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Evasion of mucosal defenses during *Aeromonas hydrophila* infection of channel catfish (*Ictalurus punctatus*) skin

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ABSTRACT

The mucosal surfaces of fish serve as the first line of defense against the myriad of aquatic pathogens present in the aquatic environment. The immune repertoire functioning at these interfaces is still poorly understood. The skin, in particular, must process signals from several fronts, sensing and integrating environmental, nutritional, social, and health cues. Pathogen invasion can disrupt this delicate homeostasis with profound impacts on signaling throughout the organism. Here, we investigated the transcriptional effects of virulent *Aeromonas hydrophila* infection in channel catfish skin, *Ictalurus punctatus*. We utilized a new 8 × 60 K Agilent microarray for catfish to examine gene expression profiles at critical early timepoints following challenge—2 h, 8 h, and 12 h. Expression of a total of 2,168 unique genes was significantly perturbed during at least one timepoint. We observed dysregulation of genes involved in antioxidant, cytoskeletal, immune, junctional, and nervous system pathways. In particular, *A. hydrophila* infection rapidly altered a number of potentially critical lectins, chemokines, interleukins, and other mucosal factors in a manner predicted to enhance its ability to adhere to and invade the catfish host.

1. Introduction

The mucosal surfaces of mammals have long been recognized as the first defensive barrier against infection by pathogenic microorganisms (Linden et al., 2008). While classical immunoregulatory tissues and organs such as spleen, liver, and lymphoid follicles often control the nature and scope of the secondary, systemic response, the local immune actors in the gastrointestinal, respiratory, and genitourinary tracts determine the success of critical early steps in pathogenesis including adhesion, entry, and replication. Similarly, interest in understanding components of the mucosal immune response in fish (gills, skin, gastrointestinal tract) is growing (Rombout et al., 2011; Rajan et al., 2011). Our current knowledge of mucosal immune factors in fish, while still limited, now includes a startling diversity of antimicrobial peptides, lectins, assorted pathogen recognition receptor (PRR) family members, lysozymes, and novel teleost immune respondents (Caipang et al., 2011; Li et al., 2012). Differential expression and regulation of these genes likely play critical roles in determining patterns of host resistance to the myriad of aquatic pathogens present in culture environments. Furthermore, because of the role of mucosal tissues as sensors and integrators of environmental and nutritional status cues, mucosal immune actors are sensitive, critical targets for manipulation using improved diets and topical therapeutants (Beck et al., 2012).

Catfish (*Ictalurus* sp.) are an important aquaculture organism and a long-standing research model for teleost immunology. Catfish researchers have developed a large base of biochemical information on the structure and function of catfish immunoglobulins (Igs) and antibodies (Bengten et al., 2006), while genomic approaches have helped to characterize a growing cross-section of the catfish innate immune system (Sha et al., 2009; Zhang et al., 2012; Niu et al., 2012; Rajendran et al., 2012a,b). While catfish remains the dominant aquaculture species in the United States (Hanson and Sites, 2012), the industry has faced a series of setbacks in recent years. Among these have been severe outbreaks of a motile aeromonad septicemia (MAS), whose etiological agent is *Aeromonas hydrophila*. *A. hydrophila,* a Gram-negative bacterium, is usually considered as a secondary pathogen in disease outbreaks among cultured fish species. However, in these cases, *A. hydrophila* appears to have emerged as a primary pathogen, causing the loss of more than 3 million pounds of channel catfish (*Ictalurus punctatus*) in 2009 alone (Pridgeon and Klesius, 2011).

Recently, we have successfully employed transcriptomic tools including microarrays (Peatman et al., 2007, 2008) and RNA-Seq (Li et al., 2012; Sun et al., 2012) to identify non-classical immune candidates following bacterial infection in catfish. Follow-up studies have served to further characterize roles of these candidates

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and examine their utility as potential biomarkers in genetic selection programs (Takano et al., 2008; Beck et al., 2012). In this same vein, here we utilized an 8 × 60 K Agilent microarray to examine global mucosal immune responses in the channel catfish skin following experimental challenge with virulent *A. hydrophila*.

2. Methods

2.1. Experimental animals and tissue collection

All procedures involving the handling and treatment of fish used during this study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) prior to initiation. Marion channel catfish (30 ± 1.6 g) were reared at the Auburn University Fish Genetics Research Unit prior to challenge.

Prior to experiments, fish were maintained in 30 L tanks (20 L water) and acclimatized for 5 days before immersion bath. Experimental fish were confirmed to be culture negative for bacterial infection by culturing posterior kidney tissues from representative groups of fish on tryptic soy agar (TSA) plates. A 12:12 h light:dark period was maintained and supplemental aeration was supplied by an air stone. The water temperature was controlled at 28 °C.

Fish were challenged in 3 control and 3 treatment groups per timepoint in each group. Aquaria were randomly divided into sampling timepoints-2 h treatment, 8 h treatment, 12 h treatment, 2 h control, 8 h control, and 12 h control with thirty fish in each aquarium. *A. hydrophila* bacteria were cultured from a single isolate (AL09-71), used in a trial challenge, re-isolated from a single symptomatic fish and biochemically confirmed to be *A. hydrophila*, before being inoculated into tryptic soy broth (TSB)and incubated in a shaker incubator at 28 °C overnight. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating 10 μL of 10-fold serial dilutions onto TSA agar plates.

To aid in infection, skin mucus was removed by gentle scraping with a dull spatula from an approximately 4 cm^2 area below the dorsal fin immediately prior to immersion challenge. Immersion experiments were performed in a 25 L bucket with aeration. Briefly, 50 mL of overnight bacterial cells 1.5×10^9 were added to water to give a final volume of 5 L (1.5×10^7 final exposure concentration). Thirty channel catfish were immersed in each bucket for 2 h. After the 2 h immersion, the catfish were distributed to 30 L glass aquaria. Control fish were treated in the same manner as the infected fish with mucus scraped and were held in buckets with the addition of sterilized TSB prior to transfer to aquaria.

At 2 h, 8 h and 12 h after challenge, 30 fish were collected from each of the appropriate control and treatment aquaria at each timepoint and euthanized with MS-222 (300 mg/L). The skin from 8 fish/replicate pool were pooled together in equal amounts and flash frozen in liquid nitrogen during collection and stored at −80 °C until RNA extraction. During the challenge, symptomatic treatment fish and control fish were collected and confirmed to be infected with *A. hydrophila* and pathogen-free, respectively, at the Fish Disease Diagnostic Laboratory, Auburn University.

2.2. RNA extraction and probe labeling

Samples were homogenized with mortar and pestle in the presence of liquid nitrogen. Total RNA was extracted from tissues using the RN easy Plus Universal Mini Kit (Qiagen) following manufacturer's instructions. RNA concentration and integrity of each sample was measured using NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1. Fluorescently labeled complementary RNA (cRNA) probes were generated using the Two Color Microarray Quick Labeling kit (Agilent Technologies, Palo Alto, CA, USA) and following the manufacturer's instructions. Briefly, cDNA was generated from 500 ng of each isolated RNA sample; cRNA was then made using Cy3-CTP or Cy5-CTP incorporation for labeling purposes. The fluorescently labeled cRNA probes were purified using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA), and the concentration, fluorescent intensities, and quality of labeled cRNA probes were determined using a Nano-drop spectrophotometer. At each timepoint, 3 Cy3 labeled cRNA and 3 Cy5 labeled cRNA were generated with randomized dye assignments between treatment and control samples.

2.3. Microarray design and probe hybridization

Microarray interrogations were performed using a custom-designed, Agilent-based microarray platform with 8 × 60 K probes per slide layout. Catfish cDNA contigs were collected from previous catfish EST and RNA-seq studies (Wang et al., 2010; Liu et al., 2011; Li et al., 2012). Annotation was conducted based on the NCBI zebrafish database using the BLASTX program, a cutoff *E*-value of e^{-5}, and selection of the top informative hit. In total, 29,732 contigs from channel catfish and 21,208 contigs from blue catfish were selected, and 38 genes were selected as controls and repeated 10 times across the array (Table 1). The rest of the array was populated with Agilent positive and negative controls. Specific probes were designed using Agilent's eArray online probe design tool with X-hyb potential <2 and a Base Composition (BC) content score below BC3.

Hybridization, washing and scanning were performed according to the Agilent two-color microarray-based gene expression analysis protocol (version 5.5, February 2007) by the University of Florida.

2.4. Microarray data analysis

Following hybridization, the slides were scanned using a GenePix personal 4100A Scanner (Axon Instruments) and initial analysis were performed with Feature Extraction software v9.5.3 (Agilent). Background correction of feature intensities was performed within this software. After lowess normalization of background-corrected data, normalized data was imported to ArrayStar software 5 (DNASTAR Inc., Madison, WI), and then the Moderated *t*-test was performed to detect the differently expressed genes (Smyth, 2004). At each timepoint, the expression values of the three replicates of *A. hydrophila* infected fish were compared to that of the three replicates of the control fish and used to calculate fold changes and *p*-values. The genes with fold change >2.0 and $p \leqslant 0.05$ were considered as differently expressed. Only channel catfish features (known and unknown) were used for expression analysis in this experiment. Functional groups of the differently expressed genes were identified based on GO analysis and manual literature review and were subjected to further BLAST analysis to verify their identities.

2.5. Gene ontology analysis

Gene ontology (GO) annotation analysis was performed using the zebrafish BLAST results in Blast2GO version 2.5.0 (http://www.blast2go.org/), which is an automated tool for the assignment of gene ontology terms. The final annotation file was produced after gene-ID mapping, GO term assignment, annotation augmentation and generic GO-Slim process. The annotation result was categorized with respect to Biological Process, Molecular Function, and Cellular Component at level 2.

In order to identify overrepresented GO annotations in the differentially expressed gene set compared to the whole channel microarray gene set, GO analysis and enrichment analysis of

Table 1
Summary of the probe design for a catfish Agilent 8 × 60 K two-color gene expression microarray containing features from both channel catfish and blue catfish. Known probes were designed from unique transcripts which had a significant BLAST hit (E value e-10, score >100). Probes with X-hyb potential <2 and a Base Composition (BC) content score below BC3 were included on the array.

	Channel_known	Channel_unknown	Blue_known	Blue_unknown
Probes	17,038	12,694	13,377	7,831
Total	50,940			

significantly expressed GO terms was performed using Ontologizer 2.0 (Bauer et al., 2008) using the Parent-Child-Intersection method with a Benjammini-Hochberg multiple testing correction (Grossmann et al., 2007). GO terms for each gene were obtained by utilizing zebrafish annotations for the unigene set. The frequency of assignment of gene ontology terms in the differentially expressed genes sets were compared to frequency within the overall channel catfish reference transcriptome. The threshold was set as FDR value <0.1.

2.6. Experimental validation—QPCR

Ten significantly expressed genes were selected for validation using real time QPCR with gene specific primers designed using Primer3 software. Total RNA was extracted using the RNeasy Plus Universal Mini Kit (Qiagen) following manufacturer's instructions. First strand cDNA was synthesized by iScript™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer's protocol. All the cDNA products were diluted to 250 ng/μL and utilized for the quantitative real-time PCR reaction using the SsoFast™ EvaGreen® Supermix on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed in a total volume of 20 μL with cycling conditions: 94 °C for 5 s, followed by 40 cycles of 94 °C for 5 s, 60 °C for 5 s (fluorescence measured), and a disassociation curve profile of 65–95 °C for 5 s/0.5 °C increment. Results were expressed relative to the expression levels of 18S rRNA in each sample using the Relative Expression Software Tool (REST) version 2009 (Pfaffl et al., 2002). The biological replicate fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (C_t) values, were compared and converted to fold differences by the relative quantification method. Expression differences between groups were assessed for statistical significance using a randomization test in the REST software. The mRNA expression levels of all samples were normalized to the levels of 18S ribosomal RNA gene in the same samples. A no-template control was run on all plates. QPCR analysis was repeated in triplicate runs (technical replicates) to confirm expression patterns.

3. Results

3.1. A. hydrophila challenge

Extensive pre-challenge experiments examined the ability of virulent *A. hydrophila* to infect channel catfish fingerlings following bath challenge to allow natural routes of infection vs. i.p. injection (Mu et al., 2011). Bath challenges alone, regardless of exposure concentration or conditions, were unsuccessful in initiating *A. hydrophila* infection. However, disrupting the mucus covering of the skin by gentle scraping (see Section 2), followed by immediate challenge produced an effective, reproducible challenge model. We, therefore, adopted this model here to study infection-based changes in skin following infection.

The artificial challenge with virulent *A. hydrophila* showed initial mortality of infected fish beginning at 18 h after exposure and resulting in a final cumulative mortality of 42.5% at 48 h. No control fish manifested symptoms of *A. hydrophila*, and randomly selected control fish were confirmed to be negative for *A. hydrophila* by standard diagnosis procedures. Dying fish manifested external signs associated with *A. hydrophila* infection including redness in the eyes, petechial hemorrhaging and exophthalmia. *A. hydrophila* bacteria were successfully isolated from randomly selected treatment fish.

3.2. Identification and analysis of differentially expressed genes

A total of 2168 unique genes (based on assigned identifies from the zebrafish unigene database) showed significant differential expression in skin during at least one timepoint following infection. In detail, there were 82 differentially expressed genes at 2 h after challenge relative to control, 567 genes differently expressed at 8 h after challenge relative to control, and 1744 genes differently expressed at 12 h after challenge relative to control. At 8 h and 12 h, there were much greater numbers of upregulated genes than down-regulated genes. For example, at 12 h post infection, 1559 genes were upregulated, compared to only 185 downregulated genes (Table 2). Details of the 2168 differentially expressed genes are given in Supplementary Table 1. Feature values and metadata of the experiment are archived at the NCBI Gene Expression Omnibus (GEO) under Accession GSE40733.

3.3. Gene ontology and enrichment analysis

Differently expressed genes were then used as inputs to perform gene ontology (GO) annotation by Blast2GO. A total of 4,155 GO terms including 1080 (25.99%) cellular component terms, 1026 (24.69%) molecular functions terms and 2049 (49.31%) biological process terms were assigned to 3354 unique gene matches. The percentages of annotated catfish sequences assigned to GO terms are shown in Supplementary Fig. 1. Analysis of level 2 GO term distribution showed that metabolic process (GO:0008152), cellular process (GO:0009987), binding (GO:0005488) and cell (GO:0005623) were the most common annotation terms in the three GO categories.

The differently expressed unique genes were then used as inputs to perform enrichment analysis using Ontologizer. Parent-child GO term enrichment analysis was performed for the 2168 unigenes to detect significantly overrepresented GO terms. A total of 29 terms with *p*-value (FDR-corrected) <0.1 were considered significantly overrepresented. Ten higher level GO terms were retained as informative for further pathway analysis (Table 3). The GO terms include functions and processes including cellular response to stress, receptor signaling protein activity, G-protein coupled peptide receptor activity and protein kinase C-activating G-protein coupled receptor signaling pathway. Based on enrichment analysis and manual annotation and literature searches, representative key genes were arranged into 5 categories, including antioxidant/cellular stress response, cytoskeletal rearrangement, immune response, junctional/

Table 2
Statistics of differently expressed genes at different timepoints following *A. hydrophila* challenge. Values indicate cutoff values of fold change $\geqslant 2$ ($p < 0.05$) at a least one timepoint following challenge.

	2 h	8 h	12 h
Upregulated	20	529	1559
Downregulated	62	38	185
Total	82	567	1744
Total unigenes	2168		

Table 3
Summary of GO term enrichment result of significantly expressed genes in channel catfish following *A. hydrophila* challenge. The 2168 differentially expressed genes were analyzed as the study set in comparison to all the catfish unigenes. *p*-value ⩽0.1 was considered significant. Population count is the number of genes associated with the term in the population set. Study count is the number of genes associated with the term in the study set. GO names were retained only from GO terms of levels >2.

GO ID	GO name	*p*-Value (FDR)	Population count	Study count
GO:0007167	Enzyme linked receptor protein signaling pathway	1.02E-05	190	40
GO:0035556	Intracellular signal transduction	0.000153	702	123
GO:0071841	Cellular component organization or biogenesis at cellular level	0.00438	924	180
GO:0006950	Response to stress	0.00635	497	97
GO:0033554	Cellular response to stress	0.0175	204	47
GO:0031974	Membrane-enclosed lumen	0.0425	379	73
GO:0005057	Receptor signaling protein activity	0.0425	43	11
GO:0060249	Anatomical structure homeostasis	0.0499	19	9
GO:0008528	G-protein coupled peptide receptor activity	0.0847	84	11
GO:0007205	Protein kinase C-activating G-protein coupled receptor signaling pathway	0.0961	9	4

adhesion, and neural/nervous system regulation (Table 4). Imputedputative functional roles of these genes are covered in the Section 4.

3.4. Validation of microarray profiles by QPCR

In order to validate the differentially expressed genes identified by microarray, 10 genes were selected for QPCR confirmation, selecting from those with differing expression patterns and from genes of interest based on functional enrichment and pathway results. Samples from 2 h control, 8 h control, 12 h control, and 2 h, 8 h and 12 h following challenge (with three replicate sample pools per timepoint) were used for QPCR. Primers were designed based on channel catfish contig sequences (Supplementary Table 2). Melting-curve analysis revealed a single product for all tested genes. Fold changes from QPCR were compared with the microarray expression analysis results. As shown in Table 5, QPCR results were significantly correlated with the microarray results at each timepoint (correlation coefficient $R = 0.73$, p-value <0.001). With the exception of GTPase 1 at the 12 h timepoint, all examined genes had the same direction of differential expression by both methods. No consistent bias toward higher expression levels was observed by either method. We observed that concordance between QPCR values and microarray expression levels decreased at higher fold changes. For example, in the cases of myosin light polypeptide 7 and syncollin-like, expression changes of several hundred fold were registered by microarray and QPCR, respectively, but more modest changes were observed by the alternative method. These differences likely represented differential hybridization kinetics and/or the presence of paralogues contributing to expression levels which we were unable to predict without a fully annotated genome. In spite of these discordant results in regards to some fold change levels, the microarray platform consistently indicated differentially expressed genes for further analysis.

4. Discussion

A. hydrophila is an important pathogen of a range of vertebrate species, including humans, amphibians, reptiles and both freshwater and marine fishes (Cipriano, 2001). The existence of an ever-changing array of phenotypically, genotypically, and antigenically-diverse members of the species has long complicated the study of the disease and has made it difficult to pursue development of broad prophylactic and treatment solutions (Ventura and Grizzle, 1987; Rodriquez et al., 2008). Beginning in 2009 and continuing to the present, acute *A. hydrophila* outbreaks have devastated a significant portion of the US catfish industry. Recent studies on the recovered isolates (including one used in this work) have shown that they are at least 200-fold more virulent than stock *A. hydrophila* isolates, and that they have significant molecular differences when compared with low virulence isolates (Pridgeon et al., 2011; Pridgeon and Klesius, 2011). The virulent isolates are capable of producing mass mortality <24 h after exposure, mimicking the epidemiology of natural outbreaks on catfish farms. Recently, Mu et al. (2011) examined the transcriptional levels of several key genes in the head kidney of channel catfish exposed to attenuated and virulent *A. hydrophila* by i.p. injection. We sought here to expand our understanding of the host response by utilizing microarrays for global gene expression analysis and by focusing on early responses at the mucosal surface.

A clear consensus is lacking as to the primary route of entry of *A. hydrophila* into a fish host. Early reports implicated both skin lesions (Snieszko, 1974) and the gastrointestinal tract (Amlacher, 1970). Several studies have reported the resistance of host fish to infection through an undisturbed skin/mucus layer, but high infectivity of *A. hydrophila* in abraded or otherwise disturbed skin. Ventura and Grizzle (1987) found that abrading catfish skin prior to exposure was necessary for initiating infection in normal culture conditions. Similarly, Chu and Lu (2008) found that gills and damaged skin (wounded or mucus removed) were routes of invasion in crucian carp using GFP-labeled *A. hydrophila*. They concluded that while intact skin was not a primary portal, even minor disturbances of the mucosal layer allowed invasion. Studies in zebrafish with a virulent *A. hydrophila* isolate also revealed that introducing a slight tail cut was necessary to initiate infection using a bath challenge (Rodriquez et al., 2008). Our preliminary experiments mirrored these findings. Bath challenge of 30 g fingerlings did not result in *A. hydrophila* infection, regardless of dose, feeding status, or presence of i.p. injected (*A. hydrophila* positive) cohabitants (data not shown). Infection was readily initiated through disturbance of skin mucosa, however, leading us to conclude that one route of natural infection of channel catfish may be through a disturbed skin barrier, potentially obtained through fighting, seine wounds, lesions introduced by other infections, etc. It is additionally possible that stressful pond conditions, poor nutrition and/or chronic low dissolved oxygen, for example, may change the molecular and chemical nature of mucosal surfaces and allow entry in the absence of physical disruption.

A total of 17,038 unique, annotated channel catfish features were present in the utilized 8 × 60 K Agilent microarray. Of these, expression of 2168 was significantly perturbed during at least one early timepoint following infection. While only 20 transcripts showed significant induction at 2 h, this number rose steeply to 529 by 8 h and to 1559 by 12 h (Table 2). While these timepoints were chosen to capture early, critical immune responses, they likely led to higher variability in the results. The studied isolates, while generating an acute infection, typically lead to 40–60% mortality within 24 h. Thereafter, mortality rates drop precipitously, leaving a group of surviving fish which rarely manifest clinical

Table 4
Representative key functional categories and gene members differentially expressed in skin following *A. hydrophila* challenge. Bold values indicate significant fold change ($p \leqslant 0.05$).

Gene name	Probe_ID	2 h	8 h	12 h
Antioxidant/cellular stress response				
15 kDa selenoprotein precursor	Contig39146	2.15	2.21	1.65
60 kDa heat shock protein, mitochondrial	Contig3790	**−12.80**	-3.28	**−52.40**
Cysteine and histidine-rich DCP1	Contig361	**−20.71**	−3.94	**−71.02**
FK506 binding protein 4	Contig13508	**−16.86**	−3.69	**−71.21**
Heat shock protein HSP 90-alpha 1	UN13454	2.48	−1.02	**5.78**
Heat shock protein HSP 90-beta	UN17753	1.68	1.23	**3.66**
Hsp90 co-chaperone Cdc37-like 1	Contig9447	4.61	−1.17	**2.50**
Selenoprotein O	Contig8	−1.13	1.21	**3.15**
Selenoprotein T2 precursor	UN14891	1.46	1.13	**2.95**
Selenoprotein X, 1b	UN06529	1.32	−1.52	**5.48**
Superoxide dismutase	Contig24529	2.13	1.02	**9.81**
Cytoskeletal rearrangement				
Capping protein (actin filament), gelsolin	k66_388284	2.86	1.28	**3.37**
Cell division cycle 42	k50_454763	**−9.94**	−1.36	**−12.24**
Dynein, light chain, LC8-type 2a	UN18480	**−25.54**	−1.62	**−94.55**
Ephrin type-A receptor 2	Contig17991	−1.23	1.36	**7.63**
F-actin capping protein alpha-1 subunit	Contig20689	4.32	1.17	**3.85**
Gelsolin b	UN26079	**−24.96**	1.71	**2.18**
Integrin, beta 4	Contig12705	**6.90**	1.80	**5.02**
Myosin heavy chain, fast skeletal muscle	UN07214	**−7.12**	**−2.90**	−2.06
Myosin, light polypeptide 7, regulatory	UN33792	**−13.98**	**−10.88**	**−625.18**
Neurabin-1-like	Contig18327	2.64	1.78	**6.79**
PAK-interacting exchange factor beta	Contig18296	**8.91**	2.02	2.63
Plakophilin 1	UN47575	3.83	**2.84**	**12.67**
Villin 1-like	UN82082	3.04	1.41	**3.78**
Villin-1	Contig12533	2.37	−1.12	**2.43**
Vimentin	Contig348	**−15.58**	−1.00	**2.12**
Junctional/adhesion				
Claudin 32b-like	Contig8592	4.48	1.44	**3.31**
Claudin 8	k55_469604	2.33	1.65	**2.58**
Claudin-12	Contig3397	1.30	1.30	**4.86**
Claudin-like protein ZF-A89	UN06974	**−96.11**	**4.67**	2.40
Desmocollin 1 isoform Dsc1a preproprotein	UN27141	**8.55**	1.07	1.34
Desmocollin 2 like isoform 2	Contig8394	2.49	1.76	7.11
Desmoglein 2	Contig36771	1.70	−1.07	**−4.23**
Junction plakoglobin	Contig21521	2.53	1.31	**4.22**
Junctional adhesion molecule 2a	Contig2744	−1.82	−1.02	**3.61**
Tight junction protein ZO-2 isoform 1	Contig37989	2.05	1.25	**4.82**
Neural/nervous system regulation				
Ataxin-1	Contig4206	−2.11	1.33	**−11.82**
Contactin-1	UN82293	**−26.99**	−2.76	**−93.55**
Heterogeneous nuclear ribonucleoprotein Q	Contig3267	**−19.80**	−2.55	**−81.75**
Kelch-like protein 24	Contig15304	−3.91	−2.98	**−23.03**
Kv channel interacting protein 1 b	UN09508	**−8.01**	−2.34	**−30.13**
Liprin-alpha-2	k51_531132	**−24.37**	−3.13	**−80.27**
Mitochondrial glutamate carrier 1(SLC25A22)	Contig11144	**−26.91**	−1.43	**−84.81**
Rabphilin 3A homolog (mouse), b	k58_452731	**−25.56**	−3.65	**−93.54**
Ral GTPase-activating protein subunit alpha-1	Contig6603	**−22.56**	−2.85	**−100.99**
Uromodulin-like	UN07746	**−35.74**	**6.59**	1.89
Zwilling	UN55526	**−10.05**	−1.46	**−26.11**
Immune response				
B-cell receptor C22-like	Contig4299	**−18.41**	−3.17	**−72.48**
Beta-2-glycoprotein 1	k50_611578	**10.25**	2.52	3.50
C-C chemokine receptor type 2	UN33208	**7.78**	**3.27**	3.99
C-C motif chemokine 21	UN25591	**8.78**	**4.81**	3.73
CD8 beta chain	Contig13919	2.71	**2.97**	2.24
Chemokine (C-X-C motif) ligand 12b	Contig15070	1.64	**4.13**	2.29
Chemokine CCL-C11b	k50_637838	1.86	**3.17**	2.19
Galectin-3	Contig6999	**13.45**	2.34	**9.39**
C1qb	UN16436	1.72	−2.51	**5.34**
Complement component 6-like	UN17892	1.04	**−14.88**	−1.16
Complement factor D	UN16329	5.47	**6.30**	6.26
FinTRIM family, member 6 isoform 1	Contig43055	**−14.99**	−3.45	**−70.51**
Fish virus induced TRIM protein-like	Contig12784	1.03	1.42	**6.12**
Granzyme B-like	k50_2540	2.13	2.17	−1.09
H2A histone family, member X-like	UN19029	**−27.45**	1.97	**2.03**
Interferon gamma inducible protein 30	UN13983	1.92	1.45	**3.66**
Interferon, gamma	k51_690246	2.30	**2.36**	1.65
Interferon-inducible protein Gig1-like	UN34145	**12.85**	4.33	**14.36**
Interferon-inducible protein IFI58-like	k54_927290	2.00	2.26	**4.31**

(*continued on next page*)

Table 4 (*continued*)

Gene name	Probe_ID	2 h	8 h	12 h
Interleukin 1, beta	UN20464	−7.22	**−12.34**	−2.67
Interleukin 17a/f2	k50_480774	−1.20	1.01	**2.61**
Interleukin 2 receptor, beta	k50_951131	**−16.57**	−19.02	**−758.49**
Interleukin-1 receptor type II	k50_622521	−5.75	−8.90	**−5.99**
Lectin, mannose-binding, 1	Contig32944	1.70	2.06	**2.50**
Lysozyme-like protein 2	UN30680	1.34	−2.23	**2.66**
MHC class II integral membrane protein alpha3	UN12194	1.67	1.30	**8.76**
Microfibrillar-associated protein 4	UN09372	−1.28	**−6.40**	**2.20**
Mucin-5AC	Contig13257	1.83	−1.04	**2.83**
Mucin 5 B	UN20160	1.56	**2.15**	1.60
MyD88	Contig20402	−1.14	−1.20	**6.83**
Myxovirus (influenza virus) resistance E	Contig_32861	4.32	**5.47**	**−10.32**
NLR family, pyrin domain containing 1-like	Contig20868	1.69	1.05	**6.42**
Perforin-1-like	Contig23107	2.82	1.60	**3.17**
Polymeric immunoglobulin receptor	UN06743	4.10	1.35	**5.88**
Prostaglandin E synthase	UN81767	−1.30	1.18	**13.43**
Toll-interacting protein	Contig2969	1.65	1.16	**7.87**
Toll-like receptor 5a	k78_362431	**−15.97**	**−15.07**	**−10.72**
Transforming growth factor beta 1-like isoform 2	Contig6652	1.38	−1.72	**3.46**
IgGFc-binding protein (FCGBP)	k50_877718	**18.11**	**5.51**	3.11
YWHAQBY	UN06631	**3.78**	**1.86**	**18.88**
Xanthine dehydrogenase	Contig8033	**8.45**	2.64	1.03
Zona pellucida protein 4-like	UN14757	**−137.72**	**2.64**	**1.96**

Table 5
QPCR validation details. Fold changes of selected genes are given either according to microarray or QPCR results.

Gene	2 h Fold change after infection		8 h Fold change after infection		12 h Fold change after infection	
	QPCR	Microarray	QPCR	Microarray	QPCR	Microarray
Syncollin-like	−2.49	−1.38	−307.97	−12.21	1.22	1.65
Superoxide dismutase	6.53	2.13	7.82	1.02	1.84	9.81
B-cell receptor C22-like	−2.09	−1.41	48.84	18.41	−6.96	−72.48
Very large inducible GTPase 1	−14.75	−9.97	8.28	9.97	1.65	−5.69
S100 calcium binding protein A1	−9.71	−70.44	9.38	3.92	2.23	2.53
Suppressor of cytokine signaling 6	32.36	1.13	3.94	1.29	1.90	2.44
NIMA-related kinase 1	−2.11	−1.28	1.44	1.98	2.45	2.26
Myosin, light polypeptide 7	−5.03	−13.98	−3.52	−10.88	−21.06	−625.18
YWHAQBY	1.90	3.78	3.78	1.86	3.53	18.88
Interferon-inducible protein IFI58-like	1.54	2.00	2.80	2.26	1.84	4.31

signs of *A. hydrophila.* Collection of tissues from challenged fish at early timepoints, therefore, of necessity included individuals which would have ultimately evaded acute infection and death. We utilized pooled samples for biological replicates to increase the likelihood of capturing expression signatures tied to pathogen attachment and entry. Further work is clearly needed to identify the basis of the observed pattern of intra-strain resistance and susceptibility. Candidate genes identified here may be valuable as markers for future identification of these groups prior to pathogen exposure.

We categorized differentially expressed genes into five broad categories based on GO analysis and manual literature and pathway analyses. These included antioxidant/cellular stress response, cytoskeletal arrangement, immune responses, junctional/adhesion, and neural/nervous system regulation (Table 4). Below we highlight key constituents of these categories and their potential functions in the context of host responses to virulent *A. hydrophila*.

4.1. Antioxidant/cellular stress response

Recent study of several *Aeromonas* species has highlighted that their infection pattern is characterized by stimulation of robust host production of reactive oxygen species (ROS) and nitrite oxide radical (NO), leading to loss of mitochondrial membrane potential and apoptosis (Krzyminska et al., 2011). The most potent virulence factor of *A. hydrophila* strains infecting mammalian species, cytotoxic enterotoxin Act, has been shown, upon binding, to stimulate monocyte/macrophage infiltration and to induce release of ROS (Chopra et al., 2000; Sha et al., 2002). We observed dysregulation of a number of genes involved in mitochondrial regulation and antioxidant responses, largely at 12 h following infection (Table 4). This included strong downregulation (>50-fold) of several genes with roles as chaperones including 60 kDa heat shock protein, mitochondrial and FK506 binding protein 4. By 12 h, a number of selenoproteins including SelO, SelT2, and SelX1b were upregulated suggesting a response to buildup of free radicals. Similarly, Mn-superoxide dismutase (SOD2), one of the key enzymes involved in destroying free superoxide radicals in the body (Miao and St Clair, 2009), was upregulated 9.81-fold.

4.2. Cytoskeletal arrangement

Bacterial toxins often seek to alter and disrupt the actin cytoskeleton of targeted cells in order to gain entry and/or manipulate cellular immunity (Boquet and Lemichez, 2003; Aktories and Barbieri, 2005). These disruptions themselves can often lead to cell death at sites of infection (Suarez et al., 2012). Actomyosin-driven contraction and dynamics can also be important in the context of invasion as a central switch controlling both actin polymerization (Thune et al., 2007) and regulating permeability of apical junctions (Ivanov et al., 2007; Sousa et al., 2004). In our results, we observed differential expression of several genes associated with manipulation of the

actin cytoskeleton, including several filament-associated genes (gelsolin b, villin-1) with similar patterns of induction as in *Edwardsiella ictaluri* infection in catfish intestine (Li et al., 2012). Cdc42 and β4-integrin, both known to be perturbed by *A. hydrophila* effector *AexU* (Abolghait et al., 2011; Fehr et al., 2007), showed significant expression changes at 2 h after infection. Of particular interest was the observed 6.9-fold upregulation of β4-integrin (one of only 20 significantly upregulated genes at that timepoint). Abolghait and colleagues (2011) demonstrated that β4-integrin mediates the cytotoxicity of *AexU* aiding in its internalization and progressive actin cytoskeletal disruptions.

4.3. Junctional/adhesion

Pathogens also often seek to adhere to and/or disrupt cellular junctions to gain additional routes of access into the host (Guttman and Finlay, 2009). We observed dysregulation of components of the apical junction complex (AJC), consisting of the tight junction, adherens junction, and desmosome. While most genes in this category were upregulated significantly at the 12 h timepoint, two showed large fold changes at the 2 h timepoint (Table 4). A claudin-like protein ZF-A89 was sharply downregulated at 2 h before recovering at later timepoints. Claudins are a diverse family of permeability-regulating genes in various epithelial and endothelial cell types (Angelow et al., 2008). Teleost fish claudins have expanded tremendously through duplication, resulting in teleost-specific family members such as ZF-A89. Although the functions and cell specificity of ZF-A89 and most other fish claudins are currently unknown, claudins in higher vertebrates are often manipulated or disrupted by pathogens (Guttman et al., 2006; Farquhar et al., 2012). The other junctional factor showing differential expression at 2 h post-infection was desmocollin 1a, a component of the desmosome. Several other components of desmosomes, intercellular adhesive junctions of epithelial cells, including desmoglein and plakoglobin were also perturbed at 12 h. Desmosomes are often targeted by *Staphylococcus aureus* in infectious skin diseases (Amagai, 2010). Further analyses are needed to identify the cellular locations and functional responses to bacterial infection of these junctional proteins in catfish.

4.4. Neural/nervous system regulation

A. hydrophila infections in fish have long been predicted to have impacts on the central nervous system via secretion of an extracellular acetylcholinesterase (Nieto et al., 1991), but no molecular evidence of this disruption has ever been generated. A group of genes with putative functional classification as regulators of nervous system functions was strongly differentially expressed at both the 2 h and 12 h post-infection timepoints. Strikingly, expression of these genes was broadly repressed (Table 4). For example, contactin-1, a glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein that functions as a cell adhesion molecule, was downregulated −26.99-fold at 2 h and −93.55-fold at 12 h. While little is known of the function of these genes in teleost fish, we can speculate based on their roles in mammalian species that they are, in part, regulating a complex network transmitting status signals between the mucosal surface and the brain. In mammals, the cutaneous peripheral nervous system (PNS) is now understood to play a pivotal role in skin homeostasis and disease (Roosterman et al., 2006). *A. hydrophila* infection appears to be disrupting these signaling interactions, though further work is needed to elucidate whether the observed expression patterns are the result of pathogen manipulation of the host or a protective response to shunt cell signaling resources toward the inflammatory response.

4.5. Immune response

A number of immune factors were differentially expressed in channel catfish skin in the early timepoints following *A. hydrophila* infection. The composition of the responding immune repertoire differed noticeably from other recent studies which have examined fish host gene responses to *A. hydrophila* through subtractive hybridization and RNA-seq approaches in catfish (Mu et al., 2011) and yellow croaker (Mu et al., 2010), respectively. These differences likely arise from variation in timing of post-infection sampling and in a focus on different tissues, head kidney and spleen, respectively. Of note, at least eight of the 20 upregulated genes at 2 h post-infection had clear roles in innate immunity, with a similar enrichment of immune factors in down-regulated genes at the same timepoint. Our recent examinations of mucosal immune responses to *E. ictaluri* and *Flavobacterium columnare* have suggested that these first responders are critical regulators of pathogen attachment and disease progression and are often the targets of pathogen-mediated manipulation (Li et al., 2012; Sun et al., 2012; Beck et al., 2012). We were particularly interested, therefore, in the small set of immune-related genes whose expression was significantly perturbed at the earliest point of measurement.

One such factor was Fc fragment of IgG binding protein (FCGBP), which was upregulated >18-fold at 2 h post challenge. FCGBP is a poorly-characterized mucin glycoprotein, known to associate with secreted gel-forming mucins in gastric epithelium (Johansson et al., 2008). Given the critical role of mucins in controlling the rate and nature of bacterial attachment on mucosal surfaces (Linden et al., 2008), changes in FCGBP may reflect important structural changes in the skin mucus of channel catfish following contact *with A. hydrophila* or its secreted effector proteins.

The chimera galectin, galectin-3, also responded rapidly post-challenge. Its expression was induced 13.45-fold at 2 h. While our knowledge of galectin-3 functions are limited in fish (Vasta et al., 2004), it has been well-studied in mammalian species. Li et al. (2008) reported that galectin-3, while protective of endotoxin shock, favored *Salmonella* survival. Similarly, research utilizing galectin-3 deficient mice, found that galectin-3 co-localizes with *Neisseria meningitides* and contributes to bacteraemia (Quattroni et al., 2012). Studies dealing with bacterial pathogens from numerous genuses (*Helicobacter, Pseudomonas, Proteus*), have led to the consensus that galectin-3 may act as a cell surface docking site or a cross-linking molecule promoting adhesion (Altman et al., 2001; Fowler et al., 2006; Gupta et al., 1997; Quattroni et al., 2012). Potentially of greatest relevance here is research reporting binding of *A. hydrophila* enterotoxin Act to galectin-3, contributing to host cell apoptosis (Galindo et al., 2006). Further work is warranted to examine whether channel catfish galectin-3 may play similar roles in supporting pathogen adhesion.

Other gene transcripts rapidly induced following challenge included beta-2-glycoprotein 1, recently identified as a novel component of the innate immune system responsible for neutralization and clearance of LPS (Agar et al., 2011). In-vivo evidence is still lacking, however, whether this neutralization is beneficial in the context of infection, or whether, it ultimately favors bacterial survival. Expression of several chemokines and chemokine receptors (Peatman and Liu, 2006) were induced following infection. In particular, CCL21 and CCR2 were among the genes responding at 2 h (Table 4), whereas CXCL12 and CCL-C11b were up-regulated by 8 h (Baoprasertkul et al., 2005; Peatman et al., 2006).

A suite of immune genes was also strongly down-regulated following *A. hydrophila* challenge. Interestingly, among these were several zona pellucida proteins (Table 4, Supplementary Table 1). These genes are important in immunocontraception, but little is known of broader immune roles (Gupta and Bhandari, 2011). Further work is needed to study the cellular localization of these glycoproteins in

catfish skin and to examine the consequences of their potent downregulation. Also strongly downregulated relative to control fish at both 2 h and 12 h, was the interleukin 2 receptor beta (IL2RB). Notably, IL2RB expression fell greater than 700-fold at 12 h. Such a reduction may be the result of a similar suppression of IL-2 production, but an IL-2 transcript has not been identified from catfish and was not, therefore, present on the microarray. Interleukin 2 is a lymphocyte-secreted cytokine which plays critical roles in stimulating proliferation of mucosal lymphocytes, natural killer cells, and macrophages (Secombes et al., 2011; Burchill et al., 2007). IL-2 receptors are present on the mucosal epithelium of several mammalian species (Ciacci et al., 1993; de Villiers et al., 2000), and IL-2 deletion in mice leads to thymic and mucosal dysregulation (Ehrhardt and Ludviksson, 1999). Recent work has expanded the role of IL-2 in mucosal homeostasis (Mishra et al., 2012). We would predict, therefore, that potent downregulation of IL2RB may be a key immunosuppressive strategy of *A. hydrophila* to facilitate successful infection of the skin mucosal surface. Suppression of the IL-2R (and ligand) is known to occur through the action of prostaglandins and can be restored through addition of prostaglandin synthesis inhibitors (Rappaport and Dodge, 1982; Sileghem et al., 1989). Notably, we observed the induction of several of several prostaglandin synthase genes, particularly prostaglandin E synthase, upregulated greater than 13.43-fold at 12 h (Table 4).

Several other important immune genes showed reduced expression following infection. Toll-like receptor 5a (TLR5a), known to recognize bacterial flagellin, was also significantly down-regulated beginning at 2 h post infection (Table 4). This was in contrast to other reports of upregulation following bacterial infection in catfish (Bilodeau and Waldbieser, 2005; Peatman et al., 2007; Russo, 2011; Li et al., 2012). Potential antimicrobial peptide, histone H2AX (Nam et al., 2012; Robinette et al., 1998) expression was reduced greater than 25-fold at 2 h. Expression of a finTRIM family member declined significantly at 2 h and 12 h (van der Aa et al., 2012). IL-1 beta and MFAP-4 expression was significantly downregulated at 8 h (Wang et al., 2006; Niu et al., 2012). Taken together, the immune response captured by the microarray was broadly indicative of a rapid and multifaceted pathogen-directed strategy aimed at immune-sculpting effector responses to improve chances of survival and replication (Hertzog et al., 2011).

5. Conclusion

A transcriptomic profile of responses to *A. hydrophila* infection in channel catfish skin was obtained using a new 8 × 60 K microarray. Expression of >2000 genes was perturbed, including critical members of pathways regulating innate immunity and oxidative stress responses. These early signatures serve as a foundation for understanding mechanisms involved in the binding and invasion of virulent *A. hydrophila*. Accordingly, key candidate genes identified here (e.g. galectin-3, IL2RB, B4-integrin) will be utilized to compare and contrast catfish mucosal responses to bacterial isolates with differing virulence and in catfish populations with differing susceptibility to *A. hydrophila* infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2012.11.009.

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