Microbial Pathogenesis 105 (2017) 326–333

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Interaction of *Francisella noatunensis* subsp. *orientalis* with *Oreochromis mossambicus* bulbus arteriosus cell line

Esteban Soto [a, *], Susan Yun [a], Jainee Lewis [a], Michael T. Kearney [b], John Hansen [c]

[a] University of California-Davis, Department of Medicine and Epidemiology, School of Veterinary Medicine, Davis, CA 95616, USA
[b] Louisiana State University, Department of Pathobiological Sciences, School of Veterinary Medicine, Baton Rouge, LA 70803, USA
[c] U.S. Geological Survey, Western Fisheries Research Center, Seattle, WA 98115, USA

ARTICLE INFO

Article history:
Received 19 October 2016
Received in revised form
25 January 2017
Accepted 6 March 2017
Available online 7 March 2017

Keywords:
Francisella
Endothelium
Mutant
Temperature
Tilapia

ABSTRACT

Francisella noatunensis subsp. *orientalis* (*Fno*) (syn. *F. asiatica*) is an emergent warmwater fish pathogen and the causative agent of piscine francisellosis. Although *Fno* causes septicemia and can live extracellularly in infected tilapia (*Oreochromis* spp.), the early interaction of *Fno* with vasculature endothelium is unknown. In the present study, we examined the interaction of wild-type *Fno* (WT) and two *Fno* knockout [intracellular growth loci C (Δ*iglC*) and pathogenicity determinant protein A (Δ*pdpA*)] strains with the endothelial *O. mossambicus* bulbus arteriosus cell line (TmB) at 25 °C and 30 °C. Similar amounts of WT, Δ*iglC*, and Δ*pdpA* attached and were detected intracellularly after 5 h of incubation at both temperatures; however temperature affected attachment and uptake. While significantly greater amounts of *Fno* (WT, Δ*iglC*, and Δ*pdpA*) were detected intracellularly when TmB cells were incubated at 30 °C, bacteria attached to TmBs at greater levels at 25 °C. Only WT *Fno* was able to replicate intracellularly at 25 °C, which resulted in *Fno* mediated cytotoxicity and apoptosis at 24 and 72 h post-infection. WT *Fno* incubated at 30 °C as well as Δ*iglC*, and Δ*pdpA* incubated at 25 °C and 30 °C were all defective for survival, replication, and the ability to cause cytotoxicity in TmB. Taken together, these results demonstrate that temperature plays a vital role for *Fno* intracellular survival, persistence and cytotoxicity.

1. Introduction

Aquaculture is one of the world's fastest developing food production sectors. Aquaculture is now expected to produce over half the fish consumed by humans and intensification of fish production is expected to surpass any growth in global wild fisheries in the near future [1–3]. In the last decade, global tilapia aquaculture has grown exponentially and in 2014 was valued in $10 billion, representing over 4.85×10^6 metric tons of fish [4,5].

Infectious diseases pose one of the most significant threats to aquaculture production and cost the industry millions annually [6,7]. Piscine francisellosis, caused by *Fno* is an emergent disease affecting cultured tilapia and other important warm water species in both fresh and salt water. The agent is a small, Gram-negative, facultative intracellular bacterial pathogen, characterized by an alarming lack of host specificity and is being reported in an increasing range of cultured and wild fish species from around the world [8,9]. Disease may present with acute high losses and few clinical signs or follow a more protracted subacute to chronic course [8,9]. Mortalities at aquaculture facilities vary, but can approach 90% [8,9].

Members of the genus Francisella are facultative intracellular pathogens, and although monocyte-macrophage cell lineage are considered the key cells in pathogenesis of *F. tularensis* in mammals and *Fno* in fish, other cell lines have been found susceptible to infection. Similar to *F. tularensis*, *Fno* proliferates within host macrophages, facilitated by the presence of an intracellular growth operon (*iglABCD*), pathogenicity determinant protein A gene (*pdpA*), and other virulence genes located in the Francisella pathogenicity island [10–12]. As few as 23 colony forming units (CFU) can be lethal to tilapia fingerlings, but pathogenic mechanisms that underlie the remarkable infectivity of *Fno* and its ability to cause disease in such a broad range of fish are unknown [11].

Although *Fno* causes septicemia and can live extracellularly in a tilapia (*Oreochromis* spp.) infection model, the strategy used by this pathogen to cross the endothelial barrier in the microvalsculature

* Corresponding author. University of California-Davis, 2108 Tupper Hall, Department of Medicine and Epidemiology, School of Veterinary Medicine, Davis, CA 95616, USA.
E-mail address: sotomartinez@ucdavis.edu (E. Soto).

http://dx.doi.org/10.1016/j.micpath.2017.03.003
0882-4010/

and disseminate into various organs is unknown. In the present study we used *Fno* and the Tilapia mossambica (*Oreochromis mossambicus*) immortalized bulbus arteriosus cell line (TmBs) [13,14] to determine the role of endothelial cells as targets for bacterial infection and/or as a cellular refuge in which bacteria avoid host defenses in the course of *Fno* disseminated infections. We demonstrate that wild type *Fno* is able to enter, survive, and replicate in TmBs. Importantly, mutation of the *iglC* and *pdpA* genes, renders *Fno* defective for intracellular survival and replication, as well as for induction of apoptosis and cytotoxicity. Finally we demonstrate that temperature plays an important role in intracellular survival of *Fno* in TmBs.

2. Materials and methods

2.1. Bacteria

Francisella noatunensis subsp. *orientalis* LADL 07–285A recovered from naturally infected Nile tilapia (*Oreochromis niloticus*) was used in this study [15]. Wild-type *Fno* LADL 07–285A was transformed with plasmid pKEK 1128 expressing the m-cherry protein [16] following protocols by Soto et al. [11]. Additionally, previously described Δ*iglC* [11]and Δ*pdpA* [JD Hansen, unpublished results] defined mutants were used. Both mutants were created by double homologous recombination and attenuation has been demonstrated *in vivo* in zebrafish (*Danio rerio*) and tilapia models [11, JD Hansen, unpublished results]. Frozen stocks were prepared from bacteria grown to mid-log phase in Mueller-Hinton broth (BD Biosciences) supplemented with 2% IsoVitaleX Enrichment (BD Biosciences), 0.1% glucose (MMH) [15]. For each experiment, a frozen stock was thawed and streaked on modified Thayer Martin agar plates. The bacteria were grown for 4 days at 25° to allow for colony formation. A single colony then was inoculated into the supplemented Mueller-Hinton broth and grown to late log phase for 20–24 h at 25 °C with shaking at 150 rpm. Aliquots of bacterial culture then were centrifuged, and the pellets were resuspended in PBS and diluted in minimal essential medium with 10% fetal bovine serum (MEM). The number of viable bacteria in the suspensions was determined by streaking appropriate dilutions on modified Thayer martin agar plates and counting colonies 4 days later. As a negative control, Mueller-Hinton broth alone was processed in the same manner.

Growth kinetics of isolates in MMH or MEM were investigated at 25 or 30 °C. Briefly, isolates were grown overnight in MMH at 25 °C. This suspension was diluted 1000-fold (~10^6 CFU/mL) in MMH or MEM, and 100 μL of each were added to 6 different wells of a Nunc clear flat-bottom 96-well plate with standard microtiter plate lid. A 96-well plate reader (Cytation 5, Biotek) was used to obtain optical density measurements at 600 nm, every hour hours for 96 h. Plates were incubated in the plate reader at 25 or 30 °C using a double orbital continuous 3 mm shaking amplitude. Wells on the edges of the plate were not used as experimental wells and were instead filled with sterile water.

2.2. Immortalized bulbus arteriosus cell line stability at different temperatures

In order to investigate the stability and proliferation capability of TmBs at 25, 30, and 35 °C, TmBs were plated in 24-well dishes (7×10^4 cells/well in 500 μl of MEM-2+HEPES) and quantified at 1, 3, 6 and 9 d. Briefly, at each time point the wells were examined by light microscopy using a Leitz Labovert inverted microscope. Media was removed from the well and versene-trypsin (Sigma-Aldrich St. Louis, MO) was added until cells began to detach. MEM-2+HEPES was added to the well and contents were mixed to ensure detachment of all cells before removing from the well. The volume of media in the well was measured and an aliquot was removed to count the cells using a Bright-Line hemacytometer.

2.3. Adherence of Francisella noatunensis subsp. orientalis to TmBs

In order to ascertain whether *Fno* adheres to cells, cell association assays were performed with TmBs [13,14]. TmBs were plated in 24-well dishes (2.5×10^5 cells/well in 500 μl of MEM) and grown to confluence. Aliquots (500 μl) of *Fno* were then added at a multiplicity of infection of ~100, centrifuged at 300 × *g* to facilitate interactions with cells, and incubated at 25 °C for 5 h. Cells were washed twice with phosphate-buffered saline (PBS) post-incubation, and the monolayer was solubilized with PBS containing 1% saponin before being plated. This treatment did not affect the viability of *Fno* when plated on modified Thayer-Martin (data not shown). The experiment was replicated five times.

2.4. Internalization and intracellular survival of Francisella noatunensis subsp. orientalis using immortalized bulbus arteriosus cells

In order to ascertain whether *Fno* is internalized in cells, assays were performed with TmB cells. TmBs were plated in 24-well dishes (2.5×10^5 cells/well in 500 μl of culture medium) and grown to confluence. Aliquots (500 μl) of *Fno* then were added at a multiplicity of infection of ~100, centrifuged at 300 × *g* to facilitate interactions with cells, and incubated at 25 °C for 5 h. Cells were washed twice with phosphate-buffered saline (PBS) post-incubation, and incubation was continued for an additional 1 h in medium supplemented with 50 μg/ml of gentamicin to kill remaining extracellular bacteria. This concentration and time frame of antibiotic exposure was sufficient to kill all *Fno* in control samples incubated without TmBs (data not shown). Cultures then were washed with medium lacking antibiotic, and TmBs were lysed by addition of 1% saponin. Lysates were diluted and plated to determine the numbers of intracellular bacteria. The experiments were repeated six times.

In order to ascertain whether *Fno* replicates in cells, assays were performed with TmB cells similar as in internalization assays; however, 1 h post-incubation in gentamycin, cells were washed twice with PBS post-incubation, and incubation continued for an additional 3 or 6 d in medium. Immortalized bulbus arteriosus cell line were lysed by addition of 1% saponin, diluted and plated to determine the numbers of intracellular bacteria. Cell culture media used in this study does not support growth of *Fno* (data not shown).

2.5. Viability of TmBs exposed to Francisella noatunensis subsp. orientalis

To determine whether *Fno* kills endothelial cells, TmBs were plated in 96-well plates (2.5×10^4 cells/well in 100 μl of culture medium) and grown to confluence. Aliquots (100 μl) of *Fno* then were added at a multiplicity of infection of ~10, ~100 and ~1,000, and plates were incubated at 25 °C for various times. Cytotoxicity and apoptosis of cells was measured using Vibrant™ Cytotoxicity Assay Kit (Molecular Probes, Eugene, OR) and the Caspase-Glo® 3/7 Assay (Promega, Madison, WI) following manufacturers indications, respectively in a 96-well format using a plate reader (Cytation 5, Biotek).

2.6. Confocal and electron microscopy

For confocal microscopic analysis, assays were performed with TmB cells similar to internalization assays; however, 5 h post-