AN ABSTRACT OF THE DISSERTATION OF

Julie V. Early for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on March 6, 2009.

Title: Molecular and Cellular Characterization of the Intracellular Phenotype of *Mycobacterium avium*.

Abstract approved:

Luiz E. Bermudez

Mycobacterium avium cause disseminated disease in immunocompromised people such as AIDS patients. Subsequent to crossing the intestinal epithelium, *M. avium* thrive within vacuoles in macrophages. The bacteria exhibit a different, more invasive, phenotype after being in macrophages compared to *M. avium* from laboratory conditions. We hypothesized that this intracellular phenotype contributes to disease in a variety of ways, such as influencing apoptosis of the macrophage. We further studied this intracellular phenotype, analyzing the molecular and cellular details. Using microscopy, we found that a portion of *M. avium* survive after the macrophage becomes apoptotic. From apoptotic bodies, some bacteria were observed escaping the vacuole and macrophage to the extra-cellular space and others were seen invading the macrophage ingesting the apoptotic body. We also found that macrophages infected

by *M. avium* undergo autophagy, and that bacteria from autophagic macrophages were viable. After developing an *in vitro* system that elicits the intracellular phenotype, we determined that macrophages infected by bacteria exhibiting the intracellular phenotype undergo early-onset cell-death frequently compared to macrophages infected by bacteria exposed to laboratory conditions. With the use of real time PCR and microarray analysis, several genes, including genes of the twin-arginine translocase system, were shown to be upregulated by bacteria with the intracellular phenotype, while others were downregulated. The twin-arginine translocase system of *M. avium* was further characterized, and a *tatb* antisense strain was found to enter and survive in macrophages more efficiently than the wildtype strain, but was more sensitive to β-lactam antibiotics. Finally, we analyzed four transposon mutants of *M. avium* that were impaired in their ability to be taken up by macrophages to determine if the vacuole in which they reside, an aspect partially controlled by the bacteria, and therefore a part of the intracellular behavior of *M. avium*, differs from the vacuole containing wildtype bacteria. Some of these mutants were found in vacuoles with marked difference compared to the wildtype-containing vacuoles, others were similar to the wildtype. This work has increased our understanding of the intracellular phenotype of *M. avium* and how it may contribute to aspects of disease such as cell-to-cell spread.

©Copyright by Julie V. Early

March 6, 2009

All Rights Reserved

Molecular and Cellular Characterization of the Intracellular Phenotype of *Mycobacterium avium*

by

Julie V. Early

A DISSERTATION

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented March 6, 2009

Commencement June 2009

Doctor of Philosophy dissertation of Julie V. Early presented on March 6, 2009.

APPROVED:

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.

Julie V. Early, Author

ACKNOWLEDGEMENTS

I thank Luiz Bermudez for giving me the opportunity to work in his lab. He has provided valuable insight into the life of a research scientist, fostered my professional growth as a biologist, and allowed me the freedom to make mistakes and learn from them. I also thank my committee members: Daniel Rockey, Nancy Kerkvliet, Ling Jin, and Howard Gelberg for their time and commitment, as well as Karen Guillemin for her original service on my committee.

Without the help of many past and present members of the Bermudez lab, I would not be in a position to finish. I thank all of them for providing support and using their skills to help the progress of my degree. A special thanks goes to Melanie Harriff, who was always helpful, both in and out of the lab. Other noteworthy thanks go to Lia Danelishvilli, Brandon Yuan, Allison Books, Heather Duchow, Martin Wu, Marta Hearn, Sandra Sommer, Michael McNamara, Kerrigan Gilbert, Sasha Rose and Rachel Tennant.

The talented employees in the administrative offices of the molecular and cell biology program and biomedical sciences department have been incredibly accommodating. Denny Weber, especially, provided invaluable manuscript preparation and took care of miscellaneous details throughout the entire process.

Finally, I thank my family and friends who have been encouraging and supportive during these years. I am especially grateful for my husband Jeffrey who is always there for me, and my unborn child who provided motivation to suddenly finish.

CONTRIBUTION OF AUTHORS

Chapter 2: Kay Fisher helped in preparation of electron microscopy slides and operated the electron microscope for these analyses. Luiz Bermudez performed the electron and video microscopy and determined bacterial viability from apoptotic macrophages. Chapter 5: Brandon Yuan, under my supervision, performed the confocal microscopy and vacuolar pH measurements. Martin Wu determined the intracellular growth abilities of three mutants compared to the wildtype in Raw 264.7 cells. Luiz Bermudez was my advisor on all the work.

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

Molecular and Cellular Characterization of the Intracellular Phenotype of *Mycobacterium avium*

Chapter 1. Introduction

The Genus *Mycobacterium*

Mycobacteria are immotile, aerobic bacilli that belong to the class Actinomycetes. They have a characteristic thick and waxy cell wall that contains mycolic acids, making them acid-fast. These bacteria are naturally resistant to many antibiotics and their genomes have high guanine and cytosine content. Members of *Mycobacteria* are found in multiple environments, including chlorine-treated tap water, soil, and food. The genus is often divided into fast and slow growing strains, based on the amount of time it takes to see colonies on a culture. Several members of the genus live on decaying matter while others are obligate intracellular pathogens.

Diseases caused by *Mycobacteria*

Mycobacterial species cause a variety of diseases in both animals and humans. Many of these diseases result in the formation of granulomas or in disseminated disease in the host. In most cases, the pathogenic bacteria reside inside the host cells; a notable exception is *M. ulcerans*, which causes lesions in the skin and subcutaneous tissues known as Buruli ulcer disease. In humans, *M. tuberculosis* is the primary agent of the lung disease tuberculosis and *M. leprae* cause leprosy, a granulomatous disease of the peripheral nerves and mucosa lining the respiratory tract. Other examples of virulent mycobacteria include *M. shottsii* that form granulomas in splenic tissue and *M. lepraemurium* that affect the skin and lymph nodes of cats, leading to feline leprosy.

M. bovis is the agent responsible for tuberculosis in cattle, and may be transferred to humans to cause human tuberculosis.

Mycobacterium avium complex

One group of genetically related bacteria within the genus Mycobacterium is the *Mycobacterium avium complex*. Members of this group include *Mycobacterium intracellulare, Mycobacterium avium subspecies paratuberculosis* (MAP), *Mycobacterium avium subspecies hominissius* (MAH) and *Mycobacterium avium subspecies avium* (MAC). These bacteria are commonly found in the environment in water, soil, and biofilms, and are infectious when swallowed or inhaled. MAP cause Johne's disease, an infection of the small intestine in ruminants, and are prime suspects in contributing to Crohn's disease, an inflammatory disease of the digestive system in humans (Behr and Kapur 2008). Infection by MAH and MAC can result in disseminated disease in people with impaired immune systems, pneumonia in people with underlying lung diseases and, rarely, pneumonia in people who are otherwise healthy, while *M. intracellulare* cause lung disease.

Disease associated with MAC

Prior to the AIDS epidemic, MAC were mostly associated with pulmonary disease. Since then, MAC have become a serious concern for AIDS patients. In these people, the bacteria are likely acquired via the gastrointestinal tract, where they colonize (Damsker *et al* 1985), then translocate across the mucosa primarily through epithelial cells, allowing them to subsequently infect surrounding intestinal mucosal cells or macrophages (Sangari *et al* 2000, Sangari *et al* 2001). From there, they are found mainly in macrophages and spread throughout the body by unknown mechanisms to

cause disseminated disease, infecting areas such as the spleen, liver, lymph nodes, and bone marrow. Infected patients experience fever, diarrhea, weight loss, abdominal pain, an enlarged liver and/or spleen and fatigue.

Virulence factors of MAC

The molecular details of how MAC cause disseminated disease are still undergoing discovery. The waxy cell wall contributes to natural resistance to antibiotics and plays an important role in protecting the bacteria from host cell ammunition (Rastogi *et al* 1981, Philalay *et al* 2004, Krzywinski *et al* 2005). Recently, a MAC-specific pathogenicity island that, when mutated, decreases the efficiency of uptake by macrophages was identified, though the molecular mechanisms of its role are unknown (Danelishvili *et al* 2007). To name a few specifics, fadD2, a fatty acyl synthase that interrupts cell signaling through cdc42 and prevents epithelial cell entry, (Dam *et al* 2006) and a glycine rich PPE family protein that is involved in blocking phagosome-lysosomal fusion and the ability of MAC to grow in macrophages (Li *et al* 2005) also contribute to its virulence.

MAC's survival in macrophages

In the host, MAC are normally found living and replicating inside of vacuoles in macrophages. The bacteria are ingested by macrophages by phagocytosis using complement receptors such as CR1 and CR3 and other receptors such as Fc, mannose, and transferrin (Bermudez *et al* 1991, Roecklein *et al* 1992). When in the cell, MAC resist respiratory burst (Bermudez and Young 1989) and through mechanisms that are not yet clear, but require iron and involves phosphatidylinositol 3-phosphate, MAC halt the maturation of the phagosome (Sturgill-Koszycki *et al* 1994, Kelley and

Schorey 2003, Kelley and Schorey 2004). The MAC-containing phagosome acquires early endosome markers like Rab5, but not late endosome markers such as Rab7 or LAMP-1, and fusion with the lysosome does not occur, allowing the bacteria to survive and replicate within that microenvironment (Kelley and Schorey 2003, Kelley and Schorey 2004).

Other bacteria that reside in macrophages

The ability of MAC to survive within the macrophage is not unique. Several other pathogenic bacteria, such as *Legionella, Coxiella* and *Listeria,* have also adopted mechanisms to reside within macrophages. *Legionella*, which cause Legionnaires' disease get into the macrophage via phagocytosis and, like MAC, prevent fusion of the phagosome with the lysosome, allowing a protected niche for the bacteria to multiply within alveolar macrophages (Horwitz *et al* 1983). *Coxiella*, the causative agents of Q fever, are internalized by host cells such as macrophages via phagocytosis, and are then found thriving within the harsh, and usually lethal, environment of the lysosome (Akporiaye *et al* 1983, Heinzen *et al* 1996). *Listeria* have found a way to survive in another niche. *Listeria* secrete a hemolysin to break the vacuolar membrane and escape the phagosome, allowing them access to the cytoplasm where they can replicate then usurp the host cell cytoskeleton to propel the bacteria into the nearby cell allowing the process to repeat (Gaillard *et al* 1987, Tilney and Portnoy 1989).

Host control of MAC infection

Considering that healthy individuals rarely experience disease from MAC, one would expect the immune system to be able to effectively fight off MAC. In fact, multiple facets of the immune system are involved in controlling infection. Macrophages that

have been infected by MAC undergo apoptosis, starting 3 days post infection, and reduce the number of viable bacteria (Bhattacharyya *et al* 2003, Gan *et al* 1995, Hayashi *et al* 1997, Fratazzi *et al* 1997). Infection by MAC typically results in the release of cytokines such as TNFα, GM-CSF and IL-6 from macrophages (Fattorini *et al* 1994). TNFα and GM-CSF stimulated macrophages have success in controlling the infection by MAC (Suzuki *et al* 1994, Bermudez and Young 1990, Denis 1991). Similarly, IFNγ stimulation of macrophages allows inhibition or killing of some MAC strains (Denis 1991). Considering that AIDS patients are susceptible to MAC, it is not surprising that CD4+ T-cells are necessary for fighting off MAC infection (Petrofsky *et al* 2005). Other cells of the immune system, such as natural killer cells, dendritic cells and neutrophils also contribute to controlling disease caused by MAC (Bermudez *et al* 1990, Bermudez *et al* 1990, Harshan *et al* 1991, Katz *et al* 1990, Saunder and Cheers 1996, Flórido *et al* 2003, Hartmann *et al* 2001, Mohagheghpour *et al* 2000).

Intracellular phenotype of MAC

MAC that have entered epithelial cells are more efficient at entering other epithelial cells and macrophages (Sangari *et al* 2000). Similarly, MAC that have been passed through macrophages are also more efficient at entering other macrophages (Bermudez *et al* 1997). These data suggest a bacterial phenotype change, hereafter referred to as the intracellular phenotype, that allows for more efficient cell to cell spread in the host once the bacteria have been inside of host cells. Considering the course of infection of MAC in the immunocompromised host, it is assumed the intracellular phenotype is the predominant bacterial phenotype encountered inside the body. Interestingly, MAC that have been inside of macrophages do not use CR3

receptors to enter subsequent macrophages and enter the second macrophage predominantly by macropinocytosis instead of phagocytosis (Bermudez *et al* 1997, Bermudez *et al* 2004). Accordingly, MAC were able to infect and cause signs of disease in CD18 knockout mice in a manner comparable to control mice (Bermudez *et al* 1999). MAC with the intracellular phenotype from macrophages are also found residing in a macrophage vacuole with different characteristics, such as more LAMP-1, than MAC exposed to laboratory conditions (Bermudez *et al* 2004). Other details of the intracellular MAC phenotype, and how it contributes to disseminated disease, remain unknown.

Hypotheses of this project

The intracellular phenotype of MAC is likely the main phenotype used by the bacteria to move from one cell to another, in the course of causing disseminated disease. To better understand the molecular and cellular characteristics of the intracellular MAC phenotype, four hypotheses were studied. First, we hypothesized that MAC use apoptotic macrophages as a tool for dissemination. Next, we proposed that the intracellular phenotype could be elicited *in vitro* and that MAC with this phenotype induce early-onset macrophage cell death. After learning that genes of the tat secretion system are upregulated in MAC exhibiting the intracellular phenotype, we hypothesized that the MAC tat secretion system is a virulence factor. Finally, we postulated that MAC mutants impaired in macrophage uptake reside in vacuoles that differ from vacuoles containing wildtype MAC. Together, study of these hypotheses should lead to increased understanding of how the intracellular phenotype contributes to disease.

References

Akporiaye ET, Rowatt JD, Aragon AA, Baca OG. Lysosomal response of a murine macrophage-like cell line persistently infected with Coxiella burnetii. Infect Immun. 1983 Jun;40(3):1155-62.

Bhattacharyya A, Pathak S, Basak C, Law S, Kundu M, Basu J. Execution of macrophage apoptosis by Mycobacterium avium through apoptosis signal-regulating kinase 1/p38 mitogen-activated protein kinase signaling and caspase 8 activation. J Biol Chem. 2003 Jul 18;278(29):26517-25.

Behr MA, Kapur V. The evidence for Mycobacterium paratuberculosis in Crohn's disease. Curr Opin Gastroenterol. 2008 Jan;24(1):17-21.

Bermudez LE, Goodman J, Petrofsky M. Role of complement receptors in uptake of Mycobacterium avium by macrophages in vivo: evidence from studies using CD18-deficient mice. Infect Immun. 1999 Sep;67(9):4912-6.

Bermudez LE, Kolonoski P, Young LS. Natural killer cell activity and macrophage-dependent inhibition of growth or killing of Mycobacterium avium complex in a mouse model. J Leukoc Biol. 1990 Feb;47(2):135-41.

Bermudez LE, Parker A, Goodman JR. Growth within macrophages increases the efficiency of Mycobacterium avium in invading other macrophages by a complement receptor-independent pathway. Infect Immun. 1997 May;65(5):1916-25.

Bermudez LE, Petrofsky M, Sangari F. Intracellular phenotype of Mycobacterium avium enters macrophages primarily by a macropinocytosis-like mechanism and survives in a compartment that differs from that with extracellular phenotype. Cell Biol Int. 2004;28(5):411-9.

Bermudez LE, Young LS, Enkel H. Interaction of Mycobacterium avium complex with human macrophages: roles of membrane receptors and serum proteins. Infect Immun. 1991 May;59(5):1697-702.

Bermudez LE, Young LS. Oxidative and non-oxidative intracellular killing of Mycobacterium avium complex. Microb Pathog. 1989 Oct;7(4):289-98.

Bermudez LE, Young LS. Natural killer cell-dependent mycobacteriostatic and mycobactericidal activity in human macrophages. J Immunol. 1991 Jan 1;146(1):265-70.

Bermudez LE, Young LS. Recombinant granulocyte-macrophage colony-stimulating factor activates human macrophages to inhibit growth or kill Mycobacterium avium complex. J Leukoc Biol. 1990 Jul;48(1):67-73.

Cangelosi, GA, Palermo CO, Bermudez LE. Phenotypic consequences of red-white colony type variation in Mycobacterium avium. Microbiology. 2001 Mar;147(Pt3):527-33.

Chitale S, Ehrt S, Kawamura I, Fujimura T, Shimono N, Anand N, Lu S, Cohen-Gould L, Riley LW. Recombinant Mycobacterium tuberculosis protein associated with mammalian cell entry. Cell Microbiol. 2001 Apr;3(4):247-54.

Dam T, Danelishvili L, Wu M, Bermudez LE. The fadD2 gene is required for efficient Mycobacterium avium invasion of mucosal epithelial cells. J Infect Dis. 2006 Apr 15;193(8):1135-42.

Damsker B, Bottone EJ. Mycobacterium avium-Mycobacterium intracellulare from the intestinal tracts of patients with the acquired immunodeficiency syndrome: concepts regarding acquisition and pathogenesis. J Infect Dis. 1985 Jan;151(1):179-81.

Danelishvili L, Wu M, Stang B, Harriff M, Cirillo SL, Cirillo JD, Bildfell R, Arbogast B, Bermudez LE. Identification of Mycobacterium avium pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci U S A. 2007 Jun 26;104(26):11038-43.

Denis M. Growth of Mycobacterium avium in human monocytes: identification of cytokines which reduce and enhance intracellular microbial growth. Eur J Immunol. 1991 Feb;21(2):391-5.

Denis M. Modulation of Mycobacterium avium growth in vivo by cytokines: involvement of tumour necrosis factor in resistance to atypical mycobacteria. Clin Exp Immunol. 1991 Mar;83(3):466-71.

Dheenadhayalan V, Delogu G, Brennan MJ. Expression of the PE_PGRS 33 protein in Mycobacterium smegmatis triggers necrosis in macrophages and enhanced mycobacterial survival. Microbes Infect. 2006 Jan;8(1):262-72.

Fattorini L, Xiao Y, Li B, Santoro C, Ippoliti F, Orefici G. Induction of IL-1 beta, IL-6, TNF-alpha, GM-CSF and G-CSF in human macrophages by smooth transparent and smooth opaque colonial variants of Mycobacterium avium. J Med Microbiol. 1994 Feb;40(2):129-33.

Flórido M, Correia-Neves M, Cooper AM, Appelberg R. The cytolytic activity of natural killer cells is not involved in the restriction of Mycobacterium avium growth. Int Immunol. 2003 Aug;15(8):895-901.

Fratazzi C, Arbeit RD, Carini C, Remold HG. Programmed cell death of Mycobacterium avium serovar 4-infected human macrophages prevents the

mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. J Immunol. 1997 May 1;158(9):4320-7.

Gaillard JL, Berche P, Mounier J, Richard S, Sansonetti P. In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2. Infect Immun. 1987 Nov;55(11):2822-9.

Gan H, Newman GW, Remold HG. Plasminogen activator inhibitor type 2 prevents programmed cell death of human macrophages infected with Mycobacterium avium, serovar 4. J Immunol. 1995 Aug 1;155(3):1304-15.

Gioffré A, Infante E, Aguilar D, Santangelo MP, Klepp L, Amadio A, Meikle V, Etchechoury I, Romano MI, Cataldi A, Hernández RP, Bigi F. Mutation in mce operons attenuates Mycobacterium tuberculosis virulence. Microbes Infect. 2005 Mar;7(3):325-34.

Harshan KV, Gangadharam PR. In vivo depletion of natural killer cell activity leads to enhanced multiplication of Mycobacterium avium complex in mice. Infect Immun. 1991 Aug;59(8):2818-21.

Hartmann P, Becker R, Franzen C, Schell-Frederick E, Römer J, Jacobs M, Fätkenheuer G, Plum G. Phagocytosis and killing of Mycobacterium avium complex by human neutrophils. J Leukoc Biol. 2001 Mar;69(3):397-404.

Hayashi T, Catanzaro A, Rao SP. Apoptosis of human monocytes and macrophages by Mycobacterium avium sonicate. Infect Immun. 1997 Dec;65(12):5262-71.

Heinzen RA, Scidmore MA, Rockey DD, Hackstadt T. Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of Coxiella burnetii and Chlamydia trachomatis. Infect Immun. 1996 Mar;64(3):796-809.

Horwitz, MA. The Legionnaires' disease bacterium (Legionella pneumophila) inhibits phagosome lysosome fusion in human monocytes. J Exp Med. 1983 Dec 1;158(6):2108-26.

Inderlied CB, Kemper CA, Bermudez LE. The Mycobacterium avium complex. Clin. Microbiol Rev. 1993 Jul;6(3):266-310.

Katz P, Yeager H Jr, Whalen G, Evans M, Swartz RP, Roecklein J. Natural killer cell-mediated lysis of Mycobacterium-avium complex-infected monocytes. J Clin Immunol. 1990 Jan;10(1):71-7.

Kelley VA, Schorey JS. Mycobacterium's arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. Mol Biol Cell. 2003 Aug;14(8):3366-77.

Kelley VA, Schorey JS. Modulation of cellular phosphatidylinositol 3-phosphate levels in primary macrophages affects heat-killed but not viable Mycobacterium avium's transport through the phagosome maturation process. Cell Microbiol. 2004 Oct;6(10):973-85.

Krzywinska E, Bhatnagar S, Sweet L, Chatterjee D, Schorey JS. Mycobacterium avium 104 deleted of the methyltransferase D gene by allelic replacement lacks serotype-specific glycopeptidolipids and shows attenuated virulence in mice. Mol Microbiol. 2005 Jun;56(5):1262-73.

Li Y, Miltner E, Wu M, Petrofsky M, Bermudez LE. A Mycobacterium avium PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. Cell Microbiol. 2005 Apr;7(4):539-48.

Manganelli R, Fattorini L, Tan D, Iona E, Orefici G, Altavilla G, Cusatelli P, Smith I. The extra cytoplasmic function sigma factor sigma(E) is essential for Mycobacterium tuberculosis virulence in mice. Infect Immun. 2004 May;72(5):3038-41.

Mohagheghpour N, van Vollenhoven A, Goodman J, Bermudez LE. Interaction of Mycobacterium avium with human monocyte-derived dendritic cells. Infect Immun. 2000 Oct;68(10):5824-9.

Petrofsky M, Bermudez LE. 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. 2005 May;73(5):2621-7.

Philalay JS, Palermo CO, Hauge KA, Rustad TR, Cangelosi GA. Genes required for intrinsic multidrug resistance in Mycobacterium avium. Antimicrob Agents Chemother. 2004 Sep;48(9):3412-8.

Raman S, Hazra R, Dascher CC, Husson RN. Transcription regulation by the Mycobacterium tuberculosis alternative sigma factor SigD and its role in virulence. J Bacteriol. 2004 Oct;186(19):6605-16.

Rastogi N, Frehel C, Ryter A, Ohayon H, Lesourd M, David HL. Multiple drug resistance in Mycobacterium avium: is the wall architecture responsible for exclusion of antimicrobial agents? Antimicrob Agents Chemother. 1981 Nov;20(5):666-77.

Roecklein JA, Swartz RP, Yeager H Jr. Nonopsonic uptake of Mycobacterium avium complex by human monocytes and alveolar macrophages. J Lab Clin Med. 1992 Jun;119(6):772-81.

Sangari FJ, Goodman J, Bermudez LE. 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. 2000 Dec;2(6):561-8.

Sangari FJ, Goodman J, Petrofsky M, Kolonoski P, Bermudez LE. Mycobacterium avium invades the intestinal mucosa primarily by interacting with enterocytes. Infect Immun. 2001 Mar;69(3):1515-20.

Saunders BM, Cheers C. Intranasal infection of beige mice with Mycobacterium avium complex: role of neutrophils and natural killer cells. Infect Immun. 1996 Oct;64(10):4236-41.

Senaratne RH, Sidders B, Sequeira P, Saunders G, Dunphy K, Marjanovic O, Reader JR, Lima P, Chan S, Kendall S, McFadden J, Riley LW. Mycobacterium tuberculosis strains disrupted in mce3 and mce4 operons are attenuated in mice. J Med Microbiol. 2008 Feb;57(Pt 2):164-70.

Stokes RW, Norris-Jones R, Brooks DE, Beveridge TJ, Doxsee D, Thorson LM. The glycan-rich outer layer of the cell wall of Mycobacterium tuberculosis acts as an antiphagocytic capsule limiting the association of the bacterium with macrophages. Infect Immun. 2004 Oct;72(10):5676-86.

Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, Allen RD, Gluck SL, Heuser J, Russell DG. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 1994 Feb 4;263(5147):678-81.

Suzuki K, Lee WJ, Hashimoto T, Tanaka E, Murayama T, Amitani R, Yamamoto K, Kuze F. Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) or tumour necrosis factor-alpha (TNF-alpha) activate human alveolar macrophages to inhibit growth of Mycobacterium avium complex. Clin Exp Immunol. 1994 Oct;98(1):169-73.

Tilney LG, Portnoy DA. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes. J Cell Biol. 1989 Oct;109(4 Pt 1):1597-608.

Chapter 2

Mycobacterium avium use apoptotic macrophages as a tool for dissemination

Julie Early[1,2], Kay Fisher[1], Luiz E Bermudez[1,2,3]

Infection and Immunity: *In preparation*

[1]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331
[2]Molecular and Cell Biology Program, Oregon State University, Corvallis, Oregon 97331
[3]Department of Microbiology, College of Science, Oregon State University, Corvallis, Oregon 97331

Abstract

Mycobacterium avium (MAC) live and replicate in macrophages and cause disseminated disease in immunocompromised individuals. As a host response to help control disease, many macrophages become apoptotic a few days after MAC infection. In this study, we hypothesized that MAC can survive apoptotic and autophagic macrophages and use apoptotic macrophages to aid with dissemination. We determined that MAC from apoptotic macrophages are often viable. We present evidence by electron, video, and fluorescent microscopy that MAC escape both the vacuole and the macrophage once apoptosis is triggered, leaving the bacteria free to infect nearby macrophages in the process of dissemination. Additionally, we showed that MAC move from infected apoptotic bodies to healthy macrophages involved in apoptotic body ingestion. We also establish that heat killed MAC and viable MAC induce autophagy in macrophages at similar rates and show that some MAC can survive autophagic macrophages. Based on this information, we propose a model for MAC cell-to-cell spread, in which bacteria survive autophagic and apoptotic macrophages, then escape the apoptotic macrophages, either to the extra-cellular space where they can be taken up by fresh macrophages to repeat the process, or to invade macrophages clearing the apoptotic body.

Introduction

Mycobacterium avium (MAC) are opportunistic pathogens that infect individuals with immunodeficiency such as AIDS patients, people with chronic lung conditions such as emphysema and bronchiectasis and some healthy individuals. In

immunocompromised patients, acquisition of the bacteria is thought to be primarily via the GI tract. After crossing the mucosal barrier, MAC are taken up by and replicate in macrophages, leading to disseminated disease.

In macrophages, MAC thrive in a vacuole that does not acidify and does not fuse with lysosomes (Sturgill-Koszycki *et al* 1994, Via *et al* 1997). Many MAC-infected macrophages undergo apoptosis 3 to 7 days after infection (Gan *et al* 1995), presumably as an innate defense mechanism (Fratazzi *et al* 1997). Several other bacteria, however, such as *Salmonella typhimurium* (Monack *et al* 1996), *Shigella flexneri* (Zychlinsky *et al* 1992) and *Yersinia pseudotuberculosis* (Monack *et al* 1998) have developed mechanisms to survive apoptotic cells. We therefore hypothesized that some MAC may be able to survive macrophage apoptosis.

Given that disease caused by MAC in immunocompromised patients is disseminated, elucidating the mechanism(s) MAC use to spread is critical for understanding disease development. Plasmin has been suggested to be involved during early dissemination of MAC from the lung to other organs (Sato *et al* 2003) and neutrophils (Appelberg *et al* 1995) and CD4 + T cells reduce the degree of dissemination in mice (Orme *et al* 1992, Petrofsky and Bermudez 2005). In the related organism, *Mycobacterium tuberculosis*, genes in the RD1 region, such as *esat-6*, *cfp-10*, (Gao *et al* 2004) and other genes such as *hbha* (Pethi *et al* 2001) appear to have a role in bacterial dissemination. MAC do not contain genes of close sequence similarity to genes in the RD1 region and MAC's use of *hbha* has not been characterized in terms of dissemination (Mueller-Ortiz *et al* 2001). This leaves much unknown as to how MAC move from one macrophage to another. Previous studies have shown that

MAC passed through macrophages become more efficient at entering fresh macrophages, use mechanisms independent of complement receptor 3 to be internalized, and reside in a vacuole in the secondary macrophage that differs from the vacuole in the primary macrophage (Bermudez *et al* 1997, Bermudez *et al* 2004). We assume that this more invasive phenotype is the predominant behavioral state of MAC within the host, and the phenotype utilized by the bacteria to spread. In this study, we hypothesize that MAC from apoptotic macrophages are able to disseminate to nearby macrophages.

Another process macrophages undergo to help control infection is autophagy. In fact, autophagy of macrophages infected by *Mycobacterium tuberculosis* results in less bacterial survival (Gutierrez *et al* 2004). To date, the potential role of autophagy of macrophages on MAC infection has not been elucidated. Here, we demonstrate that MAC-infected macrophages undergo autophagy and that MAC are able to survive autophagic macrophages.

Materials and Methods

Bacteria. MAC strains 101 and 104 were isolated from the blood of AIDS patients. These strains are virulent in mice (Gangadharam *et al* 1989, Torrelles *et al* 2002). For extra-cellular grown bacteria (EG), MAC from frozen stocks were cultured on 7H10 Middlebrook agar for 10 days. Intracellular grown bacteria (IG) were obtained by infecting human monocyte-derived macrophage monolayers for four days as previously reported (Bermudez *et al* 1997). Briefly, macrophage monolayers were infected using a multiplicity of infection (MOI) of 10 and extracellular bacteria

removed by washing. Four days later, intracellular bacteria were collected by lysing macrophages in sterile water.

Macrophages. Human monocyte-derived macrophages were obtained as previously reported (Bermudez and Young 1988). Briefly, blood was drawn from healthy individuals and processed by centrifugation on histopaque. The mononuclear fraction was collected, washed and counted to establish a suspension of $5x10^5$ cells/ml. Monocytes were enriched by plastic adherence and incubated for 4 days to mature to macrophages. Macrophage viability was determined as previously described (Bermudez and Young 1988). THP-1 (ATCC, Manassas, VA) phagocytes were cultured in tissue culture flasks (Costar, Corning, NY) in the presence of RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gemini, Woodland, CA). Before each experiment, THP-1 cells were treated with phorbol-ester to induce maturation. Murine Raw 264.7 macrophages were obtained from ATCC (Manassas, VA) and cultured in flasks in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% FBS. The number of cells was adjusted to $1x10^6$ cells/0.2ml.

Viability Assay. Human monocyte derived macrophage monolayers in 24-well tissue culture plates (Corning NY) were infected with $5x10^6$ MAC 101 for 1 hour then extra-cellular bacteria were removed by washing with Hank's balanced salt solution (HBSS, Gibco, Carlsbad, CA) and the cell culture media was replenished. To determine the number of viable intracellular bacteria, macrophage monolayers were lysed with sterile water for 30 minutes and the lysate plated onto 7H10 agar plates for 10 days to obtain the number of colony forming units (cfu). The percent viability for intracellular

MAC was determined by lysing macrophage monolayers and apoptotic macrophages with sterile water as above and performing the Live-Dead assay, counting 300 bacteria per sample, according to manufacturer's instructions (Molecular Probes, Eugene, OR). Viability of bacteria within apoptotic macrophages was carried out in a similar fashion, and cfu was counted on 7H10 agar plates after collecting and lysing floating macrophages five days post infection, as previously described (Bermudez *et al* 1997, Bermudez *et al* 2004, Cirillo *et al* 1997).

Video Microscopy. In order to determine if bacteria exit macrophages undergoing apoptosis, human monocyte derived macrophage monolayers were established on Lab-Tek chamber slides (Nunc Inc, Napervill, IL) and infected with MAC 101 at a MOI of 10. Four and 5 days after infection, the chambers were prepared for time-lapse video microscopy on a Nikon fluorescence microscope, and the video images were collected with an Optronics DEI-750 camera. The experiment was repeated 4 times and at least 50 infected macrophages were observed each time.

Electron Microscopy. Uninfected human monocyte derived macrophages, MAC 101 infected but non apoptotic macrophages, infected apoptotic macrophages, and macrophages that were ingesting apoptotic/infected macrophages were fixed in 2% gluteraldehyde, 1% osmium tetroxide overnight and post-fixed with 0.5% uranyl acetate overnight at 4 degree C as previously described (Bermudez *et al* 1997). Visual screening of nuclear fragmentation was used to differentiate apoptotic macrophages from non-apoptotic macrophages. To prepare the sample of macrophages clearing apoptotic/infected macrophages, macrophages were infected with MAC 101 at an MOI of 10 then incubated for four days. The detached macrophages were collected

and centrifuged at 500xg for 5 minutes then incubated on a macrophage monolayer for 1 hour, 4 hours or 24 hours at 37° C. After washing, the resulting pellets were embedded and sectioned for transmission electron microscopy (TEM).

Determination of Percent Apoptotic Macrophages. MAC 104 were used to infect fresh THP-1 monolayers in Lab-Tek slide chambers at an MOI of 10. The percent apoptosis of the detached and attached phagocytes was then quantified, separately, four days post infection using the TUNEL assay according to manufacturer's instructions (Molecular Probes, Eugene, OR). Three hundred cells were evaluated under a fluorescent microscope using the FITC filter.

Supernatant Assay. MAC 104 were grown in Middlebrook 7H9 broth supplemented with oleic acid, albumin, dextrose and catalase (OADC) (Hardy Diagnostics, Santa Maria, CA) for 3-5 days, then used to infect Raw 264.7 macrophages in a 24-well plate at an MOI of 10 for one hour. Rinsing three times with HBSS washed extra-cellular bacteria away then fresh media was added to the wells. Four days later, the supernatant was removed from the wells, centrifuged at 500xg for 5 minutes in a microcentrifuge and an aliquot of the supernatant was plated on 7H10 to determine cfu of extra-cellular bacteria.

Fluorescent microscopy. A culture of MAC 104 was grown for 3-5 days in 7H9 broth, then diluted in HBSS to approximately 6 x 10^8 bacteria/ml. Wheat germ agglutinin, Texas red X (Molecular Probes) at 0.15 mg/ml was added to this bacterial culture, and allowed to stain the bacteria for 2 hours in the dark at room temperature. Bacteria were then washed 3 times in HBSS and FBS was added to the labeled bacteria for 30 minutes at room temperature in the dark. After HBSS washing, these

MAC were used to infect Raw 264.7 cell monolayers in chamber slides at an MOI of 10 for 1 hour in the presence of 5mM 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS) (Molecular Probes). The excess HPTS and bacteria were washed away with HBSS, and the media was replenished. Four days later, the extra-cellular bacteria were killed by adding 200 μg/ml amikacin for 2 hours at 37°C. The macrophages that remained attached to the plastic, as well as the detached macrophages, were, separately, rinsed 3 times in HBSS then fixed in 4% paraformaldehyde for 30 minutes at room temperature. After three more washes in HBSS, the detached and attached cells were separately analyzed to determine the percent of bacteria that co-localize with HPTS on a Leica fluorescent microscope. As a control, fluorescent red polystyrene microspheres (0.1μM, Molecular Probes) were diluted in HBSS, opsonized as described above then passed through a 23-gauge needle before being inoculated to macrophages. Three days later, staurosporine (0.5 μM) was added to half the wells containing beads to induce apoptosis and detachment for 20 hours.

Autophagy. Raw 264.7 cells in chamber slides were infected with MAC 104 or heat killed (70 ºC for 1 h) MAC 104 at an MOI of 100. One day and 4 days later, the cells were fixed in 2% paraformaldehyde (Sigma) for 30 minutes then permeabilized with 0.1% triton X-100 (J.T.Baker) for 5 minutes. Slides were prepared for immunoflourescence using 3% bovine serum albumin as a blocking agent (Sigma), the MAPLC3 primary antibody (Santa Cruz), and a FITC-conjugated secondary antibody (Santa Cruz). For the positive control, autophagy was induced by adding 20 μg/ml Rapamycin (Calbiochem, San Diego, CA) for 7 hours, then replenishing the cell

culture media overnight before fixing. At least 300 cells were counted per treatment group each time, with cells that gave bright FITC signal counting as positive.

To determine if the infected macrophages were autophagic, MAC 104 were stained with 100 μg/ml 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (rhodamine) (Molecular Probes, Eugene OR) for 1 hour in the dark, washed three times with HBSS with centrifugation, then used to infected Raw 264.7 cells in chamber slides for one hour at an MOI of ~100. Four days later, the extra-cellular bacteria were killed with 200 μg/ml amikacin (Sigma) for 2 hours then the Raw 264.7 cells were treated for LC3 visualization as described above.

To examine whether MAC can survive the process of autophagy, Raw 264.7 cells in 24 well plates were infected with MAC 104 for 1 hour at an MOI of 10. Extra-cellular bacteria were washed away with HBSS, and macrophages were lysed with sterile water for 10 minutes to obtain a baseline cfu infection load, the media was replenished in other wells. One half of these wells were treated with rapamycin as above, while the other half were left untreated. Both the attached and detached samples were analyzed and treated from this point on. The extra-cellular bacteria were then killed in half of the samples by adding 200 μg/ml amikacin (Sigma) for 2 hours, and washing three times with HBSS. The Raw 264.7 cells were lysed with water as before, and a portion was plated on 7H10 and incubated for 7-10 days at 37°C to determine cfu.

Statistical Analysis. The assays were repeated at least twice. The study samples were compared to the control using the two-sided student's t-test at a level of significance (P value) of <0.05.

Results

Bacterial Viability. To find out if MAC that have been inside of macrophages are capable of infecting other macrophages, we compared the percent viability of MAC from broth and MAC from macrophages after both sets were used to infect fresh macrophages. MAC that had been grown in broth (EG) then used to infect macrophage monolayers increased in their viability from 83% the first day after infection to 89% on the seventh day after infection (Table 2.1). Similarly, bacteria that had previously been exposed to macrophages (IG) increased in their viability from 76% the first day after infection to 96% the seventh day, demonstrating that both EG and IG were able to survive and grow in macrophages.

Since apoptosis is induced after MAC infect macrophages (Frattazi *et al* 1997), we sought to know the outcome of MAC during apoptosis. We determined the viability of MAC in apoptotic macrophages by using the Live-Dead Assay as well as plating for cfu. The percent viability of MAC in apoptotic macrophages by Live-Dead assay increases from 40% to 84% from 0 to 7 days after infection. Similarly, the cfu increased during the same time frame from 3.6×10^4 cfu/ml to 1.3×10^5 cfu/ml, indicating that MAC are able to survive and grow after being in apoptotic macrophages (Table 2.2).

Electron and Video Microscopy. Human monocyte-derived macrophages from a healthy donor were infected with MAC and TEMs were prepared of infected non-apoptotic and apoptotic macrophages. TEMs were also prepared from uninfected macrophages that had been placed in contact with infected apoptotic macrophages.

The vacuolar membrane surrounding MAC in infected but non-apoptotic macrophages remained intact (Figure 2.1A), but in apoptotic macrophages, this membrane was compromised (Figure 2.1B). These micrographs also show that MAC escape the apoptotic macrophage to the extra-cellular space (Figure 2.1C). The escape from apoptotic macrophages was confirmed by video microscopy (Figure 2.2A), which also showed uninfected macrophages ingesting MAC-infected apoptotic bodies became infected by MAC (Figure 2.2B). Taken together, these data indicated that MAC are capable of leaving both the vacuole and macrophage after induction of apoptosis. It also shows that MAC from apoptotic bodies can infect macrophages that are actively clearing the infected apoptotic body.

Fluorescent Microscopy. We infected Raw 264.7 macrophages with Texas-Red labeled MAC 104 in the presence of the membrane impermeable dye, HPTS. Co-localization of the red bacteria with the green HPTS dye indicated an uncompromised vacuolar membrane, while the lack of co-localization suggested a leaky vacuolar membrane. Four days post infection, 59% of MAC co-localized with HPTS in attached cells, while 42% of MAC co-localized with HPTS in detached cells, supporting our previous conclusion that the vacuolar membrane integrity is sometimes compromised upon apoptosis (Figure 2.3). To determine if the loss of vacuolar membrane integrity was actively induced by the MAC, we also analyzed HPTS co-localization of red latex beads internalized by macrophages. The percent co-localization with latex beads inside of macrophages was similar to the MAC infected macrophages, 44% in attached cells, 62% in detached cells, suggesting that the compromised vacuolar membrane is not specific to MAC.

Apoptosis. The percent of apoptosis in macrophages infected with MAC was determined using the TUNEL assay on both attached and detached THP-1 macrophages four days post infection. Seventeen percent of the attached macrophages and 98 percent of the detached macrophages stained positive, indicating that apoptosis is a common effect of MAC infection (Table 2.3). Infection of Raw 264.7 cells gave similar results (data not shown).

Escape from the Macrophage. To determine how frequently MAC can escape the macrophage, extra-cellular bacteria were collected four days after infecting Raw 264.7 macrophages with MAC 104, and then plated for cfu. Due to growth that occurred during the 4 days of infection, we are unable to report the percent of bacteria that leave the macrophage, but viable bacteria were consistently recovered from the extra-cellular sample, indicating MAC escape is not uncommon 4 days post infection of macrophages (Figure 2.4).

Autophagy. We infected macrophages with the wild type and heat-killed MAC then performed immunofluorescent staining on the macrophages for the autophagy-specific marker, LC3 (Yoshimori 2004). One and two days after infection, the percent of macrophages undergoing autophagy in MAC 104-infected and heat-killed MAC 104-infected cells was not significantly different than macrophages that were uninfected (Figure 2.5). Four days post infection, however, 4.6% of uninfected macrophages stained with the LC3 antibody, while 8.1% of the MAC 104 infected, and 11.9% of the heat-killed MAC 104 infected macrophages were positive for LC3 signal. This difference was statistically significant compared to the uninfected control for

macrophages exposed to both live and heat killed MAC 104, suggesting that, at four days post infection, autophagy in macrophages is induced by MAC.

We hypothesized that the macrophages that undergo autophagy were the macrophages that were infected by MAC 104. We infected Raw 264.7 macrophages with rhodamine-labeled MAC 104, waited four days, killed extra-cellular bacteria then prepared cells for LC3 immunofluorescent microscopy. Of the cells that were infected by MAC, 21.4% also stained with the LC3 marker (Figure 2.6). We also noticed that macrophages containing multiple bacteria were more likely to stain with LC3 than macrophages singly infected by MAC or uninfected macrophages, but too few of these cells were observed to quantify. These data indicate that MAC infected macrophages sometimes undergo autophagy.

To find out if MAC are able to survive the process of autophagy, we compared the ability of MAC to survive in infected macrophages treated with rapamycin to infected but untreated macrophages. To account for possible differences in the number of detached macrophages and extracellular bacteria, we plated detached and attached macrophages and amikacin treated and untreated samples for cfu counts. Just over 20% of the bacteria recovered from untreated attached macrophages were recovered in attached cells treated with rapamycin (Figure 2.7). In detached cells, we consistently recovered more bacteria from the rapamycin treated samples than untreated samples. Taken together, these data suggest more than 20% of MAC survive the autophagic macrophage.

Discussion

Fratazzi and colleagues found that 90% of MAC from macrophages that are undergoing apoptosis are no longer able to form cfu, suggesting apoptosis as a mechanism of controlling the infection (Fratazzi *et al* 1997). On the other hand, Pais and colleagues found that MAC viability was unchanged upon treatment of macrophages with staurosporine, an inducer of apoptosis (Pais *et al* 2004). The data presented in this study suggest that some MAC can survive the apoptotic process and then escape apoptotic bodies. Even if several MAC are killed by the apoptotic process, one must consider that the bacteria able to survive may be numerous enough to cause disease.

The data in this work demonstrate that the phagosome membrane becomes damaged upon apoptosis, and, sometimes MAC escape that vacuole and also break through the macrophage in the process of macrophage to macrophage spread. Interestingly, the genomic RD1 region appears to be important for cell to cell spread of *M. tuberculosis* (Gao *et al* 2004, Guinn *et al* 2004), and MAC lack genes of close sequence similarity to genes in this region. *M. marinum* can also escape the phagosome and likely spread through the formation of actin tails (Stamm *et al* 2003). MAC's effect on actin in macrophages has previously been studied, and actin networks were disrupted in MAC infected macrophages, but tails were not observed (Guerin and de Chastellier 2000), suggesting that MAC use a different mechanism of dissemination than both *M. marinum* and *M. tuberculosis*. Future work to determine the molecular mechanisms MAC use to carry out dissemination would help us understand how MAC can cause disease in the immunocompromised host.

The data presented here suggest that the vacuolar membrane loses its integrity without aid from bacteria. We must therefore consider that MAC are exposed to the cytoplasm of the apoptotic macrophage for an unknown period of time, similar to *M. tuberculosis* (McDonough *et al* 1993) and *M. marinum* (Stamm *et al* 2003). Likewise, since MAC can exit apoptotic macrophages, MAC are exposed to the extra-cellular environment after infection of the primary macrophage and before infection of the secondary macrophage. The ability of the bacteria to survive in these conditions, and for what amount of time, is currently unknown. Considering, however, that MAC are environmental organisms, it would not be unusual for MAC to survive these conditions for short periods of time. On the other hand, MAC in apoptotic bodies were also able to cause infection to secondary macrophages ingesting the apoptotic body without exposure to the extra-cellular space. The relative occurrence of these three modes of spreading is not currently known, and further research of this aspect would shed light onto the most predominant mechanism of dissemination used by MAC.

The virulent *M. tuberculosis* strain H37Rv induces less macrophage apoptosis than the avirulent H37Ra strain, suggesting an apoptosis block by virulent *M. tuberculosis* (Keane *et al* 1998), nonetheless, apoptosis still occurs upon infection by either strain. Hayashi and colleagues (Hayashi *et al* 1997) found MAC sonicate induced apoptosis in both human monocyte derived and THP-1 macrophages. Similarly, we found murine Raw 264.7 and human THP-1 macrophages to have similar amounts of apoptosis triggered by live MAC. However, without side by side comparison of intact and lysed MAC or virulent and avirulent MAC strains, we are

unable to say if apoptosis is actively induced or inhibited by live and virulent MAC compared to heat killed or avirulent MAC.

In macrophages infected by *M. tuberculosis*, autophagy results in phagosomes that mature into phagolysosomes and inhibition of bacterial survival (Gutierrez *et al* 2004). Similarly, in this study, we found a reduction in the ability of MAC to survive once macrophages become autophagic. Further work on the maturation state of the MAC-containing vacuole when macrophage autophagy is triggered would indicate if vacuoles containing MAC in autophagic macrophages also mature completely. Consistent with the observation by Chastellier and Thilo (Chasterllier and Thilo 2006), in which MAC in cholesterol-deprived macrophages were found in double-membrane bound vacuoles, but upon the addition of cholesterol, MAC were again located in typical vacuoles, we conclude that some MAC survive autophagy in macrophages. Since MAC are sometimes located in amoeba (Steinert *et al* 1998), and amoeba undergo autophagy (Giusti *et al* 2008) it would be interesting to determine if MAC survive amoebic autophagy, and if this MAC survival mechanism in macrophages evolved due to the bacteria's environmental lifestyle.

Based on the evidence gathered in this study, we propose a model for how MAC spread from macrophage to macrophage (Figure 2.8). In this model, bacteria are phagocytosed into the macrophage, and apoptosis is induced a few days later. Upon apoptosis, some bacteria are killed, while other bacteria escape the apoptotic bodies to the extra-cellular space, where they can be efficiently phagocytosed by another macrophage and the cycle repeats. Other bacteria stay in the apoptotic bodies, and

once those apoptotic bodies are cleared by new macrophages, some bacteria effectively infect the new macrophage and others are killed.

Acknowledgements

We thank Denny Weber for manuscript preparation, and the Daniel D. Rockey lab for use of their fluorescent microscope.

References

Appelberg R, Castro AG, Gomes S, Pedrosa J, Silva MT. Susceptibility of beige mice to Mycobacterium avium: role of neutrophils. Infect Immun. 1995 Sep;63(9):3381-7.

Bermudez LE, Parker A, Goodman JR. Growth within macrophages increases the efficiency of Mycobacterium avium in invading other macrophages by a complement receptor-independent pathway. Infect Immun. 1997 May;65(5):1916-25.

Bermudez LE, Petrofsky M, Sangari F. Intracellular phenotype of Mycobacterium avium enters macrophages primarily by a macropinocytosis-like mechanism and survives in a compartment that differs from that with extracellular phenotype. Cell Biol Int. 2004;28(5):411-9.

Bermudez LE, Young LS. Tumor necrosis factor, alone or in combination with IL-2, but not IFN-gamma, is associated with macrophage killing of Mycobacterium avium complex. J Immunol. 1988 May 1;140(9):3006–3013.

Cirillo JD, Falkow S, Tompkins LS, Bermudez LE. Interaction of Mycobacterium avium with environmental amoebae enhances virulence. Infect Immun. 1997 Sep;65(9):3759-67.

Danelishvili L, Wu M, Young LS, Bermudez LE. Genomic approach to identifying the putative target of and mechanisms of resistance to mefloquine in mycobacteria. Antimicrob Agents Chemother. 2005 Sep;49(9):3707-14.

Ding Z, Christie PJ. Agrobacterium tumefaciens twin-arginine-dependent translocation is important for virulence, flagellation, and chemotaxis but not type IV secretion. J Bacteriol. 2003 Feb;185(3):760-71.

Fratazzi C, Arbeit RD, Carini C, Remold HG. Programmed cell death of Mycobacterium avium serovar 4-infected human macrophages prevents the

mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. J Immunol. 1997 May 1;158(9):4320-7.

Gan H, Newman GW, Remold HG. Plasminogen activator inhibitor type 2 prevents programmed cell death of human macrophages infected with Mycobacterium avium, serovar 4. J Immunol. 1995 Aug 1;155(3):1304-15.

Gangadharam PR, Perumal VK, Parikh K, Podapati NR, Taylor R, Farhi DC, Iseman MD. Susceptibility of beige mice to Mycobacterium avium complex infections by different routes of challenge. Am Rev Respir Dis. 1989 May;139(5):1098-104.

Gao LY, Guo S, McLaughlin B, Morisaki H, Engel JN, Brown EJ. A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. Mol Microbiol. 2004 Sep;53(6):1677-93.

Giusti C, Kosta A, Lam D, Tresse E, Luciani MF, Golstein P. Analysis of autophagic and necrotic cell death in Dictyostelium. Methods Enzymol. 2008;446:1-15.

Guerin I, de Chastellier C. Pathogenic mycobacteria disrupt the macrophage actin filament network. Infect Immun. 2000 May;68(5):2655-62.

Guinn KM, Hickey MJ, Mathur SK, Zakel KL, Grotzke JE, Lewinsohn DM, Smith S, Sherman DR. Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of Mycobacterium tuberculosis. Mol Microbiol. 2004 Jan;51(2):359-70.

Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell. 2004 Dec 17;119(6):753-66.

Hayashi T, Catanzaro A, Rao SP. Apoptosis of human monocytes and macrophages by Mycobacterium avium sonicate. Infect Immun. 1997 Dec;65(12):5262-71.

Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, Kornfeld H. Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. Infect Immun. 1997 Jan;65(1):298-304.

Laurent JP, Hauge K, Burnside K, Cangelosi G. Mutational analysis of cell wall biosynthesis in Mycobacterium avium. J Bacteriol. 2003 Aug;185(16):5003-6.

Li Y, Miltner E, Wu M, Petrofsky M, Bermudez LE. A Mycobacterium avium PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. Cell Microbiol. 2005 Apr;7(4):539-48.

McDonough KA, Kress Y, Bloom BR. Pathogenesis of tuberculosis: interaction of Mycobacterium tuberculosis with macrophages. Infect Immun. 1993 Jul;61(7):2763-73.

Monack DM, Raupach B, Hromockyj AE, Falkow S. Salmonella typhimurium invasion induces apoptosis in infected macrophages. Proc Natl Acad Sci U S A. 1996 Sep 3;93(18):9833-8.

Monack DM, Mecsas J, Bouley D, Falkow S. Yersinia-induced apoptosis in vivo aids in the establishment of a systemic infection of mice. J Exp Med. 1998 Dec 7;188(11):2127-37.

Mueller-Ortiz SL, Wanger AR, Norris SJ. Mycobacterial protein HbhA binds human complement component C3. Infect Immun. 2001 Dec;69(12):7501-11.

Orme IM, Furney SK, Roberts AD. Dissemination of enteric Mycobacterium avium infections in mice rendered immunodeficient by thymectomy and CD4 depletion or by prior infection with murine AIDS retroviruses. Infect Immun. 1992 Nov;60(11):4747-53.

Ochsner UA, Snyder A, Vasil AI, Vasil ML. Effects of the twin-arginine translocase on secretion of virulence factors, stress response, and pathogenesis. Proc Natl Acad Sci U S A. 2002 Jun 11;99(12):8312-7.

Pais TF, Appelberg R. Induction of Mycobacterium avium growth restriction and inhibition of phagosome-endosome interactions during macrophage activation and apoptosis induction by picolinic acid plus IFNgamma. Microbiology. 2004 May;150(Pt 5):1507-18.

Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, Locht C, Menozzi FD. The heparin-binding haemagglutinin of M. tuberculosis is required for extrapulmonary dissemination. Nature. 2001 Jul 12;412(6843):190-4.

Petrofsky M, Bermudez LE. 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. 2005 May;73(5):2621-7.

Pradel N, Ye C, Livrelli V, Xu J, Joly B, Wu LF. Contribution of the twin arginine translocation system to the virulence of enterohemorrhagic Escherichia coli O157:H7. Infect Immun. 2003 Sep;71(9):4908-16.

Sato J, Schorey J, Ploplis VA, Haalboom E, Krahule L, Castellino FJ. The fibrinolytic system in dissemination and matrix protein deposition during a mycobacterium infection. Am J Pathol. 2003 Aug;163(2):517-31.

Stamm LM, Morisaki JH, Gao LY, Jeng RL, McDonald KL, Roth R, Takeshita S, Heuser J, Welch MD, Brown EJ. Mycobacterium marinum escapes from phagosomes and is propelled by actin-based motility. J Exp Med. 2003 Nov 3;198(9):1361-8.

Steinert M, Birkness K, White E, Fields B, Quinn F. Mycobacterium avium bacilli grow saprozoically in coculture with Acanthamoeba polyphaga and survive within cyst walls. Appl Environ Microbiol. 1998 Jun;64(6):2256-61.

Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, Allen RD, Gluck SL, Heuser J, Russell DG. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 1994 Feb 4;263(5147):678-81.

Torrelles JB, Ellis D, Osborne T, Hoefer A, Orme IM, Chatterjee D, Brennan PJ, Cooper AM. Characterization of virulence, colony morphotype and the glycopeptidolipid of Mycobacterium avium strain 104. Tuberculosis (Edinb). 2002;82(6):293-300.

Via LE, Deretic D, Ulmer RJ, Hibler NS, Huber LA, Deretic V. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. J Biol Chem. 1997 May 16;272(20):13326-31.

Yoshimori T. Autophagy: a regulated bulk degradation process inside cells. Biochem Biophys Res Commun. 2004 Jan 9;313(2):453-8.

Zychlinsky A, Prevost MC, Sansonetti PJ. Shigella flexneri induces apoptosis in infected macrophages. Nature. 1992 Jul 9;358(6382):167-9.

Table 2.1 Viability of EG and IG phenotypes in macrophages.

Bacteria (strain 101)	Day after infection	Viability (%)
EG	0	87 ± 3
EG/Mo	1	83 ± 4
	4	90 ± 5
	7	89 ± 3
IG/Mo	0	76 ± 5
	1	61 ± 6
	4	79 ± 5
	7	96 ± 2

Human monocyte derived macrophages were lysed at different time points and viability was determined under the fluorescence microscope after staining the preparation with Live-Dead assay.

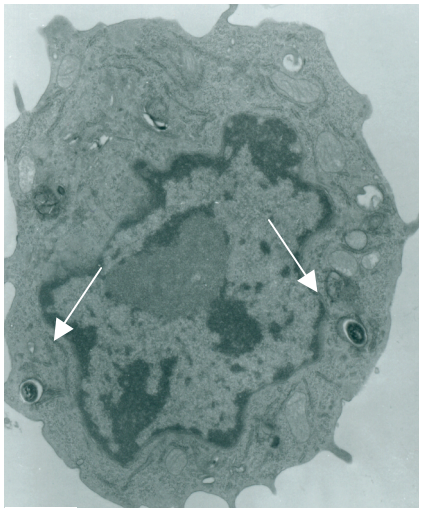
Table 2.2 Viability of IG (MAC 101) phenotype in apoptotic macrophages.

Days after infection	% viability	CFU/ml
0	40 ± 3	$3.6 \pm 0.3 \times 10^4$
1	41 ± 5	$3.8 \pm 0.5 \times 10^4$
4	67 ± 4	$8.9 \pm 0.4 \times 10^4$
7	84 ± 6	$1.3 \pm 0.6 \times 10^5$

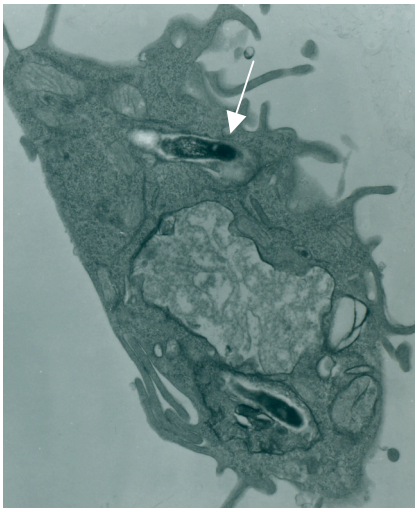
MAC 101 infected human monocyte derived macrophage monolayers were cultured for 5 days. Apoptotic macrophages were harvested, and the viability determined by Live-Dead assay and the number of CFU/ml.

Figure 2.1 Electron microscopy images of MAC in apoptotic macrophages

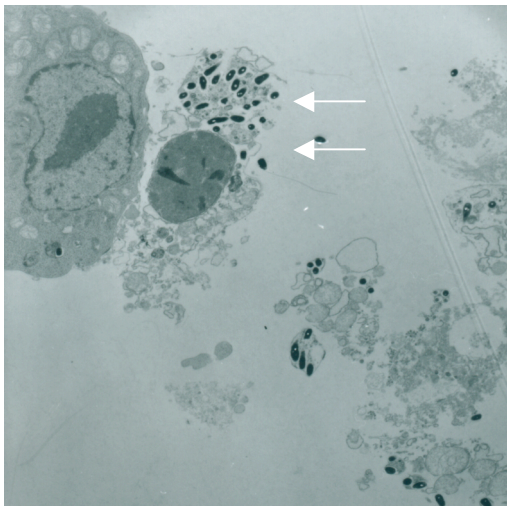
A



B



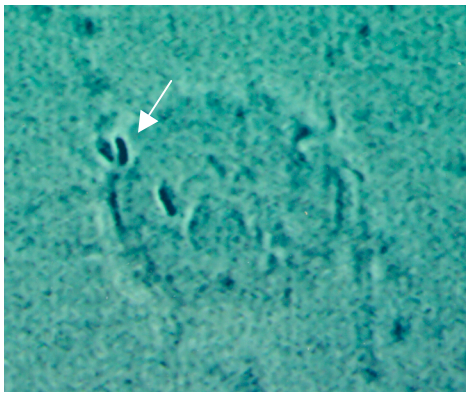
C



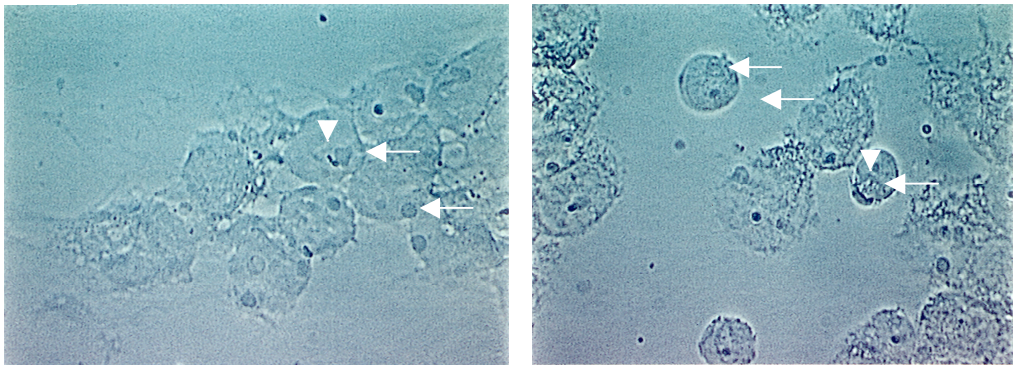
A. TEM of MAC-infected macrophages that are not apoptotic. Arrows indicate membrane bound vacuoles containing bacteria. Notice that the vacuolar membrane appears to be intact. 10,500 x magnification. B. TEM of MAC in an apoptotic macrophage. Arrow indicates an area where the integrity of the vacuole membrane appears to be compromised. 12,500 x magnification. C. TEM of MAC-infected apoptotic macrophages being ingested by healthy macrophages. Arrows indicate bacteria escaping the apoptotic macrophage. 2,500 x magnification.

Figure 2.2 Video microscopy images of MAC in apoptotic macrophages

A



B



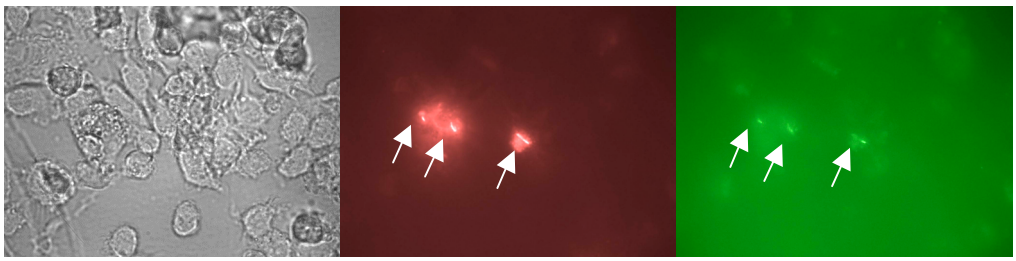
A. Representative image from video microscopy, showing MAC leaving the apoptotic macrophages. (arrow) B. Four days after the apoptotic bodies (arrows) were added to a new monolayer, MAC (arrowheads) were found inside fresh macrophages.

Figure 2.3 Fluorescent microscopy of MAC and HPTS in macrophages

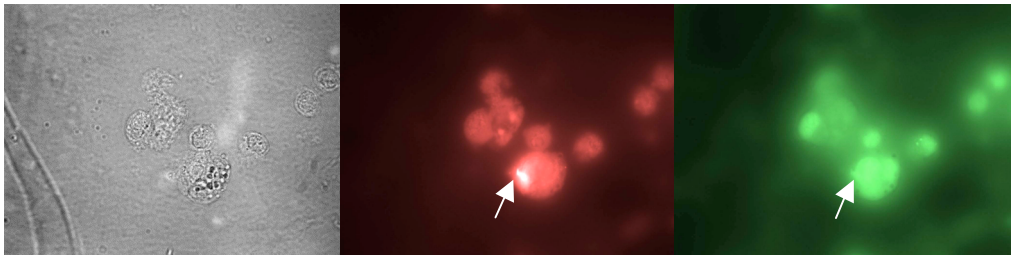
A. Percent co-localization of MAC 104 or beads with HPTS in macrophages

Strain	Detached Cells	Attached Cells
104 (WT)	42 ± 2	59 ± 6
Beads	44 ± 9	62 ± 4
Beads + staurosporine	43 ±7	58 ± 9

B



C



100 microns

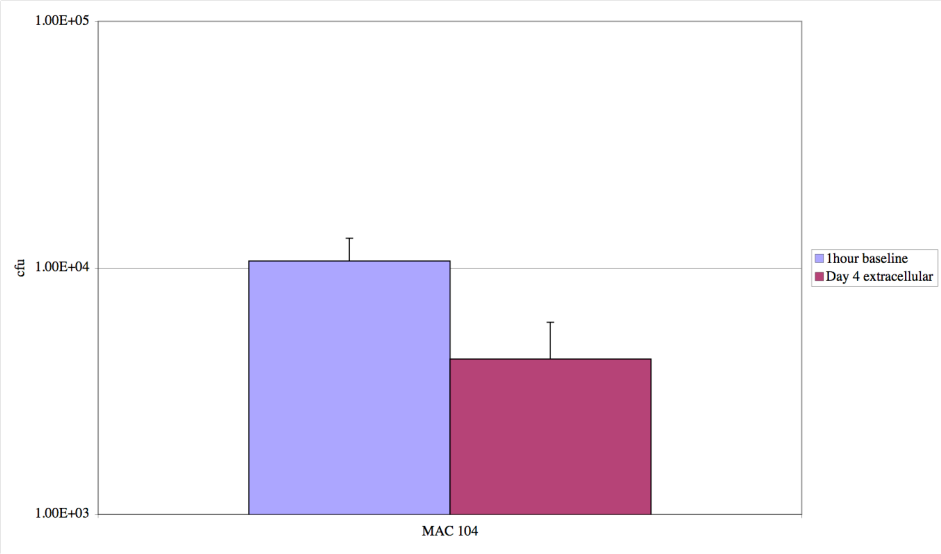
A. Four days after Raw 264.7 macrophages were infected by the wild type Texas-Red stained MAC in the presence of HPTS, attached and detached cells were, separately, collected and analyzed for co-localization on a fluorescent microscope. Values are from 3 repeats of the assay. B. Texas-Red labeled MAC 104 in attached Raw 264.7 macrophages, showing co-localization with HPTS and suggesting that the vacuolar membrane is still intact. From left to right, brightfield, Texas Red (bacteria), and FITC (HPTS). C. Texas-Red labeled MAC 104 in detached macrophages showing that the bacteria do not appear to co-localize with HPTS and suggesting that the vacuolar membrane has been compromised. From left to right, brightfield, Texas Red (bacteria), and FITC (HPTS). Arrows indicate bacteria that co-localized.

Table 2.3 Percent of MAC infected macrophages that stain by TUNEL assay

Bacterial Strain	Attached	Detached
MAC 104	17 ± 4	98 ± 2

Four days after THP-1 macrophages were MAC-infected, the TUNEL assay was performed on attached and detached macrophages, at least 300 cells of each were counted to determine the percent of apoptotic cells. The assay was repeated 3 times.

Figure 2.4 Amount of extra-cellular MAC four days post infection of macrophages



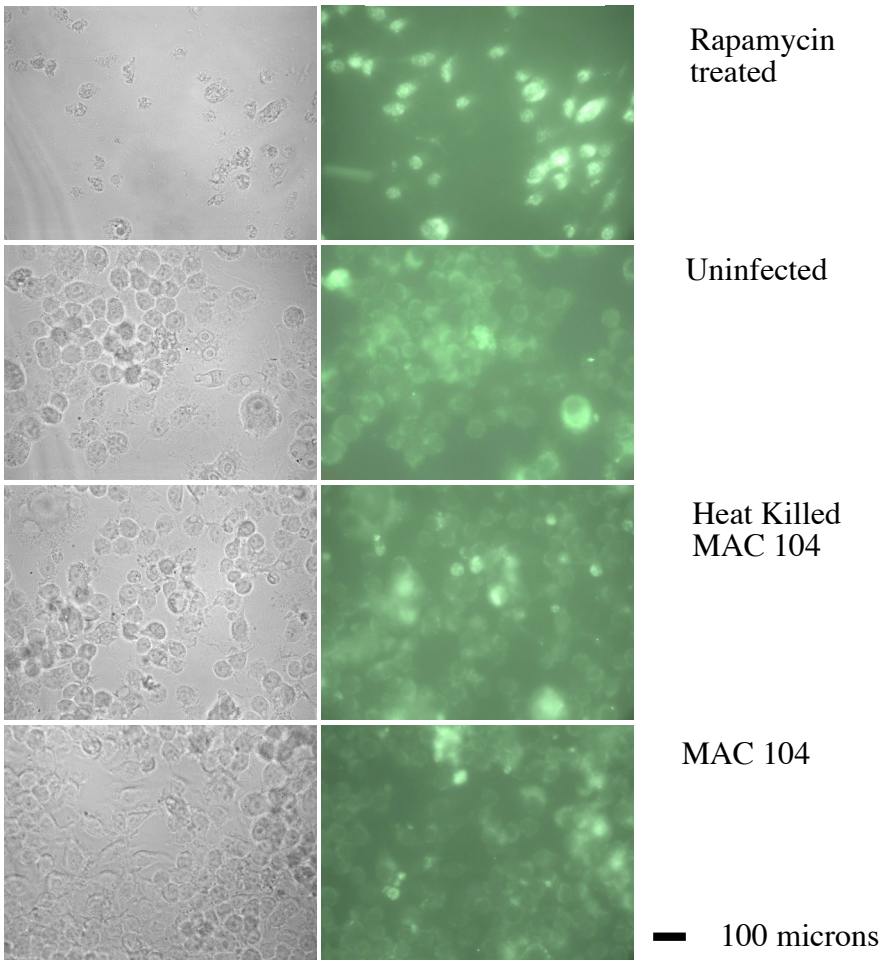
Raw 264.7 cells were infected by MAC 104, and the number of intracellular bacteria was determined one hour later by plating for cfu. Four days later, the number of extra-cellular bacteria was determined by collecting the supernatant, separating cellular debris by differential centrifugation, then plating for cfu. Bars represent mean and standard deviation values from 3 experiments.

Figure 2.5 Percent of autophagic macrophages

A

Days post infection	Rapamycin treated	Uninfected	Heat Killed MAC 104	Mac 104
1	84 ± 1.1	6.6 ± 4.1	No Data	5.4 ± 1.4
2	91 ± 2.4	4.8 ± 1.3	4.5 ± 0.1	4.3 ± 0.2
4	93 ± 4.5	4.6 ± 0.4	12 ± 0.8 *	8.1 ± 1.2 *

B



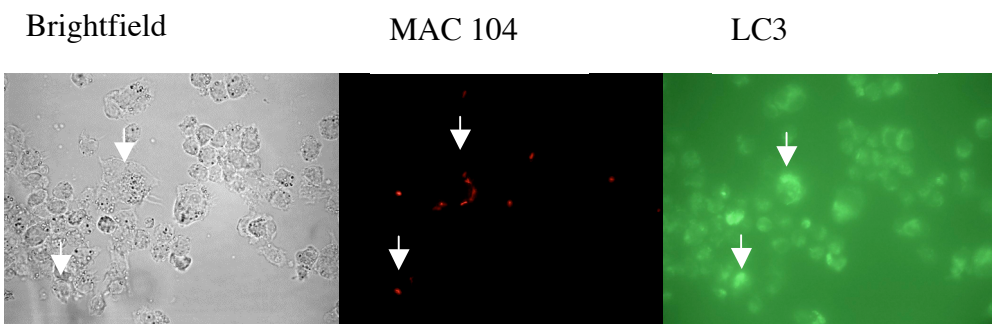
A. Raw 264.7 cells in chamber slides were infected with MAC 104, Heat-killed MAC 104, or uninfected. At indicated timepoints, the cells were analyzed for percent autophagy on a fluorescent microscope by staining for LC3. Means and standard deviations were determined from 3 assays, with at least 300 cells counted per repeat. * p value < 0.05 B. Pictures from 4 days post infection.

Figure 2.6 Microscopy to determine if MAC infected macrophages are autophagic

A

MAC 104 infected Raw 264.7 cells that stain with LC3 4 days post infection
21.4% +/- 2.3

B



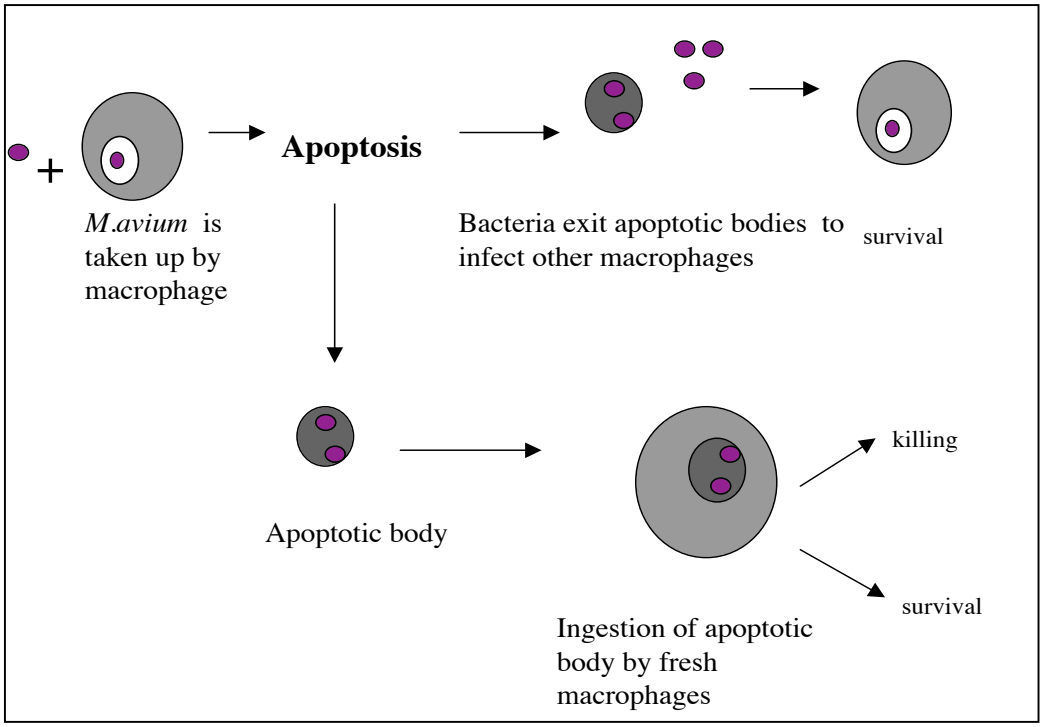
Raw 264.7 macrophages were infected with rhodamine-labeled MAC 104. Four days later, the cells were fixed, permeabolized, and processed for LC3 immunofluorescent microscopy. A. The percent of infected cells that also gave LC3 signal, based on 3 experiments with at least 100 infected cells counted per repeat. B. Representative images from the assay. Arrows point to cells that are both MAC infected and LC3 stained.

Figure 2.7 Survival of MAC in autophagic macrophages

Macrophages	Percent of cfu recovered (rapamycin treated/untreated)
Attached with amikacin	20.6 ± 6.4
Attached	22.1 ± 5.3
Detached with amikacin	184.3 ± 74.8
Detached	131.4 ± 23.6

Raw 264.7 cells in 24-well plates were infected with MAC 104 then half the infected wells were treated with rapamycin, the other half left untreated. The amount of bacteria able to survive autophagy was determined by dividing the cfu of the rapamycin treated cells by the cfu of the untreated cells with or without amikacin treatment to kill extra-cellular bacteria and in both attached and detached cells. The assay was repeated 4 times.

Figure 2.8 Model for how MAC disseminate



MAC get inside macrophages, and apoptosis is induced. Some bacteria then escape both the vacuoles and macrophages to become extra-cellular, and infection can again occur. Some other bacteria remain inside the apoptotic body, which are taken up by another macrophage in the process of clearing. During this process, some bacteria infect the clearing macrophage, while other bacteria do not survive.

Chapter 3

Mimicry of the *Mycobacterium avium* vacuole *in vitro* elicits the bacterial intracellular phenotype, including increased early-onset macrophage cell-death

Julie Early[1,2], Luiz E Bermudez[1,2,3]

Cellular Microbiology: *In preparation*

[1]Molecular and Cell Biology Program, Oregon State University, Corvallis, Oregon 97331
[2]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331
[3]Department of Microbiology, College of Science, Oregon State University, Corvallis, Oregon 97331

Abstract

Previous studies indicate that MAC within macrophages undergo a bacterial phenotype change that allows for more efficient entry into macrophages. We hypothesized that we could learn more about the intracellular phenotype by creating two solutions (mixtures) that mimicked the concentrations of metals and pH within the vacuole, one resembling the vacuole 1 hour post infection, the other similar to the vacuole 24 hours post infection. MAC were then incubated in the elemental mixtures and used to infect macrophages. These mixture-incubated MAC infected macrophages at a significantly higher rate than bacteria that were incubated in Middlebrook 7H9 broth, and were taken up by macrophages using macropinocytosis, similar to bacteria that have been passed through macrophages. Real-time PCR showed genes that have previously been reported to be upregulated in intracellular bacteria such as *Mav1365, Mav2409, Mav4487*, and *Mav0996* were upregulated in MAC incubated in the 24 hour elemental mixture compared to bacteria from 7H9 broth. Like MAC from macrophages, the vacuoles of bacteria from the 24 hour elemental mixture were more likely to contain LAMP-1. Microarray analysis of MAC that were incubated in the 24 hour elemental mixture compared to bacteria in 7H9 broth showed several changes in gene expression. Overexpression of *Mav_0460*, a gene upregulated on the array, in *M. smegmatis* resulted in decreased ability to enter macrophages, but similar ability to survive while overexpression of *Mav_5178*, another gene upregulated in the elemental mixture showed no changes in macrophage uptake and survival compared to the control. A step-wise reduction scheme of the 24 hour elemental mixture indicated that incubation in physiologically relevant concentrations of potassium chloride, calcium

chloride, and manganese chloride was sufficient to induce characteristics of the intracellular phenotype. Using the 24 hour elemental mixture, then confirming with intracellular bacteria, we also demonstrated that bacteria with the intracellular phenotype induce early-onset macrophage cell death in the second macrophage more efficiently than broth grown bacteria. This new elemental mixture has the potential to become an effective laboratory tool for the study of the MAC disease process.

Introduction

Mycobacterium avium (MAC) are environmental bacteria that are a serious concern for immunocompromised individuals such as AIDS patients. MAC are likely acquired via either the gastrointestinal or the respiratory tracts, after that, they sometimes spread to cause disseminated disease. In the host, the bacteria live and replicate in vacuoles inside of macrophages but exit phagosomes and eventually phagocytic cells, probably as a mechanism of dissemination (unpublished data). The mechanism(s) by which MAC move from one macrophage to another has not been elucidated.

Prior studies suggest that MAC sense and react to the environment within the macrophage and acquire a new phenotype that appears to have a role in its ability to spread in the host. Specifically, MAC that have been inside of macrophages infect other macrophages more efficiently than bacteria obtained from 7H9 broth, and are more resistant to killing/inhibition of growth by macrophages stimulated with TNFα (Bermudez *et al* 1997). MAC that have been passed in macrophages are more likely to use transferrin and β-1 integrin receptors and less likely to use complement receptor 3

than MAC from 7H9 broth (Bermudez *et al* 1997) to gain access to phagocytic cells. Similarly, the vacuoles containing MAC that were passed in other macrophages have a lower pH and are more likely to contain lysosomal associated membrane protein 1 (LAMP-1) than vacuoles occupied by MAC from broth (Bermudez *et al* 2004). Microscopy also indicates that MAC enter the first macrophage by phagocytosis then use macropinocytosis to be taken up by subsequent macrophages (Bermudez *et al* 2004).

Recent studies have indicated MAC actively impact the environment of the vacuole in macrophages by controlling the amount of single elements such as iron in the vacuole (Kuhn *et al* 2001, Wagner *et al* 2005 #2). Several other bacteria, such as *Salmonella* and *Pseudomonas,* are known to sense changes in metal concentrations that regulate the expression of a number of virulence determinants (Su *et al* 2008, Frank 1997). We therefore hypothesized that metals may be a key factor in sensing the environment of the macrophage. The elemental composition of the MAC vacuole was recently determined by hard x-ray microscopy at time points 1 hour and 24 hours after infection (Wagner *et al* 2005) and other work determined the vacuolar pH is 5.8-6.1 (Oh *et al* 1996). We used these data to model the condition of vacuoles that contain MAC to study the intracellular bacterial phenotype in a test tube. This type of mimicry has been used in other bacterial species, such as *Pseudomonas* (Wolfgang *et al* 2003) and *Yersinia pestis* (Straley 1981, Feodorova 2001) and has become a powerful laboratory tool.

Materials and methods

Media Recipe. Five hundred ml of Middlebrook 7H9 broth (Difco, Becton-Dickenson Sparks, MD) was prepared as usual, then the chemicals (Sigma, St. Louis MO; except $CaCl_2$ Mallinckrodt, St. Louis MO) listed in Table 1 column A were added to make a mixture that mimicked the vacuole one hour after infection and the supplements listed in column B were added to make a mixture matching the vacuole one day after infection. The pH was adjusted to 6.6 for the 1 hour mixture and 5.8 for the 24 hour mixture. Both mixtures were then autoclaved and stored at 4° C until use.

Infection assays. MAC strain 104 was obtained from the blood of an AIDS patient and grown in 7H9 broth supplemented with oleic acid, albumin, dextrose and catalase, (OADC, Hardy Diagnostics, Santa Maria, CA) for 5 days at 37°C with shaking, then diluted to $3x10^8$ bacteria/ml using MacFarland standards. The bacteria were diluted 1:10 in 7H9 broth (lacking OADC), or the indicated mixture, then incubated at 37°C with shaking for 1 hour or 24 hours. These samples were briefly vortexed, and an aliquot was used to plate for colony forming units (cfu) to determine inoculum concentration while another aliquot was used to infect Raw 264.7 (ATCC, Manassas, VA) macrophage monolayers that had been seeded in 24 well plates (Costar, Corning, NY). The Raw 264.7 cells were seeded overnight in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gemini, Woodland, CA) in a 24 well plate then infected at an multiplicity of infection (MOI) of ~10 for 1 hour. The macrophage monolayers were washed three times with Hank's balanced salt solution (HBSS) (Gibco, Carlsbad, CA) to remove extracellular bacteria, lysed with sterile water then plated on 7H10 (Difco, Becton-

Dickenson Sparks, MD) with OADC to determine cfu. This assay was repeated three times, with two wells per sample each time.

Infection control. We treated Raw 264.7 cells with the elemental mixtures for one hour then infected them with MAC from 7H9 broth at an MOI of 10. Raw 264.7 macrophage monolayers were incubated with the same concentration of 1 hour mixture, 24 hour mixture and 7H9 broth as was used in the assay described above ($1/10^{th}$ the total volume) for 1 hour, and one sample was incubated with DMEM as a control. The monolayers were washed three times with HBSS, and then fresh DMEM was added. The monolayers were infected with MAC that was grown in 7H9 broth with OADC for 5 days at 37° C with shaking at an MOI of ~10 for one hour. Bacteria were harvested and plated onto 7H10 to determine cfu. The assay was performed three times, with two wells per sample each time.

Real-Time PCR. MAC was grown in 7H9 broth supplemented with OADC for five days then exposed to the elemental mixture for 24 hours. RNA was extracted from the bacteria using phenol chloroform according to previously published methods (Danelishvili *et al* 2004, Tenant and Bermudez 2006). The RNA was DNase-treated, and first strand cDNA was synthesized using random hexamer primers and the superscript III cDNA synthesis kit according to manufacturer's instructions (Invitrogen, Carlsbad, California). Primers for genes previously described to be upregulated in macrophages (table 2) were used for real-time PCR using SYBER green master mix (BioRad, Hercules, CA) with a PCR protocol of 10 minutes at 95°C, followed by forty cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes. The threshold cycle was determined as previously described (Danelishvili *et al* 2004,

Tenant and Bermudez 2006). For real-time PCR with the 24 hour elemental mixture, *Mav4781* was used as a negative control, since its expression does not appear to be influenced by the intracellular environment (Danelishvili *et al* 2004). *Mav2036* was a positive control, since it has previously been shown to be upregulated in higher zinc concentrations (Milano *et al* 2004). For all other real-time studies, *Mav4357* was used as a negative control since its expression level was not influenced by the 24 hour elemental mixture. Data are from six real-time reactions, based on three biological samples.

Macropinocytosis. Raw 264.7 macrophages were infected as described above, except after exposure to the 24 hour elemental mixture or 7H9 broth, bacteria were stained by incubating ~$3x10^9$ MAC with 100ug/ml of 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester (rhodamine) (Molecular Probes, Eugene, OR) for 1 hour in the dark. Bacteria were diluted in HBSS before infection of macrophages in chamber slides (Nalge Nunc, Rochester, NY). The residual rhodamine was left in the presence of the bacteria during the infection to allow for visualization of the size of the compartment occupied by the bacteria. Slides were fixed in 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 30 minutes, then visualized on a Leica DM400B fluorescent microscope.

Co-localization. MAC was incubated in 7H9 broth or in the 24 hour elemental mixture for 24 hours, then a 1 mg/ml 5-(and-6)-carboxyfluorescein, succinimidyl ester (Molecular Probes, Eugene, OR) solution in HBSS was added to the MAC for 1 hour in the dark. Excess dye was removed by washing three times with centrifugation using HBSS. The stained bacteria were used to infect Raw 264.7 cells on 2-well chamber

slides at an MOI of 100 for 1 hour, and extra-cellular MAC were killed for 2 hours with 200 μg/ml amikacin (Sigma) then washed away 3 times with HBSS. The cells were fixed with 2% paraformaldehyde in HBSS for 60 minutes in the dark at room temperature, washed with HBSS, then permeabilized with 0.1% Triton X-100 (JT Baker, Phillipsburg, NJ) at room temperature for 5 minutes. Next, they were blocked for one hour with 10% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in HBSS, washed with HBSS, exposed to the appropriate primary antibody for 1 hour, washed again, and treated with Texas-Red conjugated secondary antibody. These cells were analyzed by fluorescent microscopy for co-localization of the stained bacteria with the vacuole marker, with about 40 stained bacteria per treatment group analyzed during each repeat. We used the primary antibodies rabbit anti-Rab5a (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Rab7 (Santa Cruz Biotechnology, Santa Cruz, CA) goat anti-EEA-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-LAMP-1 (BD Biosciences, San Jose, CA). Secondary antibodies were all Texas Red conjugates: (TR) donkey anti-rabbit IgG-TR (Amersham Biosciences, NJ), mouse anti-goat IgG-TR (Santa Cruz Biotechnology, Santa Cruz, CA) and sheep anti-mouse IgG-TR (Amersham Biosciences, NJ).

Microarray. Bacteria were grown, exposed to the mixtures, and RNA was extracted as in the real-time PCR. The cDNA was synthesized, labeled with Cy-3 and Cy-5, and hybridized using the 3DNA Array 900MPX kit (Genisphere, Hatfield, PA). Microarray slides were from the National Animal Disease Center (Ames, Iowa) and represented the entire set of coding sequences from *Mycobacterium avium* subsp. *paratuberculosis* strain K-10 as well as a few MAC 104 specific gene sequences with

30-mer oligonucleotides, each one in triplicate. Oregon State University's Center for Genome Research and Biocomputing ScanArray 4000 (Perkin Elmer, Waltham, MA) scanned the hybridized slides and QuantArray software (Packard Bioscience, Billerica, MA) was used to quantify the red and green intensities. Two biological repeats of the microarray, providing 6 spots per gene were used for the analysis. With the program BASE, we identified all spots that were up or downregulated by 3.0 fold, then found the corresponding spots representing the same gene and determined the average fold change and standard deviation. The oligonucleotide sequences cross-linked to the slide were individually compared to the corresponding gene in the MAC genome and to identify the sequence similarity between the two. Genes that had an average fold change of 1.5 or more and had no more than 2 mismatches in the 30-mer compared to the MAC genome were considered up or downregulated in the elemental mixture. For statistical analysis, the fold up or down regulation for the six spots of each gene was compared to the MAC subspecies *paratuberculosis* homolog of *Mav4357*, *Map4277c*; a gene that was not upregulated in our elemental mixture as determined by real-time PCR, and also showed no change in regulation on the microarray. To determine genes differentially expressed, we used the Student's t-test, with a cut off value of $p \leq .05$.

Confirmation. We confirmed a subset of those genes with changes in expression from the microarray by real-time PCR as described above. Primers were as listed in table 3.5B.

Overexpression in *M. smegmatis*. *Mav_0460* and *Mav_5178* were cloned behind the constitutive G13 promoter in pLDG13 (Danelishvili *et al* 2005) using the primer sets 5'CCGGAATTCGTGAGCAAAGTCGAGG3',

5'GGAAGATCTCTACCAACCCGACGGGTC3', and

5'CCGGAATTCATGGCAATCGACAGCGG3',

5'GGAAGATCTTCAGGCCAGGCTCAGAAAC3' respectively. These constructs, along with the empty pLDG13 vector were transformed into *M. smegmatis* mc^2155. After growing these three strains in 7H9 broth with 50 μg/ml kanamycin for 2 days, Raw 264.7 cells in 24 well plates were infected by these strains at an MOI of 100 for 1 hour at 37°C. The cells were then triply washed with HBSS and extra-cellular bacteria were killed by adding 100 μg/ml amikacin for 2 hours at 37°C. The wells were again triply washed with HBSS, then Raw 264.7 cells in some wells were lysed in sterile water for 10 minutes at room temperature and these samples were plated on 7H10 and incubated for 3 days to determine baseline cfu. The cell culture medium was replenished in the other wells, and incubated at 37°C for 1 day then the Raw 264.7 cells were rinsed with HBSS and lysed with water and plated for cfu as before. To determine efficiency of macrophage entry, the cfu at the baseline was divided by the cfu of the inoculum. To determine the ability of these strains to survive in macrophages, the cfu at 1 day post infection was divided by the cfu at the baseline.

Live-Dead Assay. Raw 264.7 cells were seeded on 2-well chamber slides and allowed to attach overnight. MAC was incubated in 7H9 broth without OADC or the 24 hour elemental mixture for 24 hours, then used to infect Raw 264.7 cells at an MOI of 1000 for bacteria from 7H9 broth, an MOI of 100 for bacteria from the elemental mixture to attain roughly the same amount of intracellular bacteria. After an 1 hour infection, the monolayers were washed three times with HBSS for removal of extracellular bacteria and the cell culture media was replenished. Two hours later, the Raw 264.7 cells were

analyzed by Live-Dead Assay (Molecular Probes, Eugene, OR) using fluorescent microscopy, according to the manufacturer's instructions. Three hundred cells were counted in each sample, per repeat. For bacteria that had been passed through Raw 264.7 cells, Raw 264.7 cell monolayers in 6-well plates were infected at an MOI of 100 for 1 hour, then monolayers were washed and media was replenished, 24 hours later, the monolayers were lysed for 10 minutes in 0.25% SDS at room temperature. The cellular debris was centrifuged for 5 minutes at 500xg, supernatant was collected, then bacteria were pelleted by centrifuging at 12000xg for 1 minute. Pelleted bacteria were resuspended in HBSS and used to infect fresh monolayers of Raw 264.7 cells seeded in chamber slides at an MOI of 50. Infection with MAC from 7H9 broth was performed using an MOI of 500. As a control, uninfected Raw 264.7 cells were also lysed, doubly centrifuged as described, and the resuspended lysate was used to inoculate fresh macrophage monolayers.

TUNEL Assay. This assay was carried out similarly to the Live-Dead Assay, except 8-well chamber slides were used instead of 2-well chamber slides, and the Fluorescein In Situ Cell Death Detection Kit was used for analysis (Roche, Indianapolis, IN).

Statistical Analysis. The two sided Student's t-test was performed comparing the experimental data to the negative control from three biological repeats using Microsoft Excel software, with a level of significance cut off value of * p value <0.05 or # p value <0.10, as indicated on the graphs.

Results

Uptake by Raw macrophages. We exposed MAC to the 1 hour elemental mixture, which mimicked single elements in the macrophage vacuole 1 hour post infection, or the 24 hour elemental mixture, which mimicked single elements in the macrophage vacuole 24 hours post infection, for 1 hour and for 24 hours then infected macrophages. In all cases, MAC from a mixture were taken up by Raw 264.7 macrophages approximately 8-fold more efficiently than bacteria that had been exposed to 7H9 broth (Figure 3.1A), similar to bacteria that have previously been exposed to macrophages before infecting other macrophages (Bermudez *et al* 1997). To ensure the difference in uptake by macrophages was not an effect of increased activation of the macrophages resulting from exposure to metals (Cheng and Sullivan 1984, Fournier *et al* 2000), macrophage monolayers were exposed to the 1 hour elemental mixture, 24 hour elemental mixture, 7H9 broth, and DMEM then washed and infected with MAC. In this assay, bacteria were taken up by macrophages with equal efficiency (Figure 3.1B), suggesting that the bacteria undergo a phenotypic change that results in more efficient macrophage uptake when exposed to the elemental mixtures.

Gene expression by real-time PCR. Real-time PCR analysis of bacteria exposed to the 24 hour elemental mixture for 24 hours indicated several genes that are upregulated in macrophages were also upregulated in the 24 hour elemental mixture (Figure 3.2). These upregulated genes were *Mav1094*, the homolog to *mprA*, which is part of a 2-component regulatory system protein that induces *sigE* expression in *M. tuberculosis (*He *et al* 2006*), Mav4487,* a PE-PGRS family protein involved in

bacteria to macrophage contact in the Bacille Calmette-Guerin strain of *M. bovis* (Brennan *et al* 2001), *Mav1365,* the homologue to *sigE*, an alternative sigma factor required for *M. tuberculosis* virulence in mice (Manganelli *et al* 2001), *Mav2420,* a hypothetical protein within a pathogenicity island (Danelishvili *et al* 2007), *Mav0996,* a gene that promotes the resuscitation of non-growing cells (Hou *et al* 2002), *Mav0660*, the homolog to *mce4D* and a member of a family of proteins potentially involved in mammalian cell entry (Hou *et al* 2002) and *Mav2409* the homolog to *tatA*, a gene of the twin-arginine translocase system, which is used to transport folded proteins across the mycobacterial membrane (Hou *et al* 2002, McDonough *et al* 2005).

Macropinocytosis. Fluorescent microscopy of macrophages infected by rhodamine-labeled MAC in the presence of unincorporated rhodamine showed large fluorescent circles and fluorescent bacteria for MAC previously incubated in the 24 hour elemental mixture, while MAC from 7H9 broth showed only fluorescent bacteria; suggesting that some MAC from the 24 hour elemental mixture were taken up by macropinocytosis, while bacteria from 7H9 broth entered by phagocytosis, similar to previously described data regarding MAC from macrophages vs. MAC from broth (Figure 3.3A,B,C) (Bermudez *et al* 2004). Taken together, these results suggest that MAC incubated in 24 hour elemental mixture behaved similarly to MAC that have been inside of macrophages.

Minimal requirements for induction of the intracellular phenotype. To determine which component(s) of the 24 hour elemental mixture induce the MAC intracellular phenotype, we made several versions of our elemental mixture, labeled mixture A through mixture F, each of which was missing one component more than the previous

version (Table 3.3). We then extracted RNA from bacteria incubated in each of the elemental mixtures and carried out real-time PCR to determine which mixture no longer induced gene upregulation. Real-time PCR on bacteria exposed to the 24 hour elemental mixture that was pH adjusted to 6.9 (similar to 7H9 broth) indicated that the *tatA* homolog *Mav2409,* was no longer upregulated, suggesting this gene was upregulated in response to the low pH. Other genes such as the alternative sigma factor *Mav1365* and a gene involved in growth of dormant cells, *Mav0996* were still upregulated, suggesting response to changes in metal concentrations (Figure 3.4). Several of the genes were upregulated similarly to the 24 hour elemental mixture until elemental mixture E, which only contained potassium chloride and calcium chloride (Figure 3.5). Similarly, bacteria incubated in the E elemental mixture did not get taken up by macrophages any more efficiently than bacteria incubated in 7H9 broth, but bacteria incubated in the D elemental mixture, which contained potassium chloride, calcium chloride and manganese chloride, had a more efficient uptake rate (Figure 3.6). Likewise, microscopy images of bacteria from the D elemental mixture indicated entry in macrophages by macropinocytosis and phagocytosis, while microscopy of bacteria from the E elemental mixture only suggested entry by phagocytosis (Figure 3.3D, E). The difference between the inducing D mixture and non-inducing E mixture was the addition of manganese chloride into mixture D. We therefore hypothesized that manganese chloride might be responsible for the induction of the intracellular phenotype.

To test manganese chloride, MAC were exposed to 7H9 broth containing the same physiologically relevant concentration of manganese chloride as the 24 hour

elemental mixture for 24 hours and real-time PCR and fluorescent microscopy analyses were done as before. As a negative control, we exposed MAC to 7H9 broth containing the same concentration of copper sulfate as the 24 hour elemental mixture. To determine if the genes were upregulated in response to manganese or chloride, we added calcium chloride at the concentration that manganese chloride was in the 24 hour elemental mixture to 7H9 broth in another sample. As expected, exposure to the copper sulfate did not induce changes in gene expression (Figure 3.7). MAC exposed to the physiological concentration of manganese chloride showed upregulation of the protein that binds the SigE promoter, *Mav1094*, PE-PGRS family protein *Mav4487*, and growth promoting protein *Mav0996*, but not the hypothetical invasion protein *Mav3301* or alternative sigma factor SigE *Mav1365*. To our surprise, MAC exposed to the calcium chloride control showed upregulation in not only the *mprA* homolog *Mav1094* and resuscitation gene *Mav0996*, but also in the hypothetical protein *Mav3301* and sigE *Mav1365,* suggesting that *Mav3301* and *Mav1365* respond to high concentrations of calcium. The fluorescent microscopy for bacteria incubated in manganese chloride showed no evidence of macropinocytosis (Figure 3.3F). To determine what role the calcium chloride had in the regulation of these genes, we also exposed MAC to a sample containing 7H9 broth with the physiologically relevant amount of calcium chloride. Only our positive control, a zinc-responding gene, *Mav2036* (Milano *et al* 2004) appeared to be upregulated under these conditions (Figure 3.7). These data suggest chloride, manganese, calcium and potassium, together, are necessary for inducing the intracellular phenotype.

Association with vacuole markers. We tested which phagosome markers were present on macrophage vacuoles occupied by bacteria incubated in 24 hour elemental mixture. Using fluorescent microscopy, FITC stained bacteria and Texas-red conjugated antibodies to phagosome marker proteins, we determined the percent co-localization between the stained bacteria and vacuolar proteins. Rab5, Rab7, and EEA-1 co-localized with the bacteria with similar rates between the group incubated in 7H9 broth and the group exposed to the elemental mixture (Figure 3.8 A,B,C). On the other hand, LAMP-1 co-localized with the bacteria exposed to 7H9 broth 30% of the time, while 60% of the stained bacteria from the elemental mixture co-localized with the LAMP-1. This trend with Rab5 and LAMP-1 is consistent with bacteria that have been passed through macrophages prior to macrophage infection, further supporting induction of the intracellular phenotype in our 24 hour elemental mixture (Bermudez *et al* 2004).

Gene expression by microarray analysis. We exposed MAC to 7H9 broth and the 24 hour elemental mixture for 24 hours, extracted RNA, and performed a microarray analysis using a *Mycobacterium avium* subp. *paratuberculosis* whole genome array to determine the genes differentially expressed in the 24 hour elemental mixture vs. 7H9 broth. Twenty-two MAC genes that were upregulated in the elemental mixture at least 1.5 fold higher than in the 7H9 broth were identified, and twenty downregulated genes (Table 3.4). Of note, *Mav3301* which is upregulated in macrophages (Danelishvili *et al* 2004) and was already tested by real-time PCR and was also upregulated by microarray, *Mav3056* was upregulated and has been previously described to be upregulated inside of macrophages (Hou *et al* 2002). None of the downregulated genes

have been described before in literature as being up or downregulated inside of macrophages in MAC. In addition to *Mav3301*, ten of the genes identified by microarray were confirmed by real-time PCR (Table 3.5A,B).

Uptake/Survival in macrophages by overexpression strains. Two genes that were upregulated on a microarray from the 24 hour elemental mixture were *Mav_0460* and *Mav_5178*, which both encode hypothetical proteins of unknown function. We hypothesized that these two genes might be involved in allowing for more efficient entry or more proficient survival in macrophages. To test this hypothesis, we upregulated these two genes, individually, in *M. smegmatis* and infected murine Raw 264.7 macrophages with these strains and a strain containing the empty vector as a control. The strain overexpressing *Mav_0460* was less efficient at entering macrophages, but able to survive up to 1 day in a manner similar to the control (Figure 3.9). The *Mav_5178* overexpression strain behaved similar to the control in this assay.

Cell death. We hypothesized that MAC that have been inside of cells induce macrophage apoptosis more efficiently than bacteria that have been grown in 7H9 broth. To test this, we exposed MAC to 7H9 broth and the 24 hour elemental mixture for 24 hours, then infected Raw 264.7 cells. Two hours later, we determined the percent cell death. Raw 264.7 cells that were infected by bacteria exposed to the elemental mixture had a death rate of 19.5%, but only 8.6% of the cells infected by bacteria from 7H9 broth were dead (Figure 3.10). To elucidate if the cell death was apoptotic, the same assay was repeated using the TUNEL assay. This time, 19.8% of cells infected by bacteria from elemental mixture gave a positive (dead) TUNEL signal, while 11% of cells from bacteria exposed to 7H9 broth indicated apoptotic

death by TUNEL assay (Figure 3.11). We also performed control tests in which the Raw 264.7 cells were treated with 7H9 broth and 24 hour elemental mixture without bacteria, and no significant difference in cell death by live-dead or TUNEL assay was observed.

To confirm that the increase in percent macrophage cell death in the elemental mixture was consistent with the intracellular phenotype, Raw 264.7 cell monolayers were infected with bacteria that were recovered from macrophages and percent cell death was compared to macrophages infected by bacteria from 7H9 broth. In these assays, only 3.4% of macrophages infected by bacteria from 7H9 broth stained dead, while 8.5% of macrophages infected by bacteria from other macrophages were dead (Figure 3.12). We found no difference in percent death between our control groups: uninfected macrophages, macrophages inoculated with 7H9 broth, and macrophages inoculated by macrophage lysate. These data suggest that bacteria of the intracellular phenotype induce apoptosis of the host macrophage more efficiently than bacteria from 7H9 broth.

Discussion

While others have not been successful mimicking the macrophage *in vitro* for MAC (Sturgill-Koszycki *et al* 1997), we attribute our success to the physiologically relevant concentrations of metals in the MAC vacuole (Wagner *et al* 2005) and the use of several metals together. Consistent with previous reports regarding MAC from macrophages, we observed that MAC from 24 hour elemental mixture was taken up by macrophages 8 times more efficiently than MAC from 7H9 broth and used

macropinocytosis in addition to phagocytosis as an entry mechanism (Bermudez *et al* 1997, Bermudez *et al* 2004). While we observed upregulation of several genes that have previously been reported to be upregulated in macrophages in our 24 hour elemental mixture, other genes were not. This could be due to differences in technique, i.e. real-time PCR vs. promoter-GFP system, or due to differences in the time point of expression inside of macrophages, but is more likely because our mimicry system only contains metals and pH changes, and these genes may require proteins, lipids, or other factors specific to the macrophage that were not included in our elemental mixture.

Despite several differences in metal concentration between the 1 hour elemental mixture and the 24 hour elemental mixture, incubation of MAC in either mixture resulted in increased uptake by macrophages. We did not examine the 1 hour elemental mixture in detail in this study, so we are unsure if other traits of the MAC intracellular phenotype were induced with the 1 hour elemental mixture. Considering the D elemental mixture, which contained potassium chloride, calcium chloride and manganese chloride at the concentrations relevant to the vacuole 24 hours post infection, was able to induce the MAC intracellular phenotype, it seems valuable to compare the concentrations of these components in the 1 hour elemental mixture to the 24 hour elemental mixture. The 1 hour elemental mixture contains higher concentrations of potassium chloride (29.4mM vs. 1.9mM) and calcium chloride (4mM vs. 2.5mM), but a lower concentration of manganese chloride (11.8mM vs. 23.8mM) than the 24 hour elemental mixture. Further study in determining which components of the 1 hour elemental mixture are necessary to induce the intracellular

phenotype will help narrow down the elemental triggers and required concentrations for inducing the MAC intracellular phenotype.

Mav2036, our positive control for real-time PCR, did not appear to be upregulated in response to physiological concentrations of zinc, but was upregulated in *M. tuberculosis* at similar zinc concentrations (Milano *et al* 2004). This gene has not been studied in MAC, and the difference in gene regulation could be one of several differences between the two species of *Mycobacteria*. Alternately, the addition of several other metals to the elemental mixture may have masked the effect of zinc on *Mav2036*.

Mav1094, the homolog to *M. tuberculosis mprA*, was upregulated in some conditions in which *Mav1365*, the homolog to *M. tuberculosis sigE* was not. In *M. tuberculosis*, *sigE* is upregulated in macrophages, and is essential for virulence (Jensen-Cain and Quinn 2001, Manganelli *et al* 2004, Mangnaelli *et al* 2001). MprA binds to the promoter region of *sigE* to lead to an increased expression of *sigE* (He *et al* 2006). In MAC, the promoter region of *sigE* contains an untested putative binding motif for MprA, but our data suggests an additional step of regulation between overexpression of *mprA* and overexpression of *sigE* since upregulation of the MprA homolog, such as in the E mixture, still showed no upregulation of the SigE homolog. Alternatively, MAC may use a different or additional method of *sigE* regulation than what is observed in *M. tuberculosis*.

Upregulation of *Mav_0460* in *M. smegmatis* resulted in a decrease of bacterial uptake by macrophages. This is a hypothetical protein, containing a transmembrane domain, with homologous genes in both *M. smegmatis (MSMEG_6182)* and *M.*

tuberculosis (Rv3669). In all three of these mycobacterial species, the gene just after it encodes for a hydrolase. The hydrolase of *M. tuberculosis*, *ephE,* is classified as a virulence/adaptation type gene and is thought to be involved in detoxification reactions following oxidative damage to lipids. It would be interesting to determine if overexpression of *Mav_0460* in *M. smegmatis* resulted in excess transport of the lipid hydrolase, resulting in an altered bacterial cell wall that hindered macrophage uptake.

The means by which manganese chloride, calcium chloride and potassium chloride together were able to induce the intracellular phenotype of MAC is unknown. Manganese contributes to virulence of other bacteria, such as *Salmonella typhimurium*, and *Bacillus anthracis* via the use of Nramp and ABC transporters. (Boyer *et al* 2002, Gat *et al* 2005, reviewed in Papp-Wallace and Maguire 2006). In *Pseudomonas aeruginosa* and *Yersinia*, type III secretion system genes are upregulated and its effectors are transported in response to low concentrations of calcium, similar to what happens when in contact with host cells (Frank 1997, Kim *et al* 2005, Vallis *et al* 1999, Straley *et al* 1993). Similarly, in *Salmonella*, potassium induces expression of the SPI1 type III secretion system and increases host cell invasion (Su *et al* 2008). While MAC have no type III secretion system, the *M. tuberculosis* potassium/proton antiporter kefB (homolog=*Mav_4198*) was found to contribute to virulence in mice (Stewart *et al* 2005, Sassetti *et al* 2003). *M avium* also contain Nramp homologs and ABC homologs, both of which are known to utilize manganese, often preferentially over other divalent metals (Jabado *et al* 2000, Kehres *et al* 2000). In *M. tuberculosis, mramp* does not appear to be required for virulence (Boechat *et al* 2002), but our study suggests an important role for manganese in MAC

virulence that may be through the nramp homolog, or some other gene(s). Likewise, the mycobacterium-specific family of PE_PGRS proteins, some of which play a role in virulence, contain calcium-binding repeats (Bachhawat *et al* 2007). Further study would potentially shed light unto the molecular details of how these genes and others respond to specific metals and are used by MAC for virulence.

The observation that bacteria sense their environment and respond, potentially in a way to enhance virulence, is not new (Gancz *et al* 2008, Altier 2005). In other bacteria, such as *Escherichia coli, Pseudomonas aeruginosa* and *M. tuberculosis*, sensor kinases of 2-component regulatory systems detect environmental changes, which leads to phosphorylation of the response regulator, this induces a conformational change that permits binding of the response regulator to promoters of the DNA, resulting in changes in gene expression and phenotype (Igo *et al* 1989, Gooderham and Hancock 2008, Saini *et al* 2004). In MAC, the *mtrA/B* system has already been shown to contribute to virulence, but its environmental cues have not been determined (Cangelosi *et al* 2006). Based on sequence similarity, other potential two component regulatory systems are present in MAC but their environmental stimuli and role in virulence have not been elucidated. The new incubation media presented in this study could become a useful laboratory tool to further decipher the sensing mechanisms of the intracellular phenotype of MAC.

Our observation that MAC from 24 hour elemental mixture and MAC from macrophages results in a higher percentage of macrophage death brings forth the question of whether the bacteria or the macrophage is responsible for inducing cell death/apoptosis, at least in the 2nd macrophage. It has previously been assumed that

apoptosis of the macrophage is induced by the host as a defense mechanism (Fratazzi *et al* 1999). One could surmise, however, that bacteria induce apoptosis, especially after passage through macrophages, as a method of macrophage escape, which would aid with the process of dissemination. Alternatively, MAC within the host may have a preference for a subpopulation of macrophages, and therefore MAC escape macrophages until they enter a macrophage with the desired characteristics, similar to the association of *Listeria monocytogenes* with a subpopulation of macrophages (Harrington-Fowler and Wilder 1982). It is also possible that an effect of the method of bacterial entry into the second macrophage, i.e. macropinocytosis, may induce apoptosis, resulting in our observed increase in percent cell death. More research with this aspect could lead to important discoveries of how MAC cause disseminated disease in AIDS patients.

Acknowledgements

The authors thank Denny Weber for manuscript assistance, Caprice Rosato for help with the microarray, and Dr. John Bannantine and Dr. Mike Paustian at the National Animal Center in Ames, IA for providing the microarray.

References

Altier C. Genetic and environmental control of salmonella invasion. J Microbiol. 2005 Feb;43 Spec No:85-92.

Bachhawat N, Singh B. Mycobacterial PE_PGRS proteins contain calcium-binding motifs with parallel beta-roll folds. Genomics Proteomics Bioinformatics. 2007 Dec;5(3-4):236-41.

Bermudez LE, Parker A, Goodman JR. Growth within macrophages increases the efficiency of Mycobacterium avium in invading other macrophages by a complement receptor-independent pathway. Infect Immun. 1997 May;65(5):1916-25.

Bermudez LE, Petrofsky M, Sangari F. Intracellular phenotype of Mycobacterium avium enters macrophages primarily by a macropinocytosis-like mechanism and survives in a compartment that differs from that with extracellular phenotype. Cell Biol Int. 2004;28(5):411-9.

Boechat N, Lagier-Roger B, Petit S, Bordat Y, Rauzier J, Hance AJ, Gicquel B, Reyrat JM. Disruption of the gene homologous to mammalian Nramp1 in Mycobacterium tuberculosis does not affect virulence in mice. Infect Immun. 2002 Aug;70(8):4124-31.

Boyer E, Bergevin I, Malo D, Gros P, Cellier MF. Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. Infect Immun. 2002 Nov;70(11):6032-42.

Brennan MJ, Delogu G, Chen Y, Bardarov S, Kriakov J, Alavi M, Jacobs WR Jr. Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. Infect Immun. 2001 Dec;69(12):7326-33.

Cangelosi GA, Do JS, Freeman R, Bennett JG, Semret M, Behr MA. The two-component regulatory system mtrAB is required for morphotypic multidrug resistance in Mycobacterium avium. Antimicrob Agents Chemother. 2006 Feb;50(2):461-8.

Cheng TC, Sullivan JT. Effects of heavy metals on phagocytosis by mollusca hemocytes. Mar. Envir. Res, 1984 Jan(14):305-15.

Danelishvili L, Poort MJ, Bermudez LE, Identification of Mycobacterium avium genes up-regulated in cultured macrophages and in mice. FEMS Microbiol Lett. 2004 Oct 1;239(1):41-9.

Danelishvili, L, Wu M, Stang B, Harriff M, Cirillo JD, Bildfell R, Arbogast B, Bermudez LE. Identification of Mycobacterium avium pathogenicity island important for macrophage and amoeba infection. Proc natl Acad Sci. 2007 June 26;104 (26);11038-43.

Danelishvili L, Wu M, Young LS, Bermudez LE. Genomic approach to identifying the putative target of and mechanisms of resistance to mefloquine in mycobacteria. Antimicrob Agents Chemother. 2005 Sep;49(9):3707-14.

Feodorova VA, Devdariani ZL. Expression of acid-stable proteins and modified lipopolysaccharide of Yersinia pestis in acidic growth medium. J Med Microbiol. 2001 Nov;50(11):979-85.

Fournier M, Cyr D, Blakley B, Boermans H, Brousseau P. Phagocytosis as a biomarker of immunotoxicity in wildlife species exposed to environmental xonobiotics. American Zoologist 2000 40(3):412-20.

Frank DW. The exoenzyme S regulon of Pseudomonas aeruginosa. Mol Microbiol. 1997 Nov;26(4):621-9.

Fratazzi C, Arbeit RD, Carini C, Balcewicz-Sablinska MK, Keane J, Kornfeld H, Remold HG. Macrophage apoptosis in mycobacterial infections. J Leukoc Biol. 1999 Nov;66(5):763-4.

Gancz H, Jones KR, Merrell DS. Sodium chloride affects Helicobacter pylori growth and gene expression. J Bacteriol. 2008 Jun;190(11):4100-5.

Gao LY, Pak M, Kish R, Kajihara K, Brown EJ. A mycobacterial operon essential for virulence in vivo and invasion and intracellular persistence in macrophages. Infect Immun. 2006 Mar;74(3):1757-67.

Gat O, Mendelson I, Chitlaru T, Ariel N, Altboum Z, Levy H, Weiss S, Grosfeld H, Cohen S, Shafferman A. The solute-binding component of a putative Mn(II) ABC transporter (MntA) is a novel Bacillus anthracis virulence determinant. Mol Microbiol. 2005 Oct;58(2):533-51.

Gooderham WJ, Hancock R. Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa. FEMS Microbiol Rev. 2008 Oct 13;33(2):279-94.

Haile Y, Caugant DA, Bjune G, Wiker HG. Mycobacterium tuberculosis mammalian cell entry operon (mce) homologs in Mycobacterium other than tuberculosis (MOTT). FEMS Immunol Med Microbiol. 2002 Jun 3;33(2):125-32.

Harrington-Fowler L, Wilder MS. Fate of Listeria monocytogenes in murine peritoneal macrophage subpopulations. Infect Immun. 1982 Jan;35(1):124-32.

He H, Hovey R, Kane J, Singh V, and Thomas C. Zahrt. MprAB Is a Stress-Responsive Two-Component System That Directly Regulates Expression of Sigma Factors SigB and SigE in Mycobacterium tuberculosis. Journal of Bacteriology. 2006 Mar. 188(6):2134-2143.

Hou JY, Graham JE, Clark-Curtiss JE. Mycobacterium avium genes expressed during growth in human macrophages detected by selective capture of transcribed sequences (SCOTS). Infect Immun. 2002 Jul;70(7):3714-26.

Igo MM, Ninfa AJ, Silhavy TJ. A bacterial environmental sensor that functions as a protein kinase and stimulates transcriptional activation. Genes Dev. 1989 May;3(5):598-605.

Iyer R, Iverson TM, Accardi A, Miller C. A biological role for prokaryotic ClC chloride channels. Nature. 2002 Oct 17;419(6908):715-8.

Jabado N, Jankowski A, Dougaparsad S, Picard V, Grinstein S, Gros P. Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane. J Exp Med. 2000 Nov 6;192(9):1237-48.

Jensen-Cain DM, Quinn FD. Differential expression of sigE by Mycobacterium tuberculosis during intracellular growth. Microb Pathog. 2001 May;30(5):271-8.

Kehres DG, Zaharik ML, Finaly BB, Maguire ME. The NRAMP proteins of Salmonella typhimurium and Escherichia coli are selective manganese transporters involved in the response to reactive oxygen. Mol. Microbiol. 2000 Jun;36(5):1085-100.

Kim J, Ahn K, Min S, Jia J, Ha U, Wu D, Jin S. Factors triggering type III secretion in Pseudomonas aeruginosa. Microbiology. 2005 Nov;151(Pt 11):3575-87.

Kuhn DE, Lafuse WP, Zwilling BS. Iron transport into mycobacterium avium-containing phagosomes from an Nramp1(Gly169)-transfected RAW264.7 macrophage cell line. J Leukoc Biol. 2001 Jan;69(1):43-9.

Li Y, Miltner E, Wu M, Petrofsky M, Bermudez LE. A Mycobacterium avium PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. Cell Microbiol. 2005 Apr;7(4):539-48.

Manganelli R, Fattorini L, Tan D, Iona E, Orefici G, Altavilla G, Cusatelli P, Smith I. The extra cytoplasmic function sigma factor sigma(E) is essential for Mycobacterium tuberculosis virulence in mice. Infect Immun. 2004 May;72(5):3038-41.

Manganelli R, Voskuil MI, Schoolnik GK, Smith I. The Mycobacterium tuberculosis ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. Mol Microbiol. 2001 Jul;41(2):423-37.

McDonough JA, Hacker KE, Flores AR, Pavelka MS Jr, Braunstein M. The twin-arginine translocation pathway of Mycobacterium smegmatis is functional and required for the export of mycobacterial beta-lactamases. J Bacteriol. 2005 Nov;187(22):7667-79.

Milano A, Branzoni M, Canneva F, Profumo A, Riccardi G. The Mycobacterium tuberculosis Rv2358-furB operon is induced by zinc. Res Microbiol. 2004 Apr;155(3):192-200.

Oh YK, Straubinger RM. Intracellular fate of Mycobacterium avium: use of dual-label spectrofluorometry to investigate the influence of bacterial viability and opsonization on phagosomal pH and phagosome-lysosome interaction. Infect Immun. 1996 Jan;64(1):319-25.

Papp-Wallace K, Maguire, M. Manganese Transport and the Role of Manganese in Virulence. Annu. Rev. Microbiol. 2006. 60:187-209.

Plum G, Brenden M, Clark-Curtiss JE, Pulverer G. Cloning, sequencing, and expression of the mig gene of Mycobacterium avium, which codes for a secreted macrophage-induced protein. Infect Immun. 1997 Nov;65(11):4548-57.

Saini DK, Malhotra V, Tyagi JS. Cross talk between DevS sensor kinase homologue, Rv2027c, and DevR response regulator of Mycobacterium tuberculosis. FEBS Lett. 2004 May 7;565(1-3):75-80.

Sassetti CM, Rubin EJ. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A. 2003 Oct 28;100(22):12989-94.

Stewart GR, Patel J, Robertson BD, Rae A, Young DB. Mycobacterial mutants with defective control of phagosomal acidification. PLoS Pathog. 2005 Nov;1(3):269-78.

Straley SC, Brubaker RR. Cytoplasmic and membrane proteins of yersiniae cultivated under conditions simulating mammalian intracellular environment. Proc Natl Acad Sci U S A. 1981 Feb;78(2):1224-8.

Straley SC, Plano GV, Skrzypek E, Haddix PL, Fields KA. Regulation by Ca2+ in the Yersinia low-Ca2+ response. Mol Microbiol. 1993 Jun;8(6):1005-10.

Sturgill-Koszycki S, Haddix PL, Russell DG. The interaction between Mycobacterium and the macrophage analyzed by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis. 1997 Dec;18(14):2558-65.

Su J, Gong H, Lai J, Main A, Lu S. The potassium transporter Trk and external potassium modulate Salmonella enterica protein secretion and virulence. Infect Immun. 2009 Feb;77(2):667-75.

Tenant R, Bermudez LE. Mycobacterium avium genes upregulated upon infection of Acanthamoeba castellanii demonstrate a common response to the intracellular environment. Curr Microbiol. 2006 Feb;52(2):128-33.

TranVan Nhieu G, Clair C, Grompone G, Sansonetti P. Calcium signalling during cell interactions with bacterial pathogens. Biol Cell. 2004 Feb;96(1):93-101.

Vallis AJ, Yahr TL, Barbieri JT, Frank DW. Regulation of ExoS production and secretion by Pseudomonas aeruginosa in response to tissue culture conditions. Infect Immun. 1999 Feb;67(2):914-20.

Wagner D, Maser J, Lai B, Cai Z, Barry CE 3rd, Honer Zu Bentrup K, Russell DG, Bermudez LE. Elemental analysis of Mycobacterium avium-, Mycobacterium tuberculosis-, and Mycobacterium smegmatis-containing phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. J Immunol. 2005 Feb 1;174(3):1491-500.

Wagner D, Maser J, Moric I, Boechat N, Vogt S, Gicquel B, Lai B, Reyrat JM, Bermudez L. Changes of the phagosomal elemental concentrations by Mycobacterium tuberculosis Mramp. Microbiology. 2005 Jan;151(Pt 1):323-32.

Wolfgang MC, Lee VT, Gilmore ME, Lory S. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev Cell. 2003 Feb;4(2):253-63.

Table 3.1 Recipe for 1 hour and 24 hour elemental mixtures

Supplement	A (1 hour elemental mixture)	B (24 hour elemental mixture)
1 M Potassium Chloride	14.7ml	0.925ml
1 M Calcium Chloride	2ml	1.25ml
1 M Manganese Chloride	5.9ml	11.9ml
1 M Copper Sulfate	1.85µl	5.5µl
1 M Zinc Chloride	33µl	58.7µl
0.25 M Ferric Pyrophosphate	288µl	2ml
1 M Nickel Chloride	5µl	5µl

The elements listed in column A were added to 500 mL of 7H9 broth to make a mixture that mimics the environment of the vacuole 1 hour post infection. Similarly, column B elements were added to make the 24 hour elemental mixture.

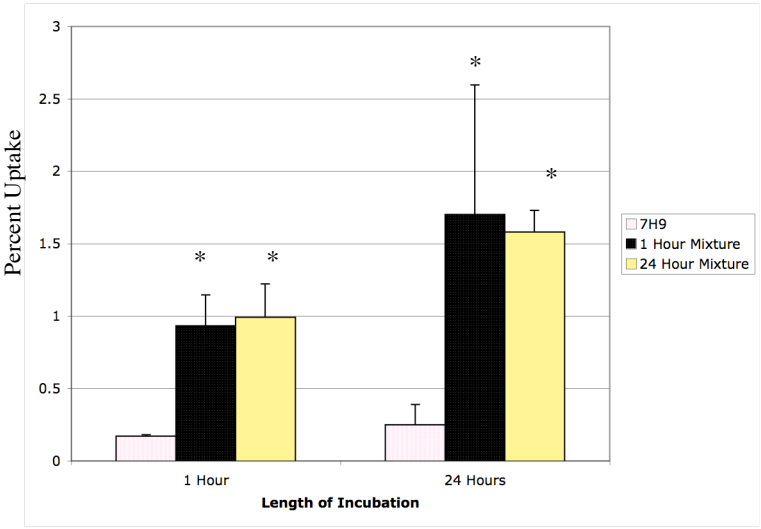
Table 3.2 Primers used for real time PCR in elemental mixture

MAC 104 gene	H37Rv gene	Primers	Reference
Mav1094	Rv0981	5'CTGGAAATGCTGATCGCCAACC3'	He *et al*. 2006
		5'TCACGGCGGCGTCTCCCGAAGCACGTA3'	
Mav3301	Rv1477	5'ATGAGACGCACACGCTGG3'	Gao *et al*. 2004
		5'CTTGTTGACGCTTTCCTG3'	
Mav4487	Rv1818c	5'ATGTCGTTCGTTCAAGCGACTC3'	Brennan *et al*. 2001
		5'GTTCATCAACTGCACGAACTGG3'	
Mav1365	Rv1221	5'GATCTCCTCATACGACAG3'	Manganelli *et al*. 2001
		5'GACCTTCATCCGGGTGTTC3'	
Mav5014	Rv0170	5'CAGACTCAGCATCTTCTC3'	Haile *et al*. 2002
		5'GTCGACCAGCTGCACCGAAC3'	
Mav3270	Rv1592	5'ATGGCAACCGAACTCCGAAAG3'	Danelishvili *et al*. 2007
		5'GAAAACGAAACGACGGGTCGT3'	
Mav2420	Rv3902	5'CACTTGGTGGAGGTGCACCCGACTC3'	Danelishvili *et al*. 2007
		5'GGACGTGATGTTTCCGCTTATTC3'	
Mav1600		5'ATGTCCGACACCACAACAG3'	Plum *et al*. 1997
		5'CTTCGACGAATTCGTTCCC3'	
Mav0996	Rv0867	5'CACCACTTCCAGTGTCAG3'	Hou *et al*. 2002
		5'GTGTTGATGCCCCAGTTG3'	
Mav0660	Rv3496	5'GACTGTTTGACCTCGTCCCAC3'	Hou *et al*. 2002
		5'GTGTATGTGCTGACGTCGCAAG3'	
Mav2409	Rv2096c	5'GTGGGCAGTCTTAGTCCGTG3'	Hou *et al*. 2002
		5'CTTGAAGATCCGCATCGACTT3'	
Mav1424	Rv1275	5'CTGACGGGCTGTTCCCGCTCG3'	Danelishvili *et al* 2004
		5'CTAGGAATTGGTCGCCAGCGT3'	
Mav0467	Rv3663	5'ATGACCACCCCGCTGCTGTCG3'	Danelishvili *et al* 2004
		5'GCCACCTGGTCGACGAGGTCG3'	
Mav3275	Rv1497	5'GCTGGAGCTGGCCGA3'	Danelishvili *et al* 2004
		5'CGGCACGTACAGGTTTCGGTC3'	
Mav2928	Rv1787	5'ACGAATTCACGGTGTTTGACTTCGGAGCG3'	Li *et al*. 2005
		5'ACAAGCTTGTGGACTCTCGGTCGTGTTGAGG3'	
Mav4781	Rv0359	5'CGGCTGGCTGGTGTCGCTGTG3'	Danelishvili *et al* 2004
		5'ACGCCGGAGAAGTCGAAC3'	
Mav2036	Rv2359	5'CGACGGGGCAGGAGTCAG3'	Milano *et al*. 2004
		5'ATGGTGTGGCTGACGTCGGAGAA3'	
Mav4357	Rv3412	5'GACCACCTCCCACCGGGTTT3'	Danelishvili *et al* 2004
		5'TTGAGTGAGGCGTCGGCGTAG3'	

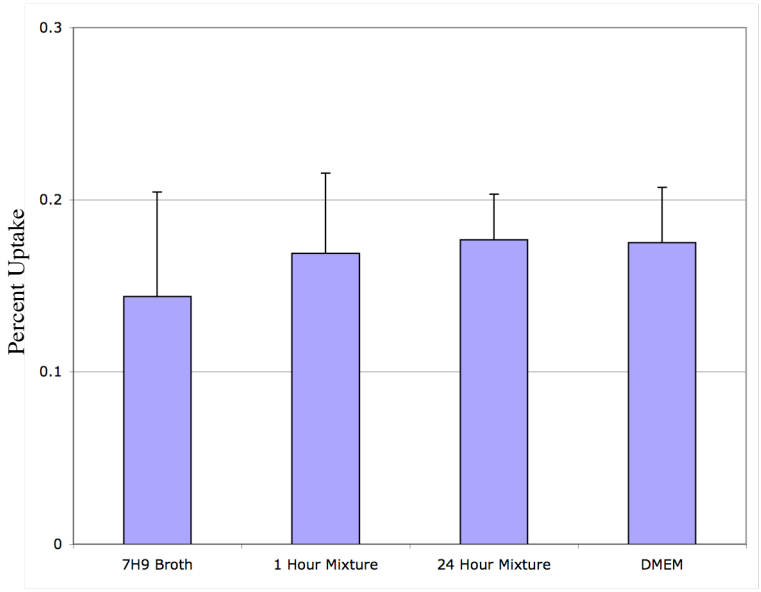
Primers used for real time PCR in the 24 hour elemental mixture, along with the nearest *M. tuberculosis* homolog and reference in which the genes have been proven to be upregulated when MAC are inside of cells.

Figure 3.1 Uptake of MAC incubated in the elemental mixture by macrophages

A

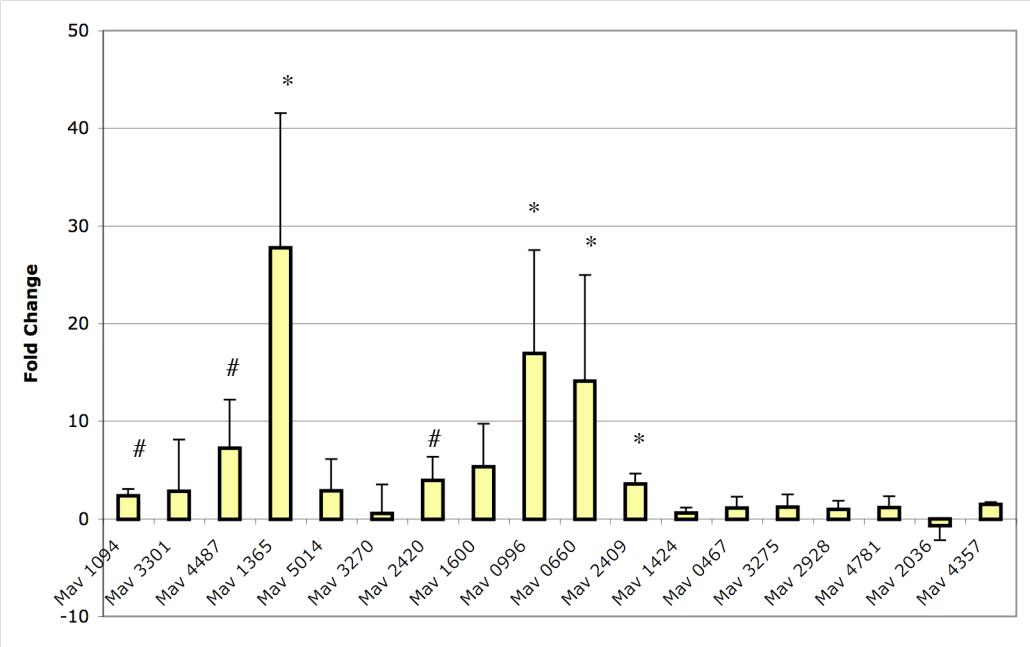


B



A. MAC were incubated in 7H9 broth, the 1 hour elemental mixture and the 24 hour elemental mixture for 1 hour and 24 hours, then used to infect Raw macrophages. Bars indicate the ratio of number of bacteria inside cells divided by inocula from 3 repeats. Bacteria that were incubated in either mixture infected macrophages more efficiently than bacteria in 7H9 broth. * p value <0.05 B. Raw macrophages were incubated in the indicated solution for 1 hour, washed, then infected with MAC. Incubation of macrophages prior to infection had no effect on the rate up bacterial uptake. The assay was repeated 3 times.

Figure 3.2 Real time PCR of MAC 104 exposed to the 24 hour elemental mixture



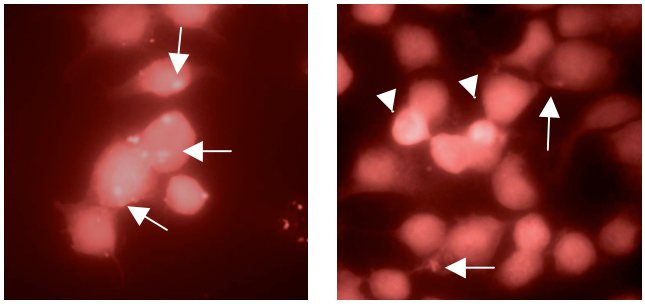
Total bacterial RNA was extracted from MAC 104 exposed to the 24 hour elemental mixture for 24 hours and bacteria exposed to 7H9 broth as a control. cDNA was made from the RNA and used for real-time PCR. Bars represent means and standard deviations from 3 repeats. # p value <0.10
* p value <0.05

Figure 3.3 Uptake of MAC by phagocytosis and macropinocytosis

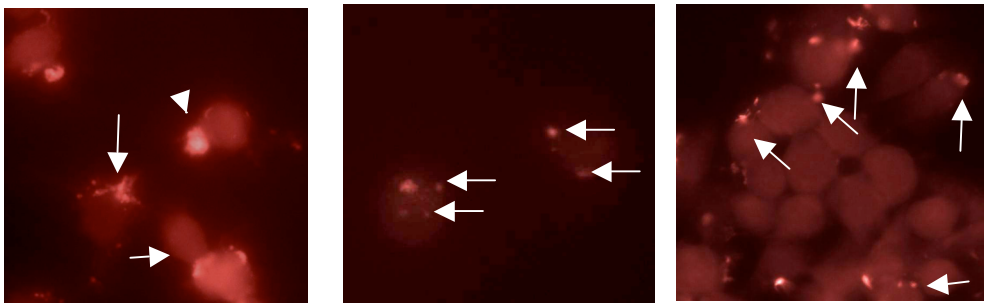
A

	7H9 Broth	24 hour Elemental mixture	D Elemental mixture	E Elemental mixture	Manganese Chloride
Percent of macrophages with punctate bacteria	22	13	26	18	19
Percent of macrophages with large circles	0	27	0	24	0

B C



D E F



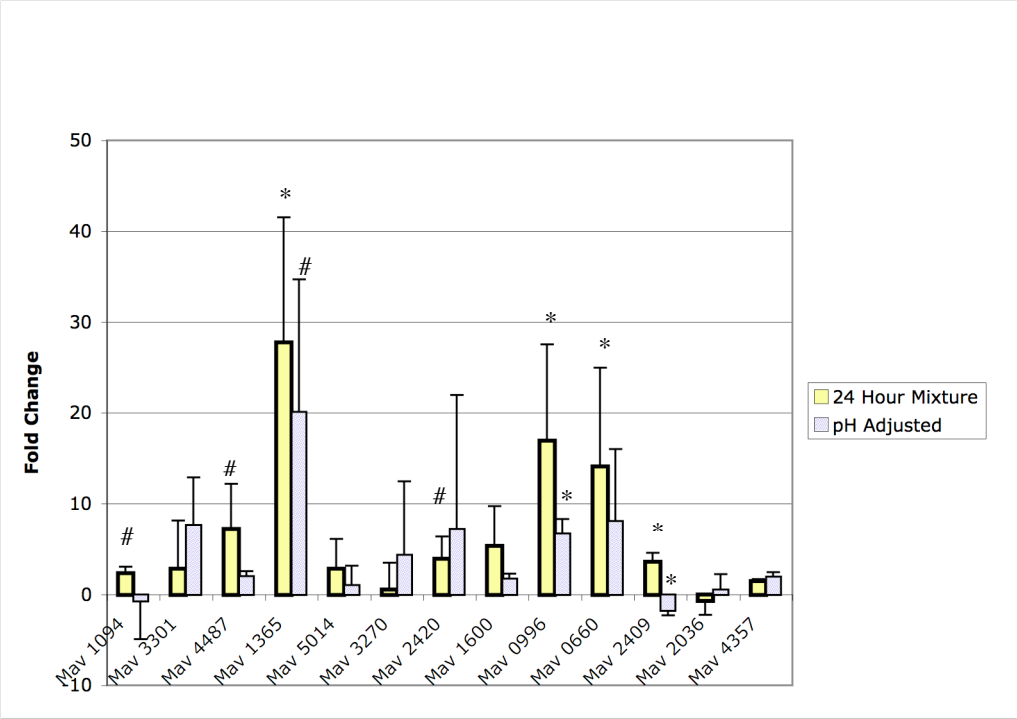
A. Percent of total MAC 104 infected macrophages containing punctate bacteria (first row) or large circles with bacteria inside (second row) following bacterial incubation in indicated solution. B. Raw 264.7 macrophages were infected by rhodamine-labeled MAC incubated in 7H9 broth prior to infection in the presence of rhodamine. C. Bacteria incubated in 24 hour mixture before rhodamine staining. D. Incubation in D mixture. E. Incubation in E mixture. F. Incubation in Manganese Chloride. The experiment was repeated 3 times. Arrows point to evidence of bacterial entry by phagocytosis, arrowheads to evidence of macropinocytosis.

Table 3.3 The reduction scheme used to determine which elements trigger the MAC intracellular phenotype

Supplement	24 hour Elemental mixture	pH adjusted	Mixture A	B	C	D	E	F
pH	5.8	6.9	6.9	6.9	6.9	6.9	6.9	6.9
1 M KCl	0.925ml	0.925ml	0.925ml	0.925ml	0.925ml	0.925ml	0.925ml	0.925ml
1 M $CaCl_2$	1.25ml	1.25ml	1.25ml	1.25ml	1.25ml	1.25ml	1.25ml	
1 M Mn_2Cl	11.9ml	11.9ml	11.9ml	11.9ml	11.9ml	11.9ml		
1 M $CuSo_4$	5.5µl	5.5µl	5.5µl	5.5µl	5.5µl			
1 M $ZnCl_2$	58.7µl	58.7µl	58.7µl	58.7µl				
0.25 M $FePO_4$	2ml	2ml	2ml					
1 M $NiCl_2$	5µl	5µl						

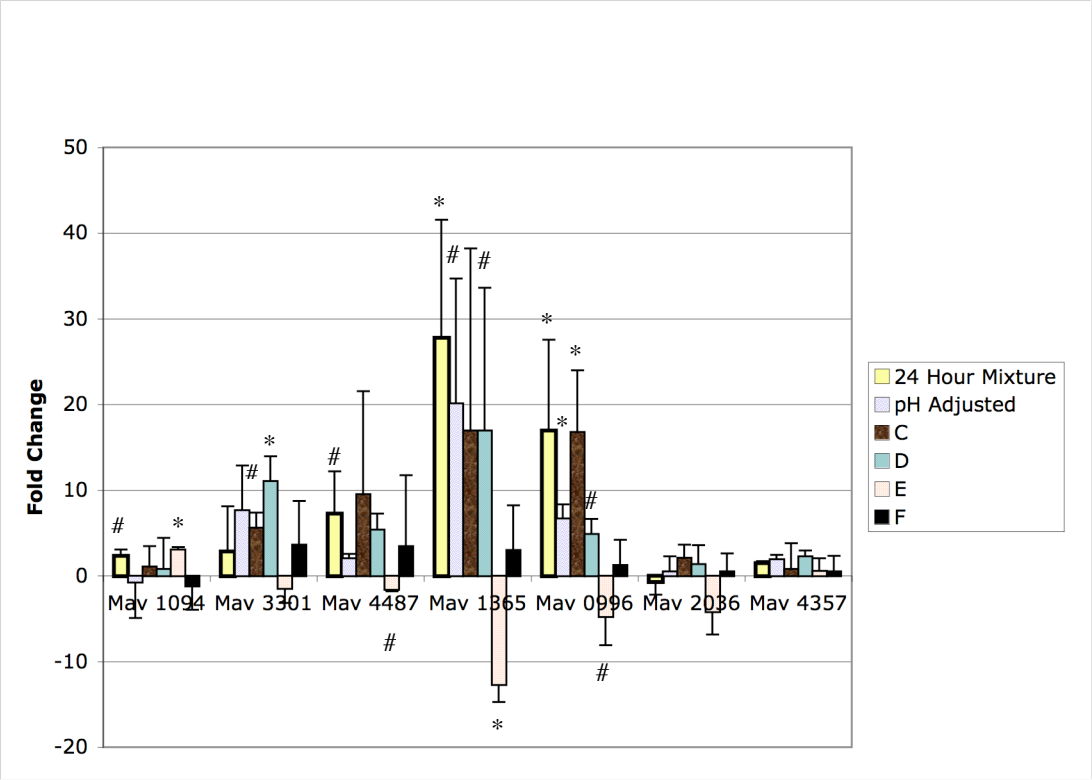
Several versions of the 24 hour elemental mixture were made, each one missing one component more than the previous one.

Figure 3.4 Real time PCR of MAC 104 in pH adjusted 24 hour elemental mixture



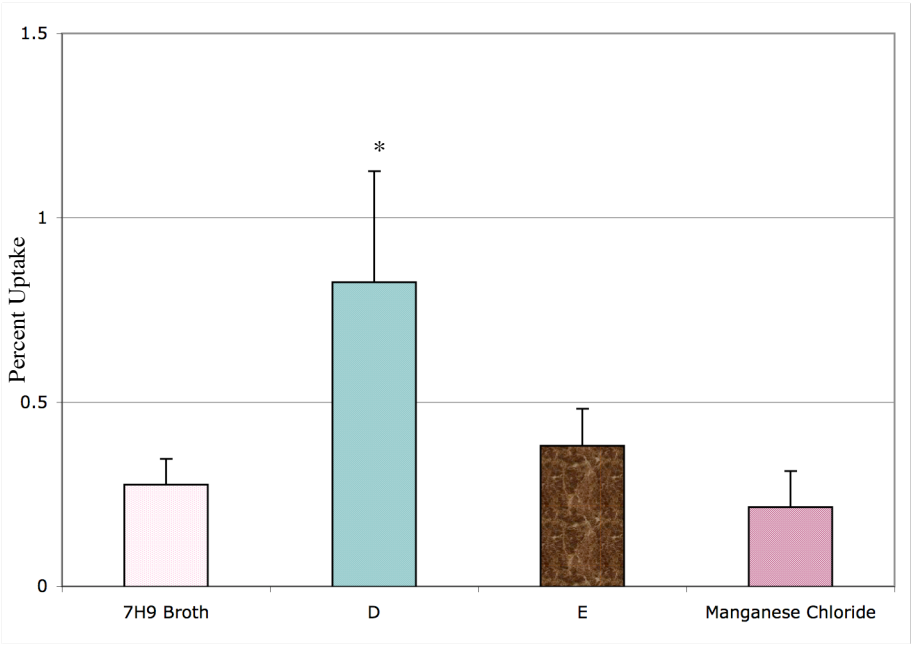
Total bacterial RNA was extracted from bacteria exposed to the 24 hour mixture or the pH adjusted 24 hour mixture for 24 hours and bacteria exposed to 7H9 as a reference. cDNA was made from the RNA and used for real-time PCR. The assay was repeated 3 times. # p value <0.10 * p value <0.05

Figure 3.5 Real time PCR of MAC 104 in the subtracted 24 hour elemental mixtures



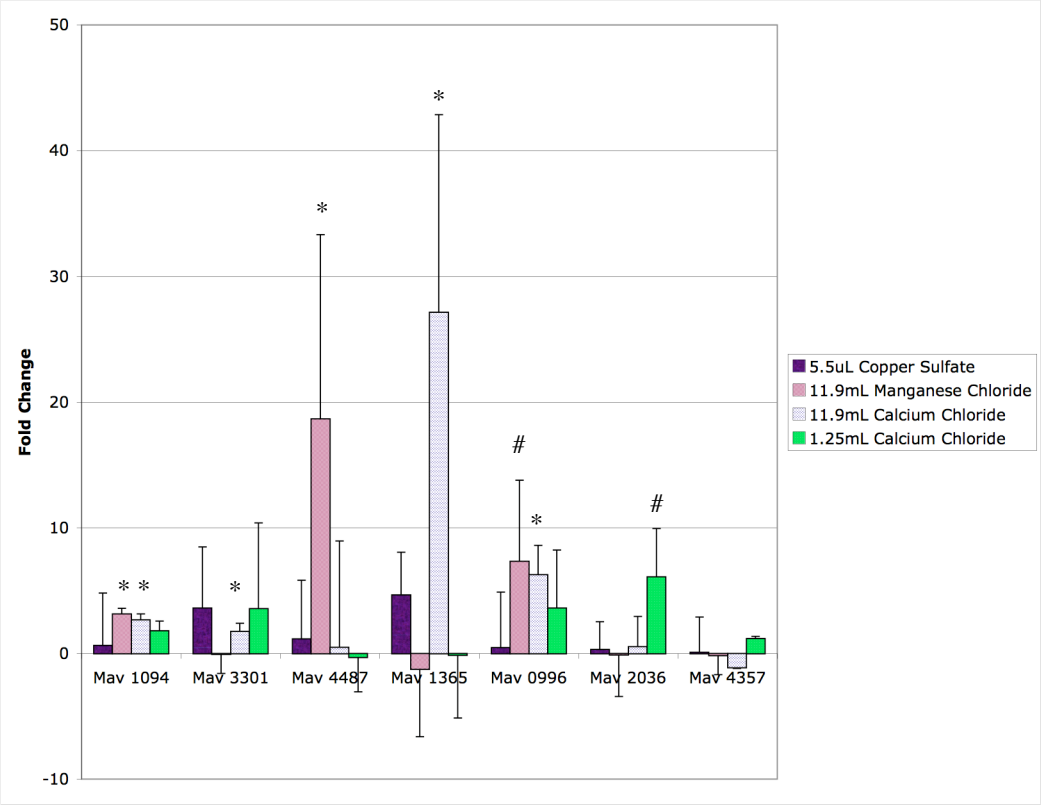
Total bacterial RNA was extracted from bacteria exposed to the indicated mixture for 24 hours and bacteria exposed to 7H9 as a reference. cDNA was made from the RNA and used for real time PCR. Bars indicate means and standard deviations from 3 repeats. # p value <0.10 * p value <0.05

Figure 3.6 Uptake of MAC 104 by macrophages after incubation in subtraction elemental mixtures



MAC were incubated in the indicated mixture for 24 hours, then used to infect Raw 264.7 macrophage monolayers. Percent uptake was determined by dividing the number of bacteria that were inside macrophages after a 1 hour infection by the number of bacteria that were added to the monolayer, then multiplying by 100. The experiment was repeated 3 times. * p value <0.05

Figure 3.7 Real time PCR of MAC 104 incubated in single metals



MAC were exposed to 7H9 broth containing the indicated volume of a 1M concentration of the indicated metal per 500ml of 7H9 broth for 24 hours, then RNA was extracted and used for cDNA synthesis and real time PCR compared to MAC exposed to 7H9 broth. Bars on the graph represent means and standard deviations from 3 repeats. # p value <0.10 * p value <0.05

Figure 3.8 Co-localization of MAC 104 incubated in 7H9 broth or 24 hour elemental mixture with vacuole markers

A

Percent Co-localization				
	Rab5	Rab7	EEA-1	LAMP-1
7H9 Broth	63±16	22±7	46±14	30±13
24 Hour Elemental mixture	71±16	25±7	46±24	60±9*

Raw 264.7 cells were infected with Rhodamine labeled MAC that had previously been incubated in either 7H9 broth or 24 hour elemental mixture for 24 hours. Cells were then washed, treated with amikacin to kill extracellular bacteria, then fixed and permeabolized and appropriately stained for analysis on a fluorescent microscope. The assay was repeated 3 times, with about 40 stained bacteria analyzed per sample per time. A. The percent co-localization determined from this assay. * p value <0.05 B. Representative photos from bacteria incubated in 7H9 broth prior to infection. C. Photos from MAC incubated in 24 hour elemental mixture prior to infection. Arrows and brackets indicate bacteria that co-localize.

Figure 3.8 Continued

B

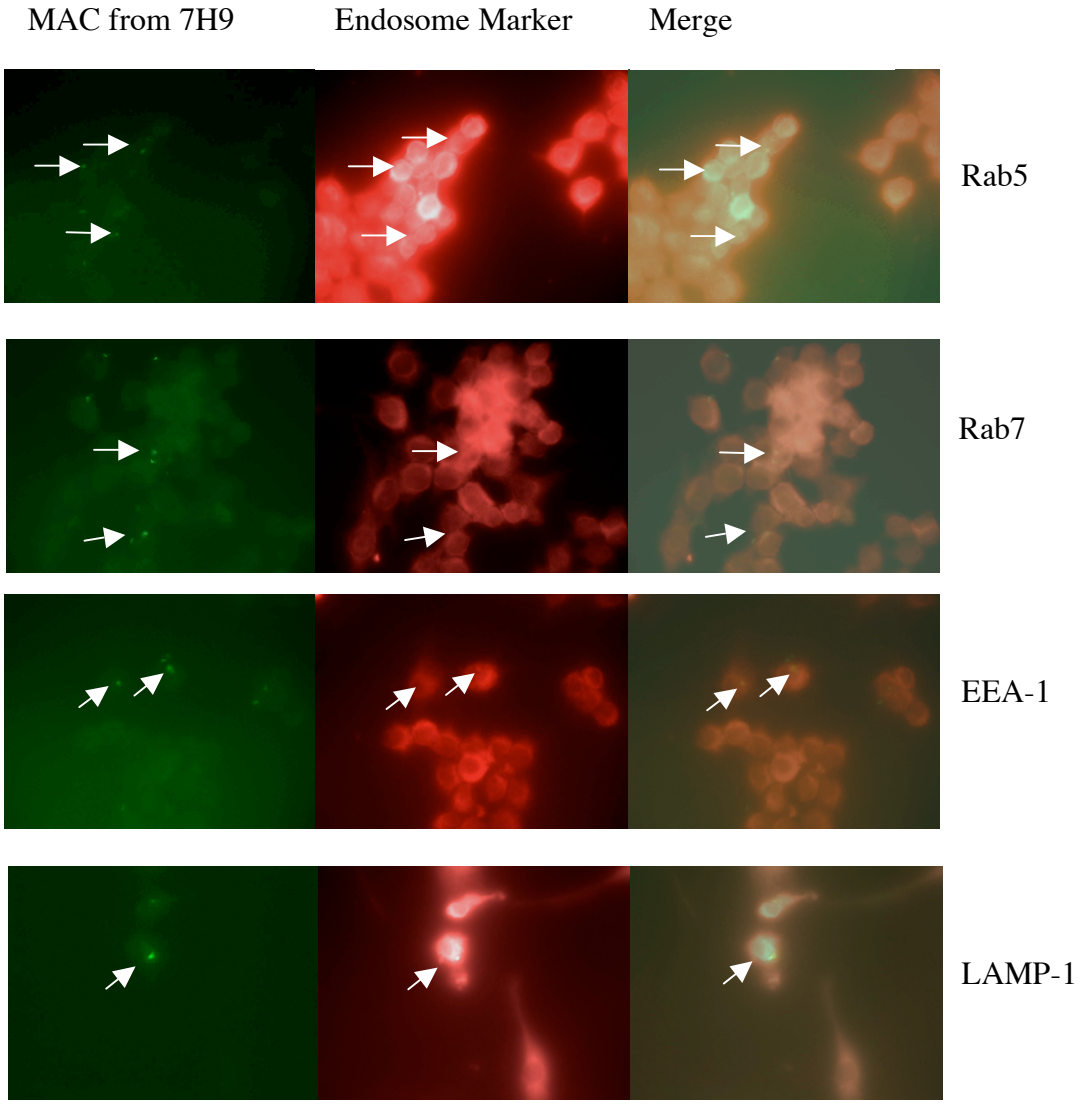


Figure 3.8 Continued

C

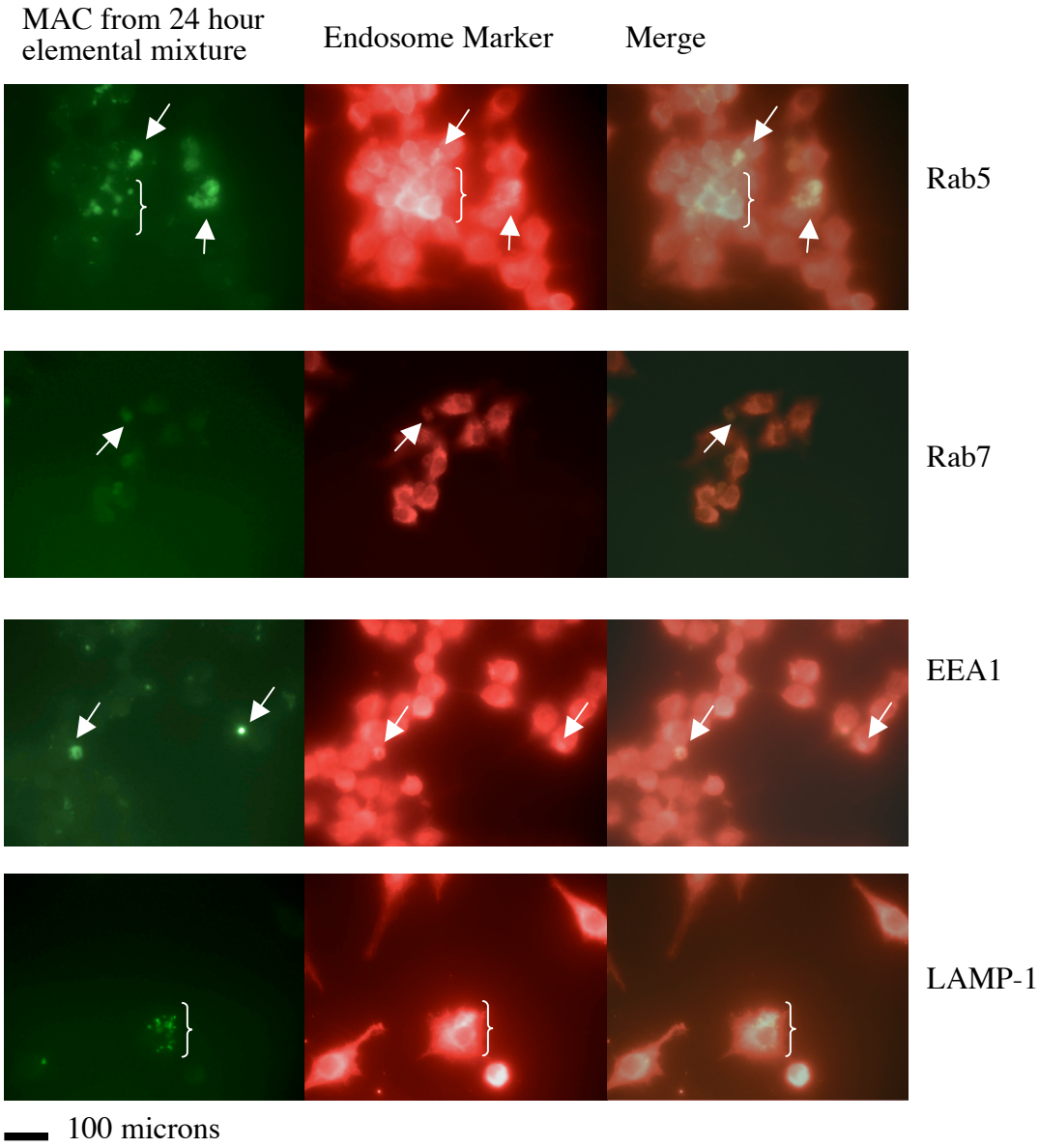


Table 3.4 MAC 104 genes up or down regulated in the 24 hour elemental mixture

Gene	Fold Change	Function
MAV_1134	7.97 ± 2.09	hypothetical protein, no conserved domains
MAV_1222	6.36 ± 1.66	glpX, type II fructose 1,6-bisphosphatae, binds manganese
MAV_5178	5.79 ± 2.06	hypothetical protein, one conserved but uncharacterized domain
MAV_4500	5.35 ± 1.83	FadE8, putative acyl-CoA dehydrogenase
MAV_3301	5.28 ± 0.98	Hypothetical invasion protein, cell wall associated hydrolases
MAV_5258	4.54 ± 2.24	hypothetical protein, conserved domain of unknown function
MAV_4158	4.45 ± 0.62	hypothetical protein, tRNA hydroxylase domain
MAV_3185	4.10 ± 1.19	HisB, imidazoleglycerol-phosphate dehydratase
MAV_3056	3.95 ± 1.54	linear gramicidin synthetase subunit D
MAV_2619	3.95 ± 1.59	acyl-CoA dehydrogenase domain protein
MAV_3055	3.68 ± 2.09	asnB Asparagine synthase
MAV_2646	3.59 ± 2.24	alcohol dehydrogenase B
MAV_0460	3.49 ± 1.39	hypothetical protein
MAV_1960	3.30 ± 1.65	pyruvate phosphate dikinase
MAV_3357	3.17 ± 2.61	hypothetical protein, conserved domain of unknown function
MAV_0491	3.14 ± 1.43	hypothetical protein, conserved cupin domain
MAV_4548	2.97 ± 1.74	virulence factor Mce family protein, Ttg2c ABC transporter domain
MAV_0750	2.96 ± 0.60	Glycine cleavage T-protein aminomethyl transferase
MAV_4326	2.94 ± 1.95	ABC Transporter, ATP Binding domain,
MAV_1181	2.81 ± 1.44	hypothetical protein, methyltransferase conserved domain
MAV_1967	2.76 ± 1.31	hypothetical protein, conserved domain of unknown function
MAV_2042	2.55 ± 1.68	deoxyguanosinetriphosphate triphosphohydrolase-like protein
MAV_2674	-8.43 ± 1.45	hypothetical protein, no conserved domains
MAV_4880	-3.84 ± 1.39	hypothetical protein, conserved domain of unknown function
MAV_4061	-3.76 ± 1.35	hydrolase, α/β fold protein, lysophospholipase and esterase_lipase domain
MAV_4701	-3.25 ± 2.32	hypothetical protein, no conserved domains
MAV_4573	-3.17 ± 1.59	putative leucine corboxyl methyltransferase, hypothetical protein
MAV_5213	-3.05 ± 0.66	oxidoreductase, short chain, fabG, 3-ketoacyl reductase and AdoHcyase
MAV_3234	-2.98 ± 0.91	hypothetical protein, restriction endonuclease like conserved domain
MAV_1281	-2.52 ± 0.69	hypothetical protein, nucleoside diphosphate sugar epimerase, and AdoHcyase
MAV_4305	-2.51 ± 1.24	D-alanyl-D-alanine carboxypeptidase
MAV_2093	-2.34 ± 1.87	hypothetical protein, carboxymuconolactone decarboxylase family domain
MAV_4566	-2.12 ± 1.86	hypothetical protein, conserved domain of unknown function
MAV_1305	-2.10 ± 1.86	narJ nitrate reductase molybdenum cofactor assembly chaperone
MAV_4919	-2.04 ± 1.60	transcriptional regulator, TetR family protein
MAV_3562	-2.02 ± 1.84	acetyltransferase, gnat family protein, hypothetical protein
MAV_2882	-1.97 ± 1.30	hypothetical protein, conserved domain of unknown function
MAV_0776	-1.92 ± 0.66	transcriptional regulator, ArsR family protein
MAV_5291	-1.90 ± 1.84	hypothetical protein, no conserved domains
MAV_3632	-1.73 ± 1.84	hypothetical protein, no conserved domains
MAV_3475	-1.56 ± 1.24	ruvC Holliday junction resolvase
MAV_1391	-1.49 ± 1.20	short-chain dehydrogenase/reductase SDR, fabG, and AdoHcyase domain

Differentially regulated genes were discovered using 2 biological repeats of a *Mycobacterium avium* subspecies *paratuberculosis* whole genome array.

Table 3.5 Confirmation of genes differentially expressed on microarray by real time PCR

A

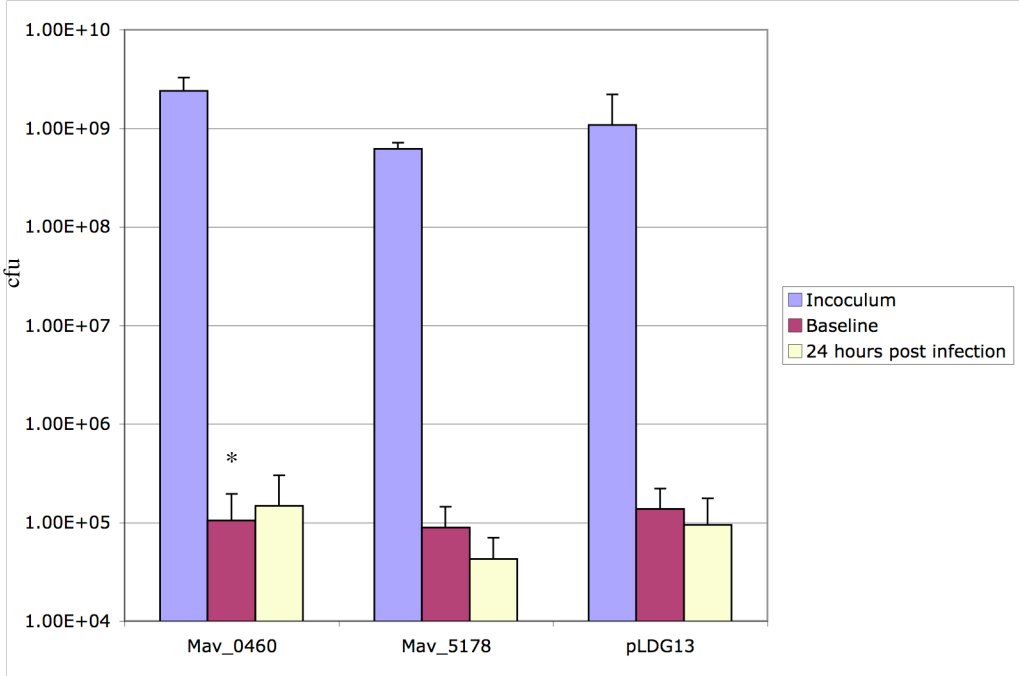
MAC 104 gene	Fold change (microarray)	Fold change (real-time)
Mav_1134	7.97	5.71
Mav_5178	5.79	1.72
Mav_4500	5.35	5.07
Mav_5258	3.91	1.21
Mav_3055	3.67	1.85
Mav_4548	2.97	2.08
Mav_4326	2.94	5.97
Mav_1967	2.76	5.95
Mav_1281	-2.52	-2.00
Mav_4880	-3.84	-1.33

B

MAC Gene	Primers
Mav_1134	5'GATGCCGTTCCGCAACCAC3'
	5'GACGATCAGCGAAAGCGGAC3'
Mav_5178	5'CAGGGCCGCAGCTGCAAGGAC3'
	5'GAAACAGTTTTTCCAGCTCATCG3'
Mav_4500	5'GAGCTGGACACCATCCAGTACCG3'
	5'TCAGCCCTTTACCAGGGCCCGCTC3'
Mav_5258	5'GACCGTGGTTCATCGGCCGGTGAC3'
	5'TCACGAACCGTCCGTCTCGGCGGTG3'
Mav_3055	5'GTGTGCTCAAACAGGCTGCGCCAC3'
	5'TCATGCAACCGGGCCCGTCACAC3'
Mav_4548	5'CGCGGCGACTACGTCAACGTC3'
	5'TCATTCGCCCCTTTCCACCAGC3'
Mav_4326	5'ACGGTGGACCTGTGGACGGTGTC3'
	5'CAACCGGATGCGCCAGCGCACCAG3'
Mav_1967	5'GGGCACTACTTCCATCCGCCGGGAC3'
	5'CAGTCCTCGAAGAAGACCTCGACTG3'
Mav_1281	5'ATGCGGTATGTGGGAGATCG3'
	5'ACCTTGTTCACCAGCCCCAG3'
Mav_4880	5'GTGCGCGAGTATCTGTATCC3'
	5'TCGTAGAGCAGGACGTTGAA3'

A. Fold change of genes up or down regulated in mixture by microarray and Real Time PCR, based on 2 biological repeats. B. Primers used for real time PCR confirmation.

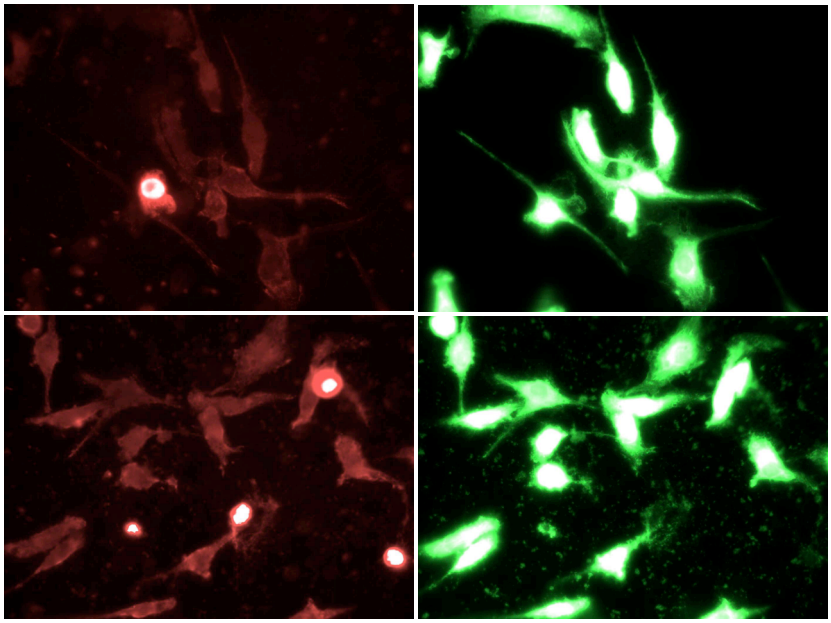
Figure 3.9 Uptake and survival of *M. smegmatis* overexpression strains by macrophages



Raw 264.7 macrophages were infected with *M. smegmatis* that was overexpressing the *Mav_0460* or *Mav_5178* gene, and the rate uptake and ability to survive for 24 hours was compared to the empty vector control, based on 3 repeats. The *Mav_0460* overexpression strain does not enter macrophages as efficiently, but survives as well as the control, and the *Mav_5178* overexpression strain behaves similar to the control. p value <0.05

Figure 3.10 Live dead assay of macrophages infected by MAC 104 from 7H9 broth and 24 hour elemental mixture

Inoculum	Percent of Dead Macrophages
7H9 Broth	4.02 ± 1.6
24 hour elemental mixture	5.04 ± 2.2
MAC from 7H9 Broth	8.58 ± 4.4
MAC from 24 hour elemental mixture	19.52 ± 4.9*

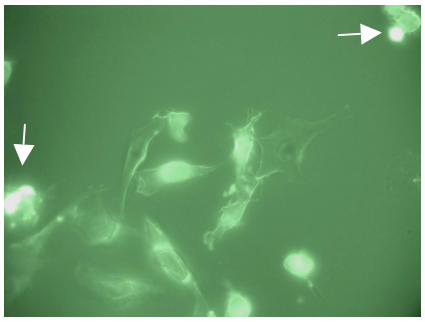


Raw cells infected by MAC exposed to 24 hour elemental mixture have a higher percent cell death. After exposure of MAC to 7H9 broth and the 24 hour elemental mixture, Raw 264.7 cells were infected. Two hours post infection, live-dead assay was performed. The experiment was repeated 3 times. *p value <0.05

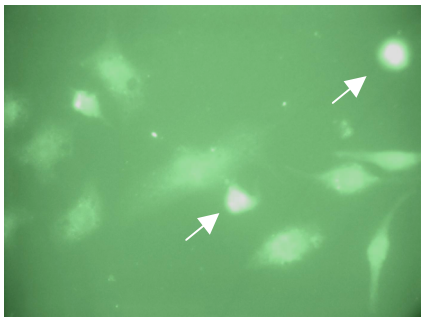
Figure 3.11 TUNEL assay of macrophages infected by MAC 104 from 7H9 broth and 24 hour elemental mixture

Inoculum	Percent of Cells Stained by TUNEL assay
7H9 Broth	12.3 ± 5.0
24 hour elemental mixture	15.8 ± 2.1
MAC from 7H9 Broth	11.0 ± 2.1
MAC from 24 hour elemental mixture	19.8 ± 6.8*

MAC from 7H9 Broth



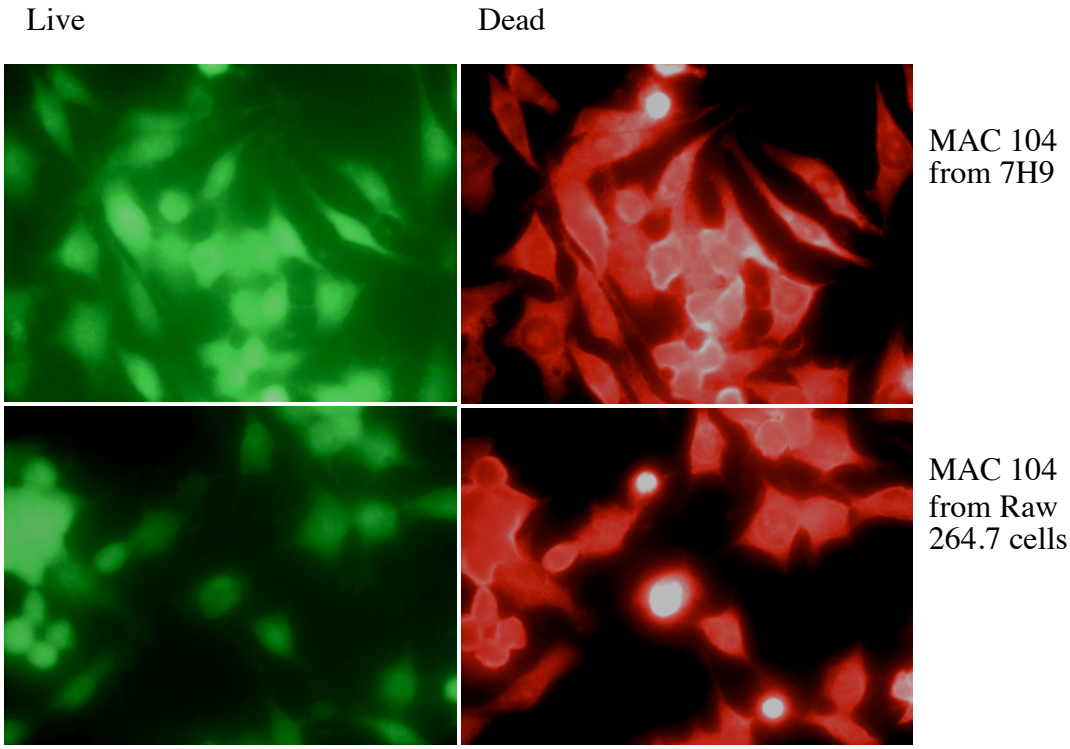
MAC from 24 hour elemental mixture



Two hours post infection, Raw cells infected by MAC from 24 hour elemental mixture undergo apoptosis more frequently than Raw cells infected by MAC from 7H9 broth. The assay was repeated 3 times. Arrows indicate TUNEL-positive cells. *p value <0.05

Figure 3.12 Live dead assay of macrophages infected by MAC 104 from 7H9 broth and other macrophages

Inoculum	Uninfected	7H9 Broth	Raw 264.7 lysate	MAC from 7H9 Broth	MAC from Raw 264.7 cells
Average Inoculum	N/A	N/A	N/A	1.40E+10	8.10E+08
Percent of Dead Cells	2.1 ± 0.9	1.9 ± 0.7	1.7 ± 0.5	3.4 ± 0.8	8.5 ± 3.0*



Bacteria from 7H9 broth or collected from macrophages 24 hours post infection were used to infect fresh macrophage monolayers. Two hours later, live-dead assay was performed to determine percent macrophage cell death. Values represent mean and standard deviation from 3 repeats. *p value <0.05

Chapter 4

Characterization of the tat secretion system in *Mycobacterium avium*

Julie Early[1], Luiz E Bermudez[1,2,3]

Journal of Bacteriology: *In preparation*

[1]Molecular and Cell Biology Program, Oregon State University, Corvallis, Oregon 97331
[2]College of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331
[3]Department of Microbiology, College of Science, Oregon State University, Corvallis, Oregon 97331

Abstract

Mycobacterium avium is of serious concern for immunocompromised patients. When in the host, MAC live within macrophage vacuoles that are modified by the pathogen. For MAC to survive in this environment, proteins from the pathogen are undoubtedly exported, but the methods of transport have not been well studied. Here, we characterize MAC's twin-arginine translocase system, a mechanism used to transport folded proteins that contributes to virulence in many other bacteria. First, we identified the tat genes within the genome, and determined that they are expressed in standard growth media and upregulated under intracellular conditions. Next, we created an antisense construct to reduce the expression of these genes and studied the phenotype. Our anti-tatB strain had an altered colony morphology, and grew slowly in 7H9 broth, but was taken up more efficiently and grew faster than the wildtype strain in macrophages. It was also more sensitive to SDS and β-lactam antibiotics. We found no evidence that this strain remains in the macrophage or the macrophage vacuole more than the wildtype, but, four days post infection, it appeared to have an impaired ability to survive in macrophages that have been activated by TNFα compared to unactivated macrophages.

Introduction

Mycobacterium avium (MAC) are acid-fast bacteria that cause disseminated disease in immunocompromised individuals. MAC live and replicate inside vacuoles in macrophages and modify the vacuolar environment (Pietersen *et al* 2004, Wagner *et al* 2005, deChastellier and Thilo 2002) but the methods for import and export of

proteins and other molecules across their unique cell wall remain unclear. MAC contain the genes required to encode for the general secretory apparatus and ABC transporters but no other major secretion systems have been identified (reviewed in DiGiuseppe and Cox 2007). In *M. tuberculosis*, export of virulence factors CFP10 and ESAT-6 is partially encoded for by the RD1 region, but no closely homologous region has been found in MAC. Study of the twin-arginine translocase secretion system (tat), a conserved transport method typically used to export proteins in their folded state, has implicated a role in virulence in *M. tuberculosis* and a role in antibiotic resistance in *M. smegmatis*, but the pathway has not yet been examined in MAC (McDonough *et al* 2005, Posey *et al* 2006, Saint-Joanis *et al* 2006, McDonough *et al* 2008). In this study, we hypothesize that the tat system of MAC may contribute to virulence and characterize its role.

The tat system is a virulence factor shared by a number of bacteria such as *E. coli, P. aeruginosa* and others (Pradel *et al* 2003, Ochsner *et al* 2002, Voulhoux *et al* 2001). In *E. coli*, for example, a tat knock out strain had reduced virulence in part due to diminished secretion of Shiga toxin 1 and a failure to synthesize flagellin (Pradel *et al* 2003). In *P. aeruginosa*, a tat knock out strain did not form typical lesions in rat lungs and several proteins were found to rely on the tat system, including likely virulence factors such as phospholipases (Ochsner *et al* 2002). This secretion system typically consists of three genes, *tatA, tatB* and *tatC* and is used to transport proteins and protein complexes across the bacterial membrane (DeLisa *et al* 2003, Rodrigue *et al* 1999). A tat consensus sequence, S/T-R-R-X-F-L-K, typically present on the N-terminus of the protein secreted by the machinery, has been determined (Berks 1996).

A current model for secretion involves recognition of this consensus signal by the TatC protein, which is followed by movement of the membrane-bound TatC and TatB complex into close proximity with the membrane-bound TatA complex. Next, the substrate is translocated through the pore-forming TatA complex and cleaved upon release (Palmer *et al* 2004). Here, we identify the MAC homologs of the *tat* genes and examine the MAC tat secretion system using an antisense approach.

Materials and Methods

Bacterial Strains. MAC strain 104 was obtained from the blood of an AIDS patient and grown on Middlebrook 7H10 agar plates (Difco, Becton-Dickenson, Sparks, MD) with oleic acid, albumin, dextrose and catalase, (OADC, Hardy Diagnostics, Santa Maria, CA) or 7H9 liquid broth (Difco, Becton-Dickenson, Sparks, MD) with OADC. When appropriate, 400 μg/ml kanamycin was added for selection.

Gene Expression. Reverse transcriptase PCR was carried out similar to previously published methods (Danelishvili *et al* 2004, Tenant and Bermudez 2006). Briefly, RNA was extracted from MAC 104 grown in 7H9 broth for 5 days then exposed for 24 hours to 7H9 broth lacking OADC or to a mixture mimicking the environment of the macrophage vacuole 24 hours post infection (unpublished data). Following RNA extraction and DNase treatement, first strand cDNA was synthesized using the superscript III cDNA synthesis kit and random hexamers, according to the manufacturer's instructions (Invitrogen, Carlsbad, California). PCR fragments were amplified using the primer set 5'GTGGGCAGTCTTAGTCCGTG3' and 5'CTTGAAGATCCGCATCGACTT3' for *tatA* and

5'CAGGTGGCGTCGGCGTCGAAC3' and 5'GAGCGGCTGCCGGGCGCCATC3' for *tatB* using the PCR parameters of 5 minutes at 95°C, followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes.

Cloning. An open reading frame on the opposite strand of the *tatB* gene was amplified from MAC 104 genomic DNA using the primer set 5'CCGGAATTCGTGCTTGGTCAGCGCCG3' and 5'CGGGGTACCTCGTGGTGGGCCTAGTG3'. Similarly, the primer set 5'CCGGAATTCATGGCGCAGCCACTCGCCG3' and 5'CGGGGTACCTCAGCGCGCACATCCGGCC3' was used to amplify an open reading frame on the opposite strand of the *tatC* gene. The amplicons were digested with KpnI and EcoRI and cloned into pLDG13 (Danelishvili *et al* 2005). The two constructs were then electroporated into MAC to create the anti-tatB and anti-tatC strains.

Growth Curve in Broth. Wildtype MAC, anti-tatB and anti-tatC strains were diluted in Hank's balanced salt solution (HBSS, Gibco, Carlsbad, CA) to approximately 10^5 bacteria per ml, then placed in 10 x volume of 7H9 broth. These cultures were placed in a shaking incubator at 37°C for 1 week. A portion of the cultures were removed every other day, diluted, and plated to determine the colony forming units/ml (cfu) at each time point.

Uptake and Intracellular Growth Curve. Wildtype, anti-tatB and anti-tatC strains were grown in 7H9 broth for 5 days, then diluted to $3x10^8$ bacteria/ml using MacFarland standards. An aliquot was used to plate for cfu to determine the inoculum while another aliquot was used to infect Raw 264.7 macrophage monolayers (ATCC,

Manassas, VA) in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gemini, Woodland, CA) in a 24 well plate (Costar, Corning, NY) at an MOI of ~10 for one hour. The macrophage monolayers were then washed three times with HBSS to remove extracellular bacteria and macrophages were lysed with sterile water then plated on 7H10 to determine the amount of uptake by cfu. For the intracellular growth curve, bacteria were collected from the Raw 264.7 macrophages 1 hour, 1 day and 4 days after infection and plated on 7H10. We used two wells per sample on each timepoint for each repeat. For statistical analysis, the cfu at day 1 and day 4 was divided by the cfu at 1 hour, then the ratio of the antisense strains were compared to the wildtype.

Association with vacuole. Cultures of the wildtype and anti-tatB strain were grown for 5 days in 7H9 broth then diluted to10^8 bacteria/ml in HBSS. Wheat germ agglutinin, Texas red X (Molecular Probes) at 0.15 mg/ml was added to the bacteria then incubated together for an hour in the dark at room temperature. Excess dye was washed away 3 times using HBSS with centrifugation then FBS was added to the labeled bacteria for 30 minutes at room temperature in the dark. These labeled and opsonized bacteria were used to infect Raw 264.7 cell monolayers in chamber slides at an MOI of ~100 for 1 hour in the presence of 5mM 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS) (Molecular Probes). The excess HPTS and bacteria were washed away with HBSS, and the media was replenished. Four days later, the extra-cellular bacteria were killed by adding 200 μg/ml amikacin for 2 hours at 37°C. Separately, both the attached Raw 264.7 cells and the detached Raw 264.7 cells were rinsed 3 times with HBSS, fixed in 4% paraformaldehyde (Sigma) for 30 minutes at

room temperature, then rinsed again before analysis on a Leica DM4000B fluorescent microscope, about 40 stained bacteria were analyzed for co-localization per sample each time.

Escape the macrophage assay. Raw 264.7 cells were infected at an MOI of 10 with the wildtype and anti-tatB strains as described above. The number of intracellular bacteria was determined 1 hour post infection by lysing the Raw 264.7 cells with sterile water for 10 minutes at room temperature, plating onto 7H10, and counting for cfu. Four days post infection, the supernatant was collected, cell debris was centrifuged at 500xg for 5 minutes in a microcentrifuge and an aliquot of the supernatant was plated on 7H10 to determine the number of extracellular bacteria by cfu. The ratio of the amount outside the macrophage at day 4 divided by the number of bacteria inside the cells 1 hour post-infection was calculated.

Sensitivity to SDS and antibiotics. As estimated by MacFarland standards, a spot containing ~ 10^3 wildtype MAC and another spot with the same amount of the anti-tatB strain were placed onto 7H10 plates containing varying concentrations of sodium dodecyl sulfate (SDS), carbenicillin, or ampicillin (all Sigma, St. Louis, MO) side by side. To ensure the spots contained similar number of bacteria, one spot of each strain for each chemical was spread out on 7H10 to count for cfu. All plates were incubated at 37°C for 10 days then visually analyzed, comparing the growth on 7H10 alone to the growth with chemicals. The plates containing chemicals of minimum inhibitory concentration to affect growth of the antisense strain were photographed and repeated three times.

Survival in activated macrophages. Raw 264.7 cells monolayers in 24 well plates were treated with 300 units/ml mouse IFNγ (AbD Serotech) or 180 ng/ml mouse TNFα (Invitrogen) for 4 hours. The cytokine was triply washed away with HBSS and new DMEM was added. These activated macrophages were infected by wildtype and anti-tatB MAC for 1 hour at an MOI of 10, then extra-cellular bacteria were washed away with HBSS. The Raw 264.7 cells were then lysed in sterile water for 10 minutes at room temperature and plated to obtain cfu 1 hour, 2 days, and 4 days post infection. To determine the amount of survival/growth, the cfu at day 2 (or day 4) was divided by the cfu at 1 hour and the anti-tatB strain was compared to the wildtype at the same timepoint and treatment.

***In silico* analysis**. To identify putative tat-dependent substrates, the annotated protein coding sequences of MAC 104, available through the National Center for Biotechnology Information, were uploaded and analyzed using the TATFIND software version 1. 4 (http://signalfind.org/tatfind.html) (Rose *et al* 2002).

Statistical Analysis. In all assays, we performed at least 3 repeats, and the 2 sided Student's t-test was performed comparing the anti-tatB and anti-tatC strains to the MAC 104 strain using Microsoft Excel software, with a level of significance cut off value of $p < 0.05$.

Results

Gene organization. Genes that code for the tat secretion system proteins were identified based on sequence similarity to tat genes in other bacteria using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The *tatB* gene

is *Mav_1367*, the last gene in a region containing 3 genes in the same orientation, *Mav_1365*, the alternative sigma factor SigE, and *Mav_1366*, trypsin (Figure 4.1). The *tatA* and *tatC* genes, *Mav_2409* and *Mav_2410* respectively, are located together, near the end of a large region of genes in the same orientation, preceded by genes coding for proteosome proteins and transcriptional regulators, and followed by *Mav_2411*, a pseudogene and *Mav_2412*, a hypothetical protein of unknown function. The *tatD* gene, *Mav_1146* was not included in the analysis because its homolog in *E. coli* is not involved in translocation of twin-arginine containing proteins (Wexler *et al* 2000).

Gene expression. We determined that the *tatA* and *tatB* genes are expressed in 7H9 growth media by reverse transcriptase PCR (Figure 4.2, lanes 2 and 4). Both *tatA* and *tatB* are upregulated in a cell-free system that mimics the environment of the macrophage 24 hours post infection (Figure 4.2 lanes 3 and 5 vs. lanes 2 and 4 and unpublished data). The *tatA* and *tatC* transcript is also upregulated when MAC is inside of macrophages (Hou *et al* 2002).

Growth phenotype. To create the antisense strains, we cloned an open reading frame of the non-coding DNA strand of *tatB* and *tatC* behind the strong, constitutively active G13 promoter, and transformed this plasmid into the wildtype strain (Barker *et al* 1999). Wildtype colonies usually appear rounded and somewhat moist. The anti-tatB colonies appeared large, flat, and dry compared to the wildtype colonies (Figure 4.3 B and C). The anti-tatC colonies were smooth, moist and yellow, similar to the wildtype, although they appeared slightly smaller (Figure 4.3D). The anti-tatB strain also grew in clumps in 7H9 broth (Figure 4.3A middle), while the wildtype (Figure 4.3A left) and anti-tatC strains dispersed (Figure 4.3A right). The anti-tatB strain grew slower in

7H9 broth than the wildtype, while the anti-tatC strain had no growth rate defect (Figure 4.4).

Uptake/Surivival in macrophages. We then determined how these strains interact with the murine Raw 264.7 macrophage-like cell line. The anti-tatB strain was taken up by Raw 264.7 macrophages more efficiently than the wildtype, while the anti-tatC strain was similar to the wildtype (Figure 4.5). Both the anti-tatB and anti-tatC strains survived in Raw 264.7 macrophages for four days, the anti-tatC was similar to the wildtype while the anti-tatB showed a slight advantage to surviving in the macrophage over the wildtype (Figure 4.6).

Association with vacuole. To determine if the anti-tatB strain remains inside the vacuole of the macrophage more frequently than the wildtype, we infected Raw 264.7 cells with rhodamine-labelled MAC 104 and anti-tatB bacteria in the presence of a membrane impermeable green fluorescent dye, HPTS. Four days later, we determined the percent of red bacteria that co-localize with the green dye in attached and unattached cells, separately. A lack of co-localization suggests leakage of the dye, and an interruption to the integrity of the vacuolar membrane. In both the wildtype and anti-tatB strains, we found 59% of the stained bacteria co-localized with HPTS in attached cells, while 50% co-localized in detached cells for both strains (Figure 4.7) Therefore, we concluded the anti-tatB strain does not appear to remain inside the vacuole of the macrophage any more or less frequently than the wildtype.

Escape the macrophage. We also investigated if the anti-tatB strain stays inside the macrophage more frequently than the wildtype. Raw 264.7 cells were infected with the wildtype and anti-tatB strain, and the baseline infection amount was determined 1

hour post infection by plating for cfu. Four days later, the number of bacteria outside of the macrophages was determined by plating a portion of the cell culture medium for cfu after differential centrifugation. In both cases, we computed a ratio of about 0.1 (Table 4.1), indicating that the anti-tatB strain exits the macrophage with similar frequency compared to the wildtype.

Sensitivity to chemicals. To test if the anti-tatB strain of MAC was more sensitive to SDS and β–lactam antibiotics, we plated 10^3 of the wildtype and the anti-tatB strain in a spot on 7H10 containing varying concentrations of SDS, carbenicillin and ampicillin, and compared the growth of the spot to a spot grown on 7H10 without any chemicals after 10 days of incubation at 37°C. The anti-tatB strain was consistently more sensitive to 0.05% SDS, 0.025 mg/ml carbenicillin and 5 μg/ml ampicillin under these conditions (Figure 4.8).

Uptake/Survival in activated macrophages. We activated murine Raw 264.7 macrophages for four hours with recombinant IFNγ or TNFα then infected them with the wildtype and anti-tatB strains. Consistent with our previous work, the anti-tatB strain was able to survive and grow better than the wildtype strain in untreated cells (Figure 4.9). This enhanced ability to grow appeared to apply to activated macrophages also, as the anti-tatB strain had statistically significantly more growth than the wildtype at both day 2 and day 4 with macrophages activated by either IFNγ or TNFα. However, comparing the growth from day two to day four (by dividing the cfu at 4 days by the cfu at 2 days) for the anti-tatB bacteria that infected untreated macrophages compared to macrophages activated by TNFα showed a statistically significant reduction in the ability of this strain to grow in TNFα activated

macrophages (p-value=0.03), while the wildtype showed no significant difference (p-value=0.91). This suggests the anti-tatB strain has more sensitivity to TNFα-activated macrophages than unactivated macrophages.

Identification of putative tat substrates. Using the program TATFIND 1.4, we identified 27 MAC genes that contain putative tat secretion signals (Table 4.2). The *M. tuberculosis* homologs of the following genes were also identified as putative tat secreted proteins by the same program (McDonough *et al* 2008): *Mav_0719, Mav_1199, Mav_1648* & *Mav_2754* (both align with *Rv2525*), *Mav_2476, Mav_2761, Mav_3455,* and *Mav_4971*. None of the genes identified by TATFIND in MAC were matches to those identified in *M. smegmatis* (Posey *et al* 2006). Interestingly, *Mav_4759*, a hypothetical protein identified by TATFIND has no homologous proteins in either *M. tuberculosis* or *M. smegmatis*

Discussion

Like *M. tuberculosis* and *M. smegmatis*, the *tatA* and *tatC* genes of MAC are located together in the genome and *tatB* is in a separate operon, near *sigE* (Posey *et al* 2005, Saint-Joanis *et al* 2006*)*. These *tat* genes contribute to virulence in other bacteria such as *Escherichia coli, Pseudomonas, Legionella*, and *Yersinia pseudotuberculosis* (Pradel *et al* 2003, Caldelari *et al* 2006, Bronstein *et al* 2005 Rossier and Cianciotto 2005, Lavander *et al* 2007). Consistent with the possibility that the tat system is a virulence factor in MAC, MAC*'s* tat genes are expressed at low levels in 7H9 broth, but upregulated in the intracellular environment (Hou *et al* 2002, Figure 4.2, and unpublished data).

Disruption of the tat secretion system in *M. smegmatis* results in slower growing bacteria, an altered colony morphology and sensitivity to SDS and antibiotics (Posey *et al* 2005, McDonough *et al* 2005). Similarly, the anti-tatB strain of MAC used in this study grew in clumps, with low flat colonies on plates, grew slowly in 7H9 broth and exhibited sensitivity to SDS, carbenicillin and ampicillin. In *M. smegmatis* the increased sensitivity to β-lactams is at least partially due to the inability of BlaS to be translocated by the tat system (Posey *et al* 2006). Similarly, in *M. tuberculosis,* a tat dependent *blaC* gene contributes to the bacterium's resistance to β–lactams (McDonough *et al* 2005). MAC has no genes of close sequence similarity to *blaS* or *blaC*, but two genes with sequences similar to *Rv2525*, *Mav_1648* and *Mav_2754* were both identified as putative tat substrates. *Rv2525* is a hypothetical protein that is potentially involved in peptidoglycan synthesis, has been shown to be tat-dependent and contribute to resistance to β-lactams in *M. tuberculosis* (DiGiuseppe *et al* 2007, Saint-Joanis *et al* 2006). Likewise, these two MAC genes may be involved in MAC's resistance to β-lactams.

Our anti-tatB strain was both taken up by and survived more efficiently in macrophages than the wildtype strain. The differences in interaction of the anti-tatB strain with Raw 264.7 macrophages compared to the wildtype may be due, at least in part, to the anti-tatB strain growing in clumps. MAC in clumps, like clumps of *Staphylococcus aureus*, may be taken up more efficiently than well-dispersed bacteria (Lammers *et al* 1999). Similarly, clumps may be more resistant to the initial killing usually seen one day after infection of Raw 264.7 macrophages, and therefore grow

more efficiently in Raw 264.7 cells than non-clumpy bacteria, explaining the increased survival of the anti-tatB strain compared to the wildtype in macrophages.

Due to the technical challenge of creating knock-out mutants in MAC, as well as the concern that the *tatA* and *tatC* genes may be essential for MAC survival, as they are in *M. tuberculosis*, we decided to study the tat secretion system using antisense technology (Saint-Joanis, *et al* 2006). Antisense by overexpression of the complement strand has previously been used in *M. smegmatis* for the *hisD* gene, and we used a similar approach (Parish and Stoker 1997). However, our attempts to show that the *tatB* gene product was downregulated in the anti-tatB strain, or the *tatC* gene product in the anti-tatC strain by real time PCR were unsuccessful. This may be because the expression level of the *tatB* and *tatC* genes in 7H9 broth is low, therefore detecting a reduction in expression was technically challenging even by strand specific real time PCR. Alternately, the method of TatB or TatC inhibition may not have been due to RnaseH degradation of the mRNA, but due to steric hindrance preventing the translation of the respective protein because of the double stranded RNA molecule. Parish and Stoker used this method of antisense in *M. smegmatis* previously, but they did not determine the process by which *Mycobacteria* downregulate the protein product, so it is difficult to surmise what is happening in MAC (Parish and Stoker 1997).

While the anti-tatB strain exhibited a phenotype consistent with other tat mutants in *M. smegmatis* (Posey *et al* 2005, McDonough *et al* 2005), the anti-tatC strain appeared to behave quite similarly to the wildtype. We speculate that the antisense mechanism employed in this study was not an effective method of

downregulation for TatC and the use of more standard antisense technology involving oligonucleotides or overexpression of a different open reading frame of the opposite strand might be effective. It is also possible that TatC is essential for MAC survival, as it is in *M. tuberculosis* and halophilic archaea (Saint-Joanis *et al* 2006, Thomas and Bolhuis 2006) and that MAC found a way to overcome the antisense mechanism to survive. Given our success with the anti-tatB strain, *tatB* in MAC may be required for the full range of substrate export but not essential for all substrates as in *E. coli* (Ize *et al* 2002), or simply not required but potentially still helpful for functional tat secretion, like in *Bacillus subtilis* (Pop *et al* 2002).

Of the putative tat secreted genes, three (*Mav_0105, Mav_2069, Mav_4547*) belong to the MCE family, a group of proteins potentially involved in allowing mammalian cell entry and therefore contributing to virulence (Gioffré *et al* 2005, Flesselles *et al* 1999). Several enzymes likely involved in various aspects of cell wall metabolism were identified: Mav_0216, Mav_0719, Mav_1199, Mav_2761, Mav_3455, Mav_4412, Mav_4844, Mav_5121, and Mav_5248, given the importance of the MAC cell wall to virulence (Kansal *et al* 1998), these putative substrates may be a focus for further research. Two transporters, Mav_2079 and Mav_4306 as well as two oxidoreductase proteins, Mav_3034, Mav_4796, were identified. Consistent with other tat-translocated proteins, these two transporters may require the tat system to be properly embedded in the membrane (Debuck *et al* 2007, Sargent *et al* 2002) and oxidoreductases are a common class of tat substrates (Voordouw G. 2000). Five hypothetical proteins that have not been previously studied with no recognizable conserved domains Mav_1648, Mav_2699, Mav_2754, Mav_4759, and Mav_4971 all

contain putative tat secretion signals, as well as a few other proteins not belonging to any of the aforementioned functional classes. Further study of these proteins, as well as the tat genes, may lead to valuable insight as to how MAC survive and communicate in the intracellular environment.

Acknowledgements

We thank Denny Weber for manuscript preparation.

References

Barker LP, Porcella SF, Wyatt RG, Small PL. The Mycobacterium marinum G13 promoter is a strong sigma 70-like promoter that is expressed in Escherichia coli and mycobacteria species. FEMS Microbiol Lett. 1999 Jun 1;175(1):79-85.

Berks BC.A common export pathway for proteins binding complex redox cofactors? Mol Microbiol. 1996 Nov;22(3):393-404.

Bronstein PA, Marrichi M, Cartinhour S, Schneider DJ, DeLisa MP. Identification of a twin-arginine translocation system in Pseudomonas syringae pv. tomato DC3000 and its contribution to pathogenicity and fitness. J Bacteriol. 2005 Dec;187(24):8450-61.

Caldelari I, Mann S, Crooks C, Palmer T. The Tat pathway of the plant pathogen Pseudomonas syringae is required for optimal virulence. Mol Plant Microbe Interact. 2006 Feb;19(2):200-12.

Danelishvili L, Poort MJ, Bermudez LE, Identification of Mycobacterium avium genes up-regulated in cultured macrophages and in mice. FEMS Microbiol Lett. 2004 Oct 1;239(1):41-9.

Danelishvili L, Wu M, Young LS, Bermudez LE. Genomic approach to identifying the putative target of and mechanisms of resistance to mefloquine in mycobacteria. Antimicrob Agents Chemother. 2005 Sep;49(9):3707-14.

De Buck E, Vranckx L, Meyen E, Maes L, Vandersmissen L, Anne J, Lammertyn E. The twin-arginine translocation pathway is necessary for correct membrane insertion of the Rieske Fe/S protein in Legionella pneumophila. FEBS Lett. 2007 Jan 23;581(2):259-64.

de Chastellier C, Thilo L. Pathogenic Mycobacterium avium remodels the phagosome membrane in macrophages within days after infection. Eur J Cell Biol. 2002 Jan;81(1):17-25.

DeLisa MP, Tullman D, Georgiou G. Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. Proc Natl Acad Sci U S A. 2003 May 13;100(10):6115-20.

DiGiuseppe Champion PA, Cox JS. Protein secretion systems in Mycobacteria. Cell Microbiol. 2007 Jun;9(6):1376-84.

Flesselles B, Anand NN, Remani J, Loosmore SM, Klein MH. Disruption of the mycobacterial cell entry gene of Mycobacterium bovis BCG results in a mutant that exhibits a reduced invasiveness for epithelial cells. FEMS Microbiol Lett. 1999 Aug 15;177(2):237-42.

Gioffré A, Infante E, Aguilar D, Santangelo MP, Klepp L, Amadio A, Meikle V, Etchechoury I, Romano MI, Cataldi A, Hernández RP, Bigi F. Mutation in mce operons attenuates Mycobacterium tuberculosis virulence. Microbes Infect. 2005 Mar;7(3):325-34.

Hou JY, Graham JE, Clark-Curtiss JE. Mycobacterium avium genes expressed during growth in human macrophages detected by selective capture of transcribed sequences (SCOTS). Infect Immun. 2002 Jul;70(7):3714-26.

Ize B, Gérard F, Zhang M, Chanal A, Voulhoux R, Palmer T, Filloux A, Wu LF. In vivo dissection of the Tat translocation pathway in Escherichia coli. J Mol Biol. 2002 Mar 29;317(3):327-35.

Kansal RG, Gomez-Flores R, Mehta RT. Change in colony morphology influences the virulence as well as the biochemical properties of the Mycobacterium avium complex. Microb Pathog. 1998 Oct;25(4):203-14.

Lammers A, Nuijten PJ, Smith HE. The fibronectin binding proteins of Staphylococcus aureus are required for adhesion to and invasion of bovine mammary gland cells. FEMS Microbiol Lett. 1999 Nov 1;180(1):103-9.

Lavander M, Ericsson SK, Bröms JE, Forsberg A. Twin-arginine translocation in Yersinia. Adv Exp Med Biol. 2007;603:258-67.

McDonough JA, Hacker KE, Flores AR, Pavelka MS Jr, Braunstein M. The twin-arginine translocation pathway of Mycobacterium smegmatis is functional and required for the export of mycobacterial beta-lactamases. J Bacteriol. 2005 Nov;187(22):7667-79.

McDonough JA, McCann JR, Tekippe EM, Silverman JS, Rigel NW, Braunstein M. Identification of functional Tat signal sequences in Mycobacterium tuberculosis proteins. J Bacteriol. 2008 Oct;190(19):6428-38.

Ochsner UA, Snyder A, Vasil AI, Vasil ML. Effects of the twin-arginine translocase on secretion of virulence factors, stress response, and pathogenesis. Proc Natl Acad Sci U S A. 2002 Jun 11;99(12):8312-7.

Palmer T, Sargent F, Berks BC. Light traffic: photo-crosslinking a novel transport system. Trends Biochem Sci. 2004 Feb;29(2):55-7.

Parish T, Stoker NG. Development and use of a conditional antisense mutagenesis system in mycobacteria. FEMS Microbiol Lett. 1997 Sep 1;154(1):151-7.

Pietersen R, Thilo L, de Chastellier C. Mycobacterium tuberculosis and Mycobacterium avium modify the composition of the phagosomal membrane in infected macrophages by selective depletion of cell surface-derived glycoconjugates. Eur J Cell Biol. 2004 May;83(4):153-8.

Pop O, Martin U, Abel C, Müller JP. The twin-arginine signal peptide of PhoD and the TatAd/Cd proteins of Bacillus subtilis form an autonomous Tat translocation system. J Biol Chem. 2002 Feb 1;277(5):3268-73.

Posey JE, Shinnick TM, Quinn FD. Characterization of the twin-arginine translocase secretion system of Mycobacterium smegmatis. J Bacteriol. 2006 Feb;188(4):1332-40.

Pradel N, Ye C, Livrelli V, Xu J, Joly B, Wu LF. Contribution of the twin-arginine translocation system to the virulence of enterohemorrhagic Escherichia coli O157:H7. Infect Immun. 2003 Sep;71(9):4908-16.

Rodrigue A, Chanal A, Beck K, Muller M, Wu LF. Co-translocation of a periplasmic enzyme complex by a hitchhiker mechanism through the bacterial tat pathway. J Biol Chem. 1999 May 7;274(19):13223-8.

Rose RW, Brüser T, Kissinger JC, Pohlschröder M. Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. Mol Microbiol. 2002 Aug;45(4):943-50.

Rossier O, Cianciotto NP. The Legionella pneumophila tatB gene facilitates secretion of phospholipase C, growth under iron-limiting conditions, and intracellular infection. Infect Immun. 2005 Apr;73(4):2020-32.

Saint-Joanis B, Demangel C, Jackson M, Brodin P, Marsollier L, Boshoff H, Cole ST. Inactivation of Rv2525c, a substrate of the twin-arginine translocation (Tat) system of Mycobacterium tuberculosis, increases beta-lactam susceptibility and virulence. J Bacteriol. 2006 Sep;188(18):6669-79.

Sargent F, Berks BC, Palmer T. Assembly of membrane-bound respiratory complexes by the Tat protein-transport system. Arch Microbiol. 2002 Aug;178(2):77-84.

Tenant R, Bermudez LE. Mycobacterium avium genes upregulated upon infection of Acanthamoeba castellanii demonstrate a common response to the intracellular environment. Curr Microbiol. 2006 Feb;52(2):128-33.

Thomas JR, Bolhuis A. The tatC gene cluster is essential for viability in halophilic archaea. FEMS Microbiol Lett. 2006 Mar;256(1):44-9.

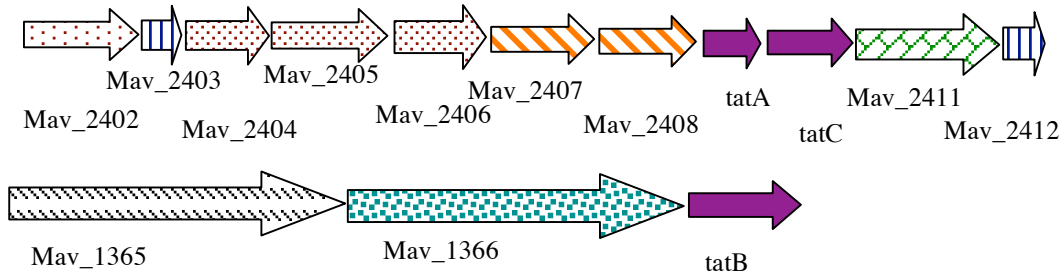
Voordouw GA. universal system for the transport of redox proteins: early roots and latest developments. Biophys Chem. 2000 Aug 30;86(2-3):131-40.

Voulhoux R, Ball G, Ize B, Vasil ML, Lazdunski A, Wu LF, Filloux A. Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. EMBO J. 2001 Dec 3;20(23):6735-41.

Wagner D, Maser J, Lai B, Cai Z, Barry CE 3rd, Höner Zu Bentrup K, Russell DG, Bermudez LE. Elemental analysis of Mycobacterium avium-, Mycobacterium tuberculosis-, and Mycobacterium smegmatis-containing phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. J Immunol. 2005 Feb 1;174(3):1491-500.

Wexler M, Sargent F, Jack RL, Stanley NR, Bogsch EG, Robinson C, Berks BC, Palmer T. TatD is a cytoplasmic protein with DNase activity. No requirement for TatD family proteins in sec-independent protein export. J Biol Chem. 2000 Jun 2;275(22):16717-22.

Figure 4.1 The arrangement of genes including and surrounding the tat genes of MAC 104.




tat gene


proteosome component


hypothetical protein


predicted transcriptional regulator

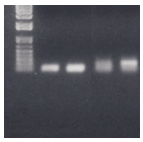

pseudogene


alternative sigma factor, sigE


trypsin

The *tata*, *tatb* and *tatc* genes of MAC were identified using BLAST, and putative functions identified for surrounding genes in the same orientation.

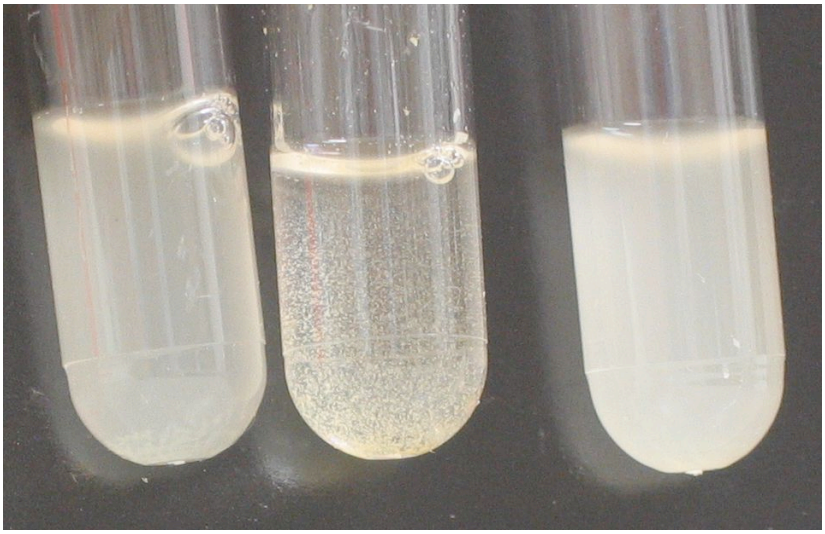
Figure 4.2 Reverse transcriptase PCR of *tatA* and *tatB*



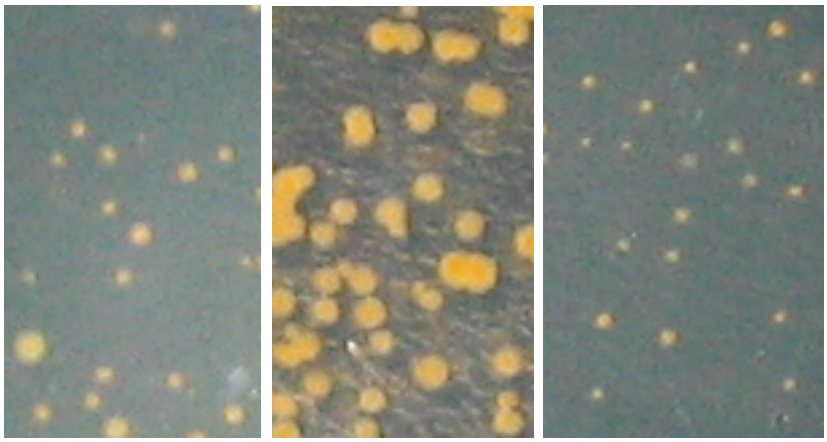
Lane 1. DNA ladder. Lane 2. *tatA* amplicon amplified from MAC 104 in 7H9 broth. Lane 3. *tatA* from bacteria in conditions that mimic the macrophage vacuole 24 hours post infection. Lane 4. *tatB* amplicon from MAC 104 in 7H9 broth. Lane 5. *tatB* from MAC 104 in conditions that mimic the macrophage vacuole 24 hours post infection.

Figure 4.3 Growth characteristics of anti-tatB and anti-tatC in 7H9 broth and on 7H10 plates

A

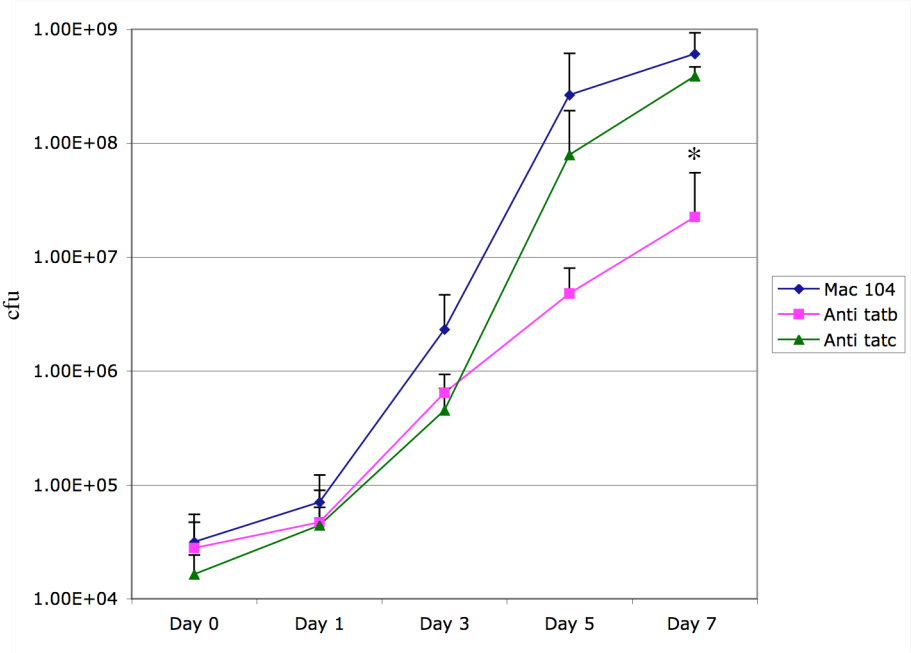


B C D



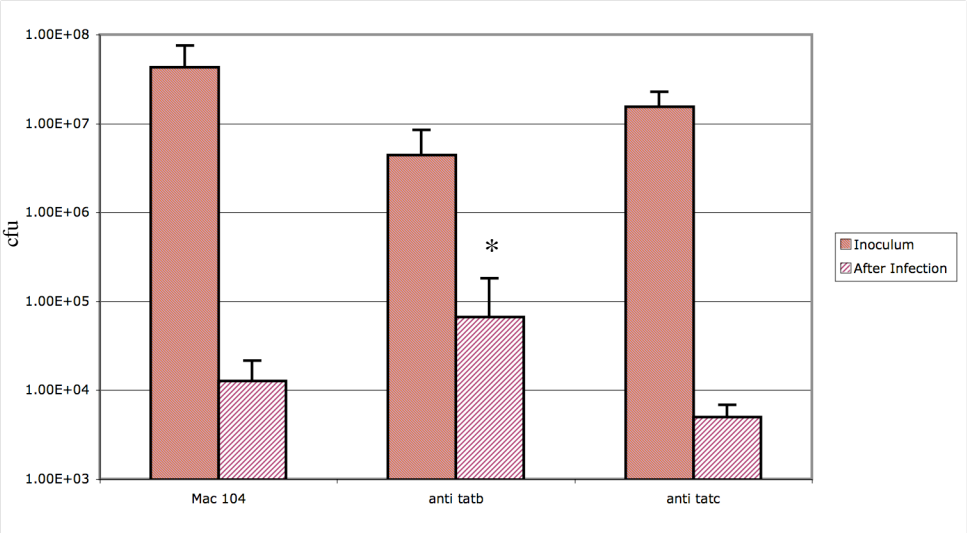
A. MAC 104, anti-tatB and anti-tatC (from left to right) strains were grown in 7H9 broth for 5 days then photographed. The anti-tatB strain grows in clumps, usually at the bottom of the tube, while the wildtype and anti-tatC strains grow dispersed throughout the media. B. MAC 104 C. anti-tatB and D. anti-tatC strains were grown on 7H10 for 10 days then photographed. The anti-tatB strain grows in low, flat, dry colonies compared to the wildtype strain. The anti-tatC strain colonies appear yellow, rounded, smooth, and moist, similar to the wildtype, except they may be smaller.

Figure 4.4 Growth curve of anti-tatB and anti-tatC in 7H9 broth



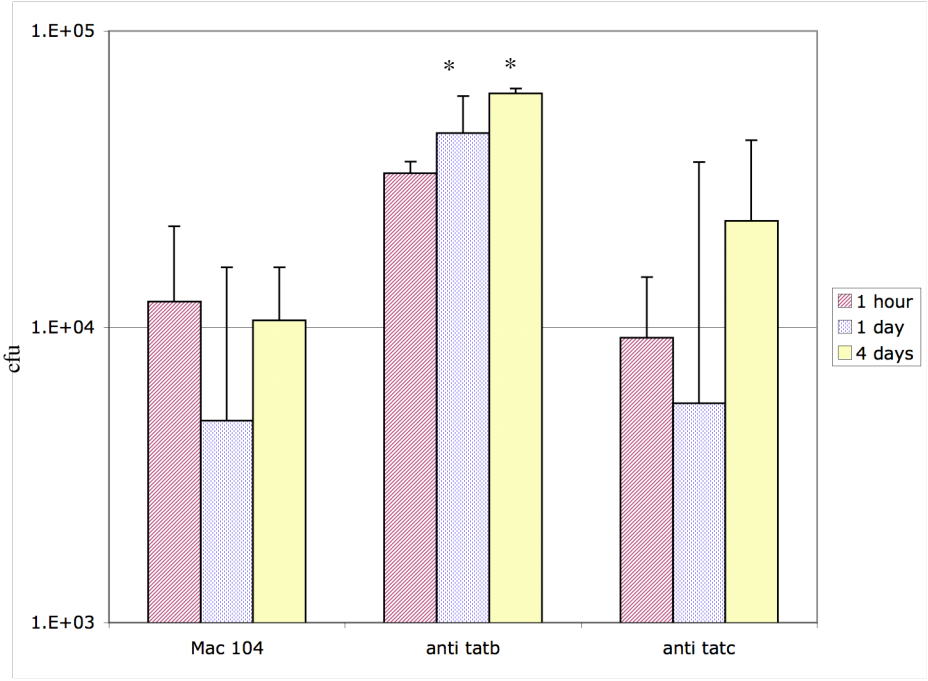
The wildtype and 2 antisense strains were grown in 7H9 broth lacking antibiotics for 7 days, and a portion of the culture was plated for cfu every other day. The experiment was repeated 3 times. The anti-tatB strain has a growth defect in 7H9 broth compared to the wildtype at 7 days. *p value <0.05

Figure 4.5 Uptake by Raw 264.7 macrophages of anti-tatB and anti-tatC



Raw 264.7 macrophages in 24-well plates were infected by the wildtype and 2 antisense strains for 1 hour at 37°C. Inocula and the amount of intracellular bacteria were determined by plating for cfu. Bars represent mean and standard deviation values from 3 repeats. The anti-tatB strain is taken up by Raw 264.7 macrophages more efficiently than the wildtype strain. * p value <0.05

Figure 4.6 Survival/Growth of anti-tatB and anti-tatC in Raw 264.7 macrophages



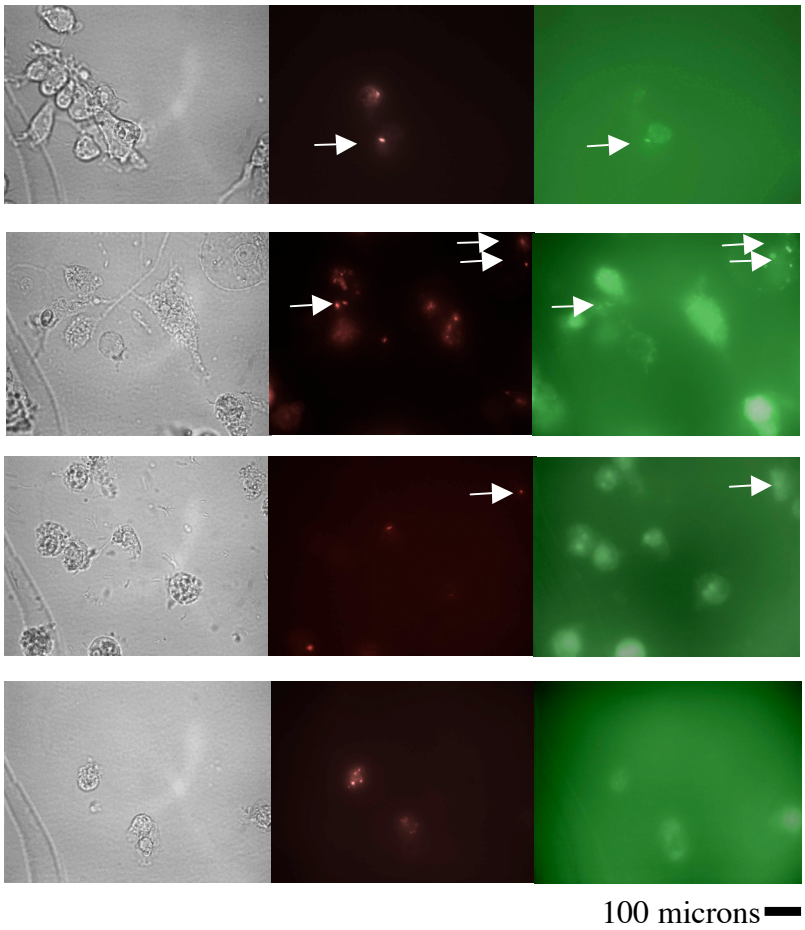
Raw 264.7 cells in 24-well plates were infected by the wildtype and antisense strains for 1 hour, extra-cellular bacteria was washed away, and the amount of intracellular bacteria determined by lysing the macrophages in water and plating for cfu at 1 hour, 1 day, and 4 days post infection. The assay was repeated 3 times. As a ratio of the number of intracellular bacteria 1 day or 4 days post infection divided by the number 1 hour after infection, the anti-tatB strain survived and grew in Raw 264.7 macrophages better than the wildtype strain. * p value <0.05

Figure 4.7 Percent Co-localization of anti-tatB with HPTS in macrophages

A

Strain	Attached Cells	Detached Cells
MAC 104 (WT)	59 ± 5	50 ± 8
Anti-tatB	59 ± 10	49 ± 10

B



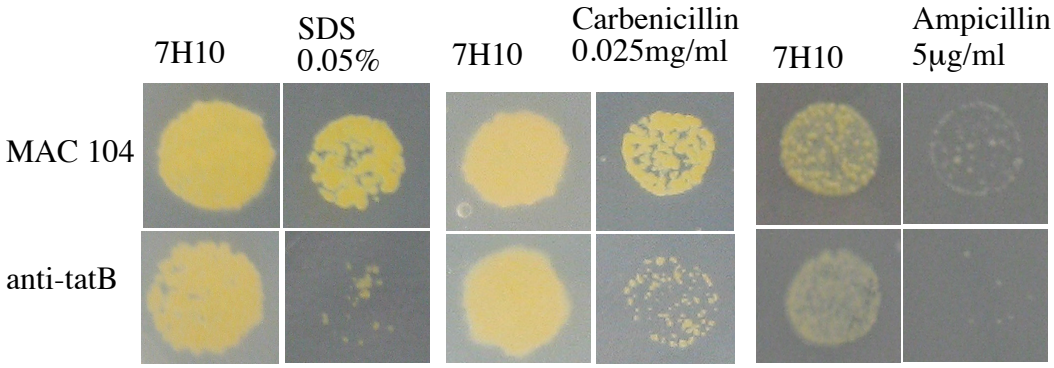
Raw 264.7 macrophages were infected by rhodamine-labeled MAC 104 or anti-tatB in the presence of the green fluorescent membrane impermeable dye HPTS. A. Four days later, the percent co-localization between red bacteria and green dye was determined for both attached and detached macrophages. Values represent mean and standard deviations from 3 repeats. We observed no difference in percent co-localization between the two strains. B. Representative images from the analysis. Arrows indicate bacteria that co-localized.

Table 4.1 Amount of anti-tatB bacteria that get out of macrophages

Strain	Ratio
MAC 104	0.09 ± .08
Anti-tatB	0.10 ± .21

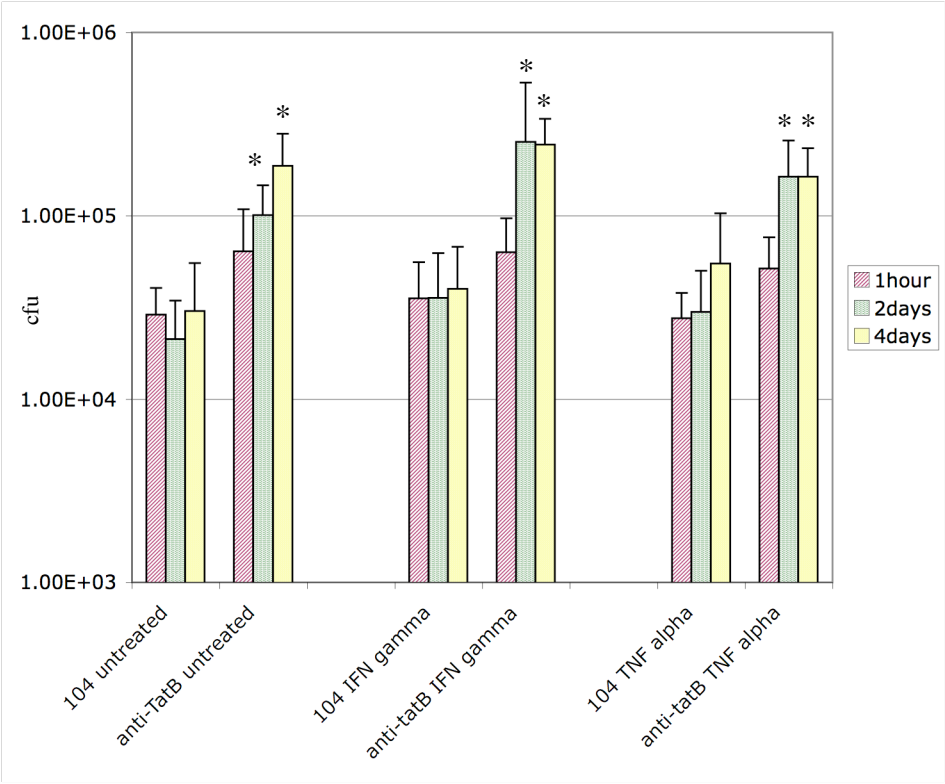
Raw 264.7 macrophages were infected by the wildtype and anti-tatB strains. Four days later, the relative number of extra-cellular bacteria was determined by collecting the total volume of cell media, doing a differential centrifugation to pellet cellular debris, and plating the supernatant for cfu, then dividing that value by the number of intracellular bacteria just after the 1 hour infection. The assay was repeated 3 times. There was no difference in the number of viable extra-cellular bacteria between the wildtype and anti-tatB strains, suggesting the anti-tatB strain exits the macrophage with similar frequency to the wildtype strain.

Figure 4.8 Sensitivity of anti-tatB to SDS, carbenicillin and ampicillin



A spot containing 10^3 wildtype bacteria was plated beside a spot containing 10^3 anti-tatB bacteria on 7H10 containing no antibiotics, and 7H10 containing varying concentrations of SDS, carbenicillin or ampicillin. Ten days later, the size of the spots was compared between the plate lacking chemical and the plates with chemicals. Images are representative from 3 repeats of the experiment. The anti-tatB strain was more sensitive to 0.05% SDS, 0.025mg/ml carbenicillin, and 5µg/ml ampicillin than the wildtype.

Figure 4.9 Uptake and survival/growth of anti-tatB in IFN-γ and TNF-α activated macrophages



Raw 264.7 cells in 24-well plates were treated with 300 units/ml IFNγ or 180 ng/ml TNFα for 4 hours, washed 3 times, then infected with MAC 104 or anti-tatB bacteria at an MOI of 10 for 1 hour. The Raw 264.7 cells were lysed with water at the indicated timepoints to determine cfu. Bars represent mean and standard deviation from 3 repeats. The anti-tatB strain grows better than the wildtype in activated and unactivated macrophages, but appears stunted in growth between day 2 and day 4 in TNFα treated macrophages compared to unactivated macrophages. *p value <0.05

Table 4.2 Putative MAC tat substrates

Mac 104 Gene	Function
Mav_0105	Mce protein
Mav_0216	cutinase
Mav_0445	secreted protein, phosphatase domain
Mav_0719	putative esterase superfamily protein
Mav_1199	hypothetical protein, feeA/B esterase/lipase domain
Mav_1648	hypothetical protein, secreted
Mav_2069	Mce protein
Mav_2079	transporter, major facilitator family protein
Mav_2140	LppO
Mav_2168	N-acetylmuramoyl-L-alanine amidase
Mav_2476	sugar ABC transporter, sugar binding protein
Mav_2485	Tat-translocated enzyme; Dyp_peroxidase family
Mav_2699	hypothetical protein
Mav_2754	hypothetical protein
Mav_2761	glycosyl hydrolase family protein
Mav_3034	oxidoreductase
Mav_3122	andenylate and guanylate cyclase catalytic domain
Mav_3455	metallophosphoesterease
Mav_4306	transporter, major facilitator family protein
Mav_4412	metallophosphoesterease
Mav_4547	Mce protein
Mav_4759	hypothetical protein
Mav_4796	FAD dependent oxidoreductase domain
Mav_4844	glycosyl hydrolase family protein
Mav_4971	hypothetical protein
Mav_5121	HpcH/HpaI aldolase/citrate lyase family protein
Mav_5248	polysaccharide deacetylase domain

The entire set of MAC 104 open reading frames were analyzed by TATFIND 1.4 to identify putative tat substrates.

Chapter 5

Mycobacterium avium mutants with impaired uptake by macrophages occupy intracellular vacuoles that differ from vacuoles containing wildtype bacteria

Julie Early[1,2], Brandon Yuan[1], Martin Wu[1,3] and Luiz E. Bermudez[1,2,4]

Infection and Immunity: *In preparation*

[1]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331
[2]Molecular and Cell Biology Program, Oregon State University, Corvallis, Oregon 97331
[3]Current address, California Pacific Medical Center Research Institute, San Francisco, California 94120
[4]Department of Microbiology, College of Science, Oregon State University, Corvallis, Oregon 97331

Abstract

The environmental pathogen *Mycobacterium avium* is of grave concern for immunocompromised individuals, who frequently develop disseminated infection. In the host, MAC live inside vacuoles in macrophages. We previously identified mutants that have impaired ability to enter macrophages and hypothesized that, because some of these mutants do not enter macrophages as efficiently but grow equally to the wildtype bacterium *in vitro*, they may reside in vacuoles that differ from the wildtype vacuole. In this study, we examined for the ability to block acidification of the vacuole and analyzed Rab5, EEA-1 and LAMP-1 located on the vacuoles of four transposon mutants with impaired ability to enter macrophages. The mutants' vacuoles were slightly more alkaline during the initial 4 h of infection than the wildtype bacterium vacuole. Some vacuoles, however, became more acidic than the vacuole of the wildtype bacterium at 24 h after infection while others were similar to the wildtype. In three of the four mutants, the presence of some vacuole markers differed from markers in vacuoles with wildtype bacteria. Some mutants did not replicate in macrophages in culture as well as the wildtype strain. We also found that one mutant was unable to survive in both unactivated and activated murine macrophages as well as the wildtype strain. Finally, we investigated the association of Rab5, Rab7, EEA-1 and LAMP-1 with vacuoles containing two different wildtype strains of MAC, 101 and 109 and found no differences between vacuoles of the two strains. These findings support the idea that phagosome trafficking might depend, at least partially, upon the membrane receptor and/or the signaling pathways stimulated by the bacteria.

Introduction

Mycobacterium avium are opportunistic pathogens found throughout the environment in water and soil (Falkinham 1996, Inderlied *et al* 1993). The bacteria are a serious concern for immunocompromised people, such as AIDS patients. Likewise, MAC infection is a complication encountered in patients with chronic lung diseases, such as emphysema and bronchiectasis (Aksamit 2002). When inside of the host, MAC live within non-acidic vacuoles in macrophages and often cause disseminated disease.

The mechanisms MAC use to enter the macrophage have been the focus of many studies, however are still not well understood. Previous reports have shown that, *in vitro,* MAC interact with macrophages through integrin receptors, mainly CR3 and CR4 (Bermudez *et al* 1991). Other membrane receptors, such as mannose receptor, have also been associated with macrophage uptake (Bermudez *et al* 1991). Although blocking these receptors *in vitro* has resulted in a decrease in MAC phagocytosis, several *in vivo* studies could not confirm this finding (Bermudez *et al* 1999, Bohlson *et al* 2001).

Recently, screening of a MAC transposon bank for mutants impaired in uptake by macrophages resulted in the identification of several clones. The MAC mutants identified also had impaired uptake by splenic macrophages *in vivo* (Danelishvili *et al* 2007), but the intracellular growth in macrophages *in vitro* was comparable among several of the mutants and the wildtype bacterium. The clones 8H8, 9B9, 9C3 and 9E6, which were identified in this screen, contain mutations found in two distinct regions of the chromosome. The interrupted gene in the 8H8 mutant is in an operon

involved in the synthesis of glycopeptidolipids (Eckstein *et al* 2003). The genes interrupted in clones 9B9, 9C3 and 9E6 are found in a newly identified pathogenicity island (Danelishvili *et al* 2007). The interrupted gene in mutant 9B9 is a gene of unknown function, while mutants 9C3 and 9E6 both contain the transposon in the same gene, another gene of unknown function, with its nearest match (at 41% sequence similarity) to *Mycobacterium tuberculosis Rv2074*.

Recent findings have suggested that the components of the vacuole membrane and its content may be influenced by the mechanism of entry. For example, during spreading of the infection, after leaving the originally infected macrophage, MAC are ingested by other macrophages by mechanisms other than the complement receptors and the rate of entry is greater than in the first macrophage (Bermudez *et al* 1997, Bermudez *et al* 2004). In these macrophages, bacteria are encountered in vacuoles that differ significantly from the vacuole in the first macrophage (Bermudez *et al* 1997, Bermudez *et al* 2004). Therefore, it is plausible to hypothesize that the mutants impaired in macrophage uptake, because of their inability to direct the interaction with macrophages by pathways typically used by *Mycobacteria*, are likely to be internalized by different mechanisms of uptake and reside in vacuoles that are different from the vacuoles occupied by the wildtype bacterium.

To study the vacuoles in which the 8H8, 9B9, 9C3 and 9E6 mutants reside, we analyzed the pH of the vacuoles and identified protein markers present on the vacuoles. When macrophages ingest bacteria, most microorganisms are found in phagosomes that undergo a series of maturation steps ultimately resulting in fusion with lysosomes and destruction of the phagosomal contents (Desjardins *et al* 1994).

MAC, like *Salmonella* (Beuzon *et al* 2000), *Leishmania* (Holm *et al* 2001, Jacques and Bainton 1978), and *Mycobacterium tuberculosis* (Via *et al* 1997), avoid destruction by stalling endosome maturation that precedes fusion with the lysosome. Typically, the pH of phagosomes decreases to around 5.0 within 10 min of ingestion (Geisow *et al* 1981, Desjardins *et al* 1994), such as with uptake of *Salmonella typhimurium* or heat-killed MAC (Rathman *et al* 1996). The pH of viable *Mycobacteria*-containing phagosomes decreases to only 5.7 – 6.5 (Sturgill-Koszycki *et al* 1994). Identification of proteins that exist on the phagosomal membrane such as members of the rab family of small GTP-ases (Zerial and Stenmark 1993) also indicate maturation state of the phagosome. Phagosomes containing *Mycobacteria* acquire Rab5, but not EEA-1 (Via *et al* 1997), markers of early endosomes. They do not acquire LAMP-1 (Fratti *et al* 2001, Sturgill-Koszycki *et al* 1994), a marker for late endosomes.

Materials and Methods

Bacteria and reagents. MAC strains (MAC) 109 and 101 were isolated from the blood of AIDS patients. The 8H8, 9B9, 9C3 and 9E6 transposon mutants are isogenic strains of MAC 109 selected by screening for impaired macrophage invasion (Danelishvili *et al* 2007). Briefly, 8H8 has inactivation of a gene (*orfB*) in the GPL synthesis operon. This operon is only present in MAC and has been described previously (Eckstein *et al* 2003). Genes of a pathogenicity island only present in MAC are interrupted in mutants 9B9, 9C3 and 9E6. These genes have no known function. Inactivation of genes in this region leads to impaired ability to enter macrophages.

The 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (rhodamine) and 5-(and-6)-carboxyfluorescein, succinimidyl ester (fluorescein) were obtained from Molecular Probes (Eugene OR). Middlebrook 7H10 agar plates and 7H9 broth (Difco) were supplemented with oleic acid, albumin, dextrose, and catalase (OADC; Hardy Scientific) and used to grow MAC cultures as previously described (Bermudez *et al* 1997). Primary antibodies used were rabbit anti-Rab5a (Santa Cruz Biotechnology, Santa Cruz CA), goat anti-EEA-1 (Santa Cruz Biotechnology, Santa Cruz CA) and mouse anti-LAMP-1 (BD Biosciences, San Jose CA). Secondary antibodies used were all Texas Red conjugates (TR) and included donkey anti-rabbit IgG-TR (Amersham Biosciences, NJ), mouse anti-goat IgG-TR (Santa Cruz Biotechnology, Santa Cruz CA), and sheep anti-mouse IgG-TR (Amersham Biosciences, NJ). Two-well chamber slides from Nalge Nunc (Rochester NY) were employed in preparation of cells for confocal microscopy, and 24-well tissue culture plates (Corning NY) were used to prepare macrophage monolayers for the cytofluorometer assay.

Determination of vacuole pH. The pH of macrophage vacuoles containing different mutants was determined by cytofluorometry. The procedure was originally described by Murphy and colleagues (Murphy *et al* 1984), then modified for MAC by Oh *et al*. (Oh and Straubinger 1996), and recently adapted by Li and colleagues (Li *et al* 2005). In this procedure, bacteria were dual-labeled with two fluorescent dyes. One dye, fluorescein, has a pH-dependent emission spectra, while the fluorescence of the second dye, rhodamine, is largely independent of pH. The ratio of these signals was used to determine the pH of the vacuole. MAC 109 and transposon mutants 8H8,

9B9, 9C3 and 9E6 were cultured on Middlebrook 7H10 agar with OADC at 37 ºC. Pure colonies of these strains were transferred to Middlebrook 7H9 broth with OADC, grown at 37 ºC for 3 days, pelleted by centrifugation, then resuspended in Hank's Balanced Salt Solution (HBSS, Gibco) at a density of 3×10^9 bacteria/ml, as estimated by MacFarland turbidity standards. To label bacteria with fluorescent probes, 100 μg/ml of rhodamine and 1 mg/ml of fluorescein were both added to 1 ml of bacteria at room temperature for 1 h in the dark then briefly spun down to separate clusters of free fluorescent dye from the bacterial suspension. The supernatant was collected and centrifuged at 13,000 RPM for 2 min, and the pellet was washed 3 times with centrifugation with HBSS to dispose of unincorporated dye. Peritoneal Raw 264.7 macrophage monolayers that had grown to confluence in a 24-well plate in Dubleco's Modified Eagle Medium (DMEM, Gibco) with 10% heat inactivated fetal bovine serum (FBS) were infected for 2 h with the dual-labeled bacteria at an MOI of ~1,000 with the four transposon mutants and an MOI of ~100 with MAC 109 to achieve a comparable number of intracellular bacteria. Labeling the bacteria does not alter the uptake into macrophages (data not shown). Each well was washed with HBSS before replenishing the media and adding 200 μg/ml of amikacin (Sigma) to eliminate extra-cellular bacteria. After 2 h, the wells were washed 3 times with HBSS and fresh media was added. At time points of 1 h, 4 h and 24 h, media was replaced with HBSS and the fluorescein and rhodamine emission intensity was measured with a CytoFluor II cytofluorometer. The signal for fluorescein was determined using an excitation wavelength of 480 nm and an emission of 530 nm; while rhodamine was excited at 545 nm with emission at 580 nm. To obtain a standard curve, 8 monolayers that were

infected with dual–labeled MAC 109 and had extra-cellular bacteria eliminated as described above, were then filled with sodium acetate or sodium phosphate buffer with pHs between 4.0 and 7.5. These buffers contained 10 μM Nigericin (Sigma) to equilibrate the intracellular pH to the extra-cellular pH. The fluorescence from these wells was measured as described above, and the data were used to create a standard curve to estimate the pH of the vacuoles containing MAC 109 and each of the four transposon mutants.

Endosomal protein co-localization analysis of mutants by confocal microscopy. Human U937 macrophages (ATCC) were cultured in RPMI-1640 (Gibco) media supplemented with heat-inactivated 10% FBS. We added 5×10^5 U937 cells and 2 μg/ml phorbol myristate acetate (PMA) to trigger cell differentiation to each well of a 2-chamber slide (Nalge Nunc, Naperville IL). Two days later, the unattached cells and excess PMA were removed by washing with HBSS and media replenished. MAC 109 and the four mutants were grown and labeled as described above, with the exception that only fluorescein at 200 μg/ml was added to each sample. U937 macrophages were infected with the labeled MAC 109, labeled heat-killed (70 ºC for 1 h) MAC 109, and the four labeled mutants at an MOI of 100 for 1 h. The chambers were then washed 3 times with sterile phosphate buffered saline (PBS) to remove any extracellular bacteria, and the media was replenished. Infected macrophages were incubated for 4 h, washed with PBS, fixed by adding 4% paraformaldehyde (Sigma) in PBS at room temperature in the dark for 1 h, then permeabilized in cold 0.1% Triton X-100 (J.T. Baker, Phillipsburg, NJ) and 0.1% sodium citrate for 2 min on ice. The cells were then washed with PBS and blocked with 10% bovine serum albumin (BSA,

Sigma) in PBS for 1 h at room temperature. After washing with PBS, the appropriate primary antibody was added to the chamber for 1 h. All antibodies were prepared in 10% BSA in PBS to prevent non-specific binding. The cells were washed again, and exposed to the appropriate TR conjugated secondary antibody. After washing and air-drying the chambers, Aqua-mount mounting media (Learner Laboratories) and cover slips (Corning NY) were added. MAC infected U937 cells were visualized with a Carl Zeiss LSM510 META motorized confocal microscope. An argon ion laser was used to excite fluorescein at 488 nm, and a HeNe543 laser was used to excite TR at 543 nm. Fluorescein's emission spectra were collected at 505-545 nm and TR's emission was collected above 561 nm, with the multi-track function activated to prevent crosstalk. The microscope was operated by LSM Software v.3.2 from Carl Zeiss on a Windows based system. The microscope and computer are maintained by the Center for Genome Research and Biocomputing at Oregon State University. The percent of green and red labeling that co-localized in at least five randomly selected fields was determined.

Endosome protein co-localization of wildtype strains by fluorescent microscopy. Infection of macrophages and preparation of slides were carried out as described above. Murine Raw 264.7 cells were infected with MAC strains, and a fluorescent microscope was employed for the analysis. Approximately 100 stained bacteria were analyzed per sample, and the assay was repeated three times.

Bacterial growth in macrophages. To examine whether these mutants could grow was well as the wildtype in murine macrophages, 2×10^5 Raw 264.7 macrophages were seeded in 24-well tissue culture plates (Costar) and 24 h later infected with MAC

strains, according to reported methods (Bermudez *et al* 1997, Bermudez *et al* 2004) using a MOI of 10. To obtain a baseline, monolayers of some wells were lysed 1 h after infection according to published protocol (Bermudez *et al* 1997, Bermudez *et al* 2004), and the lysate was plated onto 7H10 agar plates. The plates were incubated at 37 ºC for 8 days, when the number of colonies was determined. Other wells were followed for 4 days and the number of viable intracellular bacteria determined (Bermudez *et al* 1997, Bermudez *et al* 2004). The difference between the number of intracellular organisms at 1 h and 4 days represents the amount of intracellular growth or killing.

Bacterial growth in activated macrophages. The assay was carried out as described above, except the Raw 264.7 cells were activated with either 100 ng of recombinant mouse TNFα (Roche, Indianapolis, IN) or 1.5 x 10^3 units of recombinant mouse IFNγ (Biosource International, Camarillo, CA) for four hours, then triply washed with HBSS prior to the 1 hour MAC infection.

Statistical analysis. The pH of the vacuoles occupied by the four mutants was compared to the pH of the wildtype vacuole using the ANOVA test at a level of significance (p value) of < 0.05. Other measures represent the mean ± standard deviation and the p values were calculated using the Student's *t*-test.

Results

Environmental pH of bacteria determined by cytofluorometry. To ascertain the pH of the phagosomes containing mutant bacteria with impaired uptake by macrophages, we infected Raw 264.7 macrophages with fluorescein/rhodamine-

labeled MAC strains and measured the fluorescence of each dye on a cytofluorometer 1 h, 4 h, and 24 h later. The fluorescence intensity ratios were compared to a standard curve to estimate the pH. As shown in Table 5.1, the phagosome of the mutants had a pH slightly higher than the wildtype vacuoles for the first 4 h of infection. While the wildtype bacterium vacuoles became slightly more basic from 1 h to 24 h, the mutant vacuoles became more acidic over the same time period. The lowest pH observed was of the vacuoles containing the 9C3 mutant at 24 h, with a pH of 5.93, which was significantly lower than the wildtype vacuoles at 6.47 at the same time point.

Presence of Early Endosome Markers. To examine whether the vacuole of the low invasion MAC mutants contained Rab5, we labeled MAC 109, heat-killed MAC 109, and the four transposon mutants 8H8, 9B9, 9C3 and 9E6 with fluorescein then infected macrophages. Four hours later, the cells were stained for Rab5 using antibodies and visualized by confocal immuno-fluorescence microscopy. The heat-killed MAC 109 sample was found to co-localize with rab5 in 83% ($p < 0.05$ compared with the wildtype bacterium) of the cases studied, while the control MAC 109 co-localized 51% of the time. Mutant 9C3 co-localized in only 47% of the vacuoles studied, while 9B9 and 8H8 co-localized at 58% and 67 % respectively ($p < 0.05$ for both compared with the wildtype bacterium). 9E6 stained bacteria colocalized with rab5 in 68% ($p < 0.05$ compared with the wildtype bacterium) of the bacteria analyzed (Figure 5.1). Apart from 9C3, the vacuoles with mutants appeared to contain Rab5 more frequently than vacuoles of the wildtype bacterium, but less frequently than heat-killed MAC 109.

To determine whether the vacuoles of the four mutants and the wildtype bacterium contained EEA-1 on the vacuole membrane, we again infected Raw 264.7 macrophages with fluorescein-labeled MAC strains, waited 4 h, then utilized antibodies to stain for EEA-1 and performed confocal microscopy. Wildtype MAC 109 co-localized in 31.4% of vacuoles studied, while the heat-killed MAC 109 co-localized 96% of the time. All four of the mutants scored between the wildtype MAC 109 and heat-killed MAC 109, but the highest co-localization observed was with 9C3 at 42% ($p < 0.05$ compared with the wildtype bacterium), indicating that the majority of the vacuoles occupied by the four mutants do not contain EEA-1 (Figure 5.2).

Presence of Late Endosome Markers. To examine the percent of low invasion transposon mutants that have LAMP-1 on their vacuoles, macrophages were infected with fluorescein labeled MAC strains, i.e., MAC 109, heat-killed MAC 109, 8H8, 9B9, 9C3 and 9E6; and 4 h later, cells were stained for LAMP-1 using antibodies then analyzed by confocal microscopy. Only 28.8% of the vacuoles with the viable wildtype MAC 109 were found to have LAMP-1. By contrast, heat-killed MAC 109 was observed with LAMP-1 79.5% of the time. The mutants 9B9 and 9E6 appeared not to co-localize with LAMP-1, as they scored in at 27% and 28.8%, respectively (Figure 5.3). Mutants 8H8 and 9C3 were associated with LAMP-1 more often than the wildtype bacterium, 36.1% and 38.9% respectively ($p < 0.05$ for both clones compared with the wildtype bacterium).

Association of wildtype strains MAC 101 and MAC 109 with Rab5, Rab7, EEA-1, and LAMP-1. We sought to determine if there is a difference in proteins present on the phagosome between vacuoles containing the MAC 101 and MAC 109 strains since

previous studies of this type have used the MAC 101 strain (Bermudez *et al* 2004, Kelley and Schorey 2003). In our hands, both the MAC 101 and MAC 109 strains were associated with Rab5 in 65% of the vacuoles at 4 hours post infection (Figure 5.4). In macrophages infected with MAC 101, 26% of the bacteria were associated with Rab7, while in MAC 109 infected macrophages Rab7 was observed in 28% of the vacuoles. EEA-1 was associated with 35% of MAC 101 vacuoles and 34% of MAC 109 phagosomes. LAMP-1 was found in 30% of MAC 101 phagosomes and 27% of the MAC 109 compartments. Taken together, these results suggest no significant difference in these phagosome features between the MAC 101 and MAC 109 strains.

Growth in macrophages. To evaluate if the 8H8, 9B9 and 9E6 mutants grow in Raw 264.7 macrophages, we infected monolayers with the mutants and the wildtype bacterium at a MOI of 10. One hour and 4 days after infection, the number of intracellular bacteria was determined. As shown in Table 5.2, the intracellular growth of the mutants, except 8H8, was comparable to the growth of MAC 109.

Growth of 9C3 in unactivated and activated macrophages. Since the 9C3 strain had decreased uptake by splenic macrophages in mice (Danelishvili *et al* 2007), we examined if the 9C3 strain was able to survive and grow in either unactivated or activated macrophages as well as the wildtype strain. Raw 264.7 macrophages were activated with IFNγ or TNFα for 4 hours prior to infection, and we found that 9C3 was able to survive and replicate in a manner similar to the wildtype for the first two days (Figure 5.5). By four days post infection, however, 9C3 showed less

growth/survival compared to the wildtype strain in macrophages that were untreated and macrophages activated by either cytokine.

Discussion

MAC and *M. tuberculosis* target mononuclear phagocytes, the chief host cell infected by the bacteria (Sturgill-Koszycki *et al* 1994). One could explain the preference by the bacterium for macrophages or monocytes as an evolving ability to survive in those cells. Since mononuclear phagocytes are cells specialized in the killing of microorganisms, it would be predictable that virulent mycobacteria evolved complex mechanisms to neutralize the bactericidal activity of host mononuclear phagocytes.

Once taken up by macrophages, virulent mycobacteria alter the trafficking of the vacuole, preventing docking of molecules such as EEA-1 and LAMP-1 (Via *et al* 1997). They also prevent the cleavage and consequent activation of other molecules such as cathepsin D (Sturgill-Koszycki *et al* 1994). Maturation of the endosome does not occur. Although elegant work by several investigators has begun to elucidate the mechanisms responsible for the blocking of vacuole maturation, as well as the prevention of fusion between the phagosome and the lysosome, much remains to be understood.

Because the phagosome derives from the cell membrane, it is possible that, depending on the membrane receptor and the pathway of internalization the bacterium follows, a somewhat different phagosome would be formed. Zimmerli and colleagues (Zimmerli *et al* 1996) investigated this possibility many years ago, by using specific

antibodies to block membrane receptors of macrophages used by mycobacteria. The authors showed that the intracellular growth of *M. tuberculosis* did not depend on the receptor used by the bacteria to be phagocytized by the macrophage. The choice of receptors to block was based on several studies that identified the complement receptors CR3, CR4 and CR1, as well as the mannose receptor as the main ligands for MAC and *M. tuberculosis* on macrophages (Bermudez *et al* 1999, Schlesinger *et al* 1990). More recently, however, a number of laboratories using β-integrin knockout mice could not confirm the essential need of complement receptors for macrophage infection (Bermudez *et al* 1999, Bohlson *et al* 2001). In fact, independent of the presence or absence of functional complement receptor or the C3a fraction of the complement, virulent mycobacteria can be ingested by macrophages *in vivo* in a comparable manner (Bermudez *et al* 1999). Interestingly, MAC that leave the originally infected macrophage and infect another macrophage *in vitro*, in the process mimicking dissemination, bypass the complement and mannose receptors, and are ultimately associated with vacuoles that differ from the vacuoles in the first macrophage (Bermudez *et al* 1997, Bermudez *et al* 2004). Further investigation to determine the receptors used to enter macrophages by the four mutants impaired in macrophage uptake and investigated in this study, and how they differ from the receptors used by the wildtype, might help shed light on how the receptor used for macrophage entry affects the vacuole in which the bacteria reside.

We have identified several mutants of MAC that are significantly impaired in their ability to enter macrophages (Danelishvili *et al* 2007). The interrupted genes of mutants 9B9, 9C3 and 9E6 all mapped to a region of the chromosome with G+C

content of 53%, in contrast with the usual MAC G+C content of 69% to 72%, indicating the presence of a pathogenicity island. This pathogenicity island is only present in MAC, not in *M. tuberculosis* and *Mycobacterium avium* subspecies *paratuberculosis*. The other evaluated mutant, 8H8, has inactivation of a gene involved in glycopeptidolipid synthesis. The 8H8 strain initially exhibited a vacuole of higher pH than the wildtype, but by 24 hours, the vacuolar pH appeared similar to the wildtype strain vacuoles. In addition to its lower ability to enter macrophages, this strain did not grow in macrophages or mice as well as the wildtype (Danelishvili *et al* 2007), and vacuoles were more likely to contain Rab5, EEA-1, and LAMP-1 than the vacuoles occupied by wildtype bacteria, suggesting that this mutant had a marked behavioral difference in macrophages compared to the wildtype. The 9B9 strain initially had vacuoles of higher pH than the wildtype, but by 24 hours was similar to the wildtype. It also grew in macrophages in a manner similar to the wildtype and had no significant difference in the association of its vacuole with Rab5, EEA-1 or LAMP-1 compared to vacuoles containing wildtype bacteria, suggesting its intracellular phenotype was comparable to the wildtype, despite a lower ability to invade macrophages. The pH of vacuoles containing the 9C3 strain was initially like the wildtype vacuoles, but by 24 hours post infection, became significantly lower. This strain was unable to survive in IFNγ or TNFα-activated or unactivated murine Raw 264.7 macrophages as well as the wildtype strain and vacuoles occupied by this strain were more likely to contain both EEA-1 and LAMP-1 than vacuoles containing wildtype bacteria, indicating a behavioral difference inside of macrophages between the 9C3 strain and wildtype strain. Finally, vacuoles of the 9E6 strain had a higher pH

than the wildtype vacuoles 1 hour post infection, but were similar to the wildtype 4 hours and 24 hours post infection. The 9E6 strain was able to grow in macrophages comparable to the wildtype strain, but vacuoles occupied by 9E6 were more likely to be associated with Rab5 and EEA-1 than vacuoles with wildtype bacteria. Taken together, these data suggest the intracellular phenotype of the 9E6 strain was somewhat different than the wildtype strain. It is of note that mutants 9C3 and 9E6 have the same gene interrupted and yet behave differently in some of our assays. This could be due to the different location of the transposon within the gene. In fact, the 9C3 strain seems to be more severely impaired, and the transposon interrupts in the beginning of the gene in this strain, while the transposon is located near the end of the gene in the 9E6 strain, raising the possibility of a somewhat functional truncated protein.

In the current study, the vacuoles of macrophages occupied by four phagocytosis-impaired mutants were examined for the presence of membrane markers as well as pH, and have shown to differ in several aspects of the vacuole from the parent strain. For example, the 9E6 mutant strain was more likely to contain early endosome markers on its vacuole than the wildtype vacuole. Nonetheless, it grew inside of macrophages in a manner similar to the wildtype strain. This observation agrees with the findings of Zimmerli and colleagues, and might support, at least partially, their observation regarding the ability of mycobacteria to replicate in macrophages despite differences in pathway of uptake. This also suggests that virulent mycobacteria do not live only in a restricted environment, but can adapt to diverse conditions, such as phagosomes of different characteristics. Recent studies by El-Etr

and colleagues (El-Etr *et al* 2004) have identified two chromosomal regions in *Mycobacterium marinum* that have an important role in the uptake by macrophages. It would be important to determine if mutations in those loci are associated with different vacuoles in macrophages.

Acknowledgements

We would like to thank Tamara Fraley for her confocal microscopy expertise and Denny Weber for manuscript preparation. This work was supported by the grants #AI40710 and #AI43199 from the National Institute of Allergy and Infectious Diseases.

References

Aksamit TR. Mycobacterium avium complex pulmonary disease in patients with pre-existing lung disease. Clin Chest Med. 2002 Sep;23(3):643-53.

Bermudez LE, Goodman J, Petrofsky M. Role of complement receptors in uptake of Mycobacterium avium by macrophages in vivo: evidence from studies using CD18-deficient mice. Infect Immun. 1999 Sep;67(9):4912-16.

Bermudez LE, Parker A, Goodman JR. Growth within macrophages increases the efficiency of Mycobacterium avium in invading other macrophages by a complement receptor-independent pathway. Infect Immun. 1997 May;65(5):1916-25.

Bermudez LE, Petrofsky M, Sangari F. Intracellular phenotype of Mycobacterium avium enters macrophages primarily by a macropinocytosis-like mechanism and survives in a compartment that differs from that with extracellular phenotype. Cell Biol Int. 2004;28(5):411-19.

Bermudez LE, Young LS, Enkel H.Interaction of Mycobacterium avium complex with human macrophages: roles of membrane receptors and serum proteins.Infect Immun. 1991 May;59(5):1697-702.

Beuzón CR, Méresse S, Unsworth KE, Ruíz-Albert J, Garvis S, Waterman SR, Ryder TA, Boucrot E, Holden DW. Salmonella maintains the integrity of its intracellular vacuole through the action of SifA. EMBO J. 2000 Jul 3;19(13):3235-49.

Bohlson SS, Strasser JA, Bower JJ, Schorey JS. Role of complement in Mycobacterium avium pathogenesis: in vivo and in vitro analyses of the host response to infection in the absence of complement component C3. Infect Immun. 2001 Dec;69(12):7729-35.

Danelishvili L, Wu M, Stang B, Harriff M, Cirillo SL, Cirillo JD, Bildfell R, Arbogast B, Bermudez LE. Identification of Mycobacterium avium pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci U S A. 2007 Jun 26;104(26):11038-43.

Desjardins M, Huber LA, Parton RG, Griffiths G. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J Cell Biol. 1994 Mar;124(5):677-88.

Eckstein TM, Belisle JT, Inamine JM. Proposed pathway for the biosynthesis of serovar-specific glycopeptidolipids in Mycobacterium avium serovar 2. Microbiology. 2003 Oct;149(Pt 10):2797-807.

El-Etr SH, Subbian S, Cirillo SL, Cirillo JD. Identification of two Mycobacterium marinum loci that affect interactions with macrophages. Infect Immun. 2004 Dec;72(12):6902-13.

Falkinham JO 3rd. Epidemiology of infection by nontuberculous mycobacteria. Clin Microbiol Rev. 1996 Apr;9(2):177-215.

Fratti RA, Backer JM, Gruenberg J, Corvera S, Deretic V. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. J Cell Biol. 2001 Aug 6;154(3):631-44.

Geisow MJ, D'Arcy Hart P, Young MR. Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. J Cell Biol. 1981 Jun;89(3):645-52.

Holm A, Tejle K, Magnusson KE, Descoteaux A, Rasmusson B. Leishmania donovani lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKCalpha and defective phagosome maturation. Cell Microbiol. 2001 Jul;3(7):439-47.

Inderlied CB, Kemper CA, Bermudez LE. The Mycobacterium avium complex. Clin Microbiol Rev. 1993 Jul;6(3):266-310.

Jacques YV, Bainton DF. Changes in pH within the phagocytic vacuoles of human neutrophils and monocytes. Lab Invest. 1978 Sep;39(3):179-85.

Kelley VA, Schorey JS. Mycobacterium's arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. Mol Biol Cell. 2003 Aug;14(8):3366-77.

Li Y, Miltner E, Wu M, Petrofsky M, Bermudez LE. A Mycobacterium avium PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. Cell Microbiol. 2005 Apr;7(4):539-48.

Murphy RF, Powers S, Cantor CR. Endosome pH measured in single cells by dual fluorescence flow cytometry: rapid acidification of insulin to pH 6. J Cell Biol. 1984 May;98(5):1757-62.

Oh YK, Straubinger RM. Intracellular fate of Mycobacterium avium: use of dual-label spectrofluorometry to investigate the influence of bacterial viability and opsonization on phagosomal pH and phagosome-lysosome interaction. Infect Immun. 1996 Jan;64(1):319-25.

Rathman M, Sjaastad MD, Falkow S. Acidification of phagosomes containing Salmonella typhimurium in murine macrophages. Infect Immun. 1996 Jul;64(7):2765-73.

Schlesinger LS, Bellinger-Kawahara CG, Payne NR, Horwitz MA. Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. J Immunol. 1990 Apr 1;144(7):2771-80.

Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, Allen RD, Gluck SL, Heuser J, Russell DG. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 1994 Feb 4;263(5147):678-81

Via LE, Deretic D, Ulmer RJ, Hibler NS, Huber LA, Deretic V. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. J Biol Chem. 1997 May 16;272(20):13326-31.

Zerial M, Stenmark H. Rab GTPases in vesicular transport. Curr Opin Cell Biol. 1993 Aug;5(4):613-20.

Zimmerli S, Edwards S, Ernst JD. Selective receptor blockade during phagocytosis does not alter the survival and growth of Mycobacterium tuberculosis in human macrophages. Am J Respir Cell Mol Biol. 1996 Dec;15(6):760-70.

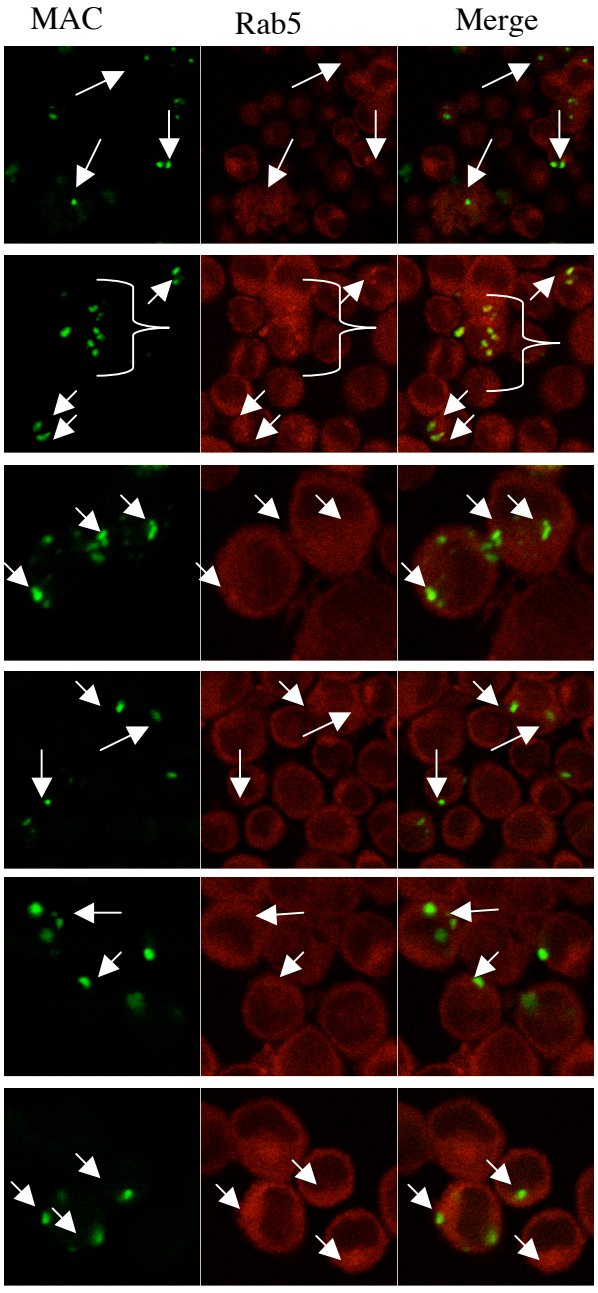
Table 5.1 Vacuolar pH values of the four mutants in Raw 264.7 macrophages

Time After Infection	MAC 109	8H8	9B9	9C3	9E6
1 h	6.33 ± 0.4	6.87 ± 0.6*	7.05 ± 0.7*	6.37 ± 0.7	6.73 ± 1.4*
4 h	6.53 ± 1.8	7.03 ± 0.5*	7.17 ± 1.5*	6.50 ± 1.1	6.67 ± 0.8
24 h	6.47 ± 0.6	6.70 ± 0.3	6.53 ± 1.9	5.93 ± 1.6*	6.23 ± 1.7

The assay was repeated 3 times, with 2 wells per strain each time. A pH standard curve was used to estimate the vacuolar pH, as described in materials and methods. *p value <0.05 compared to the MAC 109 wildtype strain at the same time point.

Figure 5.1 Co-localization of MAC and Rab5

A



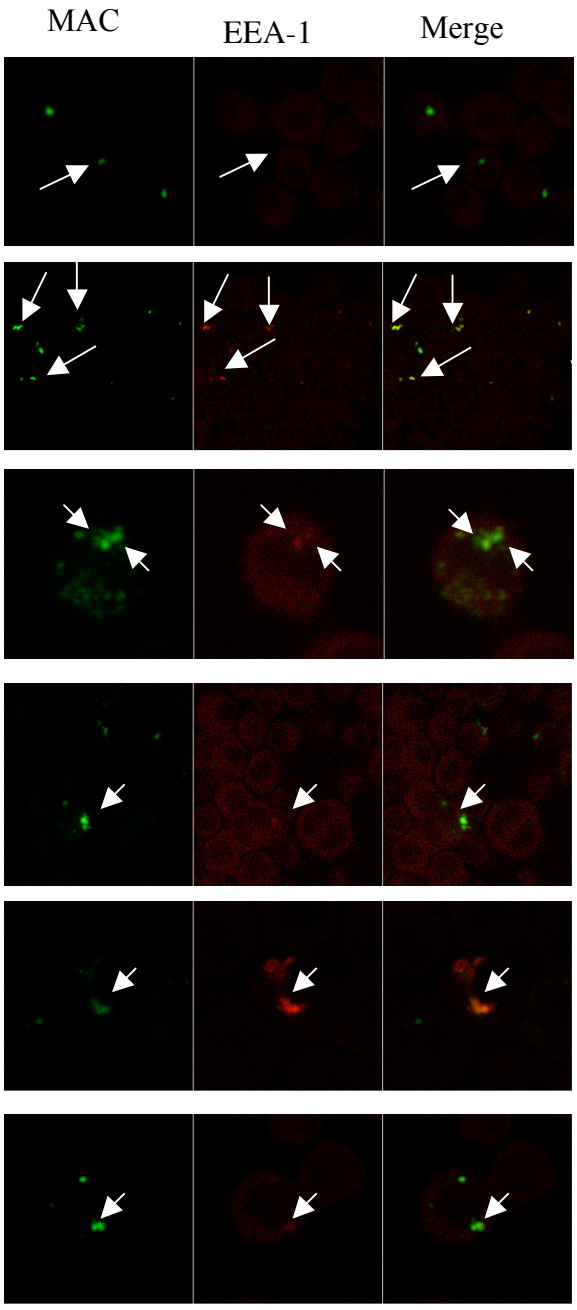
B

MAC 109	51.1	
Heat-killed MAC 109	82.6	*
8H8	66.5	*
9B9	58.3	
9C3	47.2	
9E6	68.1	*

A. Confocal microscopy images of U937 cells infected with fluorescein-labeled MAC, and immunofluorescently stained for Rab5. Arrows and brackets indicate co-localization. B. The percent of fluorescein-labeled MAC that co-localized with Rab5. The number of bacteria analyzed per strain ranged from 29 to 110 in 3 repeats. * p value <0.05

Figure 5.2 Co-localization of MAC and EEA-1

A



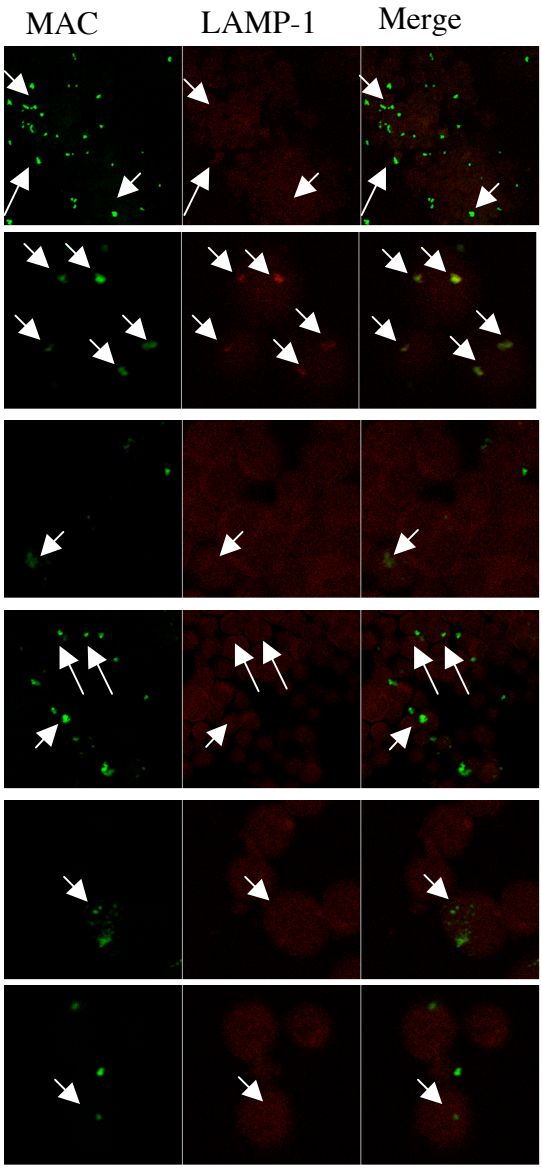
B

MAC 109	31.4	
Heat-killed MAC 109	96	*
8H8	36.7	*
9B9	33.2	
9C3	42	*
9E6	38	*

Confocal microscopy images of U937 macrophages infected by fluorescein-labeled MAC and immunofluorescently labeled for EEA-1. B. Percent of labeled MAC that colocalize with EEA-1. From 3 repeats, at least 30, and at most 140 bacteria were counted for each strain. * p value <0.05 compared with MAC 109.

Figure 5.3 Co-localization of MAC and LAMP-1

A



B

MAC 109	28.8	
Heat-killed MAC 109	79.5	*
8H8	36.1	*
9B9	27	
9C3	38.9	*
9E6	28.8	

A. Confocal microscopy images of human U937 macrophages infected with fluorescein-labeled MAC then immunofluorescently stained for LAMP-1. Arrows indicate bacteria that co-localized with LAMP-1. B. The percent of labeled MAC that was associated with LAMP-1 antibody. The assay was repeated 3 times, with a range of 33 to 167 bacteria analyzed for each strain. * p value <0.05.

Figure 5.4 Co-localization of MAC 101 and 109 with endosome markers

A

Strain	Percent Co-localization			
	Rab5	Rab7	EEA-1	LAMP-1
MAC 101	65 ± 4	26 ± 6	35 ± 2	30 ± 8
MAC 109	65 ± 2	28 ± 5	34 ± 3	27 ± 9

B

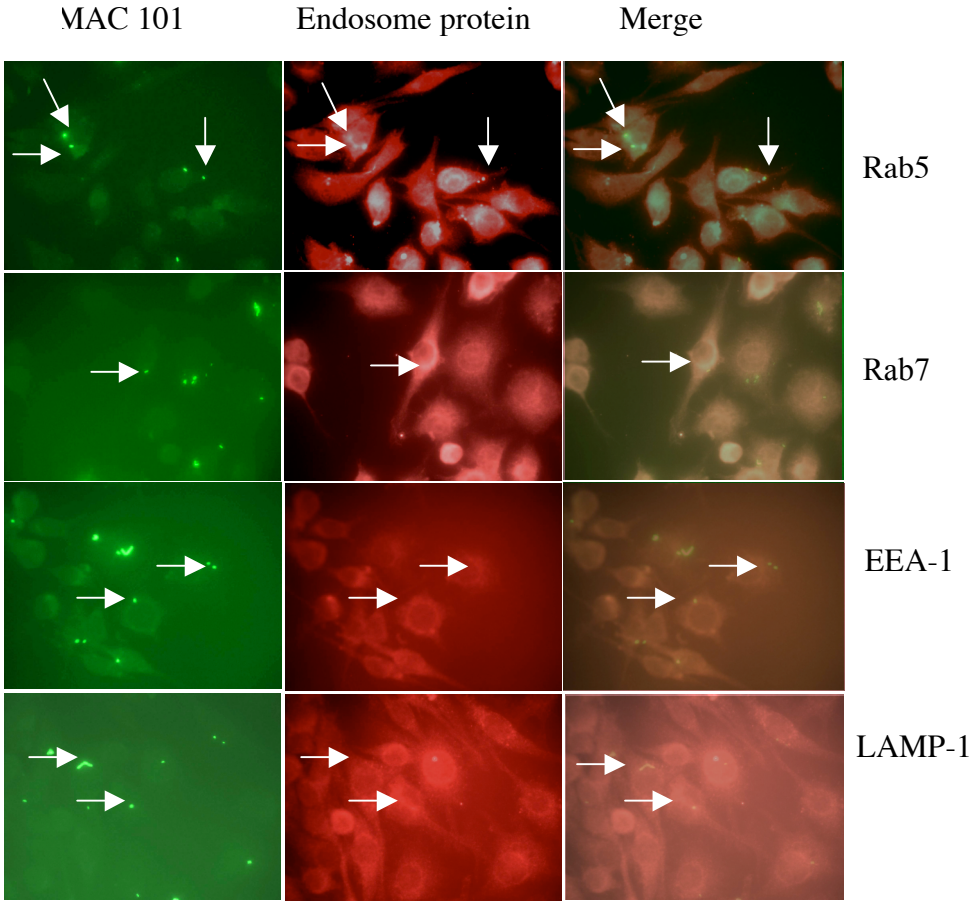
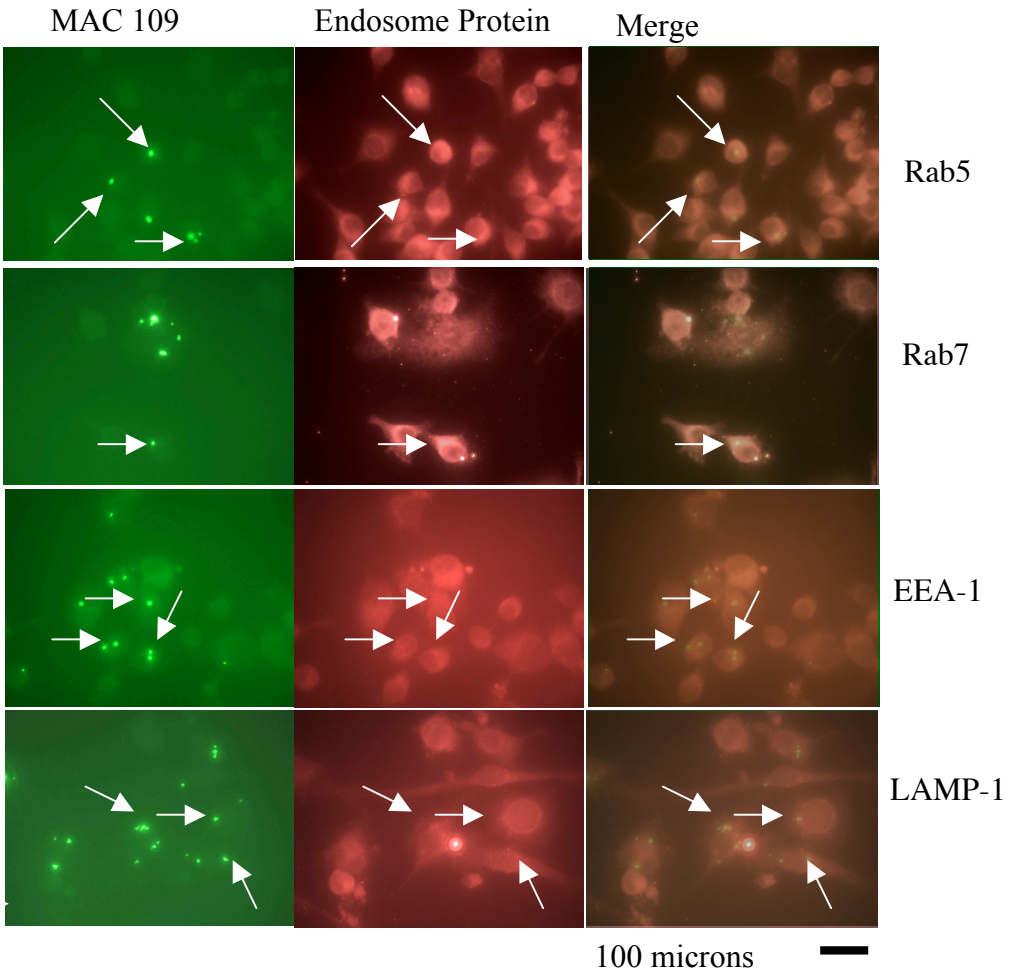


Figure 5.4 Continued

C



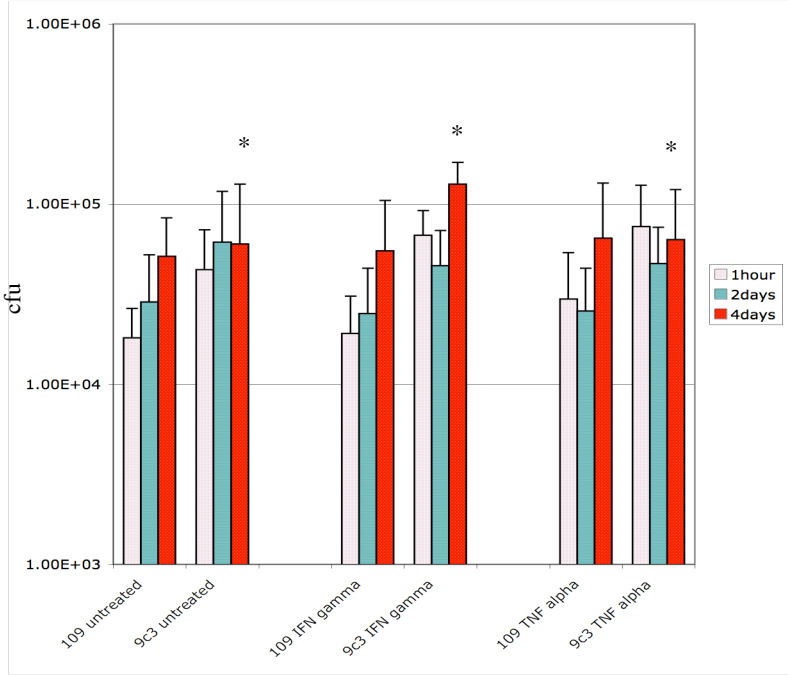
MAC-infected Raw cell monolayers were appropriately treated with the indicated endosome-specific antibodies and assayed for percent co-localization using a fluorescent microscope. A. Percent co-localization ± standard deviation based on 3 experiments. B. MAC 101 images. C. MAC 109 images. Arrows indicate bacteria that co-localize. No differences were observed between these two strains.

Table 5.2 Intracellular growth of MAC 109 and invasion mutants in Raw 264.7 macrophages

Strain	Number of bacteria/ 10^5 macrophages	
	1 hour	4 days
Wildtype MAC 109	$3.7 \pm 0.4 \times 10^5$	$4.7 \pm 0.3 \times 10^6$
8H8	$2.5 \pm 0.6 \times 10^4$	$3.9 \pm 0.3 \times 10^4$
9B9	$2.1 \pm 0.3 \times 10^4$	$6.3 \pm 0.5 \times 10^5$
9E6	$3.6 \pm 0.3 \times 10^4$	$5.5 \pm 0.2 \times 10^5$

Monolayers were seeded with approximately 10^5 Raw 264.7 cells and 24 h later infected with MAC strains for 1 h. Values represent the mean ± standard deviation of 3 different experiments.

Figure 5.5 Survival of the 9C3 strain in activated macrophages



The 9C3 strain was unable to survive in murine macrophages as well as the wildtype. Monolayers of Raw cells were seeded in 24 well plates, activated for 4 hours with either 100 ng of TNFα or 1.5 x 10^3 units of IFNγ, then infected at an MOI of 10 with the 109 or 9C3 strains. One hour, 2 days, and 4 days later, the Raw 264.7 cells were lysed, and bacteria were plated to determine cfu. Bars represent mean and standard deviation values from 3 experiments. *p value <0.05 compared with MAC 109 at the same time point and cytokine treatment.

Chapter 6. Discussion

The overall objective was to investigate the intracellular phenotype of *Mycobacterium avium* (MAC) at cellular and molecular levels. MAC that have been inside macrophages exhibit a different, more invasive, phenotype than bacteria exposed to laboratory conditions (Bermudez *et al* 1997, Bermudez *et al* 2004). This phenotype is important to MAC survival in macrophages and in the course of MAC moving from one macrophage to another, in the process of causing disseminated disease. Therefore, understanding of this intracellular phenotype is crucial to our comprehension of the disease process and could potentially be used for the development of new treatments. We used several approaches to learn about the details of how MAC adapts when inside the macrophage. First, we examined the relationship between MAC and the apoptotic macrophage, and discovered that a portion of the bacterial population survives apoptosis and can subsequently infect nearby macrophages. We also determined that MAC-infected macrophages sometimes undergo autophagy, and that MAC are able to survive the autophagic macrophage. We developed a cell-free system to study the intracellular phenotype of MAC, and discovered that bacteria exhibiting the intracellular phenotype induce macrophage cell death efficiently. Upon learning that genes of the tat secretion system are upregulated in bacteria exhibiting the intracellular phenotype, we also analyzed the tat secretion system of MAC and examined its role in virulence. Finally, we characterized the vacuole in which MAC transposon mutants that were unable to efficiently enter the macrophage reside.

Conclusion 1: *Mycobacterium avium* use apoptotic macrophages as a tool for dissemination. After using a variety of microscopy methods and learning that MAC use apoptosis to assist in spreading from cell to cell (chapter 2), we thought it would be important to identify MAC genes that allow for movement from one macrophage to another. Two significant attempts were made to identify MAC mutants that could not disseminate. In the first attempt, I used an existing signature-tagged-mutagenesis (STM) library and tried to find mutants that were able to infect the first macrophage but less efficient at entering the second macrophage. I discovered that several of the tags in the STM library cross-reacted and several others were missing altogether. I then worked to make my own STM library, and created a PCR-based screening system that allowed for faster screening than the standard dot blot technique. Unfortunately, once the plasmid that contained the transposon and tag was transformed into MAC, an important portion of the plasmid was excised and the STM library could not be formed. A post-doc in our lab, Marta Alonso-Hearn, had similar issues with this plasmid in *Mycobacterium avium* subspecies *paratuberculosis*, even after reversing the orientation of the mycobacterium origin of replication and changing the location of the STM tag. Why or how this occurs in Mycobacteria is still a mystery. The second attempt involved screening a transposon library by pooling mutants together, passing them through macrophages several times then collecting and plating the MAC that were unable to enter the last set of macrophages in 1 hour. Luiz Bermudez screened several mutants and identified four with this phenotype. Infection assays suggested that these four mutants were impaired because they stalled inside the apoptotic macrophage. I determined that all four mutants were able to exit the phagosome of an

apoptotic macrophage as efficiently as the wildtype, suggesting that these four mutants were left in the cytoplasm more frequently than the wildtype. Lia Danelishvilli, a research assistant, and I tried to determine where the transposon was inserted in these four mutants, but conflicting results arose. Sometimes I was able to amplify various portions of the transposon by PCR, other times I could not. Some probes I used for Southern blotting hybridized as expected while other probes did not in the same mutant. One mutant consistently suggested insertion in the gene *Mav_2120*, but I was able to amplify across the indicated insertion site with more than one set of PCR primers. After several confusing attempts, we sought help from Jeffrey Cirillo in another lab, who said he was unable to find the kanamycin gene, and therefore the transposon, in any of the mutants. At this point we are unsure if the mutants were all spontaneous kanamycin resistant mutants from the beginning, or if the transposon, or portions of it, were lost at some point in the process of screening and culturing.

With so many questions about the behavior of the transposon in MAC, we refocused our attention to the wildtype strain and its interaction with host macrophages. It has been assumed that apoptosis is a host mechanism used to control the infection by MAC (Fratazzi *et al* 1997), and the work in chapter 2 does not dispute or confirm this assumption. However, given the association of apoptosis and MAC spreading, further research into whether the host cells or the bacteria are what trigger apoptosis is warranted.

Conclusion 2: Mimicry of the *Mycobacterium avium* vacuole *in vitro* elicits the bacterial intracellular phenotype, including increased early-onset macrophage

cell death. The intracellular phenotype, which is important for the dissemination of MAC, was successfully induced using a cell-free system that mimicked the environment of macrophage vacuoles in which MAC reside (chapter 3). Using this cell-free system, we wanted to identify molecular changes that result in the intracellular phenotype.

We hypothesized that genes that were upregulated in the *in vitro* conditions (mixture) were directly involved in allowing for more efficient uptake by macrophages. To test this, I individually cloned *Mav_1365* (SigE) and *Mav_0660* (Mce4d) into a construct for constitutive overexpression then transformed those constructs into MAC. First, I infected macrophages and tested these overexpression strains for invasion and survival, but they showed no change in the ability to invade or survive in macrophages through 4 days. At the same time, I was interested in purifying RNA from the *Mav_1365* overexpression strain to determine the SigE regulon in MAC. The first time I extracted RNA and did real time PCR to confirm upregulation indicated that *Mav_1365* was well overexpressed. However, after attempts to grow new *Mav_1365* overexpression cultures that were either passed on plates or frozen in a glycerol stock, the overexpression was lost even though the plasmid was present. Several times, I attempted to freshly transform MAC with this construct, with the hope that a newly transformed culture would exhibit the upregulation, but was unable to find a second transformant that overexpressed *Mav_1365*. Given the issues with the *Mav_1365* overexpression strain, it is unknown if the macrophage infection assays that indicated no difference in infection or survival rates compared to wildtype are accurate or not, i.e. we are unsure if the strains were still overexpressing the gene of

interest at the time of the assays. With this fickle expression in mind, I decided not to overexpress any other genes in MAC or repeat the work with *Mav_0660*. Since then, it has become common knowledge within the Bermudez lab that many *Mycobacterium* strains lose their overexpression after being cultured.

We also hypothesized that the intracellular phenotype of MAC involves changes in protein phosphorylation of MAC proteins. To determine differences in MAC protein phosphorylation profiles upon incubation in 24 hour elemental mixture compared to incubation in 7H9 broth, I did immunoprecipitation with total bacterial cell lysates using phospho-threonine and phospho-tyrosine antibodies, then cut out bands that were consistently differentially Coomassie stained on a SDS-PAGE gel. A band present in bacteria from 7H9 broth, but absent in bacteria from the pH-adjusted 24 hour elemental mixture was trypsin digested and sent for identification by mass spectrometry. It was about 25kDa, p-tyrosine associated, and identified as the DNA binding protein HupB homolog. This protein contains no tyrosine residues and is involved in multiple essential cell survival functions, such as binding DNA, binding laminin and contributing to survival under cold-shock (Marques *et al* 2001, Shires and Steyn 2001, Lee *et al* 1998). While it is possible HupB was pulled down in a complex with some other tyrosine phosphorylated protein in the MAC incubated in 7H9 broth, the wide-ranging functions of HupB left us no obvious clues about where to focus for the next few steps of research, so this result was not pursued any further.

This cell free system discussed in chapter 3 should allow a feasible means to further identify molecular changes in intracellular MAC, leading to more information about how MAC cause disseminated disease. More research would determine which

metals are sensed and the sensors and regulators used to elicit the MAC intracellular phenotype.

Conclusion 3: Characterization of the tat secretion system in *Mycobacterium avium*. MAC genes of the tat secretion system are upregulated when MAC is inside of macrophages or in the cell free system studied in chapter 3. We explored the role of the MAC tat secretion system in virulence (chapter 4).

After identification of the MAC genes that encode for tat secretion system proteins, we wanted to demonstrate that this system is operating in MAC. Functionality of the tat system in *M. smegmatis* was illustrated by cellular location of green fluorescent protein (gfp) in a tat mutant vs. gfp in the wildtype strain (Posey *et al* 2006). I tried to create MAC that expressed either gfp or red fluorescent protein (rfp). I created several constructs, placing gfp, rfp, and monomeric rfp under control of two different promoters, but none of the constructs gave enough fluorescent signal in MAC to be useful in our studies, despite confirmation of intact plasmid. Interestingly, Michael McNamara, a graduate student in the lab, has successfully created glowing strains of *M. smegmatis* using similar constructs.

Since demonstration of downregulation of the *tatB* RNA product was not successful (chapter 4), I tried to show downregulation of the TatB protein. With no access to an antibody against MAC TatB, I opted to follow TatB expression by fusing it with a 6XHIS tag, and expressing it in the wildtype and anti-tatB strains using its native promoter. As a control, I also created an overexpression HIS-tagged TatB fusion protein. For reasons that are not clear, after growth of these transformed MAC

strains and protein purification, I was unable to detect the HIS-tagged fusion protein in any of these samples.

I also wanted to identify substrates of the tat system, and tried more than one method to do so. I concentrated proteins present in the growth media of the anti-tatB strain and wildtype strain. These proteins were run on a SDS PAGE gel, and stained by Coomassie. However, I did not find any differences in the protein profile between the two strains. This may be because the tat system is still somewhat functional in the anti-tatB strain, but is more likely because the amount of protein that was successfully concentrated by either TCA precipitation or Centricon column concentration was quite low, and that a 2-D SDS PAGE gel was needed to properly separate the proteins. I also tried to identify substrates of the tat system by screening the bacterial two hybrid system employed by Singh *et al*, using the TatB protein as a bait and MAC genomic library as prey (Singh *et al* 2006). Despite screening several thousands of colonies, I found no positively interacting peptides.

Two MAC proteins identified using an older set of criteria for *in silico* tat consensus signals, Mav_1189 and Mav_0719 each contained conserved domains that suggested a possible function as a cytolysin. The Mav_1189 protein contains a putative patatin like domain, similar to the cytolysin ExoU from *Pseudomonas aeruginosa* (Sato and Frank 2004, Sato *et al* 2003) and Mav_0719 is an exported protein with a putative lipolytic domain. I created constructs overexpressing these genes to individually transfect into macrophages, with the hypothesis that the transfected cells would die quickly compared to cells transfected with the empty vector. However, the background level of cell death due to the process of transfection

was high enough that measuring for cell death, either by release of lactate dehydrogenase (LDH) or counting using Live-Dead assay was not feasible in these transfected Raw 264.7 or U937 macrophages. Next, I tried to overexpress the Mav_1189 protein, fused with a 6xHIS tag in *E. coli* and in *M. smegmatis*. The *M. smegmatis* construct containing this protein contained an inducible promoter, but I was never able to detect Mav_1189 by western blotting after growth under inducing conditions. On the other hand, the HIS-tagged Mav_1189 encoded protein was successfully purified from *E. coli* using nickel beads that interacted with the 6xHIS tag, and this purified protein was sent to Sarah Parker at the University of Colorado for use in phospholipase analyses. Despite several attempts with a variety of substrates, she never found evidence of phospholipase activity. I also infected Raw 264.7 cells with this *E. coli* strain under inducing and non-inducing conditions, to see if the Raw 264.7 cells would die quickly upon infection of *E. coli* under inducing conditions, but never saw an increase in cell death. Similarly, exposure of the induced *E. coli* to red blood cells, followed by measurement of LDH release, gave no evidence of cytolysin activity. Upon closer examination of the patatin domain of Mav_1189, it appears to contain the classic GXSXG motif, but the surrounding amino acids do not have sequence similarity to other phospholipases, so it may not be active. It is also possible that Mav_1189, like ExoU, requires a co-factor (Sato *et al* 2005, Sato *et al* 2006), or that Mav_1189 was not purified in its functional conformation from *E. coli* and/or not correctly post-translationally modified in *E. coli*.

Further study of the MAC tat secretion system would increase our understanding of how proteins are secreted and how the tat secretion system contributes to disease in MAC.

Conclusion 4: *Mycobacterium avium* mutants with impaired uptake by macrophages occupy intracellular vacuoles that differ from vacuoles containing wildtype bacteria. Bacteria from macrophages enter fresh macrophages with different receptors and are found in vacuoles that differ from the vacuole of the primary macropahge (Bermudez *et al* 1997, Bermudez *et al* 2004). Similarly, we found transposon mutants that did not enter macrophages as efficiently as the wildtype reside in vacuoles that differ from the wildtype vacuoles, suggesting a difference in method of uptake is linked to a difference in intracellular phenotype of the bacteria (chapter 5).

In an effort to further understand the 9C3 mutant's intracellular phenotype, we studied the effect of 9C3 on autophagy of Raw 267.4 macrophages. The 9C3 mutant cannot grow as well as the wildtype in unactivated or activated macrophages, and its phagosome is more likely to contain EEA-1 and LAMP-1 (chapter 5). Unlike the wildtype strain, the 9C3 mutant does not phosphorylate the skeletal muscle and kidney inositol phosphate 1 (SKIP1) protein (Danelishvili *et al* 2007), a protein that has similarity to the autophagy protein ATG1. Both two days and four days post infection, we found the 9C3 strain to induce autophagy of macrophages at a rate similar to the wildtype and heat-killed MAC (3% and 11% respectively), suggesting that this interaction with SKIP1 is not essential for the induction of autophagy.

Consistent with the idea that MAC can adapt and survive in multiple microenvironments within the macrophage, vacuoles containing MAC are rarely characterized as all containing or all lacking specific endosome markers. In addition to the percent co-localization data presented in chapter four, other researchers have reported percent co-localization that isn't 100% or 0% for several markers, despite making general statements that imply complete association or blockage. For example, Kelley and Schorey suggest that phagosome maturation is blocked after Rab5 but before Rab7 association, however the percent co-localization with Rab5 was reported at 100%, while Rab7 was still found in 30% of the MAC vacuoles (Kelley and Schorey 2000). Other researchers have found Rab5 to be associated with MAC vacuoles less frequently, 60% by Bermudez and colleagues (Bermudez *et al* 2004) and 63% in chapter 3 of this work; both of these are more consistent with the 51% reported in chapter 5 than 100% reported by Kelley and Schorey. Interestingly, Kelley and Schorey and Bermudez and colleagues used the same MAC strain, and work presented in chapter 5 showed no differences in vacuolar characteristics with two different MAC wildtype strains. However, these studies used different macrophages, raising the possibility that the macrophage has an influence on the vacuolar environment in which MAC reside. A review of the literature also shows disagreement in the degree of co-localization of MAC vacuoles with the late endosome marker LAMP-1. Kelley and Schorey reported 10% of MAC vacuoles to contain LAMP-1 48 hours post infection, while Bermudez and colleagues reported 20% at 1 hour post infection and 40% at 24 hours post infection. Chapter 4 of this study reported 29% co-localization 4 hours post infection, while Pais *et al* reported LAMP-1 associated MAC vacuoles 48% of the

time 3 days post infection (Pais *et al* 2004), and Kuehnel *et al* reported 90% or more at timepoints between 1 hour and 1 day post infection (Kuehnel *et al* 2001). Kuehnel and colleagues, who reported the highest percent association, utilized electron microscopy for their calculations, while all other associations were determined by fluorescent or confocal microscopy, raising a question about the consistency between these techniques. Even still, the percent co-localization between vacuoles containing MAC and LAMP-1 as determined by fluorescent or confocal microscopy seems to vary depending on the researcher.

The importance of gaining a more detailed understanding of the intracellular phenotype of MAC led me to the general goal of this work. Despite several failed studies discussed above, we have shown that MAC use apoptotic macrophages to aid with dissemination, developed a new laboratory tool to help examine the predominant phenotype MAC use for dissemination, characterized the tat secretion system, and studied the vacuoles of mutants impaired in macrophage uptake. These data should lead to more in depth studies of how MAC survive within the macrophage and potentially lead to the development of new treatments.

References

Bermudez LE, Parker A, Goodman JR. Growth within macrophages increases the efficiency of Mycobacterium avium in invading other macrophages by a complement receptor-independent pathway. Infect Immun. 1997 May;65(5):1916-25.

Bermudez LE, Petrofsky M, Sangari F. Intracellular phenotype of Mycobacterium avium enters macrophages primarily by a macropinocytosis-like mechanism and survives in a compartment that differs from that with extracellular phenotype. Cell Biol Int. 2004;28(5):411-19.

Danelishvili L, Wu M, Stang B, Harriff M, Cirillo SL, Cirillo JD, Bildfell R, Arbogast B, Bermudez LE. Identification of Mycobacterium avium pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci U S A. 2007 Jun 26;104(26):11038-43.

Fratazzi C, Arbeit RD, Carini C, Remold HG. Programmed cell death of Mycobacterium avium serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. J Immunol. 1997 May 1;158(9):4320-7.

Kelley VA, Schorey JS. Mycobacterium's arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. Mol Biol Cell. 2003 Aug;14(8):3366-77.

Kuehnel MP, Goethe R, Habermann A, Mueller E, Rohde M, Griffiths G, Valentin-Weigand P. Characterization of the intracellular survival of Mycobacterium avium ssp. paratuberculosis: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. Cell Microbiol. 2001 Aug;3(8):551-66.

Lee BH, Murugasu-Oei B, Dick T. Upregulation of a histone-like protein in dormant Mycobacterium smegmatis. Mol Gen Genet. 1998 Dec;260(5):475-9.

Marques MA, Mahapatra S, Sarno EN, Santos S, Spencer JS, Brennan PJ, Pessolani MC. Further biochemical characterization of Mycobacterium leprae laminin-binding proteins. Braz J Med Biol Res. 2001 Apr;34(4):463-70.

Pais TF, Appelberg R. Induction of Mycobacterium avium growth restriction and inhibition of phagosome-endosome interactions during macrophage activation and apoptosis induction by picolinic acid plus IFNgamma. Microbiology. 2004 May;150(Pt 5):1507-18.

Posey JE, Shinnick TM, Quinn FD. Characterization of the twin-arginine translocase secretion system of Mycobacterium smegmatis. J Bacteriol. 2006 Feb;188(4):1332-40.

Sato H, Feix JB, Hillard CJ, Frank DW. Characterization of phospholipase activity of the Pseudomonas aeruginosa type III cytotoxin, ExoU. J Bacteriol. 2005 Feb;187(3):1192-5.

Sato H, Feix JB, Frank DW. Identification of superoxide dismutase as a cofactor for the pseudomonas type III toxin, ExoU. Biochemistry. 2006 Aug 29;45(34):10368-75.

Sato H, Frank DW. ExoU is a potent intracellular phospholipase. Mol Microbiol. 2004 Sep;53(5):1279-90.

Sato H, Frank DW, Hillard CJ, Feix JB, Pankhaniya RR, Moriyama K, Finck-Barbançon V, Buchaklian A, Lei M, Long RM, Wiener-Kronish J, Sawa T. The

mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. EMBO J. 2003 Jun 16;22(12):2959-69.

Shires K, Steyn L. The cold-shock stress response in Mycobacterium smegmatis induces the expression of a histone-like protein. Mol Microbiol. 2001 Feb;39(4):994-1009.

Singh A, Mai D, Kumar A, Steyn AJ. Dissecting virulence pathways of Mycobacterium tuberculosis through protein-protein association. Proc Natl Acad Sci U S A. 2006 Jul 25;103(30):11346-51.

Bibliography

Akporiaye ET, Rowatt JD, Aragon AA, Baca OG. Lysosomal response of a murine macrophage-like cell line persistently infected with Coxiella burnetii. Infect Immun. 1983 Jun;40(3):1155-62.

Aksamit TR. Mycobacterium avium complex pulmonary disease in patients with pre-existing lung disease. Clin Chest Med. 2002 Sep;23(3):643-53.

Altier C. Genetic and environmental control of salmonella invasion. J Microbiol. 2005 Feb;43 Spec No:85-92.

Appelberg R, Castro AG, Gomes S, Pedrosa J, Silva MT. Susceptibility of beige mice to Mycobacterium avium: role of neutrophils. Infect Immun. 1995 Sep;63(9):3381-7.

Bachhawat N, Singh B. Mycobacterial PE_PGRS proteins contain calcium-binding motifs with parallel beta-roll folds. Genomics Proteomics Bioinformatics. 2007 Dec;5(3-4):236-41.

Barker LP, Porcella SF, Wyatt RG, Small PL. The Mycobacterium marinum G13 promoter is a strong sigma 70-like promoter that is expressed in Escherichia coli and mycobacteria species. FEMS Microbiol Lett. 1999 Jun 1;175(1):79-85.

Bhattacharyya A, Pathak S, Basak C, Law S, Kundu M, Basu J. Execution of macrophage apoptosis by Mycobacterium avium through apoptosis signal-regulating kinase 1/p38 mitogen-activated protein kinase signaling and caspase 8 activation. J Biol Chem. 2003 Jul 18;278(29):26517-25.

Behr MA, Kapur V. The evidence for Mycobacterium paratuberculosis in Crohn's disease. Curr Opin Gastroenterol. 2008 Jan;24(1):17-21.

Berks BC.A common export pathway for proteins binding complex redox cofactors? Mol Microbiol. 1996 Nov;22(3):393-404.

Bermudez LE, Goodman J, Petrofsky M. Role of complement receptors in uptake of Mycobacterium avium by macrophages in vivo: evidence from studies using CD18-deficient mice. Infect Immun. 1999 Sep;67(9):4912-6.

Bermudez LE, Kolonoski P, Young LS. Natural killer cell activity and macrophage-dependent inhibition of growth or killing of Mycobacterium avium complex in a mouse model. J Leukoc Biol. 1990 Feb;47(2):135-41.

Bermudez LE, Parker A, Goodman JR. Growth within macrophages increases the efficiency of Mycobacterium avium in invading other macrophages by a complement receptor-independent pathway. Infect Immun. 1997 May;65(5):1916-25.

Bermudez LE, Petrofsky M, Sangari F. Intracellular phenotype of Mycobacterium avium enters macrophages primarily by a macropinocytosis-like mechanism and survives in a compartment that differs from that with extracellular phenotype. Cell Biol Int. 2004;28(5):411-9.

Bermudez LE, Young LS, Enkel H. Interaction of Mycobacterium avium complex with human macrophages: roles of membrane receptors and serum proteins. Infect Immun. 1991 May;59(5):1697-702.

Bermudez LE, Young LS. Oxidative and non-oxidative intracellular killing of Mycobacterium avium complex. Microb Pathog. 1989 Oct;7(4):289-98.

Bermudez LE, Young LS. Natural killer cell-dependent mycobacteriostatic and mycobactericidal activity in human macrophages. J Immunol. 1991 Jan 1;146(1):265-70.

Bermudez LE, Young LS. Recombinant granulocyte-macrophage colony-stimulating factor activates human macrophages to inhibit growth or kill Mycobacterium avium complex. J Leukoc Biol. 1990 Jul;48(1):67-73.

Bermudez LE, Young LS. Tumor necrosis factor, alone or in combination with IL-2, but not IFN-gamma, is associated with macrophage killing of Mycobacterium avium complex. J Immunol. 1988 May 1;140(9):3006–3013.

Beuzón CR, Méresse S, Unsworth KE, Ruíz-Albert J, Garvis S, Waterman SR, Ryder TA, Boucrot E, Holden DW. Salmonella maintains the integrity of its intracellular vacuole through the action of SifA. EMBO J. 2000 Jul 3;19(13):3235-49.

Boechat N, Lagier-Roger B, Petit S, Bordat Y, Rauzier J, Hance AJ, Gicquel B, Reyrat JM. Disruption of the gene homologous to mammalian Nramp1 in Mycobacterium tuberculosis does not affect virulence in mice. Infect Immun. 2002 Aug;70(8):4124-31.

Bohlson SS, Strasser JA, Bower JJ, Schorey JS. Role of complement in Mycobacterium avium pathogenesis: in vivo and in vitro analyses of the host response to infection in the absence of complement component C3. Infect Immun. 2001 Dec;69(12):7729-35.

Boyer E, Bergevin I, Malo D, Gros P, Cellier MF. Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. Infect Immun. 2002 Nov;70(11):6032-42.

Brennan MJ, Delogu G, Chen Y, Bardarov S, Kriakov J, Alavi M, Jacobs WR Jr. Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. Infect Immun. 2001 Dec;69(12):7326-33.

Bronstein PA, Marrichi M, Cartinhour S, Schneider DJ, DeLisa MP. Identification of a twin-arginine translocation system in Pseudomonas syringae pv. tomato DC3000 and its contribution to pathogenicity and fitness. J Bacteriol. 2005 Dec;187(24):8450-61.

Caldelari I, Mann S, Crooks C, Palmer T. The Tat pathway of the plant pathogen Pseudomonas syringae is required for optimal virulence. Mol Plant Microbe Interact. 2006 Feb;19(2):200-12.

Cangelosi GA, Do JS, Freeman R, Bennett JG, Semret M, Behr MA. The two-component regulatory system mtrAB is required for morphotypic multidrug resistance in Mycobacterium avium. Antimicrob Agents Chemother. 2006 Feb;50(2):461-8.

Cangelosi, GA, Palermo CO, Bermudez LE. Phenotypic consequences of red-white colony type variation in Mycobacterium avium. Microbiology. 2001 Mar;147(Pt3):527-33.

Cheng TC, Sullivan JT. Effects of heavy metals on phagocytosis by mollusca hemocytes. Mar. Envir. Res, 1984 Jan(14):305-15.

Chitale S, Ehrt S, Kawamura I, Fujimura T, Shimono N, Anand N, Lu S, Cohen-Gould L, Riley LW. Recombinant Mycobacterium tuberculosis protein associated with mammalian cell entry. Cell Microbiol. 2001 Apr;3(4):247-54.

Cirillo JD, Falkow S, Tompkins LS, Bermudez LE. Interaction of Mycobacterium avium with environmental amoebae enhances virulence. Infect Immun. 1997 Sep;65(9):3759-67.

Dam T, Danelishvili L, Wu M, Bermudez LE. The fadD2 gene is required for efficient Mycobacterium avium invasion of mucosal epithelial cells. J Infect Dis. 2006 Apr 15;193(8):1135-42.

Damsker B, Bottone EJ. Mycobacterium avium-Mycobacterium intracellulare from the intestinal tracts of patients with the acquired immunodeficiency syndrome: concepts regarding acquisition and pathogenesis. J Infect Dis. 1985 Jan;151(1):179-81.

Danelishvili L, Poort MJ, Bermudez LE, Identification of Mycobacterium avium genes up-regulated in cultured macrophages and in mice. FEMS Microbiol Lett. 2004 Oct 1;239(1):41-9.

Danelishvili L, Wu M, Stang B, Harriff M, Cirillo SL, Cirillo JD, Bildfell R, Arbogast B, Bermudez LE. Identification of Mycobacterium avium pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci U S A. 2007 Jun 26;104(26):11038-43.

Danelishvili L, Wu M, Young LS, Bermudez LE. Genomic approach to identifying the putative target of and mechanisms of resistance to mefloquine in mycobacteria. Antimicrob Agents Chemother. 2005 Sep;49(9):3707-14.

De Buck E, Vranckx L, Meyen E, Maes L, Vandersmissen L, Anne J, Lammertyn E. The twin-arginine translocation pathway is necessary for correct membrane insertion of the Rieske Fe/S protein in Legionella pneumophila. FEBS Lett. 2007 Jan 23;581(2):259-64.

de Chastellier C, Thilo L. Pathogenic Mycobacterium avium remodels the phagosome membrane in macrophages within days after infection. Eur J Cell Biol. 2002 Jan;81(1):17-25.

DeLisa MP, Tullman D, Georgiou G. Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. Proc Natl Acad Sci U S A. 2003 May 13;100(10):6115-20.

Desjardins M, Huber LA, Parton RG, Griffiths G. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J Cell Biol. 1994 Mar;124(5):677-88.

DiGiuseppe Champion PA, Cox JS. Protein secretion systems in Mycobacteria. Cell Microbiol. 2007 Jun;9(6):1376-84.

Denis M. Growth of Mycobacterium avium in human monocytes: identification of cytokines which reduce and enhance intracellular microbial growth. Eur J Immunol. 1991 Feb;21(2):391-5.

Denis M. Modulation of Mycobacterium avium growth in vivo by cytokines: involvement of tumour necrosis factor in resistance to atypical mycobacteria. Clin Exp Immunol. 1991 Mar;83(3):466-71.

Dheenadhayalan V, Delogu G, Brennan MJ. Expression of the PE_PGRS 33 protein in Mycobacterium smegmatis triggers necrosis in macrophages and enhanced mycobacterial survival. Microbes Infect. 2006 Jan;8(1):262-72.

Ding Z, Christie PJ. Agrobacterium tumefaciens twin-arginine-dependent translocation is important for virulence, flagellation, and chemotaxis but not type IV secretion. J Bacteriol. 2003 Feb;185(3):760-71.

Eckstein TM, Belisle JT, Inamine JM. Proposed pathway for the biosynthesis of serovar-specific glycopeptidolipids in Mycobacterium avium serovar 2. Microbiology. 2003 Oct;149(Pt 10):2797-807.

El-Etr SH, Subbian S, Cirillo SL, Cirillo JD. Identification of two Mycobacterium marinum loci that affect interactions with macrophages. Infect Immun. 2004 Dec;72(12):6902-13.

Falkinham JO 3rd. Epidemiology of infection by nontuberculous mycobacteria. Clin Microbiol Rev. 1996 Apr;9(2):177-215.

Fattorini L, Xiao Y, Li B, Santoro C, Ippoliti F, Orefici G. Induction of IL-1 beta, IL-6, TNF-alpha, GM-CSF and G-CSF in human macrophages by smooth transparent and smooth opaque colonial variants of Mycobacterium avium. J Med Microbiol. 1994 Feb;40(2):129-33.

Feodorova VA, Devdariani ZL. Expression of acid-stable proteins and modified lipopolysaccharide of Yersinia pestis in acidic growth medium. J Med Microbiol. 2001 Nov;50(11):979-85.

Flesselles B, Anand NN, Remani J, Loosmore SM, Klein MH. Disruption of the mycobacterial cell entry gene of Mycobacterium bovis BCG results in a mutant that exhibits a reduced invasiveness for epithelial cells. FEMS Microbiol Lett. 1999 Aug 15;177(2):237-42.

Flórido M, Correia-Neves M, Cooper AM, Appelberg R. The cytolytic activity of natural killer cells is not involved in the restriction of Mycobacterium avium growth. Int Immunol. 2003 Aug;15(8):895-901.

Fournier M, Cyr D, Blakley B, Boermans H, Brousseau P. Phagocytosis as a biomarker of immunotoxicity in wildlife species exposed to environmental xonobiotics. American Zoologist 2000 40(3):412-20.

Frank DW. The exoenzyme S regulon of Pseudomonas aeruginosa. Mol Microbiol. 1997 Nov;26(4):621-9.

Fratazzi C, Arbeit RD, Carini C, Balcewicz-Sablinska MK, Keane J, Kornfeld H, Remold HG. Macrophage apoptosis in mycobacterial infections. J Leukoc Biol. 1999 Nov;66(5):763-4.

Fratazzi C, Arbeit RD, Carini C, Remold HG. Programmed cell death of Mycobacterium avium serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. J Immunol. 1997 May 1;158(9):4320-7.

Fratti RA, Backer JM, Gruenberg J, Corvera S, Deretic V. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. J Cell Biol. 2001 Aug 6;154(3):631-44.

Gaillard JL, Berche P, Mounier J, Richard S, Sansonetti P. In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2. Infect Immun. 1987 Nov;55(11):2822-9.

Gan H, Newman GW, Remold HG. Plasminogen activator inhibitor type 2 prevents programmed cell death of human macrophages infected with Mycobacterium avium, serovar 4. J Immunol. 1995 Aug 1;155(3):1304-15.

Gancz H, Jones KR, Merrell DS. Sodium chloride affects Helicobacter pylori growth and gene expression. J Bacteriol. 2008 Jun;190(11):4100-5.

Gangadharam PR, Perumal VK, Parikh K, Podapati NR, Taylor R, Farhi DC, Iseman MD. Susceptibility of beige mice to Mycobacterium avium complex infections by different routes of challenge. Am Rev Respir Dis. 1989 May;139(5):1098-104.

Gao LY, Pak M, Kish R, Kajihara K, Brown EJ. A mycobacterial operon essential for virulence in vivo and invasion and intracellular persistence in macrophages. Infect Immun. 2006 Mar;74(3):1757-67.

Gao LY, Guo S, McLaughlin B, Morisaki H, Engel JN, Brown EJ. A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. Mol Microbiol. 2004 Sep;53(6):1677-93.

Gat O, Mendelson I, Chitlaru T, Ariel N, Altboum Z, Levy H, Weiss S, Grosfeld H, Cohen S, Shafferman A. The solute-binding component of a putative Mn(II) ABC transporter (MntA) is a novel Bacillus anthracis virulence determinant. Mol Microbiol. 2005 Oct;58(2):533-51.

Geisow MJ, D'Arcy Hart P, Young MR. Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. J Cell Biol. 1981 Jun;89(3):645-52.

Gioffré A, Infante E, Aguilar D, Santangelo MP, Klepp L, Amadio A, Meikle V, Etchechoury I, Romano MI, Cataldi A, Hernández RP, Bigi F. Mutation in mce operons attenuates Mycobacterium tuberculosis virulence. Microbes Infect. 2005 Mar;7(3):325-34.

Giusti C, Kosta A, Lam D, Tresse E, Luciani MF, Golstein P. Analysis of autophagic and necrotic cell death in Dictyostelium. Methods Enzymol. 2008;446:1-15.

Gooderham WJ, Hancock R. Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa. FEMS Microbiol Rev. 2008 Oct 13;33(2):279-94.

Guerin I, de Chastellier C. Pathogenic mycobacteria disrupt the macrophage actin filament network. Infect Immun. 2000 May;68(5):2655-62.

Guinn KM, Hickey MJ, Mathur SK, Zakel KL, Grotzke JE, Lewinsohn DM, Smith S, Sherman DR. Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of Mycobacterium tuberculosis. Mol Microbiol. 2004 Jan;51(2):359-70.

Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell. 2004 Dec 17;119(6):753-66.

Haile Y, Caugant DA, Bjune G, Wiker HG. Mycobacterium tuberculosis mammalian cell entry operon (mce) homologs in Mycobacterium other than tuberculosis (MOTT). FEMS Immunol Med Microbiol. 2002 Jun 3;33(2):125-32.

Harrington-Fowler L, Wilder MS. Fate of Listeria monocytogenes in murine peritoneal macrophage subpopulations. Infect Immun. 1982 Jan;35(1):124-32.

Harshan KV, Gangadharam PR. In vivo depletion of natural killer cell activity leads to enhanced multiplication of Mycobacterium avium complex in mice. Infect Immun. 1991 Aug;59(8):2818-21.

Hartmann P, Becker R, Franzen C, Schell-Frederick E, Römer J, Jacobs M, Fätkenheuer G, Plum G. Phagocytosis and killing of Mycobacterium avium complex by human neutrophils. J Leukoc Biol. 2001 Mar;69(3):397-404.

Hayashi T, Catanzaro A, Rao SP. Apoptosis of human monocytes and macrophages by Mycobacterium avium sonicate. Infect Immun. 1997 Dec;65(12):5262-71.

He H, Hovey R, Kane J, Singh V, and Thomas C. Zahrt. MprAB Is a Stress-Responsive Two-Component System That Directly Regulates Expression of Sigma Factors SigB and SigE in Mycobacterium tuberculosis. Journal of Bacteriology. 2006 Mar. 188(6):2134-2143.

Heinzen RA, Scidmore MA, Rockey DD, Hackstadt T. Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of Coxiella burnetii and Chlamydia trachomatis. Infect Immun. 1996 Mar;64(3):796-809.

Holm A, Tejle K, Magnusson KE, Descoteaux A, Rasmusson B. Leishmania donovani lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKCalpha and defective phagosome maturation. Cell Microbiol. 2001 Jul;3(7):439-47.

Horwitz, MA. The Legionnaires' disease bacterium (Legionella pneumophila) inhibits phagosome lysosome fusion in human monocytes. J Exp Med. 1983 Dec 1;158(6):2108-26.

Hou JY, Graham JE, Clark-Curtiss JE. Mycobacterium avium genes expressed during growth in human macrophages detected by selective capture of transcribed sequences (SCOTS). Infect Immun. 2002 Jul;70(7):3714-26.

Igo MM, Ninfa AJ, Silhavy TJ. A bacterial environmental sensor that functions as a protein kinase and stimulates transcriptional activation. Genes Dev. 1989 May;3(5):598-605.

Inderlied CB, Kemper CA, Bermudez LE. The Mycobacterium avium complex. Clin. Microbiol Rev. 1993 Jul;6(3):266-310.

Iyer R, Iverson TM, Accardi A, Miller C. A biological role for prokaryotic ClC chloride channels. Nature. 2002 Oct 17;419(6908):715-8.

Ize B, Gérard F, Zhang M, Chanal A, Voulhoux R, Palmer T, Filloux A, Wu LF. In vivo dissection of the Tat translocation pathway in Escherichia coli. J Mol Biol. 2002 Mar 29;317(3):327-35.

Jabado N, Jankowski A, Dougaparsad S, Picard V, Grinstein S, Gros P. Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane. J Exp Med. 2000 Nov 6;192(9):1237-48.

Jacques YV, Bainton DF. Changes in pH within the phagocytic vacuoles of human neutrophils and monocytes. Lab Invest. 1978 Sep;39(3):179-85.

Jensen-Cain DM, Quinn FD. Differential expression of sigE by Mycobacterium tuberculosis during intracellular growth. Microb Pathog. 2001 May;30(5):271-8.

Kansal RG, Gomez-Flores R, Mehta RT. Change in colony morphology influences the virulence as well as the biochemical properties of the Mycobacterium avium complex. Microb Pathog. 1998 Oct;25(4):203-14.

Katz P, Yeager H Jr, Whalen G, Evans M, Swartz RP, Roecklein J. Natural killer cell-mediated lysis of Mycobacterium-avium complex-infected monocytes. J Clin Immunol. 1990 Jan;10(1):71-7.

Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, Kornfeld H. Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. Infect Immun. 1997 Jan;65(1):298-304.

Kehres DG, Zaharik ML, Finaly BB, Maguire ME. The NRAMP proteins of Salmonella typhimurium and Escherichia coli are selective manganese transporters involved in the response to reactive oxygen. Mol. Microbiol. 2000 Jun;36(5):1085-100.

Kelley VA, Schorey JS. Mycobacterium's arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. Mol Biol Cell. 2003 Aug;14(8):3366-77.

Kelley VA, Schorey JS. Modulation of cellular phosphatidylinositol 3-phosphate levels in primary macrophages affects heat-killed but not viable Mycobacterium avium's transport through the phagosome maturation process. Cell Microbiol. 2004 Oct;6(10):973-85.

Kim J, Ahn K, Min S, Jia J, Ha U, Wu D, Jin S. Factors triggering type III secretion in Pseudomonas aeruginosa. Microbiology. 2005 Nov;151(Pt 11):3575-87.

Krzywinska E, Bhatnagar S, Sweet L, Chatterjee D, Schorey JS. Mycobacterium avium 104 deleted of the methyltransferase D gene by allelic replacement lacks serotype-specific glycopeptidolipids and shows attenuated virulence in mice. Mol Microbiol. 2005 Jun;56(5):1262-73.

Kuehnel MP, Goethe R, Habermann A, Mueller E, Rohde M, Griffiths G, Valentin-Weigand P. Characterization of the intracellular survival of Mycobacterium avium ssp. paratuberculosis: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. Cell Microbiol. 2001 Aug;3(8):551-66.

Kuhn DE, Lafuse WP, Zwilling BS. Iron transport into mycobacterium avium-containing phagosomes from an Nramp1(Gly169)-transfected RAW264.7 macrophage cell line. J Leukoc Biol. 2001 Jan;69(1):43-9.

Lammers A, Nuijten PJ, Smith HE. The fibronectin binding proteins of Staphylococcus aureus are required for adhesion to and invasion of bovine mammary gland cells. FEMS Microbiol Lett. 1999 Nov 1;180(1):103-9.

Laurent JP, Hauge K, Burnside K, Cangelosi G. Mutational analysis of cell wall biosynthesis in Mycobacterium avium. J Bacteriol. 2003 Aug;185(16):5003-6.

Lavander M, Ericsson SK, Bröms JE, Forsberg A. Twin-arginine translocation in Yersinia. Adv Exp Med Biol. 2007;603:258-67.

Lee BH, Murugasu-Oei B, Dick T. Upregulation of a histone-like protein in dormant Mycobacterium smegmatis. Mol Gen Genet. 1998 Dec;260(5):475-9.

Li Y, Miltner E, Wu M, Petrofsky M, Bermudez LE. A Mycobacterium avium PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. Cell Microbiol. 2005 Apr;7(4):539-48.

Manganelli R, Fattorini L, Tan D, Iona E, Orefici G, Altavilla G, Cusatelli P, Smith I. The extra cytoplasmic function sigma factor sigma(E) is essential for Mycobacterium tuberculosis virulence in mice. Infect Immun. 2004 May;72(5):3038-41.

Manganelli R, Voskuil MI, Schoolnik GK, Smith I. The Mycobacterium tuberculosis ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. Mol Microbiol. 2001 Jul;41(2):423-37.

Marques MA, Mahapatra S, Sarno EN, Santos S, Spencer JS, Brennan PJ, Pessolani MC. Further biochemical characterization of Mycobacterium leprae laminin-binding proteins. Braz J Med Biol Res. 2001 Apr;34(4):463-70.

McDonough JA, Hacker KE, Flores AR, Pavelka MS Jr, Braunstein M. The twin-arginine translocation pathway of Mycobacterium smegmatis is functional and required for the export of mycobacterial beta-lactamases. J Bacteriol. 2005 Nov;187(22):7667-79.

McDonough JA, McCann JR, Tekippe EM, Silverman JS, Rigel NW, Braunstein M. Identification of functional Tat signal sequences in Mycobacterium tuberculosis proteins. J Bacteriol. 2008 Oct;190(19):6428-38.

McDonough KA, Kress Y, Bloom BR. Pathogenesis of tuberculosis: interaction of Mycobacterium tuberculosis with macrophages. Infect Immun. 1993 Jul;61(7):2763-73.

Milano A, Branzoni M, Canneva F, Profumo A, Riccardi G. The Mycobacterium tuberculosis Rv2358-furB operon is induced by zinc. Res Microbiol. 2004 Apr;155(3):192-200.

Mohagheghpour N, van Vollenhoven A, Goodman J, Bermudez LE. Interaction of Mycobacterium avium with human monocyte-derived dendritic cells. Infect Immun. 2000 Oct;68(10):5824-9.

Monack DM, Raupach B, Hromockyj AE, Falkow S. Salmonella typhimurium invasion induces apoptosis in infected macrophages. Proc Natl Acad Sci U S A. 1996 Sep 3;93(18):9833-8.

Monack DM, Mecsas J, Bouley D, Falkow S. Yersinia-induced apoptosis in vivo aids in the establishment of a systemic infection of mice. J Exp Med. 1998 Dec 7;188(11):2127-37.

Mueller-Ortiz SL, Wanger AR, Norris SJ. Mycobacterial protein HbhA binds human complement component C3. Infect Immun. 2001 Dec;69(12):7501-11.

Murphy RF, Powers S, Cantor CR. Endosome pH measured in single cells by dual fluorescence flow cytometry: rapid acidification of insulin to pH 6. J Cell Biol. 1984 May;98(5):1757-62.

Oh YK, Straubinger RM. Intracellular fate of Mycobacterium avium: use of dual-label spectrofluorometry to investigate the influence of bacterial viability and opsonization on phagosomal pH and phagosome-lysosome interaction. Infect Immun. 1996 Jan;64(1):319-25.

Orme IM, Furney SK, Roberts AD. Dissemination of enteric Mycobacterium avium infections in mice rendered immunodeficient by thymectomy and CD4 depletion or by prior infection with murine AIDS retroviruses. Infect Immun. 1992 Nov;60(11):4747-53.

Ochsner UA, Snyder A, Vasil AI, Vasil ML. Effects of the twin-arginine translocase on secretion of virulence factors, stress response, and pathogenesis. Proc Natl Acad Sci U S A. 2002 Jun 11;99(12):8312-7.

Pais TF, Appelberg R. Induction of Mycobacterium avium growth restriction and inhibition of phagosome-endosome interactions during macrophage activation and apoptosis induction by picolinic acid plus IFNgamma. Microbiology. 2004 May;150(Pt 5):1507-18.

Palmer T, Sargent F, Berks BC. Light traffic: photo-crosslinking a novel transport system. Trends Biochem Sci. 2004 Feb;29(2):55-7.

Papp-Wallace K, Maguire, M. Manganese Transport and the Role of Manganese in Virulence. Annu. Rev. Microbiol. 2006. 60:187-209.

Parish T, Stoker NG. Development and use of a conditional antisense mutagenesis system in mycobacteria. FEMS Microbiol Lett. 1997 Sep 1;154(1):151-7.

Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, Locht C, Menozzi FD. The heparin-binding haemagglutinin of M. tuberculosis is required for extrapulmonary dissemination. Nature. 2001 Jul 12;412(6843):190-4.

Petrofsky M, Bermudez LE. 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. 2005 May;73(5):2621-7.

Philalay JS, Palermo CO, Hauge KA, Rustad TR, Cangelosi GA. Genes required for intrinsic multidrug resistance in Mycobacterium avium. Antimicrob Agents Chemother. 2004 Sep;48(9):3412-8.

Pietersen R, Thilo L, de Chastellier C. Mycobacterium tuberculosis and Mycobacterium avium modify the composition of the phagosomal membrane in infected macrophages by selective depletion of cell surface-derived glycoconjugates. Eur J Cell Biol. 2004 May;83(4):153-8.

Plum G, Brenden M, Clark-Curtiss JE, Pulverer G. Cloning, sequencing, and expression of the mig gene of Mycobacterium avium, which codes for a secreted macrophage-induced protein. Infect Immun. 1997 Nov;65(11):4548-57.

Pop O, Martin U, Abel C, Müller JP. The twin-arginine signal peptide of PhoD and the TatAd/Cd proteins of Bacillus subtilis form an autonomous Tat translocation system. J Biol Chem. 2002 Feb 1;277(5):3268-73.

Posey JE, Shinnick TM, Quinn FD. Characterization of the twin-arginine translocase secretion system of Mycobacterium smegmatis. J Bacteriol. 2006 Feb;188(4):1332-40.

Pradel N, Ye C, Livrelli V, Xu J, Joly B, Wu LF. Contribution of the twin arginine translocation system to the virulence of enterohemorrhagic Escherichia coli O157:H7. Infect Immun. 2003 Sep;71(9):4908-16.

Raman S, Hazra R, Dascher CC, Husson RN. Transcription regulation by the Mycobacterium tuberculosis alternative sigma factor SigD and its role in virulence. J Bacteriol. 2004 Oct;186(19):6605-16.

Rastogi N, Frehel C, Ryter A, Ohayon H, Lesourd M, David HL. Multiple drug resistance in Mycobacterium avium: is the wall architecture responsible for exclusion of antimicrobial agents? Antimicrob Agents Chemother. 1981 Nov;20(5):666-77.

Rathman M, Sjaastad MD, Falkow S. Acidification of phagosomes containing Salmonella typhimurium in murine macrophages. Infect Immun. 1996 Jul;64(7):2765-73.

Rodrigue A, Chanal A, Beck K, Muller M, Wu LF. Co-translocation of a periplasmic enzyme complex by a hitchhiker mechanism through the bacterial tat pathway. J Biol Chem. 1999 May 7;274(19):13223-8.

Roecklein JA, Swartz RP, Yeager H Jr. Nonopsonic uptake of Mycobacterium avium complex by human monocytes and alveolar macrophages. J Lab Clin Med. 1992 Jun;119(6):772-81.

Rose RW, Brüser T, Kissinger JC, Pohlschröder M. Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. Mol Microbiol. 2002 Aug;45(4):943-50.

Rossier O, Cianciotto NP. The Legionella pneumophila tatB gene facilitates secretion of phospholipase C, growth under iron-limiting conditions, and intracellular infection. Infect Immun. 2005 Apr;73(4):2020-32.

Saini DK, Malhotra V, Tyagi JS. Cross talk between DevS sensor kinase homologue, Rv2027c, and DevR response regulator of Mycobacterium tuberculosis. FEBS Lett. 2004 May 7;565(1-3):75-80.

Saint-Joanis B, Demangel C, Jackson M, Brodin P, Marsollier L, Boshoff H, Cole ST. Inactivation of Rv2525c, a substrate of the twin-arginine translocation (Tat) system of Mycobacterium tuberculosis, increases beta-lactam susceptibility and virulence. J Bacteriol. 2006 Sep;188(18):6669-79.

Sangari FJ, Goodman J, Bermudez LE. 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. 2000 Dec;2(6):561-8.

Sangari FJ, Goodman J, Petrofsky M, Kolonoski P, Bermudez LE. Mycobacterium avium invades the intestinal mucosa primarily by interacting with enterocytes. Infect Immun. 2001 Mar;69(3):1515-20.

Sargent F, Berks BC, Palmer T. Assembly of membrane-bound respiratory complexes by the Tat protein-transport system. Arch Microbiol. 2002 Aug;178(2):77-84.

Sassetti CM, Rubin EJ. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A. 2003 Oct 28;100(22):12989-94.

Sato H, Feix JB, Hillard CJ, Frank DW. Characterization of phospholipase activity of the Pseudomonas aeruginosa type III cytotoxin, ExoU. J Bacteriol. 2005 Feb;187(3):1192-5.

Sato H, Feix JB, Frank DW. Identification of superoxide dismutase as a cofactor for the pseudomonas type III toxin, ExoU. Biochemistry. 2006 Aug 29;45(34):10368-75.

Sato H, Frank DW. ExoU is a potent intracellular phospholipase. Mol Microbiol. 2004 Sep;53(5):1279-90.

Sato H, Frank DW, Hillard CJ, Feix JB, Pankhaniya RR, Moriyama K, Finck-Barbançon V, Buchaklian A, Lei M, Long RM, Wiener-Kronish J, Sawa T. The mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. EMBO J. 2003 Jun 16;22(12):2959-69.

Saunders BM, Cheers C. Intranasal infection of beige mice with Mycobacterium avium complex: role of neutrophils and natural killer cells. Infect Immun. 1996 Oct;64(10):4236-41.

Sato J, Schorey J, Ploplis VA, Haalboom E, Krahule L, Castellino FJ. The fibrinolytic system in dissemination and matrix protein deposition during a mycobacterium infection. Am J Pathol. 2003 Aug;163(2):517-31.

Schlesinger LS, Bellinger-Kawahara CG, Payne NR, Horwitz MA. Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. J Immunol. 1990 Apr 1;144(7):2771-80.

Senaratne RH, Sidders B, Sequeira P, Saunders G, Dunphy K, Marjanovic O, Reader JR, Lima P, Chan S, Kendall S, McFadden J, Riley LW. Mycobacterium tuberculosis strains disrupted in mce3 and mce4 operons are attenuated in mice. J Med Microbiol. 2008 Feb;57(Pt 2):164-70.

Shires K, Steyn L. The cold-shock stress response in Mycobacterium smegmatis induces the expression of a histone-like protein. Mol Microbiol. 2001 Feb;39(4):994-1009.

Singh A, Mai D, Kumar A, Steyn AJ. Dissecting virulence pathways of Mycobacterium tuberculosis through protein-protein association. Proc Natl Acad Sci U S A. 2006 Jul 25;103(30):11346-51.

Stamm LM, Morisaki JH, Gao LY, Jeng RL, McDonald KL, Roth R, Takeshita S, Heuser J, Welch MD, Brown EJ. Mycobacterium marinum escapes from phagosomes and is propelled by actin-based motility. J Exp Med. 2003 Nov 3;198(9):1361-8.

Steinert M, Birkness K, White E, Fields B, Quinn F. Mycobacterium avium bacilli grow saprozoically in coculture with Acanthamoeba polyphaga and survive within cyst walls. Appl Environ Microbiol. 1998 Jun;64(6):2256-61.

Stewart GR, Patel J, Robertson BD, Rae A, Young DB. Mycobacterial mutants with defective control of phagosomal acidification. PLoS Pathog. 2005 Nov;1(3):269-78.

Stokes RW, Norris-Jones R, Brooks DE, Beveridge TJ, Doxsee D, Thorson LM. The glycan-rich outer layer of the cell wall of Mycobacterium tuberculosis acts as an antiphagocytic capsule limiting the association of the bacterium with macrophages. Infect Immun. 2004 Oct;72(10):5676-86.

Straley SC, Brubaker RR. Cytoplasmic and membrane proteins of yersiniae cultivated under conditions simulating mammalian intracellular environment. Proc Natl Acad Sci U S A. 1981 Feb;78(2):1224-8.

Straley SC, Plano GV, Skrzypek E, Haddix PL, Fields KA. Regulation by Ca2+ in the Yersinia low-Ca2+ response. Mol Microbiol. 1993 Jun;8(6):1005-10.

Sturgill-Koszycki S, Haddix PL, Russell DG. The interaction between Mycobacterium and the macrophage analyzed by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis. 1997 Dec;18(14):2558-65.

Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, Allen RD, Gluck SL, Heuser J, Russell DG. Lack of acidification in Mycobacterium

phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 1994 Feb 4;263(5147):678-81.

Su J, Gong H, Lai J, Main A, Lu S. The potassium transporter Trk and external potassium modulate Salmonella enterica protein secretion and virulence. Infect Immun. 2009 Feb;77(2):667-75.

Suzuki K, Lee WJ, Hashimoto T, Tanaka E, Murayama T, Amitani R, Yamamoto K, Kuze F. Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) or tumour necrosis factor-alpha (TNF-alpha) activate human alveolar macrophages to inhibit growth of Mycobacterium avium complex. Clin Exp Immunol. 1994 Oct;98(1):169-73.

Tenant R, Bermudez LE. Mycobacterium avium genes upregulated upon infection of Acanthamoeba castellanii demonstrate a common response to the intracellular environment. Curr Microbiol. 2006 Feb;52(2):128-33.

Thomas JR, Bolhuis A. The tatC gene cluster is essential for viability in halophilic archaea. FEMS Microbiol Lett. 2006 Mar;256(1):44-9.

Tilney LG, Portnoy DA. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes. J Cell Biol. 1989 Oct;109(4 Pt 1):1597-608.

Torrelles JB, Ellis D, Osborne T, Hoefer A, Orme IM, Chatterjee D, Brennan PJ, Cooper AM. Characterization of virulence, colony morphotype and the glycopeptidolipid of Mycobacterium avium strain 104. Tuberculosis (Edinb). 2002;82(6):293-300.

TranVan Nhieu G, Clair C, Grompone G, Sansonetti P. Calcium signalling during cell interactions with bacterial pathogens. Biol Cell. 2004 Feb;96(1):93-101.

Vallis AJ, Yahr TL, Barbieri JT, Frank DW. Regulation of ExoS production and secretion by Pseudomonas aeruginosa in response to tissue culture conditions. Infect Immun. 1999 Feb;67(2):914-20.

Via LE, Deretic D, Ulmer RJ, Hibler NS, Huber LA, Deretic V. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. J Biol Chem. 1997 May 16;272(20):13326-31.

Voordouw GA. universal system for the transport of redox proteins: early roots and latest developments. Biophys Chem. 2000 Aug 30;86(2-3):131-40.

Voulhoux R, Ball G, Ize B, Vasil ML, Lazdunski A, Wu LF, Filloux A. Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. EMBO J. 2001 Dec 3;20(23):6735-41.

Wagner D, Maser J, Lai B, Cai Z, Barry CE 3rd, Honer Zu Bentrup K, Russell DG, Bermudez LE. Elemental analysis of Mycobacterium avium-, Mycobacterium tuberculosis-, and Mycobacterium smegmatis-containing phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. J Immunol. 2005 Feb 1;174(3):1491-500.

Wagner D, Maser J, Moric I, Boechat N, Vogt S, Gicquel B, Lai B, Reyrat JM, Bermudez L. Changes of the phagosomal elemental concentrations by Mycobacterium tuberculosis Mramp. Microbiology. 2005 Jan;151(Pt 1):323-32.

Wexler M, Sargent F, Jack RL, Stanley NR, Bogsch EG, Robinson C, Berks BC, Palmer T. TatD is a cytoplasmic protein with DNase activity. No requirement for TatD family proteins in sec-independent protein export. J Biol Chem. 2000 Jun 2;275(22):16717-22.

Wolfgang MC, Lee VT, Gilmore ME, Lory S. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev Cell. 2003 Feb;4(2):253-63.

Yoshimori T. Autophagy: a regulated bulk degradation process inside cells. Biochem Biophys Res Commun. 2004 Jan 9;313(2):453-8.

Zerial M, Stenmark H. Rab GTPases in vesicular transport. Curr Opin Cell Biol. 1993 Aug;5(4):613-20.

Zimmerli S, Edwards S, Ernst JD. Selective receptor blockade during phagocytosis does not alter the survival and growth of Mycobacterium tuberculosis in human macrophages. Am J Respir Cell Mol Biol. 1996 Dec;15(6):760-70.

Zychlinsky A, Prevost MC, Sansonetti PJ. Shigella flexneri induces apoptosis in infected macrophages. Nature. 1992 Jul 9;358(6382):167-9.