Video Article

Tractable Mammalian Cell Infections with Protozoan-primed Bacteria

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Abstract

Many intracellular bacterial pathogens use freshwater protozoans as a natural reservoir for proliferation in the environment. *Legionella pneumophila*, the causative agent of Legionnaires' pneumonia, gains a pathogenic advantage over *in vitro* cultured bacteria when first harvested from protozoan cells prior to infection of mammalian macrophages. This suggests that important virulence factors may not be properly expressed *in vitro*. We have developed a tractable system for priming *L. pneumophila* through its natural protozoan host *Acanthamoeba castellanii* prior to mammalian cell infection. The contribution of any virulence factor can be examined by comparing intracellular growth of a mutant strain to wild-type bacteria after protozoan priming. GFP-expressing wild-type and mutant *L. pneumophila* strains are used to infect protozoan monolayers in a priming step and allowed to reach late stages of intracellular growth. Fluorescent bacteria are then harvested from these infected cells and normalized by spectrophotometry to generate comparable numbers of bacteria for a subsequent infection into mammalian macrophages. For quantification, live bacteria are monitored after infection using fluorescence microscopy, flow cytometry, and by colony plating. This technique highlights and relies on the contribution of host cell-dependent gene expression by mimicking the environment that would be encountered in a natural acquisition route. This approach can be modified to accommodate any bacterium that uses an intermediary host as a means for gaining a pathogenic advantage.

Video Link

The video component of this article can be found at https://www.jove.com/video/50300/

Introduction

Numerous bacterial pathogens have adapted generalized strategies to exploit host cells for survival and replication in an intracellular compartment. In many instances, pathogenic mechanisms are similar between protozoan and metazoan cells. However, these two microenvironments are very different and can result in differential expression of virulence factors[1-4]. The Legionnaires' disease bacterium *Legionella pneumophila* is ubiquitously associated with freshwater environments worldwide[5]. Importantly, *L. pneumophila* cultivated in protozoan cells prior to infection of human monocytes gain a pathogenic advantage, suggesting that global gene expression profiles of the bacterium exiting a protozoan cell are different than that of the *in vitro* cultivated organism[6-8]. In nature, freshwater amoebae provide nutrient rich confines for rapid amplification of an invading bacterium. Human acquisition of *L. pneumophila* is most often attributed to inhalation of contaminated water droplets that contain the bacterium. It is likely that these droplets harbor protozoan cell-associated bacteria; where protozoan cells are more resistant to conventional water treatment practices[9,10]. Infection of lung alveolar macrophages proceeds in a manner nearly identical to the intracellular life cycle of the bacterium in protozoan host cells[11-13].

In order to survive and replicate in eukaryotic cells, *L. pneumophila* uses a specialized type IVb secretion system termed Dot/Icm to deliver nearly 300 'effector' proteins into the cytosol of the host cell[14-16]. These effector proteins collectively function to subvert cellular processes in order to generate a replication permissive compartment for the bacterium[17,18]. Deletions in any of the 26 genes that comprise the Dot/Icm transporter result in strains defective for intracellular multiplication[19-23]. Historically, deletion of individual effector encoding genes rarely resulted in strains attenuated for intracellular growth. This phenomenon has been attributed to several hypotheses including redundant function and paralogous copies of effectors.

Some virulence factors are only expressed in the context of host cell-associated intracellular growth[24]. We rationalized that if a particular effector was only expressed in the context of protozoan infection, then the contribution of the effector could not be compared with a wild-type strain when both were cultured *in vitro*. *L. pneumophila* transitions from a replicative to a transmissive phase as it enters stationary phase in culture[25]. The phase switching phenotype represents the nutrient depletion encountered during intracellular growth and is exemplified through assembly of flagella for motility[26]. Because *L. pneumophila* is more invasive and virulent when harvested from protozoan cells, we sought to develop an assay that more faithfully represented the pathogenic state of the bacterium when it encountered host macrophages.

To this end, we developed a versatile protozoan priming assay that can accommodate any suitable host for both the first (priming cell) and second (target cell) stage infections. The infection process is tractable through use of bacteria stably expressing green fluorescent protein (GFP). The infection model for the protozoan *Acanthamoeba castellanii* follows a methodology widely used in the field[27]. For the priming step,

L. pneumophila strains are cultivated *in vitro* to stationary phase in liquid media to produce labeled 'transmissive' bacteria (**Figure 1A**). Bacteria are next used to infect monolayers of *A. castellanii* for 18 hr to achieve a late stage of the intracellular life cycle. Large vacuoles containing bacteria can be visualized at this time point using fluorescence microscopy (**Figure 1A**). Protozoan cells are then lysed and bacteria recovered from the lysate are measured for emission at 512 nm using a fluorescence plate reader. Fluorescence is correlated with optical density to calculate multiplicity-of-infection (MOI) for the infection of target cells (**Figure 1**, *Correlation Curve). After invasion (T_0) and 18 hr post-invasion (T_{18}), target cells are quantified for fluorescence, representing intracellular bacteria. Fluorescence can be monitored by microscopy and flow cytometry, and viable counts can be measured through colony plating. The priming assay is always accompanied by infections with wild-type *L. pneumophila* and a strain defective in the Dot/Icm type IV secretion system (Δ*dotA*) (**Figure 1A**). This importantly provides internal controls for direct comparisons between wild-type and any isogenic mutant strains used in the infection process. The inclusion of the avirulent Δ*dotA* strain during the priming stage sets a threshold for observation of attenuated growth phenotypes associated with isogenic mutant strains that are cultured *in vitro*.

Protocol

1. Preparation of *Legionella pneumophila* Cultures for Priming Stage Infections

1. Transform all *L. pneumophila* strains used in the assay with the plasmid pAM239, encoding an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible green fluorescent protein (GFP)[28]. Streak the bacterial strains onto iron and cysteine supplemented N-(2-Acetamido)-2-aminoethanesulfonic acid **(**ACES) buffered charcoal yeast extract agar (CYEA) containing 6.25 µg/ml chloramphenicol (CM) (for plasmid maintenance) and incubate for 72 hr at 37 °C.
2. Transfer an inoculum of the bacterial strain into supplemented ACES buffered yeast extract broth (AYE) with 6.25 µg/ml CM and 1 mM IPTG and cultivate to stationary phase (O/N) on an orbital shaker at 37 °C.
3. Confirm GFP expression in the *in vitro* cultures using fluorescence microscopy. Transfer 10 µl of culture onto a glass slide under a cover slip and image using a 60X objective with GFP excitation / emission cube (AMG EVOS fl).
4. Dilute a 100 µl aliquot of each *in vitro* culture to 1:10 using sterile H_2O. Make a blank using 100 µl AYE media with 1 mM IPTG and 6.25 µg/ml cm and water. Take OD600 measurements of the dilutions using a spectrophotometer (Bio-Rad Smart Spec Plus). Calculate the volume necessary to infect a well at an MOI = 20 for each *in vitro* culture: V = [(amoeba seeded) x MOI]/[OD_{600} x (dilution factor) x (constant)] = [(1 X 10^6) x (20)]/[OD_{600} x (10) x (1 x 10^6)] = 2/OD_{600}. Concentration of bacteria is determined as OD_{600} = 1.0 = 1 x 10^9 CFU/ml.

2. Priming Stage Infection Using *Acanthamoeba castellanii*

1. Maintain and cultivate the amoebae in ATCC 712 PYG medium in 175 cm^2 flasks at RT.
2. Replace media in amoebae cultures 24 hr before starting the bacterial liquid cultures. On the same day liquid bacterial cultures are started, collect and count cells on a light microscope using a hemocytometer.
3. Dilute the amoebae with fresh media to a final concentration of 1 x 10^6 cells/ml. Seed 12-well cell culture plates with 1 ml aliquots of the amoebae using a repeat pipettor and incubate at RT O/N.
4. After incubation, wash the wells of the 12-well cell culture plates 3x with 1 ml sterile PBS using a 10 ml serological pipette and a manual pipette aid.
5. After aspiration of PBS, add 1ml of infection media (ATCC 712 PYG media minus glucose, peptone, and yeast extract) with 1 mM IPTG and 6.25 µg/ml cm to each well. Incubate the plates at RT for 1 hr.
6. Infect wells at an MOI = 20. Centrifuge the plate at 400 x g for 5 min (Eppendorf 5810R) and float in a 37 °C water bath for 5 min. Transfer the plate to a 37 °C, 5% CO_2 incubator for 18 hr.
7. Confirm the infections using live cell imaging on a fluorescence microscope prior to host cell lysis and harvest of bacteria.

3. Seeding THP-1 Cells for Target Cell Stage Infection

1. (Begin this process 24 hr prior to setting up liquid bacterial cultures). Cultivate THP-1 cells in a 75 cm^2 culture flasks to near-confluency in RPMI 1640 media with 10% heat-inactivated fetal bovine serum (FBS).
2. Count the suspension cells using a hemocytometer, dilute the THP-1 cells in RPMI 1640 media with 10% FBS and 100 ng/ml phorbol-12-myristate-13-acetate (PMA) to a concentration of 1 x 10^6 cells/ml, and plate in 1 ml aliquots on 12-well cell culture plates. Incubate the plates for 48 hr at 37 °C, 5% CO_2.

4. Processing of Bacteria for Target Cell Stage Infection after the Priming Stage Infection

1. Aspirate the media from the primed amoebae.
2. Lyse the amoebae using 500 µl of ice cold, sterile ultra-filtered (UF) H_2O and incubate at RT for 10 min.
3. Pool the lysates according to strain type and take an $E_{512\ nm}$ measurement for each of the pooled lysates using a fluorescence plate reader (Molecular Devices Spectramax Gemini EM).
4. Calculate an OD_{600} measurement for the pooled lysates: calcOD_{600} = 0.0008(E_{512} - lysate background) + 0.0019. The formula was previously determined by graphing a direct comparison of both the OD_{600} and the $E_{512\ nm}$ measurements of dilutions of *L. pneumophila* wild-type expressing GFP from stationary phase *in vitro* culture (**Figure 1***). The lysates of uninfected amoeba, subject to the same experimental conditions as the infected amoeba, are used as a blank and provided a correction value (*e.g.* lysate background), which was incorporated into the equation. The volume necessary to infect a well at an MOI = 20 is calculated for each lysate pool: V = [(THP-1 seeded) x MOI] / [calcOD_{600} x (constant)] = [(1 X 10^6) x (20)] / [calcOD_{600} x (1 x 10^6)] = 20 / calcOD_{600}.
5. Wash the THP-1 cells 3x with PBS and add 1 ml fresh RPMI 1640 (10% FBS) to each well. Incubate THP-1 cells for 1 hr at 37 °C, 5% CO_2.

6. Infect the THP-1 cells at the calculated MOI using the pooled lysates. Allow a set of wells to remain uninfected, serving as a negative control for flow cytometric analysis. Processing of the priming stage should be completed in less than 30 min. The lysates are kept on ice to limit any changes in bacterial gene expression before infection.
7. Centrifuge the plate, float to raise temperature, and incubate as in the priming stage.
8. One hour post-infection, remove the media by aspiration and wash wells 3x with PBS to remove extracellular bacteria.
9. Add 1 ml fresh RPMI 1640 (10% FBS) to wells and return the plate to the incubator. The time immediately after media replacement serves as time zero (T_0). Incubate the THP-1 cells for 14-16 hr at 37 °C, 5% CO_2.

5. Experimental Analysis

5.1 Live cell imaging

Image the infected wells using a fluorescence microscope (AMG EVOS fl) at 10X or 20X magnification (**Figures 1A-D**). The images can be compared either qualitatively or quantitatively to determine levels of infection in both the priming stage and target cell stage infections.

5.2 Flow cytometry

1. The emission peaks of different infections can be compared by flow cytometry (BD FACS Calibur). Trypsinize the infected THP-1 cells and gently wash them from the wells by mixing in PBS with a pipette.
2. Pool the cells by strain type into 15 ml Falcon tubes and pellet at 1,800 x g for 2 min in the table top centrifuge.
3. Suspend the pellets in 1 ml PBS and transfer to 1.5 ml microcentrifuge tubes.
4. Centrifuge the suspensions at 1,800 x *g* (Eppendorf 5424) and re-suspend the pellets in 1 ml PBS. If the resulting suspensions are highly turbid ($OD_{600} \geq 1.0$) they must be diluted in additional PBS (1:3) to prevent clogging of the lines in the flow cytometer.
5. Use sterile filtered PBS for equilibration of the flow cytometer and for wash steps between sample injections. Plot the forward / side scatter of uninfected target cells prior to injection of target cell infection samples.
6. Collect 20,000 total events for each condition using 488 nm laser excitation and the FL1 channel.
7. Gate post-capture cell populations to exclude uninfected cells and plot fluorescence intensity in a histogram (FlowJo) (**Figure 1E**).

5.3 CFU plating

1. Examine the efficiency of infection by CFU plating of the cells harvested for flow cytometry. Prepare serial dilutions of the samples in ultra-filtered H_2O at 10^{-1}, 10^{-2}, and 10^{-3}.
2. Plate 20 µl of each dilution onto 1/3 of a CYEA plate with 6.25 µg/ml CM.
3. Incubate the plates at 37 °C for 72 hr.
4. Count the colonies using a toothpick and cell counter.

Representative Results

A typical result for the entire infection process is outlined in **Figure 1**. Live cell fluorescence micrographs depicting monolayers of *A.castellanii* infected with wild-type *L. pneumophila* during the priming stage is shown in **Figure 1A**. A successful measure of the priming step would lead to a population of approximately 90% of the host cells containing large vacuoles populated with GFP labeled bacteria at this MOI. At 18 hr post-infection, most *A. castellanii* cells have achieved maximum bacterial loads yet lysis of the population is minimal. Under light microscopy, a successful priming infection will reveal rounded and detached amoebae. Gentamycin [25 µg/ml] can be added to the culture medium 30 min to 1 hr post infection to eliminate the contribution of extracellular bacteria. The Δ*dotA* mutant strain, which cannot support Dot/Icm-mediated translocation of effectors, should not grow in *A. castellanii* and although the *in vitro* culture should fluoresce as well as the wild-type strain, minimal fluorescence should be detected with this strain at 18 hr post infection. Amoebae will appear flat and the monolayer should be intact.

Total lysates harboring *L. pneumophila* and host cell debris are immediately subjected to spectrophotometric analysis in order to calculate concentration of bacteria in the sample. MOI can be calculated using the correlation curve (**Figure 1***). Target cells must be prepared for infection such that primed bacteria are used less than 30 min after lysis to preserve the integrity of gene expression profiles. Once infected, target cells are incubated for 14-18 hr before they are processed. Fluorescence micrographs in **Figure 1B** demonstrate the pathogenic advantage acquired by protozoan-primed wild-type *L. pneumophila* in target cell stage infections of *A. castellanii* when compared to the identical strain that was limited to *in vitro* cultivation prior to infection. Target cell stage infections are less robust than the priming stage due to the inclusion of a wash step 1-2 hr post-infection (target cell-dependent). This media replacement step removes non-invasive bacteria, synchronizing the infection and reducing background fluorescence in subsequent applications. **Figures 1C-D** demonstrate fluorescence micrographs of a typical wild-type *L. pneumophila* two-stage infection scenario, where bacteria are first primed through infection of *A. castellanii*, harvested from lysed protozoan cells, quantified using the correlation curve and used to infect THP-1 macrophages for 14 hr.

After microscopy, cells are trypsinized for flow cytometry (BD FACS Calibur). Uninfected cells are used to calibrate the machine for forward and side scatter. Infections with each strain are processed with 20,000 events recorded. FlowJo 7.6.1 software is used to analyze the raw data. Typically, fluorescence (488 nm excitation) is first plotted against side scatter to identify the background fluorescence of the uninfected cell population. Plots are gated to exclude this population and re-plotted as a histogram of total counts versus fluorescence intensity. Because each bacterial cell represents a unit of fluorescence, intensity of the signal gives a representation of the vacuolar load in each infected cell. A typical plot of a wild-type infection is shown in **Figure 1E**. Overall, the relative number of infected cells detected by the flow cytometer is lower than anticipated when comparing with microscopy data. Live cell flow cytometry is sensitive enough however to detect strain variances.

Quantification of total CFU's in the samples is performed by generating serial dilutions of the cells prepared for flow cytometry and subsequent plating on CYEA media. After 72 hr at 37 °C, single colonies should be evident and sparse enough for counting.

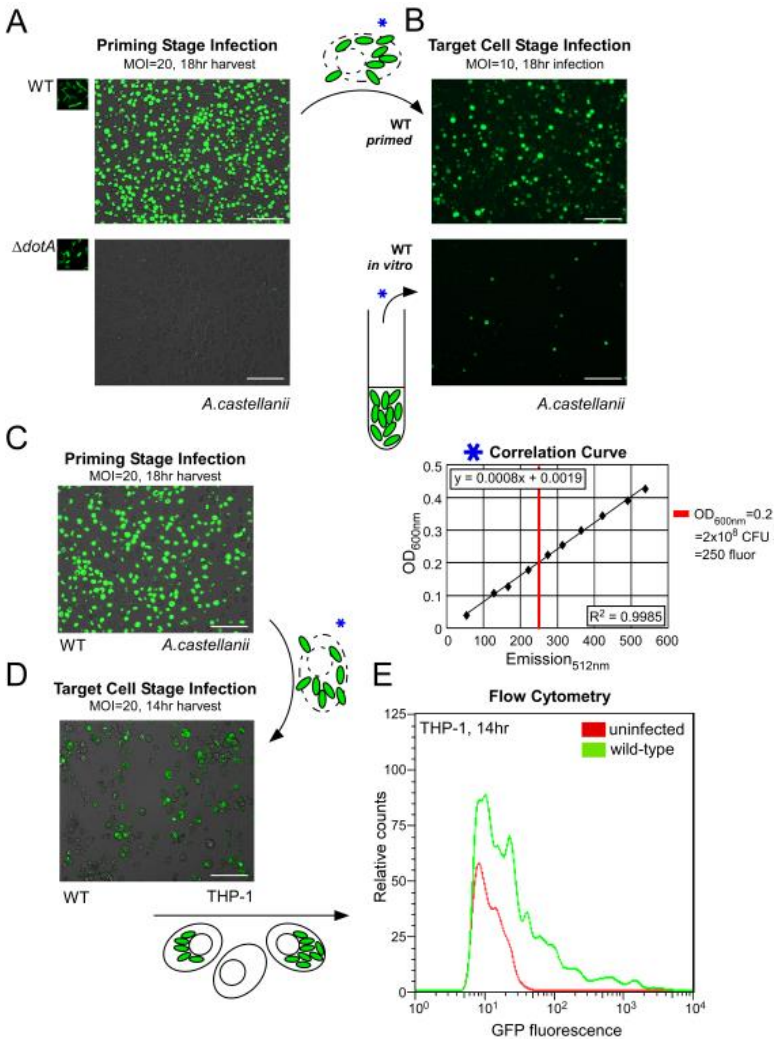

Figure 1. Protozoan priming and target cell stage infections using *Legionella pneumophila*. A. Wild-type or type IV secretion deficient (Δ*dotA*) *L. pneumophila* strains were induced to express GFP through *in vitro* culture (small frames) and used to infect *A. castellanii* monolayers for 18 hr at MOI=20. Fluorescence micrographs of infections with each *L. pneumophila* strain are shown (large frames) as a merge of phase contrast and $E_{512\ nm}$ fluorescence images with a 20X objective on a fluorescence microscope (AMG EVOS fl). Green crescents are representative of intracellular vacuolesharboring *L. pneumophila*. **B.** GFP fluorescence micrographs of 18 hr target cell stage infection of *A. castellanii* with wild-type *L. pneumophila* harvested from the protozoan priming stage infection (top) or *in vitro* cultured (bottom), where the MOI=10 for each was calculated using the equation derived from the correlation curve shown (blue*). The curve was produced using serial dilutions of wild-type *L. pneumophila* expressing GFP from an 18 hr *in vitro* culture. **C.** Fluorescence micrograph of a priming stage infection of *A. castellanii* with wild-type *L. pneumophila* as in **(A)**. **D.** Fluorescence micrograph of a 14 hr target cell stage infection of PMA differentiated THP-1 macrophages with wild-type *L. pneumophila* harvested from the protozoan priming stage infection in **(C)**. The MOI=20 was calculated using the equation derived from the correlation curve shown (blue*). The frame shown is a merge of phase contrast and E512 nm fluorescence images as in **(A)**. **E.** Histogram plot of flow cytometry data comparing uninfected THP-1 macrophages (red) and cells infected with protozoan primed wild-type *L. pneumophila* (green). Scale bar = 100 µm. Click here to view larger figure.

Discussion

Bacterial gene expression is tightly controlled through a combination of life cycle progression and response to signals in the surrounding microenvironment. Vacuolar pathogens such as *L. pneumophila* respond to a multitude of host cell-derived cues when compartmentalized in a phagosome. As a collective result of nutrient exhaustion in the host cell, the bacterium compensates by expressing factors required for successful dissemination to a subsequent host cell[25]. *L. pneumophila* adapted to efficiently parasitize a wide variety of protozoan cells and therefore harbors an extensive catalog of effector proteins to drive this process. These effector proteins are translocated directly into the host cytoplasm during infection by the Dot/Icm type IVb secretion system[16]. Effector protein translocation is required, as mutations that result in a defective transporter completely abrogate intracellular growth in any host cell type. Curiously, individual deletions in particular effector proteins rarely resulted in intracellular growth attenuation. Nearly 300 effector proteins, or almost 10% of the protein encoding genome have been validated as Dot/Icm substrates[14,15]. Several hypotheses have emerged to explain the lack of observable phenotypes for effector deletions, including the presence of paralogous copies or the horizontal acquisition of orthologous enzymes resulting from the wide host range of *L. pneumophila*.

Because *L. pneumophila* gains a pathogenic advantage when pre-cultured in protozoan cells (**Figures 1A-B**), we reasoned that *in vitro* cultured bacteria would not display the same gene expression profile apparent in bacteria exiting the protozoan host. Therefore, expression of particular effector proteins could only be induced in the context of intracellular growth in protozoan cells. If a particular effector was not induced for expression during *in vitro* culture, than the contribution of the deleted effector could not be effectively evaluated in a traditional infection scenario. Phenotypes for virulence factors important for the establishment of infection or dissemination would only be detected in prolonged infections that allowed for cell to cell spread. We therefore sought to develop an assay that could measure the contribution of potential virulence factors in the context of a sequential infection. In the case of natural *L. pneumophila* acquisition, it is likely that a human host would encounter a bacterium that had been primed for infection in a protozoan cell; where protozoans can be more resistant to traditional municipal water treatment practices[10].

In order to optimize the partial purification of sufficient quantities of primed bacteria, we first established a visually tractable strain of *L. pneumophila* using IPTG inducible *gfp* using plasmid borne copies of the *gfp* locus. We also generated a single copy chromosomal insertion of IPTG inducible *gfp* on the wild-type genome. Although both strains fluoresce *in vitro* and during infection, the higher copy number of the plasmid-borne *gfp* produced sufficient signal for downstream applications in the assay. We established that using broth cultured *L. pneumophila* at an MOI = 20 was sufficient to heavily infect *A. castellanii* monolayers. Eighteen hours of incubation was determined as optimal to visualize large fluorescent vacuoles without reaching a stage of host cell lysis (**Figure 1A**). Protozoan monolayers are not adhered to cell culture plastics, and are therefore easily disturbed with washing steps. 12-well cell culture plates produced the most stable monolayers when compared with other vessels (6 or 24 well, 10 cm dish). Because *L. pneumophila* cannot grow in the infection medium used in the priming step, we chose to abandon any wash step to remove extracellular bacteria. This greatly improves the yield of infected amoeba in the monolayer. Gentamycin is also often used to kill extracellular bacteria. We found protozoan toxicity effects at concentrations above 25 µg/ml, and found no differences in the total number of bacteria recovered from the priming stage infection at 18 hr. Lysis of the protozoan cell monolayer was performed with ice cold ultra-filtered H_2O, a solvent that osmotically compromises the host cell without harming *L. pneumophila*. If adapted for use with pathogens that are not stable in H_2O, 0.1% Triton X-100 in PBS can be used to lyse the amoebae with similar results.

In order to directly compare strain to strain differences in subsequent target cell infections, an equation was generated to correlate optical density of bacteria at 600 nm with fluorescence emission of GFP at 512 nm (**Figure 1***). We chose to use fluorescence emission as a means for quantification due to the large contribution of host cell debris in OD_{600} measurements from eukaryotic cell lysates. This high background contrasted with negligible values observed for emission at 512 nm from the same lysates. Values of OD_{600} = 1.0 = $1x10^9$ CFU/ml were predetermined for *L. pneumophila*, allowing for calculation of bacterial concentration over a wide range of fluorescence values. Many organisms have established CFU values for OD_{600} = 1.0, which would allow for applying this technique to any other pathogen through generation of a correlation curve based on fluorescence emission. We chose to avoid a bacterial purification step during lysis of the protozoan cells in order to 1) maximize the yield of primed bacteria available for the target cell infection and 2) minimize the time between harvest and subsequent infection to preserve global gene expression patterns.

In vitro cultured wild-type *L. pneumophila* was induced to express GFP for 18 hr with 1 mM IPTG. Serial dilutions of this culture were used for the spectrophotometric measurements. Using the derived equation, target cell infections were routinely performed at variable MOI's with consistent output. An MOI of 10 produced approximately 1/2 the number of total infected cells when compared to infections that received an MOI of 20 (**Figures 1A-B**). We have tested three target cell hosts with this assay; *A. castellanii*, murine J774 macrophages and human PMA-differentiated THP-1 macrophages. In each instance, the resulting infection was never as robust as the priming infection at the same MOI (**Figures 1C-D**). We determined that that an additional wash step during the target cell stage infection, which is performed to both synchronize the target cell infection and reduce extracellular bacteria, is responsible for this observation. Nevertheless, it is crucial that the experiment is internally controlled with a wild-type strain for both the priming and target cell stage infections. Bacterial quantities recovered in the priming step is rather limiting for subsequent infections, therefore only a single target cell type should be used per experiment.

Quantification of the target cell infection was achieved using three methods in this assay. Fluorescence microscopy was used with a 20X objective. Images of vacuoles harboring *L. pneumophila* were manually counted from several fields for each strain tested. Flow cytometric analysis provided a sensitive measure for differences in total cells infected, as well as an approximation of vacuolar load; where a higher fluorescence signal was correlated with the contribution of GFP signal from increasing numbers of *L. pneumophila* cells. Serial dilution plating provided quantitative values for total number of CFU's per volume in the cell samples collected for flow cytometry. Collectively, these methods allow for fully tractable and quantifiable results in a sequential infection scenario.

The priming assay is quite versatile and is suited for adaptation to any bacterial pathogen that uses freshwater protozoans as an environmental intermediate[29,3]. The choice of target cell can be catered to fit the natural infection route for the particular pathogen. Importantly, a sequential infection model can be used to examine mutant strains that did not present with a growth phenotype in a traditional infection model using *in vitro* cultured bacteria. Transcriptional profiling *in vitro* cultured bacteria for presumed virulence factors could reveal key differences in the profile of expression in the infected intermediate host. This could open a new line of investigation for the function of uncharacterized proteins and their contribution to natural infection routes.

Disclosures

The authors declare that they have no competing financial interests.

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