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# Characterisation of the outer membrane proteome of *Francisella noatunensis* subsp. *orientalis*

**Running headline:** *Fno* outer membrane proteome characterisation.

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## Abstract

**Aims:** The aims of the current study were to characterise the outer membrane proteins (OMPs) of *Francisella noatunensis* subsp. *orientalis* (*Fno*) STIR-GUS-F2f7, and identify proteins recognised by sera from tilapia, *Oreochromis niloticus*, (L) that survived experimental challenge with *Fno.*

**Methods and Results:** The composition of the OMPs of a virulent strain of *Fno* (STIR-GUS- F2f7), isolated from diseased red Nile tilapia in UK, was examined. The sarcosine-insoluble OMP fraction was screened with tilapia hyper-immune sera by western blot analysis following separation of the proteins by 1D SDS-PAGE. Liquid chromatography-electrospray ionisation-tandem mass

spectrometry (LC-ESI-MS/MS) was used to identify the various proteins present in the OMP profile. Two hundred and thirty-nine proteins were identified, of which 44 were found in the immunogenic band recognised by the tilapia hyperimmune serum. *In silico* analysis was performed to predict the function and location of the OMPs identified by MS.

**Conclusions:** Using a powerful proteomic-based approach in conjugation with western immunoblotting, proteins comprising the outer membrane fraction of *Fno* STIR-GUS-F2f7 were identified, catalogued and screened for immune recognition by tilapia sera.

**Significance and Impact of the study:** The current study is the first report on the characterisation of *Fno* OMPs. The findings here provide preliminary data on bacterial surface proteins that exist in direct contact with the host's immune defences during infection and offer an insight into the pathogenesis of *Fno*.

**Keywords:** *Francisella noatunensis* subsp. *orientalis*; Outer membrane proteins; SDS PAGE; Hyperimmune sera; LC-ESI-MS/MS.

## Introduction

*Francisella noatunensis* subsp. *orientalis* (*Fno*) is the causative agent of piscine francisellosis in a wide range of warm water fish species (Colquhoun and Dudu, 2011). Recently, *Fno* has emerged as a major threat to tilapia aquaculture where chronic infection by this organism has been reported in different geographical regions (Soto *et al.*, 2009a; Qiang *et al.,* 2015; Ortega *et al.,* 2016), with high morbidity and associated mortalities of up to 95% (Birkbeck *et al.,* 2011; Rodrigues *et al.,* 2017). To date, there is no commercially available vaccine or prophylaxis for *Fno* infection on fish farms.

The outer membrane proteins (OMPs) are specific highly conserved components of Gram-negative bacterial cells that include those associated with bacterial pathogenicity (Seltman and Holst, 2002), nutrient uptake (*e.g.* iron), antimicrobial peptide resistance and other proteins required for *in vivo* survival in the host environment (Koebnik *et al.,* 2000). Their location on the surface of the bacteria facilitate interaction with the host immune system and thus antibodies raised against these proteins are likely to result in neutralising activity against target microorganisms (Lin *et al.,* 2002). The OMPs of a variety of fish-pathogenic bacteria have previously been characterised, including those of *Flavobacterium columnare* (Liu *et al.,* 2008; Luo *et al.,* 2016), *Streptococcus iniae* (Cheng *et al.,* 2010), *Edwardisella tarda* (Kumar *et al.,* 2009; Sun *et al.,* 2011), *Edwarsiella ictaluri* (Dumpala *et al.,* 2009), *Aeromonas hydrophila* (Wang *et al.,* 2013), *Aeromonas salmonicida* (Ebank *et al.,* 2005), *Vibrio harveyi* (Yu *et al.,* 2013) and *Vibrio alginolyticus* (Qian *et al.,* 2008). A more comprehensive characterisation of this vital group of proteins facilitated development of a new generation of diagnostic and prophylactic tools for various bacterial diseases of economic importance to farmed and ornamental fish species (Maji *et al.,* 2006; Maiti *et al.,* 2011; Thangaviji *et al.,* 2012; Yu *et al.,* 2013; Divya *et al.,* 2015).

Immuno-proteomics is a powerful approach used to highlight and identify immuno-reactive components of the bacterial outer membrane proteome where innately hydrophobic membrane associated proteins are solubilised and separated in the presence of an anionic detergent during 1-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D-SDS PAGE). This initial separation step is followed by western immunoblotting using immune sera to highlight reactive

areas within the protein profile, downstream mass spectrometry and database mining to identify individual proteins within immuno-reactive complexes (Boyce *et al.,* 2006).

Previous studies have identified some potential *Fno*-pathogenicity determinants including those genes responsible for intracellular localisation, survival and replication using genomics and proteomic approaches (Sridhar *et al.,* 2012; Soto *et al.,* 2013; Lagos *et al.,* 2017), however the functions of the conserved proteins representing these genes are not fully-understood. Given the increasing economic impact of francisellosis on commercial tilapia farming, there is a growing need to better understand the mechanisms by which *Fno* causes disease and develop sustainable solutions to control infection in farmed fish. To this end, the OMP profile of *Fno* (STIR-GUS-F2f7) was catalogued and proteins recognised by pooled hyper-immune sera collected from infected tilapia were identified using the approach outlined above.

## Materials and methods

### Bacterial isolate, culture media and growth conditions

*Fno* STIR-GUS-F2f7, a highly virulent strain that was isolated in 2012 from a moribund red Nile tilapia, *Oreochromis niloticus* (L.) farmed in England (Ramirez-Paredes *et al.,* 2017a) was used in this study. The strain had been previously identified by conventional biochemical tests and conventional PCR (Ramirez-Paredes *et al.,* 2017a) and its annotated whole genome sequence was published (Ramirez-paredes *et al.,* 2017b). Cultivation of the bacterium was performed on Cysteine Heart Agar containing 2% bovine haemoglobin (CHAH) (DIFCO, USA) following incubation at 28 °C for 72 h. For liquid cultures, aliquots of Modified Mueller Hinton broth (MMHB) containing 2% Isovitalex and 0.1% glucose (DIFCO, BD, USA) were inoculated with a single bacterial colony from CHAH plates and incubated at 28 °C for 18 h with shaking at 150 rpm.

**Extraction of OMPs**

OMPs were obtained by the method of Gauthier *et al.* (2003), with slight modifications. Briefly, *Fno* cells in 20 ml of liquid culture were harvested by centrifugation at 5,000 *g* for 15 min at 4 °C; supernatant was discarded, and the cell pellet was washed three times with 10 ml of chilled 50 mmol $l^{-1}$ Tris HCl (pH 7.0) at 3,000 *g* for 10 min. The wash buffer was discarded, and the pellet re-suspended in 1 ml of 50 mmol $l^{-1}$ Tris HCl (pH 7.0) containing 20% (w/v) sucrose, 10 mmol $l^{-1}$ Na-EDTA, 10 μg $ml^{-1}$ lysozyme (Sigma-Aldrich UK) and 10 μl of protease inhibitor cocktail (Sigma-Aldrich, UK). The cell suspension was then transferred to 1.5 ml micro-centrifuge tubes (Eppendorf, Germany) containing 0.1 ml Zirconium silica beads (Thistle scientific, UK), and cells were disrupted in a FastPrep homogeniser B101011 (MP Biomedicals, USA) for 6×30 sec, with cooling on ice for 5 min between each cycle. The lysate was then transferred to a fresh 1.5 ml micro-centrifuge tube and cell debris were removed by centrifugation at 16,000 *g* for 2 min at 4 °C. The insoluble material containing the membrane proteins was obtained by ultracentrifugation at 100,000 *g* for 40 min at 4 °C, the supernatant was discarded, and the pellet washed by addition of 1 ml of 10 mmol $l^{-1}$ Tris HCl (pH 7.0), without re-suspension, and incubation on ice for 1 min prior to discarding the supernatant once more. The cell pellet was then re-suspended in 1 ml of 10 mmol $l^{-1}$ Tris HCl (pH 7.0) containing 0.5% (w/v) N-lauryl-sarcosine and centrifuged at 100,000 *g* for 40 min at 4 °C. The supernatant was discarded, and the cell pellet was washed in 1 ml of 10 mmol $l^{-1}$ Tris HCl (pH 7.0), as previously described, before the supernatant was discarded, and pellets were air dried at room temperature (RT) (~22 $^{o}$C) for 30 sec. Finally, the pellet was re-suspended in 1 ml of 10 mmol $l^{-1}$ Tris HCl (pH 7.0) containing 0.5% (w/v) N-lauroylsarcosine and 0.1% (w/v) Sodium dodecyl sulfate (SDS) on a variable

speed vortex mixer (Cole-parmer, UK) for 30 sec. The concentration of OMPs was determined by BCA assay (Pierce BCA protein assay Kit; Thermo, USA) according to the manufacturer's instructions, and adjusted to 50 µg $\mu l^{-1}$ using Milli-Q water (Thermo, UK). Aliquots of 20 µl were prepared and stored at -80 °C until used.

**Fish challenge and serum samples**

Healthy Nile tilapia, *Oreochromis niloticus,* (14±2 g) were obtained from a commercial fish farm in East of Thailand. Fish were divided into two groups of duplicate static 15 L tanks with 10 fish each. Group one was intraperitoneally injected (i.p.) with 0.1 ml of $1x10^6$ cfu $ml^{-1}$ ($LD_{60}$) of *Fno* (STIR-GUS-F2f7) while the second group received i.p. injection of PBS as control. The bacterial inoculum was prepared using MMHB containing 2% Isovitalex and 0.1% glucose as previously described (Soto *et al.,* 2009a) and the challenge dose was selected based on a previous experiment by Ramirez-Paredes (2015). Fish were maintained at 23±2 °C for 21 days and dead fish were autopsied to determine the cause of death. The presence of *Fno* in the tissues was determined by bacterial culture in CHAH supplemented with Polymixin B 100 U $ml^{-1}$ as previously described (Soto *et al.,* 2009a). Blood was collected from survivor and control tilapia 21 days post challenge (dpc) and the serum IgM level was measured using an indirect enzyme-linked immunosorbent assay (ELISA). In Brief, 96-well ELISA plates (Immulone®4 HBX-USA) were coated with 100 µl of 1% w/v poly-lysine in carbonate-bicarbonate buffer (Sigma-Aldrich, UK) and incubated at room temperature (RT) (~22°C) for 1 h. The plates were then washed three times by low salt wash buffer (LSWB: 0.02 mol $l^{-1}$ Trizma base, 0.38 mol $l^{-1}$ NaCl, 0.05% v/v Tween 20, pH 7.2). 100 µl of *Fno* in PBS at $OD_{600}$ 0.4 (~$1x10^9$ cfu $ml^{-1}$) was added to each well and plates were incubated at 4 °C overnight. The plates were washed three times with LSWB and bacteria were fixed by adding 100 µl/well of 0.05% v/v glutaraldehyde (Sigma-Aldrich, UK) in LSWB and incubated for 20 min at RT. Plates were washed as before with LSWB. Endogenous peroxidase activity was prevented by adding 100 µl/well of 1:10 of 30% stock solution of hydrogen peroxide (Sigma-Aldrich, UK) and plates were incubated for 1 h at RT. Washing was done three times as before and non-specific antibody binding was blocked by adding 250 µl/well of 5% w/v dried skimmed milk (Marvel, Premier Foods Group Ltd, UK) in distilled water (DW) for 3 h at RT. After washing the plates three times with LSWB, 100 µl/well of serum from challenged and control fish 21 dpc at 1:500 in LSWB with 1% bovine serum albumin (BSA) (Fisher scientific, UK) were then incubated overnight at 4°C. *Fno* positive and negative sera were included on each plate as assay controls. After incubation, the plates were washed 5 times with high salt wash buffer (HSWB: 0.02 mol $l^{-1}$ Trizma base, 0.5 mol $l^{-1}$ NaCl, 0.01% v/v Tween 20, pH 7.4) with a 5 min soak on the last wash to ensure removal of unbound antibodies. 100 µL/well of anti-tilapia IgM monoclonal antibodies (Mab) at a dilution of 1:75 in PBS (Fo4, Aquatic Diagnostic Ltd, UK) were added and the plates were incubated at RT for 1 h. Washing was repeated using HSWB as before and 100 µL/well of goat anti-mouse IgG-HRP MAb (Sigma, UK) at a dilution of 1:3000 in LSWB with 1% BSA were added and the plates were then incubated for 1 h at RT. Washing was done once with HSWB and the colour was developed with tetramethyl-benzidine (TMB) (Amresco, MA, USA) for 5 min at RT and the reaction was stopped by adding 50 µL/well of 2 mol $l^{-1}$ $H_2SO_4$ (Sigma, UK). The absorbance was measured at $OD_{450}$ using a micro-plate reader (Biotek Synergy HT, USA) and serum samples with high specific anti-*Fno* IgM levels 21 dpc were selected for performing immunoblotting. All procedures utilizing fish were carried out in accordance with the UK Animal (Scientific Procedures) Act 1986 and the University of Stirling Animal Welfare and Ethical Review Body (AWERB) regulations.

**1D SDS-PAGE**

A 100 µg sample of the OMPs was resolved on a 12% NuPAGE™ Novex® Bis-Tris Gel (NuPAGE ™, Invitrogen, USA) in a NuPAGE™ MES SDS Running Buffer (20x) (Thermo Fisher scientific, UK) at 200 V (constant voltage) for 45 min, according to the manufacture's protocol. After electrophoresis, the separated proteins were stained with SimplyBlue Safe Stain (Invitrogen, USA) following the manufacturer's instructions and the gel scanned using an Epson expression 1680 artist scanner (Epson, USA). The image obtained was evaluated using Irfanview software (http://www.irfanview.com). Two technical replicates of SDS-PAGE were performed to ensure reproducibility.

**Western blot analyses**

Following electrophoresis as described above, the separated OMPs were transferred to a nitrocellulose membrane (Invitrogen, USA) at 30 V for 45 min using 1x NuPAGE™ Transfer buffer (ThermoFisher Scientific, UK), following the manufacture's protocol for two replicate gels. Following transfer, the membranes were washed for 5 min in TBS (50 mmol $l^{-1}$ Tris, 150 mmol $l^{-1}$ NaCl, pH 7.4) and blocked overnight at 4 °C in TBS with 5% (w/v) dried skimmed milk (Marvel, Premier Foods Group Ltd, UK). After washing 3 times with TBST (50 mmol $l^{-1}$ Tris, 150 mmol $l^{-1}$ NaCl, 0.1% Tween-20, pH 7.5) for 10 min on each wash, the membranes were incubated for 3 h at RT (~22 °C) with continuous agitation with 5 ml of pooled infected (*n*=5) and control (*n*=5) fish sera at a dilution of 1:50 in TBS with 1% (w/v) BSA (Sigma-Aldrich, UK), respectively. Washing was repeated as described before, then 5 ml of 1:50 mouse anti-tilapia IgM Mab (Fo4, Aquatic Diagnostic Ltd, UK) in TBS was added to each membrane and incubated with continuous shaking at RT for 1 h. Following washing, 5 ml of Goat anti-mouse HRP MAb (Sigma, UK) at a dilution of 1:200 in TBS was added to each membrane with incubation for 1 h at 22 °C. The membranes were then washed 3 times with TBST and once with TBS for 5 min, before the reaction was developed by adding 5 ml of ImmPACT DAB peroxidase substrate (Vector laboratories Ltd, USA) to each membrane for 2 min. The reaction was stopped by addition of 5 ml of distilled water. Membranes were left to dry then scanned using an Epson expression 1680 artist scanner (Epson, USA). Two technical replicate immunoblots were prepared to ensure reproducibility.

**In-gel digestion and *LC-ESI-MS/MS***

Gel lanes containing OMPs were excised and sliced horizontally from top to bottom to yield a series of equal slices of 2.5 mm depth. Each of the resulting gel slices was then subjected to standard in-gel destaining, reduction, alkylation and trypsinolysis procedures (Shevchenko *et al.,* 1996). LC-ESI-MS/MS was performed as described by Watson *et al.* (2014), with instrument parameters based on those used previously by Batycka *et al.* (2006).

**Data analysis and Database Mining**

Deconvoluted MS/MS data in Mascot generic format (mgf) was imported into ProteinScape™ V3.1 (Bruker Daltonics, USA) for downstream database mining of the available annotated cognate chromosomal and plasmid *Fno* protein database derived from genomic sequences available at the National Centre for Biotechnology Information (NCBI, Genbank), (http://www.ncbi.nlm.nih.gov) (Table 1) and the NCBInr *Fno* sub-database, utilising the Mascot™ V2.5.1 (Matrix Science, London, UK) search algorithm (Perkins *et al.,* 1999). The protein contents of individual gel slices and the entire gel lane were established using the "Protein Search" and "Protein Compilation features of the ProteinScape™ software", respectively, and the separate compilation of the proteins contained in the gel slices of each of gel replicates was formed using the "Protein Extractor" feature of the software. Data was searched specifying Trypsin and Trypsin/P. Spectra used for protein

identifications were re-searched against the entire NCBInr database to ensure accurate peptide assignments. Mascot search parameters were set in accordance with published guidelines (Taylor and Goodlett, 2005). Fixed (carbamidomethyl "C") and variable (oxidation "M" and deamidation "N,Q") modifications were selected along with peptide (MS) and secondary fragmentation (MS/MS) tolerance values of 0.5 Da, whilst allowing for a single $^{13}C$ isotope. Molecular weight search scores (MOWSE) attained for individual protein identifications were inspected manually and considered significant only if two or more peptides were matched for each protein, and each matched peptide contained an unbroken "b" or "y" ion series represented by a minimum of four contiguous amino acid residues.

## Bioinformatics analyses

The PSORTb algorithm (http://www.psort.org) was used to predict the subcellular location of identified proteins. The putative functional classification of the identified proteins was obtained by comparison of predicted proteins against clusters of orthologous groups of proteins (COGs) database using the EggNOG v4.5 server (http://eggnog.embl.de). Lipoproteins were identified using the LipoP 1.0 server (http://www.cbs.dtu.dk/services/LipoP-1.0/) and the presence of signal peptides sequence was searched using SignalP 2.0 server (http://www.cbc.dtu/services/Signal/).

## Results

### 1D PAGE and immunoblotting

Following electrophoretic separation of *Fno* STIR-GUS-F2f7 OMPs (Figure 1A), immunoblotting was performed using either convalescent immune sera from *Fno*-infected tilapia or control tilapia sera. An abundant protein band was observed between 17 and 28 kDa on the stained gel, and similarly, the pooled immune sera reacted with an equivalent sized band on the Western blot (Figure 1B). No immuno-reactivity was detected in this region by the control fish sera (Figure 1C).

### Protein identification by LC-MS/MS

Mass spectrometric analysis facilitated the confident identification of a total of 239 proteins in the OMP fraction (Table S1), including 44 proteins in the immunogenic band (17-28 kDa) (Table S2) highlighted by the immune tilapia serum pool. The top 20 protein IDs of the OMP fraction are listed in Table 2 and the immunogenic OMP proteins in Table 3. The full protein lists can be found in supporting information (Table S1 and S2).

### Prediction of function, subcellular localisation and lipoproteins of the identified proteins

EggNOG v4.5, PSORTb® v.2.0, LipoP v.1.0, SignalP v.2.0 servers were used to predict the function, sub-cellular location and lipoprotein nature of the 239 proteins identified in the OMP preparation. Proteins associated with translation, ribosomal structure and biogenesis were the most abundant (42%), followed by those involved in energy production and conversion (31%), and those associated with cell wall biogenesis and post-transitional modification (20%). The potential subcellular localisation of about 82.8% of the *Fno* OMPs was identified, with the cytoplasmic proteins representing the majority of proteins (62%). These were followed by cytoplasmic membrane proteins (8.8%), outer membrane proteins (5.8%), periplasmic proteins (2.5%) and extracellular proteins (0.8%). The lipoproteins in the *Fno* OMPs represented 16.7%. *In silico* analysis of the top 20 OMP fraction protein IDs are listed in Table 4. A full summary of bioinformatics analysis of the identified proteins is shown in Figure 2 and Tables S3 in supporting information.

**Discussion**

Outer membrane proteins play an important role in the pathobiology of various bacteria by facilitating their adaptation to a wide range of different environments. Due to their prominence at the host-pathogen interface, the OMPs represent antigens with the potential to induce protective humoral and cellular immune response in the host capable of inactivating the bacteria (Lin *et al.,* 2002; Mukhopadhaya *et al.,* 2006). Despite their potential importance, to date, no studies have been conducted on membrane proteins of *Fno*. We therefore analysed the 1D SDS-PAGE profile of OMPs extracted from the bacterium in combination with immunoblotting and LC-ESI-MS/MS to identify and catalogue the proteins present in this outer membrane enriched fraction of *Fno* STIR-GUS-F2f7.

In the current study, a total of 239 confidently identified *Fno* OMPs were highlighted (Table S1). Interestingly, many of these were observed to share similarities with proteins found in the *Fno*-derived outer membrane vesicles (OMVs) described by Lagos *et al.* (2017), where 52 % of the OMV proteins identified were predicted to be cytoplasmic, while the outer membrane and extracellular proteins were 5% and 1%, respectively, compared to 5.8% and 0.8% in this study, respectively.

The presence of cytoplasmic, periplasmic or inner membrane proteins in the current *Fno*-OMP preparation can be attributed to the fact that most of the bacterial outer membranes are involved in the transportation of substances between the intracellular or extracellular membranes. This may allow contact between the OMPs and other membrane proteins or the periplasmic proteins as an essential component of the membrane associated-enzyme complex (Vipond *et al.,* 2006). Identification of different classes of non-outer membrane proteins in OMP preparations have been previously reported (Liu *et al.,* 2008; Kumar *et al.,* 2009; Watson *et al.,* 2014). The reason for this is unknown, but as shown for other bacteria, *Fno* may express non-classically associated outer membrane "moonlighting" proteins on its surface, which are known to have more than one function both within the cytoplasm and extracellularly, and which have been reported to be associated with bacterial virulence (Henderson and Martin, 2011). Definitive assignment of OMPs to specific subcellular locations within Gram-negative bacteria remains unclear. This may be due to the OMPs spanning the three layers of bacterial cell membrane as β-barrel trans-membrane proteins associated with the transportation of ions and other micro-molecules (Wimely, 2003; Pavkova *et al.,* 2005). Alternatively, post-transitional modification may enable the OMPs to associate with other proteins including lipoproteins and glycoproteins (Santoni *et al.,* 2000). This may explain the high percentage of non-OMPs in the extracted *Fno*-OMPs preparation. Further studies are needed to confirm the identity and biological functions of these non-OMPs.

Functional analysis of the proteins identified using EggNOG v. 4.5 revealed that most of the abundant proteins were involved in vital biological functions including energy production, cell wall/cell membrane formation, post-transitional modification, and metabolism of various cellular components (including protein, carbohydrate and lipid metabolism), transcription and transport activities. This highlights the diversity of biological functions associated with OMPs and may reflect the importance of the OMPs in the pathobiology of *Fno*. It has already been reported that proteins responsible for translation/transcription, catalytic activity, and transporting activity are the most abundant proteins found in *Fno*-derived OMVs (Lagos *et al.,* 2017).

The presence of lipoproteins in the current *Fno*-OMPs was predicted using LipoP server v0.2 of which 24 were predicted to be cleaved by signal peptidase I and 16 by signal peptidase II. In addition to their role in the acquisition of nutrients, it has been suggested that lipoproteins have the ability to switch-on the host's immune response by interacting with Toll-like receptor 2 (Nguyen and Götz, 2016). Moreover, 31 ribosomal proteins, mainly 30s and 50s, were detected in the OMPs of

*Fno*. Their presence has also been reported in OMP preparations of other bacteria such as *F. tularensis* (Janovska *et al.,* 2007a), *F. columnare* (Liu *et al.,* 2008) and *Pasturella multocida* (Boyce *et al.,* 2006). Herskovits *et al.* (2002) reported the importance of ribosomal proteins in the biogenesis and translocation of integral membrane proteins.

In our study, PdpD, IglA, IglB and IglC, outer membrane-A family protein (FopA), peptidoglycan associated lipoprotein (PAL), GroEl and ClpB displayed high scores in comparison to the other proteins identified in the *Fno*-OMPs. Interestingly, all of these proteins have already been detected in various protein preparations, including OMPs and OMVs, from different *Francisella* spp., including *Fno* (Lagos *et al.,* 2017)*, F. noatunensis* subsp. *noatunensis* (*Fnn*) (Pierson *et al.,* 2011) and *Francisella tularensis* (*Ft*) (Melillo *et al.,* 2006; Huntley *et al.,* 2007; Hickey *et al.,* 2011). Homologues of some of the *Fno* OMPs identified in this study have previously been described as immunogenic in the *F. tularensis* live vaccine strain (LVS), as demonstrated by western blotting using sera from tularemic patients (Janovska *et al.,* 2007).

The PdpA, PdpB, PdpD, IglA, IglB and IglC proteins represent the core elements of the *Francisella* pathogenicity island (FPI), which itself constitutes the major determinant associated with bacterial virulence and intracellular replication within host macrophages (Nano and Schmerk, 2007; Bröms *et al*., 2010). PdpA suppresses cell signalling by macrophages including growth factors, cytokines and adhesion ligands, thus suppressing the macrophage's ability to recruit and stimulate other immune cells (Nano *et al.,* 2004). Ludu *et al.* (2008) reported that PdpD protein is localised to the outer membrane of *Francisella novicida* and is involved in the extracellular virulence of this pathogen by affecting the localisation of other FPI proteins including IglA, IglB, IglC and T6SS. IglA and IglB are two cytoplasmic proteins that constitute an essential part of the type VI secretion system in *F. novicida* and both are required for intra-macrophage growth through stimulating secretion of effector molecules, that affect host cell processes (Barker *et al.,* 2009). It has also been demonstrated that IglA is required for virulence and supporting the growth of the bacterium inside macrophages (De Bruin et *et al.,* 2007).

The IglC protein, which was associated with the immunoreactive band (17-28 kDa) in our *Fno* OMP, is one of the important proteins that is upregulated during intracellular growth of *Francisella* spp. in macrophages (Golovliov *et al.,* 1997, 2003). Earlier studies reported that IglC protein, with its regulator MglA, assist the ability of *F. tularensis* to modulate biogenesis of the phagosome, preventing the formation of the phagolysosome, and thus facilitating escape of the bacteria into the cytoplasm of the host cell following replication (Clemens *et al.,* 2004; Santic *et al.,* 2005). Furthermore, IglC has been reported to play a role in inducing the production of inflammatory cytokines (Telepnev *et al.,* 2003) and subsequent induction of cell apoptosis (Lai *et al.,* 2004). Mutations of this protein alter bacterial virulence and impair intracellular growth in human derived macrophages (Santic *et al.,* 2005) as well as tilapia macrophages (Soto *et al.,* 2009b, 2011).

The *Francisella* outer membrane-A family protein (FopA) identified within the immunogenic band of *Fno* OMPs is the predominant outer membrane protein which is highly expressed on the cell surface and has been found to be highly immunogenic in *F. tularensis* (Fulop *et al.,* 1996; Huntley *et al.,* 2007). Readily accessible to different antibodies, it provided good protection when tested as a candidate subunit vaccine antigen against human tularemia in mice exposed to lethal intradermal and intranasal *F. tularensis* SchuS4 challenge (Hickey *et al.,* 2011). The GroEL chaperone protein, is a heat shock protein that has been found to be up-regulated in mice vaccinated with mutant LVS (Bakshi *et al.,* 2008) and in association with other heat shock proteins like DnaK and GroES, is

thought to affect the long-lasting recall of $CD4^+$ and $CD8^+$ T cells by stimulating specific anti-tularemic antibodies (Havlasova *et al.,* 2002; Lee *et al.,* 2006).

PALs detected in OMPs of *Fno* in the current study, are ubiquitous proteins, found in many pathogenic Gram-negative bacteria including *Escherichia coli* (Hellman *et al.,* 2002), *Vibrio cholerae* (Heilpern and Waldor, 2000) and *F. novicida* (McCaig *et al.,* 2013). The PALs are thought to perform virulence-related functions and assist in survival of pathogenic bacteria by modulating the host's immune response and initiating the release of pro-inflammatory cytokines (Buwitt-Beckmann *et al.,* 2006; Oscarsson *et al.,* 2008; Godlewska *et al.,* 2009).

A previous genomic study performed by Sridhar *et al.,* (2012) revealed major differences between human pathogenic *F. tularensis* and fish pathogenic *Fno* genomes, especially in their pathogenicity island (FPI) where *Ft* possess two copies of FPI, but *Fno* contains only one copy. More importantly, the number of protein coding genes are lower in *Fno* (*n*=1595) than *Ft* (*n*=1664) where *pdpC*, encoding one of the FPI proteins, was one of the most important genes missing in *Fno* and it was reported to be crucial for growing of *Francisella* spp. in mammalian cells (Hazlett and Cirillo, 2009). Interestingly, our proteomic approach is in agreement with the later findings, where the PdpC protein was not detected. This highlights the value and importance of proteomic approaches in complementing genomic studies for establishing valid and definitive information about the microbial phenotype, especially in selection of candidates for therapeutic or diagnostic applications.

When the OMP profile of *Fno* STIR-GUS-F2f7 was examined by immunoblotting using hyper-immune sera from convalescent tilapia, an immunoreactive region was observed between 17-28 kDa, while no immunoreactivity was seen with the control sera. Similar patterns were obtained by Shrøder *et al.* (2009), who screened *Fnn* with serum from Atlantic cod (*Gadus morhua,* L.) immunised with either a monovalent *Fnn* vaccine or a multivalent vaccine containing *Fnn* and *Vibrio anguillarum,* then challenged with either *Fnn* alone or *Fnn* and *V. anguillarum*. These authors also reported an immunogenic band between 20-25 kDa after probing the *Fnn* whole cell protein extract with a polyclonal rabbit serum raised against *Francisella* spp. In a separate study by Kay *et al.* (2006), polyclonal antisera raised against *Fno* recognised an immuno-dominant band of approximately 20 kDa in the large lipo-oligosaccharide fraction (LOS) of the proteinase-K treated whole cell protein lysate. The presence of the immunoreactive band (~17-28 kDa) in the OMP fraction in our study may support the results obtained in previous studies. More importantly, establishing the proteins present in the *Fno* outer membrane proteome may enable a greater understanding of which proteins are involved in stimulating the fish's immune system in response to *Fno* infection.

To our knowledge, this is the first report describing the characterisation and identification of proteins comprising the OMP fraction of *Fno*. Interestingly, most of these proteins were previously reported to be immunogenic in *F. tularensis* (Havlasova *et al.,* 2002; Lee *et al.,* 2006). When taken together, these results give more insight into our understanding of the pathogenicity of *Fno* and highlight the potential of OMPs in future diagnostic and control strategies for the management of *Fno* infection in farmed tilapia.

It is worth mentioning that, some of the pathogenicity-related proteins that showed high score in the current *Fno*-OMP preparation, such as PdpD and FopA were not recognised by the sera from challenged tilapia. However, they were previously described as immunogenic antigens in *F. tularensis* using sera from tularemic patients (Hickey *et al.,* 2011; Huntly *et al.*, 2007; Janovska *et al.,* 2007). This anomaly may be attributed to the limited resolution offered by 1D-SDS-PAGE and its inability to separate complex mixtures of proteins that co-migrate as a single band. To this end, the use of higher resolution 2-D gel electrophoresis together with immunoblotting and downstream

mass spectrometry may facilitate a more precise characterisation of the protein complement of the *Fno* OMP fraction. In summary, the data presented here offers a first insight into OMPs of *Fno*, which help build our understanding of how this organism is able to cause disease.


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## Conflict of interest

The authors declare no conflict of interest.

**References**


Batycka, M., Inglis, N.F., Cook, K., Adam, A., Fraser-Pitt D., Smith, D, et al. (2006) Ultra-fast tandem mass spectrometry scanning combined with monolithic column liquid chromatography increases throughput in proteomic analysis. *Rapid Commun Mass Spectrom* **20**, 2074–2080. doi: 10.1002/rcm.2563.

Baik, S.C., Kim, K.M., Song, S.M., Kim, D.S., Jun, J.S., Lee, S.G., et al. (2004) Proteomic analysis of the sarcosine-insoluble outer membrane fraction of *Helicobacter pylori* strain 26695. *J Bacteriol* **186**(4), 949-955. doi: 10.1128/JB.186.4.949-955.2004.

Bakshi, C.S., Malik, M., Mahawar, M., Kirimanjeswara, G.S., Hazlett, K.R.O, et al. (2008) An improved vaccine for prevention of respiratory tularemia caused by *Francisella tularensis* SchuS4 strain. *Vaccine* **26**(41), 5276–5288. doi.org/10.1016/j.vaccine .2008.07.051

Barker, J.R., Chong, A, Wehrly, T.D., Yu, J.J., Rodriguez, S.A., Liu, J., et al. (2009) The *Francisella tularensis* pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. *Mol Microbiol* **74**(6), 1459–1470. PMID: 20054881.

Birkbeck, T.H., Feist, S.W. and Verner-Jeffreys, D.W. (2011) *Francisella* infection in fish and shellfish. *J Fish Dis* **34**,173-178. doi: 10.1111/j.1365-2761.2010.01226.x.

Berven, F.S., Staume, A.H., Murrell, K.F., Lillehuag, A.F. and Jensen, H.B. (2006) Analysing the outer membrane subproteome of *Methylococcus capsulatus* using proteomic and novel bicomputing tools. *Arch Microbiol* **184**, 362-377. doi: 10.1007/s00203-005-0055-7.

Boyce, J.D., Cullen, P.A., Nguyen, V., Wilkie, I. and Alder, B. (2006) Analysis of the *Pasturella multocida* outer membrane sub-proteome and its response to the invivo environment of the natural host. *Proteomics* **6**, 870-880. doi:10.1002/pmic.200401342.

Bröms, J.E., Sjöstedt, A. and Lavander, M. (2010). The role of the *Francisella tularensis* pathogenicity island in type VI secretion, intracellular survival, and modulation of host cell signaling. *Front Microbiol* **1**, 1–17. doi.org/10.3389/fmicb.2010.00136.

Buwitt-Beckmann, U., Heine, H., Wiesmuller, K.H., Jung, G., Brock, R., Akira, S, et al. (2006) TLR1- and TLR6-independent recognition of bacterial lipopeptides. *J Biol Chem* **281**, 9049–9057. doi: 10.1074/jbc.M512525200.

Cheng, S., Hu, Y.H., Jiao, X.D. and Sun, L. (2010) Identification and immunoprotective analysis of a *Streptococcus iniae* subunit vaccine candidate. *Vaccine* **28**(14), 2636-2641. doi: 10.1016/j.vaccine.2010.01.016.

Clemens, D.L., Yu, L.B. and Horwitz, M.A. (2004) Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. *Infect Immun* **72**(6), 3204–3217. doi.org/10.1128/IAI.72.6.3204-3217.2004

De Bruin, O.M., Jagjit, S. and Nano, F.E. (2007) The *Francisella* pathogenicity island protein IglA localizes to the bacterial cytoplasm and is needed for intracellular growth. *BMC Microbiol* **7**, 1. doi.org/10.1186/1471-2180-7-1

Divya, S., Thangaviji, V., Velmurugan, S., Michaelbabu, M. and Citarasu, T. (2015) Comparative study on bacterial outer membrane protein (BOMP) and bacterial outer membrane gene (BOMPG) vaccination to Goldfish *Carassius Auratus* against *Aeromonas hydrophila*. *J Vaccines Vaccin* **6**, 3. doi: 10.4172/2157-7560.1000281

Dumpala, P.R., Lawrence, M.L. and Karsi, A. (2009) Proteome analysis of *Edwardisella ictaluri*. *Proteomics* **9**, 1535-1363. doi: 10.1002/pmic.200800652.

Ebanks, R.O., Goguen, M., Mckinnon, S., Pinto, D.M. and Ross N.W. (2005) Identification of the majour outer membrane proteins of *Aeromonas salmonicida*. *Dis Aquat Organ* **68**, 29-38. doi: 10.3354/dao068029.

Figueiredo, H.C.P., Leal, C.A.G, Pereira, F.L., Soares, S.C., Gonçalves, L.A., Dorella, F.A., et al. (2016). Whole-genome sequence of *Francisella noatunensis* subsp. *orientalis* strain FNO01 isolated from diseased Nile tilapia in Brazil. *Genome Announc* **4**(1), e01603-15. doi:10.1128/genomeA.01603-15.

Fulop, M., Manchee, R. and Titball, R. (1996) Role of two outer membrane antigens in the induction of protective immunity against *Francisella tularensis* strains of different virulence. *FEMS Immunol Med Microbiol* **13**, 245–247. PMID: 8861037

Gauthier, A., Puente, J.L. and Finlay, B.B. (2003) Secretin of the enteropathogenic *Escherichia coli* type III secretion system requires components of the type III apparatus for assembly and localization. *Infect Immun* **71**, 3310-3319. PMID: 12761113.

Godlewska, R., Wisniewska, K., Pietras, Z. and Jagusztyn-Krynicka, E.K. (2009) Peptidoglycan - associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis. *FEMS Microbiol Lett* **298**, 1–11. doi: 10.1111/j.1574-6968.2009.01659.x.

Golovliov, I., Ericsson, M., Sandstrom, G., Tarnvik, A., and Sjostedt, A. (1997). Identification of proteins of *Francisella tularensis* induced during growth in macrophages and cloning of the gene encoding a prominently induced 23-kilodalton protein. *Infect Immun* **65**, 2183–2189. PMID: 9169749.

Golovliov, I., Baranov, V., Krocova, Z., Kovarova, H., and Sjostedt, A. (2003). An attenuated strain of the facultative intracllular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. *Infect Immun* **71**, 5940–5950. doi: 10.1128/IAI.71.10.5940-5950.2003

Gonçalves, L.A., Soares, S.D.C, Pereira, F.L., Dorella, F.A., De Carvalho, A.F., Magno, G., et al. (2016) Complete genome sequences of *Francisella noatunensis* subsp *orientalis* strains FNO12, FNO24 and FNO 190: a fish pathogen with genomic clonal behaviour. *Stand Genomic Sci* 1–8. doi.org/10.1186/s40793-016-0151-0.

Havlasova, J., Hernychova, L., Halada, P., Pellantova, V., Krejsek, J., Stulik, J., et al. (2002). Mapping of immunoreactive antigens of *Francisella tularensis* live vaccine strain. *Proteomics* **2**, 857–867. doi: 10.1002/1615-9861(200207)2:7<857:AID-PROT857> 3.0.CO;2-L.

Havlasova, J., Hernychova, L., Brychta, M., Hubalek, M., Lenco, J., Larsson, P., et al. (2005). Proteomic analysis of anti-*Francisella tularensis* LVS antibody response in murine model of tularemia. *Proteomics* **5**, 2090–2103. doi: 10.1002/pmic.200401123.

Hazlett, K.R., Cirillo, K.A. (2009) Environmental adaptation of *Francisella tularensis*. *Microbes Infect* **11**, 828–834. doi: 10.1016/j.micinf.2009.06.001

Heilpern, A., and Waldor, M. (2000) CTX ϕ infection of *Vibrio cholerae* requires the tolQRA gene products. *J Bacteriol* **182**, 1739-1747. PMCID: PMC94473.

Hellman, J., Roberts, J.D.J., Tehan, M.M., Allaire, J.E. and Warren, H.S. (2002). Bacterial peptidoglycan-associated lipoprotein is released into the bloodstream in Gram-negative sepsis and causes inflammation and death in mice. *J Biol Chem* **277**, 14274–80. doi:10.1074/ jbc.M109696200.

Henderson, B. and Martin, A. (2014) Bacterial Virulence in the Moonlight: Multitasking Bacterial moonlighting proteins are virulence determinants in infectious Disease. *Infect Immun* **79**(9), 3476–3491. doi: 10.1128/IAI.00179-11

Herskovits, A.A., Shimoni E., Minsky A. and Bibi, E. (2002) Accumulation of endoplasmic membranes and novel membrane bound ribosome-signal recognition particle receptor complexes in Escherichia coli. *J Cell Biol* **159**, 403–410. doi: 10.1083/jcb.200204144

Hickey, A.J., Hazlett, K.R.O., Kirimanjeswara, G.S., Metzger, D.W. (2011). Identification of *Francisella tularensis* outer membrane protein A (FopA) as a protective antigen for tularemia. *Vaccine* **29**(40), 6941–6947. /doi.org/10.1016/j.vaccine.2011.07.075

Huntley, J.F., Conley, P.G., Hagman, K.E., Norgard, M.V. (2007). Characterization of *Francisella tularensis* outer membrane proteins. *J Bacteriol* **189**(2), 561–574. doi.org/10.1128/JB.01505-06

Janovska, S., Pavkova, I., Hubalek, M., Lenco, J., Macela, A. and Stulik, J. (2007) Identification of the immunoreactive antigens in the membrane enriched fraction from *Francisella tularensis* LVS. *Immunol Lett* **108**, 151-159. doi: 10.1016/j.imlet.2006.12.004.

Kay, W., Petersen, B.O., Duus, J.Ø., Perry, M.B. and Vinogradov, E. (2006) Characterization of the lipopolysaccharide and β-glucan of the fish pathogen *Francisella victoria*. *FEBS J* **273**, 3002-3013. doi: 10.1111/j.1742-4658.2006.05311.x.

Koebnik, R., Locher, K.P. and Van Gelder, P. (2000) Structure and function of bacterial outer membrane proteins: Barells in nutshell. *Mol Microbiol* **37**, 239-253. PMID: 10931321

Kumar, G., Sharma, P., Rathore, G., Bisht, D. and Sengupta, U. (2009) Proteomic analysis of outer membrane proteins of *Edwardisella tarda*. *J Appl Microbiol* **108**, 2214-2221. doi: 10.1111/j. 1365-2672.2009.04627.x

Lee, B.Y., Horwitz, M.A. and Clemens, D.L. (2006). Identification, recombinant expression, immunolocalization in macrophages, and T-cell responsive- ness of the major extracellular proteins of *Francisella tularensis*. *Infect Immun.* **74**(7), 4002–4013. doi.org/10.1128/IAI.00257-06.

Lagos, L., Tandberg, J.I., Repnik, U., Perben, B., Ropstad, E., Vareky, D., et al. (2017) Characterization and vaccine potential of membrane vesicle produced by *Francisella noatunensisi* subsp. *orientalis* in adult Zebrafish Model. *Clin Vaccine Immuno* **24**(5), pii: e005517-16. doi: 10.1128/CVI.00557-16.

Lai, X.H., Golovliov, I. and Sjöstedt, A. (2004) Expression of IglC is necessary for intracellular growth and induction of apoptosis in murine macrophages by *Francisella tularensis*. *Microb pathog* **37**, 225-230. doi: 10.1016/j.micpath.2004.07.002.

Lin, J., Huang, S. and Zhang, Q. (2002) Outer membrane proteins: key players for bacterial adaptation in host niches. *Microbe Infect* **44**, 325e31. PMID: 11909743

Liu, G.Y., Nie, P., Zhang, J. and Li, N. (2008) Proteomic analysis of the sarcosine-insoluble outer membrane fraction of *Flavobacterium columnare*. *J Fish Dis* **31**(4), 269-276. doi: 10.1111/j.1365-2761.2007.00898.x.

Ludu, J.S., De Bruin, O.M., Duplantis, B.N., Schmerk, C.L., Chou, A.Y., Elkins, K.L., et al. (2008) The Francisella pathogenicity island protein PdpD is required for full virulence and associates with homologues of the type VI secretion system. *J Bacteriol* **190**, 4584-4595. doi:10.1128/JB.00198-08.

Luo, Z., Liu, Z., Fu, J., Zhang, Q., Huang, B. and Nie, P. (2016) Immunogenicity and protective role of

antigenic regions from five outer membrane proteins of *Flavobacterium columnare* in grass carp *Ctenopharyngodon idella*. *Chinese J Oceanol Limnol* **34**, 1247. doi.org/10.1007/s003-016-5096-z.

Maiti., B, Shetty, M., Shekar, M., Karunasagar, I., Karunasagar, I. (2011) Recombinant outer membrane protein A (OmpA) of *Edwardsiella tarda*, a potential vaccine candidate for fish, common carp. *Microbiol Res* **167**(1), 1-7. doi: 10.1016/ j.micres.2011.02.002

Maji, S., Mali, P. and Joardar, S.N. (2006) Immunoreactive antigens of the outer membrane protein of *Aeromonas hydrophila*, isolated from goldfish, *Carassius auratus* (Linn.) *Fish Shellfish immunol* 462-473. doi: 10.1016/j.fsi.2005.06.003

McCaig, W.D., Koller, A. and Thanassi, D.G. (2013) Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. *J Bacteriol* **195**, 1120-1132. doi: 10.1128/JB.02007-12.

Melillo, A., Sledjeski, D.D., Lipski, S., Wooten, R.M., Basrur, V. and Lafontaine, E.R. (2006). Identification of a *Francisella tularensis* LVS outer membrane protein that confers adher ence to A549 human lung cells. *FEMS Microbiol Lett* **263**, 102–108. doi: 10.1111/j.1574-6968.2006.00413.x.

Mukhopadhaya, A., Mahalanabis, D. and Chakrabarti, M.K. (2006) Role of *Shigella flexneri* 2a 34 kDa outer membrane protein in in-duction of protective immune response. *Vaccine* **24**, 6028-6036. doi:10.1016/j.vaccine.2006.03.026 .

Nano, F.E., Zhang, N., Cowely, S.C., Klose, K.E., Cheung, K.K., Roberts, M.J., Ludu, J.S., Letendre, G.W., Meiervonics, A.I., Stephens, G., Elkins, K.L. (2004) A *Francisellsa tularensis* pathogenicity island required for intramacrophage growth. *J Bacteriol* **186,** 6430-6436. doi: 10.1128/JB.186.19.6430-6436.2004

Nguyen, M.T. and Götz, F. (2016) Lipoproteins of Gram-Positive Bacteria: Key players in the immune response and virulence. *Microbiol Mol Biol Rev* **80**, 891-903. doi: 10.1128/MMBR.00028-16.

Ortega, C., Mancera, G., Enriquez, R., Vargas, A., Martinez, S., Fajardi, R., et al. (2016) First identification of *Francisella noatunensis* subsp. *orientalis* causing mortality in Mexican tilapia *Oreochromis spp. Dis Aquat Org* **120**, 205–215. doi: 10.3354/dao02999.

Oscarsson, J., Karched, M., Thay, B., Chen, C. and Asikainen, S. (2008) Pro inflammatory effect in whole blood by free soluble bacterial components released from planktonic and biofilm cells. *BMC Microbiol* **8**(206), 13. doi: 10.1186/1471-2180-8-206.

Pavkova, I., Hubalek, M., Zechovska, J., Lenco, J., Stulik, J. (2005) *Francisella tularensis* live vaccine strain: Proteomic analysis of membrane protein enriched fraction. *Proteomics* **5**, 2460-2467. doi: 10.1002/pmic.200401213.

Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551–67. PMID:10612281

Qian, R.H., Xiao, Z.H., Zhang, C.W., Chu, W., Wang, L.S., Zhou, H., et al. (2008) A conserved outer membrane protein as an effective vaccine candidate from *Vibrio alginolyticus*. *Aquaculture* **278**, 5–9. doi.org/10.1016/j.aquaculture.2008.03.010.

Qiang, L., Ningqiu, L., Xiaozhe, F., Qiandong, H., Chang, O., Lihui, L., et al. (2015) An outbreak or granulomatous inflammation associated with *Francisella noatunensis* subsp. *orientalis* in farmed tilapia (*Oreochromis niloticus* x *O. aureus*) in China. *Chin J Ocean and Limnol* **34**, 460–466. doi.org/10.1007/s00343-016-4311-2.

Ramirez-Paredes, J.G. (2015) The fish pathogen *Francisella orientalis*: characterisation and vaccine development. PhD Thesis. University of Stirling, UK. http://hdl.handle.net/1893/21822

Ramirez-Paredes, J.G., Thompson, K.D., Metselaar, M., Shahin, K., Soto, E., Richard, R.H., et al. (2017a) A polyphasic approach for phenotypic and genetic characterisation of the fastidious aquatic pathogen *Francisella noatunensis* subsp. *orientalis* (2017). *Front Microbiol* **12**, 8-2324. doi: 10.3389/fmicb.2017.02324.

Ramirez-paredes, J.G., Larsson, P., Wehner, S., Thompson, K.D., Richards, H., Penman, J., et al. (2017b). Draft Genome Sequence of Francisella noatunensis subsp. orientalis STIR-GUS-F2f7, a Highly Virulent Strain Recovered from Diseased Red Nile Tilapia Farmed in Europe. *Genome Announc* **5**, e01555-16. doi.org/10.1128/genome A.01555-16.

Rodrigues, M.V., Francisco, C.J., David, G.S., Da Silva, D.J., Falcone-Dias, M.F., Júnior, J.P. (2017) Monitoring of *Francisella noatunensis* subsp. *orientalis* in farmed Nile tilapia (*Oreochromis niloticus*) in Brazil. *Aquac Int* **26**(1), 127-138. doi.org/10.1007/s10499-017-0204-4.

Santic, M., Molmeret, M., Klose, K.E., Jones, S., and Kwaik, Y.A. (2005). The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. *Cell Microbiol* **7**, 969–979. doi: 10.1111/j.1462-5822.2005.00526.x.

Santoni, V., Molloy, M. and Rabilloud T (2000) Membrane proteins and proteomics: Un amour impossible? *Electrophoresis* **21**(6), 1054:1070. PMID: 10786880

Schrøder, M.B., Ellingsen, T., Mikkelsen, H., Norderhus, E.A. and Lund, V. (2009) Comparison of antibody responses in Atlantic cod (*Gadus morhua* L.) to *Vibrio anguillarum, Aeromonas salmonicida* and *Francisella sp. Fish Shellfish Immunol* **27**, 112-119. doi: 10.1016/j.fsi.2008.11.016.

Seltman, G. and Holst, O. (2002). The bacterial cell wall. In: *The outer membrane of Gram Negative Bacteria and their compunds.* Editor: Fischer, 9-40. Berlin, Heiderberg: Springer.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analyt Chem* **68**, 850–858. PMID: 8779443.

Soto, E., Hawke, J.P., Fernandez, D. and Morales, J.A. (2009a) *Francisella* sp., an emerging pathogen of tilapia, *Oreochromis niloticus* L., in Costa Rica. *J Fish Dis* **32**, 713-722. doi: 10.1111/j.1365-2761.2009.01070.x.

Soto, E., Fernandez, D. and Hawke, J.P. (2009b) Attenuation of the fish pathogen *Francisella* sp. by mutation of iglC gene. *J Aquat Anim Health* **21**, 140-149. doi: 10.1577/H08056.1.

Soto, E., Wiles, J., Elzer, P., Macaluso, K. and Hawke, J.P. (2011) Attenuated *Francisella asiatica* iglC mutant induces protective immunity to francisellosis in tilapia. *Vaccine* **29**, 593-598. doi: 10.1016/j.vaccine.2010.06.040.

Sridhar, S., Sharma, A., Kongshaug, H., Nilsen, F. and Jonassen, I. (2012) Whole genome sequencing of the fish pathogen *Francisella noatunensis* subsp. *orientalis* Toba04 gives novel insights into Francisella evolution and pathogenicity. *BMC Genomics* **13**, 598. doi:10.1186/ 1471-2164-13-598

Sun, Y., Liu, C.S. and Sun, L. (2011) Comparative study of the immune effect of *Edwardisella tarda* antigens in two forms: subunit and DNA vaccine. *Vaccine* **29**, 2051-2057. doi: 10.1016/j.vaccine.2011.01.013.

Taylor, G.K. and Goodlett, D.R. (2005). Rules governing protein identification by mass spectrometry. *Rapid Commun Mass Spectrom* **19**(23), 3420. doi: 10.1002/rcm.2225.

Thangaviji, V., Michaelbabu, M., Anand, SB., Gunasekaran, P., Citarasu, T. (2012) Immunization with the Aeromonas OMP Provides Protection against A*eromonas hydrophila* in Gold fish (Carassius

auratus). *J Microbial Biochem Technol* **4**, 2. doi: 10.4172/1948-5948.1000070

Telepnev, M., Golovliov, I., Grundström, T.,Tärnvik, A. and Sjöstedt, A. (2003) *Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-α and IL-1 from murine macrophages. *Cell Microbiol* **5**, 41-51. PMID: 12542469

Vipond, V., Suker, J., Jones, C., Tang, C., Feavers, I.M. and Wheelers, J.X. (2006) Proteomic analysis of a meningeococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. *Proteomics* **6**, 3400-3413. doi: 10.1002/pmic.200500821.

Wang, N., Yang, Z., Zang, M., Liu, Y. and Lu, C. (2013) Identification of Omp38 by immunoproteomic analysis and evaluation as a potential vaccine antigen against *Aeromonas hydrophila* in Chinese breams. *Fish Shellfish immunol* **34**, 74-78. doi: 10.1016/j.fsi.2012.10.003.

Watson, E., Sherry, A., Inglis, N.F., Lainson, A., Jyothi, D., Yaga, R., et al. (2014) Proteomic and genomic analysis reveals novel *Campylobacter jejuni* outer membrane proteins and potential heterogeneity. *EuPA Open Proteomics* **4**, 184–194. doi: 10.1016/j.euprot.2014.06.003

Wimley, W.C. (2003) The versatile beta-barel membrane proteins. *Curr Opin Struct Biol* **13,** 404-411. PMID: 12948769.

Yu, L.P., Hu, Y., Sun, B. and Sun, L. (2013) Immunological study of the outer membrane proteins of *Vibrio harveyi*: Insights that link immunoprotectivity to interference with bacterial infection. *Fish Shellfish immunol* **35**(4), 1293-300. doi: 10.1016/j.fsi.2013.07.043.

**Tables**

**Table 1.** Genomes used in this study

| Bacteria ID | Source | Gene bank accession no. | Genome status | Reference |
|---|---|---|---|---|
| *Fno* STIR-GUS-F2f7 | Tilapia (UK) | LTD00000000.1 | Complete | Ramirez-paredes *et al.*,2017b. |
| FNO01 | Tilapia (Brazil) | CP012153.2 | Complete | Figueiredo *et al.*, 2016. |
| FNO12 | Tilapia (Brazil) | CP011921 | Complete | Gonçalves *et al.*, 2016 |
| FNO24 | Tilapia (Brazil) | CP011922 | Complete | Gonçalves *et al.*, 2016 |
| FNO190 | Tilapia (Brazil) | CP011923 | Complete | Gonçalves *et al.*, 2016 |
| Fno Toba-04 | Tilapia (Indonesia) | NC_017909 | Complete | Sridhar *et al.*, 2012. |

**Table.2** List of top 20 proteins identified in the outer membrane proteome of *Francisella noatunensis* subsp. *orientalis*, isolate *Fno* STIR-GUS-F2f7

| No. | NCBI accession no. | Protein ID | MW [kDa] | pI | Mascot Score | No. of peptides matched | Sequence coverage (%) |
|---|---|---|---|---|---|---|---|
| 1 | gi\|300193845\| | PdpD | 139.9 | 6.2 | 4477.2 | 72 | 61.4 |
| 2 | gi\|386872131\| | Chaperone ClpB | 96.0 | 5.4 | 2588.9 | 49 | 57.7 |
| 3 | gi\|169589436\| | PdpD | 139.6 | 6.1 | 2478.8 | 39 | 32.1 |
| 4 | gi\|300193842\| | IglC | 22.1 | 5.3 | 2388.4 | 14 | 84.2 |
| 5 | gi\|386871181\| | Chaperonin GroEL | 57.1 | 4.9 | 1857.1 | 33 | 63.7 |
| 6 | gi\|103012949\| | Ribosomal L29e protein family | 126.8 | 8.9 | 1839.5 | 42 | 46.6 |
| 7 | gi\|386871126\| | Elongation factor | 43.3 | 5.0 | 1585.1 | 36 | 75.6 |
| 8 | gi\|386872079\| | Bifunctional proline dehydrogenase/pyrroline-5-carboxylate | 149.5 | 7.8 | 1539.6 | 33 | 34.6 |
| 9 | gi\|300193843\| | IglB | 57.5 | 4.7 | 1526.5 | 30 | 59.9 |
| 10 | gi\|386870689\| | 30S ribosomal protein S1 | 61.5 | 5.2 | 1389.2 | 26 | 58.1 |
| 11 | gi\|386870694\| | Cell division protein FtsZ | 39.3 | 4.6 | 1354.0 | 24 | 85.4 |
| 12 | gi\|386871696\| | Outer membrane associated protein | 41.3 | 5.2 | 1307.4 | 22 | 42.8 |
| 13 | gi\|386871889\| | Chorismate binding family protein | 120.0 | 5.6 | 1243.3 | 26 | 31.3 |
| 14 | gi\|386870866\| | OmpA family protein | 47.2 | 6.0 | 1090.3 | 10 | 32.1 |
| 15 | gi\|860224409\| | Non-ribosomal peptide synthetase | 249.4 | 5.2 | 1046.5 | 27 | 18.2 |
| 16 | gi\|386871950\| | Ribonuclease E | 101.4 | 8.3 | 1036.3 | 19 | 27.4 |
| 17 | gi\|300193844\| | IglA | 20.4 | 8.6 | 1010.3 | 12 | 47.2 |
| 18 | gi\|386871082\| | Alpha-ketoglutarate decarboxylase | 105.5 | 6.1 | 1009.2 | 21 | 27.9 |
| 19 | gi\|386870797\| | Heat shock protein 90 | 72.2 | 5.3 | 992.4 | 21 | 36.9 |
| 20 | gi\|386871083\| | 2-oxoglutarate dehydrogenase complex, E2 component | 52.5 | 5.0 | 991.1 | 18 | 45.6 |

MW: molecular mass; pI: isoelectric point

**Table.3** List of the top 20 proteins identified in the immunogenic band (17-28 kDa) of the outer membrane proteome of *Francisella noatunensis* subsp. *orientalis*, *Fno* STIR-GUS-F2f7

| No. | NCBI accession no. | Protein ID | MW [kDa] | pI | Mascot Score | No. of peptides matched | Sequence coverage (%) |
|---|---|---|---|---|---|---|---|
| 1 | gi\|300193842\| | IglC | 22.1 | 5.3 | 2388.4 | 14 | 84.2 |
| 2 | gi\|504527828\| | IglA | 20.4 | 8.6 | 754.5 | 12 | 74.2 |
| 3 | gi\|504527329\| | OmpA family peptidoglycan-associated lipoprotein | 23.4 | 4.8 | 753.9 | 9 | 64.9 |
| 4 | gi\|504527815\| | beta-ketoacyl-ACP reductase | 26.3 | 9.6 | 601.2 | 10 | 55.9 |
| 5 | gi\|504527529\| | succinate dehydrogenase iron-sulfur subunit | 26.5 | 8.8 | 540.4 | 11 | 54.9 |
| 6 | gi\|504527238\| | 50S ribosomal protein L5 | 20.0 | 9.7 | 526.4 | 11 | 61.5 |
| 7 | gi\|504527915\| | AhpC/TSA family peroxiredoxin | 21.8 | 5.0 | 470.0 | 7 | 57.8 |
| 8 | gi\|504528404\| | enoyl-ACP reductase | 27.7 | 5.5 | 449 | 11 | 55.4 |
| 9 | gi\|386871251\| | Hypothetical protein OOM-0776 | 22.5 | 9.8 | 388.6 | 9 | 41.3 |
| 10 | gi\|504527834\| | hypothetical protein | 24.3 | 5.6 | 376.8 | 5 | 33.5 |
| 11 | gi\|504527577\| | 50S ribosomal protein L1 | 24.5 | 9.5 | 374.1 | 9 | 39.4 |
| 12 | gi\|504527226\| | 50S ribosomal protein L3 | 22.1 | 9.5 | 359.4 | 6 | 40.5 |
| 13 | gi\|504527216\| | 30S ribosomal protein S2 | 26.5 | 8.8 | 333.7 | 5 | 21.3 |
| 14 | gi\|855345305\| | Transcription termination/antitermination protein nusG | 20.0 | 6.8 | 333.1 | 7 | 41.2 |
| 15 | gi\|855345177\| | Hypothetical protein | 27.9 | 9.4 | 330.4 | 6 | 27.2 |
| 16 | gi\|504527578\| | 50S ribosomal protein L10 | 18.7 | 9.1 | 274.5 | 8 | 45.3 |
| 17 | gi\|504527053\| | chorismate mutase | 20.3 | 9.2 | 267.1 | 5 | 40.6 |
| 18 | gi\|504527683\| | DNA-binding response regulator | 25.5 | 6.2 | 261.9 | 5 | 32.9 |
| 19 | gi\|504527248\| | 30S ribosomal protein S4 | 23.2 | 10.4 | 245.0 | 4 | 24.8 |
| 20 | gi\|504527363\| | LemA-like protein | 21.9 | 6.0 | 235.4 | 6 | 37.2 |

MW: molecular mass; pI: isoelectric point

**Table 4.** Bioinformatic analysis of the top 20 proteins identified in the outer membrane enriched fraction of *Francisella noatunensis* subsp. *orientalis*, *Fno* STIR-GUS-F2f7

| No | Protein ID. | PSORTb[a] | COGs[b] | LipoP[c] | SignalP[d] |
|---|---|---|---|---|---|
| 1 | PdpD | Outer membrane | S | N | N |
| 2 | Chaperone ClpB | Cytoplasmic | O | N | N |
| 3 | PdpD | Outer membrane | S | N | N |
| 4 | **IglC *** | Unknown | S | N | N |
| 5 | Chaperonin GroEL | Cytoplasmic | O | N | N |
| 6 | Ribosomal L29e protein family | Outer membrane | S | N | N |
| 7 | **OmpA family peptidoglycan-associated lipoprotein *** | Outer membrane | M | Y | Y (SpII) |
| 8 | Bifunctional proline dehydrogenase | Cytoplasmic | C | N | N |
| 9 | IglB | Cytoplasmic | S | N | N |
| 10 | 30S ribosomal protein S1 | Cytoplasmic | J | N | N |
| 11 | Cell division protein FtsZ | Cytoplasmic | D | N | N |
| 12 | Outer membrane associated protein | Outer membrane | M | Y | Y (SpI) |
| 13 | PdpA | Unknown/multiple localization | S | N | N |
| 14 | OmpA family protein | Outer membrane | M | Y | Y (SpII) |
| 15 | PdpB | Outer membrane | M | N | N |
| 16 | Ribonuclease E | Cytoplasmic | E | N | N |
| 17 | **IglA *** | Cytoplasmic | S | N | N |
| 18 | Alpha-ketoglutarate decarboxylase | Cytoplasmic | G | N | N |
| 19 | Heat shock protein 90 | Cytoplasmic | O | N | N |
| 20 | 2-oxoglutarate dehydrogenase complex, E2 component. | Cytoplasmic | C | N | N |

[a] Subcellular localization as predicted by PSORTb v. 2.0 (http://psort.org/).

[b] Functional classification of the tentative proteins as predicted by EggNOG v. 4.5 server (http://eggnog.embl.de). The COGs functional categories are: C: energy production and conversion; D: Cell cycle control, cell division and chromosome partitioning; E: amino acid transport and metabolism; F: Nucleotide transport and metabolism; G: carbohydrate transport and metabolism; H: Coenzyme transport and metabolism; I: lipid transport and metabolism; J: Translation, ribosomal structure and biogenesis; K: Transcription; L: replication, recombination and repair; M: cell wall/membrane biogenesis; O: Post-translational modification, protein turnover, and chaperones; P: Inorganic ion transport and metabolism; Q: Secondary metabolites biosynthesis, transport, and catabolism; S: Unknown function (includes Category R with general function and category N not in known COGs); T: Signal transduction mechanisms; U: Intracellular trafficking, secretion, and vesicular transport; V: Defence mechanisms.

[c] Lipoproteins prediction by LipoP v. 1.0 (http://www.cbs.dtu.dk/services/Lipo/) , Y: Yes, N: No.

[d] Signal peptide sequence prediction by SignalP v. 2.0 (http://cbs,dtu.dk/services/Signal P/), Y: Yes, N: No, SpI: Signal peptides cleave by signal peptidase I, SpII: Signal peptides cleaved by signal peptidase II.

[*] Immuno-reactive proteins (In bold).

**Figure legends**

**Figure 1.** SimplyBlue Safe stained 1D PAGE of *Francisella noatunensis* subsp. *orientalis*, *Fno* STIR-GUS-F2f7 outer membrane proteins (OMP) (A) and 2 representative immunoblots (B) and (C). Immunoreactivity was seen between the pooled-anti *Fno* sera from tilapia surviving challenge and *Fno* OMPs (B), while no reaction was observed with control tilapia sera (C). The arrow refers to an abundant protein band between 17-28 kDa in the OMP profile (A) and the immune-reactive protein band on blot (B). M: Protein molecular weight standard. The gel and the 2 blots are representatives of two replicates.

**Figure 2.** Summary of bioinformatics analysis of proteins identified in *Francisella noatunensis* subsp. *orientalis*, *Fno* STIR-GUS-F2f7 OMP fraction. (A) Prediction of biological functions [Transitional, ribosomal structure and biogenesis (17.6%), Not related to COGs (16.7%), Energy production and conversion (13%), Cell wall, membrane and envelope biogenesis (8.4%), Post transitional modification and protein turnover (8.4%), Carbohydrate transport and metabolism (5.8%), Amino acid transport and metabolism (5.4%), Lipid transport and metabolism (5.4%), Nucleotide transport and metabolism (2.9%), Transcription (2.9%), Intracellular trafficking, secretion and vesicular transport (2.5%), Co-enzyme transport and metabolism (2%), Inorganic ion transport and metabolism (2%), Replication, recombination and repair (2%), Cell cycle control and cell division (1.7%), Secondary metabolites biosynthesis (1.7%), Signal transduction mechanism (0.8%), T Defense mechanism (0.4%)], (B) Subcellular localization [Cytoplasmic (62%), Cytoplasmic membrane (11.7%), Unknown (11%),

■ Outer membrane (5.8%), ■ Unknown/multiple locations (5.4%), ■ Periplasmic (2.5%), ■ Cytoplasmic/multiple locations (0.8%), ■ Extracellular (0.8%) and (C) cleavage by signal peptidases [■ Unknown (83.3%), ■ Signal peptidase I (spI) (10%), ■ Signal peptidase II (spII) (6.7%)]

**Supporting information**

**Table S1.** Outer membrane proteins of *Francisella noatunensis* subsp. *orientalis* (STIR-GUS- F2f7) identified by LC/ESI/MS/MS analysis.

**Table S2.** Immunogenic outer membrane proteins of *Francisella noatunensis* subsp. *orientalis* (STIR-GUS-F2f7) identified by LC/ESI/MS/MS analysis.

**Table S3.** Bioinformatic predictions of the proteins identified in the outer membrane enriched fraction of *Francisella noatunensis* subsp. *orientalis* (STIR-GUS-F2f7)