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Intra-vacuolar proliferation of *F. novicida* within *H. vermiformis*

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Francisella tularensis is a gram negative facultative intracellular bacterium that causes the zoonotic disease tularemia. Free-living amebae, such as *Acanthamoeba* and *Hartmannella*, are environmental hosts of several intracellular pathogens. Epidemiology of *F. tularensis* in various parts of the world is associated with water-borne transmission, which includes mosquitoes and amebae as the potential host reservoirs of the bacteria in water resources. *In vitro* studies showed intracellular replication of *F. tularensis* within *A. castellanii* cells. Whether ameba is a biological reservoir for *Francisella* in the environment is not known. We used *Hartmannella vermiformis* as an amebal model system to study the intracellular life of *F. novicida*. For the first time we show that *F. novicida* survives and replicates within *H. vermiformis*. The *iglC* mutant strain of *F. novicida* is defective for survival and replication not only within *A. castellanii* but also in *H. vermiformis* cells. In contrast to mammalian cells, where bacteria replicate in the cytosol, *F. novicida* resides and replicates within membrane-bound vacuoles within the trophozoites of *H. vermiformis*. In contrast to the transient residence of *F. novicida* within acidic vacuoles prior to escaping to the cytosol of mammalian cells, *F. novicida* does not reside transiently or permanently in an acidic compartment within *H. vermiformis* when examined 30 min after initiation of the infection. We conclude that *F. tularensis* does not replicate within acidified vacuoles and does not escape into the cytosol of *H. vermiformis*. The *Francisella* pathogenicity island locus *iglC* is essential for intra-vacuolar proliferation of *F. novicida* within *H. vermiformis*. Our data show a distinct intracellular lifestyle for *F. novicida* within *H. vermiformis* compared to mammalian cells.

Keywords: *Francisella novicida*, *Hartmannella vermiformis*, vacuolar replication, LysoTracker, *iglC*

INTRODUCTION

Francisella tularensis is a gram negative, facultative intracellular bacterium that causes the zoonotic disease tularemia in humans and animals, and various recent reviews in this special topic issue have discussed various aspects of *Francisella* (Chong and Celli, 2010; Meibom and Charbit, 2010; Akimana and Abu Kwaik, 2011; Asare and Abu Kwaik, 2011; Bosio, 2011; Bröms et al., 2011; Cremer et al., 2011; Dai et al., 2011; Gavrilin and Wewers, 2011; Jones et al., 2011; Zogaj and Klose, 2011). Tularemia is a zoonotic disease of the northern hemisphere. Humans acquire infection by exposure to infected arthropod vectors, or by handling, ingesting, or inhaling infectious materials. *F. tularensis* has been isolated from over 250 animal species, including fish, birds, amphibians, rabbits, squirrels, hares, voles, ticks, and flies (Santic et al., 2010; Akimana and Abu Kwaik, 2011). Three closely related subspecies of *F. tularensis* have been identified: *tularensis*, *holarctica*, and *mediasiatica* (Forsman et al., 1994). Recently *F. novicida* has been accepted as new species (Sjöstedt, 2005). It has been suggested that *holarctica* ssp. has a strong association with water-borne disease (Greco et al., 1987; Thelaus et al., 2009; Broman et al., 2011). An *in vitro* study showed that *F. tularensis* subsp. *holarctica* can survive and grow within *Acanthamoeba castellanii* (Abd et al., 2003). In addition, *F. tularensis* subsp. *holarctica* was found within amebal cysts, suggesting potential for long-term survival and an important environmental reservoir for tularemia. The isolation of the bacterium from a water eco-system, as well as from natural spring water (Thelaus et al., 2009; Willke et al., 2009; Broman et al., 2011), supports the hypothesis that protozoa may serve as a reservoir for *F. tularensis* in nature (Morner, 1992; Thelaus et al., 2009; Broman et al., 2011). Very little is known about the *F. tularensis*–ameba interaction.

It has been shown that within mammalian and arthropod-derived cells, the *Francisella* containing phagosome (FCP) transiently matures to an acidified late endosomal stage with limited fusion to lysosomes, followed by rapid bacterial escape into the host cell cytosol (Clemens et al., 2004; Chong et al., 2008; Santic et al., 2008, 2009; Asare and Abu Kwaik, 2011). The FCP is acidified by the vATPase proton pump within 15–30 min of phagosome biogenesis, which is essential for subsequent rapid disruption of the FCP and escape of *F. tularensis* into the host cell cytosol, where the bacterium replicates (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011; Dai et al., 2011). Inhibition of the vATPase proton pump causes a significant delay in phagosomal escape and blocks bacterial proliferation (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011), indicating a major role for acidification of the FCP in rapid bacterial escape into the cytosol and subsequent replication (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011).

A gene cluster, the *Francisella* pathogenicity island (FPI), that regulates phagosomal escape and intracellular survival of *F. tularensis* within macrophages, has been identified (Nano et al., 2004; Nano and Schmerk, 2007; Meibom et al., 2009). It has been suggested to encode a type VI-like secretion system (de Bruin et al., 2007; Nano and Schmerk, 2007; Bingle et al., 2008; Ludu et al., 2008; Barker et al., 2009; Bröms et al., 2011). It has also been shown that IglC is essential for avoiding lysosomal fusion (Santic et al., 2005b; Bonquist et al., 2008) and for bacterial escape into the host cytosol (Lindgren et al., 2004; Santic et al., 2005a) in macrophages. In addition, the *iglC* mutation diminishes intracellular replication in *A. castellanii* (Lauriano et al., 2004).

Free-living amebae such as *Acanthamoeba* and *Hartmannella* are environmental hosts of several intracellular pathogens such as *Legionella*, *Chlamydia*, and *Mycobacterium* (Amann et al., 1997; Abu Kwaik et al., 1998; Steinert et al., 1998; Molmeret et al., 2005). It has been shown that legionellae interact with their protozoan hosts and mammalian cells in a similar way (Harb et al., 2000). Since the host reservoir of *F. tularensis* in water systems is not known, we used *H. vermiformis*, which is the most predominant non-pathogenic ameba in water resources, as an amebal model system to study the intracellular life of *F. novicida*. Our data indicate that *F. novicida* survives within *H. vermiformis* and that the bacteria do not escape into the cytoplasm, which is very distinct from the lifestyle of *F. novicida* within mammalian cells. The *iglC* bi-cistronic locus plays an important role in intra-vacuolar replication in *H. vermiformis*.

MATERIALS AND METHODS

BACTERIA AND PROTOZOAN STRAINS AND MEDIA

The wild type (wt) *F. novicida* strain U112 and it isogenic *iglC* mutant were grown on buffered-charcoal yeast extract (BCYE) agar plates and have been described previously (Santic et al., 2005b). Construction of ΔiglC::ermC has been described previously (Lauriano et al., 2003). The *iglD* gene was not affected. The tetracycline-resistant plasmid pKK214, encoding green fluorescent protein (GFP), was introduced into *F. novicida* (Abd et al., 2003).

Acanthamoeba castellanii and *H. vermiformis* were obtained from the American Type Culture Collection, 30234 and 1034, respectively. The amebae were grown in medium 30234 and 1034 at 25°C, as described elsewhere (Pedersen et al., 2001; Viswanathan et al., 2002).

INFECTION AND INTRACELLULAR SURVIVAL ASSAY IN AMEBAL CELLS

Infection of protozoan strains with *F. novicida* has been described previously (Abu Kwaik, 1996; El-Etr et al., 2009). Briefly, triplicate cultures of protozoan strains were seeded into 96-well plates at 1×10^5 amebal cells/well and allowed to adhere for a few hours at 25°C. The amebae were washed and infected with *F. novicida* at a multiplicity of infection (MOI) of 10. After coincubation for 15 min, the cells were washed once and incubated with 100 μg gentamicin/ml for 1 h at 37°C and 5% CO_2, followed by gentamicin treatment at the end of all time points examined. The amebae were then washed once to remove gentamicin and lysed with Triton-X100 (0.1%) for 10 min. The number of *F. novicida* in each well was determined by plating serial dilutions on BCYE agar plates.

CONFOCAL LASER SCANNING AND ELECTRON MICROSCOPY

For confocal microscopy, acidification of the *Francisella* containing vacuoles (FCVs) was determined using the lysosomotropic agent LysoTracker Red DND-99 (Molecular Probes). *H. vermiformis* cells were grown on glass cover slips in 24-well plates and then used for subsequent invasion assays with live or heat-killed bacteria. Briefly, triplicate cultures of protozoan strains were seeded into plates at 1×10^5 amebal cells/well and allowed to adhere for a few hours at 25°C. The amebae were washed and infected with *F. novicida* at a MOI of 10. After coincubation for 15 min, the cells were washed once and incubated with 100 μg gentamicin/ml for 1 h at 37°C and 5% CO_2. Thirty minutes prior to the time point, the amebal cells were washed and incubated with 1 ml of 1 μM LysoTracker, washed three times with PBS, fixed with 4% paraformaldehyde, and then mounted on glass slides for confocal microscopy analysis. All confocal microscopy analyses were performed on one hundred infected amebal cells from three different cover slips, for each time point in each experiment, and all experiments were performed three times. The analysis of colocalization has been performed on individual optical sections. The quantification was performed manually on a FV1000 Olympus confocal microscope. The images shown in the figures are stacks of 15 one-micron-thick Z-series sections.

For electron microscopy, triplicate cultures of protozoan strains were seeded into 12-well plates at 1×10^5 amebal cells/well and allowed to adhere for a few hours at 25°C. The amebae were washed and infected with *F. novicida* at a MOI of 10. After coincubation for 15 min, the cells were washed once and incubated with 100 μg gentamicin/ml for 1 h at 37°C and 5% CO_2. At several time intervals, the infected and uninfected monolayers were fixed for transmission electron microscopy with glutaraldehyde, post fixed with OsO_4 in Sorenson's buffer (pH 7.4), dehydrated with ethanol, and embedded in Epon (Miller-Stephenson), as described previously (Santic et al., 2005b). The sections were stained with lead citrate and uranyl acetate and examined with a Phillips Morgany transmission electron microscopy.

RESULTS

INTRACELLULAR REPLICATION OF *F. NOVICIDA* WITHIN *A. CASTELLANII* AND *H. VERMIFORMIS*

Previous studies have shown that *F. tularensis* subsp. *holarctica and tularensis*, and *F. novicida* survive and replicate in *A. castellanii* (Abd et al., 2003; Greub and Raoult, 2004; Lauriano et al., 2004; Hazlett et al., 2008; El-Etr et al., 2009). Since *H. vermiformis* is the most predominant non-pathogenic ameba in water supplies, we determined the ability of *F. novicida* to replicate within *H. vermiformis* and compared that to *A. castellanii* during early and late stages of infection. In addition, it has been shown that *iglC* is required for growth in macrophages and *Acanthamoeba* (Abd et al., 2003; Greub and Raoult, 2004; Lauriano et al., 2004; Hazlett et al., 2008; El-Etr et al., 2009), but there is no evidence about the intracellular replication of *F. novicida* within *H. vermiformis*.

The amebal cells (1×10^5 amebal cells/well) were infected with *F. novicida* and/or the *iglC* mutant at a MOI of 10 for 15 min, followed by treatment with gentamicin for 1 h to kill extracellular bacteria,

followed by further incubation. The time at the end of 15 min of infection was considered T_0. At different time points after infection (2, 24, 48, and 72 h), the amebal cells were lysed and bacteria were grown on agar plates to determine the number of colony forming units (CFU).

The data showed that *F. novicida* exhibited robust replication and bacterial numbers increased by 10,000-fold by ~48 h after infection within *A. castellanii* cells (**Figure 1A**). We also determined the ability of *F. novicida* to replicate within *H. vermiformis*. *F. novicida* replicated in *H. vermiformis* cells at a much faster rate than in *A. castellanii* cells, and the bacterial number increased by 10,000-fold within 24 h after infection (**Figure 1B**). The *iglC* mutant was unable to multiply in *A. castellanii* or *H. vermiformis*, and their viability decreased dramatically by 48 h post-infection (**Figures 1A,B**). Only around 10% of cells were lysed by 48 h after infection (data not shown). The above data showed that *F. novicida* survives and replicates intracellularly within *A. castellanii* and *H. vermiformis*, and that the *iglC* bi-cistronic locus is necessary for intracellular growth and survival within *H. vermiformis*.

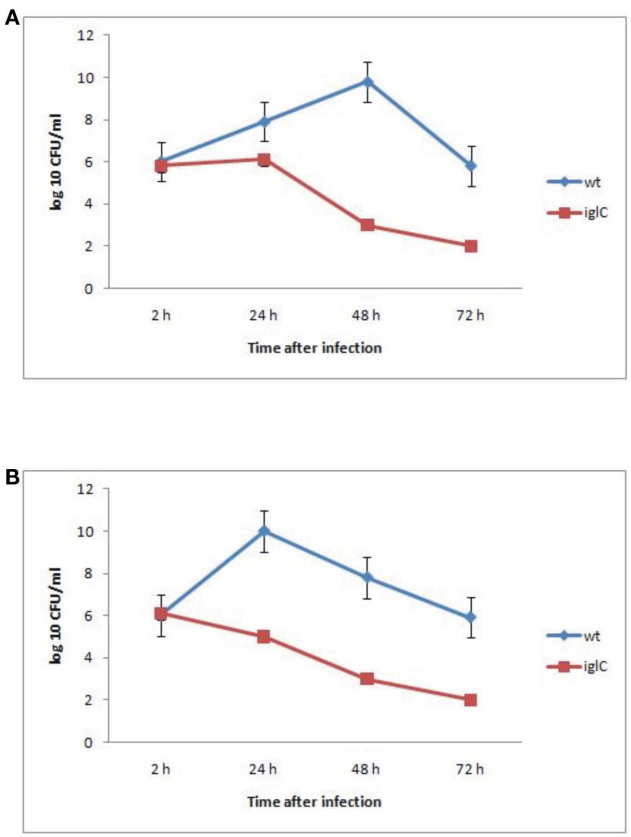

FIGURE 1 | Intracellular growth of *F. novicida* and its isogenic mutant *iglC* in *A. castellanii* (A) and in *H. vermiformis* (B). The cells were infected for 15 min, followed by gentamicin treatment, and determination of the number of intracellular bacteria at the indicated time points. The error bars represent standard deviations of triplicate samples and results shown are representative of three independent experiments.

F. TULARENSIS REPLICATES IN VACUOLES WITHIN *H. VERMIFORMIS* CELLS

It has been shown that the FCP matures to a late-endosome-like-phagosome prior to bacterial escape into the cytosol of macrophages, where bacterial proliferation occurs (Clemens et al., 2004; Santic et al., 2005b, 2010; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). The process of phagosomal disruption is rapid and occurs within 30 min. of infection in mammalian cells (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). The *iglC* locus is essential for bacterial escape into the cytosol of macrophages (Lindgren et al., 2004; Santic et al., 2005b; Asare and Abu Kwaik, 2011). Therefore, we examined at the ultra-structural level the intracellular infection of *H. vermiformis* with *F. novicida*.

Up to 2 h after infection by *F. novicida*, the bacteria were localized in intact vacuoles within *H. vermiformis* (**Figures 2A,B**), and efficient phagocytosis of bacteria by the amebae was observed. Almost 95% of the vacuoles harboring *F. novicida* had intact vacuolar membranes. By 6 h after infection, the bacteria were still enclosed in intact vacuoles within amebal cells, and still 90% of *H. vermiformis* harboring the bacteria had intact vacuolar membranes (**Figures 2A,B**). There was clear evidence that by 6 h bacterial replication had been initiated, since only one bacterium per vacuole was detected at 2 h while two to six bacteria per vacuole were detected by 6 h post-infection. During all time points examined more than 90% of bacteria were intra-vacuolar. Only 10% of the intracellular bacteria were localized in the cytosol.

By 24 h after infection ~90% of the vacuoles were intact with a clear and distinct vacuolar membrane (**Figures 2A,B**). Similar results to intra-vacuolar localization of the wt strain have been also observed for the *iglC* mutant. The mutant resides in intact vacuoles within *H. vermiformis* cells at all time points after infection (**Figures 2A,B**). However, the *iglC* mutant failed to replicate within the vacuole. In addition, *H. vermiformis* did not differentiate into cysts during the time points examined. There have been just a few cysts containing multiplying bacteria.

We conclude that in contrast to mammalian cells, where bacteria do not replicate in the vacuole but escape into the cytosol where they replicate, *F. novicida* does not escape into the cytosol, but replicates within the vacuoles in *H. vermiformis*.

F. TULARENSIS DOES NOT RESIDE IN AN ACIDIC COMPARTMENT WITHIN *H. VERMIFORMIS*

Previous data in human macrophages showed that within the first 15 min after infection, ~90% of the FCPs acquire the lysosomotropic dye LysoTracker, which concentrates in acidic compartments (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). Colocalization of phagosomes harboring the *iglC* mutant with the LysoTracker dye was persistent, which is consistent with fusion to the lysosomes and failure of the *iglC* mutant to escape into the macrophage cytosol (Lindgren et al., 2004; Santic et al., 2005b; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). The acquired lysosomotropic dye is gradually lost by 30–60 min post-infection, which coincides with bacterial escape into the cytosol of human monocyte derived macrophages (hMDMs; Chong et al., 2008; Santic et al., 2008;

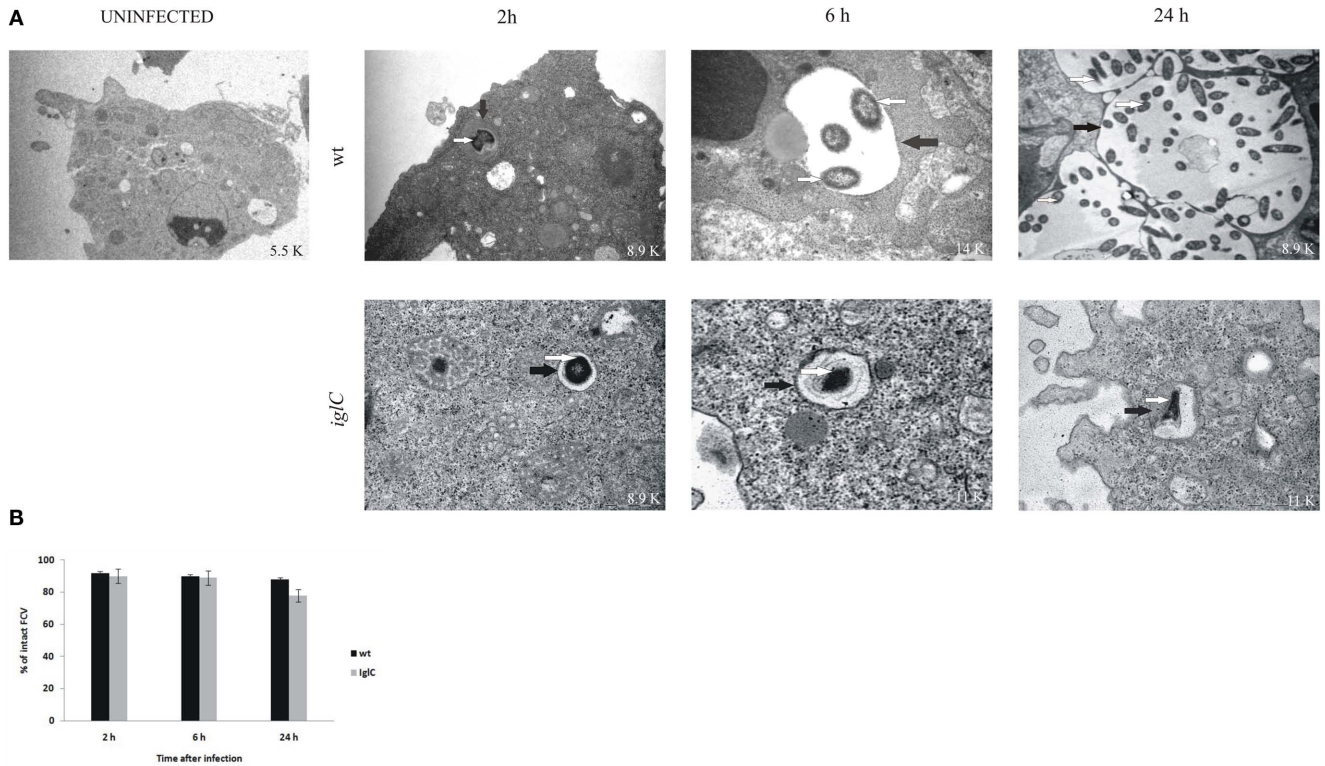

FIGURE 2 | *Francisella novicida* replicates in vacuoles of *H. vermiformis*. **(A)** Representative electron micrographs of *H. vermiformis* infected with wt *F. tularensis* subsp. *novicida* or the *iglC* mutant at 2, 6, and 24 h after infection. Thin black arrows show intact vacuolar membranes and white arrows show bacteria. **(B)** Quantitative analysis of the integrity of vacuolar membranes containing wt *F. novicida* or *iglC* in *H. vermiformis* cells. The percentage of disrupted vacuoles harboring bacteria at different time points.

Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). This transient acidification is essential for subsequent bacterial escape and replication in the macrophage cytosol (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). Based on our ultra-structural studies, we did not observe dramatic changes between 1 and 2 h after infection and 6 and 12 h after infection; therefore we monitored the acidification of the vacuoles at 30 min, 1, and 12 h by using the lysosomotropic agent LysoTracker Red DND-99, which concentrates in acidified vesicles and compartments.

Our results showed that 90% of the wt bacteria in FCVs did not co-localize with the LysoTracker Red DND-99 dye at 30 min, 1, or 12 h post-infection (**Figures 3A,B**). In contrast, many of the *iglC* mutant-containing vacuoles acquired the LysoTracker Red DND-99 dye (~55% colocalization) at 12 h after infection (**Figures 3A,B**). We conclude that *F. tularensis* does not reside transiently or permanently in acidic compartments within *H. vermiformis*, which is distinct from mammalian cells.

DISCUSSION

Epidemiology of *F. tularensis* in various parts of the world is associated with water-borne transmission, which includes mosquitoes and amebae as the potential host reservoirs of the bacteria in water resources (Thelaus et al., 2009; Chong and Celli, 2010; Akimana and Abu Kwaik, 2011; Asare and Abu Kwaik, 2011; Bosio, 2011; Broman et al., 2011; Bröms et al., 2011; Dai et al., 2011; Gavrilin and Wewers, 2011; Jones et al., 2011; Zogaj and Klose, 2011). However, the main aquatic reservoir of the bacterium is still not known, but likely includes mosquitoes and amebae. Recently, it has been shown that *F. novicida* and LVS utilize *A. castellanii* as a natural reservoir (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). In addition, *F. tularensis* LVS and *F. novicida* survive in *A. castellanii* for weeks of infection (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). Surprisingly, *F. novicida* multiplied to a much higher degree in *H. vermiformis* in comparison to what has been found in *A. castellanii* (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). Another explanation is that we used rich 30234 medium compared to the El-Etr et al. (2009) study, where they used High Salt buffer, which does not support *F. novicida* replication. At 24 h after infection there were 10^{10} CFU/ml in *H. vermiformis*. In addition, there was a sudden drop in bacterial numbers, probably due to lysis by amebae. El-Etr et al. (2009) showed that by 30 min post-infection, the bacteria were in spacious vacuoles and continued to replicate until 24 h after infection. They suggested that spacious vacuoles are not lysosomal in nature, and that enclosure within these vacuoles may provide a survival advantage to *F. novicida*, SCHU S4 and other virulent strains of *F. tularensis*. However, it was not examined whether the bacteria replicated within vacuoles, and whether

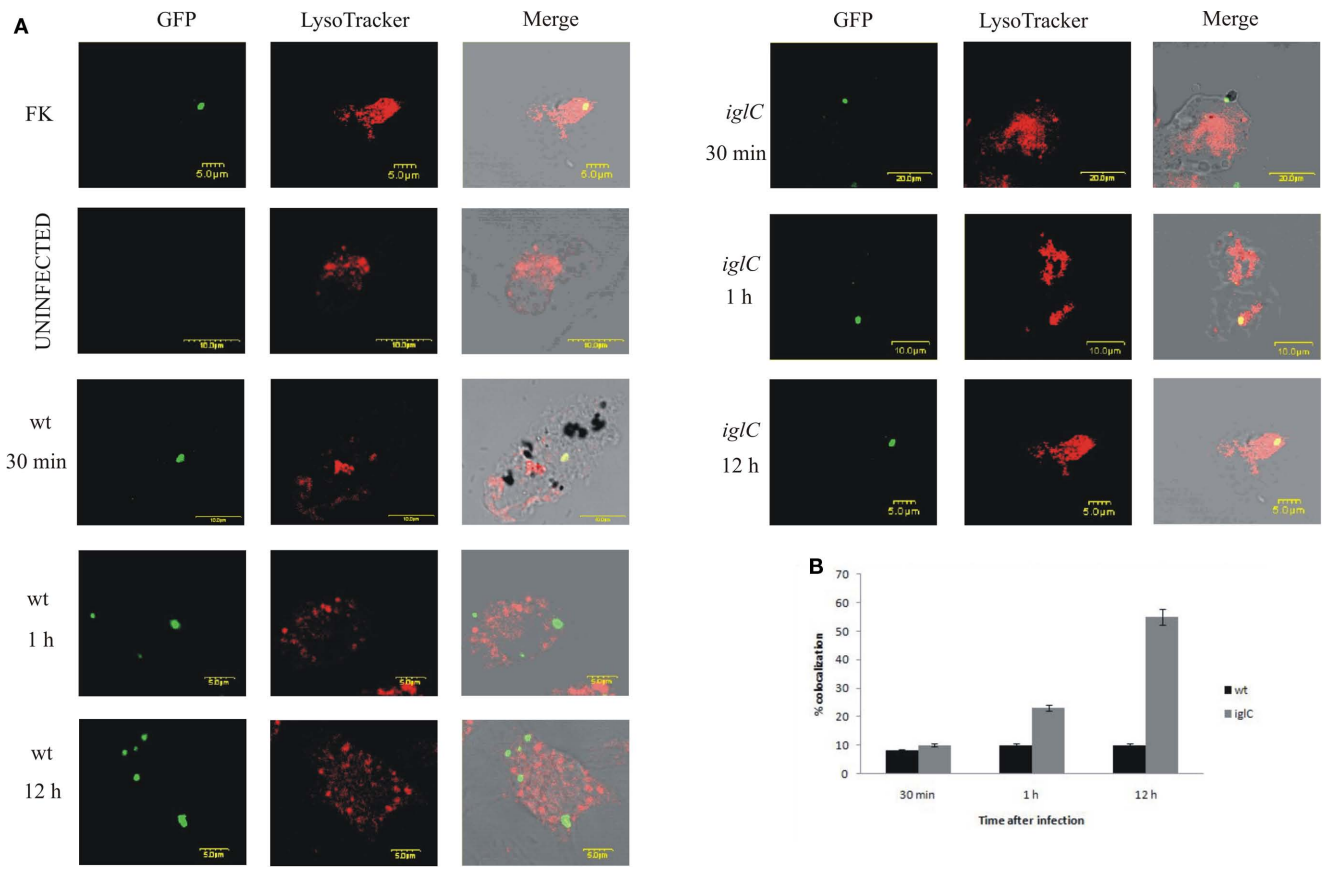

FIGURE 3 | *Francisella novicida* does not reside in acidic compartments within *H. vermiformis* cells. (A) Representative confocal microscopy images of colocalization of FCVs with the LysoTracker dye by the GFP-expressing wt *F. novicida* and *iglC* at 30 min, 1, and 12 h post-infection is shown. Uninfected cells were used as a negative control, while formalin killed bacteria served as a positive control. The images are representatives of 100 infected cells examined from three different cover slips. The results shown are representative of three independent experiments. **(B)** Quantification of colocalization of the LysoTracker DND-99 dye with the FCVs of the bacterium at 30 min, 1, and 12 h post-infection is shown. The results shown are representative of three independent experiments, and error bars represent standard deviations of triplicate samples.

formation of vacuoles was necessary for efficient replication (El-Etr et al., 2009). The long-term survival of pathogenic *F. tularensis* in amebae is dependent on induction of amebal cyst formation. In comparison to *A. castellanii*, *H. vermiformis* did not differentiate into cysts. There have been just a few cysts containing multiplying bacteria, which is very different from *A. castellanii*. The same culture conditions have been used as in the El-Etr et al. (2009) study. It is possible that the encystation process is delayed in *H. vermiformis* in comparison to *A. castellanii* infected with *F. novicida*.

Lauriano et al. (2004) showed that *F. tularensis mglA* and *iglC* mutant strains are not only defective for survival and replication within the macrophage-like cell line, but also within *A. castellanii*. In the present study we examined the interaction of *F. novicida* with *H. vermiformis* and the role of the FPI gene *iglC* in this interaction. The results show, for the first time, that *F. novicida* can survive and grow within *H. vermiformis*. The bacteria replicate and grow in vacuolar structures inside the trophozoites of *H. vermiformis*. Our ultra-structural studies showed that the vacuoles are tight and intact at 2, 6, and 24 h after infection. The *iglC* locus plays an important role in survival and replication of bacteria within *H. vermiformis* cells.

Other previous studies have shown that within mammalian cells, *F. tularensis* resides in acidic vacuoles before escaping to the cytosol where it replicates (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). Recently El-Etr et al. (2009) have shown that ~40% of the FCVs harboring *F. novicida* co-localized with the acidic dye, LysoTracker Red, in *A. castellanii* vacuoles at 2 h after infection. However, our results in *H. vermiformis* show that only 10% of the FCVs harboring *F. novicida* co-localize with the acidic dye at 30 min, 1, and 12 h after infection. The effect of the lysotracker was examined in uninfected cells as well as in *H. vermiformis* cells with formalin killed bacteria. In both cases *H. vermiformis* cells were in the trophozoite stage, and the lysotracker did not affect the physiology of the amebae. Whether the lysotracker affects cyst formation or vice versa has not been examined in this study. El-Etr et al. (2009) also showed that there was little difference between colocalization of the FCVs with LysoTracker Red for the different *Francisella* subsp. (*holarctica*-derived LVS and *novicida*). Our data show that many of the *iglC* mutant-containing FCVs acquired the LysoTracker Red DND-99 dye at a late stage of infection

of *H. vermiformis*, which likely coincided with loss of bacterial viability (**Figure 1**). Similar observations have been described in macrophages (Santic et al., 2005b). Our results clearly show that *F. novicida* does not reside transiently or permanently in acidic compartments within *H. vermiformis* cells after 30 min of initiation of the infection.

CONCLUSION

There are major differences in the life style of *F. tularensis* within various protozoa and macrophages. The bacteria multiply to a higher degree in *H. vermiformis* in comparison to *A. castellanii* cells (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). The formation of cysts is not significant in *H. vermiformis* cells compared to *Acanthamoeba* (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). Our results showed intra-vacuolar replication of *F. novicida* within *H. vermiformis*. This is very different from mammalian cells, where cytosolic location of bacteria is a key component in productive intracellular replication. In *H. vermiformis* the bacteria do not escape from the vacuole into the cytosol to replicate. In contrast, they are enclosed in vacuoles. Despite the major differences between the intracellular lifestyles within *H. vermiformis* and macrophages, the IglC protein is essential for bacterial proliferation in both hosts. It is clear that this protein is essential for intra-vacuolar proliferation in amebae, but is also essential for evasion of lysosomal fusion/and or bacterial escape into the cytosol in macrophages. It is likely that these two events in the evolutionarily distant host cells are due to a secondary effect of the biological function of IglC, which still remains to be determined.

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