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Conserved Mechanisms of *Mycobacterium marinum* Pathogenesis within the Environmental Amoeba *Acanthamoeba castellanii*

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***Mycobacterium marinum* is a waterborne mycobacterial pathogen. Due to their common niche, protozoa likely represent natural hosts for *M. marinum*. We demonstrate that the ESX-1 secretion system is required for *M. marinum* pathogenesis and that *M. marinum* utilizes actin-based motility in amoebae. Therefore, at least two virulence pathways used by *M. marinum* in macrophages are conserved during *M. marinum* infection of amoebae.**

Mycobacterial pathogens are responsible for some of the leading causes of death by infectious disease. The majority of these deaths are caused by mycobacterial species within the *Mycobacterium tuberculosis* complex (MTC) (3). However, mycobacterial species in the environment, including atypical or nontuberculous mycobacteria (NTM), pose an emerging disease threat (19). Little is understood about the basic molecular virulence mechanisms employed by environmental mycobacterial pathogens.

Mycobacterium marinum is a waterborne pathogen that causes a tuberculosis (TB)-like infection in ectotherms and is an occasional opportunistic human pathogen (21). *M. marinum* is related to *M. tuberculosis* and is used to model aspects of MTC pathogenesis (4, 7, 21, 23, 27). Free-living amoebae (FLA), including *Acanthamoeba castellanii*, are professional phagocytes (12). Pathogenic bacteria, including *M. marinum* and other NTM, have been recovered from samples of water colonized by free-living amoebae (8, 26). Several mycobacterial species are established amoeba-resistant bacteria (ARB) and resist destruction by FLA (1, 6, 12, 17, 18). It has been posited that protozoa serve as a reservoir for mycobacteria in the environment (17).

It is probable that *M. marinum* naturally interacts with protozoa, including *A. castellanii*, that share an environmental niche. It was demonstrated that *M. marinum* are pathogenic to *A. castellanii* (6, 17, 20, 28). The molecular mechanisms underlying this interaction have not been well established.

We hypothesized that virulence mechanisms required for infection of macrophages by *M. marinum* would also be required for pathogenesis of *A. castellanii*. The ESX-1 protein secretion system is required by mycobacteria and other Gram-positive pathogens to cause disease (2, 11, 13, 16, 24). Amoebae were infected (multiplicity of infection [MOI] of 1) with either the wild-type (WT) strain or an attenuated RD1 deletion (ΔRD1) strain of *M. marinum*, which bears a deletion in components and substrates of the ESX-1 system (Fig. 1A) (27). *M. marinum* replicated roughly three logs in *A. castellanii* over the 72-h experiment, with an average generation time of 7 h. The ΔRD1 strain replicated approximately two logs in *A. castellanii* over the 72-h experiment, with an average generation time of 12 h. Following an early rapid growth phase for both strains, the bacteria were maintained at relatively constant levels throughout the experiment. However, the ΔRD1 strain failed to reach the levels of growth achieved by the wild-type strain (Fig. 1A). Significant differences in growth between the WT and ΔRD1 strains were observed at 48 ($P < 0.035$) and 72 ($P < 0.002$) hours postinfection (hpi) (Fig. 1A).

Next, we infected *A. castellanii* with WT or ΔRD1 *M. marinum* expressing DsRed at an MOI of 1, 5, and 10 and monitored bacterial uptake and survival. We performed fluorescence microscopy and determined the percentage of amoebae infected with *M. marinum*. At each time point, the numbers of infected amoebae were counted and compared to the total number of amoebae in that field (see Fig. S1 in the supplemental material). Five fields of approximately 100 amoebae were counted at each time point. We found that with increasing MOI, the fraction of infected amoebae increased (Fig. 1B; see also Fig. S2 in the supplemental material). At 4 hpi, an MOI of 1 resulted in 20 to 25% of *A. castellanii* cells becoming *M. marinum* infected (see Fig S2). At MOIs of 5 and 10, roughly 60% and 80% of *A. castellanii* cells became infected, respectively (Fig. 1B; see also Fig S2). Importantly, our data indicate that once *M. marinum* is phagocytosed by amoebae, the proportion of *A. castellanii* cells infected remains constant for at least 48 h (Fig. 1B). In contrast, although the ΔRD1 strain is not defective for uptake relative to the wild-type strain (Fig. 1B, 4 hpi), the population of infected amoebae significantly decreased over time at all MOIs tested relative to the wild-type infection (Fig. 1B; 12 hpi, $P = 0.005$; 24 hpi, $P = 0.010$; 48 hpi, $P = 6.547 \times 10^{-5}$).

It was previously reported that *Legionella pneumophila* infection of *A. castellanii* results in amoeba lysis and reduced viability (5, 9, 28). To determine if we observed a similar decrease in amoeba viability, we counted amoebae during infection with wild-type *M. marinum* at an MOI of 10 and normalized the total number of amoebae each day to the number plated at time zero. The total number of amoebae remained constant when infected with wild-type *M. marinum* (Fig. 1C; see also Fig. S3 in the supplemental material). In contrast, infection with ΔRD1 *M. mari-*

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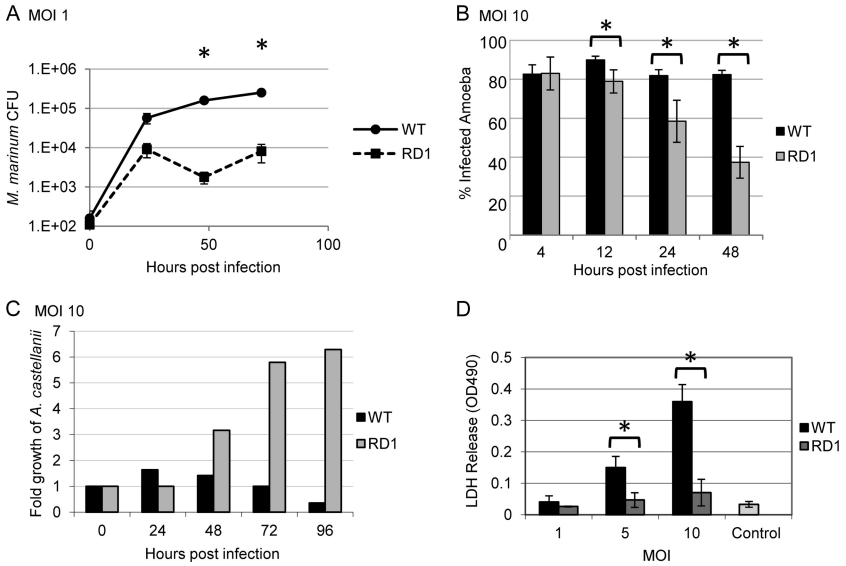

FIG 1 *M. marinum* pathogenesis within *A. castellanii* requires the ESX-1 secretion system. (A) *M. marinum* replicates and persists within *A. castellanii*, while the *M. marinum* strain lacking ESX-1 is attenuated. Amoebae were infected (MOI of 1) and treated with gentamicin (150 μg/ml). Amoebae were lysed and bacteria were plated for CFU at the indicated times. Error bars represent standard deviations. Asterisks represent statistically significant differences based on an unpaired tailed Student *t* test ($P < 0.05$) (48 hpi, $P = 0.035$; 72 hpi, $P = 0.002$). (B) Infection by *M. marinum* results in a stable percentage of infected amoebae, while the population of infected amoebae decreased over time in the absence of the ESX-1 system. *A. castellanii* was infected with *M. marinum* and ΔRD1 *M. marinum* expressing DsRed at an MOI of 10. Monolayers were imaged using a 20× objective on a Zeiss AxioObserver microscope. The percentage of infected amoebae was established after counting >100 cells in each of five different fields at the indicated time points (12 h, $P = 0.005$; 24 h, $P = 0.010$; 48 h, $P = 6.547 \times 10^{-5}$). (C) Infection of *A. castellanii* results in a relatively constant number of amoebae over time, while infection by *M. marinum* lacking ESX-1 leads to amoeba growth. At each time point following infection, the amoebae were counted and the number was normalized to the initial number of amoebae plated. See Fig. S3 in the supplemental material for amoeba counts. (D) Infection with *M. marinum* results in amoeba lysis in an ESX-1-dependent manner. MOI of 5, $P = 0.009$; MOI of 10, $P = 0.001$. The control is uninfected amoebae after 72 h of incubation.

num resulted in amoeba replication over time (Fig. 1C; see also Fig. S3). Because the amoebae infected with the attenuated strain continue to replicate during the course of the infection, growth arrest is likely due to infection with wild-type *M. marinum* rather than nutrient exhaustion. To confirm that we were observing amoeba death, we performed a lactate dehydrogenase (LDH) release assay at various MOIs 72 hpi. Infection with increasing MOI of wild-type *M. marinum* resulted in significantly increased amoebal lysis as measured by LDH release (Fig. 1D; MOI of 5, $P = 0.009$; MOI of 10, $P = 0.001$). Importantly, we did not observe detectable lysis during infection by the ΔRD1 deletion strain of *M. marinum* at any MOI tested or of uninfected amoebae.

One of the features of *M. marinum* pathogenesis is the ability of the bacteria to access the macrophage cytosol and utilize host actin for motility (22). Interestingly, *M. marinum* fails to exhibit actin-based motility in the soil amoeba *Dictyostelium discoideum* and instead escapes via nonlytic ejection (14, 15). To determine if *M. marinum* forms actin tails in *A. castellanii*, we infected amoebae at an MOI of 5 and visualized actin tail formation using immunofluorescence microscopy. We observed actin tail formation by wild-type *M. marinum* at approximately 22 hpi (Fig. 2). As was reported in macrophages, we observed that only a subset of mycobacteria form actin tails (22). Similarly, a single amoeba cell infected with multiple bacteria harbors bacteria with and without tails. We were unable to visualize actin tails in bacteria lacking the ESX-1 system (data not shown).

In conclusion, we demonstrate that two virulence mechanisms used by *M. marinum* to infect macrophages are also required for pathogenesis of the amoeba *A. castellanii* under laboratory conditions. The *M. marinum* strain lacking the ESX-1 secretion system was attenuated for growth in amoebae, as previously shown in macrophages and zebrafish (7, 10, 25, 27). Moreover, infection by *M. marinum* resulted in lysis of the amoeba host in an ESX-1-dependent manner. We observed actin tail formation by *M. marinum* but were unable to observe actin tail formation in the ESX-1-deficient strains (data not shown), consistent with the requirement of ESX-1 for phagosomal escape by *M. marinum* (22, 23). Our findings contribute to the basic molecular understanding of the interaction between *A. castellanii* and *M. marinum*.

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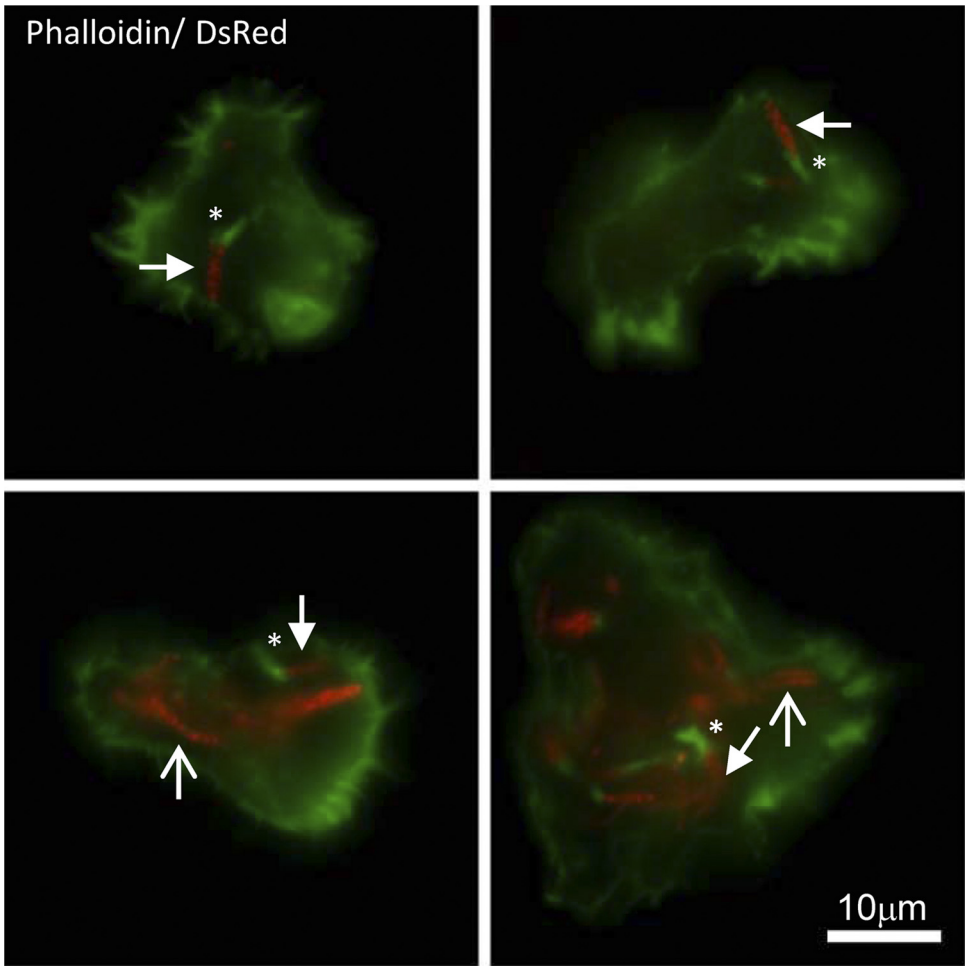

FIG 2 Wild-type *M. marinum* forms actin tails in *A. castellanii.* Florescence microscopy demonstrating actin tail formation by *M. marinum* at 22 hpi, MOI of 5. *M. marinum* is expressing DsRed, and Alexa 488 phalloidin is green. Four representative images are shown. The scale bar is 10 μm. Images were acquired with an Evolution QEi charge-coupled device (CCD) (Media Cybernetics) on a Nikon Eclipse TE300 microscope (60× objective) using IPLab software (Scanalytics). Examples of bacteria bearing tails are indicated with filled arrows. Tails are indicated with asterisks. Examples of bacteria without tails are indicated with open arrows.

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