Characterization of the outer membrane proteome of Francisella noatunensis subsp. orientalis

K. Shahin, K.D. Thompson, N.F. Inglis, K. Mclean, J.G. Ramirez-Paredes, S.J. Monaghan, R. Hoare, M. Fontaine, M. Metselaar and A. Adams

1 Faculty of Natural Sciences, Institute of Aquaculture, University of Stirling, Stirling, Scotland, UK
2 Aquatic Animals Diseases Lab, Aquaculture Division, National Institute of Oceanography and Fisheries, Suez, Egypt
3 Moredun Research Institute, Pentlands Science Park, Penicuik, Midlothian, UK
4 Benchmark Animal Health, Bush House, Edinburgh Technopole, Edinburgh, Midlothian, UK

Abstract

Aims: The aims of the current study were to characterize the outer membrane proteins (OMPs) of Francisella noatunensis subsp. orientalis (Fno) STIR-GUS-F2f7, and identify proteins recognized 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 the United Kingdom, was examined. The sarcosine-insoluble OMPs fraction was screened with tilapia hyperimmune sera by western blot analysis following separation of the proteins by 1D SDS-PAGE. Liquid chromatography-electrospray ionization-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 recognized 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 conjunction 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 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.

Introduction

Francisella noatunensis subsp. orientalis (Fno) is the causative agent of piscine francisellosis in a wide range of warm water fish species (Colquhoun and Duodu, 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 neutralizing activity against target micro-organisms (Lin et al. 2002). The OMPs of a variety of fish pathogenic bacteria have previously been characterized, including those of Flavobacterium columnare (Liu et al. 2008; Luo et al. 2016), Streptococcus iniae (Cheng et al. 2010), Edwardsiella tarda (Kumar et al. 2009; Sun et al. 2011), Edwardsiella ictaluri (Dumpala et al. 2009), Aeromonas hydrophila (Wang et al. 2013), Aeromonas salmonicida (Ebanks et al. 2005), Vibrio harveyi (Yu et al. 2013) and Vibrio alginolyticus (Qian et al. 2008). A more comprehensive characterization 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; Thangavijji et al. 2012; Yu et al. 2013; Divya et al. 2015).

Immunoproteomics is a powerful approach used to highlight and identify immunoreactive components of the bacterial outer membrane proteome where innately hydrophobic membrane-associated proteins are solubilized 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 immunoreactive complexes (Boyce et al. 2006).

Previous studies have identified some potential Fno-pathogenicity determinants including those genes responsible for intracellular localization, 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 OMPs profile of Fno (STIR-GUS-F2f7) was catalogued and proteins recognized 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) following incubation at 28°C for 72 h. For liquid cultures, aliquots of modified Mueller–Hinton broths (MMHB) containing 2% isovitalex and 0-1% glucose (Difco, BD) were inoculated with a single bacterial colony from CHAH plates and incubated at 28°C for 18 h with shaking at 150 rev min⁻¹.

Extraction of OMPs

Outer membrane proteins 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 5000 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 3000 g for 10 min. The wash buffer was discarded, and the pellet resuspended 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, Dorset, UK) and 10 μl of protease inhibitor cocktail (Sigma-Aldrich, Dorset, UK). The cell suspension was then transferred to 1.5 ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing 0.1 ml Zirconium silica beads (Thistle Scientific, Glasgow, UK), and cells were disrupted in a FastPrep homogenizer B101011 (MP Biomedicals, Bedford, UK) for 6 × 30 s, with cooling on ice for 5 min between each cycle. The lysate was then transferred to a fresh 1.5-ml microcentrifuge 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 resuspension, and incubation on ice for 1 min prior to discarding the supernatant once more. The cell pellet was then resuspended 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°C) for 30 s. Finally, the pellet was resuspended in 1 ml of 10 mmol l⁻¹ Tris HCl (pH 7-0) containing 0-5% (w/v) N-laurylsarcosine and 0-1% (w/v) sodium dodecyl sulphate (SDS) on a variable speed vortex mixer (Cole-parmer, St. Neots, UK) for 30 s. The concentration of OMPs was determined by BCA assay (Pierce BCA protein assay
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Fish challenge and serum samples

Healthy Nile tilapia, O. 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 1 × 10^6 CFU per ml (LD<sub>50</sub>) of Fno (STIR-GUS-F217) while the second group received i.p. injection of PBS as control. The bacterial inoculum was prepared using MMHB containing 2% Isosol 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 polymyxin B 100 U ml<sup>-1</sup>, 0.5% v/v Tween 20, pH 7.0 washed three times by low salt wash buffer (LSWB: 0.02 mol l<sup>-1</sup> Trizma base, 0.38 mol l<sup>-1</sup> NaCl, 0.05% v/v Tween 20, pH 7.2). One hundred microlitres of Fno in PBS at OD<sub>600</sub> 0.4 (~1 × 10<sup>6</sup> CFU per ml) 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 per well of 0.05% v/v glutaraldehyde (Sigma-Aldrich, Dorset, UK) in LSWB and incubated for 20 min at RT. Plates were washed as stated earlier with LSWB. Endogenous peroxidase activity was prevented by adding 100 μl per well of 1:10 of 30% stock solution of hydrogen peroxide (Sigma-Aldrich, Dorset, UK) and plates were incubated for 1 h at RT. Washing was done three times as stated earlier and nonspecific antibody binding was blocked by adding 250 μl per well of 5% w/v dried skimmed milk (Marvel, Premier Foods Group Ltd, Liverpool, UK) in distilled water (DW) for 3 h at RT. After washing the plates three times with LSWB, 100 μl of serum per well from challenged and control fish 21 dpc at 1:500 in LSWB with 1% bovine serum albumin (BSA) (Fisher Scientific, Paisely, 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 five times with high salt wash buffer (HSWB: 0.02 mol l<sup>-1</sup> Trizma base, 0.5 mol l<sup>-1</sup> 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. One hundred microlitres per well of anti-tilapia IgM monoclonal antibodies (Mab) at a dilution of 1:75 in PBS (Fo4; Aquatic Diagnostic Ltd, Stirling, UK) was added and the plates were incubated at RT for 1 h. Washing was repeated using HSWB as stated earlier and 100 μl per well of goat antimouse IgG-HRP MAb (Sigma-Aldrich, Dorset, UK) at a dilution of 1:3000 in LSWB with 1% BSA was 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 (Amresco, MA, USA) for 5 min at RT and the reaction was stopped by adding 50 μl per well of 2 mol l<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> (Sigma-Aldrich, Dorset, UK). The absorbance was measured at OD<sub>450</sub> using a microplate reader (Biotek Synergy HT, Swindon, UK) 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, CA, USA) in a NuPAGE MES SDS running buffer (20x) (Thermo Fisher Scientific, Paisely, 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, CA, USA) following the manufacturer’s instructions and the gel scanned using an Epson expression 1680 artist scanner (Epson, Suwa, Japan). 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, CA, USA) at 30 V for 45 min using 1x NuPAGE Transfer buffer (ThermoFisher Scientific, Paisely, UK), following the manufacturer’s protocol for two replicate gels. Following transfer, the membranes were washed for 5 min in TBS (50 mmol l<sup>-1</sup> Tris, 150 mmol l<sup>-1</sup> NaCl, 0.1% v/v Tween 20, 0.05% w/v poly-lysine in carbonate-bicarbonate buffer (Sigma-Aldrich, Dorset, UK) and incubated at RT (~22°C) for 1 h. The plates were then washed three times by low salt wash buffer (LSWB: 0.02 mol l<sup>-1</sup> Trizma base, 0.38 mol l<sup>-1</sup> NaCl, 0.05% v/v Tween 20, pH 7.2). One hundred microlitres of Fno in PBS at OD<sub>600</sub> 0.4 (~1 × 10<sup>6</sup> CFU per ml) 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 per well of 0.05% v/v glutaraldehyde (Sigma-Aldrich, Dorset, UK) in LSWB and incubated for 20 min at RT. Plates were washed as stated earlier with LSWB. Endogenous peroxidase activity was prevented by adding 100 μl per well of 1:10 of 30% stock solution of hydrogen peroxide (Sigma-Aldrich, Dorset, UK) and plates were incubated for 1 h at RT. Washing was done three times as stated earlier and nonspecific antibody binding was blocked by adding 250 μl per well of 5% w/v dried skimmed milk (Marvel, Premier Foods Group Ltd, Liverpool, UK) in distilled water (DW) for 3 h at RT. After washing the plates three times with LSWB, 100 μl of serum per well from challenged and control fish 21 dpc at 1:500 in LSWB with 1% bovine serum albumin (BSA) (Fisher Scientific, Paisely, 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 five times with high salt wash buffer (HSWB: 0.02 mol l<sup>-1</sup> Trizma base, 0.5 mol l<sup>-1</sup> 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. One hundred microlitres per well of anti-tilapia IgM monoclonal antibodies (Mab) at a dilution of 1:75 in PBS (Fo4; Aquatic Diagnostic Ltd, Stirling, UK) was added and the plates were incubated at RT for 1 h. Washing was repeated using HSWB as stated earlier and 100 μl per well of goat antimouse IgG-HRP MAb (Sigma-Aldrich, Dorset, UK) at a dilution of 1:3000 in LSWB with 1% BSA was 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 (Amresco, MA, USA) for 5 min at RT and the reaction was stopped by adding 50 μl per well of 2 mol l<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> (Sigma-Aldrich, Dorset, UK). The absorbance was measured at OD<sub>450</sub> using a microplate reader (Biotek Synergy HT, Swindon, UK) 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.
pH 7.4) and blocked overnight at 4°C in TBS with 5% (w/v) dried skimmed milk (Marvel, Premier Foods Group Ltd, Liverpool, UK). After washing three 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, Dorset, UK) respectively. Washing was repeated as described before, then 5 ml of 1 : 50 mouse antitilapia IgM Mab (Fo4; Aquatic Diagnostic Ltd, Stirling, UK) in TBS was added to each membrane and incubated with continuous shaking at RT for 1 h. Following washing, 5 ml of goat antimouse HRP MAb (Sigma-Aldrich, Dorset, 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 three 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, CA, USA) to each membrane for 2 min. The reaction was stopped by addition of 5 ml of DW. Membranes were left to dry then scanned using an Epson expression 1680 artist scanner (Epson, Suwa, Japan). Two technical replicate immunoblots were prepared to ensure reproducibility.

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

Gel lanes containing Fno-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 was imported into ProteinScape™ ver. 3.1 (Bruker Daltonics, MA, 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 NCBI Fno subdatabase, utilizing the Mascot™ ver. 2.5.1 (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 gel replicates was formed using the ‘Protein Extractor’ feature of the software. Data were searched specifying Trypsin and Trypsin/P. Spectra used for protein identifications were researched against the entire NCBI Fno 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, while allowing for a single 13C 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 ver. 4.5 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/).

Table 1 Genomes used in this study

| Bacteria ID | Source | Gene bank accession no. | Genome status | Reference |
|-------------|--------|-------------------------|---------------|-----------|
| Fno STR-GUS-F217 | Tilapia (UK) | LT00000000.1 | 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 | Snidhar et al. (2012) |
Results

1D PAGE and immunoblotting

Following electrophoretic separation of Fno STIR-GUS-F2f7-OMPs (Fig. 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 (Fig. 1b). No immunoreactivity was detected in this region by the control fish sera (Fig. 1c).

Protein identification by LC-MS/MS

Mass spectrometric analysis facilitated the confident identification of a total of 239 proteins in the OMPs 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 OMPs fraction are listed in Table 2 and the immunogenic OMPs proteins in Table 3. The full protein lists can be found in supporting information (Tables S1 and S2).

Prediction of function, subcellular localization and lipoproteins of the identified proteins

EggNOG ver. 4.5, PSORTb® ver. 2.0, LipoP ver. 1.0, SignalP ver. 2.0 servers were used to predict the function, subcellular location and lipoprotein nature of the 239 proteins identified in the OMPs 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 localization 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%), OMPs (5.8%), periplasmic proteins (2.5%) and extracellular proteins (0.8%).

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-OMPs 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...
### Table 2 List of top 20 proteins identified in the outer membrane proteome of *Francisella noatunensis* subsp. *orientalis*, isolate *Fno STIR-GUS-F217*

| 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|368672131| 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| IgIC      | 22.1    | 5.3 | 2388.4      | 14                      | 84.2                  |
| 5   | gi|368671181| Chaperonin GroEL | 57.1   | 4.9 | 1857.1      | 33                      | 63.7                  |
| 6   | gi|103011294| Ribosomal L29e protein family | 126.8  | 8.9 | 1839.5      | 42                      | 46.6                  |
| 7   | gi|368671126| Elongation factor | 43.3   | 5.0 | 1585.1      | 36                      | 75.6                  |
| 8   | gi|368672079| Bifunctional proline dehydrogenase/pyrroline-5-carboxylate | 149.5  | 7.8 | 1539.6      | 33                      | 34.6                  |
| 9   | gi|300193843| IgIB      | 57.5    | 4.7 | 1525.6      | 30                      | 59.9                  |
| 10  | gi|368670689| 30S ribosomal protein S1 | 61.5   | 5.2 | 1389.2      | 26                      | 58.1                  |
| 11  | gi|368670694| Cell division protein FtsZ | 39.3   | 4.6 | 1354.0      | 24                      | 85.4                  |
| 12  | gi|368671696| Outer membrane associated protein | 41.3   | 5.2 | 1307.4      | 22                      | 42.8                  |
| 13  | gi|368671889| Chorismate binding family protein | 120.0  | 5.6 | 1243.3      | 26                      | 31.3                  |
| 14  | gi|368670866| OmpA family protein | 47.2   | 6.0 | 1090.3      | 10                      | 32.1                  |
| 15  | gi|860224409| Nonribosomal peptide synthetase | 249.4  | 5.2 | 1046.5      | 27                      | 18.2                  |
| 16  | gi|368671950| Ribonuclease E | 101.4  | 8.3 | 1036.3      | 19                      | 27.4                  |
| 17  | gi|300193844| IgIA      | 20.4    | 8.6 | 1010.3      | 12                      | 47.2                  |
| 18  | gi|368671082| Alpha-ketoglutarate decarboxylase | 105.5  | 6.1 | 1009.2      | 21                      | 27.9                  |
| 19  | gi|368670797| Heat shock protein 90 | 72.2   | 5.3 | 992.4       | 21                      | 36.9                  |
| 20  | gi|368671083| Z-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-F217*

| 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-sulphur subunit | 26.5   | 8.8 | 540.4       | 11                     | 54.9                  |
| 6   | gi|504527238| SOS ribosomal protein L5 | 20.0   | 9.7 | 526.4       | 11                     | 61.5                  |
| 7   | gi|504527919| AhpC/TSA family peroxiredoxin | 21.8   | 5.0 | 470.0       | 7                      | 57.8                  |
| 8   | gi|504528404| Enoyl-ACP reductase | 27.7   | 5.5 | 449.4       | 11                     | 55.4                  |
| 9   | gi|368671251| 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| SOS ribosomal protein L1 | 24.5   | 9.5 | 374.1       | 9                      | 39.4                  |
| 12  | gi|504527226| SOS ribosomal protein L3 | 22.1   | 9.5 | 359.4       | 6                      | 40.5                  |
| 13  | gi|504527216| SOS 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|504527576| SOS 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| SOS 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.
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-OMPs in OMPs 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 nonclassically associated outer membrane 'moon-lighting' 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 transmembrane proteins associated with the transportation of ions and other micromolecules (Wimley 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 ver. 4.5 revealed that most of the abundant 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.

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* | COGs† | LipoP‡ | SignalP§ |
|----|------------|---------|-------|--------|---------|
| 1  | PdpD       | Outer membrane | S      | N      | N       |
| 2  | Chaperone ClpB | Cytoplasmic | O      | N      | N       |
| 3  | PdpD       | Outer membrane | S      | N      | N       |
| 4  | IgIC       | 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  | IgIB       | 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 (SpII) |
| 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 | IgIA       | 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       |

Immunoreactive proteins previously identified in *F. tularensis* (Havlasova et al. 2002; Lee et al. 2006) are highlighted in bold.

*Subcellular localization as predicted by PSORTb ver. 2.0 (http://psort.org/).

†Functional classification of the tentative proteins as predicted by EggNOG ver. 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.

‡Lipoproteins prediction by LipoP ver. 1.0 (http://www.cbs.dtu.dk/services/Lipo/), Y: Yes, N: No.

§Signal peptide sequence prediction by SignalP ver. 2.0 (http://cbs.dtu.dk/services/SignalP/), Y: Yes, N: No, SpI: signal peptides cleaved by signal peptidase I, SpII: signal peptides cleaved by signal peptidase II.
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 ver. 0.2 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. 2007), F. culmipnare (Liu et al. 2008) and Pasteurella multocida (Boye 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 sp., 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 tularaemic patients (Janovska et al. 2007).

The PdpA, PdpB, PdpD, IgIA, IgIB and IgIC 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 localized to the outer membrane of Francisella novicida and is involved in the extracellular virulence of this pathogen by affecting the localization of other FPI proteins including IgIA, IgIB, IgIC and T6SS. IgIA and IgIB 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 IgIA is required for virulence and supporting the growth of the bacterium inside macrophages (De Bruin et al. 2007).

The IgIC protein, which was associated with the immunoreactive band (17–28 kDa) in our Fno-OMPs, is one of the important proteins that is upregulated during intracellular growth of Francisella sp. in macrophages (Golovliov et al. 1997, 2003). Earlier studies reported that IgIC protein, with its regulator MglA, assist the ability of F. tularensis to modulate biogenesis of the phagosome, preventing the formation of the phagolysosome, thus facilitating the escape of the bacteria into the cytoplasm of the host cell following replication (Clemens et al. 2004; Santic et al. 2005). Furthermore, IgIC 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 OMP 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 upregulated 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 antitularaemic antibodies (Havlasova et al. 2002; Lee et al. 2006).

Peptidoglycan-associated lipoproteins 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 (McCag 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 proinflammatory cytokines (Buwitt-Beckmann et al. 2006; Oscarsson et al. 2008; Godlewksa 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 sp. 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 OMPs profile of Fno STIR-GUS-F2F7 was examined by immunoblotting using hyperimmune 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 Schroder et al. (2009), who screened Fnn with serum from Atlantic cod (Gadus morhua, L.) immunized 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 sp. In a separate study by Kay et al. (2006), polyclonal antisera raised against Fno recognized an immuno-dominant band of approximately 20 kDa in the large lipo-oligosaccharide fraction of the proteinase-K-treated whole cell protein lysate. The presence of the immunoreactive band (~17–28 kDa) in the OMPs fraction in our study may support the results obtained in previous studies. More importantly, establishing
the proteins present in the \textit{Fno} outer membrane proteome may enable a greater understanding of which proteins are involved in stimulating the fish’s immune system in response to \textit{Fno} infection.

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

It is worth mentioning that, some of the pathogenicity-related proteins that showed high score in the current \textit{Fno}-OMPs preparation, such as PdpD and FopA were not recognized by the sera from challenged tilapia. However, they were previously described as immunogenic antigens in \textit{F. tularensis} using sera from tularemic patients (Huntley \textit{et al.} 2007; Janovska \textit{et al.} 2007; Hickey \textit{et al.} 2011). This anomaly may be attributed to the limited resolution offered by 1D SDS-PAGE and its inability to separate complex mixtures of proteins that comigrate as a single band. To this end, the use of higher resolution 2D gel electrophoresis together with immunoblotting and downstream mass spectrometry may facilitate a more precise characterization of the protein complement of the \textit{Fno}-OMPs fraction. In summary, the data presented here offers a first insight into OMPs of \textit{Fno}, which help build our understanding of how this organism is able to cause disease.

\section*{Acknowledgements}

This work was supported by the PhD research grant from Egyptian Ministry of Higher Education and Scientific Research (MHESR) (grant no. 1582014) and Benchmark Animal Health Ltd (BAHL) (grant no. 0306-064-2325290). The authors thank Dr Eleanor Watson and Dr Anita Jaglarz (Moredun Research Institute, UK) for their technical assistance. We gratefully acknowledge Dr Andy Shinn (Fish Vet Group Asia ltd.) for his assistance during the fish challenge experiment.

\section*{Conflict of Interest}

The authors declare no conflict of interest.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Outer membrane proteins of *Francisella noatunensis* subsp. *orientalis* (STIR-GUS- F27) 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)