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SHORT COMMUNICATION

Skin mucus proteins of rainbow trout (*Oncorhynchus mykiss*) in response to mucosal vaccination and challenge with *Flavobacterium psychrophilum*

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Flavobacterium psychrophilum is one of the most important pathogens affecting rainbow trout (*Oncorhynchus mykiss*) worldwide at the fry stage of their life cycle. Studies have shown disruption of the fish's skin mucus to be a critical element in the establishment of *F. psychrophilum* infection in rainbow trout (Henriksen et al., 2013; Madetoja et al., 2000; Martínez et al., 2004). Mucus is the first barrier (biochemical and physical) of the fish, playing a vital role in the protection against pathogens, and contains various antibacterial and inflammatory factors such as lysozyme, immunoglobulin, complement, lectins, acute-phase proteins and proteases (Shunsuke, 2016; Subramanian et al., 2007). Furthermore, the study of external fish mucus provides non-lethal alternatives for the early detection of infections.

In the current study, we examined the proteins of skin mucus of rainbow trout following immersion vaccination with *F. psychrophilum*. The influence of the route of infection on mucus proteins was also investigated by comparing skin mucus collected from fish challenged with *F. psychrophilum* by intramuscular injection to fish challenged with immersion. Skin mucus was subjected to 2D sodium dodecyl sulphate–polyacrylamide gel electrophoresis (2D SDS-PAGE) and spots differentially expressed between vaccinated and control fish were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Proteomic studies to elucidate host responses in fish vaccinated and/or infected with *F. psychrophilum* are limited, and to our knowledge, this is the first report on proteomics of skin mucus of trout in response to mucosal vaccination and mucosal challenge with this pathogen.

Rainbow trout fry (3–4 g) and 15-g rainbow trout were maintained in flow-through (5 L/min) dechlorinated tap water at 15°C at the Institute of Aquaculture, University of Stirling, UK. The fish were fed at 2% body weight/day (Inicio feed, BioMar). The *F. psychrophilum*-free status of the fish was determined by streaking samples of head kidney and spleen onto the modified Veggietone medium followed by a nested PCR (Toyama et al., 1994). Fry were immersion-vaccinated with a polyvalent *F. psychrophilum* vaccine (Hoare et al., 2017); controls were immersed in tank water using the same procedure. At 630 degree-days post-vaccination (dd pv), the fish were starved for 24 hr prior to immersion challenge according to Ref. (Hoare et al., 2017). The mucus of fish (vaccinated and unvaccinated) was sampled post-vaccination prior to challenge, and again 32 days after the challenge as described by Ref. (Hoare et al., 2017). Briefly, skin mucus was sampled by placing three fish (from each duplicate tank giving 2 pools of mucus/group) into a plastic bag containing 5 ml of Tris-buffered saline (TBS: 10 mM Tris base and 0.5 M NaCl, pH 7.5) and gently massaging for 2 min. Fish were removed, and the mucus was collected into a centrifuge tube and placed on ice. Any mucus samples contaminated with blood were discarded. The mucus was vortexed vigorously (10 s) and centrifuged at 4000 x *g* for 15 min, and the supernatant was filtered through

a 0.45-μm syringe filter (Sigma-Aldrich, USA), aliquoted into sterile tubes and stored at −70°C until use.

For the intramuscular challenge, five rainbow trout (unvaccinated, 15 ± 2.3 g) were starved for 24 h, anaesthetized with benzocaine and injected intramuscularly (0.05 ml/fish) with a virulent isolate of *F. psychrophilum* (1×10^6 CFU/ml; AVU-1T/07). The fish were maintained in 100-L flow-through tanks with aeration at 15°C and monitored for 32 days. Prior to sampling, fish were killed with an overdose of benzocaine and mucus sampled as for fry, but with five fish per bag in 10 ml of Tris-buffered saline.

Mucus supernatants were defrosted on ice and concentrated 10X using a 10K MWCO centrifuge tube (Merck Millipore), and protease inhibitor (1%; Cytiva, Buckinghamshire, UK) and nuclease mix (1%; Cytiva) were added and mixed by gentle pipetting. Protein concentration was determined using a 2D Quant Kit (Cytiva) according to the manufacturer's instructions. Mucus samples were treated with the 2D Clean-up Kit (Cytiva) according to the manufacturer's instructions. Isoelectric focusing (IPG) strips (pH 4–7, Cytiva) were rehydrated with 60 μg of protein samples initially mixed in a total volume of 125 ml IPG rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG Buffer, 40 mM DTT, 25 ml] and allowed to swell overnight in an Immobiline® DryStrip IPGbox (Cytiva). The following isoelectric focusing (IEF) parameters were applied using an Ettan IPGphor 3 IEF System (GE Healthcare, UK): 300v for 30 min (0.2kVh), 1000v for 30 min (0.3 kVh), 5000v for 130 min (4.5kVh) and 5000v for 25 min (1–3 kVh). After the focusing was complete, IPG strips were sequentially equilibrated in two equilibration buffers: buffer I (6 M urea, 75 mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 50 mM dithiothreitol and 0.002% bromophenol blue) and buffer II (6 M urea, 75 mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue and 125 mM iodoacetamide). The second-dimension separation was performed on 12% SDS-PAGE (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK) for 1.5 h under a constant voltage of 100 V. Following the electrophoretic runs, gels were stained with QC Colloidal Coomassie G-250 (Bio-Rad, USA). Spot detection and matching were performed using ImageMaster 2D Platinum (Cytiva, UK). Following removal of background, the spot volumes were normalized to the total protein detected for each gel. Spot intensity of proteins between the groups (vaccinated/unvaccinated; injection-challenged/immersion-challenged) was analysed for significance using one-way ANOVA.

Differentially expressed spots were excised from gels, destained and digested overnight with trypsin (Promega Porcine trypsin) at 37°C. Digests were analysed on a Bruker Ultraflex II MALDI-TOF mass spectrometer (Bruker Daltonics) and by scanning the 600- to 5000-dalton region in reflectron mode producing monoisotopic resolution. The spectra generated were mass-calibrated using known standards. Masses obtained were then database-searched using the MASCOT (Version 2.5.1) search engine. The data were searched in two custom databases: the larger database 'F_psych_All_NCBI', which contains all the *F. psychrophilum* entries as of 07/07/17 in NCBI and comprised 102,119 sequences; and the smaller database F_psych_CSF2593, which was constructed using the genome entry for *F. psychrophilum* CSF2593 and comprised 2634 sequences. Where no significant match was obtained using peptide mass fingerprinting (PMF), the samples were subjected to MS/MS analysis and searched again using the MASCOT search engine.

Differences in the mucus proteome of immersion-unvaccinated and immersion-vaccinated fish were apparent post-immersion challenge with *F. psychrophilum* (Figure 1), and one spot (Henriksen et al., 2013) was found to be significantly different between these groups ($p = .04$, nominal mass (NM): 34.7). This differentially expressed spot was identified following MALDI-TOF MS as annexin A5-like. The neighbouring spot (Madetoja et al., 2000) was identified as annexin A1-like (NM: 37.2).

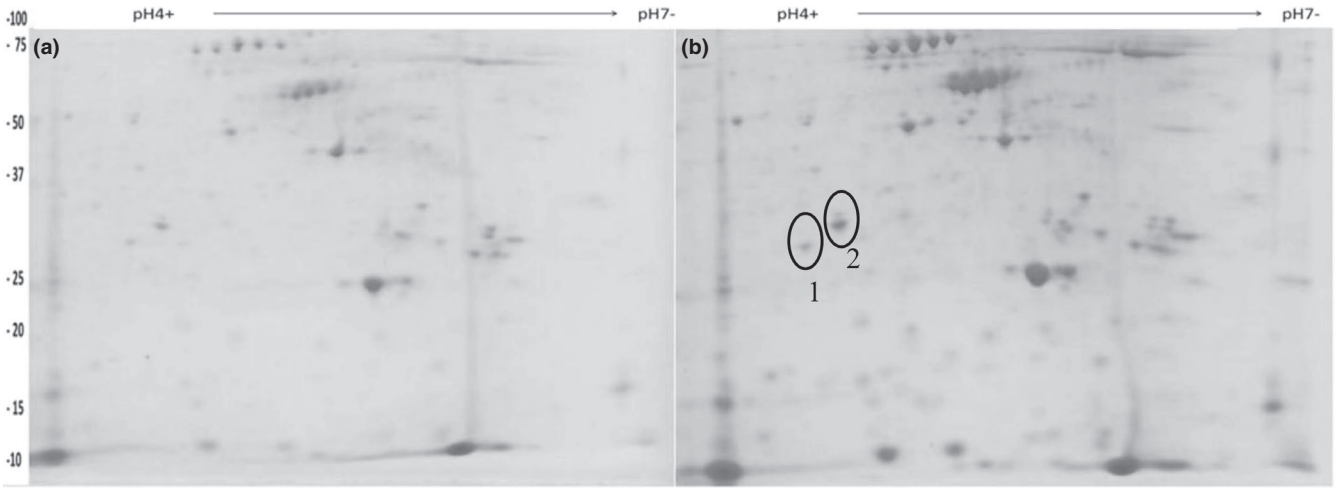

FIGURE 1 2D gel electropherograms of mucus (60 μg protein) indicating protein spots that were excised and used for mass spectrometry analysis. Spot 1 was identified as annexin A5-like (NM 34.7); spot 2 was identified as annexin A1-like (NM: 37.2). Mucus was isolated from rainbow trout vaccinated with *Flavobacterium. psychrophilum* (A: non-vaccinated; B: vaccinated) post-immersion challenge with virulent *F. psychrophilum* (AVU-1T/07). For more information including Mascot scores and p*I*, see Table S1. Duplicate gels were run for each treatment group as technical replicates

Annexins have been found in many species of eukaryotes, including Atlantic salmon (*Salmo salar*) (Hwang et al., 2007) and zebrafish (*Danio rerio*) (Farber et al., 2003). Annexins are usually cytosolic proteins (Mirsaeidi et al., 2016) and have diverse functions in inflammation, membrane trafficking, opsonization and phagocytosis, transduction of mitogenic signals and cell–matrix interactions, among others (Munoz et al., 2007). The low number of significantly different protein spots detected in this study could be due to high abundant proteins being over-represented in the gels, which can mask the detection of low-abundant molecules. Future studies should attempt to increase the protein concentration applied to each 2D gel, and the use of IPG strips with a broader pH range may result in better resolution of the separated proteins. In addition, the use of other techniques such as iTRAQ could overcome these limitations.

Thirty-five spots were detected in mucus proteins sampled from immersion- and injection-challenged unvaccinated fish, with 11 significantly different spots following analysis (Figure 2). Of these, 3 spots were chosen for MS analysis (spots 3, 4 and 5; Table 1), as the others were deemed to have too low protein concentration to enable MS detection. Beta-actin was the dominant protein found in significantly higher amounts in the immersion-challenged fish when compared to the injection-challenged fish ($p \leq .02$). Spots with NM of approximately 41.75 (spots 3 and 5) were identified as the intracellular protein, β-actin, and were present in the 2D gels in relatively high abundance. Other significant matches included actin, cytoplasmic 1 isoform (spot 4; NM 42.75) in the immersion-challenged fish, which was not apparent in injection-challenged fish.

Actins are highly conserved proteins that are involved in cell motility, cytoplasmic streaming and phagocytosis (Weeds, 1982). Beta-actin has been shown to activate endothelial NOS (eNOS), also known as nitric oxide synthase 3, thereby increasing nitric oxide (NO) production (Kondrikov et al., 2010). The ability of macrophages to migrate and to invade the extracellular matrix is based on their adaptable morphology, and the local degradation of matrix components. The higher levels of actin in the mucus of immersion infected fish compared to fish infected by injection is interesting and suggests that the presence of actin may not simply be due to contamination from damaged cells, but that it may have a role in mucosal immunity by potentiating the macrophage response to pathogens. Extracellular localization for actin has previously been described following sea louse infection in Atlantic salmon (Easy & Ross, 2009). A cluster of unidentified basic proteins (~p*I* 6–6.5) were seen in immersion-infected fish, which did not appear in injection-challenged fish (Figure 2a: spots in large blue circle, $p \leq .01$). Future studies should endeavour to identify these proteins.

These results highlight the importance of the route of infection in challenge studies with *F. psychrophilum*, which is suspected to have a mucosal route of entry. Many challenge experiments in fish are conducted using intraperitoneal or intramuscular injection as these methods allow a more standardized infection model to be used. However, they completely bypass the primary natural barriers of mucus and epithelium, thereby promoting an 'unnatural' infection. Recent research has highlighted the importance of mucosal immunity in fish (Cordero et al., 2017; Kato et al., 2018; Salinas & Magadán, 2017). New technology has allowed the visualization of bacterial (*Vibrio anguillarum*) spread in a zebrafish model (Schmidt et al., 2017), revealing the tissues and organs where bacteria were detected differed significantly depending on the route of infection. In this study, as with *F. psychrophilum*, significantly higher numbers of bacteria were needed to cause infection and morbidity/mortality by the mucosal route than by injection, revealing how effective the mucosal barriers of fish are to prevent infection. These studies also highlight the importance of investigating both mucosaland humoral immune parameters when developing vaccines for fish.

The results of this study contribute to our knowledge of mucosal immune reactions in rainbow trout following vaccination and

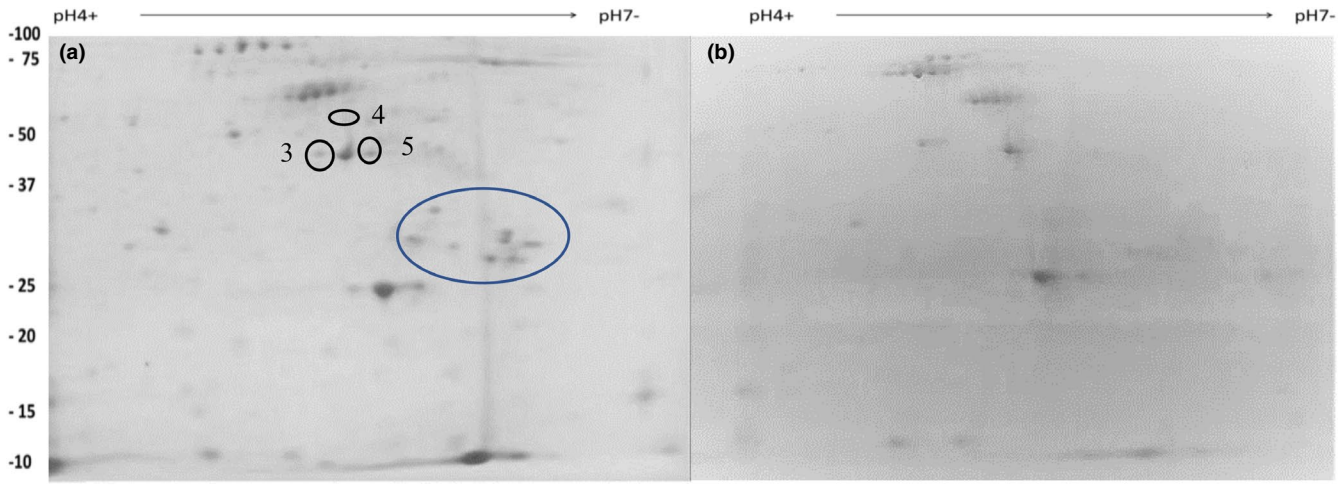

FIGURE 2 2D gel electropherograms of mucus (60 μg protein) indicating protein spots that were excised and used for mass spectrometry analysis (spots 3–5). Mucus was isolated from unvaccinated rainbow trout challenged with *F. psychrophilum* by: (A) immersion and (B) intramuscular injection. The large blue circle indicates significantly different spots observed on gel A (immersion-challenged) but not on gel B (injection-challenged) with insufficient protein present to be analysed by MS. For more information including Mascot scores and p*I*, see Table S1. Duplicate gels were run for each treatment group as technical replicates

TABLE 1 Mucus proteins identified by mass spectrophotometry following vaccination and/or challenge

Spot	Protein (BLAST)	Nominal Mass	~ pI	Sequence coverage	Mascot scores	Spot intensity				Stats (ANOVA)
						Immersion-vaccinated (non-challenged)	Immersion-unvaccinated (non-challenged)	I.P. injection-challenged (unvaccinated)	Immersion-challenged (unvaccinated)	
1	Annexin A5-like [*Oncorhynchus mykiss*] XP_021450040.1|	34,765.7	5.3	MS: 50.8% MS/MS: 8.5%	103	35	22	NA	NA	**
2	Annexin A1-like [*Oncorhynchus mykiss*] XP_021461147.1|	37,214.3	6.1	MS: 54.6%	143	71	49	NA	NA	***
3	Actin beta [*Oncorhynchus mykiss*] NP_001117707.1|	41,755.7	5.2	MS: 39.2%	74	NA	NA	X	74	***
4	Actin, cytoplasmic 1 isoform X1 [*Oncorhynchus mykiss*] XP_021451454.1|	42,966.4	5.4	MS: 41.9%	73	NA	NA	X	73	***
5	Actin beta [*Oncorhynchus mykiss*] NP_001117707.1|	41,755.7	5.2	MS: 62.9%	101	NA	NA	X	101	***

Note: MASCOT scores >61 are deemed significant. Asterisks denote significance difference between groups (vaccinated/unvaccinated; injection-challenged/immersion-challenged): *$p < .05$; **$p < .01$; ***$p < .001$; and ****$p < .0001$. X: absent and NA: not applicable.

infection indicating a role for annexins and actins in potentiating the innate mucosal immune response. Further studies are needed to fully characterize the mucus proteome following mucosal vaccination and challenge in juvenile fish and its significance to the immune response elicited by different routes of vaccination and challenge.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

RH, KT and AA conceived and designed the experiments. RH, KM and KS carried out the analysis. KM, KS and RH analysed the data. RH, KT and KS wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are included in the manuscript or the supplementary information.

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SUPPORTING INFORMATION

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