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Genes associated with an effective host response by Chinook salmon to *Renibacterium salmoninarum*

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ABSTRACT

An effective host response to *Renibacterium salmoninarum*, the etiologic agent of bacterial kidney disease, is poorly characterized. Using suppression subtractive hybridization, we exploited the difference in early host response in the pronephros of fish challenged by an attenuated strain (MT239) or a virulent strain (ATCC 33209) of *R. salmoninarum*. Among the 132 expressed sequence tag (EST) clones that were sequenced, 20 were selected for expression analysis at 24 and 72 h after challenge. ESTs matching two interferon inducible genes (IFN-inducible GBP and VLIG1), the ligand GAS6, and the kinase VRK2 were upregulated in fish exposed to MT239, but downregulated or unchanged in fish exposed to 33209. A second group of ESTs matching genes involved in apoptosis (caspase 8) and immune function (IκBα, $p47^{phoX}$, EMR/CD97) were more slowly upregulated in fish exposed to 33209 compared to fish exposed to MT239. The ESTs displaying elevated expression in MT239-exposed fish may represent important cellular processes to bacterial challenge, and may be useful indicators of an effective host response to *R. salmoninarum* infection.

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1. Introduction

Chronic or cryptic bacterial infections present difficult treatment situations. These difficulties can be compounded when infections involve bacteria capable of intracellular residence. Bacterial kidney disease (BKD) is caused by the Gram-positive *Renibacterium salmoninarum*, which can survive within macrophages [1] possibly by exhausting the macrophage response before engulfment [2]. Infection by *R. salmoninarum* is widely observed among free-living Pacific salmon [3,4], and BKD is one of the most common chronic infectious disease problems in hatcheries and culture programs. Although infected salmon can mount vigorous antibody production to this bacterium [5–7], this humoral response is typically ineffective or can even be deleterious [8].

R. salmoninarum possesses an abundant, 57-kDa extracellular protein called major soluble antigen (MSA). MSA has been implicated in agglutination [9,10], immunomodulation [11–14], and it is the only demonstrated virulence factor for *R. salmoninarum* [15,16]. MT239 is a spontaneous mutant with attenuated pathogenicity that expresses greatly reduced levels of MSA [9,17,18]. Chinook salmon exposed to MT239 appear to rapidly (within a week of challenge) and effectively clear the bacterium after intraperitoneal exposure, whereas fish challenged with the virulent ATCC type strain 33209 exhibit increasing bacterial burdens and lesions [19]. This difference suggests that an early, effective host response is mounted against MT239.

There are only a few studies of host response to *R. salmoninarum* that were not focused on antibody titers or innate humoral factors [20,21]. The absence of a consistent association between antibody titers and immunity suggests that a cell-mediated immune response may be crucial against BKD [2], and that the response with a few days after exposure would be important in controlling infection. We hypothesized that the difference in host response to the attenuated MT239 strain and virulent ATCC 33209 could be exploited to identify markers associated with an early, effective immune response to *R. salmoninarum* infection. We used suppression subtractive hybridization (SSH) to isolate sequences associated with the response to MT239 in anterior kidney, a target organ for infection. A subset of these expressed sequence tags (ESTs) were tested for differential expression in anterior kidney at 24 and 72 h after challenge. The ESTs identified in this study offer insight into the interaction between *R. salmoninarum* and its host, and can serve as proximal indicators of the effectiveness of the host response.

2. Materials and methods

2.1. Fish

Fish were yearling Chinook salmon (*Oncorhynchus tshawytscha*) of George Adams stock. Twenty fish sampled at the start of the experiment (time 0) had an average length of 110.7 mm (S.D. 16.9 mm) and an average weight of 15.3 g (S.D. 7.1 g). Fish were

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maintained in isolation in 1.5 m diameter tanks receiving ultraviolet light-treated seawater at a rate of 4 gpm, and fed Rangen no. 3 salmon grower feed (Rangen) at a rate of 2.5% g body weight^{-1} day^{-1}. During the post-challenge period (up to 6 days after challenge), no mortalities occurred. Sampled fish were euthanized with tricaine methanesulphonate (MS222, Alpharma), followed by branchial exsanguination and cervical severance. In addition to tissue collection, fish were weighed and measured for length, and the gender was determined. Ten fish from each bacterial exposure group and five fish from the peptone–saline control group were sampled at each timepoint.

2.2. Bacterial strains and bacterial challenge

Bacterial strains used were the ATCC type strain 33209 and the spontaneous variant MT239 [9]. The type strain 33209 exhibits a virulent phenotype, whereas the variant strain MT239 exhibits an attenuated phenotype in Chinook salmon [19]. Individual colonies were cultured in modified KDM broth (1% Bacto-Peptone, w/v, 0.05% yeast extract, w/v; 0.05% L-cysteine, w/v; adjusted to pH 7.5) at 15 °C until an optical density at 525 nm (OD_{525}) ranging between 0.8 and 1.0. Cells were washed with peptone–saline (0.1% Bacto-Peptone, 0.85% NaCl), and cell concentration determined by the membrane fluorescent antibody technique [22]. Cell concentration was adjusted to a final concentration of 10^7 cells ml^{-1} with peptone–saline and stored at 4 °C for less than 2 h before use.

Fish were anesthetized with tricaine methanesulfonate (Finquel; Argent) and injected intraperitoneally with a total volume of 0.1 ml of inoculum (i.e., bacterial suspension or peptone–saline alone). Challenge dose was 10^6 bacterial cells per fish.

2.3. Quantitative fluorescence antibody technique (qFAT)

Posterior kidney was removed at necropsy, placed into a Whirl-Pak bag, and stored on ice until slide preparation, later in the same day. Slides were prepared and stained by the method of Cvitanich [23], using a goat, polyclonal, anti-*R. salmoninarum* antibody conjugated to FITC (Kirkegaard and Perry). Slides were examined at 1000× magnification using an Axiophot epifluorescence microscope (Zeiss), and all bacterial cells within 400 fields were counted.

2.4. Suppression subtractive hybridization library

Pronephros (anterior kidney) was immediately submerged into liquid nitrogen and stored at −70 °C until RNA extraction. Total RNA was isolated with Trizol reagent (InVitrogen) according to the manufacturer's instructions, and RNA concentrations determined by spectrophotometry at 260 nm. RNA from fish exposed to the attenuated strain was designated as the tester. RNA from fish exposed to the virulent ATCC type strain 33209 was designated as the driver. RNA used in the SSH was a pool of equal amounts of RNA from two animals from each exposure group. To use samples with the most comparable bacterial exposure, individual fish with similar qFAT levels at 72 h after exposure were selected (200–500 cells per kidney). Poly A+ RNA was obtained from total RNA with an oligo-dT spin column (Ambion).

SSH was performed using the BD PCR-Select cDNA subtraction kit (BD Biosciences-Clontech), following the manufacturer's instructions. The SSH library was directly cloned into the pCR2.1 vector (InVitrogen) after the secondary enrichment amplification and transformed into Top10F' *E. coli* (InVitrogen). Plasmid DNA was isolated from individual clones with silica-based spin columns (Qiagen). Both strands of each cloned insert was subjected to sequencing using BigDye terminator mix v3.1 (Applied Biosystems), and analyzed with an ABI3100 (Applied Biosystems). Sequencher v. 4.7 (Gene Codes Corp.) was used for sequence editing and alignments, and consensus sequences compared to GenBank databases with BLAST [24]. The significant *e* value was set at 10^{-13}.

2.5. Transcript abundance analysis

Complementary DNA (cDNA) was synthesized (15 ng RNA; 0.125 mM each dATP, dTTP, dCTP, and dGTP; 3.8 μM random hexamers; 10 mM dithiothreitol; 6 units RNase inhibitor; 1× Superscript II buffer; 37.5 units Superscript II [InVitrogen]) following the manufacturer's recommended thermal conditions. Real-time quantitative PCR (RT-qPCR) with cDNA using TaqMan chemistry was performed in 25 μl volumes (3 μl cDNA; 0.6 μM each primer [Operon]; 0.25 μM probe [5′ 6-FAM, 3′ Black Hole Quencher 1; Integrated DNA Technologies]; 1× TaqMan Universal PCR Master Mix [Applied Biosystems]) using at least duplicate reactions. Cycling (50 °C, 2 min; 95 °C, 10 min; 45 cycles of 95 °C, 15 s and 60 °C, 1 min; reading at the end of 60 °C incubation) and analysis was performed on the Mx3005P QPCR system (Stratagene). Standard curves for each EST used seven serial dilutions of cognate plasmid, ranging from 12 pg to 2.4×10^{-5} pg. Standards and no template controls were run in triplicate and were included for each qPCR run. Primer-probe sets used are shown in Table 1. RT-qPCR results were normalized by acidic ribosomal phosphoprotein P0 (ARP) [25] for each cDNA synthesis. Although DNA was not detectable in RNA preparations by spectrophotometry, each cDNA synthesis was checked by qPCR without reverse transcription for each EST primer-probe set.

2.6. Statistical analysis

Strain and timepoint differences in bacterial cell counts from qFAT analysis were compared by two-way analysis of variance and Bonferroni post-tests. Normalized levels of transcripts for ESTs from RT-qPCR analysis were compared by the Kruskal–Wallis test. Because multiple tests were performed for each EST, a Bonferroni adjustment for type I error was applied and the critical *p* value was 0.0056.

3. Results

3.1. Kidney infection after bacterial challenge

For each anterior kidney sample collected for SSH, a corresponding posterior kidney sample was collected and analyzed by qFAT. Although there was wide variation in infection severity among fish at certain timepoints, there were significant differences in the number of bacterial cells between the two challenge strains and among the timepoints after challenge (0, 24, 48, 72, and 144 h after exposure) for each strain (Fig. 1). For both strains, there was a significant increase in bacterial cells at the 72- and 144-h timepoints, relative to the earlier timepoints (Dunn's multiple comparison, $p < 0.001$). However, at the 72- and 144-h timepoints, the infection in fish challenged with 33209 was significantly greater than the infection in fish challenged with MT239 (Bonferroni post-test, $p < 0.01$). The percentage of degenerate bacterial cells did not vary across the sampling timepoints for fish challenged with either strain, and there was no difference in percentage of degenerate bacterial cells between the strains after challenge (*G*-test, $p > 0.05$; data not shown).

3.2. SSH library results

A library of clones derived from the forward subtraction (i.e., MT239 minus 33209) was generated, and 132 clones from the library were sequenced. Average insert size was 435 bp, ranging