Establishing *Caenorhabditis elegans* as a model for *Mycobacterium avium* subspecies *hominissuis* infection and intestinal colonization

By
Navid Ziaie

A PROJECT

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(Honors Scholar)

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AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____

Luiz E. Bermudez

Mycobacterium avium subspecies *hominissuis* (MAH) causes potentially lethal opportunistic infections in immunocompromised individuals. Lack of a good animal model system currently hinders *in vivo* study of MAH virulence. Here we applied the tractable organism *Caenorhabditis elegans* (*C. elegans*), as a surrogate host to study the virulence of MAH. Worms were fed MAH and assayed for ability of MAH to infect intestinal epithelium and for the cytotoxic effects of the bacterial infection on *C. elegans*. It was observed that viable MAH number increases, during feeding, in the intestinal lumen in a time dependent manner. Ingestion of MAH was deemed non-toxic to worms as MAH-fed populations had similar survival curves to those *C. elegans* fed *E. coli* strain OP50. Pulse-chase analysis using *E. coli* strain OP50 revealed that MAH colonize the intestinal tract, and viable MAH remain within the intestine. Using histopathology and transmission electron microscopy we demonstrate that MAH localizes in the intestinal lumen, and establishes an interaction with intestinal epithelium. Bacterial colonization appears to have a detrimental effect on the microvilli of the intestinal epithelial cells. Previous studies have identified the MAH ΔGPL strain, containing a mutation in glycopeptidolipid production, as deficient in binding to human epithelial cells (HEp-2), as well as deficient in its

ability to bind to and colonize the intestinal tract of *C. elegans* as efficiently as wild-type MAH. These data indicate the *C. elegans* may serve as a useful model system for studying MAH pathogenesis and in determining the mechanisms used by MAH during infection and colonization of the intestinal epithelium.

Key Words: *Mycobacterium avium*, *Caenorhabditis elegans*, virulence, intestinal colonization

Corresponding e-mail: ziaien@onid.oregonstate.edu

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APPROVED:

Luiz E. Bermudez, Mentor, representing Biomedical Sciences

Lia Danelishvili, Committee Member, representing Biomedical Sciences

Katharine G. Field, Committee Member, representing Microbiology

Toni Doolen, Dean, University Honors College

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

Navid Ziaie, Author

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Introduction

The genus *mycobacterium* includes many disease causing pathogens, most notably *Mycobacterium tuberculosis*, the causative agent of tuberculosis, as well as *M. leprae*, the causative agent of leprosy. *Mycobacterium avium* subsp. *hominissuis* (MAH) is a ubiquitous environmental bacterium, most commonly found in water and soil. It is a member of the *Mycobacterium avium* complex (MAC) and can cause disease in immunocompromised humans, particularly people with AIDS or other immunodeficiency, and individuals with chronic lung conditions (1, 2). Mycobacteria can colonize their hosts without symptoms of disease. Previous research has shown that MAH infects its host by two manners, through the gastrointestinal as well as by the respiratory epithelia. The bacterium ultimately invades and infects sub-mucosal macrophages. Within the macrophages, the bacterium establishes an intracellular niche where it can disseminate via lymph nodes and the blood (3). Mycobacterial infections are difficult to treat not only due to this evasion of the host's immune system, but also due to their unique cell wall rich with mycolic acid, which provides resistance to many antibiotics and desiccation. Work is currently being undertaken to understand how MAH is transmitted and colonizes its host. Little is known about how MAH is transmitted from an infected host to a non-infected host. Discovery of specific proteins expressed by bacterium within the host could be seen as potential candidates for development of novel approaches to treat and prevent the disease and its transmission.

Caenorhabditis elegans (*C. elegans*) is a transparent nematode which lives in soil and feeds on bacteria. *C. elegans* has been established as a useful model organism for studying genetics, the nervous system, and host-pathogen

interactions. The nematode has been recently been used to model infections of *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Enterococcus faecalis* (3, 4). Because *C. elegans* and MAH share the soil environment, there's a natural interaction between the two organisms, with a considerable chance for *C. elegans* to encounter the pathogen. Additionally, *C. elegans* makes a good model organism because the intestinal epithelium of *C. elegans* and human intestinal epithelial cells are very similar, as they share similar morphology and functions, which includes acting as a first defense against invading pathogenic bacteria. We describe here how *C. elegans* feeds on MAH without significant consequences on its health or lifespan. Our results also demonstrate that MAH is able to colonize the intestinal tract of the worm in a stable non-transient manner, and that colonization of the gut results from a close association between the pathogen and the apical membrane and microvilli of the intestinal epithelial. Establishing *C. elegans* as a model system for MAH infection allows for the further characterization of the pathogenic mechanisms employed by MAH, and for increased progress towards therapeutic and prevention strategies.

Methods and Materials

Nematode propagation. *Caenorhabditis elegans* (*C. elegans)* strain N2 was kindly provided by the laboratory of Dr. Dee Denver at Oregon State University. Nematodes were maintained in monoxenic cultures with the addition of *Escherichia coli* strain OP50 and propagated on nematode growth medium (NGM) agar plates at 25°C as previously described (7).

Bacterial culture. *E. coli* strain OP50 was grown in Luria-Bertani (8) broth overnight prior to inoculation of NGM plates. *Mycobacterium avium* subspecies *hominissuis* (MAH) strain 104 and strain A5 was grown on Middlebrook 7H10 agar supplemented with 10% w/v oleic acid-albumin-dextrose-catalase (OADC; Hardy Diagnostics; Santa Maria, CA) for 10 days at 37°C. MAH A5 ΔGPL mutant (described by Yoshitoka et al, 2006) was grown on 7H10 medium described above supplemented with kanamycin sulfate (400 μg/ml). Bacterial suspensions were processed through a 23-gauge syringe, clumps allowed to settle for 10 minutes, and top 2 ml of suspension collected and used for assays.

Mammalian cell culture. Human epithelial (HEp-2; CCL-23) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products; West Sacramento, CA) in 37°C with 5% CO_2.

Nematode MAH feeding assays. NGM plates were prepared as described above, or supplemented with 400 μM 5-Fluoro-2'-deoxyuridine, a DNA synthesis inhibitor which allows for the synchronization of worm cultures (FUdR; Sigma-Aldrich; St. Louis, MO) (9). Agar plates with and without FUdR were seeded with 10^8 MAH-td104, or appropriate MAH strain as per experimental design, and inoculated with equal volume synchronized nematode cultures at the L4 stage of growth. Plates were incubated for 1, 3, or 5 days at 25°C prior to sample collection. At each timepoint, worms were collected in M9 salt solution, pelleted, and anesthetized using 70% ethanol. Samples were spotted onto glass slides, and

visualized on a DM4000B Leica microscope. Images were captured and analyzed using QCapture Pro7 software.

Pulse-chase feeding assay. Pulse-chase analysis was modified for mycobacterial isolation and conducted as previously described (10). Synchronized worms at the L4 growth stage were collected in 1x M9 saline solution and washed twice. Individual NGM plates supplemented with FUdR (400 μM) were seeded with 10^8 MAH as per experimental design. Each plate was seeded with equivalent number of worms and incubated at 25°C. After 5 days, worms were collected in M9 saline, washed twice, and seeded onto new NGM-FUdR plates containing a lawn of *E. coli* OP50. After 24 hours of incubation, worms were collected in M9 saline supplemented with 25 mM of levamisole hydrochloride (Sigma-Aldrich) for paralysis and prevention of expulsion or uptake of bacteria during washes. Worms were collected at 225 × g for 2 minutes, washed twice in M9 saline solution, treated with amikacin sulfate (200 μg/ml) for 2 hours at room temperature to kill all extracellular bacteria surrounding the worms, and washed twice with HBSS. For visualization, suspensions were analyzed under fluorescent microscopy. To quantify pulse-chase assays, suspensions were homogenized using a handheld motorized pestle (VWR; Radnor, PA) for 1 minute in 0.1% triton X-100 and deionized water, and samples were serially diluted and quantified.

Nematode survival curve. Synchronized nematode cultures were prepared and 30 worms were picked using a platinum wire pick and transferred to NGM plates supplemented with FUdR (400 μM) which were seeded with either 10^8 *E. coli* strain OP50 or 10^8 MAH strain 104. Cultures were monitored and scored for

nematode survival. Worms that did not move or respond to gentle agitation by platinum wire pick were deemed dead, and worms found on the plastic sides and lid of petri plate as well as worms that burst were censored from the data counts. Survival of populations was assessed for 30 days and analyzed using a Kaplan-Meier survival analysis.

Histology and transmission electron microscopy. *Specimen Preparation.* Synchronized nematodes at the L4 growth stage were picked and seeded onto NGM agar plates supplemented with FUdR (400 μM) for 24 hours in the absence of any seeded bacteria to remove any extracellular or residual *E. coli* from the worm cultures. Worms were then picked and seeded onto plates containing either 10^8 MAH-td104 for experimentally fed samples or containing no bacteria for starved control samples and allowed to incubate for 5 days at 25°C.

Histology. Worms were collected in M9 saline solution and washed twice at 50 × g for 2 minutes to remove any extracellular bacteria in suspension. Nematodes were fixed in 10% buffered formalin for 5 minutes at room temperature, washed in M9 solution, suspended in 1% low-melt agarose. Agarose-encased worms were embedded in resin, and sections mounted onto glass slides by the Veterinary Diagnostic Lab at Oregon State University. Specimens were acid-fast stained and visualized. *Transmission Electron Microscopy.* Worms were collected in M9 solution, washed twice, and pellet was suspended in fixative buffer of 2.5% glutaraldehyde, 1% paraformaldehyde, and 0.1 M sodium cacodylate. Worms were cut in half and incubated in fixative buffer overnight at 4°C. Specimen sections were stained, dehydrated, and infiltrated for TEM visualization by the Electron Microscopy Facility at Oregon State University as previously described (11).

HEp-2 binding assay. Bacterial suspensions were prepared in Hank's Balanced Salt Solution (HBSS; Corning; Corning, NY) and HEp-2 cells were infected at an MOI of 10:1. Infections were synchronized at 225 × g for 5 minutes at 4°C. Plates were incubated for 1 hour at 4°C, washed 3 times with PBS, and monolayers lysed for 15 minutes in 0.1% triton X-100 in water. Lysates were serially diluted and quantified using CFU counts.

Statistical analysis and data interpretation. Results are reported as the mean of at least 2 independent experiments ± standard error. For binding and pulse-chase assays statistical comparisons between experimental groups and control groups were determined using the Student's *t* test with $p<0.05$ denoting statistical significance. Survival curve data was analyzed using Kaplan Meier Survival Analysis. GraphPad Prism version 6.0 software was used for the construction of graphs, data interpretation, and all statistical analyses.

Results

C. elegans is able to feed on MAH

It is well documented that *C. elegans* can be used as an infection model for a variety of pathogens (3, 12). Previous work has determined that nematodes can be cultivated on fast growing strains of *Mycobacterium fortuitum* and *Mycobacterium marinum* (3). Our investigation first examined whether nematodes would feed on MAH. NGM plates were seeded with 10^8 MAH strain 104 containing the plasmid pJDC60-tdTomato which contains a tomato red

fluorescent protein under a constitutive mycobacterial L5 promotor for identification of MAH within the intestinal tract of the nematode (MAH-td104) by fluorescent microscopy. Synchronized worms in the L4 growth stage were seeded onto MAH-td104 containing plates and allowed to feed for 1, 3, and 5 days. Visualization of worms at each timepoint demonstrated that *C. elegans* fed on MAH-td104 in a time-dependent manner, with pharynx-, grinder-, and intestinal-localized MAH-td104 increasing in intensity over time (data not shown). During the 5 days incubation, worms were able to readily feed, mate, and produce apparently healthy progeny (observation; data not shown). In order to determine if the variety of life stages played a role in uptake and feeding on MAH-td104, 5-Fluoro-2'-deoxyuridine (FUdR) was added to MAH seeded NGM plates. The addition of FUdR inhibits DNA synthesis, thus preventing progeny from being produced, allows for the maintenance of a synchronous population during experiments, and prevents the over population of a plate during longer timepoints. Synchronized cultures at the L4 stage of growth seeded onto NGM-FUdR plates demonstrated similar feeding trends on MAH-td104 during an identical feeding time course (Figure 1).

Previous work demonstrated that fluorescence does not indicate viability, as GFP can still be detected within the intestine after bacteria are killed and digested for nutrients (13). To identify whether intestinal-localized MAH-td104 was viable, worms fed for 5 days were collected, treated with levamisole to maintain the state of bacterial uptake by preventing pharyngeal uptake or expulsion by the worms, and treated with amikacin to remove residual extracellular MAH from the feeding assays. Quantification of worm lysates indicated that the intracellular MAH visualized after a 5-day feeding was viable, as greater than 10 MAH were isolated from each culture of MAH-fed nematodes. From these data, we determined that *C. elegans* would feed on MAH

if it is the sole source of bacteria during growth, and that the bacteria reside within the digestive tract after ingestion.

C. elegans survival upon MAH-td104 feeding

While *C. elegans* can feed on a variety of bacteria during the experimental studies, pathogens, notably *Salmonella* and *Pseudomonas,* are rapidly lethal (1). It is unknown whether the high fatty acid, lipid-rich composition of mycobacteria has an effect on the longevity of worms in culture. Feeding on MAH can be visualized for 5 days, as indicated by fluorescent microscopy (Figure 1); however, that timeframe may not be adequate to establish whether MAH is toxic or lethal to the nematodes. To determine lethality of MAH on *C. elegans,* a survival curve was conducted in order to compare the lifespan of *C. elegans* fed on standard *E. coli* strain OP50 cultures to the lifespan of those fed on MAH-td104. Equal numbers of synchronized worms were picked and fed on *E. coli* or MAH in the presence of FUdR for 35 days. Kaplan-Meier survival analysis indicated that feeding on MAH-td104 had no observable effect on worm lifespan, as median survival of both the MAH-fed animals and OP50-fed animals was 18 days, after the start of feeding (Figure 2). Furthermore, there was no significant difference between the total lifespan of OP50-fed worms (31 days) and that of MAH-fed worms (35 days).

MAH colonization of the *C. elegans* intestine

C. elegans is capable of ingesting a wide variety of environmental bacteria for nutrients. As the nematodes can feed on MAH, and the colonization appears to remain within the intestinal tract at 5 days post-infection, we analyzed

whether the presence of MAH within the intestine was transient, or if it was a longer-lived, more permanent state of colonization. To answer this question, we used pulse-chase analysis during feeding. Nematodes were fed MAH-td104 for 5 days and then placed onto a clean NGM plate for 2 hours to remove any MAH that was on the outside of the worm bodies. Worms were then placed onto a plate containing *E. coli* strain OP50 and fluorescent microscopy indicated that after 24 hours of pulse-chase feeding on *E. coli*, red MAH-td104 was still readily visible within the intestinal lumen of the worms (Figure 3).

Visualization of the MAH-colonized *C. elegans* intestine

Fluorescent microscopy analysis of MAH-fed worms indicated that the bacteria are capable of colonizing the intestine of the worms during infection. As MAH is an intracellular pathogen known for its ability to invade and survive within host cells, we wanted to determine whether the red fluorescent MAH seen in our photos were in the lumen of the intestine or if the bacteria were able to invade the intestinal epithelium and cause an intracellular infection within the intestinal tract. To analyze the cross-sections of MAH-fed animals, worms were seeded onto plates and fed bacteria as described above. Worms were picked at 5-days post-infection and fixed for histology. As a control, worms were seeded onto plates in the absence of any bacteria for the same incubation time and processed as described. Acid-fast stained cross-sections indicated that MAH was capable of colonizing the lumen of the intestine at high levels during feeding and infection (Figure 4c – 4f), while starved worms showed an absence of pink acid-fast bacilli, or any bacilli within the lumen of the intestine (Figure 4a and 4b)

In order to visualize whether MAH interacts with the intestinal epithelium of *C. elegans* following feeding, MAH-fed and starved worms were prepared and

visualized using transmission electron microscopy. As a healthy and uninfected control, starved worms demonstrated an absence of bacteria within the lumen, which appeared compact in size (Figure 4a and 4b). An undisturbed intestinal tract could be clearly seen in the control worms, as intact and tightly clustered microvilli line the apical membrane of the intestinal epithelial cells. Alternatively, MAH-fed worms present with a grossly distended lumen that was filled with colonizing MAH bacteria (Figure 4c – 4f). MAH could be seen both in the luminal space, as well as in direct contact with the microvilli located on the apical membrane of the intestinal epithelium. Both at the site of contact, as well as in the vicinity of these sites, it can be appreciated that the neatly layered, tightly clustered microvilli seen in the control sections are shortened, appear damaged, and in a much looser association in the presence of MAH colonization (Figure 4c – 4f).

Binding and colonization of MAH glycopeptidolipid mutant ΔGPL/4B2

We next aimed to determine if the *C. elegans* model could identify MAH mutants and lead to the understanding of bacterial components responsible for the colonization of the intestinal epithelium within the nematode. The previously described MAH ΔGPL/4B2 mutant (14) was analyzed for binding to HEp-2 epithelial cells during a 1 hour infection (Figure 5a). Compared to the parental MAH A5 strain, the MAH ΔGPL/4B2 mutant is deficient in its ability to bind to human HEp-2 cells during infection. Worms were allowed to feed on the wild-type and mutant MAH strains for 5 days and intestinal binding was quantified to assess the ability of worms to feed on and retain each bacterial strain (Figure 5b). The MAH ΔGPL/4B2 mutant was found at significantly lower levels than the wild-type MAH strain within the intestinal tract of the worms. After 5 days of

feeding on each bacterial strain, nematodes underwent pulse-chase analysis with a 24-hour feeding on *E. coli* strain OP50 prior to intestinal MAH quantification (Figure 5c). The ability of the MAH ΔGPL/4B2 mutant to colonize the *C. elegans* intestinal epithelium was lower compared to the wild-type MAH infection, as indicated by a significant decrease in the amount of bacteria localized in the intestine after pulse-chase analysis.

Discussion

C. elegans has been established as an extremely useful model organism in a variety of scientific disciplines, ranging from genetics and cell biology, to host-microbe interactions and pathogenesis. The transparent nature of the nematode and simple body structure provides a simplified *in vivo* model to mimic physiological and pathogenic mechanisms that occur during infection. *C. elegans* are inexpensive to maintain and can be simply manipulated. Additionally, there are a variety of tools available for studying the processes occurring within the nematode. The bacterium is known to primarily invade enterocytes of the intestinal epithelium, rather than the Peyer's Patches and M-cells (15). Likewise, the relatively similar physiology and epithelial make-up of the intestinal epithelium between *C. elegans* and humans, provides an opportunity to develop a model to examine the many aspects of the interaction between MAH and the intestinal mucosa. Nematodes naturally feed on environmental bacteria for nutrients and we show that the ability of populations *C. elegans* to feed on MAH is no exception. Unlike the feeding on other pathogens, such as Pseudomonas and Salmonella, the ingestion of MAH is not rapidly toxic, suggesting that MAH does not exude rapidly toxic components within the intestinal tract, resulting in a

longer-lived infection within the *C. elegans* host. In fact, this finding should not represent a surprise, since both MAH and *C. elegans* co-inhabit the same environmental niche.

It is described that MAH first colonizes the host intestinal epithelium prior to being observed as systemic bacterial infection (8). Our histopathological images and the pulse-chase data demonstrate that MAH are found within the lumen of the intestinal tract over the 5-day infection, quickly increasing in number and colonizing in a stable, non-transient manner. These data are supported by TEM images of MAH-infected worms, which indicate that MAH establish a direct interaction during colonization with the intestinal epithelium. This interaction results in damage to the villi on the epithelial cells which has been observed during mouse infection (16). As the current experimental infection only progressed until 5-days post-feeding (obtained images), it is possible that longer infection of *C. elegans* with MAH would cause progression of the intestinal pathology. These observations suggest that MAH is able to colonize the intestinal epithelium to very likely, establish a more long-term and chronic infection within the host.

Past experimental data indicates that the MAH ΔGPL/4B2 mutant, which expresses a decreased ability to form biofilm on surfaces (17), and an impaired capacity to bind to human epithelial HEp-2 cells, also shows a decreased interaction with the intestinal tract of *C. elegans*, with deficiency in binding and colonization. The model, therefore, may be quite useful to screen and identify mutant with impaired ability to colonize the host mucosal surface. In fact, many studies have identified bacterial molecules associated with invasion of the host mucosal by bacteria, but not much is known about the ability of MAH to anchor to structures on the epithelium (18, 19). Ultimately, we aim to understand the

bacterial mechanisms and factors that are responsible for MAH colonization and infection of the host. The identification of such factors used to initiate the disease could be useful in developing therapeutic approaches to prevent the infection from establishing or to treat infections that are in the early stages of colonization.

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Figures

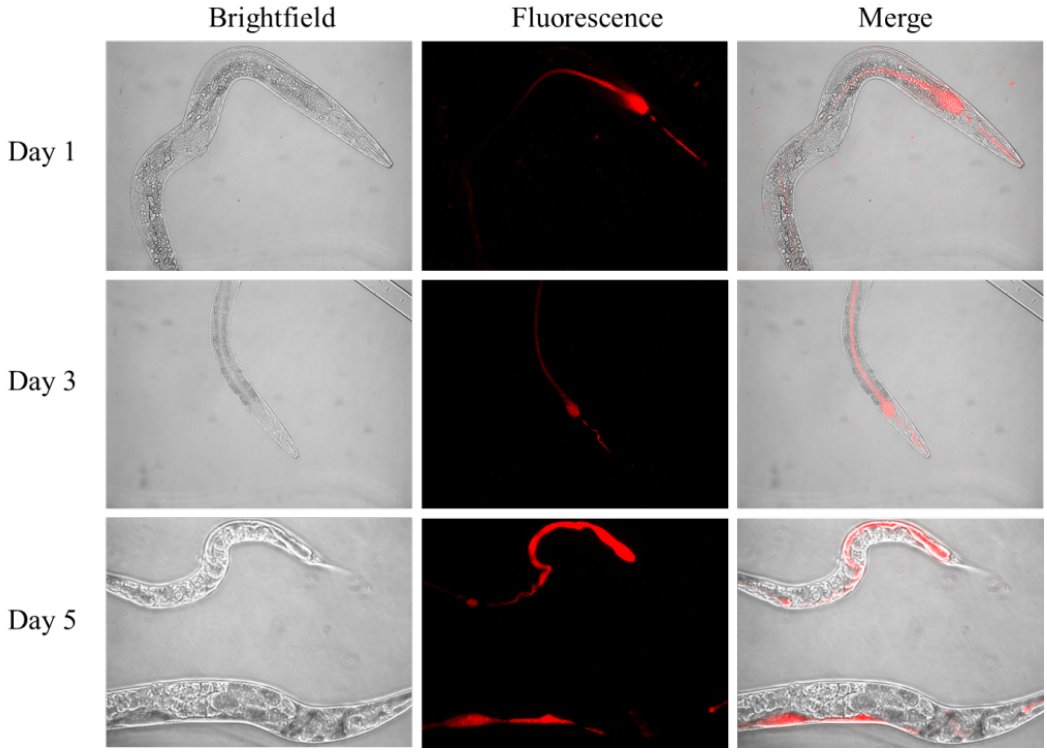

Figure 1: *C. elegans* feed on MAH

C. elegans were seeded onto NGM plates supplemented with FUdR (400 μM) and seeded with MAH expressing a fluorescent tomato red marker. Worms were allowed to feed for 1, 3, or 5 days at which time worms were collected, washed, and mounted on glass slides for microscopic observation. Images are representative of 20 worms visualized per experiment and independently repeated 5 times. All images are shown at 400x magnification.

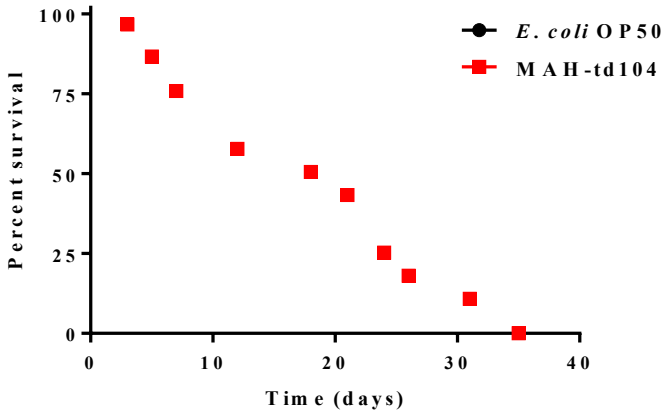

Figure 2: MAH-td104 does not affect median or total lifespan of *C. elegans*

Worms were picked and incubated on NGM agar to remove external bacteria for 3 hours. Thirty worms were individually placed onto NGM plates supplemented with FUdR (400 μM) and seeded with either 10^8 *E. coli* strain OP50 or MAH-td104. Worms were scored every 2 days for survival and worms that ruptured or crawled up the sides of the plate were censored and removed from the study. Kaplan-Meier statistics were used to construct and analyze growth characteristics. Data represents survival from one experiment and is representative of 2 independently completed experiments.

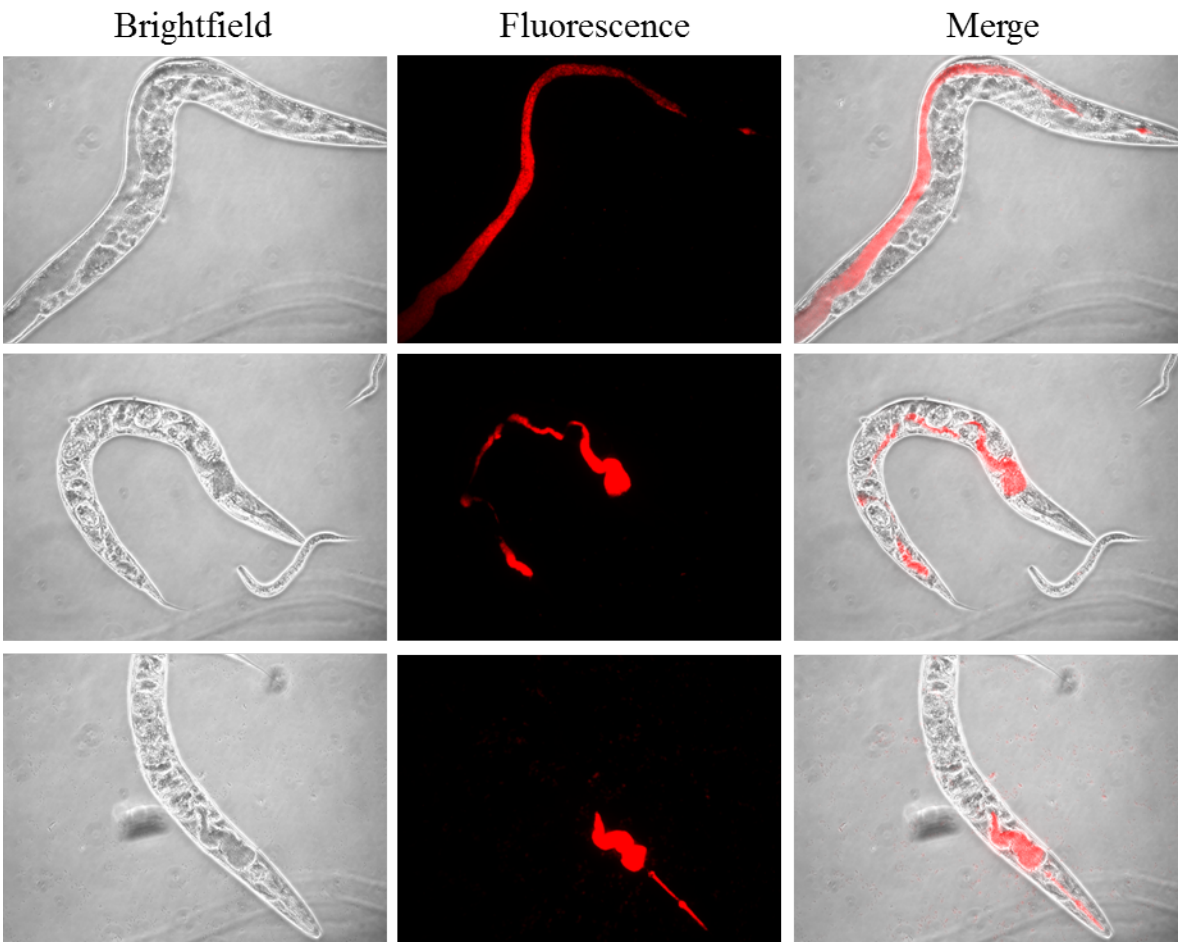

Figure 3: MAH colonization persists after pulse-chase with *E. coli* strain OP50

C. elegans were placed onto NGM-FUdR (400 µg/ml) plates and seeded with 10^8 MAH-td104 and allowed to feed for 5 days. Worms were collected and moved to a new plate to remove extracellular bacteria. Worms were then transferred to a plate seeded with *E. coli* strain OP50, and allowed to feed for 24 hours. Nematodes were collected and mounted onto glass slides for microscopic observation. Images are representative of 20 worms visualized per experiment and independently repeated 3 times. All images are shown at 400x magnification.

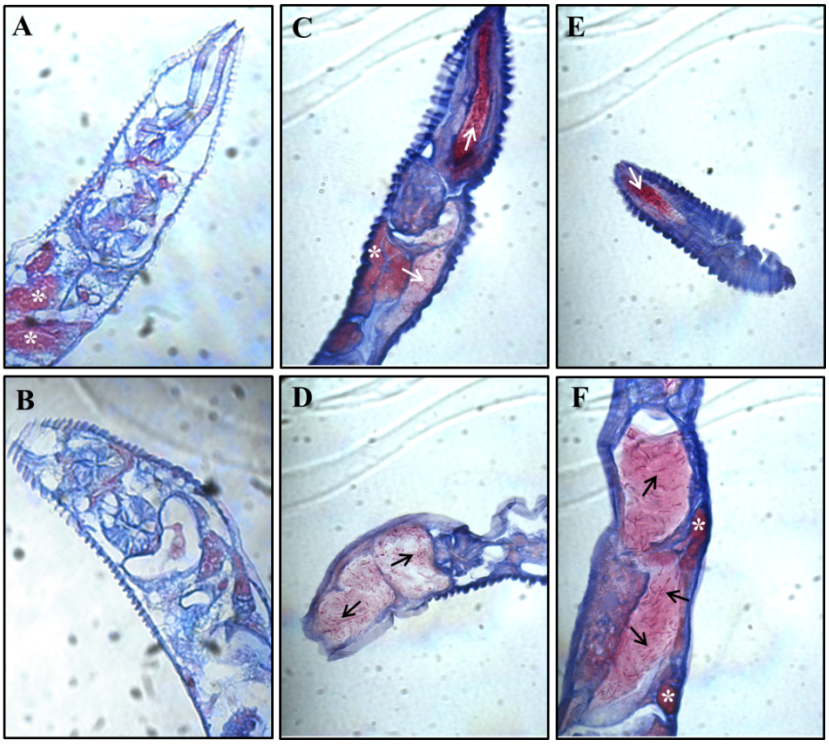

Figure 3: MAH colonize lumen of *C. elegans* intestinal tract

Worms were seeded onto NGM plates supplemented with FUdR (400 μM) for 5 days and samples were fixed, set into agarose blocks and paraffin, sectioned, and acid-fast stained. Starved worms were seeded onto plates in the absence of bacteria (A and B) onto plates containing MAH (D – F). Acid-fast positive bacilli within the intestinal space are indicated by black/white arrows. Non-specific staining of fat deposits by carbol-fuchsin is indicated by white asterisks (*). Images are representative of 10 worms sectioned and analyzed per treatment. All images are shown at 630x magnification.

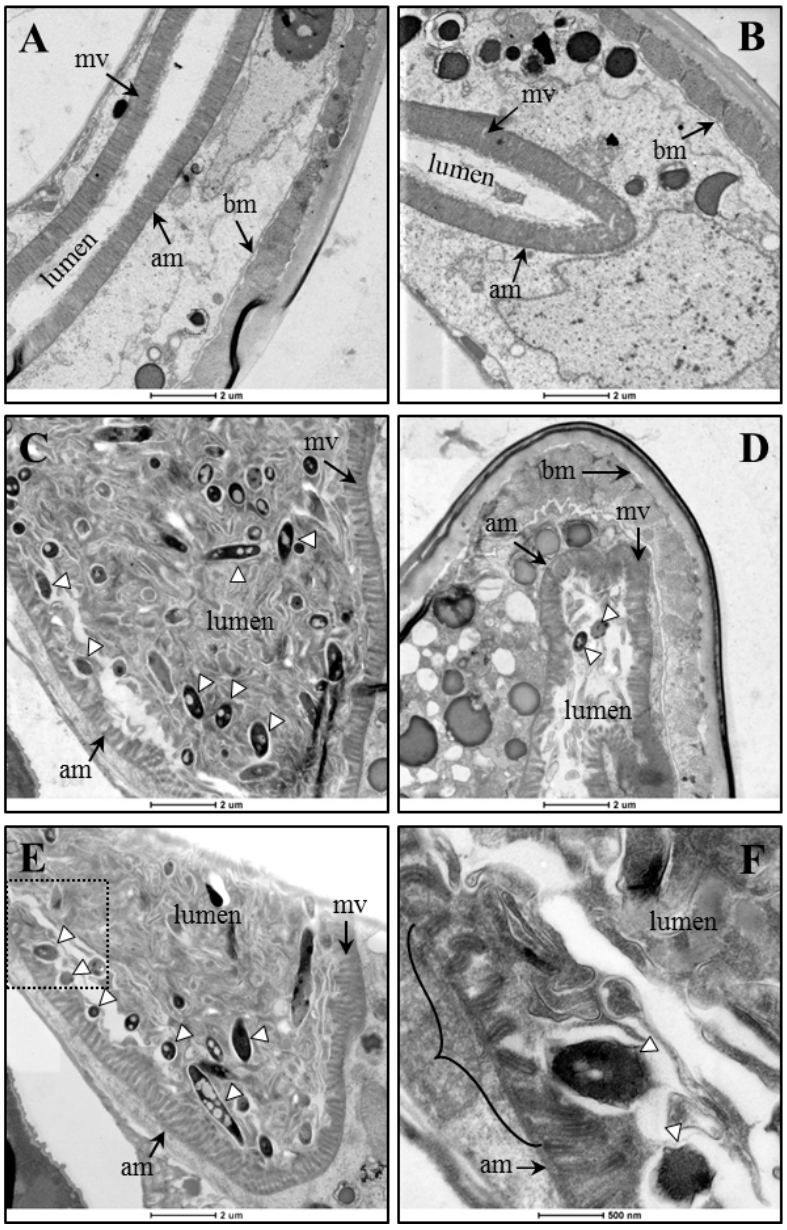

Figure 4: Transmission electron microscopy of MAH-colonized *C. elegans*

Worms were seeded onto NGM plates supplemented with FUdR (400 μM) for 5 days and samples were fixed, processed, and visualized by transmission electron microscopy on an FEI Titan 80-200 microscope. Starved worms were seeded onto plates in the absence of bacteria (A and B) or onto plates seeded with 10^8 MAH (C – F). Panel F illustrates a magnified view taken from panel E (dotted line) and shows disruption on the microvilli (bracket). Key: white arrowheads - MAH, lumen – luminal space, mv – microvilli, am – apical epithelial membrane, bm –

basal epithelial membrane. Panels A – E scale bar is 2 μm, Panel F scale bar is 500 nm.

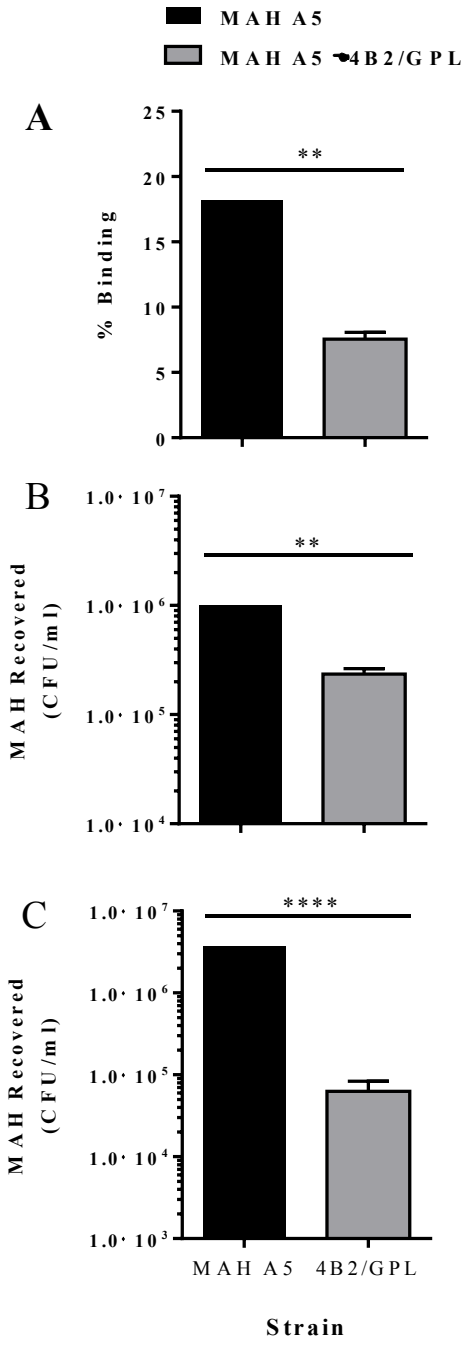

Figure 5: Binding of HEp-2 cells and colonization of *C. elegans* by MAH ΔGPL/4B2 mutant

The MAH ΔGPL/4B2 mutant and the parental strain MAH A5 were used for HEp-2 binding assays (A). HEp-2 cells were infected at an MOI of 10:1 with each

strain and binding was allowed to progress for 1 hour at 4°C. Wells were lysed and quantified for percent of bound bacteria to the surface of epithelial cells during assay. Equivalent numbers of *C. elegans* were seeded onto NGM-FUdR (400 μM) plates containing 10^8 of each mutant strain of MAH, and allowed to feed at 25°C for 5 days. Worms were collected, washed with levamisole (25 mM), treated with amikacin (200 μg/ml), and lysed for quantification of intracellular bacteria. Worms were homogenized immediately after MAH feeding to determine binding ability after feeding (B). Analysis for colonization using pulse-chase analysis was conducted by transferring worms to NGM-FUdR plates seeded with *E. coli* strain OP50 for 24 hours prior to homogenization and quantification (C). Data represent the mean ± SEM of 2 independent experiments each performed in triplicate (**p-value<0.01, ****p-value<0.0001 as determined by Student's *t*-test).

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