Establishment of an *in vitro* model to study persistence in *Mycobacterium avium* subspecies *hominissuis*

by

Elyssa Armstrong

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented November 17, 2016
Commencement June 2017
AN ABSTRACT OF THE THESIS OF

Elyssa Armstrong for the degree of Honors Baccalaureate of Science in Microbiology presented on November 17, 2016. Title: Establishment of an in vitro model to study persistence in *Mycobacterium avium* subspecies *hominissuis*.

Abstract approved:________________________________________________________

Luiz Bermudez

*Mycobacterium avium* subspecies *hominissuis* (MAH) is the most common pathogen among non-tuberculous mycobacteria, causing disease in immunocompromised individuals. An intracellular bacterium, MAH resides within the phagosome, a vesicle formed by macrophages as they engulf invading pathogens. Here, a subpopulation of MAH regresses into a nonreplicative state called persistence, allowing them to tolerate antimicrobial treatment. Unlike resistant bacteria, they are genetically identical to wild-type bacteria that cannot tolerate antibiotics. Persistent bacteria contribute to the development of chronic infection. Little is known about the mechanisms that allow MAH to lapse into this metabolic state.

We investigated whether a previously established model with trace elemental mixtures representing the phagosome environment could be used to study persistence in MAH. We determined which mixture would yield the most persistent colony-forming units, then removed individual elements from full-spectrum mixture to determine which metals might be involved in inducing persistence. We established an *in vitro* model to study persistence in MAH based on media mimicking the phagosome environment after 24 hours of infection. Using this model, we found that iron might play a role in inducing...
persistence. Our model will enable researchers to study the mechanisms of persistence, contributing to current understanding of pathogenesis in MAH.

Key Words: *Mycobacterium avium*, persistence, pathogenesis

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

______________________________________________
Elyssa Armstrong, Author
ACKNOWLEDGEMENTS

I would like to thank Dr. Luiz Bermudez, my mentor through the Pre-Veterinary Scholars, for his support over the past few years. He welcomed me into his lab and gave me the incredible opportunity to work on this project as an undergraduate. Thank you for letting me contribute to your field, and for helping me learn the scientific skills necessary to be a well-rounded and effective medical practitioner.

I would also like to give special thanks to Dr. Lia Danelishvili, who trained me during my first months in the lab and taught me everything that I know about essential laboratory techniques. Thank you for believing in me, and for your unrelenting patience and support throughout this project.

I would like to thank Dr. Brian Dolan for lending his expertise to my committee. I am grateful to have had the opportunity to work with you.

I would like to thank Dr. Sasha Rose for answering all my questions, for providing valuable feedback on my presentation and discussion, and for helping me navigate all the processes of research.

I would like to thank everyone in the Bermudez laboratory for their camaraderie and for welcoming me into their community. I also want to acknowledge all my friends for always being there for me. The study sessions, baking nights, dinners, impromptu adventures, and laughter kept me strong and steady on my path. Finally, I want to thank my parents for being in my corner and encouraging me to follow my passion in the fields of science and veterinary medicine.
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INTRODUCTION

Persistent bacteria contribute to the development of chronic infections, creating a significant public health issue by adding to the issue of antibiotic resistance. Bacterial persistence was first observed in Staphylococcus aureus, which forms persistent biofilms and causes recurring infections in skin and soft tissue (1). Persistence is also observed in Pseudomonas aeruginosa, the most common source of recurrent pneumonia in patients with cystic fibrosis (CF). While treatment with antibiotics is standard for treating pneumonia in patients with CF, this method may not be effective. In a recent study, administration of broad-spectrum antibiotics for two weeks did little to change the composition of the microbial community in the lungs of CF-affected patients. These results imply the presence of drug tolerance within the microbial community of the adult lung affected with CF (2). Studies with P. aeruginosa and Escherichia coli demonstrate that the persistent state is closely associated with the SOS response and the stringent response, both processes which are triggered by environmental stressors (3). Thus, bacterial persistence can be induced by the introduction of antibiotics or by the presence of certain environmental factors.

Mycobacteria are among the best-characterized persistent pathogens. Mycobacterium avium subspecies hominissuis (hereafter referred to as MAH) is the most common pathogen among non-tuberculous mycobacteria. A ubiquitous environmental organism found in soil and water, it causes disease in animals and humans with weakened immune systems, such as patients with acquired Human Immunodeficiency Virus and existing chronic respiratory diseases (4). MAH enters the host through the respiratory or gastrointestinal tract. It primarily infects macrophages, which ingest invading pathogens.
through phagocytosis. During phagocytosis, the macrophage encloses bacterial cells inside a phagosome. Normally, phagocytosed bacteria are killed in the phagosome, but MAH deters host killing mechanisms by inhibiting acidification of the phagosome. This process allows bacteria to survive by preventing the phagosome from maturing into a phagolysosome, an organelle that degrades bacterial cells (5). This strategy to avoid host defense mechanisms is also observed in Mycobacterium tuberculosis, the causative agent of tuberculosis (6).

In addition to arresting phagosome development, both M. tuberculosis and MAH are able to transform into the persistent state. The combination of these strategies leads to high rates of relapse and prolonged rounds of drug treatment. Treatment of M. tuberculosis can last as long as 12 months, while treating MAH requires up to 2 years of antibiotic therapy (7). While many studies have scrutinized the mechanisms of bacterial resistance to antibiotics, far less is known about those of bacterial persistence. Unlike resistant bacteria, persisters are genetically identical to wild-type, non-tolerant bacteria; in essence, persistence is a nonheritable phenotype. This unique characteristic makes it challenging to identify persistent bacteria with current clinical diagnostic methods (7).

The concentrations of trace elements within the phagosomes of macrophages at 1 hour and 24 hours after MAH infection have been determined using hard X-ray microscopy (8). Trace elemental mixtures resembling the phagosome environment were made using this information. Prior to infecting macrophages, selected cultures of MAH were incubated in elemental mixtures, while others were incubated in Middlebrook 7H9 broth. MAH incubated in elemental mixtures infected macrophages at a significantly higher rate than MAH incubated in Middlebrook 7H9 broth. These results suggest that
MAH retains its virulence phenotype when incubated in these trace elemental mixtures (9). While this system of elemental mixtures is an effective in vitro model to study virulence in MAH, it is not known whether it can be used to study persistence in MAH.

We hypothesized that trace elements in the phagosome environment play a role in one of the mechanisms that can trigger the persistent state of MAH. To test the hypothesis, we first determined if MAH could become persistent following exposure to metals. Then, we identified trace elements that induced the persistent phenotype in MAH.

Results determined that trace elements in the phagosome environment contribute to the induction of persistence in MAH after antibiotic treatment. MAH could transform into the persistent state after antibiotic treatment when incubated in 1-h and 24-h post-infection elemental mixtures. The established in vitro persistence model was based on 24-h post-infection mixture, which yielded the most persisters. A stepwise reduction pattern of the 24-h elemental mixture implied that iron may induce persistence in MAH in conjunction with unknown elements. Using an in vitro model to study bacterial persistence allows recovery of the subpopulation of nonreplicating persistent bacteria. Moreover, this type of model allows removal of individual trace elements to better characterize their contributions to persistence. Establishing this model would allow biomedical researchers to better understand the pathogenesis of persistent MAH, aiding in the development of therapeutic strategies to treat human and animal patients.
MATERIALS AND METHODS

Bacterial cultures. *Mycobacterium avium* subsp. *hominissuis* strain 104 (MAH) was originally isolated from the blood of an AIDS patient. This strain was cultured on Middlebrook 7H10 agar with 10% oleic acid, albumin, dextrose, and catalase supplement (OADC; Hardy Diagnostics, Santa Maria, CA) at 37°C for 7 to 14 days, until colony-forming units (CFUs) were able to be visually quantified on agar media without a microscope. Three ml of liquid medium was inoculated with $3.0 \times 10^8$ CFU/ml in 50 ml conical centrifuge tubes. After an initial incubation period of 24 hours, selected bacterial cultures were exposed to antibiotics for 24 hours. All cultures were then centrifuged at 2000 g for 15 minutes and resuspended in 1 ml of Hank’s Balanced Salt Solution (HBSS; Cellgro, Manassas, VA). Serial dilutions were then plated to quantify CFUs yielded from each condition. Fifty colonies from each treatment were picked and plated on agar media with added rifampicin, clarithromycin, and ciprofloxacin to determine whether they were truly persistent and to ensure that resistant CFUs were not counted in the assays.

Antibiotics and reagents. Selected bacterial strains were challenged with 125 μg/ml rifampicin (stock concentration 10 mg/ml in dimethyl sulfoxide), 200 μg/ml clarithromycin (stock concentration 25 mg/ml in acetone), and 125 μg/ml ciprofloxacin (stock concentration 25 mg/ml in sterile water). Rifampicin was chosen to inhibit DNA-dependent RNA synthesis, clarithromycin to inhibit protein synthesis, and ciprofloxacin to inhibit cell division. Rifampicin and clarithromycin were purchased from Sigma-Aldrich (St. Louis, MO, USA), and ciprofloxacin was purchased from Miles Laboratories (Elkhart, IN, USA).
Medium recipes. 500 ml of Middlebrook 7H9 broth (Difco, Becton-Dickinson, Sparks, MD) was prepared according to manufacturer instructions. Supplements listed in Table 1 were added to make each elemental mixture. The pH of the 1-h elemental mixture was adjusted to 6.6, while the pH of 24-h elemental mixture and single-element dropout mixtures was adjusted to 5.8. All solutions were autoclaved and stored at 4°C until use.
Establishing an *in vitro* persistence model.

**Figure 1. MAH survival in post-infection elemental mixtures.** Bacterial cultures were subjected to one of the three following treatments: (A) Incubated in thermal shaker for 1 hour in 1-h elemental mixture mimicking the phagosome after 1 hour of infection, then exposed to antibiotics for 24 hours before plating serial dilutions. (B) Incubated for 1 hour in 1-h elemental mixture, then transferred bacteria into 24-h elemental mixture mimicking the phagosome after 24 hours of infection. After incubating in new medium for 24 hours, exposed to antibiotics for 24 hours before plating serial dilutions. (C) Incubated for 24 hours in 24-h elemental mixture, then exposed to antibiotics for 24 hours before plating serial dilutions.
Media matching the elemental composition of the phagosome at 1 hour and 24 hours post-infection were used to incubate MAH (Table 1). MAH was initially incubated in one or both types of media in a thermal shaker to allow the bacteria to adjust to the simulated phagosome environment. Antibiotics were added to the cultures to kill replicating bacteria and isolate the subpopulation of nonreplicating persistent bacteria. Cultures were then incubated for an additional 24 hours. At this time point, cultures were centrifuged at 2000 g for 15 minutes and resuspended in 1 ml of Hank’s Balanced Salt Solution before plating serial dilutions.

Figure 2. Comparing MAH incubated in 24-h post-infection elemental mixture with MAH incubated in 7H9.
To determine whether trace elements in phagosome contribute to persistence in MAH, bacteria were incubated in Middlebrook 7H9 broth or 24-h elemental mixture (Table 1). After initial incubation in a thermal shaker for 24 hours, selected cultures exposed to both conditions were centrifuged, diluted, and plated. Two groups of cultures were set aside to incubate for an additional 24 hours. Antibiotics were added to the first set, while the second set without antibiotics served as controls. After the second 24-hour incubation period, the control and treatment cultures were centrifuged at 2000 g for 15 minutes and resuspended in 1 ml of Hank’s Balanced Salt Solution before plating serial dilutions.
Trace elements inducing the persistent state of MAH. In addition to growing MAH in Middlebrook 7H9 broth and 24-h post-infection elemental mixture, MAH was grown in seven types of single-element dropout media based on our full-spectrum 24-h elemental mixture. These mixtures were made by removing one of the seven elements used to make the full-spectrum 24-h elemental mixture (Table 1). Cultures were incubated in a thermal shaker for 24 hours. At this time point, selected cultures were centrifuged, diluted, and plated. Two groups of cultures were set aside to incubate for an additional 24 hours. Antibiotics were added to the first set, while the second set without antibiotics served as controls. After the second 24-hour incubation period, the control and treatment cultures were centrifuged at 2000 g for 15 minutes and resuspended in 1 ml of Hank’s Balanced Salt Solution before plating serial dilutions.

### TABLE 1. Recipes for elemental mixtures and single-element dropout media

<table>
<thead>
<tr>
<th>Supplement</th>
<th>1-h elemental mixture</th>
<th>24-h elemental mixture</th>
<th>- Ni</th>
<th>- Fe</th>
<th>- Zn</th>
<th>- Cu</th>
<th>- Mn</th>
<th>- Ca</th>
<th>- K</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M KCl (ml)</td>
<td>14.7</td>
<td>0.925</td>
<td>0.925</td>
<td>0.925</td>
<td>0.925</td>
<td>0.925</td>
<td>0.925</td>
<td>0.925</td>
<td>-</td>
</tr>
<tr>
<td>1 M CaCl₂ (ml)</td>
<td>2</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>-</td>
<td>1.25</td>
</tr>
<tr>
<td>1 M Mn₄Cl₂ (ml)</td>
<td>5.9</td>
<td>11.9</td>
<td>11.9</td>
<td>11.9</td>
<td>11.9</td>
<td>11.9</td>
<td>-</td>
<td>11.9</td>
<td>11.9</td>
</tr>
<tr>
<td>1 M CuSO₄ (μl)</td>
<td>1.85</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>-</td>
<td>5.5</td>
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<td>5.5</td>
</tr>
<tr>
<td>1 M ZnCl₂ (μl)</td>
<td>33</td>
<td>58.7</td>
<td>58.7</td>
<td>58.7</td>
<td>-</td>
<td>58.7</td>
<td>58.7</td>
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</tr>
<tr>
<td>0.25 M FePO₄ (ml)</td>
<td>288</td>
<td>2</td>
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<td>2</td>
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<td>2</td>
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<td>2</td>
</tr>
<tr>
<td>1 M NiCl₂ (μl)</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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</table>

*Amounts of elements listed were added to 500 ml Middlebrook 7H9 broth.*
Statistical analysis. Data were evaluated for statistical significance with Student’s $t$-test using GraphPad’s online $t$-test calculator. Data with a p-value less than 0.05 were considered statistically significant.
RESULTS

Survival assay in post-infection elemental mixtures. Serial dilutions were plated to quantify CFUs after the initial incubation before adding antibiotics and after the second incubation with added drugs. After antibiotic treatment, 24-h elemental mixture gave $9.3 \pm 3.8 \times 10^3$ CFUs, while 1-h elemental mixture alone gave $1.3 \pm 3.7 \times 10^2$ CFUs (P<0.05). Because 24-h elemental mixture alone gave significantly more CFUs after antibiotic treatment than the 1-h mixture alone, this type of media was chosen for the model to study persistence in MAH (Figure 1). Selected colonies did not yield observable growth on antibiotic agar media, suggesting that the survivors were persistent.

![Bar graph showing survival of MAH in post-infection elemental mixtures](image)

**Figure 3. Comparing survival of MAH in post-infection elemental mixtures.** After initial incubation period in their respective elemental mixtures, bacteria were exposed to antibiotics for 24 hours before plating dilutions to quantify CFUs. Data represent the mean ±SDev of 2 independent experiments (*, p<0.05, the significance of difference as determined by Student’s t-test).
Contribution of phagosome trace elements to the induction of persistence. We compared survival of MAH in 7H9 broth and 24-h elemental mixture after antibiotic treatment. After antibiotic treatment, 7H9 broth gave $1.1 \pm 2.0 \times 10^1$ CFUs, and 24-h elemental mixture gave $2.0 \pm 1.8 \times 10^4$ CFUs. Although not statistically significant ($P=0.2540$), the 24-h elemental mixture gave more CFUs than 7H9 broth after antibiotic treatment. Moreover, selected colonies did not yield observable growth on antibiotic agar media, suggesting that the observed CFUs were persistent. These results suggest that the trace elements in the phagosome contribute to the induction of persistence in MAH.

**Figure 4. Effects of phagosome trace elements on induction of persistence.** After initial 24-hour incubation period, selected groups were exposed to antibiotics for 24 hours before plating dilutions to quantify CFUs from all groups. 24-hour post-infection elemental mixture gives more persisters after multi-antibiotic treatment. Data represent the mean ± SDev of 2 independent experiments.
**Phagosome elements inducing persistence.** MAH was incubated in seven types of single-element dropout metal media to determine which specific elements induce persistence in MAH. Each medium was based on the 24-h elemental mixture, with one of the seven trace elements removed (Table 1). After antibiotic treatment, full-spectrum 24-h elemental mix gave $7.5 \pm 3.0 \times 10^4$ CFUs. Dropout medium without iron gave $1.5 \pm 8.0 \times 10^2$ CFUs after antibiotic treatment – significantly fewer than full-spectrum 24-hour elemental mix ($P<0.05$). We did not observe growth from selected colonies on antibiotic agar media, suggesting that the survivors were persistent. These results suggest that iron may contribute to the induction of persistence in MAH.

![Figure 5](image)

**Figure 5. Examining persistence-inducing phagosome elements.** After initial 24-hour incubation period, selected groups were exposed to antibiotics for 24 hours before plating dilutions to quantify CFUs from all groups. Data represent the mean ±SDev of 2 independent experiments (*, $p<0.05$, the significance of difference as determined by Student’s $t$-test).
DISCUSSION

The objective of this study was to establish an in vitro model to investigate persistence in MAH. Observations from a previous work determined that the metals in the phagosome environment trigger bacterial virulence (9). This study successfully established a model for studying the bacterial persistence phenotype based on 24-h post-infection elemental mixture, and evidenced that trace elements in the phagosome contribute to the induction of the persistent phenotype in MAH. A stepwise reduction scheme, in which individual elements were removed from full-spectrum 24-h elemental mix, indicated that iron is necessary for the expression of bacterial persistence phenotype.

The elemental mixtures reproducing the environmental conditions of the phagosome have not been applied heavily in the study of intracellular bacteria outside of MAH. Culture medium mimicking the phagosome environment has been used to characterize targeted protein degradation in Salmonella enterica, but this study did not examine the role of individual elements in the pathogenesis of this organism (10). The in vitro model in our project has previously been used to characterize the upregulation of virulence genes in MAH (9). A follow-up study confirmed that MAH incubated in these elemental mixtures produced proteins identified from M. avium culture supernatant, and that these proteins that were also intracellularly secreted (11). Further work characterizing the transcriptome of MAH with RNA sequencing found that MAH putatively upregulates ESX-3 when incubated in 24-h elemental mixture (Unpublished Data). ESX-3 is a secretion system belonging to the Type VII apparatus necessary for iron homeostasis in M. tuberculosis (12). This transport system is dependent on iron as well as other metals found in the phagosome (13, 14). The upregulation of this system in
24-h elemental mixture suggests the role of iron in MAH pathogenesis, and the results from our study specifically identify the potential role of iron in the induction of persistence.

Iron plays a role in several bacterial metabolic functions, including DNA replication, and pathogenic bacteria possess factors such as siderophores to obtain iron from host proteins (15). For instance, iron regulates intracellular growth of *Chlamydia psittaci*, *Chlamydia trachomatis*, and *Legionella pneumophila* in macrophages (16). A stepwise reduction scheme of full-spectrum 24-h elemental mixture facilitated investigation of the role of iron in the induction of persistence. Dropout medium without iron gave significantly fewer persistent CFUs than full-spectrum 24-h elemental mixture following antibiotic treatment. This observation suggests that iron in the phagosome environment is potentially involved in persistence-inducing mechanisms, most likely in combination with other trace elements. In addition, because persistent bacteria do not undergo cellular division and DNA replication (15), it is unexpected that the absence of iron would affect expression of the persistent phenotype in bacteria. Alternative explanations for this observation are that the incubation period was not sufficient to observe significant growth in bacteria exposed to dropout medium without iron, or that the presence of other trigger factors is important in the induction of bacterial persistence. In either case, future work might involve longer incubation periods, as well as continued work to determine the roles of other elements with dropout media by removing individual metals in a stepwise fashion.

The induction of bacterial persistence is closely related to stress responses, including the stringent response and Toxin-Antitoxin (TA) systems (3). The frequency of
persisters and cellular levels of the stringent response alarmone \((p)p\text{Gpp}\) increase with decreased growth rate. In turn, this molecule induces persister formation by activating TA molecules (3). Degradation of antitoxins during stress releases toxins, blocking major bacterial metabolic processes such as cellular division. Bacteria induced into this nonreplicating state can survive drug treatment due to inactivation of target sites for the antibiotics (3). \textit{In vitro} models for studying the molecular mechanisms of persistence in \textit{M. tuberculosis} have been established to characterize molecular events under various conditions, including exposure to antibiotics (17). However, mRNA expression of bacteria cultured in these conditions is inconsistent across different models, bringing the biological relevance of these models into question (3).

A study of cytokine-stimulated macrophages infected with MAH found the existence of static, non-replicating subpopulations of bacteria that grew when plated on 7H10 agar (18). In each of the experiments done in this study, selected colonies did not yield observable growth on antibiotic agar media. These results suggest that the surviving bacteria expressed the persistent phenotype, and that no resistant bacteria were counted when recording observed CFUs. This notion suggests that the model established in this project is successfully able to isolate bacteria that express the persistent phenotype. Another important observation throughout the experiments is the presence of a significant subpopulation of bacteria that survive antibiotic treatment with three different antibiotics against three different targets – rifampicin to inhibit RNA synthesis, clarithromycin to inhibit protein synthesis, and ciprofloxacin to inhibit DNA replication – at high doses. Given that most patients receiving this treatment for mycobacterial infection have been chronically infected for months, as opposed to a few hours, the persistent phenotype is far
more prevalent in the clinical setting. The model established in this study is critical to helping researchers better understand the mechanisms of mycobacterial persistence, and discover therapeutic targets for new treatments. By identifying trace elements in the phagosome involved in the induction of persistence, researchers can develop strategies to target these factors and block their uptake by pathogenic bacteria to prevent the formation of persistent subpopulations, consequently preventing their contribution to chronic infection.
References


