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Francisella noatunensis subsp. *noatunensis* invades, survives and replicates in Atlantic cod cells

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ABSTRACT: Systemic infection caused by the facultative intracellular bacterium *Francisella noatunensis* subsp. *noatunensis* remains a disease threat to Atlantic cod *Gadus morhua* L. Future prophylactics could benefit from better knowledge on how the bacterium invades, survives and establishes infection in its host cells. Here, facilitated by the use of a gentamicin protection assay, this was studied in primary monocyte/macrophage cultures and an epithelial-like cell line derived from Atlantic cod larvae (ACL cells). The results showed that *F. noatunensis* subsp. *noatunensis* is able to invade primary monocyte/macrophages, and that the actin-polymerisation inhibitor cytochalasin D blocked internalisation, demonstrating that the invasion is mediated through phagocytosis. Interferon gamma (IFNγ) treatment of cod macrophages prior to infection enhanced bacterial invasion, potentially by stimulating macrophage activation in an early step in host defence against *F. noatunensis* subsp. *noatunensis* infections. We measured a rapid drop of the initial high levels of internalised bacteria in macrophages, indicating the presence and action of a cellular immune defence mechanism before intracellular bacterial replication took place. Low levels of bacterial internalisation and replication were detected in the epithelial-like ACL cells. The capacity of *F. noatunensis* subsp. *noatunensis* to enter, survive and even replicate within an epithelial cell line may play an important role in its ability to infect live fish and transverse epithelial barriers to reach the bacterium's main target cells — the macrophage.

KEY WORDS: Bacterial invasion · Gentamicin protection assay · Macrophages · Intracellular bacteria · IFNγ

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INTRODUCTION

The intracellular bacteria belonging to the genus *Francisella* have in recent years emerged as severe pathogens of both wild and farmed fish species worldwide, demonstrating that *Francisella* infections have a broader geographical distribution than previously recognised (Kamaishi et al. 2005, Nylund et al. 2006, Olsen et al. 2006, Ostland et al. 2006, Birkbeck et al. 2007, Hsieh et al. 2007, Mauel et al. 2007, Soto et al. 2010). At the same time, there are no commercial vaccines available to limit the spread of francisellosis in aquaculture. Bacterial strains isolated from warm water fish species are mainly *Francisella noatunensis* subsp. *orientalis*, while isolates from cold-water species belong to *Francisella noatunensis* subsp. *noatunensis* (*F.n.n.*). Francisellosis is a systemic granulomatous inflammatory disease and is associated with high morbidity causing variable mortality levels (reviewed in Colquhoun & Duodu 2011). The

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absence of effective treatment options has created a critical need for increased knowledge about host–pathogen interactions. New knowledge concerning the pathogenesis of this bacterium could identify future targets for therapeutic intervention and vaccine development to combat the disease.

Infections with *F.n.n.* predispose for high levels of granulomas in visceral organs in Atlantic cod *Gadus morhua L.* and particularly in the hematopoietic organs (Olsen et al. 2006). Bacterial colonisation has been found in or around phagocytic cells associated with epithelial cells in diseased cod (Nylund et al. 2006) and macrophages are a target for the intracellular infection with *F.n.n.* in cod (Bakkemo et al. 2011, Furevik et al. 2011, Vestvik et al. 2013). During the initial phase of infection, *F.n.n.* is found enclosed within a tight phagosomal membrane, while at later stages of the infection an apparently disintegrated membrane surrounds bacteria in cytosol (Bakkemo et al. 2011). It was also observed that intracellular *F.n.n.* releases bacterial-derived vesicles, probably as a virulence mechanism to escape phagosomes (Bakkemo et al. 2011), and these vesicles have shown promise as a vaccine candidate in a zebrafish model (Brudal et al. 2015).

The abilities to attach to, invade and subsequently replicate in phagocytic cells are essential to the virulence of intracellular pathogens. Phagocytosis in mammals is triggered by interactions between membrane receptors on the phagocyte surface and the bacteria or opsonin-coated bacteria (Aderem & Underhill 1999). The receptor for the Fc domain of immunoglobulin G (FcR), the receptor for complement factor iC3b (C3bR; a cleavage product of complement 3 receptor) and the scavenger receptor A (SR-A) are all involved in the uptake of opsonised bacteria (Balagopal et al. 2006, Chong & Celli 2010). Mannose receptor (MR) and surface-exposed nucleolin seems to mediate phagocytosis of non-opsonised bacteria (Santic et al. 2010).

The invasion of the human pathogen *Francisella tularensis* into macrophages is mediated by looping phagocytosis after the transduction of an invasion signal to induce rearrangements of the host actin cytoskeleton (Clemens et al. 2005). Inside the macrophages, the bacteria are able to degrade the phagosomal membrane and escape to cytosol for replication (Chong & Celli 2010). Following replication in cytosol, *F. tularensis* has a post-replication stage that involves re-entering the endocytic compartment through an autophagy-mediated process (Checroun et al. 2006). Studies have shown that the route of entry could impair intracellular survival and replication by limiting phagosomal escape and intracellular replication (Geier & Celli 2011, Jones et al. 2012).

Paradoxically, intracellular bacteria have created favourable strategies for replication inside professional phagocytic cells whose primary role is to provide the first line of host defence by recognising bacteria and by performing phagocytosis (Clemens & Horwitz 2007). The innate immune system senses invasion of bacteria through recognition of characteristic pathogen-associated molecular patterns by Toll-like receptors and nucleotide-binding oligomerisation domain-like receptors (reviewed in Akira et al. 2006, Delbridge & O'Riordan 2007). We previously proposed that *F.n.n.* evade the innate immune response in cod macrophages by inducing an anti-inflammatory response (Bakkemo et al. 2011). Activation of cell mediated immune response has been assigned a key role in the eradication of intracellular infections and the different T cells play distinct and complementary functions in immunity. The cytokine interferon gamma (IFNγ) has an important dual role by directly activating macrophages for increased killing of intracellular bacteria and polarisation of T cells (Schroder et al. 2004). There are several reports of IFNγ mediated killing of intracellular *F. tularensis* (Fortier et al. 1992, Lindgren et al. 2004, Edwards et al. 2010). As a survival mechanism, *F. tularensis* has developed strategies to delay, bypass and/or suppress host immune responses (reviewed in Sjöstedt 2006, Gillette et al. 2014).

In the present work, we adapted the use of an *F.n.n.* susceptible monocyte/macrophage model (Bakkemo et al. 2011) for studies on internalisation, intracellular survival and replication of *F.n.n.,* the francisellosis-causing agent in Atlantic cod. This model was employed to gain more insight into the intracellular lifestyle of *F.n.n.* in monocyte/macrophages and a cod cell line derived from Atlantic cod larvae (ACL cells) (Jensen et al. 2013). Bacterial invasion studies in macrophages were carried out at different temperatures or in the presence of recombinant IFNγ to examine the effect of these parameters, the first of which has direct relevance to seasonal changes of conditions in nature.

MATERIALS AND METHODS

Bacterial strain and growth conditions

The *Francisella noatunensis* subsp. *noatunensis* (NCIMB 14265) strain used in this study was isolated from diseased farmed cod (Olsen et al. 2006). Frozen

stock culture stored at −80°C in broth medium containing 20% glycerol (v/v) was thawed on Cystine Heart Agar (Difco®) containing 5% human blood (CHAB) and incubated for 10 d at room temperature. Bacteria inoculum was further cultured in Eugon Broth (Difco) supplemented with 0.2 M $FeCl_3$ and incubated with shaking (120 rpm; KS 501digital, IKA) for 24 h at 20°C. Bacteria in exponential growth with optical density (OD_{600nm}) ranging from 1.4 to 2.2 were adjusted to approximately 5×10^8 colony forming units (CFU) ml^{-1} in Leibovitz (L) cell medium 15+ (L-15 [PAA Laboratories] buffered with 25 mM HEPES and supplemented with 2 mM L-glutamine, 13.7 mM NaCl, 1.8 mM glucose, 4.2 mM $NaHCO_3$) and 5% fetal bovine serum [FBS Gold; PAA Laboratories]). The L-15+ medium was adjusted to 360 mOsmol kg^{-1} using a semi-micro osmometer (Knauer).

Isolation of head kidney monocytes/macrophages

Atlantic cod (approx. 500 to 1300 g) were obtained from the Aquaculture Research Station in Tromsø, Norway. Fish were kept in circular 900 l tanks filled with seawater (3.4%) at natural seawater temperature (3 to 9°C) in a 24 h light regime and fed ad libitum with commercial feed (Amber Neptun). Monocytes/macrophages were isolated from head kidney using established protocols (Steiro et al. 1998, Seppola et al. 2007, Bakkemo et al. 2011) with minor modifications. Fish were rapidly killed by cranial concussion and blood was removed by bleeding the fish from the caudal vein. Head kidneys were aseptically removed and transferred to L-15++ (L-15+ with antibiotics; 20 U ml^{-1} penicillin and 20 µg ml^{-1} streptomycin) supplemented with 10 U ml^{-1} heparin (LEO Pharma AS) and kept on ice. Head kidneys were homogenised using the gentleMACS™ dissociator (Miltenyi Biotech), minced through a 100 µm nylon Falcon cell strainer (BD Biosciences) and diluted in 30 ml L-15++ with 10 U ml^{-1} heparin. The cell suspensions were placed on discontinuous 28/45% Percoll gradients (GE Healthcare) and separated by centrifugation (400 × *g*, for 40 min) at 4°C. The interface, enriched for monocytes/macrophages, was washed twice in 50 ml L-15++ followed by centrifugation (300 × *g*, for 10 min) at 4°C. In the last washing step, cells were diluted in L-15++ with 2% FBS Gold. Cells were seeded at a density of 5 to 8×10^6 cells $well^{-1}$ in 24-well culture plates (Nunc). Then the cells were allowed to adhere to plastic for 16 to 24 h at 12°C and washed twice with L-15+ to remove non-adherent cells and residual antibiotics before use.

ACL cells

ACL cells (Jensen et al. 2013) at passage number 50 were cultured in minimum essential medium (MEM; PAA Laboratories) supplemented with 10% (v/v) FBS Gold and incubated in 5% CO_2 at 20°C. ACL cells (3×10^5 cells $well^{-1}$) were cultured in 24-well plates (Nunc) for 2 d without antibiotics prior to infection.

Sensitivity of *F.n.n.* to gentamicin

The sensitivity of *F.n.n.* to gentamicin was tested in cell culture media for macrophages (L-15 with 5% FBS Gold) and ACL cells (MEM with 10% FBS Gold) at 12 and 20°C, respectively. Bacteria (10^5) were diluted in culture media and subjected to different gentamicin concentrations (100, 50, 25, 12.5, 6.25 and 3.125 µg ml^{-1}) for 1 h. The culture media were serially diluted and spread onto CHAB plates to determine CFU ml^{-1}.

Gentamicin protection assay

Isolated monocytes/macrophages (n = 4 to 6 fish, diluted in duplicate wells) or ACL cells (n = 4 parallel wells) were infected with 1 ml *F.n.n.* (macrophages: diluted in L-15+ with 5% FBS Gold; ACL cells: MEM with 10% FBS Gold) at a multiplicity of infection (MOI) of 50 to 100. Uninfected cells were incubated with cell media/FBS Gold alone, and otherwise treated similarly as infected cells. The cell culture plates were centrifuged (500 × *g*, 5 min) to enhance initial bacterial contact, and incubated for 2 h at 12 or 20°C. After infection, cells were washed 3 times in L-15+ (or MEM) and pulse-treated for 1 h with 50 µg ml^{-1} gentamicin (Sigma-Aldrich) to kill extracellular bacteria. The cells were then washed 3 times to remove gentamicin and, depending on the experiment, the cells were either lysed immediately to determine the invasion rate or incubated further for replication studies. Monocytes/macrophages were lysed by adding 100 µl 0.1% Triton X-100 (22°C; Sigma-Aldrich) diluted in phosphate-buffered saline (PBS; Gibco). After 2 min incubation with the detergent, 900 µl PBS were added, mixed and tenfold serial diluted in 0.9% NaCl. The toxic effect of Triton X-100 was carefully validated. Serially diluted lysates (10^{-2} and 10^{-3}) were plated on duplicate CHAB plates to determine viable counts (CFU ml^{-1}) after incubation for 7 to 8 d at 20°C. Cell culture media prior to cell lysis were

also spread on agar plates for evaluating the bactericidal effect of the gentamicin treatment.

Treatment of macrophages with cytochalasin D

Cytochalasin D, an inhibitor of actin polymerisation, was used to examine if actin polymerisation influences *F.n.n.* internalisation of monocytes/macrophages. Isolated monocytes/macrophages (n = 5 fish, diluted in duplicate wells) were pre-treated with 0.5 or 5 µg ml^{-1} cytochalasin D (Invitrogen) for 30 min at 12°C. Monocytes/macrophages were infected with *F.n.n.* using the gentamicin protection assay for determination of the invasion rate as described above. Control macrophages were not treated with cytochalasin D, but otherwise treated similarly. Cytochalasin D showed no negative effect on cell viability of macrophages or on the growth rate of *F.n.n.*

Activation of macrophages by recombinant IFNγ

A recombinant (r) cod IFNγ was produced as described by Seppola et al. (2016). The purified protein was resolved by 4 to 20% SDS-PAGE and then visualised by Coomassie Blue staining. Protein concentration was determined by comparing the protein band density with a standard protein. Western blot analysis was performed to confirm the identity of the rIFNγ using anti-His antibody (Genscript). The endotoxin level was determined by the limulus amebocyte lysate method.

To study the gene expression induced by rIFNγ, isolated monocytes/macrophages (n = 4 fish, diluted in duplicate wells) were treated for 24 h with 1000 ng ml^{-1} rIFNγ and rCtr (diluted similarly). Cells were harvested in 1× lysis buffer (Applied Biosystems) and RNA was isolated for expression analysis of the IFNγ-inducible protein IRF1 (Grayfer et al. 2010, Sun et al. 2011) as described below.

For studies of the direct effect of IFNγ, monocyte/macrophage cultures (n = 4 fish, diluted in duplicate wells) were pre-treated for 24 h with 1000 ng ml^{-1} IFNγ, rCtr or left untreated (Ctr). Macrophages were then infected with *F.n.n.* using the gentamicin protection assay as described above. Cell cultures were incubated for different time points (6 and 10 h) before lysis, and the lysate were plated on CHAB as described above. For bacterial quantification using real-time PCR analysis, monocytes/macrophages (n = 4 fish, diluted in duplicate wells) were infected and treated as previously described. Instead of CFU enumeration, cells were lysed in 1× lysis buffer and all samples were stored at −80°C for further gene expression analysis as described below.

Bacterial uptake at different temperatures

The ability of *F.n.n.* to enter monocytes/macrophages at different temperatures was studied. Isolated monocytes/macrophages (n = 6 fish, diluted in duplicate wells) were infected with *F.n.n.* using the described gentamicin protection assay and incubated at 3 different temperatures (12, 16 and 20°C). The invasion rate was determined in CFU ml^{-1}.

Intracellular replication assay

Monocytes/macrophages (n = 4 fish, diluted in duplicate wells) and ACL cells (n = 4 parallel wells) were infected with *F.n.n.* using the described gentamicin protection assay for determination of bacterial invasion. After infection, the cells were pulse-treated for 1 h with 50 µg ml^{-1} gentamicin and washed 3 times before further incubation in cell media. The cell media was supplemented with 5% FBS Gold (monocytes/macrophages) or 10% FBS Gold (ACL cells). To quantify the number of viable bacteria at succeeding time points, infected cells were either subjected to a second pulse-treatment with gentamicin (50 µg ml^{-1} for 1 h) before lysis or incubated with gentamicin (5 µg ml^{-1}) in the cell media during the experiment. Excessive gentamicin was removed before lysis by 3 washing steps to ensure that antibiotics did not impair bacterial quantification. CFU ml^{-1} was determined as described above. To evaluate the amount of cell death or detachment during the experiment, single-cell suspensions were enumerated by an automated cell counter (Scepter^{TM} 2.0; Millipore) in duplicate wells for each individual at all time points.

For bacterial quantification using real-time PCR analysis, monocytes/macrophages (n = 4 fish, diluted in duplicate wells) were infected and treated as previously described. Instead of CFU enumeration, cells were lysed in 1× lysis buffer at sequential time points (4, 8, 24, 48 and 72 h). All samples were stored at −80°C for further gene expression analysis as described below.

Gene expression studies following infection of macrophages

Total RNA was isolated using an ABI Prism 6100 nucleic acid prep station (Applied Biosystems) with the

recommended on-column DNAse treatment. Reverse transcription was performed using the high capacity RNA to cDNA master mix or high capacity cDNA reverse transcription kit (Applied Biosystems) with the addition of 2.5 mM poly dT primer (Promega). The reaction conditions were 25°C (5 to 10 min), 42°C (60 to 120 min) and 85°C (5 min) and the cDNA was diluted 1:30 in nuclease free water (Ambion) for further use in quantitative real-time PCR. The absence of genomic DNA was verified by subjecting RNA samples to real-time PCR analyses without prior cDNA synthesis. Real-time PCR was performed in duplicates in 384 well plates using the 7900HT Fast real-time PCR system and Power SYBR green PCR master mix according to the manufacturers description (Applied Biosystems). Real-time PCR primers for the *F.n.n.* bacterial outer membrane protein FopA and Fc50 targeting the 16S rRNA gene (Ottem et al. 2008), IRF1 (Jensen et al. 2013) and elongation factor 1α (eF1α) (Seppola et al. 2008) have been reported earlier. Gene expression data were analysed with the SDS 2.3 software (Applied Biosystems) and exported to Microsoft Excel for further analysis. Quantification of relative gene expression levels were performed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). The expression of target genes was normalised to eF1α and the expression level was calibrated to non-stimulated control cells. From relative quantification values obtained from cells, the mean quantity ± SEM was calculated.

Statistical analysis

Statistical analyses were performed using Prism 5 (GraphPad Software). The results were analysed with 1-way ANOVA where data followed the Gaussian distribution, and difference between groups was tested using Tukey's multiple comparison test. Student's *t*-test and Kruskal-Wallis were used for data that were non-parametric. The data are presented as mean ± SEM; $p < 0.05$ was considered significant.

RESULTS

Adjusting the gentamicin concentration in the protection assay

Efficient removal of extracellular bacteria is a perquisite prior to quantification of intracellular bacteria and can be accomplished by the use of the gentamicin protection assay. The *Francisella noatunensis* subsp. *noatunensis* strain used in this study replicates in cell culture media (MEM and L-15) at both 12 and 20°C, but at a considerably higher rate (~1 $\log_{10}$) at 20°C. The gentamicin concentration necessary to kill extracellular *F.n.n.* was thus evaluated to obtain reliable quantification of intracellular bacteria in the 2 Atlantic cod cell types. The ACL cells are adapted to cultivation at 20°C, while the primary cod macrophages prefer low temperatures with an optimum between 8 and 12°C (Steiro et al. 1998). Subsequently, the bactericidal effect of gentamicin (3.125 to 100 µg ml^{-1}) was tested at 12°C in L-15 and at 20°C in MEM. At 12°C, viable *F.n.n.* could not be recovered from bacterial cultures containing gentamicin concentrations of 50 µg ml^{-1} or higher (Fig. 1). This confirms that a pulse treatment with 50 µg ml^{-1} gentamicin for 1 h is sufficient to kill all extracellular bacteria at this low temperature. Gentamicin did not completely kill *F.n.n.* in MEM at 20°C at any of the tested concentrations. However, only 0.2% of the initial bacteria survived a gentamicin concentration of 50 µg ml^{-1}.

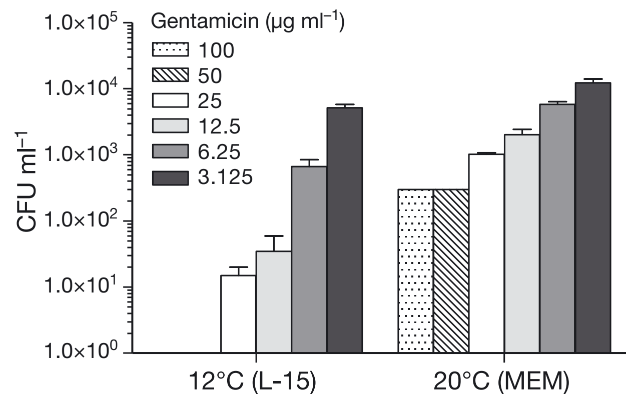

Fig. 1. Sensitivity of *Francisella noatunensis* subsp. *noatunensis* (*F.n.n.*) to gentamicin: 10^5 bacteria were incubated in cell culture medium resembling the conditions for macrophages (Leibovitz cell medium 15+ [L-15] with 5% Fetal Bovine Serum Gold [FBS] at 12°C) and Atlantic cod larvae (ACL) cells (minimum essential medium [MEM] with 10% FBS Gold in 5% CO_2 at 20°C), with various concentrations of gentamicin for 1 h followed by quantification of CFU ml^{-1}. Results are mean (+SEM) CFU ml^{-1}

For invasion studies of macrophages, a pulse treatment with 50 µg ml^{-1} was therefore used before lysis and counting CFU of internalised bacteria. ACL cells, in addition to the pulse treatment, also received a low dose of gentamicin (5 µg ml^{-1}) in the culture medium after infection due to the higher culturing temperature and the risk of bacterial growth and reinfection. The absence of extracellular bacteria in cell culture media was verified by CFU counting before lysis of infected ACL cells.

Bacterial invasion in macrophages

Invasion assays revealed that *F.n.n.* was able to enter cod monocytes/macrophages *in vitro*. When infecting cells with 50 to 100 MOI of *F.n.n.*, a high invasion rate (~10^5 CFU) was obtained 2 h post-infection (Fig. 2). To verify that *F.n.n.* were internalised by phagocytosis, the actin microfilament inhibitor cytochalasin D (Goddette & Frieden 1986) was used. Monocytes/macrophages were treated with 2 doses of cytochalasin D (0.5 and 5 µg ml^{-1}) prior to and during infection, followed by CFU counting. Cytochalasin D at 0.5 µg ml^{-1} reduced the amount of retrieved intracellular bacteria by more than 50% (Fig. 2). A near complete inhibition of the *F.n.n.* uptake and a recovery of less than 100 CFU ml^{-1} was seen when the cytochalasin D dose was increased to 5 µg ml^{-1}. This confirms that the obtained bacterial counts in cod macrophages were dependent on the uptake of bacteria by phagocytosis into the intracellular compartment.

Effect of IFNγ on bacterial internalisation

A previously produced recombinant cod IFNγ (Seppola et al. 2016) was used to study macrophage activation and bacterial internalisation. Fig. 3A shows the His-tagged 23.3 kDa recombinant protein analysed by SDS-PAGE and Western blotting. Monocytes/macrophages were treated with rIFNγ or with a control extract from *E. coli* transformed with an empty vector (i.e. rCtr), which was diluted similarly as rIFNγ. Real-time PCR analysis showed that rIFNγ stimulation induced gene expression of the rIFNγ-inducible IRF1, while the rCtr did not alter the expression of IRF1 (Fig. 3B), which verifies the production of a functionally active cod IFNγ.

To study the effect of recombinant IFNγ on *F.n.n.* infection, monocytes/macrophages were treated prior to infection with rIFNγ and rCtr. Pre-treatment of cells with rIFNγ resulted in a more than 10-fold increase in *F.n.n.* invasion 6 h after infection compared to rCtr-treated cells and the non-treated control cells (Fig. 3C). Ten hours after infection, the amount of viable intracellular bacteria was reduced 20-fold in the cells pre-treated with IFNγ, indicating activation of bactericidal activity in macrophages. Results were also confirmed using real-time PCR analysis (Fig. 3D). Experiments including later sampling points showed that 72 h after infection, viable bacteria could still be recovered from pre-treated cells, indicating that IFNγ activation of macrophages does not completely eradicate intracellular bacteria (data not shown).

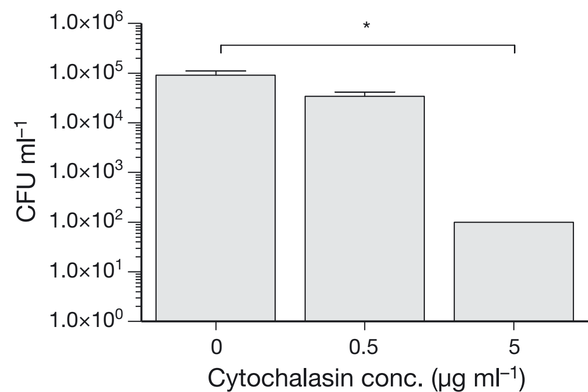

Fig. 2. Internalisation of *Francisella noatunensis* subsp. *noatunensis* (*F.n.n.*) by cod monocytes/macrophages treated with cytochalasin D (n = 5 fish) and infected with *F.n.n.* (see 'Materials and methods: Gentamicin protection assay'). Results are mean (+SEM) CFU ml^{-1}; a repetition of the experiment produced similar results. *Significant difference at $p < 0.05$ (Kruskal-Wallis with Dunn's Multiple Comparison test)

Effect of temperature on bacterial uptake in macrophages

The potential influence of temperature on bacterial uptake was studied in isolated monocytes/macrophages during infection with *F.n.n.* The invasion of *F.n.n.* was significantly higher at 16°C compared to 12°C, demonstrating that bacterial uptake increases with temperature (Fig. 4). At 20°C, the invasion displayed an intermediate bacterial count between the first 2 temperatures, most likely reflecting highly impaired cell quality at such high temperature. The use of elevated culture temperatures for cod monocyte/macrophage cells has a severe impact on cell viability. We repeatedly experienced high levels of cell detachment at similar levels in both control and infected cells at elevated temperatures, making long-term experiments at such temperatures difficult to perform.

Intracellular survival and replication of *F.n.n.* in macrophages

The gentamicin assay was used to examine survival and replication of *F.n.n.* in primary monocyte/macrophage cultures (12°C). The amount of intracellular bacteria in monocytes/macrophages was re-

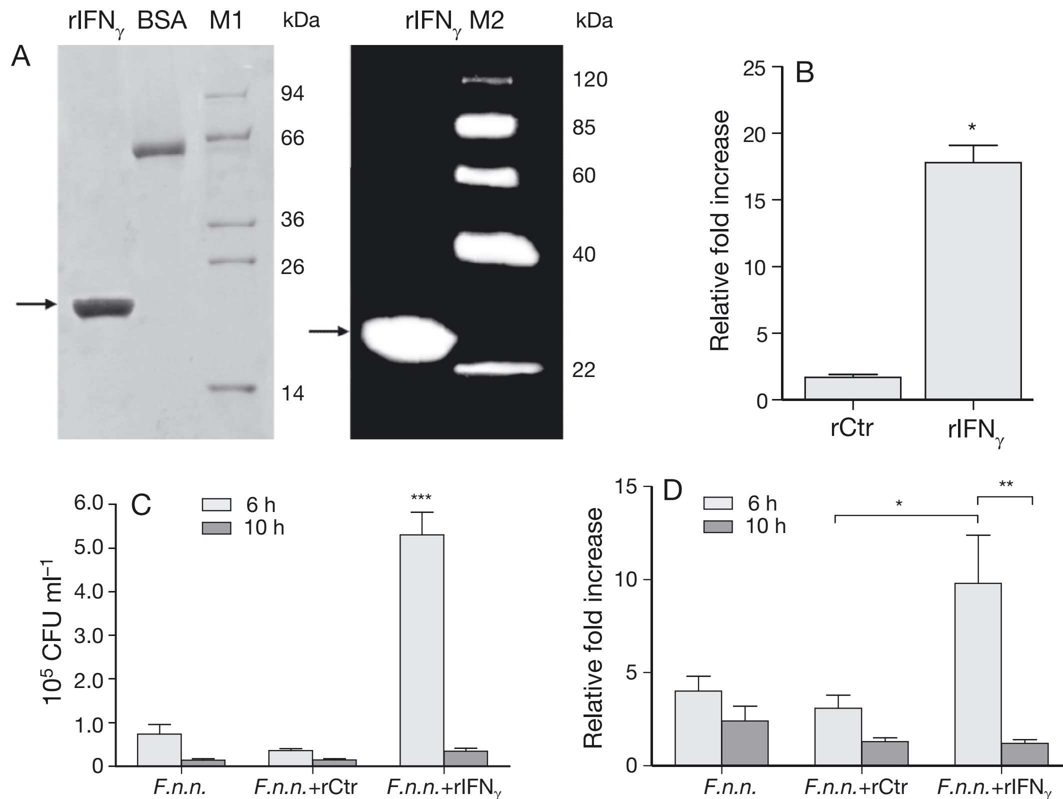

Fig. 3. Gel electrophoresis, Western blot and functional analyses of recombinant interferon gamma (rIFNγ). (A) Purified rIFNγ was analysed by SDS-PAGE and stained with Coomassie Blue and Western blot using anti-His antibody. Bovine serum albumin (BSA) was included in SDS-PAGE for comparison, and 2 different protein MW standards (M1 and M2) were used. (B) Monocytes/macrophages (n = 4 fish) were treated with recombinant control (rCtr; background proteins isolated from *E. coli* containing an empty vector) and rIFNγ followed by gene expression analysis of IRF1. Results are mean (+SEM) relative expression levels; *significant difference (Student's *t*-test; $p < 0.05$) compared to rCtr. (C,D) Monocytes/macrophages (n = 4 fish) were either infected with *Francisella noatunensis* subsp. *noatunensis* (*F.n.n.*), pre-treated with rCtr and infected (*F.n.n.* + rCtr) or pre-treated with rIFNγ followed by infection (*F.n.n.* + rIFNγ). Cells were subjected to a gentamicin protection assay (see 'Materials and methods'), and results (from the same experiment) are mean (+SEM) (C) CFU ml^{-1} and (D) relative fold increase of Fc50 after gene expression analysis. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ compared to all groups at both time points, tested by (C) 1-way ANOVA with Tukey's Multiple Comparison test or (D) Student's *t*-test

duced more than 2-fold between 2 and 24 h, while CFU increased between 24 and 48 h (Fig. 5A). The latter shows that *F.n.n.* is able to survive and replicate in monocyte/macrophage cultures. Similarly, in a different experiment, intracellular CFU increased from 8 to 48 h (Fig. 5B). In the same experiment, quantification of intracellular bacterial levels using real-time PCR with primers specific for the *F.n.n.* outer membrane protein FopA and Fc50 targeting 16S rRNA gene were used. Gene expression analysis revealed an initial significant drop (5-fold) in bacterial transcripts 8 h after infection followed by a slight increase during the succeeding sampling points (8 to 72 h) (Fig. 5B). Thus, expression analyses confirmed the live bacteria counting results and strongly indicated that the bacteria are metabolically active after invasion.

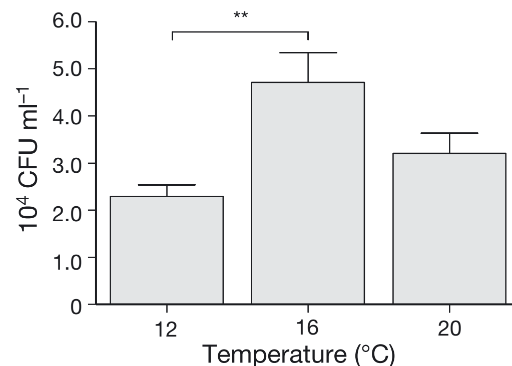

Fig. 4. Invasion rate of *Francisella noatunensis* subsp. *noatunensis* (*F.n.n.*) in monocytes/macrophages (n = 6 fish) cultured at different temperatures and infected with *F.n.n.* (see 'Materials and methods: Gentamicin protection assay'). Results are mean (+SEM) CFU ml^{-1}; **$p < 0.01$ (Kruskal-Wallis with Dunn's Multiple Comparison test)

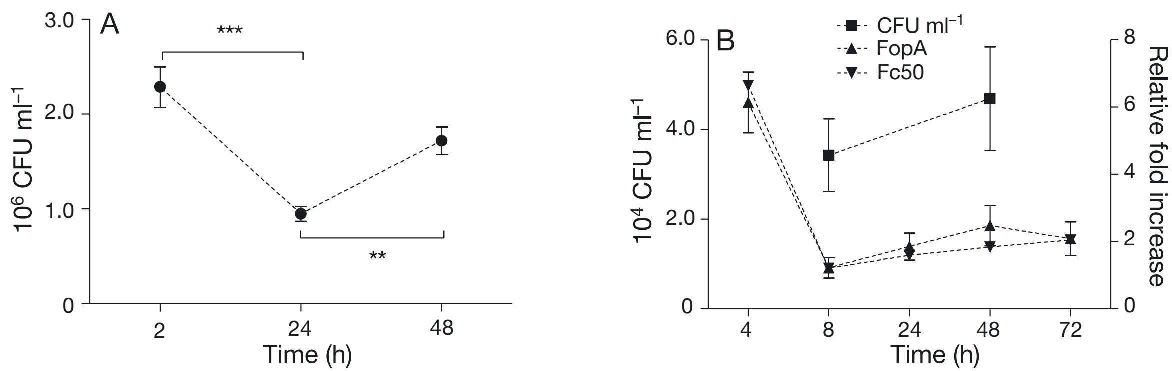

Fig. 5. Intracellular replication of *Francisella noatunensis* subsp. *noatunensis* (*F.n.n.*) in monocytes/macrophages (n = 4 fish per experiment) (see 'Materials and methods: Intracellular replication assay'). Infected cells were either (A) subjected to a second pulse-treatment with gentamicin (50 µg ml^{-1}) at succeeding time points or (B) incubated with gentamicin (5 µg ml^{-1}) in the cell medium. (A) Cells were harvested and the results are mean (±SEM) CFU ml^{-1}. (B) Infected cells were subjected to gene expression analysis of FopA and Fc50. The relative fold increase results are from 4 individual fish. Additionally, CFUs were detected at 8 and 48 h post-infection. ***$p < 0.001$; **$p < 0.01$ (1-way ANOVA with Tukey's Multiple Comparison test). The figures represent results from 2 different experiments

Two different approaches were used when removing extracellular bacteria before lysis: either a second pulse-treatment with gentamicin (50 µg ml^{-1}) (Fig. 5A) or incubation of cells with gentamicin (5 µg ml^{-1}) in the cell medium (Fig. 5B). Using both methods, the bacterial number (i.e. CFU) in the cell medium represented only 0.1 to 1% of the detected intracellular CFU and were considered not to interfere with the quantification of intracellular CFU.

Cell death or detachments of macrophages did increase with time, but correction of CFU against cell number did not have an impact on the results. Both infected and non-infected (control) cells were counted at all time points. Control wells were treated similarly as infected cells and the number of cells did not differ between controls and infected cells.

Intracellular internalisation, survival and replication of *F.n.n.* in ACL cells

The invasion assay was also applied in an epithelial-like cell line derived from ACL cells to study whether *F.n.n.* is able to gain access to cell types other than macrophages. Fig. 6 shows that *F.n.n.* invaded ACL cells, although in much lower numbers than in macrophages (2 vs. 5 $\log_{10}$ CFU). The marked initial drop in bacterial numbers experienced in macrophages was not observed in the ACL cells. The amount of intracellular bacteria increased strongly during incubation, demonstrating that *F.n.n.* can replicate in ACL cells at 20°C. After 3 d the number of detected bacteria decreased, indicating that *F.n.n.* does not persist in ACL cells for longer periods.

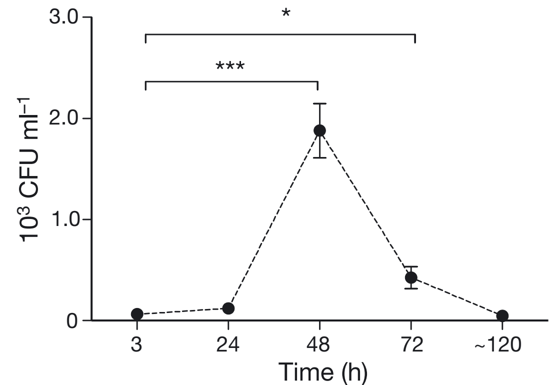

Fig. 6. Intracellular survival of *Francisella noatunensis* subsp. *noatunensis* (*F.n.n.*) in Atlantic cod larvae cells (n = 4 wells) (see 'Materials and methods: Intracellular replication assay'), incubated with gentamicin (5 µg ml^{-1}) in the cell medium. Results are mean (±SEM) CFU ml^{-1}; a repetition of the experiment produced similar results. ***$p < 0.001$; *$p < 0.05$ (Kruskal-Wallis with Dunn's Multiple Comparison test)

When using the cell line, no increase in cell death was detected when comparing cell counts of control and infected cells.

DISCUSSION

Phagocytic uptake of bacteria, followed by intracellular killing, is an important defence mechanism against several types of invasive bacterial infections. Here, we have shown efficient internalisation of *Francisella noatunensis* subsp. *noatunensis* in primary cod macrophages. Bacterial internalisation is usually associated with rearrangement of the host cytoskeletal structure, resulting in phagocytosis of

bacteria. Although bacterial uptake typically occurs through phagocytosis, alternative mechanisms that are independent of actin polymerisation have been described (Oelschlaeger et al. 1993, Lai et al. 2010). We were able to demonstrate that entry of *F.n.n.* in macrophages was dose-dependently inhibited by cytochalasin D, thus showing that bacterial uptake occurs through phagocytosis. Prior to phagocytosis, bacterial internalisation is commonly enhanced by opsonins acting as attachment sites which aid receptor-mediated phagocytosis of pathogens. While complement receptors (C3bR) and FcR receptors (FcR) have been implicated in the uptake of *Francisella tularensis* in mammals (Clemens et al. 2005, Schulert & Allen 2006), it is unknown what receptors are involved in the uptake of *F.n.n.* We observed that the use of sera from cod were toxic to the cod macrophages (results not shown). Such toxic effects were not apparent using bovine sera (FBS Gold), and we observed a 4-fold increased bacterial uptake with FBS present (results not shown). However, it is questionable whether fish complement receptors are capable of recognising mammalian factors due to the relatively large phylogenetic difference. Several reports have demonstrated that macrophage priming by IFNγ mediates killing of intracellular *F. tularensis* (Fortier et al. 1992, Lindgren et al. 2004, Edwards et al. 2010). In the present work, monocytes/macrophages had a significantly higher uptake of *F.n.n.* when treated with recombinant cod IFNγ prior to infection. Ten hours later, the amount of live intracellular bacteria was reduced almost to the level in controls that did not receive IFNγ, suggesting killing of a large proportion of the internalised bacteria. Still, a small percentage of bacteria survived and viable bacteria were detected in IFNγ-treated macrophages even after 72 h (results not shown). This demonstrates that macrophage activation does not contribute to complete eradication of internalised *F.n.n.* Similar effects on increased uptake in primary macrophages after IFNγ treatment have previously been reported in other species, and it was proposed that receptors involved in internalisation are somehow modified by IFNγ (Maródi et al. 1993, Gordon et al. 2005). More recently, Bosedasgupta & Pieters (2014) showed that IFNγ reprograms the mammalian macrophage endocytic pathway from receptor-mediated phagocytosis to micropinocytosis independent of receptors, enabling macrophages to internalise large amounts of cargo for direct transfer to lysosomes. However, our results were obtained with cod macrophages incubated at a low temperature, which leads to low levels of bacterial replication of *F.n.n.* and this might have affected efficient transfer to lysosomes and subsequent bacterial removal. Further studies are mandatory to reveal if IFNγ stimulates micropinocytosis and if these *in vitro* observations relate to pathogenesis *in vivo* with *F.n.n.*.

Atlantic cod is a cold water species. Primary cod macrophages in cultures prefer low temperatures, with an optimum at 8 to 12°C (Steiro et al. 1998), whereas the growth optimum for *F.n.n.* is 22°C in traditional bacterial EB growth medium (Olsen et al. 2006). Raising the culture temperature for macrophages from 12 to 16°C resulted in a 2-fold increase in bacterial uptake, but also severely impaired cell quality. We conclude that culture temperatures above 12°C for these primary cells have a severe impact on cell viability. Thus, long-term experiments to determine how bacterial replication is affected in macrophages by increasing temperatures are difficult to perform. Long-term infection studies of macrophages at 12°C showed bacterial invasion followed by a 2-fold reduction of viable bacteria and a 5-fold reduction in the outer membrane protein (FopA) gene expression. Similar initial bacterial decline results have also been obtained for other intracellular bacteria using macrophages or macrophage-like cell lines (Lissner et al. 1983, Larsen et al. 2013), and could be explained by efficient transfer of bacteria to lysosomes. Following the decline, the number of viable bacteria increased, showing that *F.n.n.* can replicate intracellularly in macrophages even at low temperatures. Our invasion assay confirmed the ability of *F.n.n.* to replicate in phagocytes as described by Vestvik et al. (2013) using flow cytometry and green fluorescent protein labelled bacteria.

Invasion studies also demonstrated efficient invasion of *F.n.n.* in ACL cells at 20°C, but at a considerably lower level (2 to 3 $\log_{10}$ CFU) than in macrophages, most likely reflecting that macrophages are the preferred host cell type. The observation that cultured macrophages presumably have an intact cellular immune defence, which gives a measurable initial drop in bacterial amounts, was not evident in the epithelial-like ACL cells. Macrophages represent professional phagocytes, normally possessing efficient intracellular means of killing bacteria, and thus the observed differences between the 2 cell types in internalisation and permitting bacterial replication probably reflect their respective abilities in processes for bacterial invasion and bactericidal mechanisms. In contrast to macrophages, the higher amount of bacterial replication observed in ACL cells might be attributable to a temperature dependency for intracellular bacterial growth. This could be coherent

with the fact that Atlantic cod is adapted for a life at cold temperatures, and outbreaks of francisellosis have not been observed unless the sea water temperature exceeds 15°C, although the bacterium has been found both in farmed and wild populations at lower temperatures (Nylund et al. 2006, Colquhoun & Duodu 2011).

The ability of *F.n.n.* to enter, survive and even replicate within an epithelial cell line (i.e. ACL cells) may play an important role in bacterial persistence in cod. Epithelial cells line the mucosal surfaces in the mouth/gills, the intestine and the skin, and represent a likely portal of bacterial entry. Other virulent intracellular bacterial strains are also able to invade cultured epithelial cells (Hall et al. 2007, Acosta et al. 2009), although macrophages are regarded as the main target cell type. Our findings revealed that the efficiency of internalisation differed greatly between cell types, whereby the invasion rate was lower in ACL cells compared to macrophages. However, and albeit at low frequency, the invasion, survival and replication in epithelial cells provide sufficient time and milieu to build a reservoir and to encourage bacterial spread to internal organs and target cells.

Taken together, we have established *in vitro* models of internalisation and intracellular survival for *F.n.n.* causing fransicellosis in cod. We showed that *F.n.n.* is able to invade, survive and replicate in both macrophages and epithelial-like cells, although a lower invasion rate accompanied by higher replication levels was detected in the latter cell type. The observed cell type differences regarding bacterial replication are possibly biased by a temperature-dependency for intracellular bacterial growth. On the other hand, the results might be explained by the high capacity of macrophages to efficiently kill intracellular *F.n.n.* Bacterial internalisation was greatly increased when cod macrophages were treated with IFNγ prior to infection, indicating an important role for IFNγ in cellular defence against *F.n.n.* infections.

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