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Identification and analysis of differentially expressed genes in immune tissues of Atlantic cod stimulated with formalin-killed, atypical *Aeromonas salmonicida*

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[1]*Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland;* [2]*Fisheries and Oceans Canada, Pacific Biological Station, Nanaimo, British Columbia;* [3]*The Atlantic Genome Centre;* [4]*Institute for Marine Biosciences, National Research Council;* and [5]*Department of Process Engineering and Applied Science, Dalhousie University, Halifax, Nova Scotia, Canada*

Feng CY, Johnson SC, Hori TS, Rise M, Hall JR, Gamperl AK, Hubert S, Kimball J, Bowman S, Rise ML. Identification and analysis of differentially expressed genes in immune tissues of Atlantic cod stimulated with formalin-killed, atypical *Aeromonas salmonicida. Physiol Genomics* 37: 149–163, 2009. First published February 24, 2009; doi:10.1152/physiolgenomics.90373.2008.—Physiological changes, elicited in animal immune tissues by exposure to pathogens, may be studied using functional genomics approaches. We created and characterized reciprocal suppression subtractive hybridization (SSH) cDNA libraries to identify differentially expressed genes in spleen and head kidney tissues of Atlantic cod (*Gadus morhua*) challenged with intraperitoneal injections of formalin-killed, atypical *Aeromonas salmonicida*. Of 4,154 ESTs from four cDNA libraries, 10 genes with immune-relevant functional annotations were selected for QPCR studies using individual fish templates to assess biological variability. Genes confirmed by QPCR as upregulated by *A. salmonicida* included interleukin-1β, interleukin-8, a small inducible cytokine, interferon regulatory factor 1 (IRF1), ferritin heavy subunit, cathelicidin, and hepcidin. This study is the first large-scale discovery of bacteria-responsive genes in cod and the first to demonstrate upregulation of IRF1 in fish immune tissues as a result of bacterial antigen stimulation. Given the importance of IRF1 in vertebrate immune responses to viral and bacterial pathogens, the full-length cDNA sequence of Atlantic cod IRF1 was obtained and compared with putative orthologous sequences from other organisms. Functional annotations of assembled SSH library ESTs showed that bacterial antigen stimulation caused changes in many biological processes including chemotaxis, regulation of apoptosis, antimicrobial peptide production, and iron homeostasis. Moreover, differences in spleen and head kidney gene expression responses to the bacterial antigens pointed to a potential role for the cod spleen in blood-borne pathogen clearance. Our data show that Atlantic cod immune tissue responses to bacterial antigens are similar to those seen in other fish species and higher vertebrates.

Gadus morhua; bacterial antigen; immune response; transcriptome; gene expression

AEROMONAS SALMONICIDA SUBSP. SALMONICIDA is a Gram-negative bacterium that is the causative agent of typical furunculosis, a bacterial septicemia of coldwater fish. In addition to this subspecies, a large number of other subspecies exist that produce atypical forms of this disease. These subspecies, which are referred to as atypical strains, infect a wide range of fish hosts in a wide variety of environments (87). Both typical and atypical *A. salmonicida* are known to infect and cause disease in aquaculture-reared Atlantic cod (*Gadus morhua*) (67).

Lipopolysaccharides (LPS), unmethylated CpG motifs, peptidoglycan, and flagellin are common pathogen-associated molecular patterns (PAMPs) associated with Gram-negative bacteria, which can be recognized by a variety of host pattern recognition receptors (PRRs). These PRRs include a number of Toll-like receptors as well as other cell-surface and cytosolic receptors that, upon stimulation, modulate immunity (58, 83). In higher vertebrates, these receptors, their signaling pathways, and the immunological pathways that they stimulate, are relatively well characterized compared with our current understanding of lower vertebrates such as fish. To add to our knowledge of the immune system of fish, we are undertaking research aimed at fully characterizing the Atlantic cod immunome. This research is being conducted as part of the Genome Canada-funded Atlantic Cod Genomics and Broodstock Development Program (CGP, http://www.codgene.ca) and aims to develop a complete understanding of the genes and molecular pathways involved in Atlantic cod responses to pathogens and thus to facilitate the development of management practices, markers and methods for selecting disease-resistant broodstock, and new vaccines and therapeutics to combat disease outbreaks in Atlantic cod aquaculture.

To this end, we constructed reciprocal suppression subtractive hybridization (SSH) cDNA libraries enriched for genes that were differentially expressed in the spleen and hematopoietic kidney (head kidney) of juvenile Atlantic cod following stimulation with formalin-killed, atypical *A. salmonicida*. Sequencing of the expressed sequence tags (ESTs), the development of an EST database, as well as the development of quantitative reverse transcription-polymerase chain reaction (QPCR) protocols, enabled us to partially characterize, functionally annotate, and study the expression of genes involved in primary immune responses (2–72 h poststimulation) to these

bacterial antigens. The use of SSH libraries has been previously demonstrated to be an effective method for identifying Atlantic cod genes that are differentially expressed during immune responses to virus-like antigens (59, 71). We decided to study the head kidney and spleen due to their roles in fish as primary and secondary lymphoid organs, respectively (57). *A. salmonicida* was selected due both to its importance as a disease-causing organism, as well as the interest in developing vaccines against this pathogen for use in marine fishes (24, 43).

MATERIALS AND METHODS

Bacterial antigen preparation. Single colonies of atypical *A. salmonicida*, originally isolated from a Norwegian Atlantic cod [strain #aAs4099 (IMB #05-2)], were grown in 100 ml of tryptic soy broth overnight at 17°C, with shaking (100 rpm). Cultures were centrifuged (10 min, 2,000 g, 4°C), and the resulting pellet was washed twice with 40 ml of cold, sterile phosphate-buffered saline (PBS). After washing, the bacteria were resuspended in PBS to give an OD_{600} of 1.0 and inactivated by the addition of formalin (4% formalin wt/vol). Following inactivation, the bacteria were centrifuged to form a pellet (10 min, 2,000 g at 4°C), washed twice with 40 ml of PBS, and resuspended in PBS to an OD_{600} of 1.0. Inactivation was confirmed by plating 0.5 ml of the suspension onto tryptic soy agar and incubating for 24 h at room temperature. The inactivated cells were stored at −20°C until use.

Fish husbandry, bacterial antigen stimulation, and tissue sampling. We divided equally 150 passive integrated transponder-tagged, juvenile, healthy-appearing Atlantic cod (~25 g) from a single family (family 32, CGP 2006 year class) into three 500-liter tanks and maintained them in flowing seawater (10°C, >90% O_2 saturation) under a 12 h light-12 h dark photoperiod. The fish were fed daily (at 1.5% body mass/day) with a commercial fish feed and acclimated to the experimental system for 17 days before experimentation.

Immediately prior to stimulation, eight individuals from each tank (0 h controls) were captured with a dip net and rapidly killed by a lethal dose (0.4 g/l) of tricaine methanesulphonate (TMS) (Syndel Laboratories, Vancouver, BC, Canada). Brain, head kidney, and spleen tissues were placed individually in RNase-free 1.5 ml tubes, flash-frozen in liquid nitrogen, and stored at −80°C until RNA extraction. Dissecting tools were cleaned with RNAseZap (Ambion, Austin, TX) for spleen and head kidney excision or heat-sterilized for brain excision, between individuals to prevent cross contamination between samples. After 0 h control samples were taken, the remaining individuals received one of the following treatments: no handling (referred to as undisturbed control, or "UC"), an intraperitoneal (ip) injection of 100 μl of sterile PBS (referred to as PBS), or ip injection of 100 μl of formalin-inactivated *A. salmonicida* suspended in PBS (referred to as ASAL). Prior to injection, fish were captured with a dip net and lightly anaesthetized in an aqueous solution of 0.1 g/l TMS. At ~2 h, 6 h, 24 h, and 72 h postinjection (HPI), individuals from all three groups (UC, PBS, and ASAL) were captured, euthanized, and sampled as previously described for the 0 h control individuals (Supplemental Fig. S1*A*).[1]

Nodavirus testing. To determine if individuals used in library construction were asymptomatic carriers of nodavirus, a reverse transcription-polymerase chain reaction (RT-PCR) test for nodavirus (59) was conducted on individual brain RNA samples obtained from the UC and ASAL groups. This test was performed to avoid the use of nodavirus carriers as UCs (in SSH library construction) and to determine if nodavirus carrier status of brain tissue influenced gene expression in immune tissues (in QPCR studies). The results of the nodavirus testing, and the nodavirus carrier status of individuals

utilized in SSH library construction, are shown in Supplemental Fig. S1*B*.

SSH cDNA library construction and sequencing. Spleen and head kidney reciprocal SSH libraries were constructed to identify genes involved in the response to ip injection with bacterial antigens. For each tissue (spleen, head kidney) and condition (UC, ASAL), DNase I-treated, cleaned total RNA (10 μg per sample) from five individuals sampled at each time point (2, 6, 24, and 72 HPI) was used to generate the mRNA pools for the UC and ASAL groups (Supplemental Fig. S1*B*). Samples used to create the ASAL mRNA pool were taken from nodavirus carriers and noncarriers, whereas samples for the UC mRNA pool were taken only from noncarrier fish. This strategy was employed to maximize the difference between ASAL and UC pool transcriptomes, thereby maximizing the utility of resulting SSH libraries for immune gene discovery. The individuals utilized for building spleen and head kidney SSH libraries, with nodavirus carrier status information, are shown in Supplemental Fig. S1*B*. For each tissue, the ASAL mRNA pool was the tester in the forward subtraction, and the driver in the reverse subtraction; the UC mRNA pool was the driver in the forward subtraction and the tester in the reverse subtraction. Therefore, the forward SSH libraries were enriched for transcripts that were upregulated due to injection stress, exposure to *A. salmonicida* antigens, and/or the presence of nodavirus (in brain) in an asymptomatic carrier state. The reverse SSH libraries were enriched for transcripts that were downregulated by these factors (Supplemental Fig. S1*B*).

In brief, SSH library construction was performed using the PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA) following the manufacturer's instructions as previously described (59). A brief description of the method and any differences from Ref. 59 follows. Total RNA was extracted from spleen and head kidney samples of individual fish (Supplemental Fig. S1) using TRIzol Reagent (Invitrogen, Burlington, ON, Canada). Total RNA was treated with DNase I (RNase-Free DNase Set; QIAGEN, Mississauga, ON, Canada) to remove residual genomic DNA and column purified (RNeasy MinElute Cleanup Kit, QIAGEN) as described (59). Poly(A)$^+$ RNA (mRNA) was isolated from UC and ASAL tissue total RNA pools (Supplemental Fig. S1*B*) using the MicroPoly (A) Purist Small Scale mRNA Purification Kit (Ambion, Austin, TX) as described below.

The SSH libraries were amplified using the Advantage 2 Polymerase Kit (Clontech) following the manufacturer's protocol and purified using the MinElute PCR Purification Kit (QIAGEN). The resulting cDNA libraries were TA cloned into pGEM-T-Easy (Promega, Madison, WI), and the transformations were performed using MAX Efficiency DH5α Chemically Competent Cells (Invitrogen).

DNA sequencing, sequence assembly, and gene identification. The methods used for sequencing the SSH libraries and for assembly and annotation of ESTs arising from these libraries were previously described (59). Briefly, DNA extracted from individual bacterial clones was amplified using Templiphi DNA polymerase and sequenced using ET terminator chemistry (GE Healthcare, Piscataway, NJ) on MegaBACE capillary sequencers. The resultant ESTs were first trimmed with PHRED (20, 21), then screened, and clustered using Paracel Transcript Assembler (PTA; Paracel, Pasadena, CA). Both contigs (contiguous consensus sequences) and singletons (individual sequence reads) generated by the PTA clustering procedure were annotated using AutoFACT (36). In our AutoFACT annotation, BLASTn was used to identify ribosomal RNA sequences in large and small subunit databases, while BLASTx was used for all other alignments. For both BLASTx and BLASTn, the BLAST hits with bit scores >40 were considered significant. All EST sequences have been deposited in GenBank dbEST (See Table 1 for accession numbers and EST library statistics). In addition, these sequences and their AutoFACT annotations can be accessed through the CGP EST database (http://ri.imb.nrc.ca/codgene).

QPCR. For 10 immune-relevant genes identified in the SSH libraries, transcript (mRNA) expression was studied in PBS and ASAL

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[1] The online version of this article contains supplemental material.

Table 1. *Statistics for ESTs generated from the spleen and head kidney SSH libraries*

Library Name	S_Forward	S_Reverse	HK_Forward	HK_Reverse
Tissue	spleen	spleen	head kidney	head kidney
Direction[1]	forward	reverse	forward	reverse
CGP identifier[2]	sb_gmnlsfas	sb_gmnlsras	sb_gmnlkfas	sb_gmnlkras
Accession numbers	EY974820–EY975867	EY975868–EY976954	EY972492–EY973450[3]	EY973692–EY974677
ESTs, n	1,048	1,087	1,033	986
Average EST length, bp[4]	375	400	333	460
Contigs,[5] n	136	157	172	229
Singletons, n	685	677	570	268
Nonredundant ESTs,[6] n	821	834	742	497
Redundancy, %[7]	21.7	23.3	28.2	49.6

[1]The forward suppression subtractive hybridization (SSH) libraries were constructed to enrich genes upregulated by the *Aeromonas salmonicida* injection, and the reverse SSH libraries were constructed to enrich genes downregulated by the *A. salmonicida* injection. S, spleen; [2]The identifiers of the SSH libraries in the Atlantic Cod Genomics and Broodstock Development Program (CGP) expressed sequence tag (EST) database: http://codgene.ca. [3]For head kidney (HK) forward library, 74 ESTs were not submitted to GenBank as they were rejected during the Paracel Transcript Assembler (PTA) clustering process. [4]The ESTs were trimmed with PHRED (20, 21) with the trim_alt and trim_cut-off fixed at 0.06, followed by the removal of known contaminant sequences and short sequences (<75bp), and the average EST length was calculated based on edited sequences. [5]Sequences generated were then clustered using PTA, with the cluster threshold set at 100 for relatively stringent clustering. [6]The number of nonredundant ESTs is the sum of the number of contigs plus the number of singletons. [7]The percent redundancy is the proportion of redundant ESTs in each library, calculated as [1 minus (number of nonredundant ESTs/total number of ESTs)] multiplied by 100.

tissues (spleen and head kidney) from five time points (2, 6, 24, 72 HPI, and 0 h preinjection control) using Power SYBR Green I dye chemistry and the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). With the exception of the 0 h control PBS group ($n = 5$), six fish from each group, tissue, and time point were used in the QPCR study. QPCR primers, designed from EST sequences (Tables 2–5 and Supplemental Table S1) using the Primer 3 program (65) (available at http://frodo.wi.mit.edu), are listed in Table 6. Dissociation curves were run to ensure that primer pairs amplified single products, and no-template controls were run to ensure that primer dimers were absent. The amplification efficiencies of primer pairs for SCYA, interferon regulatory factor 1 (IRF1), and 18S ribosomal RNA were determined previously (59). The amplification efficiencies of the other primer sets were determined as described (59). Expression levels of the genes of interest were normalized to 18S ribosomal RNA, which was stably transcribed in all samples involved in the QPCR study.

For each sample, 1 μg of DNase I-treated and column-purified total RNA was reverse-transcribed in a final reaction volume of 20 μl as in Rise et al. (59), and the resulting cDNA was diluted with nuclease-free H_2O to a final volume of 100 μl. PCR amplifications were performed using a 7300 Real Time PCR detection system (Applied Biosystems) using 25 μl reactions that contained 1 μl of diluted cDNA (10 ng input total RNA), 50 nM each of forward and reverse primer, and 1× Power SYBR Green PCR Master Mix (Applied Biosystems). The amplification program consisted of 1 cycle of 95°C for 10 min, 40 cycles of (95°C for 15 s and 60°C for 1 min) (primer annealing and extension stage), with the fluorescent signal from SYBR green measured at the end of each 60°C step. For each sample, the target transcript (gene of interest) and the normalizer (18S rRNA) were each run in duplicate on the same plate. The fluorescence thresholds and baseline were determined automatically using the 7300 PCR Detection System SDS Software Relative Quantification Study Application (version 1.2.3, Applied Biosystems). Thresholds were set manually if the software did not place them at the exponential phase of amplification with minimal variation between technical replicates. C_T (threshold cycle) values were obtained and used for calculation of relative quantity (RQ) of each transcript with the $2^{-\Delta\Delta C_T}$ quantification method and assuming 100% amplification efficiencies for gene of interest and normalizer primer pairs (41).

QPCR data analysis. All RQ data are presented as means ± SE. RQ values were subjected to a two-way (main effects group and sampling time) analysis of variance (ANOVA). In addition, one-way ANOVA (for each group and sampling time) with Tukey posttests were conducted to determine: *1*) whether PBS control sample gene

expression (RQ values) at 2, 6, 24, and 72 HPI differed significantly from gene expression in the 0 h control group from the PBS tank; *2*) if gene expression of ASAL group at each time point differed significantly from levels of gene expression in the 0 h control group from the ASAL tank; and *3*) if gene expression differed significantly between the PBS and ASAL group at each individual time point (2, 6, 24, 72 HPI, and 0 h). Differences in spleen and head kidney constitutive gene expression between asymptomatic carriers of nodavirus and noncarriers were examined by one-way ANOVA of RQ values obtained for all genes studied by QPCR at 0 h (prior to injection) as in Rise et al. (59). All statistical tests were performed using Systat 12.0 (Systat Software) with the *P* value set at ≤0.05.

Atlantic cod IRF1 characterization. The 5′ and 3′ ends of IRF1 cDNA were amplified using a commercial kit for RNA ligase-mediated-RACE, GeneRacer Kit (Invitrogen, Burlington, ON, Canada). IRF1-specific primers (Supplemental Table S2) were designed based upon IRF1 assembled EST sequences from the forward spleen SSH library (contig sb_gmnlsfas.73.C1, containing ESTs with accession numbers EY975211 and EY975084; Supplemental Table S1*A*). Briefly, 250 ng of the same mRNA from ASAL fish used in spleen SSH library construction (Supplemental Fig. S1*B*) was used as the RNA template. For 5′ rapid amplification of cDNA ends (RACE), touch-down PCR was performed with GeneRacer 5′ primer and IRF1_5′RACE1, followed by a nested PCR conducted with GeneRacer 5′ nested primer and IRF1 _5′RACE2. For 3′RACE, only one round of touch-down PCR was carried out with GeneRacer 3′primer and IRF1_3′RACE. The cycling conditions of both touch-down PCR and nested PCR are as specified in the GeneRacer Kit manual with the extension time set to 3 min for all cycles. Nested primer pairs (IRF1_F1, IRF1_R1, IRF1_F2, and IRF1_R2; Supplemental Table S2) were designed in the 5′ and 3′ untranslated regions to amplify the open reading frame (ORF). The cycling conditions for both PCRs were 1 cycle of 2 min at 94°C, 25 cycles of (30 s at 94°C, 30 s at 70°C, 3 min at 72°C), and 1 cycle of 10 min at 68°C.

All PCR amplifications were performed using the Advantage 2 Polymerase kit (Clontech) and all PCR products were gel extracted using the QIAQuick Gel Extraction kit (QIAGEN), ethanol precipitated, washed and cloned into PCR4-TOPO (Invitrogen). The clones were transformed into One Shot TOP10 competent cells, and plated with Luria broth (LB)/carbenicillin (50 μg/ml). Individual colonies were grown overnight at 37°C in LB/carbenicillin (50 μg/ml), and plasmid DNA samples were isolated in the 96-well format using standard methods. The insert sizes of recombinant plasmids were determined by *Eco*RI (Invitrogen) digestion prior to sequencing. For

Table 2. *Selected[1] transcripts identified in the forward spleen SSH library (designed to be enriched for genes upregulated by bacterial antigens)*

Accession Number[2]	Name of BLASTx hit (species)	% ID (align)	E-value	ESTs, *n*	Gene Ontology[4] or Function of BLASTx Hit[5]
	BLASTx Identification[3] of Cod cDNAs				
EY974899	small inducible cytokine SCYA104 (African cichlid)	42 (27/63)	1e-10	12	CC chemokine activity
EY974843	cathelicidin 1 (Atlantic cod)	97 (121/124)	9e-64	10	defense response
EY975257	ferritin heavy subunit (Atlantic salmon)	89 (157/176)	3e-89	5	ferroxidase activity; iron ion homeostasis; oxidoreductase activity
EY975281	ferritin middle subunit (Atlantic salmon)	75 (125/166)	2e-67	4	ferroxidase activity; iron ion homeostasis; oxidoreductase activity
EY975262	myeloid cell leukemia sequence 1b (zebrafish)	50 (61/121)	1e-23	3	negative regulation of apoptosis (Ref. 42)
EY975464	hepcidin (Atlantic cod)	100 (98/98)	9e-43	2	iron homeostasis antimicrobial activity (Ref. 72)
EY975498	interleukin 8 (Atlantic cod)	96 (96/99)	2e-43	2	CXC chemokine activity
EY975450	serum lectin isoform 4 (spotted halibut)	58 (31/53)	8e-12	2	sugar binding; complement pathway activation (Ref. 52)
EY974936	cathepsin L (barramundi)	41 (43/103)	4e-53	2	cysteine-type endopeptidase activity; antigen processing (Ref. 90)
EY975712	DNA damage-inducible transcript 4 (African clawed frog)	53 (50/94)	1e-17	2	DNA damage and/or p53 induced (Ref. 17)
EY975030	proliferating cell nuclear antigen (channel catfish)	96 (128/133)	1e-65	2	regulation of transcription
EY975027	BH3-interacting domain death agonist protein (zebrafish)	33 (48/142)	8e-13	2	positive regulation of apoptosis
EY975211	interferon regulatory factor 1 (snakehead)	41 (43/103)	3e-13	2	regulation of transcription
EY975863	basic transcription factor 3 (African clawed frog)	87 (152/173)	4e-72	2	regulation of apoptosis (Ref. 38)
EY975549	goose-type lysozyme 2 (Atlantic cod)	95 (89/93)	1e-44	1	peptidoglycan catabolism
EY975542	probable Bax inhibitor 1 (Japanese flounder)	88 (136/153)	9e-66	1	negative regulation of apoptosis
EY975059	caspase 3B (pufferfish)	72 (66/91)	2e-32	1	apoptosis
EY975676	interleukin-1β (Atlantic cod)	97 (41/42)	9e-16	1	inflammatory response
EY975713	BCL2-like10 (zebrafish)	81 (53/65)	4e-22	1	regulation of apoptosis (Ref. 33)
EY975339	CC chemokine type 3 (Atlantic cod)	95 (21/22)	1e-04	1	immune response; chemokine activity
EY975733	Toll-like receptor 8 (pufferfish)	84 (27/32)	1e-11	1	transmembrane receptor activity
EY975550	CXC chemokine receptor type 3B (rainbow trout)	66 (110/166)	1e-45	1	CXC chemokine receptor activity
EY974897	CXC chemokine receptor (rainbow trout)	71 (66/92)	3e-15	1	CXC chemokine receptor activity
EY975110	serum lectin isoform 3 precursor (spotted halibut)	62 (43/69)	6e-19	1	sugar binding
EY975124	heme oxygenase 1 (European sea bass)	65 (31/47)	1e-08	1	heme oxygenase activity
EY975780	natural killer-enhancing factor (Japanese flounder)	87 (131/150)	2e-75	1	antioxidant activity

[1]Criteria for selection of contigs and singletons are discussed in RESULTS. [2]For each contig (i.e., cluster containing at least 2 ESTs), the accession number for a representative EST is given. All contigs and singletons from this SSH library were annotated using AutoFACT (36). The additional information (e.g., functional annotations, BLASTx statistics, and GenBank accession numbers for contributing ESTs) is listed in online Supplemental Table S1*A*. [3]The top BLASTx hit with a gene name (e.g., not "hypothetical," "predicted," "unnamed," or "novel protein") is shown. The BLASTx statistics in this table were collected on October 8, 2008, and reflect the state of the GenBank nonredundant sequence databases on that date. The length of the BLASTx alignment (i.e., the number of amino acid residues translated from the cod cDNA that are aligned with the best BLASTx hit), percent identity (% ID) over the aligned region, and E-value are shown. [4]Only the "molecular function" and "biological process" Gene Ontology terms are included in this table. [5]Putative functions are assigned based on citations of previous studies.

each PCR product, four individual clones were sequenced in both directions using the ABI 3730 DNA Analyzer using standard techniques.

Atlantic cod IRF1 amino acid sequence analysis and phylogenetic tree construction. The amino acid (AA) sequence of Atlantic cod IRF1 was deduced based on the cDNA sequence using the SeqBuilder function of Lasergene 7.20 software package (DNASTAR, Madison, WI), and the mRNA features such as Sm sites, snRNP binding sites, and polyadenylation signal were predicted using the RNA analyzer (4) (available at http://rnaanalyzer.bioapps.biozentrum.uni-wuerzburg.de/). The IRF1 DNA binding domain model was predicted and visualized by Swiss-model and Swiss-PdbView software (2, 25, 35, 69) (available at http://swissmodel.expasy.org/). The deduced Atlantic cod IRF1 AA sequence was compared with the orthologous AA sequences from other vertebrates. The multiple alignments were performed using the CLUSTALX (version 2.09) program, and the unrooted phylogenetic tree for IRF1 was constructed by the neighbor-joining method and

was bootstrapped 10,000 times. The phylogenetic trees were plotted using the MEGA4 (78).

RESULTS

Screening of cod immune tissue transcripts responsive to stimulation with bacterial antigens. To identify genes important in the response of Atlantic cod to bacteria, juvenile fish were stimulated with formalin-killed, atypical *A. salmonicida*, and reciprocal SSH libraries from spleen and head kidney were constructed and sequenced. The single family of fish that was used was the same family utilized in a previous report (59). Although this family was selected due to their good growth and high survival in the laboratory, there were asymptomatic carriers of nodavirus within this family (Supplemental Fig. S1). Nodavirus screening by RT-PCR on 32 individual fish brain

samples from each of UC and ASAL group revealed that 20 and 32.5% of fish in the UC and ASAL groups, respectively, were carriers of nodavirus (Supplemental Fig. S1). For SSH library construction, we utilized *A. salmonicida*-stimulated spleen and head kidney samples from both nodavirus carriers and noncarriers. UC tissues were obtained only from nodavirus negative fish. The resulting libraries, although biased toward genes involved in the response to the bacterial antigens, may also contain genes that are responsive to nodavirus carrier status and stress associated with the ip injection. With respect to immune-related genes, our selection of early time points (2, 6, 24, and 72 HPI) biased our results toward identification of genes involved in innate immunity.

We obtained a total of 4,154 good quality ESTs including: 1,048 from the forward spleen library (designated "sb_gmnlsfas" in Table 1, Supplemental Table S1, and the cod gene website), 1,087 from the reverse spleen library (sb_gmnlsras), 1,033 from the forward head kidney library (sb_gmnlkfas), and 986 from the reverse head kidney library (sb_gmnlkras) (Table 1; www.codgene.ca). Our ESTs are 3′ biased and relatively short, averaging 300–500 bp in length (Table 1). The presence of short coding sequences is in part responsible for some of the higher E-values (up to 1e-4) reported in Tables 2–5. With the exception of the head kidney reverse library, which showed 49.6% redundancy, the libraries were relatively complex ($<$ 29% redundancy) (Table 1). Selected contiguous sequences (contigs) and singletons from the forward spleen, reverse spleen, forward head kidney, and reverse head kidney libraries are shown in Tables 2, 3, 4, and 5, respectively. These data are limited to contigs and singletons with immune-related functional annotations. Complete lists of assembled sequences in these libraries, with contributing EST accession numbers and functional annotations, are found in online Supplemental

Table S1, *A* (forward spleen library), *B* (reverse spleen library), *C* (forward head kidney library), and *D* (reverse head kidney library).

The deepest contigs (i.e., having the highest numbers of contributing ESTs) in the forward spleen library were identified as a small inducible cytokine (SCYA, 12 contributing ESTs), and cathelicidin antimicrobial peptide (CAMP, 10 ESTs) (Table 2; Supplemental Table S1*A*). Other contigs present in this library were identified as ferritin heavy subunit (FTH, 5 ESTs), ferritin middle subunit (FTM, 4 ESTs), and myeloid cell leukemia 1 (MCL1, 3 ESTs). In the head kidney forward library, FTM (8 ESTs), FTH (6 ESTs), CAMP (3 ESTs), proteasome activator subunit 2 (3 ESTs), and goose-type lysozyme 1 (3 ESTs) were among the most common sequences, not including unclassified sequences (i.e., no significant BLAST hit) and transcripts found in both forward and reverse libraries (e.g., hemoglobin subunits) (Table 4; Supplemental Table S1*C*). Although the two forward libraries shared some transcripts in common, numerous genes were only identified in one of the libraries. For example, transcripts identified as SCYA, IRF1, hepcidin antimicrobial peptide (HAMP), interleukin 8 (IL8), basic transcription factor 3 (BTF3), DNA-damage-inducible transcript 4, interleukin 1 beta (IL1β), and serum lectin isoforms 1 and 2, were unique to the forward spleen library (Table 2; Supplemental Table S1*A*). Transcripts identified as proteasome activator subunit 2, translationally controlled tumor protein, CD84 molecule, LPS binding protein, interleukin 5 receptor alpha, and inhibitor of nuclear factor kappa B alpha, were unique to the forward head kidney library (Table 4; Supplemental Table S1*C*).

Within the spleen and head kidney reverse libraries multiple heat shock protein (HSP) transcripts were identified, including transcripts for HSP 90. Within the reverse libraries, several

Table 3. *Selected[1] transcripts identified in reverse spleen SSH library (designed to be enriched for genes downregulated by bacterial antigens)*

| Accession Number[2] | BLASTx Identification[3] of Cod cDNAs | | | | |
	Name of BLASTx hit (species)	% ID (align)	E-value	ESTs, n	Gene Ontology[4] or Function of BLASTx Hit[5]
EY976151	acetylserotonin (zebrafish)	38 (81/210)	2e-21	4	LPS responsive in isolated macrophages (Ref. 23)
EY975983	upstream transcription factor 1 (USF1) (zebrafish)	71 (49/69)	2e-16	2	regulation of transcription; immune response (Ref. 14)
EY976034	interleukin-1 receptor-like protein precursor (Atlantic salmon)	65 (30/46)	5e-11	2	interleukin-1 receptor activity
EY976089	novel immune-type receptor 4 (rainbow trout)	50 (77/153)	6e-26	2	receptor activity
EY976541	CD63 (rainbow trout)	65 (75/114)	3e-21	2	protein binding; cell adhesion regulation
EY976556	Toll-like receptor 23 (pufferfish)	75 (104/138)	2e-58	1	transmembrane receptor activity; protein binding
EY976136	lymphocyte antigen 75 (cow)	39 (64/163)	2e-31	1	receptor-based antigen processing for MHC class I presentation (Ref. 9)
EY976820	cell division cycle and apoptosis regulator 1 (CCAR1) (zebrafish)	41 (71/170)	3e-22	1	apoptosis (Ref. 60); apoptosis regulation (Ref. 46)
EY976002	E3 ubiquitin-protein ligase Itchy (mouse)	81 (122/150)	1e-67	1	regulation of p73 stability; downregulated by DNA damage (Ref. 64)
EY976504	leukocyte elastase inhibitor (rainbow trout)	70 (46/65)	5e-38	1	involved in caspase-independent apoptosis (Ref. 82)
EY976417	caspase 8 (dog)	48 (35/72)	3e-08	1	caspase activity; regulation of apoptosis
EY976348	mitogen-activated protein kinase 14a (zebrafish)	73 (36/49)	4e-14	1	kinase activity
EY976608	MIP1alpha (Japanese flounder)	43 (38/88)	4e-14	1	CC chemokine activity
EY976661	heat shock protein 90 (pink stalk borer)	93 (28/30)	6e-08	1	regulation of progression through cell cycle; protein folding
EY976096	complement receptor-like protein 1 (rainbow trout)	40 (43/106)	1e-18	1	receptor activity

[1]All contigs and singletons from this SSH library were annotated using AutoFACT (36). The additional information (e.g., functional annotations, BLASTx statistics, and GenBank accession numbers for contributing ESTs) is listed in online Supplemental Table S1*B*. [1–5]See footnotes for Table 2.

Table 4. *Selected [1] transcripts identified in forward head kidney SSH library (designed to be enriched for genes upregulated by bacterial antigens)*

	BLASTx Identification[3] of Cod cDNAs				
Accession Number[2]	Name of BLASTx hit (species)	% ID (align)	E-value	ESTs, n	Gene Ontology[4] or Function of BLASTx Hit[5]
EY972828	ferritin middle subunit (Atlantic salmon)	81 (127/155)	2e-65	8	ferroxidase activity; iron ion homeostasis; oxidoreductase activity
EY972657	ferritin heavy subunit (Atlantic salmon)	90 (158/174)	3e-89	6	ferroxidase activity; iron ion homeostasis; oxidoreductase activity
EY973285	proteasome activator subunit 2 (common carp)	71 (113/158)	4e-45	3	antigen cleavage and presentation (Ref. 73)
EY972595	cathelicidin 1 (Atlantic cod)	98 (117/119)	3e-62	3	defense response
EY972694	cathepsin L (Japanese ricefish)	77 (98/126)	2e-58	3	antigen processing (Ref. 90)
EY972725	goose-type lysozyme 1 (Atlantic cod)	97 (143/146)	3e-73	3	lysozyme activity
EY973198	translationally controlled tumor protein (common carp)	66 (104/156)	8e-55	2	B cell growth factor (Ref. 32); interleukin production (Ref. 7)
EY972718	probable Bax inhibitor 1 (Japanese flounder)	88 (105/118)	9e-54	2	negative regulation of apoptosis
EY972894	cellular FLICE-like inhibitory protein (pig)	63 (26/41)	4e-07	1	caspase activity; regulation of apoptosis
EY972979	cyclin L1 (African clawed frog)	96 (24/25)	8e-07	1	cell division
EY972692	myeloid cell leukemia sequence 1b (zebrafish)	46 (49/106)	1e-16	1	negative regulation of apoptosis (Ref. 42)
EY972562	CD84 (mouse)	26 (48/180)	1e-05	1	lymphocyte proliferation, macrophage activation (Ref. 79)
EY972791	Src family-associated phosphoprotein 2 (Skap2) (*Astatotilapia burtoni*)	65 (78/120)	6e-25	1	regulation of leukocyte adhesion (Ref. 81)
EY973092	lipopolysaccharide-binding protein variant b (Atlantic cod)	98 (94/95)	6e-37	1	LPS binding (Ref. 76)
EY973172	interleukin 5 receptor-α (rat)	25 (32/124)	2e-06	1	interleukin-5 receptor activity
EY972529	inhibitor of nuclear factor-κB α (rainbow trout)	88 (23/26)	7e-05	1	LPS-inducible (Ref. 68)
EY972872	complement receptor-like protein 1 precursor (rainbow trout)	44 (34/76)	8e-15	1	receptor activity

[1]All contigs and singletons from this SSH library were annotated using AutoFACT (36). The additional information (e.g., functional annotations, BLASTx statistics, and GenBank accession numbers for contributing ESTs) is listed in online Supplemental Table S1*C*. [1–5]See footnotes for Table 2.

novel transcripts with gene names and functional annotations suggesting involvement with kinase or receptor activity were identified such as tyrosine kinase 2, mitogen-activated protein kinase 14a, scavenger receptor class B member 2, interleukin 1 receptor-like protein precursor (IL1R), lymphocyte antigen 75, complement receptor-like protein 1, a novel immune-type receptor 4, and Toll-like receptor 23 (Tables 3 and 5; Supplemental Table S1, *B* and *D*). In addition, transcripts with gene names and functional annotations suggesting involvement in apoptosis regulation (e.g., caspase 8, leukocyte elastase inhibitor, and cell division cycle and apoptosis regulator 1) were identified in the reverse spleen library (Table 3, Supplemental Table S1*B*).

Gene ontology annotation of nonredundant transcripts from SSH libraries. The nonredundant ESTs from each of the SSH libraries were assigned biological process gene ontology (GO) terms using AutoFACT and Goblet (26) as described (59). We were able to assign 57 and 49 GO terms for sequences from the forward and reverse spleen library, respectively (Fig. 1*A*; Supplemental Table S3, *A* and *B*). For the forward and reverse head kidney libraries (Fig. 1*B*; Supplemental Table S3, *C* and *D*), 58 and 27 GO terms were assigned, respectively. For the spleen libraries, the highest numbers of sequences were assigned to categories "immune response" (forward library) and "transport" (reverse library). For the forward head kidney library, the highest numbers of sequences were assigned to "protein biosynthesis," while for the reverse head kidney library, the highest numbers of sequences were assigned to "protein biosynthesis" and "protein folding" (Fig. 1*B*). In the spleen, "apoptosis"-annotated ESTs were more abundant in the forward library (Fig. 1*A*), and in the

head kidney, "regulation of apoptosis" and "apoptosis" were among the GO terms that were associated exclusively with ESTs in the forward library (Fig. 1*B*). A comprehensive list of assembled ESTs from these libraries, and their GO annotations, can be found at http://ri.imb.nrc.ca/codgene/.

Gene expression patterns following injection of formalin-killed A. salmonicida or PBS. Nine genes (IRF1, CAMP, HAMP, SCYA, IL1β, IL8, FTH, MCL1, and BTF3) from the forward spleen and head kidney libraries, and one gene (IL1R) from the spleen reverse library, were subjected to QPCR to study the magnitude and timing of their expression following ip stimulation with formalin-killed, atypical *A. salmonicida* (Fig. 2; Table 6; see Supplemental Table S4 for all relative quantification data and calculations). These genes were selected to investigate the influence of bacterial antigen stimulation on the expression of genes involved in the following biological processes: cytokine signaling (IL1β, IL1R, IL8, SCYA, and IRF1); apoptosis (BTF3, MCL1); iron homeostasis (FTH and HAMP); and antibacterial defense response (CAMP and HAMP). The CAMP QPCR was designed to study the overall expression of cathelicidin transcripts (i.e., all known paralogs) by utilizing primers in conserved regions (i.e., common to all cathelicidin-like ESTs represented in these SSH libraries).

The genes IRF1, CAMP, HAMP, and SCYA showed similar patterns of expression in the spleen and head kidney samples from the ASAL group, with highest levels of expression at 24 HPI, followed by a large reduction in expression by 72 HPI (Fig. 2, *A–H*). Of these, at 72 HPI, only CAMP had significantly higher expression in tissues (both spleen and head kidney) of *A. salmo-*

Table 5. *Selected[1] transcripts identified in reverse head kidney SSH library (designed to be enriched for genes downregulated by bacterial antigens)*

Accession Number[2]	BLASTx Identification[3] of Cod cDNAs			ESTs, n	Gene Ontology[4] or Function of BLASTx Hit[5]
	Name of BLASTx hit (species)	% ID (align)	E-value		
EY973973	cyclin B2 (rainbow trout)	51 (82/158)	4e-30	5	regulation of progression through cell cycle
EY974252	TRAF4-associated factor 1 (human)	30 (45/147)	6e-08	4	signal transduction; downregulated in nitric oxide-exposed human monocytic cells (Ref. 84)
EY974460	tyrosine kinase 2 (human)	67 (66/98)	1e-32	2	Signal transduction; Jak-STAT signaling pathway
EY974533	heat shock 60 kDa protein 1 (chicken)	57 (57/100)	4e-25	2	response to stress
EY974525	heat shock 90 kDa protein 1 β-isoform b (rainbow trout)	98 (98/100)	8e-49	2	response to stress
EY974305	scavenger receptor class B member 2 (cow)	50 (45/90)	1e-23	1	receptor activity
EY974340	HSP90 co-chaperone Cdc37 (green pufferfish)	74 (117/157)	3e-43	1	regulation of cell cycle; protein folding
EY974279	cathepsin B (Atlantic halibut)	46 (31/66)	8e-07	1	regulation of catalytic activity; cysteine-type endopeptidase activity
EY974635	cytochrome P450 (European sea bass)	60 (89/146)	2e-48	1	monooxygenase activity; iron ion binding; oxidoreductase activity; heme binding

[1]All contigs and singletons from this SSH library were annotated using AutoFACT (36). The additional information (e.g., functional annotations, BLASTx statistics, and GenBank accession numbers for contributing ESTs) is listed in online Supplemental Table S1*D*. [1–5]See footnotes for Table 2.

nicida-stimulated animals compared with PBS controls (Fig. 2, *C* and *D*). The proinflammatory cytokines IL1β and IL8 had similar patterns of expression in the spleen and head kidney of stimulated animals. In both tissues, there were significantly higher levels of expression for both of these genes at 2 and 6 HPI in ASAL individuals compared with the PBS controls (Fig. 2, *I–L*). Maximum expression of these genes relative to their appropriate 0 h controls occurred at 6 HPI [IL1β (684.3 fold) and IL8 (33.8-fold) for spleen; IL1β (356.3-fold) and IL8 (70.6-fold) for head kidney] after which time levels of expression declined (Fig. 2, *I–L*). Expression of FTH was highest at 24 HPI in spleens from ASAL individuals (Fig. 2*M*). Levels of FTH expression in head kidney were similar in magnitude between the PBS and ASAL groups and there was no trend in FTH expression over time in either group (Fig. 2*N*). Levels of MCL1 expression were relatively low and similar in magnitude between spleen and head kidney samples (Fig. 2, *O–P*). In addition, there were no trends in expression over time in either tissue. Expression of MCL1 was significantly higher in spleens from ASAL individuals compared with PBS controls at 6 and 24 HPI but significantly lower at 72 HPI (Fig. 2*O*). Head kidneys from stimulated animals had significantly higher levels of expression compared with the PBS controls at 6 h, but significantly lower levels of expression before stimulation (0 h control) and at 72 HPI (Fig. 2*P*). Two genes selected for QPCR studies from the spleen SSH libraries (BTF3 and IL1R) were not significantly dysregulated by *A. salmonicida* stimulation (data given in Supplemental Table S4, *Q–R*, but not presented in Fig. 2).

We also examined whether the presence of nodavirus, as detected by RT-PCR in brain samples, had an effect on constitutive expression of these 10 genes in spleen and head kidney. For both immune tissues, there was no significant effect of nodavirus carrier status on the constitutive expression of these genes (data not shown).

Characterization and phylogenetic analysis of Atlantic cod IRF1. The putative translation of one EST from the spleen forward SSH library (EY975211) shared 40% identity to the IRF1 of snakehead (*Channa argus*) over 103 aligned AAs (GenBank accession number ABN42504). Additional sequence was obtained from this transcript by 5′RACE (807 bp) and 3′RACE (680 bp) using mRNA from the spleens of *A. salmonicida*-

stimulated fish. These sequences were assembled to generate a 1,465 bp cDNA sequence that encodes a 306-AA protein. The Atlantic cod IRF1 sequence characterized in this study was deposited in GenBank under accession number FJ346564. Structural modeling of Atlantic cod IRF1 shows that its DNA binding domain (DBD) consists of three α-helixes, four β-sheets, and three long loops (see Supplemental Fig. S2*A* for detailed information). Alignment of IRF1 amino acid sequences from Atlantic cod (deduced protein sequence) and several other vertebrate species shows that the sequences all contain a highly conserved (>60% identity; Supplemental Table S5) DNA binding domain at the NH_2 terminus with six conserved tryptophan repeats (Supplemental Fig. S2*B*). Overall, the Atlantic cod IRF1 amino acid sequence is 42–55% identical to the IRF1 orthologs of other teleosts, and 34–37% identical to IRF1 orthologs from nonteleost vertebrates (Supplemental Table S5). A phylogenetic tree whose construction is based on the IRF1 multiple sequence alignment shows that the Atlantic cod IRF1 is distinct from other teleost IRF1 orthologs (Supplemental Fig. S2*C*).

DISCUSSION

Subspecies of the Gram-negative bacterium *A. salmonicida* are the causative agents of a serious disease in Atlantic cod (61, 67) and many other fishes (40, 54, 62). This disease is classified as either typical furunculosis, caused by *A. salmonicida* subsp. *salmonicida* or atypical furunculosis, caused by atypical variants of *A. salmonicida*. To date, a number of studies have examined the transcriptional response of salmonids challenged or vaccinated with the typical subspecies (*A. salmonicida* subsp. *salmonicida*) (19, 22, 47, 51). In addition, the immune response of zebrafish to challenge with the related species *Aeromonas hydrophila* has also been recently examined in detail (63). In this study we used an atypical variant of *Aeromonas salmonicida* that was obtained from an atypical furunculosis outbreak in Atlantic cod. To identify and characterize genes with transcriptional changes related to the early immune response (2–72 h) to bacterial antigens, we ip injected Atlantic cod with formalin-killed, atypical *A. salmonicida* and constructed, sequenced, and characterized reciprocal SSH li-

Fig. 1. Gene classification of spleen (*A*) and head kidney (*B*) reciprocal suppression subtractive hybridization (SSH) libraries of Atlantic cod based on Gene Ontology (GO) (biological process terms). GO annotations were obtained using AutoFACT and Goblet analysis of clusters. For the complete lists of GO annotations by biological process, see Supplemental Table S3, *A–D*. Superscripts: [1]G protein-coupled receptor (GPCR) protein signaling pathway. [2]For the spleen SSH libraries, "other categories" include 33 and 28 assembled expressed sequence tags (ESTs) with GO biological process terms in the forward and reverse libraries, respectively. [3]For the head kidney SSH libraries, "other categories" include 38 and 14 assembled ESTs with GO biological process terms in the forward and reverse libraries, respectively. [4]For a given GO biological process term, if the number of ESTs present in the forward SSH library was 2 and/or 50% more than in the reverse SSH library. [5]For a given GO biological process term, if the number of ESTs present in the reverse SSH library was 2 and/or 50% more than in the forward SSH library. For a given GO biological process term, if the difference between the numbers of ESTs present in the SSH libraries was <2 and/or 50% of the smaller number.
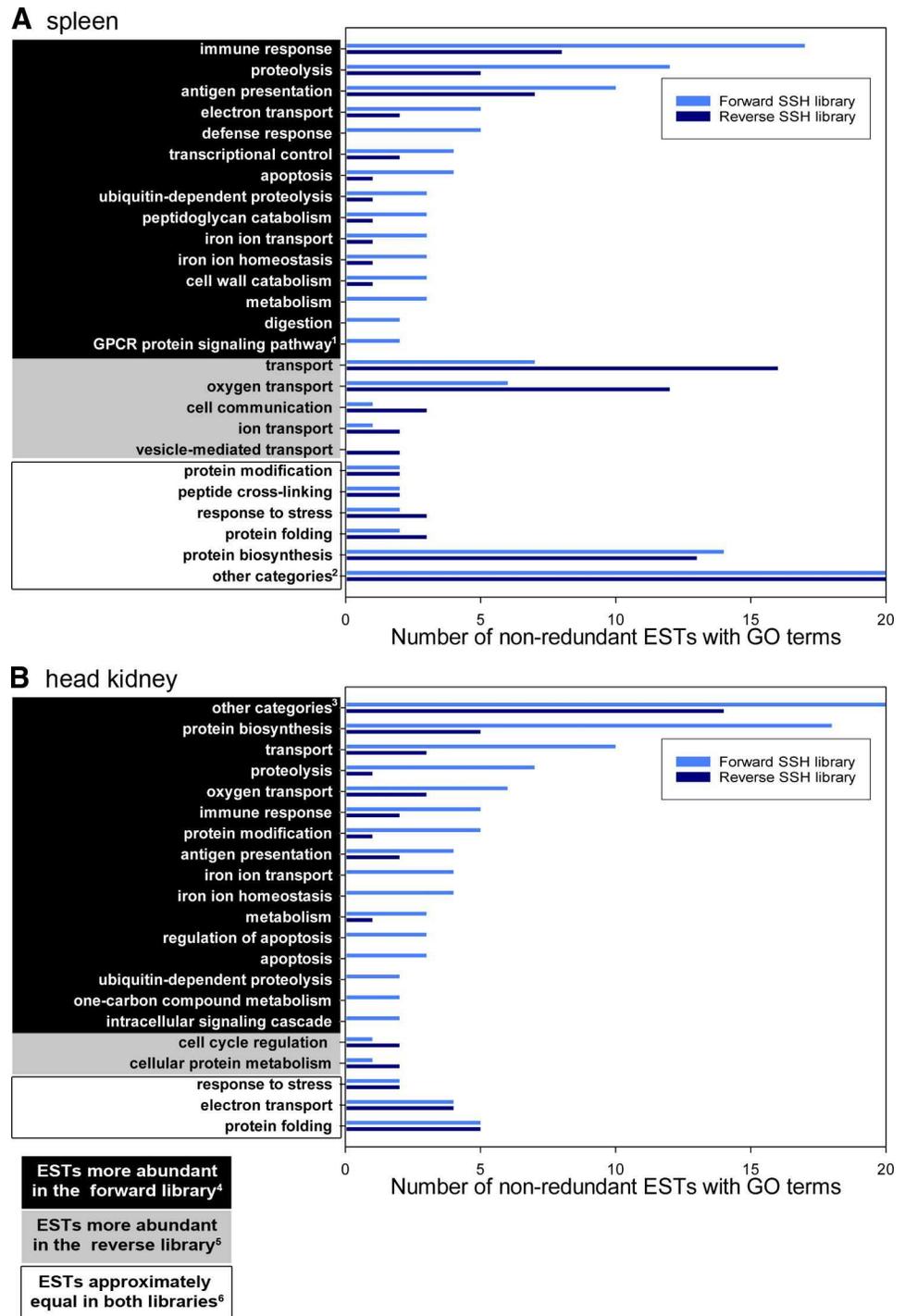
braries for the spleen and head kidney. From these libraries, we generated 4,154 ESTs that have enabled us to identify a large number of immune-related genes for which sequence information was previously not available for Atlantic cod.

Functional annotation and analysis of the ESTs generated from the SSH libraries revealed that the killed-*A. salmonicida* stimulation induced changes in the expression profiles of genes involved in a variety of physiological processes in spleen and

Fig. 2. QPCR analyses of selected genes identified in the SSH libraries. Gene expression data are presented as means ($\pm$ SE). RQ (relative quantity) values were normalized to 18S ribosomal RNA and calibrated to the individual with the lowest gene of interest expression. Within each gene of interest study, identical letters [uppercase for *Aeromonas salmonicida*-treated (ASAL) gene expression data, lowercase for saline-injected control (PBS) data] indicate no significant difference ($P > 0.05$) between the groups at the different time points postinjection. *Significant ($P \leq 0.05$) differences between *A. salmonicida*-treated and saline-injected control groups at a particular time point. For each condition and time point (e.g., ASAL, 24 h), fold upregulation was calculated as (average RQ)/(average RQ for the appropriate 0 h control group), and fold downregulation where appropriate was calculated as the inverse of fold upregulation.
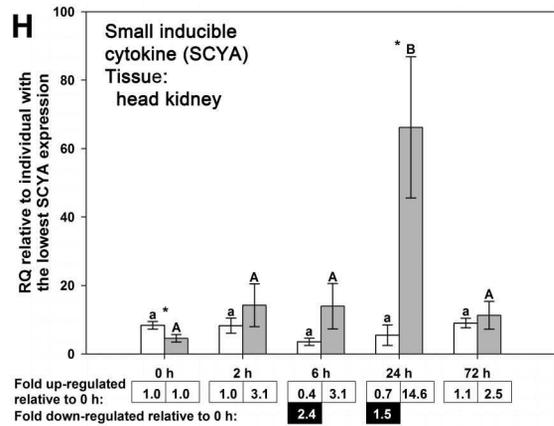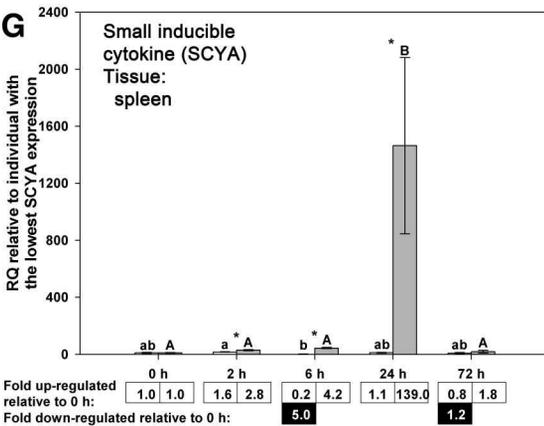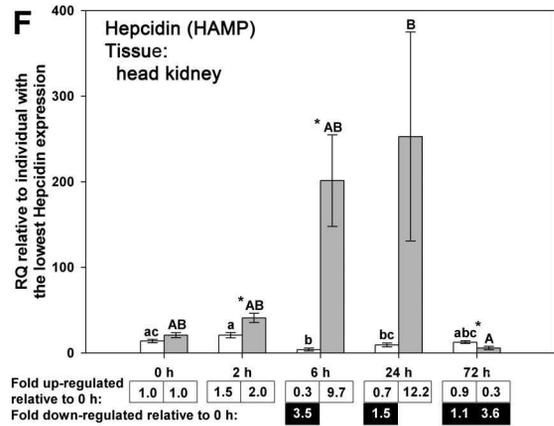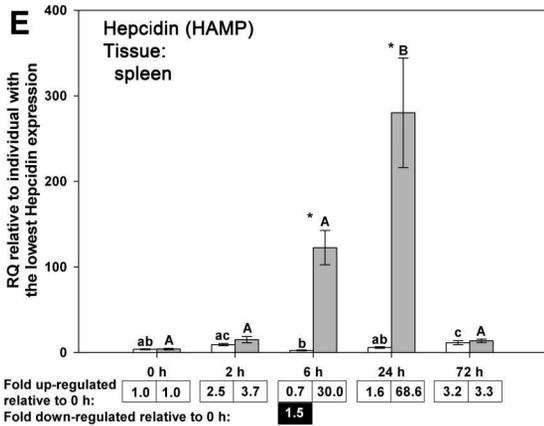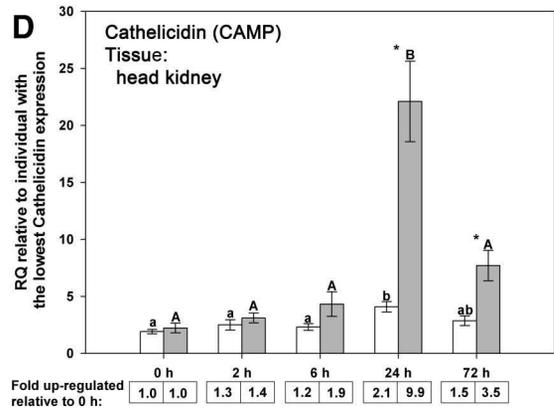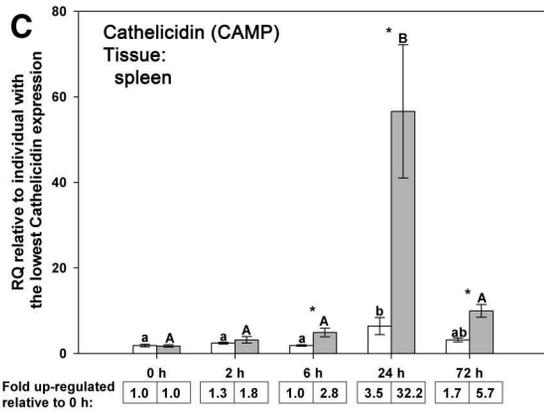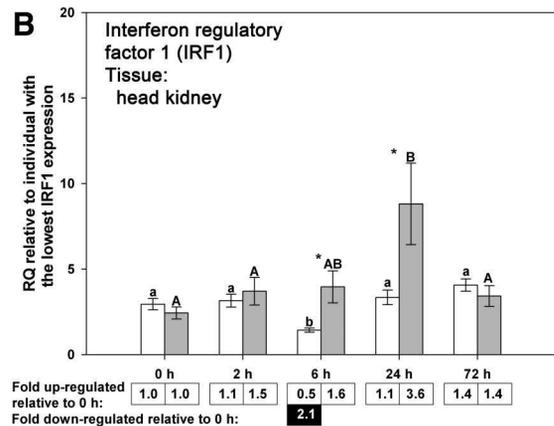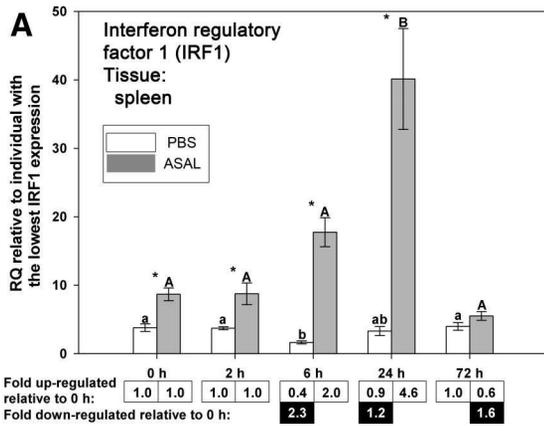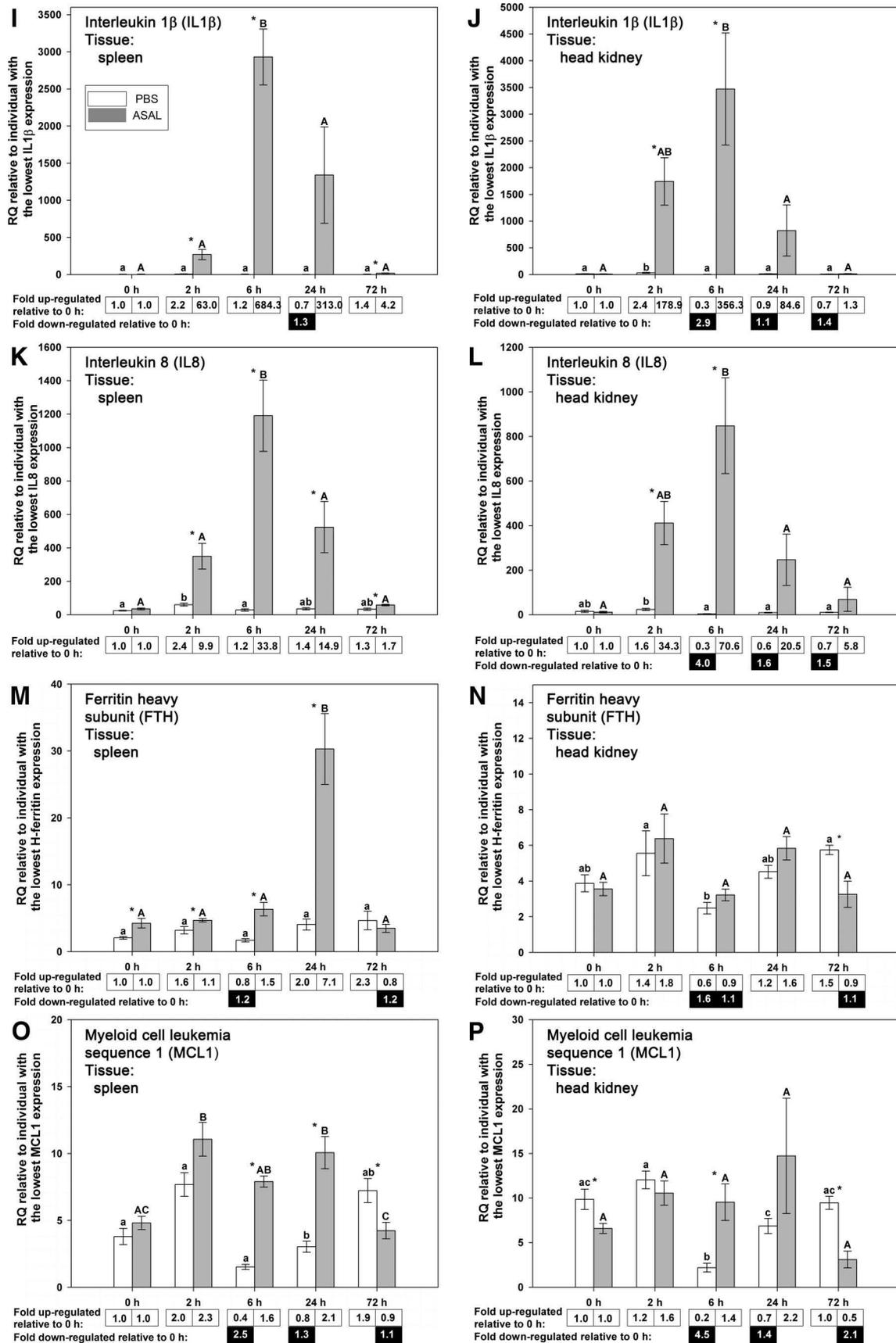
Fig. 2—Continued

Table 6. *Primers used for QPCR*

Primer Name[1]	QPCR Oligonucleotide Sequences (5′-3′)	Gene Name of the Top BLASTx Hit	Amplicon Size, bp	Efficiency,[2] %
CAMP-f	ATTGCAATTTCACCCTGAGC	cathelicidin	118	94
CAMP-r	CCAGACCTGCTCCTTCTCAC			
IL8-f	CCAATCTGACGGCTCTCTGT	interleukin 8	116	103
IL8-r	ATCGGCTCCCTACTGGTTCT			
FTH-f	TCGAGAAAGTGGGTCTCGAT	ferritin heavy subunit	168	97
FTH-r	AGACGTCAGGAAGCCAGAAA			
IRF1-f	AGAAGGACGCCAGTCTGTTCAA	interferon regulatory factor 1	100	86
IRF1-r	GCGGAAGTTGGCTTTCCATT			
SCYA-f	CTCAAACCTCTGCATCGTCA	small inducible cytokine SCYA	188	96
SCYA-r	CACGGAGAGGTAAGCAGCTC			
IL1βR-f	ACATCATGCAGCGCTTCTC	interleukin 1β receptor-like precursor	101	86
IL1βR-r	TTTGCCCTCAAGGTCCTG			
IL1β-f	ACAGGAAGTGCACCATGTCA	interleukin 1β	107	95
IL1β-r	GTCGTGCACACAGAAAGCAG			
MCL-f	CGCAGACAGCACAACAAACT	myeloid cell leukemia sequence 1	102	101
MCL-r	GACACGCAGCCTTCTTTACC			
HAMP-f	CCACAGGCTCCTCTCAAGTC	hepcidin	146	89
HAMP-r	CTGCAACTGCAATGCTGAAT			
BTF3-f	AGCTCGGCGTCAACAATATC	basic transcription factor 3	159	89
BTF3-r	GCATCTCTGTCAGCTGCTTG			
18S-f	ATGGCCGTTCTTAGTTGGTG	18S ribosomal RNA (normalizer gene)	180	109
18S-r	GGACATTTAAGGGCGTCTCA			

[1]Primer direction is denoted by "f" or "r" following the gene name for forward or reverse, respectively. [2]The calculation of amplification efficiency using a standard curve is described in MATERIALS AND METHODS.

head kidney. In addition to the physiological processes directly linked to the innate antibacterial immune response, such as antimicrobial peptide synthesis, chemotactic signaling, regulation of iron homeostasis, antigen processing and presentation, and complement pathway, other processes appeared to be dysregulated by the *A. salmonicida* stimulation including regulation of apoptosis, protein synthesis, proteolysis, DNA-dependent transcription, and stress response. Using QPCR with individual fish tissue templates (to assess biological variability), we investigated the expression of nine genes in the forward libraries with functional annotations representing a subset of these physiological processes.

One Atlantic cod contiguous sequence (contig) containing 2 ESTs from the forward spleen SSH library (enriched for genes upregulated by bacterial antigens) had significant homology to the IRF1 of snakehead (*Channa argus*), and we obtained the full-length cDNA sequence using 5′ and 3′RACE. The Atlantic cod IRF1 is encoded by an ORF of 921 bp that translates to 306 AA residues. This deduced protein sequence has ~35 and 45% identity to the human (*Homo sapiens*) and rainbow trout (*Oncorhynchus mykiss*) IRF1s, respectively (Supplemental Table S5). Phylogenetic analysis placed Atlantic cod IRF1 sequence near to the branching point of the group containing IRF1 from snakehead and Chinese perch (*Siniperca chuatsi*). Structural modeling of Atlantic cod putative IRF1 DBD suggests the presence of three α-helixes, four β-sheets, and three long loops, which is consistent with the structure of human IRF1 (18). Therefore, based on its amino acid identity, the results of the phylogenetic analysis, and structural modeling, we are confident that this sequence encodes the Atlantic cod IRF1 protein. Furthermore, the conserved DBD in Atlantic cod IRF1 suggests that it may have similar function and recognize similar DNA motifs (i.e., the interferon simulated response elements) as its human ortholog.

Most studies on fish have examined patterns of IRF1 expression following polyriboinosinic polyribocytidylic acid

(pIC)-stimulation or virus challenge (13, 30, 55, 59, 77, 89). There are few reports of IRF1 expression in fish following stimulation with bacteria or bacterial antigens. Yabu et al. (89) demonstrated induction of an interferon regulatory factor in the liver of Japanese flounders following intramuscular injection with *Edwardsiella tarda*. Although these authors could not determine whether their sequence encoded IRF1 or IRF2 due to lack of representative sequences from fish, our more recent BLASTx analysis of their sequence shows homology to other IRF1 sequences (Supplemental Fig. S2). However, Collet and Secombes (13) reported that IRF1 expression was induced only by pIC and not by LPS in rainbow trout gonad cells. More recently, Ordas et al. (55) described the IRF1 from turbot (*Scophthalmus maximus*) and seabream (*Sparus aurata*). In these species, IRF1 was reported to have low levels of constitutive expression in a variety of tissues, and its expression was induced by pIC stimulation and viral hemorrhagic septicemia virus challenge. However, the increase in IRF1 expression in head kidney of turbot following *Vibrio pelagius* challenge was not statistically significant at the single time point they studied (8 h postchallenge). In our study, Atlantic cod IRF1 was constitutively expressed at low levels, and expression was significantly elevated in spleen and head kidney at 24 h following *A. salmonicida* stimulation, with highest levels of induction being observed in the spleen. In fish, the target genes of IRF1, as well as its importance in innate immune responses, are yet to be determined. However, in rainbow trout, the expression of IRF1 in macrophages can be induced by both IFN-γ and IL1β, with IFN-γ being a much more potent inducer of IRF1 than IL1β (48). As both IFN-γ and IL1β are known to be induced in fish following a challenge with bacterial antigens, it appears that similar pathways to those of higher vertebrates exist in fish (10, 12).

Chemotactic cytokines are directly involved in leukocyte trafficking and play an important role in the innate immune response. Interleukin 8, a CXC chemokine ligand (CXCL), was

identified in the spleen forward SSH library, and its sequence was homologous (96% AA identity) to the Atlantic cod IL8 recently described by Seppola et al. (70). In our study, IL8 expression was upregulated by formalin-killed *A. salmonicida* stimulation in both spleen and head kidney at 2 h, reaching its peak at 6 h, and returning to basal levels at 72 h. Our results are in agreement with those of Seppola et al. (70), who reported IL8 upregulation in spleen and head kidney following ip injection with formalin-killed *Vibrio anguillarum* at the single time point they examined (24 h). Thus, it appears that expression of IL8 in Atlantic cod, like in other vertebrates (6, 10, 12), is induced rapidly as part of the inflammatory response to bacterial antigens.

Members of the CC chemokine subfamily are generally known for their activity targeting mononuclear cells rather than neutrophils (39). The largest contig in the spleen forward SSH library is homologous to a small inducible cytokine, SCYA104, from the African cichlid (*Paralabidochromis chilotes*). Analysis of Atlantic cod SCYA expression demonstrated that it was highly upregulated in spleen, as well as upregulated to a much smaller magnitude in the head kidney, at 24 h following *A. salmonicida* stimulation. This gene has been previously reported to be highly upregulated in the spleen of Atlantic cod at 6 and 24 h following stimulation with pIC (59). Using BLASTp analysis of Atlantic cod SCYA we determined that this gene is related to the human monocyte chemotactic protein 2 (MCP2) (23/65 aligned AA for 35% identity). Human MCP2 is a known chemoattractant of monocytes, and its expression is induced in response to various immunogenic stimuli, such as IL1β, IFN-γ, and pIC (85, 86). However, due to relatively low levels of homology between the fish CC chemokines and putative orthologs in higher vertebrates, it is possible that they will have different functions. Further work is required to assign chemoattractant function to Atlantic cod SCYA.

Several putative apoptosis regulatory transcripts were identified in the forward SSH libraries, such as members of the caspase family and the Bcl-2 antiapoptotic family. Of these, transcripts encoding myeloid cell leukemia sequence 1 (MCL1) were identified in both forward SSH libraries. Our QPCR analysis indicated that Atlantic cod MCL1 has relatively low levels of constitutive and induced expression in both the spleen and head kidney. Furthermore, it presented no obvious trends over time following stimulation with bacterial antigens. In humans, two forms of MCL1 exist as a result of differential splicing, generating a longer antiapoptotic form of MCL1 with all three exons and a shorter proapoptotic form of MCL1 containing exons 1 and 3 (8). In contrast to humans, both MCL1 paralogs identified in zebrafish have antiapoptotic activity (37). The MCL1 identified in our study is a putative ortholog of the antiapoptotic human MCL1 (87/223 aligned AAs for 39% identity), and it is more similar to zebrafish MCL1b (99/195 aligned AAs for 50% identity) than to MCL1a (97/209 aligned AAs for 46% identity) described by Kratz et al. (37). So far, there is no evidence that paralogs of MCL1 are present in the Atlantic cod genome, as all MCL1-representing sequences identified in the CGP database represent the same transcript. Even if another copy of MCL1 is present in cod, it is unlikely that the QPCR primers were amplifying both paralogs as the primers are specific to a region poorly conserved between the two zebrafish paralogs. In Atlantic salmon coexpression of MCL1 and IL1β in response to an ectoparasite infection has been documented (49).

Genes that encode for antimicrobial peptides (AMPs) and proteins that are involved in the regulation of iron homeostasis are commonly responsive in fish following stimulation with bacterial antigens or live bacterial challenge (11, 23, 45). AMPs, such as cathelicidins and hepcidins (HAMPs), are cationic peptides that lyse bacterial cells by disrupting the bilipid layer of their plasma membrane (3). In both the spleen and head kidney forward SSH libraries relatively high numbers of ESTs encoding cathelicidins were identified. Multiple alignment of these sequences indicates that they encode several putative forms of cathelicidins. Using data from these subtracted libraries, as well as other data for cathelicidins obtained from our project website (www.codgene.ca), Maier et al. (45) recently described three cathelicidins from Atlantic cod. These cathelicidins are very similar to each other with the majority of differences between them occurring within their antimicrobial peptide domain.

Due to the high similarity of the sequence encoding these cathelicidin antimicrobial peptides (CAMP), our QPCR assay was designed to amplify all forms of the cathelicidin transcripts. Using this assay, low levels of constitutive CAMP expression were evident in both the spleen and head kidney. In both of these tissues expression levels peaked at 24 h poststimulation with higher levels of upregulation seen in the spleen. Maier et al. (44) studied the expression of one form of cathelicidin at 24 h following infection with an atypical strain of *Aeromonas* bacterium (*A. salmonicida ssp. achromogenes*) using RT-PCR. These authors reported constitutive expression of CAMP in both the spleen and head kidney and no change in expression level in these tissues following infection. Interestingly, the cathelicidin that they studied was upregulated in gill, liver, pyloric caeca and intestine but not in skin. Based on our results it appears that one or both of the other forms of cathelicidin are important in the spleen and head kidney response to bacterial stimulation. Maier et al. (44) reported that DNase treatment of commercially purchased *Escherichia coli* LPS (with DNA contamination) or *A. salmonicida* lysate abolished their effects on CAMP expression in a Chinook salmon (*Oncorhynchus tshawytscha*) embryo cell line (CHSE-214), demonstrating that bacterial DNA, or perhaps both LPS and bacterial DNA, are required for the induction of fish CAMP expression. One of the defining characteristics of bacterial DNA is the presence an unmethylated CpG motif that can be recognized by the Toll like receptor 9 (TLR9) (66). Therefore, the upregulation of CAMP by *A. salmonicida* observed in our study may be associated with the TLR9 signaling cascade.

Another AMP, hepcidin, was also identified in the spleen forward SSH library (Table 2). Some fish hepcidins have been shown to have antimicrobial activity (27, 28, 29), and the upregulation of fish hepcidin expression has been observed in response to immunogens (e.g., Refs. 15, 29, 75), viral infection (e.g., Ref. 15) and bacterial infection (e.g., Ref. 28). Solstad et al. (75) characterized an Atlantic cod hepcidin that was 100% identical (over 98 aligned AA) to the sequence we obtained in this study. Upregulation of this Atlantic cod hepcidin has been observed in peritoneum, blood, liver, and head kidney following stimulation with inactivated-*Listonella anguillarum* and pIC (75). In agreement with these findings, we report that the expression of HAMP was significantly upregulated in both spleen and head kidney at 6 h with maximum levels of expression seen at 24 h poststimulation. Besides their

role as AMPs hepcidins are generally considered to be iron-regulatory hormones that modulate iron metabolism (53). In fish, several studies present evidence supporting this dual role for the hepcidins (27, 28, 29). The role that hepcidin plays in iron regulation in Atlantic cod remains to be determined.

The accumulation of free intracellular iron is toxic as it reacts with oxygen and creates H_2O_2 as a byproduct (reviewed in Ref. 80). Peroxides can cause DNA damage and ultimately lead to cell death (e.g., as reviewed in Ref. 5). To maintain iron homeostasis, ferritin captures and stores free iron in a soluble nontoxic state thereby limiting cell damage. In our study, ferritin heavy subunit (H-ferritin) and ferritin middle subunit (M-ferritin) encoding transcripts were identified in both forward SSH libraries as deep contigs. H-ferritin is known to be a generic type of ferritin that is present in all animals, while M-ferritin has only been identified in fish and amphibians (1, 16). In contrast to H-ferritin, very little is known about the roles of M-ferritin in iron metabolism or the regulatory mechanisms of M-ferritin synthesis. It is known, however, to possess a conserved ferroxidase center as the one found in H-ferritin (1). In our study we found a significant increase in H-ferritin expression in the spleen at 24 h poststimulation. In the head kidney, levels of constitutive expression were low and there was no significant change in expression over time. In fish, previous studies have shown that the expression of H-ferritin in liver can be induced by *Edwardsiella ictaluri* infection (56), and Martin et al. (48) further showed that IL1β caused upregulation of both hepcidin and H-ferritin in trout macrophages.

Many of the dysregulated transcripts in this study were found in spleen but not head kidney SSH libraries. This could be a result of the degree of success of the subtractive hybridization, transcriptome complexity, or magnitude of transcription dysregulation. For all genes studied with QPCR with the exception of IL8, the magnitude of upregulation was greater in spleen than in the head kidney. The difference in transcriptome shift between spleen and head kidney may reflect the distinct roles that these two immune tissues play in response to bacterial immunogen. Following the pathogen entry via blood stream, the resident leukocytes in spleen, predominately macrophages, trap and phagocytize the blood borne pathogens. The spleen also serves as a processing site for erythrocytes, as a result of which, the iron level within the spleen is relatively high (reviewed in Ref. 74). Since the spleen is essential for both pathogen clearance and iron storage, it is critical that splenic macrophages withhold iron from pathogens (50). The more prominent HAMP upregulation in spleen, coupled with the spleen-specific induction of ferritin by *A. salmonicida*, suggests that the fish spleen may play a key role in iron-withholding as an innate immune response to bacterial pathogens.

In this study, some of the cod used in our QPCR analysis were asymptomatic carriers of nodavirus as determined by RT-PCR on brain tissues using nodavirus-specific primers. In asymptomatic carriers, nodavirus is carried in the brain and eyes, which are immune privileged sites (31). Therefore, it is difficult to know what, if any, exposure the immune system has to this virus in the asymptomatic state. In this study, we found no significant differences in constitutive gene expression between nodavirus carriers and noncarriers in either spleen or head kidney. Using the same family of Atlantic cod that was used in the current study, Rise et al. (59) reported that asymp-tomatic nodavirus carrier status of brain did not influence the constitutive expression of 13 immune-relevant genes in the spleen, including two genes of interest from the current report (IRF1 and SCYA). In addition, they reported that there was no apparent correlation between nodavirus carrier status and pIC response in the spleen for these genes. Unfortunately, we had insufficient samples to fully examine the correlation between nodavirus carrier status and responses to *A. salmonicida* in the spleen and head kidney.

Our gene expression results may also be affected by our use of a single cod family. There is growing evidence for differences between families of fish (including Atlantic cod) in their susceptibility to disease (34, 88). In this study, we utilized a single family of Atlantic cod that were selected based on their survival and growth performance in culture. It is unknown whether this family contains individuals that are more or less susceptible to infection with atypical *A. salmonicida* than other families in our broodstock development program. Whether differences in disease resistance between families of cod will be related to differences in patterns of immune-related gene expression is unknown.

In summary, we have identified many genes in Atlantic cod that are known to be important in the innate immune response against bacteria, and have also characterized IRF1 in this species for the first time. The transcriptional innate immune response of Atlantic cod to *A. salmonicida* observed in our study is similar to many previous studies in fish and includes genes such as those involved in chemotactic signaling and AMPs.

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