphophorus hellerii*) were collected in 2002 and 2003, from two locations: Loka Waka Pond (Seaside Pond) on the island of Hawaii and Kahana Stream on the island of Oahu.

Two shortfin mollies and one green swordtail with distended abdomens were collected from Loka Waka Pond in 2002 and were preserved in Dietrich's fixative. Multiple cross sections from each fish were prepared and stained with haematoxylin and eosin (H&E) or Ziehl-Neelsen acid-fast stains. Histological analysis of theses fishes revealed numerous granulomas throughout the visceral organs, including the liver, spleen, kidney, mesenteries, and gills (Figure 1). The granulomas were well organized and surrounded by squamous, epithelioid cells, some showing centers with caseous necrosis. In the mollies, low numbers of acid-fast bacteria were seen in the majority of granulomas (Figure 2). The swordtail exhibited a more severe bacterial infection than the mollies, with the granulomas containing numerous bacteria observed by acid-fast staining.

Tissues from 10 shortfin mollies collected in 2002, and 10 shortfin mollies collected in 2003 from Loka Waka Pond were cultured for mycobacteria. All fish exhibited macroscopically visible, discrete (often mineralized) granulomas in the kidney. Aseptic inocula were cultured on Middlebrook 7H10 agar and incubated for 4 weeks at

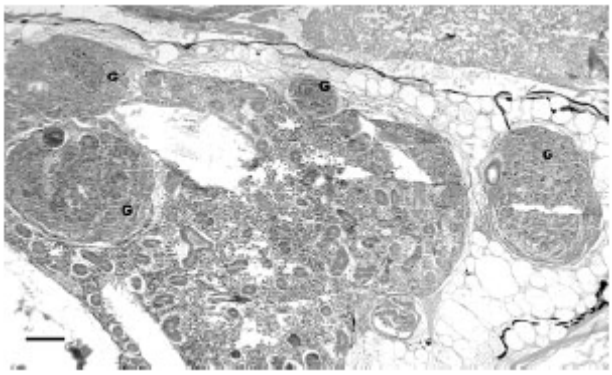

Figure 1. Multiple granulomas in the kidney and extra-renal spaces associated with *Mycobacterium* sp. in a shortfin molly, *Poecilia mexicana*. Hematoxylin and eosin. Bar = 100 μm. G = granuloma.

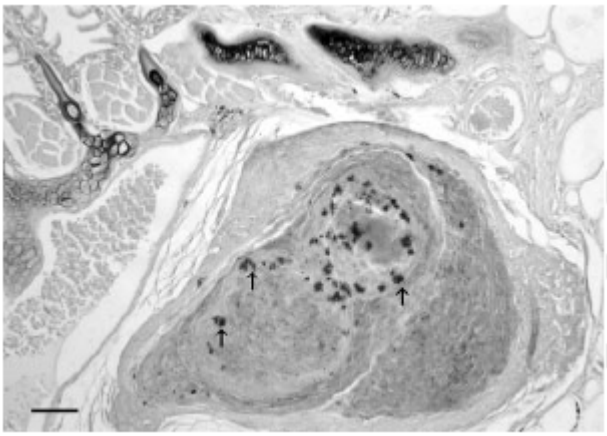

Figure 2. Coalescing granulomas at the base of the gill with aggregates of acid-fast bacilli (arrows) from a shortfin molly, *Poecilia mexicana*. Ziehl-Nelseen acid fast. Bar = 50 μm. Arrow = acid-fast bacteria.

30 ºC. A *Mycobacterium* species was cultured from one of the 20 fish. Three colonies were observed, appearing after about 5 days. Sequencing of the partial SSU rDNA (540 base pairs), as described below, identified this bacterium as *M. chelonae*.

Two mollies and one swordtail from Loka Waka Pond and one swordtail from Kahana Stream, collected in 2002, were preserved in the field in 95% ethanol. All showed internal signs suggestive of mycobacteriosis including enlarged livers, and numerous, whitish nodules consistent with granulomas observed throughout the viscera and kidney. Five additional shortfin mollies collected from Loka Waka Pond in 2003 were sent on ice to Oregon State University, and then preserved in ethanol. These fish exhibited a few focal lesions consistent granulomas in the kidney. Genomic DNA from these 9 fish samples was extracted from the kidney tissue using the DNeasy Tissue Kit (QIAGEN, Valencia CA, USA) according to manufacturers instructions with an added step of shaking tissue samples with 10mg/mL lysozyme (Sigma, St. Louis MO, USA) in a 37 °C shaking incubator overnight prior to starting the protocol. Polymerase chain

Table 1. Polymerase chain reaction primer-pair sequences for amplification and sequencing of small subunit (SSU), internal transcribed spacer (ITS), and large subunit (LSU) ribosomal DNA, and heat shock protein 65 (*hsp65*) genes from *Mycobacterium* species obtained in this study.

Name	Reference	gene/region	Position*	Sequence
T39 (F)	Talaat *et al.* (1997)	SSU	94-112	CGA ACG GGT GAG TAA CAC G
T13 (R)	Talaat *et al.* (1997)	SSU	1016-997	TGC ACA CAG GCC ACA AGG GA
16SCF	Whipps *et al.* (2003)	SSU	934-951	CGA TGC AAC GCG AAG AAC
MycoITS1R	Whipps *et al.* (2003)	ITS	1730-1711	ATG CTC GCA ACC ACT ATC CA
MycoITS1F	Whipps et al (2003)	SSU	1461-1478	GAC GAA GTC GTA ACA AGG
M23S-214R	This study	LSU	214-194	ACG GGT ACT GAG ATG TTT CAC
HSF1	Ucko *et al.* (2002)	*hsp65*	1-21	ATC CGG AGG AAT CAC TTC G
TB12	Telenti *et al.* (1993)	*hsp65*	588-607	CTT GTC GAA CCG CAT ACC CT

*Relative to GenBank accession AF330038 for SSU and ITS, AY172134 for LSU, and AF456468 for *hsp65*.

reaction (PCR) was used to amplify various regions of the small subunit (SSU) and internal transcribed sequence (ITS) ribosomal DNA (rDNA), and heat shock protein 65 (*hsp65*) genes from the DNA extracted from infected tissues. All PCR primer sequences are shown in Table 1. Conditions used for PCR amplification with T13/T39 primers were according to Talaat, Reimschuessel & Trucksis (1997). All other primer combinations employed the conditions described below. PCR amplifications were performed in 50 µL volumes containing 1.25 units of Taq DNA Polymerase (Qiagen Inc., Valencia CA, USA), 0.2 mM dNTPs, 25 pmol each primer and 1 µL DNA sample. Reactions were performed on an MJ Research DNA Engine 200 (MJ Research, Watertown MA, USA) for 35 cycles of 94 °C for 30 sec, 56 °C for 45 sec, 72 °C for 90 sec, preceded by an initial denaturation of 95 °C for 3 min, and followed by a final extension of 72 °C for 7 min. Samples were visualized on a 1.5% agarose gel run at 100V

for 1 h, and products were excised and purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia CA, USA). Direct sequencing of purified PCR products was carried out with the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1, using the ABI PRISM® 3730 DNA Analyzer (Applied Biosystems, Foster City CA, USA).

Identical sequences of 540 base pairs (bp) of SSU sequence and 284 bp of ITS were obtained from all 9 fish analyzed. For completeness, additional SSU sequence was obtained for one specimen, and the combined SSU and ITS sequence was deposited in GenBank (Accession# DQ078788). Standard nucleotide-nucleotide analysis was performed using the basic local alignment search tool (BLAST), to determine the highest sequence similarity between the DNA sequences obtained from the Hawaiian samples and all other sequences deposited in GenBank (Table 2). Small subunit sequences were most similar to *Mycobacterium florentinum, Mycobacterium triplex*, and *Mycobacterium lentiflavum*. These species were also identified as the best matches from BLAST searches of the ITS DNA sequence, although *M. lentiflavum* was the best match, not *M. florentinum* or *M. triplex*. To clarify these results, we obtained 550 bp of *hsp*65 sequence (GenBank Accession #DQ078789) directly from the tissues of one of the 9 identical samples. With the *hsp*65, BLAST results again yielded the best match as *M. lentiflavum* and *M. triplex* (Table 2). Our BLAST results highlight some of the difficulties of identifying *Mycobacterium* species by sequence alone. Small subunit sequence was 99.7% similar to *M. florentinum* and *M. triplex* and 99.0% similar to *M. lentiflavum* and *M. montefiorense* over shorter regions.

Table 2. Basic Logical Alignment Search Tool (BLAST) results for *Mycobacterium* species 16S, ITS, and *hsp65* sequences obtained from infected fish tissues of shortfin molly, *Poecilia mexicana*.

Gene	BLAST matches	GenBank accession #	Proportion of nucleotide similarity	Reference
16S	*M. florentinum*	AJ616230	1381/1385 (99.7%)	Tortoli *et al.* (2005)
	M. triplex	AJ276890	1381/1385 (99.7%)	Suomalainen *et al.* (2001)
	M. triplex	U57632	1357/1367 (99.3%)	Floyd *et al.* (1996)
	M. sherrisii	AY353699	1371/1381 (99.3%)	Selvarangan *et al.* (2004)
	M. lentiflavum	AF480583	1330/1343 (99.0%)	Turenne *et al.* (2001)
	*M. montefiorense**	AF330038	1371/1385 (99.0%)	Levi *et al.* (2003)
	Mycobacterium sp.*	AF541924	1364/1385 (98.5%)	Whipps *et al.* (2003)
ITS	*M. lentiflavum*	AF318174	272/283 (96.1%)	Niobe *et al.* (2001)
	M. florentinum	AJ616230	270/283 (95.4%)	Tortoli *et al.* (2005)
	M. triplex	AJ276890	270/283 (95.4%)	Suomalainen *et al.* (2001)
	M. triplex	AF214587	270/283 (95.4%)	Hazra *et al.* (2000)
	M. triplex	Y14189	270/283 (95.4%)	Roth *et al.* (1998)
	M. triplex	AF334028	270/283 (95.4%)	Floyd *et al.* (1996)
	*M. montefiorense**	AF330038	201/214 (93.9%)	Levi *et al.* (2003)
	Mycobacterium sp.*	AF541924	190/213 (89.2%)	Whipps *et al.* (2003)
hsp65	*M. lentiflavum*	AF547851	406/414 (98.1%)	Devulder *et al.* (2005)
	M. triplex	AF547882	400/414 (96.6%)	Devulder *et al.* (2005)
	M. triplex	AY027786	371/385 (96.3%)	Levi *et al.* (2003)
	*M. montefiorense**	AY943204	385/401 (96.0%)	Unpublished

* Fish hosts

The ITS and *hsp65* sequences, however, were most similar to *M. lentiflavum* (Table 2). All three of these species are slow-growing (Floyd *et al.,* 1996; Springer *et al.,* 1996; Tortoli *et al.,* 2005). Therefore, the conditions at which we chose to grow the genetically similar *Mycobacterium* species sequenced from the shortfin mollies (Middlebrook 7H10 agar at 30 °C) were probably inappropriate. We cannot assign the bacterium in this study to a specific species at this time because of conflicting taxonomic results using molecular methods and our inability to isolate the organism in culture. However, molecular data

clearly demonstrate that it is a member of the *M. triplex* clade, and is not closely related the more common *Mycobacterium* spp. of fishes (e.g., *M. chelonae, M. fortuitum,* and *M. marinum*) (Chinabut, 1999).

Mycobacterium triplex, *M. florentinum*, and *M. lentiflavum* are known to cause disease in humans (Floyd *et al.,* 1996; Cingolani *et al.,* 2000; Niobe *et al.,* 2001; Tortoli *et al.,* 2002; Tortoli *et al.,* 2005). In addition, *M. triplex*-like organisms have recently been reported from several fishes, such as moray eels (*Gymnothorax* spp.) (Herbst *et al.,* 2001; Levi *et al.,* 2003), rockfishes (*Sebastes* sp.) (Whipps *et al.,* 2003), and striped bass (*Morone saxatilis*) (Rhodes *et al.,* 2004). Interestingly, the bacterium sequenced here was more closely related to strains isolated from humans than to those from fish (Table 2)**.**

Although we are unaware of a published study, PGD has been recognized in the feral poeciliid fishes in Hawaii for several years. A *Mycobacterium* sp. has been suspected, but not proven, to be the cause. Our study supports the concept that the cause is mycobacterial. Histological analyses demonstrated the presence of numerous acid-fast bacilli, morphologically consistent with *Mycobacterium* sp., contained in granulomas in both poeciliid species. The data generated in this study suggest that the cause of PGD is a *M. triplex*-like organism, rather than *M. chelonae,* because: (1) we obtained identical sequences from 9 sequenced fish tissue samples, some of which had dramatic gross pathological signs consistent with mycobacteriosis, and (2) the same sequence results were obtained from 2 host species, collected from 2 islands over 2 years. We do not believe that *M. chelonae* is the primary cause of PGD because this bacterium is a fast

grower and is easy to culture, and we isolated just three colonies of this species from one fish.

As in the present report, the *M. triplex*-like bacterium from rockfish was identified by sequencing directly from tissues (Whipps *et al.,* 2003) when attempts at culture were unsuccessful. Our study reiterates that there are certain advantages in obtaining taxonomic relevant sequences directly from tissues, an approach that we have used with other outbreaks of mycobacteriosis (Whipps *et al.,* 2003; Kent *et al.,* 2004). The limitations of identifying organisms by direct sequencing from the tissue, without culture, are the inability to conduct phenotypic characterization, and to identify the organism as the etiological agent by fulfilling Koch's postulates.

It is unknown whether exotic poeciliids were introduced to Hawaii with the infection or if the bacterium is endemic in this isolated location. Nevertheless, PGD is a concern for Hawaii's native fish species, as mollies may serve as reservoirs for this unusual *M. triplex*-like bacterium. The presence of this pathogen in exotic poeciliids inhabiting Hawaiian streams warrants a careful search for possible infections in native gobioid stream fish, some of which are considered endangered species.

Acknowledgements. This study was supported in part by NIH grant 5R24RR017386-02. W. Font was funded by the Hawaii Division of Aquatic Resources and the U.S. Fish and Wildlife Service Sport Fish Restoration Program.

References

Chinabut, S. 1999. Mycobacteriosis and Nocardiosis. In: Woo, P.T.K and Bruno, D.W. (Eds.) *Fish Diseases and Disorders*, Vol 3: *Viral, Bacterial and Fungal Infections*. CAB International, New York, NY, pp 319 - 340.

Cingolani, C., M. Sanguinetti, A. Antinori, L. M. Larocca, F. Ardito, B. Posteraro, G. Federico, G. Fadda & L. Ortona. 2000. Disseminated mycobacteriosis caused by drug-resistant *Mycobacterium triplex* in a human immunodeficiency virus-infected patient during highly active antiretroviral therapy. Clinical Infectious Diseases **31,** 177–179.

Decostere, A., K. Hermans & F. Haesebrouck. 2004. Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. Veterinary Microbiology **99,** 159-166.

Devulder, G., M. Perouse de Montclos & J.P. Flandrois. 2005. A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. International Journal of Systematic and Evolutionary Microbiology **55,** 293-302.

Floyd, M.M., L.S. Guthertz, V.A. Silcox, P.S. Duffey, Y. Jang, E.P. Desmond, J.T. Crawford & W.R. Butler. 1996. Characterization of an SAV organism and proposal of *Mycobacterium triplex* sp. nov. Journal of Clinical Microbiology **34,** 2963-2967.

Font, W. F. & D. C. Tate. 1994. Helminth parasites of native Hawaiian freshwater fishes: An example of extreme ecological isolation. *Journal* of Parasitology **80,** 682–688.

Font, W.F. 1997. Distribution of helminth parasites of native and introduced stream fishes in Hawaii. Bishop Museum Occasional Papers, Records of the Hawai‘i Biological Survey for 1996, Part 2, Notes 49**:** 56–62.

Gozlan, R.E. S. St-Hilaire, S.W. Feist, P. Martin, & M.L. Kent. 2005. Disease threat to European fish. Nature **435,** 1046.

Hazra, R., S.H. Lee, J.N. Maslow & R.N. Husson. 2000. Related strains of *Mycobacterium avium* cause disease in children with AIDS and in children with lymphadenitis. Journal of Infectious Diseases **181,** 1298-1303.

Herbst, L.H., S.F. Costa, L.M. Weiss, L.K. Johnson, J. Bartell, R. Davis, M. Walsh & M. Levi. 2001. Granulomatous skin lesions in moray eels caused by a novel *Mycobacterium* species related to *Mycobacterium triplex*. Infection and Immunity **69,** 4639-46.

Hoffman, G.L. & G. Schubert. 1984. Some parasites of exotic fishes. Pages 233-261 in W.R. Courtenay Jr. and J.R. Stauffer Jr., eds. *Distribution, biology, and management of exotic fishes.* The Johns Hopkins University Press. Baltimore, MD.

Kent M.L., C.M. Whipps, J.L. Matthews, D. Florio, V. Watral, J.K. Bishop-Stewart, M. Poort & L.E. Bermudez. 2004. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. Comparative Biochemistry and Physiology - Part C: Toxicology, Pharmacology **138,** 383-390.

Levi, M.H., J. Bartell, L. Gandolfo, S.C. Smole, S.F. Costa, L.M. Weiss, L.K. Johnson, G. Osterhout & L.H. Herbst. 2003. Characterization of *Mycobacterium montefiorense* sp. nov., a novel pathogenic mycobacteria from moray eels that is related to *Mycobacterium triplex*. Journal of Clinical Microbiology **41,** 2147-52.

Niobe, S.N., C.M. Bebear, M. Clerc, J.L. Pellegrin, C. Bebear & J. Maugein. 2001. Disseminated *Mycobacterium lentiflavum* infection in a human immunodeficiency virus-infected patient. Journal of Clinical Microbiology **39,** 2030-2032.

Rhodes M.W., H. Kator, I. Kaattari, D. Gauthier, W. Vogelbein & C.A. Ottinger. 2004. Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay. Diseases of Aquatic Organisms **61,** 41-51.

Roth, A., M. Fischer, M.E. Hamid, S. Michalke, W. Ludwig & H. Mauch. 1998. Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. Journal of Clinical Microbiology **36**, 139-147.

Selvarangan, R., W.K. Wu, T.T. Nguyen, L.D. Carlson, C. K. Wallis, S.K. Stiglich, Y.C. Chen, K.C. Jost Jr, J.L. Prentice, R.J. Wallace Jr, S.L. Barrett, B.T. Cookson & M.B. Coyle. 2004. Characterization of a novel group of mycobacteria and proposal of *Mycobacterium sherrisii* sp. nov. Journal of Clinical Microbiology **42,** 52-59.

Springer, B., W.K. Wu, T. Bodmer, G. Haase, G.E. Pfyffer, R.M. Kroppenstedt, K.H. Schroder, S. Emler, J.O. Kilburn, P. Kirschner, A. Telenti, M.B. Coyle & E.C. Boltger. 1996. Isolation and characterization of a unique group of slowly growing mycobacteria; description of *Mycobacterium lentiflavum* sp. nov. Journal of Clinical Microbiology **34**, 110-7.

Suomalainen, S., P. Koukila-Kahkola, E. Brander, M.L. Katila, A. Piilonen, L. Paulin & K. Mattson. 2001. Pulmonary infection caused by an unusual, slowly growing nontuberculous *Mycobacterium*. Journal of Clinical Microbiology **39,** 2668-2671.

Talaat, A.M., R. Reimschuessel, & M. Trucksis. 1997. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. Veterinary Microbiology **58,** 229-37.

Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Böttger & T. Bodmer. 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. Journal of Clinical Microbiology. **31,**175–178.

Tortoli E., A. Bartoloni, M.L. Erba, E. Levre, N. Lombardi, A. Mantella & L. Mecocci. 2002. Human infections due to *Mycobacterium lentiflavum*. Journal of Clinical Microbiology **40**, 728-9.

Tortoli E., L. Rindi, K.S. Goh, M.L. Katila, A. Mariottini, R. Mattei, G. Mazzarelli, S Suomalainen, P. Torkko, & N. Rastogi. 2005. *Mycobacterium florentinum* sp. nov., isolated from humans. International Journal of Systematic and Evolutionary Microbiology **55,** 1101-1106.

Turenne, C.Y., L. Tschetter, J. Wolfe & A. Kabani. 2001. Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. Journal of Clinical Microbiology **39,** 3637-48

Ucko, M., A. Colomi, H. Kvitt, A. Diamant, A. Zlotkin & W.R. Knibb. 2002. Strain variation in *Mycobacterium marinum* fish isolates. Applied Environmental Microbiology **68,** 5281-7.

Vincent, A.G. & W. F. Font. 2003. Host specificity and population structure of two exotic helminthes, *Camallanus cotti* (nematoda) and *Bothriocephalus acheilognathi* (Cestoda), parasitizing exotic fishes in Waianu Stream, O'ahu Hawai'i. Journal of Parasitology **89,** 540–544.

Whipps C.M., V.G. Watral & M.L. Kent. 2003. Characterization of a *Mycobacterium* sp. in rockfish, *Sebastes alutus* (Gilbert) and *Sebastes reedi* (Westrheim & Tsuyuki), using rDNA sequences. Journal of Fish Diseases **26,** 241-5.

Appendix II

Environmental amoeba and mycobacterial pathogenesis

Melanie J Harriff[1,2] and Luiz E Bermudez[1,2]

Methods in Molecular Biology: *Accepted*

[1] Molecular and Cellular Biology Program, Center for Gene Research and Biotechnology, Agricultural and Life Sciences Building, Oregon State University, Corvallis, Oregon 97331
[2] Department of Biomedical Sciences, College of Veterinary Medicine, 105 Magruder, Oregon State University, Corvallis, OR 97331

Summary. In Chapter 4, I looked at the effect of passing *Mycobacterium marinum* and *Mycobacterium peregrinum* through environmental amoeba before infecting zebrafish. Because of this study, and other investigations with amoeba in the laboratory, I worked with Dr. Bermudez to write a chapter for this publication on methods we have used with mycobacteria and amoeba.

Abstract

Environmental amoebae have been shown to be suitable hosts to pathogenic mycobacteria. *Mycobacterium avium*, *Mycobacterium marinum* and *Mycobacterium peregrinum* can grow inside *Acanthamoeba* and other environmental amoebae. *M. avium* upregulates a number of genes once ingested by *Acanthamoeba*, many of them similar to the ones upregulated upon phagocytosis by macrophages. Mycobacteria ingested by amoebae grow intracellularly, as well as acquire an invasive phenotype, evident when the bacterium escapes the infected amoeba. Once inside of amoebae, it has been shown that mycobacteria are protected from antibiotics and disinfectants, such as chlorine.

Introduction

Mycobacterium avium is an environmental bacterium that infects immunosuppressed and immunocompetent individuals (Wolinsky, 1979; Inderlied *et al.,* 1993). In humans, *M. avium* can be associated with disseminated disease, as well as lung infection (Aksamit, 2002). The majority of *M. avium* infections are acquired through the gastrointestinal tract. Once ingested, *M. avium* can survive the harsh environment of the stomach (Bodmer *et al.,* 2000). The bacterium has been shown to invade the intestinal mucosa prior to dissemination. Bacterial genes associated with the ability to cross the intestinal mucosa have been identified (Miltner *et al.,* 2005; Dam *et al.,* 2006), and *M. avium* has been shown to survive for at least six months in mesenteric lymph node macrophages (Petrofsky and Bermudez, 2005). The bacterium also invades the bronchial mucosa, a process that seems to require the ability to form a biofilm prior to translocation (Yamazaki *et al.,* 2006a; Yamazaki *et al.,* 2006b). Bacterial genes involved in biofilm

formation have been described, and strategies to prevent the biofilm phase of infection are in the experimental phase of development (Carter *et al.,* 2004; Yamazaki *et al.,* 2006b).

As an environmental organism, *M. avium* can potentially infect protozoa, such as amoebae. Studies carried out in both mice and zebrafish have demonstrated that passage of *M. avium*, *Mycobacterium marinum* and *Mycobacterium peregrinum*, all environmental mycobacteria, in *Acanthamoeba castellanii* results in an increase of mycobacterial virulence to the host (Cirillo *et al.,* 1997; Harriff *et al.,* 2007), as well as association with enhanced resistance to antibiotics (Miltner and Bermudez, 2000). Recently, we have shown that *Acanthamoeba* infection by *M. avium* is associated with the regulation of a number of known virulence genes, as well as unknown ones (Tenant and Bermudez, 2006). The majority of the genes regulated inside amoebae are also genes required for survival in macrophages (Hou *et al.,* 2002; Danelishvili *et al.,* 2004; Tenant and Bermudez, 2006).

Materials

1. *Escherichia coli* strain DH5
2. *M. avium* and *M. smegmatis*
3. GFP plasmid, pEMC1 (Dam *et al.,* 2006)
4. Middlebrook 7H9 broth and 7H10 agar, and LB broth and agar
5. phosphate-buffered saline
6. Hank's balanced salt solution
7. 24-well and 96-well tissue culture plates

8. Triton-X 100
9. *M. avium* promoter library in pEMC1
10. cytofluorimeter
11. kanamycin
12. PCR, Real-time PCR equipment, and reagents
13. RPMI-1640, 712PYG media
14. THP-1 cells or other human monocyte-derived macrophage
15. *Acanthamoeba castellanii*
16. mice, zebrafish
17. tissue culture flasks

Methods

The methods outlined below describe (a) the infection of acanthamoebae by mycobacteria, (b) the construction and screening of a promoter GFP library and (c) the use of animal models to determine the effect on virulence of passage through amoebae.

3.1. Bacterial Culture

The culture of *M. smegmatis*, *M. avium*, *Acanthamoeba*, and infection of *Acanthamoeba* by mycobacteria are described in subheadings 3.1.1 – 3.1.3.

3.1.1. M. avium and M. smegmatis Culture

Using Middlebrook 7H9 broth or 7H10 agar, either seed the broth with *M. avium* or *M. smegmatis*, or strike the agar plate with the bacterium. On average, *M. avium* will grow in approximately 10 days and *M. smegmatis* in 4.5 days, both in agar as well as in broth. The *M. avium* colonies on the plate should have a smooth dome transparent

morphotype, or smooth opaque, with a small percent of the strains having a rough morphotype. The *M. smegmatis* phenotype is rough. After growth, obtain pure colonies and wash them in phosphate-buffered saline at 3,500 rpm/min × 10 min. The bacterial pellet obtained should be resuspended in PBS or Hank's balanced salt solution (HBSS). The number of bacteria in the suspension should be adjusted, using the McFarland standard, to 1×10^7 bacteria/ml.

3.1.2. Acanthamoeba Culture

Acanthamoeba castellanii can be cultured in 712 PYG medium available from the ATCC: www.atcc.org (*see* **Note 1**). It should be kept in the dark at room temperature. *Acanthamoeba* in culture will adhere to the bottom of the plastic flask. Amoebae should be observed daily, and medium changed every five days. If left for too long without changing media, amoebae will encyst and additional time will be required for optimal infectious conditions. To detach *Acanthamoeba*, tap the flask a couple of times. The suspended amoebae can be counted and used to establish a monolayer.

3.1.3 Acanthamoeba Infection by Mycobacteria

1. After detachment from the plastic, count the number of amoebae in suspension using a chamber of Neubauer.
2. Use 10^5 amoebae to seed a 6-well or 24-well tissue culture plate/well. Allow to sit over night for adherence.
3. Infect amoebae with mycobacteria at a multiplicity of infection (MOI) of 0.1 to 1, 1 to 1, 10 to 1.
4. After 1 or 2 hours, wash the extracellular bacteria. Amoebae will not detach easily from plastic.

5. Place the amoebae monolayers at room temperature or 37 °C for the desired time (*see* **Note 2**).
6. Lyse amoebae with 0.1% Triton X-100 for up to 30 minutes.
7. Follow the lysis of amoebae in the microscope, in order to ensure complete lysis of individual amoeba. It usually takes 15-20 minutes to see loss of membrane integrity. Pass through a 23-gauge needle to ensure complete lysis (*see* **Note 3**).
8. The lysate can now be serially diluted in sterile H_2O and plated onto solid medium (Middlebrook 7H10 agar) for quantification of the viable intracellular bacteria.
9. Depending on the bacteria, the outcome of the infection will vary. *Mycobacterium smegmatis* will be killed while *M. avium* will grow.

3.2. Mycobacterial gene regulation within amoebae

To survive within *Acanthamoeba*, *M. avium* needs to upregulate virulence genes. Starting with the premise that *M. avium* genes required to replicate and survive within environmental amoebae should be partially common to the genes important for the survival within macrophages, we screened a GFP-promoter library of *M. avium* in *Mycobacterium smegmatis* upon uptake by *Acanthamoeba castellanii* (s*ee* **Note 4**). The construction and screening of this library is described in detail in subheadings 3.2.1 – 3.2.3.

3.2.1. Construction of an M. avium GFP-promoter library in M. smegmatis

1. *M. avium* genomic DNA was extracted from 10^8-10^9 bacteria, using standard molecular biology methods with some modifications for *M. avium* (*see* **Note 5**), and then digested with 2 μl of Sau3A1 overnight at 37 °C

2. Separate fragments ranging from 300-1000 bp using agarase gel electrophoresis.
3. Purify the DNA from the segment of the gel containing the fragments from 300 to 1000 bp using a commercial kit for DNA gel extraction.
4. Digest the plasmid pEMC1 with BamH1 and insert the DNA obtained from the gel, using standard molecular biology methods. The pEMC1 plasmid contains a kanamycin-resistance gene, and the promoterless GFP gene, downstream of a cloning site. The plasmid also contains a stop site upstream of the cloning site (Figure 1).
5. After DNA fragments have been cloned in pEMC1, transform a competent strain of *Escherichia coli* and allow replication overnight. Select on LB agar supplemented with 50 μg/ml of kanamycin.
6. Purify plasmid and screen the transformants using standard plasmid extraction and digestion methods. After the ligation efficiency has been determined, transform competent *M. smegmatis* using electroporation. (See **Note** 6.)
7. Plate the *M. smegmatis* transformants onto 7H10 supplemented with 50 μg/ml kanamycin, such that > 10,000 clones are obtained.
8. Place 5 – 10 clones per well in a 96-well tissue culture plate.

3.2.2. Screening of an M. avium GFP-promoter Library for Genes Expressed in Amoeba Using M. smegmatis Clone Pools

1. Grow *Acanthamoeba* in 712 PYG medium at 24 °C in the dark.
2. Detach amoebae from the plastic by tapping the container vigorously.
3. Count amoebae/ml, either using a chamber of Neubauer or hemocytometer. Seed 10^5 amoeba in each well of the 96-well plate. Let them adhere overnight.

4. Infect each well with the pools of 5 – 10 *M. smegmatis* from clones of the *M. avium* GFP-promoter library**.** You need to have approximately 10^6 – 10^7 bacteria per monolayer. After 30 min or 1 h, wash the monolayer twice with 0.2 ml of HBSS. Add fresh medium and read the baseline emission in GFP band of a cytofluorimeter.
5. Place the plate in the dark for 15 min and then read again for GFP expression. Repeat the reading at 30 min, 1 h, 2 h, 24 h, 48 h, or as long as it is desired.
6. Clone pools that have an increase in GFP expression at least 2 times above baseline, at desired time points, should be plated onto 7H10 agar containing 50 μg/ml of kanamycin to separate the clones in the pool. Select 20 individual colonies from each well and seed them in a 96-well tissue culture plate. Repeat the infection of amoeba using isolated colonies. Those clones that are associated with 2.5-fold increase or more, compared to the baseline, should be tested again to confirm the increase in GFP expression.
7. Sequence the isolated clones using primers specific for the plasmid regions upstream of the mycobacterial promoter and downstream of the GFP sequence (Danelishvili *et al.,* 2004).
8. Confirm the expression of the genes in *M. avium* (or another environmental bacteria) by using real-time PCR.

3.2.3. Real-time PCR verification of promoter activation in M. avium

1. Grow *M. avium* in 7H9 broth.
2. Seed amoeba in a flask as described previously and infect with *M. avium* at an MOI of 100. Retain some bacterial culture in 7H9 as a control.

3. At the time point desired, *Acanthamoeba* should be lysed with Triton-X100 as described previously.
4. Centrifuge the lysate at 400 × *g* for 5 min to remove lysed amoebae from suspension containing bacteria.
5. Supernatant should then be centrifuged at 3,000 × *g* to precipitate bacteria.
6. Extract RNA by standard molecular biology methods for *M. avium* and confirm the RNA integrity and quantity (Danelishvili *et al.,* 2004; Yamazaki *et al.,* 2006).
7. Total RNA should be reversed transcribed with a commercial kit, according to manufacturer's instructions.
8. Amplify selected genes from the cDNA using a real-time PCR cycler and calculate the fold-change in the gene expression between the control bacteria and bacteria from amoebae. (See **Note** 7.)

3.3. Passage of Mycobacterium in Amoebae before Infecting Macrophages, Mice, or Zebrafish

Environmental amoebae inhabit the same environment as *M. avium* and other environmental mycobacteria. Therefore, it is plausible that infection of amoebae is part of the *M. avium* life cycle in the environment. Humans and animals can come into contact with contaminated water and ingest *M. avium* that has infected amoebae. The ingested bacteria can be free (after amoeba infection) or still be within the amoebae. The evaluation of the infectivity and virulence of strains following the encounter with environmental amoebae is crucial for the understanding of the bacterial virulence. Previous work has demonstrated that *M. avium* grows faster in human macrophages

following passage in amoebae. In addition, feeding both *M. avium* that has been released from amoebae (and still maintains the same intracellular phenotype) and *M. avium*-infected amoebae to mice led to an increase in virulence, compared with bacteria grown in 7H10 agar, as determined by the bacteria growth in spleen and liver (Cirillo *et al.,* 1997). The methods for infecting macrophages, mice, and fish with *M. avium* that has been passed through amoebae are described in subheadings 3.3.1 – 3.3.4.

3.3.1. Preparation of M. avium Inoculum with Passage through Amoebae

1. Culture *A. castellanii* in 712 PYG medium at room temperature, in the dark, as described previously.
2. Amoebae seeded in large tissue culture flasks can now be infected with *M. avium* or other mycobacteria, using an infection ratio of 20 bacteria per amoeba for 1 h. One can use 3 to 5 days of infection.
3. Wash the extracellular bacteria three times using HBSS. Remove the last wash and add 712 PYG to the monolayer. Allow the bacteria to grow in amoebae for 3 to 4 days.
4. Remove the media and then lyse the amoebae with 0.1 % Triton-X in HBSS as described previously. After observation under the microscope to ensure the lysis of the amoebae, place the lysate in a 15 ml plastic tubes and centrifuge for 5 minutes at 500 × *g*, to allow for the sedimentation of cell debris and the nucleus. All the centrifugation should be carried out at 4 °C to maintain the bacterial phenotype. *M. avium* can be found in the supernatant. As the second stage of the centrifugation, the resulting supernatant of the first centrifugation should be centrifuged at 3,500 × *g* for 20 min, to deposit the bacteria. The pelleted bacteria

should be resuspended in HBSS prior to the infection of mammalian or other host cells.

5. From another flask, scrape the amoebae from the bottom, centrifuge the whole infected amoebae, and re-suspend in HBSS so that the bacteria remain in the amoebae. Before infecting, remove a portion of the infected amoebae to lyse and count the intracellular bacterial inoculum.
6. Infection of mammalian or other host cells should be done at 2 different concentrations (dilutions) of the re-suspended pellet, since the adjustment of CFU and turbidity will lack precision. Infection should also be done as soon as possible after lysis or collection of the amoebae to maintain the phenotype.

3.3.2. Infection of Macrophages

To determine if infection of amoebae modifies the interaction of mycobacteria with the second (following) host, bacteria obtained from the first host should not be plated or seeded in culture medium.

1. Bacteria should be used directly from the amoebae lysate or whole amoebae, without passage in medium, in order to maintain the phenotype.
2. Use two different dilutions.
3. Macrophages (primary human, mouse, or THP-1 cells) should be seeded at 5×10^5 cells in a 24-well tissue culture plate. When using THP-1 cells, phorbol-myristate acetate should be used prior to infection to induce maturation of the monocytes. Add from 1.0 to 20 μg/ml per well for 4 to 24 h. Once the cells are mature (they adhere to the bottom of the well), they can be infected.

4. Use bacteria grown *in vitro* (medium) as control. The MOI of the bacteria grown in medium and the bacteria from amoebae should be as close as possible.
5. Macrophage monolayers should be infected for 1 or 2 h, and the extracellular bacteria removed by washing. Three consecutive washes with HBSS should be capable of removing 99 % of the extracellular bacteria. After infection, some monolayer wells should be lysed with sterile water (0.5 ml) and the lysate diluted (10^{-3}, 10^{-4}, 10^{-5}) and the three dilutions plated onto 7H10 agar plates.
6. The other macrophage monolayer wells should be followed for 3, 4, or 5 days, lysed as described above, and the lysate should be plated onto 7H10 agar to determine the number of intracellular bacteria.
7. The analysis of the data should be done comparing the growth of mycobacteria obtained from amoebae to mycobacteria grown *in vitro*. For each experimental group, growth at days 3, 4, or 5 should be compared with the number of bacteria inside macrophages at 1 h or 2 h (after infection).

3.3.3. Infection of Mice

1. Bacteria that will be used to orally challenge mice should be prepared from agar plates (control), amoebae, and amoebae lysate. The reason for culture in the plate is the confirmation of purity prior to challenging.
2. Bacteria from plates should be washed twice in HBSS, before the last pellet can be resuspended and the concentration adjusted using McFarland standards. Use 0.1 ml volume to infect mice by gavage.
3. To prepare bacteria obtained from amoeba will require seeding *Acanthamoeba* in culture and infecting with *M. avium* at MOI of 100. Amoebae will internalize

approximately 25 % to 30 % of the *M. avium* inoculum in 1 h, 50 % in 2 h. The percent of uptake can vary for other mycobacterial species. Once uptake has taken place, the amoebae monolayer should be washed with HBSS a couple of times to remove extracellular bacteria.

4. Infected amoebae should be kept for 3 to 4 days in culture at 24 °C or 30 °C. Either temperature would be adequate when working with *M. avium* or the majority of environmental mycobacteria.

5. Bacterial preparation for mice infection:

    A. Bacteria grown in 7H10 agar or 7H9 broth

    B. Bacteria grown in *Acanthamoeba*, but released prior to infection:

    To prepare, lyse amoeba with 0.1 % Triton-X100 for 15 min. Spin down amoebae lysate and bacteria of 10,000 rpm for 30 min at 4 °C. Re-suspend the pellet in HBSS and spin it down at 500 rpm for 5 min to separate bacteria from the amoebae nucleus, plus other organelles. Retrieve the supernatant and centrifuge it at 3,500 rpm for 20 min (4 °C) to obtain bacteria. Use the bacteria to prepare inoculum to infect mice. Plate an aliquot to determine the number of organisms in the inoculum.

    C. Bacteria intracellular in amoebae.

    Obtain an aliquot of infected amoebae for infection. Lyse a portion of the amoebae as described above and plate the lysate onto 7H10 agar to determine the number of intracellular organisms.

6. Infect mice orally, by gavage using a syringe adapted to a plastic tube, and anesthetized with halothane if necessary, depending on the skill level of the

person performing the procedure. No more than 0.5 ml of inoculum should be administered.

7. Harvest mice at different time points, according to the experimental protocol.
8. After removal, spleen tissue should be homogenized, using a hand-homogenizer.
9. The whole animal can be frozen (-20 °C to -80 °C) for plating at a later time. Freezing should not have any effect on bacterial counts for a week at -20 °C. It is preferable, with dissected mice, to freeze tissue in broth after homogenization.
10. Serially dilute the suspension and plate onto 7H10 plates.

3.4. Infection of Zebrafish

Preparation of bacterial inoculum for the infection of zebrafish is performed as described for mice. Fish should be infected by oral intubation. As an alternative, place zebrafish in a small beaker with water containing bacteria or infected amoebae. The inoculum recommended is approximately 10^7 bacteria/ml in 100-200 ml, when infecting by immersion. When infecting by intubation, use doses ranging from 10^5 to 10^8 organisms to provide the desirable results. Depending on the size of the fish, 25-50 µl can be used for intubation. Anesthetize the fish lightly with MS-223 prior to intubation. Harvest organs of the zebrafish aseptically, homogenize and serially dilute as with mice (*see* **Note 8**).

Notes

1. Amoebae can also be cultured in high-salt medium, which, although it keeps the amoebae viable, does not allow the protozoan to replicate (Cirillo *et al.,* 1997).

2. At room temperature, the bactericidal ability of amoebae will be maintained, but at 37 °C, the amoeba's ability to control bacterial growth will be impaired.
3. Triton X-100 has no harmful effect on *M. avium*, *M. marinum*, or *M. peregrinum.* There are other detergents that can be used for this purpose (e.g., SDS). If using another bacterial strain, the effect of the detergent on the bacteria should be tested first.
4. The *M. avium* genomic library in *M. smegmatis* offers a number of advantages over a GFP-*M. avium* library in *M. avium*. *M. smegmatis* transformation is significantly more efficient than transformation of *M. avium*, which makes the effort of creating 10,000 clones much easier.
5. Bacterial pellet should be re-suspended and incubated overnight at 37 °C in GTE containing 20 μg/ml lysozyme. Add 20 μg/ml proteinase K and 0.1% SDS and incubate for an additional 3 hours at 37 °C. After addition of CTAB, and 5 M NaCl/CTAB, the lysate can now be used for DNA extraction using multiple rounds of phenol:chloroform:isoamyl alcohol (25:24:1) extraction.
6. Competent mycobacteria are prepared by successive washes (at least 4) with 10% glycerol, 0.1% Tween-80. It is important to keep the bacterial cells on ice between spins and to centrifuge at 4 °C. After the successive washes, the bacterial pellet should be re-suspended at a very high concentration in the 10% glycerol, 0.1% Tween-80 solution. The cells should be transformed shortly after washing. If stored at -20 °C, the cells can be subjected to an additional wash and used within a week, and used again for transformation.

7. Several technologies can be used to perform Real-time PCR to analyze the expression of RNA transcripts. In our laboratory, we use the SYBR green technology. The run protocol for the cycler is published (Danelishvili *et al.,* 2004).
8. Because of the small size of the zebrafish, asceptic removal of individual organs is difficult. Tissues, therefore, should be homogenized in cetylpyridium to kill bacteria comprising the normal gut flora without killing the mycobacteria. Additionally, the homogenates should be plated onto 7H10 plates that contain PACT (polymixinB, amphotericinB, carbenicillin, trimetropin). This will further reduce bacterial contamination from the lumen of the intestine.

References

Aksamit, T. R. 2002. *Mycobacterium avium* complex pulmonary disease in patients with pre-existing lung disease. Clin Chest Med **23,** 643-53.

Bodmer, T., Miltner, E., and Bermudez, L. E. 2000. *Mycobacterium avium* resists exposure to the acidic conditions of the stomach. FEMS Microbiol Lett **182,** 45-9.

Carter, G., Young, L. S., and Bermudez, L. E. 2004. A subinhibitory concentration of clarithromycin inhibits *Mycobacterium avium* biofilm formation. Antimicrob. Agents Chemother **48,** 4907-10.

Cirillo, J. D., Falkow, S., Tompkins, L. S., and Bermudez, L. E. 1997. Interaction of Mycobacterium avium with environmental amoebae enhances virulence. Infect Immun **65,** 3759-67.

Dam, T., Danelishvili, L., Wu, M., and Bermudez, L. E. 2006. The fadD2 gene is required for efficient Mycobacterium avium invasion of mucosal epithelial cells. J Infect Dis **193,** 1135-42.

Danelishvili, L., Poort, M. J., and Bermudez, L. E. 2004. Identification of *Mycobacterium avium* genes up-regulated in cultured macrophages and in mice. FEMS Microbiol Lett **239,** 41-9.

Harriff, M., Bermudez, L., and Kent, M. L. 2007. Experimental exposure of zebrafish (*Danio rerio* Hamilton) to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: A potential model for environmental mycobacterial infection. J Fish Dis **29:** 1-13.

Hou, J. Y., Graham, J. E., and Clark-Curtiss, J. E. 2002. *Mycobacterium avium* genes expressed during growth in human macrophages detected by selective capture of transcribed sequences (SCOTS). Infect Immun **70,** 3714-26.

Inderlied, C. B., Kemper, C. A., and Bermudez, L. E. 1993. The Mycobacterium avium complex.Clin Microbiol Rev **6,** 266-310.

Miltner, E. C., and Bermudez, L. E. 2000. Mycobacterium avium grown in Acanthamoeba castellanii is protected from the effects of antimicrobials. Antimicrob Agents Chemother **44,** 1990-4.

Miltner, E., Daroogheh, K., Mehta, P. K., Cirillo, S. L., Cirillo, J. D., and Bermudez, L. E. 2005. Identification of Mycobacterium avium genes that affect invasion of the intestinal epithelium. Infect Immun **73,** 4214-21.

Petrofsky, M., and Bermudez, L. E. 2005. CD4+ T cells but Not CD8+ or gammadelta+ lymphocytes are required for host protection against Mycobacterium avium infection and dissemination through the intestinal route. Infect Immun **73,** 2621-7.

Tenant, R., and Bermudez, L. E. 2006. *Mycobacterium avium* genes upregulated upon infection of *Acanthamoeba castellanii* demonstrate a common response to the intracellular environment.Curr Microbiol **52,** 128-33.

Wolinsky, E. 1979. Nontuberculous mycobacteria and associated diseases. Am Rev Respir Dis **119,** 107-59.

Yamazaki, Y., Danelishvili, L., Wu, M., Hidaka, E., Katsuyama, T., Stang, B., Petrofsky, M., Bildfell, R., and Bermudez, L. E. 2006a. The ability to form biofilm influences Mycobacterium avium invasion and translocation of bronchial epithelial cells. Cell Microbiol **8,** 806-14.

Yamazaki, Y., Danelishvili, L., Wu, M., Macnab, M., and Bermudez, L. E. 2006b. Mycobacterium avium genes associated with the ability to form a biofilm. Appl Environ Microbiol **72,** 819-25.

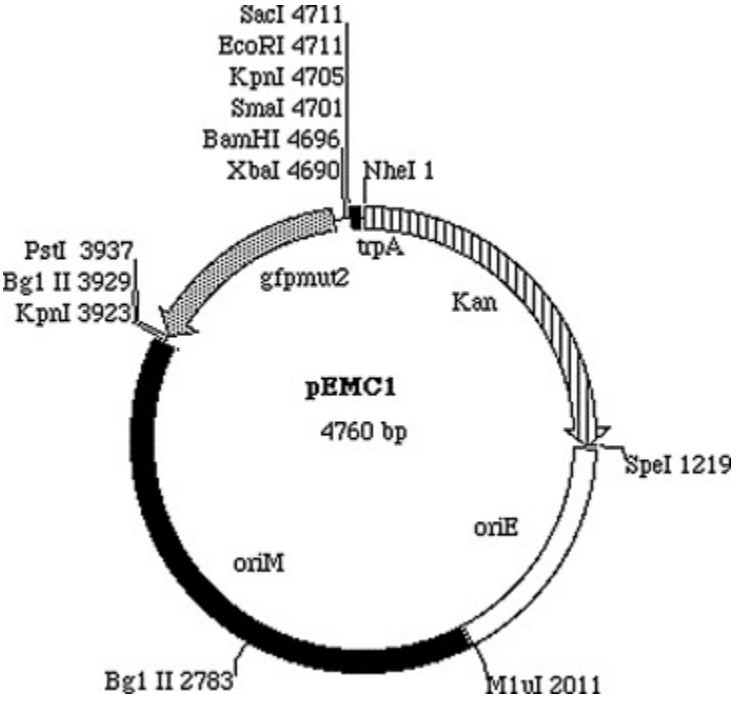

Figure 1. Plasmid map of pEMC1. This plasmid has been modified to contain a random library of 300 to 1000 bp *Sau*3AI DNA fragments of *M. avium* 104 upstream of the inserted GFP for transcriptional fusion of promoterless GFP gene. Abbreviations: *oriE*, *E. coli* origin of replication; *oriM*, mycobacterial origin of replication; *GFPmut2*, gene encoding Green Fluorescent Protein; *trpA*, transcriptional terminator.

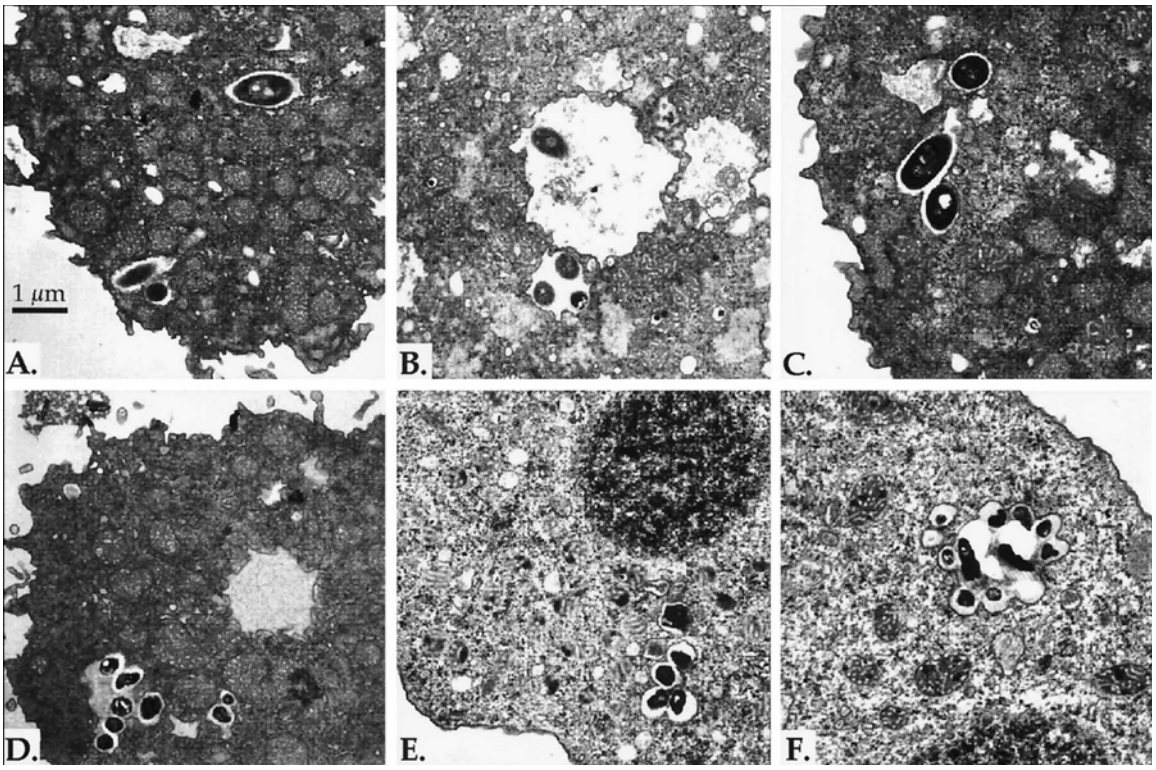

Figure 2. Transmission electron micrographs of an acanthamoeba infected with *M. avium.* TEMs show *M. avium* within *A. castellanii* at 5 min (A), 15 min (B), 1 h (C), 2 days (D), 3 days (E), and 5 days (F) following infection. The 1-mm bar shown in panel A applies to all panels.

Appendix III

Abstracts for additional publications

Mycobacteriosis in zebrafish (*Danio rerio*) research facilities

Kent, ML, Whipps, CM, Matthews JL, Florio D, Watral V, Bishop-Stewart JK, **Poort M*,** and Bermudez L. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. 2004. *Comp Biochem Physiol C Toxicol Pharmacol*. **138**: 383-90.

The Zebrafish International Resource Center was established to support the zebrafish research community, and includes a diagnostic service. One of the most common diseases that we have diagnosed is mycobacteriosis, which represented 18% of the diagnostic cases submitted from November 1999 to June 2003. We describe here the severity of the disease and associated pathological changes of 24 diagnostic cases from 14 laboratories. Identifications of the bacteria are provided for seven of these cases. For two cases in which culture of the organism was not successful, these identifications were based on ribosomal DNA (rDNA) sequence analysis obtained directly from infected tissues. Biochemical characteristics and rDNA sequence analysis from cultures are reported for the other isolates. Two severe outbreaks from different facilities on different continents were associated with an organism identified as *Mycobacterium haemophilum* based on rDNA sequence from tissues. Another severe outbreak was associated with an organism most closely related to *Mycobacterium peregrinum*. These species are recognized pathogens of humans, but this is the first report of them from fish. Bacteria identified as *Mycobacterium chelonae* or *Mycobacterium abscessus* were recovered from fish in cases categorized as moderate disease or as an incidental finding. These findings indicate that species of *Mycobacterium* previously undescribed from fish (i.e., *M. haemophilum* and

M. peregrinum) may pose significant health problems in zebrafish research facilities, whereas species and strains that are already recognized as common in fish usually cause limited disease on a population basis in zebrafish.

Identification of *Mycobacterium avium* genes up-regulated in cultured macrophages and in mice

Danelishvili L, **Poort MJ*,** Bermudez LE. 2004. Identification of *Mycobacterium avium* genes up-regulated in cultured macrophages and in mice. *FEMS Microbiol Lett*. **239**: 41-9.

To investigate *Mycobacterium avium* gene expression upon infection of macrophages, we created a *M. avium*-promoter library upstream of a promoter-less gene encoding the green fluorescent protein (GFP) in *Mycobacterium smegmatis*. Clones were evaluated for increased expression of GFP after infection of U937 macrophages. A number of *M. avium* genes were up-regulated more than 3-fold after 24 and 48 h following macrophage infection. *M. avium* genes expressed by *M. smegmatis* during growth in macrophages include genes encoding transport/binding proteins, synthesis, modification and degradation of macromolecules, and a great majority of genes for which no function is currently known. For some of the unknown genes, homologues were identified in bacteria such as *Mycobacterium leprae, Salmonella typhimurium* and *Agrobacterium tumefaciens*. In order to investigate if these genes were also expressed in *M. avium* during macrophage infection in vitro and in vivo, transcripts of selected genes were quantified using real time RT-PCR. Evaluation of most expressed genes in *M. smegmatis* confirmed their up-regulation in *M. avium* after 24 h infection of macrophages in vitro and mice.

* M. Poort's publications following these are authored under the name M.J. Harriff.

Identification of a *Mycobacterium avium* pathogenicity island important for macrophage and amoeba infection

Danelishvili L, Wu M, Stang B, **Harriff M**, Cirillo S, Cirillo J, Bildfell R, Arbogast B, and LE Bermudez. 2007. Identification of *Mycobacterium avium* pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci USA. *In press.*

The ability to infect macrophages is a common characteristic shared among many mycobacterial species. *Mycobacterium avium*, *Mycobacterium tuberculosis*, and *Mycobacterium kansasii* enter macrophages using the complement receptors CR1, CR3, CR4, and the mannose receptor. To identify *M. avium* genes and host cell pathways involved in the bacterial uptake by macrophages, we screened a *M. avium* transposon mutant library for decreased ability to enter macrophages. Uptake-impaired clones were selected. Sequence of six *M. avium* clones identified one gene involved in glycopeptidolipid biosynthesis, one gene encoding the conserved membrane protein homologue to *Mycobacterium avium* subsp. *paratuberculosis* MAP2446c gene and four others belonging to the same region of the chromosome. Analysis of the chromosome region revealed a pathogenicity island inserted between two tRNA sequences with 58 % G+C content versus 69 % in the *M. avium* genome. The region is unique for *M. avium* and is not present in *M. tuberculosis* or *M. avium* subsp. *paratuberculosis*. Although the mutants did not differ from the wild-type bacterium regarding the binding to macrophage cell membrane, analysis of macrophage proteins after 1 hr infection revealed a deficiency in the mutant to phosphorylate certain proteins upon uptake. To understand *M. avium* interaction with two evolutionarily distinct hosts, the mutants were evaluated for *Acanthamoeba castellanii* invasion. The defect in the ability of the mutants to invade

both cells was highly similar, suggesting that *M. avium* might have evolved mechanisms which are used to enter amoebas and human macrophages.