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Research Article

Immunological Responses in the Liver, Kidney, and Spleen of Atlantic Halibut Infected with atypical *Aeromonas salmonicida* using cDNA microarrays

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Abstract

We investigated the differential gene expression within Atlantic halibut (*Hippoglossus hippoglossus*) in response to atypical *A. salmonicida* infection using a 381 cDNA microarray. Atlantic halibut were infected with atypical *A. salmonicida* strain 0203 and held at 10-12°C. Liver, kidney and spleen tissues were collected at 21 days post infection (DPI), and total RNA was isolated for microarray analysis.

Of the 381 genes studied on the microarray, we identified a total of 169 differentially expressed (2-fold change, $p<0.05$) genes that were in at least one of the 3 fish samples: 46 in liver, 87 in kidney and 94 in spleen. Gene expressions of 3 fish were highly variable between fish at a mid-infection time point. Of the 46 differentially expressed genes in the liver, 28 were significantly up-regulated: betaine-homocysteine S-methyltransferase and halibut hepcidin type I were the most prominent. In the kidneys and spleens of fish 2 and 3, a greater number of adaptive immune related genes were significantly up-regulated compared to fish 1, including major histocompatibility class I receptor (MHC I) and MHC II, MHC II-associated invariant chain, proteosome activator subunit 2, heat shock protein (HSP) 70 and HSP90, t-complex polypeptide 1, CD 53-like antigen and CD 63-like antigen.

Our results indicate that a wide range of functional genes involved in adaptive immune systems of Atlantic halibut was differentially expressed at 21 DPI. Especially, genes involved in antigen-processing and presenting pathways were significantly up-regulated at mid-infection time point.

Keywords: Atlantic halibut; *Aeromonas salmonicida*; Infection; Immune; Microarrays

Introduction

The Atlantic halibut (*Hippoglossus hippoglossus*) is distributed throughout the North Atlantic and is a valuable cold-water species for aquaculture [1]. The atypical *Aeromonas salmonicida* is one of several bacterial pathogens causing serious disease outbreaks in Atlantic halibut [2]. Vaccines developed to date against typical *A. salmonicida* have provided less protection in halibut [3-4]. In a previous experiment using microarrays

and RT-PCR , we studied the gene expression of Atlantic halibut to a vaccine developed against *Listonella anguillarum* and typical *A. salmonicida*. Our findings supported previous report that the adaptive immune system of Atlantic halibut responds more slowly than that of salmonids and react differently from salmonids in processing bacteria during infection or vaccination [5]. Recent studies for immune systems of Atlantic halibut showed that activation of CD4+T-cells might be important factor in reducing nodavirus infection [6,7]. Currently, compared to other commercially important fish, there is limited information about the immune response of Atlantic halibut to bacterial pathogen exposure.

To manage disease problems in Atlantic halibut aquaculture, information on the above areas is essential. In this present experiment using our halibut cDNA microarray we determined the overall immunological response to the bacterial pathogen atypical *A. salmonicida* at 3 weeks post infection showing 20% cumulative mortality. We also determined differential expressions of three genes at 1,2,7,14,21 DPI, using qRT-PCR method.

As part of our studies on the immune system of Atlantic halibut we generated an EST library from the liver, kidney, and spleen [8]. Of the 1114 EST clones sequenced we found that 182 clones encoded cell/organism defence genes. Using these EST clones and other immunologically important genes from other individual studies, we produced an Atlantic halibut cDNA microarray. Using this Atlantic halibut microarray, we studied differentially expressed genes found in the liver, kidney, and spleen of Atlantic halibut in response to vaccination against *Listonella anguillarum* and *Aeromonas salmonicida* during 2 weeks post vaccination. In the experiment we found that stress or innate immune related genes were highly upregulated but the adaptive immune system related genes were expressed less than a 2-fold increase. However the confirmation by qRT-PCR indicated the gradual increase of adaptive immune system related genes. We had 3 main objectives in this present study: first, to find differentially expressed genes which are important for survival during an active bacterial infection; second, to observe activation of the adaptive immune system during bacterial infection; third, to compare the responses of individual fish during infection.

Here we report altered gene expression of a wide range of functional genes as well as cell/organism defence-related genes at 3 weeks post-infection. We found that several genes related to adaptive immune system were significantly up-regulated at this time point but the expression level was variable between individual fish.

Our results have produced important information relating to immunological responses of Atlantic halibut at the mid-infection time point within an atypical *A. salmonicida* infection.

Materials and Methods

Fish rearing and bacterial challenge

Atlantic halibut (ca. 150-200g) were obtained from Scotian Halibut Ltd. under Canadian Council on Animal Care guidelines. Ninety fish were stocked in 10 of 100 L tanks; 9 fish were placed in each of 6 infection tanks and each of 4 control tanks. The tanks each contained 80 L UV treated seawater, maintained at 10-12°C, in 4.3 $L \bullet min^{-1}$ water flow rate.

The atypical *A. salmonicida* strain 0203 was obtained from the Department of Fisheries and Oceans, Moncton, New Brunswick, Canada. Bacteria were inoculated in 3 replicates of 10 mL of tryptic soy broth (TSB) and then incubated in TSB overnight at 15°C with shaking. The bacteria were combined with 900 mL of TSB and incubated overnight again at 15°C. The bacteria were concentrated by centrifugation at 800 x g for 15 min then resuspended in sterile phosphate-buffered saline (PBS). Optical density (O.D.) of the bacterial cell suspension was measured at 600 nm and adjusted to an O.D. 0.2 with PBS, giving the concentration of 108 $cfu \bullet mL^{-1}$. The bacteria were serially diluted to the concentration of 2x104 $cfu \bullet mL^{-1}$.

For bacterial challenge, fish were anaesthetized with 50 $mg \bullet L^{-1}$ of tricaine methanosulphonate (TMS, Syndel Laboratories, Vancouver, British Columbia). 54 fish were injected in the intraperitoneal cavity (i.p.) with 0.5 mL (ca. 10^4 cfu) of atypical *A. salmonicida* strain 0203 and 36 control fish were injected with an equivalent volume of PBS. Fish were closely monitored for a 2-month period and moribund fish were removed daily.

Tissues sampling and total RNA isolation

In order to determine expression of genes involved in either the innate or adaptive immune systems, liver, anterior kidney and spleen tissues were collected from 5 live individuals from each of the infection and control tanks at 1, 2, 7, 14, 21 days post-infection (DPI). These samples were preserved in RNA Later (Ambion, Austin). From those collected 5 fish samples, the 3 best, high quality RNA samples were chosen for microarray analysis and qRT-PCR. For the microarray analysis, fish 1,2, and 3 were used; while, fish 1,2 and 4 or 5 were used for qRT-PCR due to RNA degradation of fish 3 at 21 DPI.

Construction of Atlantic halibut cDNA microarray

Three hundred and eighty one cDNA clones representing genes with a wide range of known and unknown functions were selected from a total of 1072 clones. Selected bacterial clones were grown overnight and one microlitre of the overnight growth was used for PCR-based amplification; with 35 cycles, using vector arm primers: T3 and T7. Amplified DNA

fragments were purified using a 96-well filtration system (Millipore, MA) and then concentrated before spotting. The concentrated template was resuspended in 50% dimethylsulfoxide (DMSO), gently shaken for 20 min. to give a final average concentration of 42 ng•uL^{-1}. These selected genes were spotted in triplicate on GAPSII coated slides (Corning, NY) at the Biotechnology Research Institute, National Research Council of Canada.

Microarray procedures and data analysis

Total RNA (20 µg) from the control and challenged fish at 21 DPI were labeled with either Cy3 dCTP or Cy5 dCTP (Amersham Biosciences, Piscataway, NJ) by a direct labeling method using SuperScript II reverse transciptase (GIBCO, Carlsbad). To control for bias due to differences in labeling efficiencies, mRNA from both groups were labeled with each of the dyes. Hybridization for the first set of slides consisted of Cy3-labeled cDNA from control (uninfected) fish and Cy5-labeled cDNA from experimentally challenged fish; hybridization for the second set of two slides consisted of Cy5-labeled control cDNA and Cy3-labeled experimental cDNA.

After purification of labelled cDNA using the Qiagen PCR Purification Kit, each of the samples were concentrated to a final volume of approximately 8 µL in a speed vacuum and combined. To block non-specific hybridization, 1 µL of poly (dA) DNA (20mg•mL^{-1}) was added to the sample. The combined sample was denatured at 95^{0}C for 3 min. and followed by the addition of 20 µL of hybridization buffer (Amersham Biosciences, Piscataway) and 40 µL of deionized formamide (Sigma, St. Louis). This sample was hybridized overnight at 42^{0}C to the halibut microarray. Slides were washed in the following order: 1x SSC with 0.2% SDS for 10 min., 0.1x SSC with 0.2% SDS for 10 min. (2 times repeat), 0.1x SSC for 5 min., and 0.1x SSC for 1-2 sec. (3 times). Microarray slides were scanned using a ScanArray 5000 XL reader and its proprietary software (Packard Bioscience) immediately after drying by spinning in a plate centrifuge for 5 min. at 500xg. The Cy3 and Cy5 signal intensities were adjusted using the signal intensities of several ribosomal genes including two internal controls, 18S ribosomal RNA genes (SL-0060, CN655605). The resulting images were imported into QuantArray software (Packard Bioscience, Boston) for quantitative analysis. These data were normalized using background subtraction and global median normalization using the QuantArray software. Only those spots with a hybridization signal intensity of greater than 500 fluorescence units for both Cy3 and Cy5 were used for analyzing expression ratios. Genes with an expression ratio ≥ 2-fold at the P<0.05 significance level were regarded as differentially expressed genes. For the statistical analysis of each gene (n=6, 3 spots and dye swap), we performed a t-test using the GraphPad (GraphPad Software, Inc. San Diego, CA). For the KEGG pathway analysis using the DAVID program (http://www.DAVID.niaid.nih.gov), we used only differentially expressed genes (≥ 2-fold at the P<0.05) from 3 tissues of fish 2 and 3.

qRT-PCR verification

To confirm gene expression changes observed from microarray results, qRT-PCR was performed on three genes (halibut hepcidin type I, MHC I and recombination activating gene-1 (RAG-1)). RNA samples were isolated from 5 infected and control fish respectively. Among isolated RNA samples, 3 non-degraded RNA samples were selected for qRT-PCR, which resulted in that one RNA sample (fish 5) in qRT-PCR was different with that (fish 3) in microarray analysis. The cDNA templates for qRT-PCR were synthesized from 500 ng poly(A) RNA of three control or infected fish. Two-step reverse transcription-RT PCR was conducted using the Superscript III qRT-PCR kit with SYBR green (Invitrogen) on an iCycler (Bio-Rad). PCR products of EF-1A, rDNA-639, hepcidin type I, MHC I and RAG-1 were cloned into a TA-cloning vector (pCR 4-TOO; Invitrogen) and sequenced to confirm cloned product. For the quantitative analysis, data having a PCR efficiency of 90-110% was used. Halibut EF-1A and halibut ribosomal gene (rDNA-639, GenBank accession no. CF931602) were used as internal housekeeping controls. The information for optimized qRT-PCR condition is given in Table 1. In our previous halibut studies, we found these two house keeping genes were the most stable in bacterial vaccination and infection experiments.

Genes Primers	GenBank Number	Nucleotide sequence (5′ → 3′)	qPCR conditions (annealing temps and time, cycles)	Amplicon size (bp)
EF-1a F EF-1a R	EU561357 EU561357	TACCTGTCGGTCGTGTTGAG ACGTATCCACGACGGATCTC	57°C for 30S, 40X	190
RAG 1 F RAG 1 R	AY454396 AY454396	CAGCTGTCCTGAGGCCTATC CGCACCATCTTCTCATCGTA	55°C for 30S, 40X	133
MHC I F MHC I R	CF931621 CF931621	CTTCATGAGGACGTGGACCT GTGGGCTTCTCTCTGTTGGA	55°C for 30S, 40X	200
rDNA-639 F rDNA-639 R	CF931602 CF931602	ACCTGTTCTCATGGATCCCACCAA CTCTGACAGTGACCCTGAATCACA	55°C for 30S, 40X	111
Hepcidin I F Hepcidin I R	CF931737 CF931737	GCCACCTTTCCTGAGGTACA TTTGATGGTTGTTGGAGCAG	55°C for 30S, 40X	212

Table 1. Primers used in quantitative RT-PCR and cycling conditions.

Results

Injection challenge

Infection with atypical *A. salmonicida* strain 0203 initiated an experimental epizootic that was monitored for 54 days and caused 37% total cumulative mortality. Atypical *A. salmonicida* was isolated from the posterior kidney of all dead fish and live fish that were used for microarray and qRT-PCR analysis. Samples for the microarray were collected at 21 DPI at which point there was 20% cumulative mortality (Figure 1). However, not all the dead fish showed overt disease signs such as furuncules or haemorrhage. No mortalities or morbidities were obtained from control tanks.

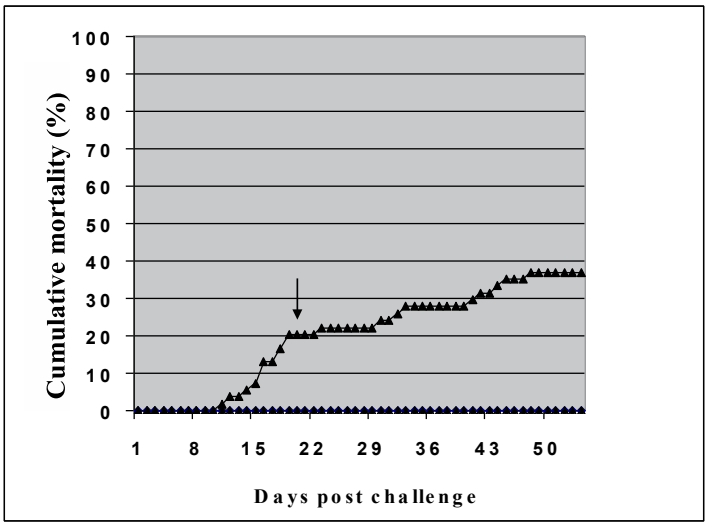

Figure 1. Cumulative mortality in Atlantic halibut (ca 150-200g) injected i.p. with 0.5 ml (ca 10^4 cfu) of atypical Aeromonas. salmonicida strain 0203 (▲) or with 0.5 ml of phosphate buffered saline (PBS) as a control (♦). Liver, kidney and spleen tissues were collected for qRT-PCR at 1, 2, 7, 14, 21 days post infection (DPI). Arrow (↓) indicates a sampling day (21 DPI) when tissues were collected for microarray analysis.

Microarray analysis

To understand the immunological response of Atlantic halibut to atypical *A. salmonicida* we studied gene expression by using microarray analysis of the liver, kidney and spleen of 3 fish at 3 weeks post infection when the cumulative mortality reached 20%. Gene expressions of these tissues sampled from 3 fish were highly variable at 3 weeks post infection. Notably, fish 1 showed a very different gene expression pattern compared to fish 2 and 3 (Table 2, 3). Fish 1 showed that a high number of genes were down-regulated and no genes were up-regulated in the anterior kidney and spleen tissues, but a number of genes were up-regulated in liver tissues (Table 2). However, fish 2 and fish 3 showed that a high number of genes were up- or down- regulated in the three tissues studied.

	Liver (46)			Kidney (87)			Spleen (94)		
	L1	L2	L3	K1	K2	K3	S1	S2	S3
Total	33	13	10	41	7	47	16	67	53
Up	24	4	8	0	3	46	0	64	48
Down	9	9	2	41	4	1	16	3	5

Table 2. Summary of the numbers of genes differentially expressed in liver (L), kidney (K) and spleen (S) of Atlantic halibut infected with atypical *Aeromonas salmonicida*. Samples were taken from 3 fish, indicated by the number associated with each tissue (e.g., L1 is the liver sample from fish 1).

Gene expressions in liver

Of the 381 genes on the microarray, we identified a total of 46 genes differentially up- or down-regulated (≥ 2-fold change, $p<0.05$) in at least one of the liver samples (Tables 2 and 3). Twenty-eight genes were significantly up-regulated, with a 2.0- to 8.6-fold change in expression, while 20 genes were down-regulated, with a 2.0- to 4.8-fold change. In the liver tissues acute phase response, innate immune response and proteinase- related genes were significantly differentially expressed, as expected.

Up-regulated genes: Of the 28 differentially up-regulated genes, betaine-homocysteine S-methyltransferase and halibut hepcidin type I showed the highest level of up-regulation with approximately 8.6-fold and 7.8-fold increases respectively (Table 3). The result of qRT-PCR for halibut hepcidin type I supports the results of the microarray analysis (Table 4). In the qRT-PCR analysis, hepcidin type I was up-regualted with 6.7-fold at 21 DPI and the highest expressions with 32.1 and 39.5-fold upregulations were obtained from the tissues at 2 and 7 DPI, respectively. Halibut hepcidin type II also showed a high level of up-regulation with a range of 3.1-5.7-fold change from fish 1 and fish 2, while fish 3 showed no significant difference. We found variable expression patterns both from hepcidin I and II. When hepcidin type I was up-regulated in fish 3, hepcidin type II was at a normal expression level. Hepcidin type II was up-regulated in fish 1 and 2 but hepcidin type I was at a normal expression level (Table 3).

Other genes that were highly up-regulated (≥ 3-fold) in at least 1 sample include: protein disulfide isomerase-related protein P5 precursor, O-methyltransferase containing protein, trypsinogen 3, chymotrypsinogen 2, alpha-2-macroglobulin-1, proteasome subunit alpha type 2, cathepsin L, and two unknowns (CF931775, CF931682) (Table 3).

A number of genes that encode for components of the acute phase-response showed significant up-regulated expression in fish 1 at 21 DPI, which included plasma protease (C1) inhibitor, alpha-2-macroglobulin-1 (Table 3). However these were at normal expression levels in fish 2 and 3.

MHC class I belonging to cell/organism defence function showed significant up-regulation. On the halibut microarray we included three sequences that share identity with MHC class I. We found only one sequence of MHC class I (CN655588) up-regulated with 2.1-fold in fish 1.

Seven unknown genes were seen by microarray analysis to be significantly up-regulated in fish 1 (Table 3).

Down-regulated genes: Among the 20 down-regulated genes, apolipoprotein E showed the most significant down-regulation

with 35.9-fold in fish 1, however the expression of this gene was normal in fish 2 and 3 (Table 3).

Accession No	Gene name	Liver			Kidney			Spleen		
	1. Cell/Organism defense (44)	**L1**	**L2**	**L3**	**K1**	**K2**	**K3**	**S1**	**S2**	**S3**
CF931751	alpha-2-macroglobulin-1	**3.92**	-1.84	-1.53	1.18	-1.25	-1.33	-1.05	-1.48	-1.21
CF931718	chemotaxin	-1.81	1.25	-1.04	-1.43	-1.14	**2.33**	-1.09	1.74	1.89
CF931651	chicken-type lysozyme	**-2.04**	1.14	-1.09	**-5.42**	1.12	1.48	1.35	**2.03**	1.02
CN655621	chicken-type lysozyme	-1.38	1.03	-1.25	**-2.63**	1.16	1.04	1.33	1.15	-1.16
CF931651	chicken-type lysozyme	-1.66	1.43	1.52	**-9.86**	1.17	1.79	1.61	1.92	1.32
CF931643	complement binding protein	-1.55	-1.51	-1.60	-1.05	-1.07	1.08	1.04	**-2.64**	1.00
CF931592	complement component C3	-1.81	-1.02	-1.07	**-3.17**	-1.18	1.12	-1.14	-1.53	-1.23
CN655688	complement component C3	-1.79	1.09	-1.03	**-3.77**	-1.20	-1.04	-1.05	-1.55	-1.20
CF931592	complement component C3	-1.38	1.14	1.06	**-3.12**	-1.29	1.10	1.03	-1.63	-1.29
CF931737	*Halibut hepcidin type I	1.34	-1.07	**7.79**	1.25	1.20	-1.60	-1.38	-1.22	1.04
CF931613	*Halibut hepcidin type II	**4.98**	**3.10**	1.05	1.15	-1.41	-1.55	-1.02	-1.72	-1.22
CN655667	*Halibut hepcidin type II	**5.70**	**5.22**	1.11	1.00	-1.18	-1.17	1.15	-1.44	-1.10
CN655682	*heat shock protein (hsp) 90, beta	-1.43	1.11	1.76	-1.40	1.16	1.71	-1.17	**2.56**	**2.40**
CN655682	*heat shock protein (hsp) 90, beta	-1.43	1.17	1.49	-1.05	1.10	1.80	-1.30	**3.59**	**2.55**
CN655718	*heat shock protein (hsp) 90, beta	-1.28	-1.04	1.13	-1.41	1.00	1.89	1.12	**3.56**	**2.03**
CF931574	*heat shock protein 70	-1.41	1.03	1.08	-1.34	1.07	1.89	-1.15	**2.92**	**2.39**
CN655590	*heat shock protein 70	-1.45	-1.24	1.32	1.12	1.07	**2.11**	-1.24	**7.11**	**2.92**
CF931748	*Hsp70-interacting protein	1.10	1.22	1.21	-1.27	1.11	**2.24**	-1.11	1.93	1.79
CN655615	*hemoglobin beta-A chain	1.48	-1.30	**-2.05**	1.27	-1.35	-1.34	1.00	1.60	1.30
CN655722	*interferon consensus sequence binding protein	1.14	-1.15	1.37	-1.19	-1.18	**2.08**	1.24	1.56	1.70
CN655606	*MHC class I	1.12	1.45	-1.02	**-2.07**	1.78	1.47	1.23	**4.67**	**2.05**
CN655588	*MHC class I	**2.06**	-1.01	1.21	-1.28	1.68	1.73	1.33	**7.17**	**2.45**
CF931621	*MHC class I	-1.34	1.35	1.13	**-2.71**	1.61	1.36	1.16	**5.77**	**2.55**
CN655650	*MHC class II	-1.06	1.69	1.54	**-2.07**	1.44	**2.11**	1.23	**2.62**	1.65
CN655713	*MHC class II	1.03	1.16	1.09	-1.07	**-2.12**	1.40	1.03	-1.47	-1.24
CN655650	*MHC class II	-1.22	1.65	1.33	**-2.70**	1.43	1.93	1.10	**2.39**	1.82
CF931837	*MHC class II, alpha	1.02	1.27	1.09	-1.07	1.96	1.50	1.87	**2.94**	**2.04**
CF931778	*MHC II-associated invariant chain	-1.19	1.41	-1.41	-1.33	1.44	1.49	1.40	**3.95**	**2.70**
CN655655	*plasma protease (C1) inhibitor precursor	**2.38**	1.26	-1.05	-1.40	1.08	1.80	1.23	**2.27**	**2.58**
CF931940	*proteasome activator subunit 2	-1.19	-1.34	1.70	-1.42	1.25	**2.63**	-1.13	-1.34	**2.25**
CN655679	*receptor for activated protein kinase C	-1.47	-1.01	1.27	-1.07	-1.06	1.52	-1.05	**2.37**	**2.66**
CF931878	*similar to CD53 antigen	-1.36	1.52	-1.07	-1.28	1.02	1.50	1.09	**6.12**	1.70
CF931913	*similar to CD63-like protein	1.04	1.07	1.00	-1.32	-1.04	1.50	1.14	**3.01**	**2.15**
CN655681	*T-complex polypeptide 1	-1.90	1.11	-1.39	1.01	-1.09	1.93	-1.11	**2.99**	**2.29**
CN655694	*T-complex protein 1 theta subunit	-1.04	1.26	-1.24	-1.28	-1.35	**2.18**	-1.24	**2.17**	1.84
CN655656	*transferrin	-1.17	1.25	-1.18	-1.15	-1.47	1.77	1.36	**2.10**	**2.24**
	2. Cell signaling/Cell communication (25)	**L1**	**L2**	**L3**	**K1**	**K2**	**K3**	**S1**	**S2**	**S3**
CF931930	signal recognition particle 54 kDa	-1.77	1.25	-1.04	**-3.13**	1.29	1.29	-1.14	1.06	-1.12
CN655603	unknown similar to Japanese flounder liver cDNA clone	**2.21**	1.98	-1.34	-1.09	-1.22	-1.36	1.12	-1.35	-1.13
CF931857	*receptor for urokinase plasminogen activator	1.14	-1.13	1.43	1.28	-1.16	1.43	1.00	1.00	**-2.08**
CN655605	CDH1-D	-1.76	1.21	1.31	-1.29	1.10	-1.02	1.20	1.54	**2.18**
	3. Cell structure/Motility (13)	**L1**	**L2**	**L3**	**K1**	**K2**	**K3**	**S1**	**S2**	**S3**
CF931792	fibrinogen	-1.81	1.25	-1.06	**-2.68**	-1.17	-1.35	-1.03	-1.62	1.00
CN655630	*beta-cytoplasmic actin2	1.04	1.09	-1.62	-1.39	1.09	**2.97**	-1.66	1.48	**2.50**
CF931740	*cytoplasmic actin	-1.80	1.24	-1.49	-1.27	1.13	1.91	-1.34	**2.95**	**2.42**
CN655616	*fibrinogen, gamma chain precursor	-1.81	1.00	-1.21	1.06	-1.43	1.72	-1.43	**2.30**	1.24
CN655663	*cofilin, muscle isoform (COFILIN 2)	-1.10	-1.27	1.43	-1.20	-1.19	**2.31**	1.53	1.04	1.04
CN655635	*tubulin, alpha	-1.16	1.60	1.26	1.22	-1.19	**2.61**	-1.41	**2.61**	**2.07**
CN655625	*tubulin, beta	1.13	1.79	1.19	-1.29	-1.12	**2.69**	-1.40	**2.06**	**2.49**
CF931649	*tubulin, beta	-1.15	**2.57**	1.37	1.13	-1.41	**2.47**	-1.32	1.44	1.93
CN655591	*tubulin, beta	1.11	1.58	1.31	1.04	-1.14	**2.95**	-1.43	**2.27**	**2.35**
CF931753	*fibrinogen-like protein A precursor	1.87	**-2.04**	-1.55	1.33	-1.91	-1.93	1.05	-1.14	1.06
CN655691	*talin	1.12	1.03	1.07	-1.42	1.01	1.48	-1.25	1.96	**2.32**
CN655659	*myosin light chain alkali, smooth-muscle isoform	-1.52	1.10	-1.16	-1.17	-1.33	0.82	1.63	**2.02**	1.96
CF931620	*glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	-1.20	1.44	1.77	**-2.23**	**2.31**	**3.65**	**-2.10**	1.85	-1.11
	4. Gene/Protein expression (93)	**L1**	**L2**	**L3**	**K1**	**K2**	**K3**	**S1**	**S2**	**S3**
CN655592	*ribosomal protein L3	-1.44	-1.10	-1.30	-1.55	1.00	1.36	1.00	**2.84**	1.95
CN655669	40S ribosomal protein S14	**-2.08**	1.00	-1.01	-1.15	1.01	-1.16	1.57	1.50	1.34
CF931808	40S ribosomal protein S24	-1.26	1.08	-1.45	**-2.13**	-1.25	-1.32	1.33	1.34	1.36
CN655614	40S ribosomal protein S26-2	-1.19	1.09	-1.58	**-2.27**	-1.03	-1.60	1.01	-1.01	1.10

CN655614	40S ribosomal protein S26-2	-1.19	1.09	-1.58	**-2.27**	-1.03	-1.60	1.01	-1.01	1.10
CN655604	*40S ribosomal protein S4	-1.03	1.07	-1.02	-1.40	1.06	1.50	1.01	1.91	**2.29**
CN655611	*40S ribosomal protein S6	-1.39	1.06	-1.19	1.21	-1.27	1.38	-1.20	**2.52**	1.50
CN655600	40S ribosomal protein S8	**-2.08**	1.24	-1.24	-1.52	1.03	-1.30	1.25	1.72	1.40
CN655658	*40S ribosomal protein Sa	-1.37	-1.10	1.58	-1.10	-1.08	1.66	1.12	1.19	**2.04**
CF931796	60S ribosomal protein L21	1.47	1.15	-1.38	**-2.07**	-1.26	1.06	-1.12	1.43	1.19
CN655601	60S ribosomal protein L35	-1.39	-1.03	-1.37	**-2.17**	-1.34	-1.49	1.33	1.50	1.47
CN655730	calpain 2	-1.13	1.13	-1.06	**-3.11**	1.59	1.06	-1.35	-1.04	-1.07
CF931840	*carboxypeptidase homolog	**2.38**	1.20	1.01	1.14	-1.03	-1.26	**-14.85**	**5.78**	-1.38
CF931685	cathepsin D precursor	**-2.20**	-1.21	1.17	**-2.18**	-1.41	1.97	**-2.32**	1.53	1.33
CF931685	cathepsin D precursor	**-2.16**	-1.16	1.32	**-2.03**	-1.15	1.89	**-2.44**	1.51	1.26
CN655706	cathepsin D precursor	-1.73	-1.18	1.29	**-2.38**	-1.14	1.79	-1.72	1.92	1.41
CF931800	*cathepsin L	-1.68	**-3.14**	-1.67	**-2.15**	-1.85	-1.01	-1.75	-1.02	1.67
CF931835	*cathepsin L precursor	**3.00**	1.62	**2.17**	1.25	1.26	-1.31	1.01	-1.51	-1.18
CF931811	*chymotrypsinogen 2	3.34	1.33	1.26	-1.14	-1.22	-1.40	**-11.64**	**3.95**	-1.34
CF931845	*elastase A precursor	1.13	1.47	1.05	1.21	-1.25	-1.14	**-8.74**	**7.04**	**-2.84**
CN655686	*elongation factor 1 alpha	-1.72	1.19	1.31	-1.22	1.10	-1.10	1.08	1.61	**2.22**
CN655607	*elongation factor 1a	**2.06**	-1.27	1.46	-1.02	1.05	**2.07**	-1.14	**3.77**	**2.42**
CF931860	*elongation factor 2	-1.41	1.05	1.21	-1.06	-1.32	1.65	1.05	**2.09**	**2.27**
CF931741	*elongation factor-1 gamma	-1.30	1.14	1.19	1.13	-1.19	1.47	-1.10	**2.76**	**2.08**
CF931891	*leukocyte elastase inhibitor	-1.33	**-3.27**	1.65	-1.59	1.48	**2.65**	1.08	**2.32**	-1.65
CF931600	*microsomal signal peptidase 25 kDa subunit	-1.09	-1.21	-1.08	-1.02	1.02	1.78	-1.03	**2.22**	1.78
CF931843	*proteasome subunit alpha type 2	**3.33**	1.44	**2.45**	-1.18	-1.17	**2.33**	1.42	**2.14**	1.98
CN655598	ribosomal protein L11	-1.41	1.10	-1.18	**-2.50**	-1.07	-1.39	1.13	1.73	1.57
CN655622	*ribosomal protein L5a	-1.48	-1.07	1.30	-1.39	-1.12	-1.11	-1.05	**2.42**	1.84
CF931833	*ribosomal protein L5b	-1.31	-1.34	-1.06	-1.05	-1.08	1.28	-1.05	**2.48**	**2.05**
CN655657	*ribosomal protein L9	1.25	1.25	-1.06	-1.31	-1.14	-1.04	1.06	1.98	**2.11**
CF931824	*transcriptional repressor CTCF	1.19	1.13	1.00	1.25	-1.36	1.48	1.06	**2.04**	1.60
CN655661	*translation elongation factor G	-1.43	**-2.52**	1.41	-1.04	1.02	-1.35	1.05	-1.63	-1.18
CN655638	*trypsinogen 2	1.93	1.25	1.92	-1.36	-1.17	-1.38	**-4.18**	**2.80**	**-2.49**
CF931595	*trypsinogen 2 precursor	1.36	1.61	1.84	1.34	-1.18	-1.51	**-7.72**	**6.47**	**-2.51**
CF931780	*trypsinogen 3	**3.13**	-1.03	1.06	1.08	-1.31	-1.47	**-7.59**	**4.33**	-1.82
CN655593	unknown (hypothetical protein)	-1.60	1.11	1.00	-1.15	1.07	-1.03	1.14	**2.67**	1.53
CN655710	*Zinc finger protein 85	-1.94	1.48	1.31	-1.28	1.06	**2.01**	1.03	**2.27**	**2.44**
	5. Metabolism (56)	**L1**	**L2**	**L3**	**K1**	**K2**	**K3**	**S1**	**S2**	**S3**
CF931571	14kDa apolipoprotein	-1.81	1.25	-1.33	**-6.93**	-1.30	1.02	-1.07	-1.05	-1.04
CF931743	14kDa apolipoprotein	-1.80	1.21	-1.04	**-6.84**	-1.18	-1.10	1.07	-1.66	-1.34
CN655583	1-phosphatidylinositol-4-phosphate 5-kinase isoform C	-1.82	1.25	-1.09	**-2.55**	-1.19	-1.36	1.02	-1.49	1.01
CF931639	ADP, ATP translocase	-1.44	-1.19	1.24	-1.47	-1.30	**2.98**	-1.01	**3.53**	1.89
CN655636	ADP, ATP translocase	-1.21	-1.02	1.13	-1.87	-1.18	**2.27**	1.05	**2.05**	1.72
CN655596	antifreeze protein precursor	-1.81	1.25	-1.04	**-4.44**	-1.51	1.08	-1.03	-1.51	-1.09
CF931577	antifreeze protein precursor	-1.92	1.03	-1.04	**-3.38**	-1.34	-1.22	-1.15	-1.28	-1.23
CF931729	apolipoprotein A-I precursor	-1.81	1.25	-1.04	**-7.11**	1.19	1.57	-1.13	-1.39	-1.21
CN655585	apolipoprotein A-I precursor	-1.82	1.25	-1.04	**-6.65**	1.16	1.48	-1.23	-1.32	-1.06
CF931735	*apolipoprotein E	**-35.87**	1.13	1.73	**-3.36**	**3.74**	**4.79**	-1.07	-1.57	1.03
CN655698	*aspartate aminotransferase	1.04	-1.02	1.03	-1.29	-1.58	**-2.11**	**-2.30**	1.65	1.72
CF931666	*aspartate aminotransferase	-1.35	1.06	1.18	1.21	1.29	**2.52**	-1.02	**2.20**	1.55
CF931742	*ATP-binding cassette transporter 1 (ABC A1)	1.80	**-2.31**	1.50	-1.16	1.10	-1.29	1.32	1.08	**2.36**
CF931569	*betaine-homocysteine S-methyltransferase	**2.68**	-1.12	**8.59**	**-13.49**	**-2.64**	1.01	-1.28	1.68	-1.38
CN655685	*cytochrome c oxidase subunit I	-1.03	-1.58	1.15	-1.24	1.08	**2.58**	-1.16	1.95	**2.94**
CN655587	*cytochrome c oxidase subunit III	-1.11	1.09	**2.17**	-1.16	1.04	**2.03**	-1.53	1.52	1.82
CN655620	*cytochrome c oxidase subunit VIa precursor	-1.02	-1.12	1.27	-1.24	-1.09	**2.11**	1.06	-1.18	1.01
CF931895	*dihydrodipicolinate synthase	1.09	-1.04	1.49	-1.23	1.72	**2.20**	-1.42	1.75	-1.14
CN655693	*erythroid 5-aminolevulinate synthase	1.61	-1.31	**-2.23**	-1.10	-1.03	-1.33	1.13	1.53	1.16
CF931659	farnesyl diphosphate farnesyl transferase 1; squalene synthase	-1.64	1.24	-1.05	**-3.24**	-1.25	-1.42	1.09	-1.51	-1.18
CN655699	*glutamate dehydrogenase	1.05	1.20	**2.84**	-1.15	1.00	**2.78**	-1.17	1.59	**2.18**
CN655680	*glutamate dehydrogenase (GDH)	1.07	1.30	1.94	-1.34	1.13	**2.04**	-1.04	1.55	1.76
CN655594	*glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	-1.20	1.52	1.70	**-2.45**	**2.24**	**3.46**	**-2.02**	1.76	-1.16
CN655639	liver-basic fatty acid binding protein	-1.03	1.64	-1.04	**-4.39**	-1.33	-1.24	1.01	-1.52	-1.17
CF931893	*lysosomal cofactor/neurotrophic factor prosaposin	-1.04	-1.07	-1.25	-1.30	1.25	1.10	-1.06	**2.30**	**2.41**
CN655690	*Methyltransferase-related protein (putative)	1.32	1.03	-1.40	1.11	-1.01	1.41	1.57	1.11	**2.44**
CF931812	O-methyltransferase containing protein (putative)	**3.09**	-1.33	1.28	1.07	-1.28	-1.38	1.06	-1.54	-1.16
CF931813	phenylalanine hydroxylase	**-2.68**	-1.37	-1.06	1.46	-1.26	-1.39	-1.03	-1.23	-1.34
CF931916	*retinol dehydrogenase type I	1.09	1.58	-1.24	-1.66	1.15	**2.59**	-1.27	1.37	-1.12
CF931889	*similar to malate dehydrogenase	**2.51**	1.87	-1.41	-1.12	**-2.12**	1.37	1.87	1.92	**2.32**
CF931986	*sodium/potassium-transporting ATPase alpha-1 chain precursor	1.00	1.11	-1.16	1.07	1.73	**3.94**	-1.29	**2.36**	1.66
CF931888	*succinyl-coA ligase [GDP-forming], alpha-chain	-1.07	1.10	1.45	-1.47	1.33	**2.35**	1.00	1.31	1.22
CF931935	*thymidine kinase 1, soluble	-1.49	1.29	-1.52	-1.50	-1.33	1.90	1.06	1.70	**2.36**
CF931795	*transketolase	1.41	-1.23	1.35	1.13	-1.76	**2.85**	1.17	1.13	-1.01
	6. Unclassified function (149)									

Table 3. Differential expression of Atlantic halibut genes upon infection with atypical *Aeromonas salmonicida*. The shaded numbers indicate differential expression. The numbers in the category of functional classification indicate the number of genes.

Days	Fish#	Hepcidin I	(Avg±STD)	MHC I	(Avg±STD)	RAG	(Avg±STD)
1	Fish 1	2.3		0.2		1.6	
	Fish 2	0.9	5.1±6.2	1.2	0.6±0.6	0.2	0.6±0.9
	Fish 3	12.2		0.3		0.0	
2	Fish 1	67.8		0.3		1.7	
	Fish 2	28.0	32.1±33.9	9.4	4.8±4.5	1.1	2.1±1.3
	Fish 3	0.4		4.6		3.5	
7	Fish 1	110.2		2.2		9.9	
	Fish 2	4.3	39.5±61.2	0.6	1.1±1.0	0.2	3.6±5.5
	Fish 5	4.1		0.4		0.6	
14	Fish 1	0.5		4.4		9.0	
	Fish 2	0.9	0.6±0.2	1.7	2.3±1.9	2.8	4.2±4.3
	Fish 4	0.6		0.8		0.8	
21	Fish 1	8.1		0.2		0.2	
	Fish 2	7.1	6.7±1.7	0.9	0.9±0.7	0.2	3.0±4.8
	Fish 5	4.8		1.7		8.5	

Table 4. Quantitative RT-PCR analysis was conducted from kidney and liver tissues collected at 1, 2, 7, 14, 21 days post infection. Fish were experimentally infected by i.p. injection with atypical *Aeromonas salmonicida* or phosphate-buffered saline (PBS) as control. MHC I and RAG-1 were selected to determine mRNA expressions in the kidney ;while, hepcidin type I was selected for the liver tissue. As control genes for qRT-PCR, EF-1A was used for MHC I and RAG-1; while, a ribosomal gene was used to determine relative expression of hepcidin I. Samples were taken from 3 fish, indicated by the number associated with each tissue (e.g., K1 is the kidney sample from fish 1).

Gene expressions in kidney

We identified a total of 87 genes, which were differentially regulated (≥ 2-fold) in at least one of the kidney samples (Table 3). Of these 46 genes were significantly up-regulated and 46 genes were down-regulated among the three fish. Levels of differential expression seen in the kidney were 2.0 to 4.8-fold increases and 2.0 to 13.5-fold decreases (Table 3). Higher numbers of genes were down-regulated in the kidney of fish 1 than fish 2 and 3, while higher numbers of genes were up-regulated in the kidney of fish 2 and 3 than fish 1.

Up-regulated genes: Forty-six genes were differentially up-regulated in fish 2 (3 genes) and fish 3 (46 genes) but no genes were seen to be up-regulated in fish 1 (Table 3). Among differentially up-regulated genes the following had more than a 2-fold change in at least one of samples: cell/organism defence genes such as MHC II, chemotaxin, heat shock protein 70, and T-complex protein 1 theta subunit (Table 3). Interferon consensus sequence binding protein was also up-regulated in fish 3 with a 2.1-fold increase. Other significantly up-regulated genes included proteasome activator subunit 2, ADP, ATP translocase, leukocyte elastase inhibitor, apolipoprotein E and a novel protein (similar to yeast and bacterial cytosine deaminase). Apolipoprotein E was up-regulated in both fish 2 and 3, while it was down-regulated in fish 1. The genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also significantly up-regulated in fish 2 and 3, while it was down-regulated in fish 1 (Table 3). The differentially expressed levels of MHC I at 21 DPI were not significant in both microarray and qRT-PCR analysis, but qRT-PCR analysis showed the significant level (≥ 2-fold) of expression from at least one fish at 2,7,14 DPI. In the qRT-PCR analysis, the expression level of RAG-1 was also significantly increased from at least one fish after 2 DPI, but expression levels of the gene in microarray analysis was not significant due to low hybridization signal intensities.

Down-regulated genes: Forty-six genes showed significant down-regulation. Most of the down-regulated genes were obtained from fish 1 (41 genes), as described above, while only five genes were down-regulated in fish 2 and 3, including aspartate aminotransferase, betaine-homocysteine S-methyltransferase, malate dehydrogenase and an unknown gene (CF931989). Of the differentially down-regulated genes in fish 1, a variety of functional genes such as metabolism, cell/organism defense and cell signaling/cell communication were observed, including MHC class I, MHC class II, complement component C3, signal recognition particle 54 kDa, 14 kDa apolipoprotein, chicken-type lysozyme, betaine-homocysteine S-methyltrasferase, apolipoprotein A-I, apolipoprotein E and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Table 3).

Gene expressions in spleen

We identified a total of 94 different genes that were differentially up- or down-regulated (≥ 2-fold change, $p<0.05$) in at least one of the spleen samples (Table 2,3). Eighty-one genes were significantly up-regulated with 2.0- to 7.2-fold changes in expression, while 20 genes were down-regulated with 2.0 to 14.9-fold changes. As in the kidney samples, fish 1 showed only down-regulated genes (16 genes), while no gene was seen to be up-regulated. Fish 2 and 3 showed the highest number of genes which were up- or down-regulated; most of those genes were up-regulated (64 up- and 3 down- in fish 2; 53 up- and 5 down- in fish 3).

Up-regulated genes: Of the 81 up-regulated genes in the spleen of fish 2 and 3, we found many genes classified as those having cell/organism defence functions.

Antigen processing and presenting system-related genes were significantly up-regulated, including MHC I, MHC II, MHC II-associated invariant chain, proteasome activator subunit 2. These genes showed a 2.1-to 7.2-fold increase. Of these genes, MHC I showed the highest signal ratio of all three MHC genes printed on the microarray, showing > 7.2-fold increases.

We found that chaperone-related genes were significantly up-regulated. On the microarray we spotted four chaperone-related genes. Of those, heat shock protein (HSP)70 and HSP90, and t-complex polypeptide 1 were significantly up-reg-

ulated with 2.0- to 7.1-fold increases.

The leukocyte antigens, CD53-like antigen and CD63-like antigen, were significantly up-regulated with 2.2- to 6.1-fold changes. The gene encoding a receptor for activated protein kinase C also showed a significant increase, showing 2.4- and 2.7-fold increases in the spleen of fish 2 and 3, respectively.

Down-regulated genes: Twenty genes were down-regulated from at least one of the samples. Most of down-regulated genes (16) were obtained from the spleen of fish 1, including the receptor for urokinase plasminogen activator, trypsinogen 2 precursor, cathepsin D precursor, aspartate aminotransferase, elastase A precursor, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), carboxypeptidase homolog, trypsinogen 2 and 3 and chymotrypsinogen 2.

Pathway analysis

We identified a total of 97 different genes that were differentially up- or down-regulated (≥ 2-fold change, $p<0.05$) in at least one of the 3 tissue samples from fish 2 and 3. Among those genes, 62 genes were matched with DAVID IDs and 36 genes were clustered into KEGG pathways. The most abundant pathways identified at 21DPI were antigen processing and presentation, and complement and coagulation cascades. In the antigen processing and presentation pathway, the following genes were included: MHC I, MHC II, proteasome activator subunit, cd74 antigen (invariant polypeptide of MHC II), cathepsin I and HSP 70. On the other hand, four genes were clustered into complement and coagulation cascades, which included serpin peptidase inhibitor, fibrinogen alpha and gamma chain and plasminogen activator. Interestingly, a talin gene was also identified in the focal adhesion pathway.

Discussion

We created cDNA microarray using clones obtained from an immune-enriched EST library constructed from the liver, kidney and spleen of Atlantic halibut [8]. This microarray was used to study differentially expressed genes within Atlantic halibut liver, kidney and spleen experimentally infected with atypical *A. salmonicida*. At 54 DPI 37% cumulative mortality was observed; 20% mortality was reached at 21 DPI when samples were taken for the microarray. Generally we have observed that flatfish, including Atlantic halibut, do not show overt clinical signs of disease nor do they demonstrate changes in feeding or other behaviours during infections. Therefore we could not differentiate between healthy or moribund fish among those sampled at 21 DPI. We would expect variation in the progression of clinical disease amongst individually infected fish, and this may be an explanation for the observed high degree of variation of gene expression among the three fish studied. However, it must be noted that even though we do not have exact data for bacterial numbers within the fish sampled for microarray and qRT-PCR analysis, culturable *A. salmonicida* was recovered from all mortalities arising from the challenge. Individual fish have been shown to have highly variable expression levels of mRNA or protein [9]. In challenges, the cohabitation method has the advantage of mimicking real infection. However this method also results in differences in the timing of infection and in the progression of clinical disease among individual fish. Pooling mRNA sample for microarray analysis has been recommended under these or other conditions, especially when the biological variability is large relative to the technical variability [10,11]. However, this method carries with it the risk of neutralizing differentially expressed genes in the microarray analysis [12]. Therefore, we used the injection method to avoid variation in disease progression and we investigated individual fish to study the difference of the response to bacterial infection. As expected, we found a wide variation in the genes expressed among the three fish analyzed. It is true that this variation may be due to differences in disease progression, even though each fish had the same initial dose of bacteria, however it is more likely that the observed differences reflected the differences in gene responses from individual fish. Notably, gene expression of fish 1 was very different compared with fish 2 and 3. We found in fish 1 that more genes, including metabolism and host defence-related genes were down-regulated than those in fish 2 and fish 3 (Table 3). Based on the expression of these genes, we can postulate that fish 1 was in a moribund stage.

In our previous vaccination study using microarray analysis, we found that many genes encoding for the acute phase response or inflammation related proteins were up-regulated at early time points such as 1 and 2 days post-vaccination against *L. anguillarum* and *A. salmonicida*. These included haptoglobin, wap65, leucin-rich alpha-2-glycoprotein and transferrin. In this present study we found that some genes encoding for the acute phase response and inflammation related proteins, such as transferrin and alpha-2-macroglobulin-1, were still up-regulated whereas wap65 had normal expression at 21 DPI. These changes varied with individual fish. A number of acute phase-response genes showed significant up-regulated expression in fish 1 at 21 DPI, including plasma protease (C1) inhibitor, alpha-2-macroglobulin-1 (Table 3) whereas these genes had normal expression in fish 2 and fish 3. *A. salmonicida* produces serine protease [13] and we found that leukocyte elastase inhibitors and protease inhibitors were differentially up-regulated in the kidney of fish 2 and spleen of fish 3. Results obtained from this study and Ewart et al. [12] support the supposition that not all genes related to acute phase response or inflammation are regulated in the same manner (reviewed in [14]).

Many genes encoding proteins belonging to the metabolism functional category were differentially up- or down-regulated

at 21 DPI. Here, we discuss two genes that encode for apolipoprotein E and cathepsin D. These two genes showed a significant difference in expression between fish 1 and fish 2 and 3. Apolipoprotein E showed the most significant down-regulation with a 35.9-fold change in the liver of fish 1, however the expression of this gene was unchanged over time in fish 2 and 3 (Table 3). Apolipoprotein E is involved in inflammation progress [15,16]. Apolipoprotein E deficient mice were more susceptible to endotoxemia than wild-type mice becuase apolipoprotein E is involved in the detoxification of LPS [17,18]. Even though the expression level has not been well studied in infection cases, serum levels of apolipoprotein E at the acute inflammation phase was significantly reduced [16]. Cathepsin D was also down-regulated in all tissues studied in fish 1, whereas its expression was unchanged in fish 2 and 3 (Table 3). Cathepsin D activity in human is highest in monocytes and the activity of this enzyme was significantly decreased in HIV-infected patients [19]. It was also found that the expression of cathepsin D was decreased in phagosome maturation [20]. Results from our study on these two genes may indicate that there was more inflammation processing occurring in fish 1 than fish 2 and 3.

With respect to genes involved in innate immunity, genes encoding hepcidins showed noteworthy results. As in multiple forms of complement C3 of rainbow trout (*Oncorhynchus mykiss*) [21,22], we found 2 forms of halibut hepcidins in the previous ESTs study [8]. In the previous study examining immune response to vaccination, we observed that hepcidin type I was more highly expressed than hepcidin type II in liver samples pooled from three fish. We, therefore, could not compare the expression differences among individual fish. In this study, we found that each fish preferentially expressed a gene for a specific type of hepcidin. In fish 1 and 2, we found that the gene expression of hepcidin type II (two probes) were significantly up-regulated in the liver, whereas hepcidin type I was at unchanged expression levels. However, fish 3 showed a different expression pattern in which halibut hepcidin type I was up-regulated whereas halibut hepcidin type II expression was stable. Even though we found that each fish preferentially expressed a specific type of hepcidin during the infection period, it is still remains to see if both types have antimicrobial function or other biological functions within Atlantic halibut during bacterial infections.

Transferrin has multiple biological functions as well as its primary role as an iron-binding protein [23-25]. Transferrin was significantly up-regulated in the spleen of fish 2 and 3 (Table 3), whereas no significant differential expression was observed in the liver and kidney (Table 3). In the our previous study, this gene was significantly up-regulated in the liver at 1 day post-vaccination, but it was not differentially expressed in the kidney during 2 weeks post-vaccination. We postulate that the up-regulation was a positive acute phase response to vaccination. In the present study, we found the gene was significantly up-regulated in the spleen. In a report [12], this gene was up-regulated in the spleen and liver of Atlantic salmon at 13 days post infection with *A. salmonicida*, whereas no differential expression was found in the head kidney. Up-regulation of this gene in the spleen at the mid-infection time point, such as was observed in this study, would be expected, if this protein has another role as an endogenous activator of the macrophage antimicrobial response [23-25].

Two genes, actin-related genes and GAPDH, were significantly up-regulated in the kidney and spleen of fish 2 and 3 (Table 3), whereas these genes were unchanged or down-regulated in fish 1. Although these genes are still used as controls for RT-PCR in many studies on gene expression in teleosts and other organisms, we found them unsuitable for use as control genes in tissues or organisms in which there is a state of physiological change [26-28]. In our study, the up-regulation of these genes may indicate that fish 2 and 3 were conducting active leukopoiesis in kidney and spleen tissues. This possibility is supported by the fact that two genes, CD53 and CD63-like proteins, were significantly up-regulated in the spleen of fish 2 and 3 (Table 3). Even though the function of these two proteins has not been characterized in fish as conclusively as they have in higher vertebrates, they are very common antigens on the surface of leukocyte [29-31], and so it is reasonable to extrapolate a leukopoietic activity. Recombination activating genes (RAG-1 and RAG-2) have important role in the rearrangement and recombination of the genes during B cells and T cells maturation [32]. We spotted halibut RAG-1 probe on the microarray, but the fluorescence signal intensity of the gene was less than minimum intensity for analysis (500 unit) from all three tissues. However, in the qRT-PCR analysis, the gene expression level was significantly increased after 2 DPI from at least one fish. This result corresponds with our previous experiment on halibut vaccination, in which RAG-1 gene was significantly up-regulated after 2 days post vaccination. These two results may indirectly indicate that Atlantic halibut also has immunological capability to generate diversity of antibodies.

We have also investigated the differential expression of heat shock proteins (HSPs): HSP70, heat shock cognate 71 kDa, HSP90. Microarray analysis showed that expressions of HSP70 and HSP90 were increased when the fish were infected with *A. salmonicida*. The spleen of fish 2 and the spleen and kidney of fish 3 showed increased expressions of HSP70 and HSP90 (Table 3). In our microarray data we have found coincident up-regulation of HSP 70, HSP 90, MHC I, MHC II and MHC II-associated invariant chain, especially in the spleen of fish 2 and fish 3 (Table 3). This up-regulation is not surprising because many studies support the fact that heat shock proteins might modulate the host immune response [33] and coincidently up-regulate with MHC I, MHC II [34]. Proteasome activator subunit 2 was also up-regulated in the kidney and spleen of

fish 3 (Table 3). It has been shown that the transcriptional level of the proteasome activator is increased by LPS stimulation and bacterial infection [35]. This up-regulation in our study seems to be related to antigen presentation. MHC class I and II are core components of the adaptive immune system. Antigenic peptides produced by cytosolic proteasome are transported by a transporter associated with antigen processing and presented to T cells by class I molecules [36]. Even though we do not have a complete set of genes on the microarray for studying antigen presentation, we have found that adaptive immune system genes including those for antigen presentation were significantly activated from two of the three infected fish at 3 weeks post infection.

Some virulent pathogenic bacteria use sophisticated type III secretion systems to inject virulence proteins into host cells [37]; while, rapid turnover and exfoliation of mucosal epithelial cells of host provide effective defense mechanisms against infection [38]. It is also known that T3SS is a virulent factor of *Aeromonas salmonicida* [39]. In our microarray analysis, a talin gene involved in focal adhesion pathway was significantly increased at 21 DPI. Focal adhesion proteins are important for cell survival. Even though talin's function as an innate defense is not studied in fishes, the defense functions of focal adhesion proteins were recently studied in human immune system [37, 38]. We postulate here that infected halibut in our study also might over express this gene to inhibit colonisation of bacteria onto cells. This will be investigated in the future studies.

This study reports the first comprehensive examination the transcription profiles of Atlantic halibut during infection with atypical *A. salmonicida* using a cDNA microarray. Despite the limitation of the number of genes printed on the halibut microarray, we have found valuable information to help further our understanding of the interactions between Atlantic halibut and atypical *A. salmonicida*. At 3 weeks post infection, each fish showed a high variation of gene expression. We found that several genes encoding adaptive immune system-related proteins, especially involved in antigen processing and presentation, were up-regulated within the spleen of two infected fish. More intensive studies using microarrays will be conducted in the future. Atypical *A. salmonicida* infections have recently become more prevalent in marine aquaculture species and therefore knowledge of host responses to these economically important pathogens will enable us to develop more efficient health management tools.

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