INTRODUCTION

Francisella tularensis survives and grows inside many types of cells, including macrophages, and similarly to diseases caused by other intracellular bacteria, the protective host response to tularemia is dependent on cell-mediated immunity (Tärnvik, 1989). The mechanisms behind this protective host response have been fairly well described. A key to the cell-mediated immunity is the ability of immunospecific T cells to enhance the microbicidal capacity of macrophages thereby enabling them to destroy the pathogen. Gamma interferon (IFN-γ) has a key role in this macrophage activation and also for in vivo survival (Anthony et al., 1992; Elkins et al., 1993; Fortier et al., 1992; Leiby et al., 1992; Sjöstedt et al., 1996). In contrast to the numerous studies on the host response to tularemia, the mechanisms behind the successful intracellular parasitism of F. tularensis are not well understood. For example, the bacterium lacks type III and type IV secretion systems utilized by other successful intracellular bacteria, e.g. Shigella, Salmonella, Chlamydia, Brucella and Legionella (Prior et al., 2001). We have demonstrated that F. tularensis shows few signs of stress when growing intracellularly in macrophages (Golovliov et al., 1997), indicating that it is well adapted to the intracellular habitat despite the fact that it is believed to reside in the hostile environment of endosomes (Anthony et al., 1991). Recently, we provided one explanation for this apparent paradox by the demonstration that F. tularensis has the ability to escape from the phagosome of murine and human monocytic cells (Golovliov et al., 2003a). In our studies, a human vaccine strain, F. tularensis LVS, belonging to subspecies holarctica has been utilized. This strain shows high virulence for mice and replicates very rapidly intracellularly and therefore appears to be an appropriate model for understanding the mechanisms of the intracellular survival of F. tularensis. Our findings were corroborated in a recent study showing that a strain of the highly virulent subspecies tularensis escaped into the cytoplasm of human monocytic cells (Clemens et al., 2004).

We have demonstrated that specific mutants can be created in F. tularensis LVS by generating a mutant designated ΔiglC (Golovliov et al., 2003b). The IgIC protein is a 23 kDa protein that we have previously shown to be one of a few proteins prominently up-regulated during intracellular growth of F. tularensis (Golovliov et al., 1997). The protein shows no similarity to other proteins in GenBank and its function is therefore elusive. We also showed that the ΔiglC strain was unable to multiply in the mouse monocytic cell line J774.A1 (Golovliov et al., 2003b). In view of this impaired intracellu-
lar multiplication, we now ask whether the ability of the mutant to escape from the phagosome was affected and if the activation status of the infected cells influences the escape.

**METHODS**

**Bacteria and fatty acid analysis.** The live vaccine strain *F. tularensis* LVS was supplied by the US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD. It was grown on modified Thayer–Martin agar at 37 °C to exponential phase and suspended in PBS before addition to cell cultures. The ΔiglC strain has been previously described and was obtained by allelic replacement of both copies of the *iglC* gene (Golovliov et al., 2003b). For complementation *in trans*, plasmid pKK214 containing the GroEL promoter of *F. tularensis* LVS (Abd et al., 2003) was used. The *iglC* gene was generated by PCR and cloned into the plasmid. The plasmid was introduced by cryotransformation (Pavllov et al., 1996). Expression of *iglC* was confirmed by Western blot analysis.

For determination of the total fatty acid content, fatty acids were extracted from freeze-dried cells according to the method of Bligh & Dyer (1959) and quantified by GC using an HP 5890 series II gas chromatograph with a 7673 autoinjector (Somerville & Dyer (1959) and quantified by GC using an HP 5890 series II gas chromatograph with a 7673 autoinjector (Somerville et al., 1996) (temperature gradient from 40 to 260 °C using a 4 °C min⁻¹ ramp). Individual fatty acids were verified by GC-MS.

**In vitro infection of mammalian cells and assay of intracellular bacterial multiplication.** Peritoneal exudate cells (PECs) were obtained from mice 3 days after intraperitoneal injection of 2 ml 3 % thioglycolate. PECs were washed with DMEM (Gibco-BRL) and resuspended in culture medium consisting of DMEM with 10 % heat-inactivated fetal calf serum. Cells were seeded out at a density of 1 × 10⁶ cells per well in a 24-well tissue culture plate (for enumeration of bacteria) or at a density of 8 × 10⁵ cells per well in a 6 cm tissue culture dish (for electron microscopy). After incubation for 2 h at 37 °C with 5 % CO₂, nonadherent cells were removed by washing with DMEM. After incubation overnight with or without 100 U IFN-γ (Peprotech) at 37 °C in 5 % CO₂, wells were washed and reconstituted with fresh culture medium. To each well, a suspension of bacteria was added and bacterial uptake was allowed to occur for a 2 h period at 37 °C and with 5 % CO₂. After bacterial uptake, the monolayer was washed twice with 0.2 ml 0.1 % sodium deoxycholate in PBS. After addition of 1-8 ml PBS, 100 µl portions of each lysate, serially diluted in PBS, were plated on modified Thayer–Martin agar for determination of viable counts.

**Electron microscopy.** To evaluate the intracellular localization of *F. tularensis* LVS by transmission electron microscopy, cells growing as monolayers in cell culture dishes with a diameter of 60 mm were infected following the aforementioned protocol. To visually estimate the content and behaviour of the bacteria in the infected cells, short ribbons of ultrathin sections (85–90 nm thickness) were collected on Formvar/carbon-coated single-hole grids. The diameter of the hole (1 mm) allowed us to analyse a series of six to seven consecutive sections of one cell, representing 500–600 nm total thickness. To determine the intracellular localization of bacteria, 200 nonconsecutive sections containing readily identifiable bacteria were counted.

**Statistical analysis.** Student's *t*-test and 2-sample test for equality of proportions with continuity correction were used.

**RESULTS**

**Growth of *F. tularensis* strains in PECs**

The intracellular bacterial numbers were determined in PECs infected with the wild-type *F. tularensis* LVS or the ΔiglC mutant. Similarly to a previous study (Golovliov et al., 2003b), we observed that the ΔiglC strain was unable to multiply in monocytic cells. However, there was only a slight but significant decrease over time, 0.8 log₁₀ (P < 0.0002), until 18 h (Table 1). In contrast, *F. tularensis* LVS replicated and the bacterial numbers increased 1.6 log₁₀ within 18 h (Table 1). Addition of IFN-γ to cultures resulted in control of the *F. tularensis* LVS infection as well and bacterial numbers decreased 1.2 log₁₀ (P < 0.0002 vs c.f.u. at 0 h) within 18 h (Table 1). The corresponding decrease of ΔiglC bacterial numbers was already highly significant within 5 h, 1.1 log₁₀ (P < 0.00001), and after 18 h, the decrease was 3.8 log₁₀ (Table 1).

**Intracellular localization of ΔiglC as determined by transmission electron microscopy**

Previously, we demonstrated by electron microscopy that *F. tularensis* LVS was able to escape from the phagosome of mouse and human monocytic cells (Golovliov et al., 2003a)

| Table 1. Growth of *F. tularensis* LVS and ΔiglC in PECs |
|---|---|---|---|
| **Time (h)** | **F. tularensis LVS** | **ΔiglC** | **F. tularensis LVS** | **ΔiglC** |
| 0 | 6.02 ± 0.05 | 6.10 ± 0.05 | 6.33 ± 0.03 | 6.15 ± 0.05 |
| 2 | 6.10 ± 0.03 | 5.86 ± 0.05 | 6.17 ± 0.02 | 5.95 ± 0.08 |
| 5 | 6.39 ± 0.02 | 5.79 ± 0.02 | 6.16 ± 0.01 | 5.07 ± 0.02 |
| 18 | 7.73 ± 0.06 | 4.93 ± 0.13 | 5.52 ± 0.10 | 2.33 ± 0.31 |
and the majority of bacteria had a cytoplasmic localization after 2 h. To this end, we determined the intracellular localization of the ΔiglC bacteria at this time point. The microscopic analysis revealed that 97% of the F. tularensis LVS bacteria were localized freely in the cytoplasm or a few were in phagosomes without an intact limiting membrane (Fig. 1a). By contrast, >99% of the ΔiglC bacteria were localized in phagosomes with intact membranes (Fig. 1b). When the ΔiglC strain was complemented with the iglC gene, the majority (60%) of the bacteria were found in the cytoplasm (Fig. 1c).

Effect of IFN-γ treatment on the intracellular localization of F. tularensis

In view of the bactericidal effect resulting from IFN-γ activation of the PECs, we asked whether this treatment influenced the intracellular localization of bacteria. As expected, >99% of the bacteria of the ΔiglC strain were found in phagosomes with intact membranes (not shown). In contrast, the majority of F. tularensis LVS bacteria were still found in the peripheral parts of the cytoplasm and a mean of one to two bacteria per section was observed. Some bacteria were present in vacuoles that were only partially surrounded by the limiting membrane (14%) and most were free in the cytoplasm (66%). However, the number of bacteria that were clearly seen within phagosomes with intact membranes (Fig. 2) was higher in treated cells than in cells without IFN-γ treatment, 20 versus 3% (P < 0.0001). Most of the intracellular F. tularensis LVS and ΔiglC bacteria exhibited an intact morphology (Figs 1 and 2).

Analysis of the lipid composition of F. tularensis LVS and the ΔiglC strain

It has been observed in previous studies that F. tularensis LVS-infected cells contain intra- and extra-phagosomal vesicles, presumably derived from surface-localized material.

![Fig. 1. Transmission electron micrographs of adherent mouse PECs infected with F. tularensis LVS or ΔiglC for 2 h. (a) Two F. tularensis LVS bacteria. One is located in the cytoplasm without any obvious phagosomal membrane surrounding it (marked with an X); the other bacterium is enclosed by a membrane with an obvious defect (the openings of the membrane are marked with arrowheads). Bar, 0.24 μm. (b) The iglC mutant is located in a phagosome with an intact membrane. Bar, 0.15 μm. (c) The complemented ΔiglC mutant is able to escape from the phagosome and is located in the cytoplasm without any surrounding membrane. Bar, 0.24 μm.](image-url)
released by the bacterium (Anthony et al., 1991; Golovliov et al., 2003a). We now asked whether there were changes in the composition of the ΔiglC mutant that could explain its lack of phagosomal escape. To this end, we investigated the total lipid composition of the two strains. The total lipid composition of *F. tularensis* LVS was similar to that reported previously (Anderson & Bhatti, 1986) and no significant differences were found between the two strains (data not shown). Thus there appear to be no changes in the lipid composition of the ΔiglC strain that could explain its lack of escape from the phagosome.

**DISCUSSION**

The strategy behind the successful intracellular parasitism of *F. tularensis* has been elusive but our and other recent work has revealed that the bacterium is capable of escaping from the phagosome of monocytic cells (Golovliov et al., 2003a; Clemens et al., 2004). This is a strategy employed by other intracellular bacterial pathogens such as *Listeria monocytogenes, Bacillus anthracis, Rickettsia* spp. and *Shigella flexneri* (Goebel & Kuhn, 2000). However, there is no evidence that the two major subspecies, *tularensis* and *holarctica*, of *F. tularensis* possess any haemolysins (Lai et al., 2003), which are utilized by *L. monocytogenes* and *B. anthracis* to escape from the phagosome (Dixon et al., 2000; Klichko et al., 2003; Portnoy et al., 2002). However, another subspecies of *F. tularensis, Francisella novicida*, possesses a haemolysin which is antigenically related to HlyA of *Escherichia coli* (Lai et al., 2003). In a previous publication we demonstrated that surface-localized material of *F. tularensis* was released intracellularly in the form of vesicles (Golovliov et al., 2003a). These vesicles were found to be associated with the phagosomal membrane and degradation of the membrane resulted. The strategy of *F. tularensis* appears to be unusual for intracellular bacterial pathogens. *Mycobacterium tuberculosis*, although confined to the phago-lysosome, is another example of an intracellular organism that releases surface material during its intracellular growth (Beatty et al., 2000). It secretes abundant amounts of predominantly lipid-containing material that profoundly affects the mammalian cell (Beatty et al., 2000). To this end, we asked whether there are differences in the fatty acid composition of *F. tularensis* LVS and ΔiglC. The analysis of total fatty acids, however, indicated no differences between the two strains. A comparative analysis in the subspecies *F. novicida* revealed no changes in the protein pattern of a ΔiglC strain besides the lack of the 23 kDa protein (Lauriano et al., 2004). Thus the findings do not give any clues that explain the function of IgIC but exclude changes in total lipid composition or regulatory effects of IgIC that affect the expression of other bacterial proteins. When we analysed the properties of the complemented ΔiglC strain, the number of bacteria free in the cytoplasm was lower than in the case of *F. tularensis* LVS (60 vs 97%). This shows that the complementation, which is dependent on expression of IgIC from a plasmid containing the GroEL promoter of *F. tularensis*, does not fully restore the wild-type phenotype. Presumably this is due to a lack of up-regulation of the plasmid expression during the intracellular phase. Nevertheless, there was a highly significant difference between bacteria of the ΔiglC strain and of the complemented strain in their ability to escape from the phagosome (< 1 vs 60 %, respectively).
The crucial role of IFN-γ for the control and eradication of tularemia resembles that of many other intracellular infections (Shtrichman & Samuel, 2001). The specific effects of IFN-γ for treatment of the cidal macrophages have been very thoroughly studied for experimental listeriosis (Prada-Delgado et al., 2001). L. monocytogenes is retained within the vacuole after IFN-γ treatment by a mechanism dependent on both reactive oxygen and nitrogen intermediates (Myers et al., 2003). A key action has been attributed to Rab5a, which causes remodelling of the phagosomal environment, affects the GTPase Rac2 and subsequently increases the NADPH oxidase activity (Prada-Delgado et al., 2001). Although we also observed an increase in the number of F. tularensis in the phagosome of IFN-γ-activated cells, the majority of bacteria still escaped. This finding appears paradoxical in view of the control and eradication that is afforded by the IFN-γ treatment and is in contrast to the findings on L. monocytogenes. Normally, it is assumed that the escape of an intracellular organism into the cytoplasm will shield it from bactericidal mechanisms (Goebel & Kuhn, 2000). However, in view of the results, we propose that the majority of the F. tularensis bacteria that escape are incapable of intracellular multiplication. It is possible that the effects of reactive nitrogen and oxidative intermediates and other effectors active in a phagolysosome of IFN-γ-activated cells will be sufficient for inducing irreversible damage during the intra-phagosomal phase that prevents subsequent bacterial multiplication but not the mechanisms required for the escape. Even though they gain access to the cytoplasm, they may be metabolically defective and eventually succumb as demonstrated by the significant decrease of bacterial numbers within 18 h. Still, the escape from the phagosome appears to be critical for avoiding rapid effects of the IFN-γ activation as evidenced by the significant killing of the mutant within 5 h.

The intracellular fate of ΔiglC was distinct from the parent strain, F. tularensis LVS. Whereas the latter is attenuated in mice when injected via the intradermal route, it is highly virulent by other routes and also capable of rapid multiplication in mouse and human monocytic cells (Golovliov et al., 2003a). In contrast, we found that the iglC mutant bacteria were incapable of multiplying intracellularly, and in IFN-γ-treated cells, almost all ΔiglC bacteria were killed within 18 h. We believe that one important explanation for the impaired ability of ΔiglC to multiply intracellularly is its inability to escape from the phagosome. However, we cannot conclude that this is the only explanation for the control and killing since we have demonstrated that ΔiglC, in contrast to F. tularensis LVS, lacks the ability to inhibit TLR-mediated activation of intracellular pathways and secretion of tumour necrosis factor alpha (Telepnev et al., 2003). Thus it is possible that ΔiglC lacks several traits of F. tularensis LVS that are important for intracellular survival and multiplication. The exact role of IgIC is still elusive and understanding this function will be an important first step in understanding the intracellular survival mechanisms of F. tularensis in general.

ACKNOWLEDGEMENTS
Grant support was obtained from the Swedish Medical Research Council and the Medical Faculty, Umeå University, Umeå, Sweden.

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