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Comparative transcriptional analysis reveals distinct expression patterns of channel catfish genes after the first infection and re-infection with *Aeromonas hydrophila*

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**Abstract**

To determine whether transcriptional levels of channel catfish (*Ictalurus punctatus*) genes are differentially regulated between a first infection with *Aeromonas hydrophila* and a re-infection, suppression subtractive hybridization (SSH) was performed in this study using anterior kidney cDNA after the re-infection as tester. Of the 96 clones isolated from the SSH library, 28 unique expressed sequence tags (ESTs) were obtained, of which eight were confirmed to be slightly but significantly (*P < 0.05*) more up-regulated by the re-infection at 6 h post infection (hpi). Expression kinetics studies at 3, 6, 12, 24, and 48 hpi revealed that the eight ESTs were significantly (*P = 0.016*) more up-regulated by the first infection, with a major peak at 3 hpi. A total of 96 genes reported in literature to be up-regulated by bacterial infections were selected and subjected to expression analysis at 3 hpi. Of the 96 selected genes, 19 were found to be significantly (*P < 0.05*) induced by *A. hydrophila* after the first infection and the re-infection. The 19 genes belonged to the following five main categories: 1) toll-like receptor (TLR2, TLR3, TLR5, TLR21); 2) antimicrobial peptide (NK-lysin type 1, NK-lysin type 2, NK-lysin type 3, cathepsin D, transferrin, hepcidin); 3) cytokine or chemokine (interleukin-1α, interleukin-1β, interleukin-10, tumor necrosis factor α, chemokine CXCL-10); 4) signaling proteins (cadherin EGF LAG seven-pass G-type receptor 1, very large inducible GTPase 1, arginine deiminase type 2, lymphokine-activated killer T-cell originated protein kinase); 5) lysozyme (lysozyme c). Overall, the total 27 genes (8 ESTs plus the 19 selected genes) were significantly (*P < 0.001*) more induced by the first infection. Peaked expression of lysozyme c and serum lysozyme activity after the first infection were seen at 24 hpi, whereas that after the re-infection were seen at 12 hpi, suggesting that both innate and adaptive immunity were involved in the defense against the re-infection of *A. hydrophila*.

**Introduction**

*Aeromonas hydrophila* is a causative agent of motile aeromonad septicaemia (MAS) [1,2]. Swelling of tissues, dropy, red sores, nerosis, ulceration, and hemorrhagic septicemia are typical symptoms of MAS [3,4]. Many fish species can be affected by MAS, including tilapia [5,6], catfish [7,8], goldfish [9,10], common carp [11,12], and eel [13]. As an opportunistic pathogen, *A. hydrophila* caused outbreaks in fish farms with high mortality rates [14–16]. In West Alabama, MAS disease outbreak caused by *A. hydrophila* in 2009 alone has led to an estimated loss of more than 3 million pounds of food size channel catfish [17]. Virulence studies have revealed that AL09–71, a 2009 West Alabama isolate of *A. hydrophila* used in this study, is highly virulent to channel catfish, killing fish within 24 h post exposure [18].

Both innate and adaptive immunity play important roles in the defense of fish against bacterial infections [9]. Immunity of fish to bacterial infections is largely mediated by cellular immune responses with humoral antibodies having a secondary role [20,21]. It was reported that live attenuated *A. hydrophila* induced the up-regulation of multiple immune genes in channel catfish [22]. In addition, infection with *A. hydrophila* induced peak up-regulation of several genes at 6 h post infection (hpi) [22]. However, it was unknown whether gene expression levels in channel catfish after re-infection with *A. hydrophila* were induced higher compared to that by the first infection. Therefore, the objectives of this study were to: 1) identify up-regulated genes in channel catfish after a second infection of *A. hydrophila* compared to a single infection at
6 hpi by suppression subtractive hybridization (SSH); 2) compare the gene expression patterns of at different time (0, 3, 6, 12, 24, and 48 hpi) after the first infection and the re-infection to determine the peak response time; and 3) screen genes that were reported in literature and identify genes up-regulated by the first infection and the re-infection at the peak response time.

2. Materials and methods

2.1. Bacteria source and growth conditions

The AL09-71 isolate of *A. hydrophila* was obtained from diseased channel catfish in 2009 from West Alabama. The isolate has been confirmed to be *A. hydrophila* through biochemical and molecular identification [17]. Bacterial cultures were grown in tryptic soy broth (TSB) (Fisher Scientific, Pittsburgh, PA) for 24 h at 28 °C.

2.2. Experimental fish

Channel catfish (26.2 ± 3.3 g) were obtained from stocks maintained at USDA-ARS, Aquatic Animal Health Research Laboratory (Auburn, AL, USA). All fish were maintained in de-chlorinated water in 340 L tanks. Prior to experiments, fish were acclimated in flow-through 57-L aquaria supplied with ~0.5 L h⁻¹ de-chlorinated water for 14 days. 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 plates. A 12:12 h light:dark period was maintained and supplemental aeration was supplied by air stones. Mean dissolved oxygen was ~5.6 mg L⁻¹ at water temperature ~27 °C, with pH ~7.1 and hardness ~100 mg L⁻¹. Fish were fed ~3% body weight daily with commercial dry fish food.

2.3. Sample collection

Prior to the experimental infection, fish were moved to 57-L flow through aquaria and acclimated for 14 days. The sub-lethal infection dose of *A. hydrophila* AL09-71 given to fish was 1 × 10⁴ colony forming unit per fish (CFU/fish) based on previous challenge result (LD₅₀ = 1 × 10⁵ CFU/fish [17]). Prior to injection, thirty untreated fish were used to collect samples at 0 h. The remaining fish were divided into three groups (150 fish/group): 1) control group [intraperitoneally (IP) injection with 100 μL PBS]; 2) Innate group (IP injection with *A. hydrophila* AL09-71 at 1 × 10⁴ CFU/fish); 3) Adaptive group (IP injection with *A. hydrophila* AL09-71 at 1 × 10⁵ CFU/fish followed by a second injection with *A. hydrophila* AL09-71 at 2 × 10⁴ CFU/fish at 28 days post first injection). Anterior kidney samples were collected from 30 fish at 0, 3, 6, 12, 24, 48 hpi and 10 tissues were pooled together as one sample (10 fish/pool, 3 pools per time point). All anterior kidney tissues were flash frozen on dry ice immediately after sampling and stored at ~80 °C until RNA extraction.

2.4. Total RNA extraction and cDNA synthesis

Total RNA was isolated from anterior kidney tissues using TRIzol Reagent (Invitrogen, Carlshad, CA) following the manufacturer’s protocol. All RNAs were treated with DNase provided by the DNA-free kit (Ambion, Austin, TX) to eliminate any DNA in the RNA sample. RNA samples were quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). The first strand cDNAs used for quantitative PCR were synthesized using 2 μg of total RNA, AMV reverse transcriptase, and Oligo-DT primer provided by the cloned AMV first strand cDNA synthesis kit (Invitrogen, Carlshad, CA).

2.5. Construction of subtractive cDNA library

For subtractive library construction, total RNAs were extracted from pooled anterior kidney samples at 6 hpi after the first infection or the re-infection of *A. hydrophila* AL09-71. The 6 hpi time point was chosen based on previous research results on gene up-regulation in channel catfish in response to *A. hydrophila* [22]. cDNAs were then synthesized using PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA). Two-step subtractive hybridizations were performed according to procedures described previously [22]. Briefly, two primary hybridization reactions (A and B) were formed by adding excess amounts of first infection cDNA (driver) to second infection cDNA (tester) samples at a 50:1 ratio. The samples were denatured for 2 min at 98 °C and allowed to anneal for 8 h at 68 °C. The remaining single-stranded, adaptor-ligated tester cDNAs were substantially enriched in each hybridization reaction for over-expressed sequences because non-target cDNAs present in the tester and driver could form hybrids. After filling in the adapter ends with DNA polymerase, over-expressed sequences (tester cDNA) had different annealing sites on their 3'- and 5'- ends. The molecules were then subjected to suppression subtraction PCR. The PCR products were then cloned into pGEM-T easy vector (Promega, Madison, WI). Plasmids were transformed into One Shot®TOP10 competent cells (Invitrogen, Carlsbad, CA). Transformed cells were plated on Luria–Bertani (LB) plates containing ampicillin (100 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml).

2.6. DNA sequencing

From the library, a total of 96 colonies were subsequently picked to grow overnight in Lysogeny broth (LB) in the presence of ampicillin (100 μg/ml) at 37 °C and 235 rpm in Innova™4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ). Overnight cultures were then sent to USDA-ARS MSA Genomics Laboratory in Stoneville, MS for plasmid DNA extraction and DNA sequencing with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Raw sequence base calling and trimming was conducted at the MSA Genomics Laboratory by using Phred with a cut-off score of Q20. Vector and adaptor sequences were manually trimmed. Trimmed cDNA sequences were then analyzed using the National Center for Biotechnology Information (NCBI) BLAST program to search for sequence homologies.

2.7. Primer design and quantitative PCR

Sequencing results of different clones were used to design gene-specific primers (Supplementary Table 1) by using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quantitative PCR (QPCR) was performed using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). For each cDNA sample, channel catfish 18S ribosomal RNA primers were included as an internal control to normalize the variation in cDNA amount as published previously [22]. All QPCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) in a total volume of 12.5 μl. The QPCR mixture consisted of 1 μl of cDNA (input RNA of 10 ng), 0.5 μl of 5 μM gene-specific forward primer, 0.5 μl of 5 μM gene-specific reverse primer and 10.5 μl of 1 x SYBR Green SuperMix. The QPCR thermal cycling parameters were 50 °C for 2 min, 95 °C for 10 min (followed by 40 cycles of 55 °C for 15 s and 60 °C for 1 min). All QPCR was run in duplicate for each pooled cDNA sample (10 kidney samples per pool; three pools were analyzed). The fluorescence intensities of the control and treatment products for each gene, as measured by cycle threshold (Ct) values, were compared and
converted to fold differences by the relative quantification method [23] using the Relative Expression Software Tool 384 v. 1 (REST) and assuming 100% efficiencies. Expression differences between control and treatment 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 sample. Expression levels of 18S were constant between all samples (<0.30 change in Ct). Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis. The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (Ct) of the sample by that of the 18S rRNA, the calibrator or internal control, as per the formula: ΔCt = Ct (sample) – Ct (calibrator). The relative expression level of a specific gene at different time points were compared to that of fish at time 0 using formula 2 –DDCt where ΔDDCt = ΔCt (time point x) – ΔCt (time point 0) as described previously [22].

2.8. Genes reported in literature screened in this study

To search for more channel catfish genes that might be differentially induced by a re-infection of *A. hydrophila* compared to that by a first infection, a total of 96 channel catfish genes reported in literature were screened in this study, including the following: 1) 43 genes induced by *Edwardsiella ictaluri* [24]; 2) 28 genes up-regulated by *Flavobacterium columnare* [25]; 3) 5 toll-like receptors (TLRs) up-regulated by *E. ictaluri* [26]; 4) 20 genes up-regulated by *A. hydrophila* [22]. Primers for these 96 genes were purchased from Sigma–Aldrich (St. Louis, MO).

2.9. Serum lysozyme activity assay

Lysozyme activity was measured using published procedures [27,28] with slight modifications. Briefly, lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich) at a concentration of 0.2 mg/ml in sodium acetate buffer (0.1 M; pH 6.0) was used as a substrate. Serum (20 μl/well in duplicate) from ten fish/group at different time points after injected with TSB or *A. hydrophila* were placed in a 96-well plate containing 250 μl of *M. lysodeikticus* cell suspension per well. Hen egg white (HEW) lysozyme was used as an external standard. The absorbance values of 0 min and 20 min post incubation (30 °C) were measured at 450 nm. The rate of reduction in absorbance of samples was converted to lysozyme concentration (μg/ml) using a standard curve of HEW lysozyme. The results were expressed as μg/ml equivalent of HEW lysozyme.

2.10. In vitro effect of serum on cell proliferation of *A. hydrophila*

The effect of serum on cell proliferation was performed using published procedures [29,30] with slight modifications. Serum samples collected at 28 days post first or second injection of *A. hydrophila* was used in this study. Briefly, logarithmic phase *A. hydrophila* AL09-71 bacterial cultures were diluted in tryptic soy broth to make a final concentration of 1.0 × 10⁸ CFU/ml based on published procedures [29,30]. The assay mixture contained 10 μl serum and 50 μl diluted bacterial culture. Heat-inactivated (70 °C, 30 min) serum samples at 28 days post first or second injection of *A. hydrophila* were used as negative controls. Plates were incubated at 28 °C for 2 h. The number of viable cells in each well was determined by CellTiter 96® AQgreen Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI). Briefly, 20 μl of solution containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-2,5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolum inner salt (MTS) and an electron coupling reagent phenazinemethosulfate (PMS) was added to each well. The MTS was then biodegraded by viable cells into a formazan product. The absorbance of the formazan product was measured at 490 nm using a BioRad 680 microplate reader (Biorad, Hercules, CA) at 0, 5, 15, 30, 60, and 180 min post MTS addition. Relative increases in OD values were calculated using the following formula: Increased OD value = OD value after incubation – OD value at 0 min of incubation.

### Table 1
List of genes isolated from the subtractive cDNA library.

| Clone     | Accession | Putative gene name | Organism                  | E-value | Identities | Insert (bp) |
|-----------|-----------|--------------------|---------------------------|---------|------------|-------------|
| A01       | JK9393536 | Voucher BHS 47559 12S ribosomal RNA gene | *Ictalurus punctatus* | 7.00E−52 | 100%       | 498         |
| A03       | JK939357 | Clone CS2 18S ribosomal RNA gene | *Ictalurus punctatus* | 0       | 100%       | 424         |
| A04       | JK939358 | C-jun-amino-terminal kinase-interacting protein 4-like | *Danio rerio* | 9.00E−25 | 75%        | 813         |
| A05       | JK939359 | Hypothetical 18K protein – goldfish mitochondrion | *Carassius auratus* | 2.00E−29 | 54%        | 812         |
| A07       | JK939340 | Mitochondrion | *Ictalurus punctatus* | 0       | 99%        | 389         |
| A08       | JK939341 | TLR 20-1 | *Ictalurus punctatus* | 1.00E−143 | 95%       | 400         |
| A09       | JK939342 | Voucher OP-10-CC-NBFR-IKO 28S ribosomal RNA gene | *Ompok pabda* | 0       | 100%       | 549         |
| A10       | JK939343 | XbaI element 5 | *Ictalurus punctatus* | 2.00E−89 | 99%        | 192         |
| B03       | JK939344 | 125 ribosomal RNA gene | *Ictalurus punctatus* | 8.00E−123 | 100%       | 428         |
| B04       | JK939345 | XbaI element 7 | *Ictalurus punctatus* | 4.00E−83 | 96%        | 625         |
| B06       | JK939346 | XbaI element 3 | *Ictalurus punctatus* | 3.00E−40 | 96%        | 184         |
| B10       | JK939347 | NADH dehydrogenase subunit 5 | *Ictalurus punctatus* | 1.00E−131 | 97%       | 1054        |
| B11       | JK939348 | 18S ribosomal RNA gene | *Ictalurus punctatus* | 5.00E−145 | 99%        | 293         |
| C02       | JK939349 | Hypothetical protein | *Danio rerio* | 2.00E−65 | 99%        | 146         |
| C04       | JK939350 | Inward rectifier potassium channel 13 | *Danio rerio* | 3.00E−104 | 74%       | 735         |
| C09       | JK939351 | Immunoglobulin heavy chain locus gene | *Ictalurus punctatus* | 6.00E−102 | 89%       | 308         |
| C10       | JK939352 | Ribosomal protein L7 mRNA | *Ictalurus punctatus* | 1.00E−110 | 100%      | 316         |
| C11       | JK939353 | Prolactin gene | *Ictalurus punctatus* | 8.00E−145 | 99%       | 296         |
| C12       | JK939354 | NADH dehydrogenase subunit 2 | *Ictalurus punctatus* | 1.00E−07 | 94%        | 157         |
| D01       | JK939355 | Cytochrome c oxidase subunit II | *Ictalurus punctatus* | 7.00E−42 | 96%        | 543         |
| D12       | JK939356 | CH1073-21K in linkage group 20 | *Danio rerio* | 3.00E−18 | 81%        | 596         |
| E05       | JK939357 | Reverse transcriptase-like protein | *Takifugu rubripes* | 1.00E−21 | 45%       | 816         |
| E06       | JK939358 | Rho GTPase-activating protein 10 | *Danio rerio* | 7.00E−42 | 88%        | 273         |
| E07       | JK939359 | 40S ribosomal protein 59 | *Ictalurus punctatus* | 5.00E−79 | 100%       | 373         |
| F01       | JK939360 | Leukocyte DNA binding receptor | *Ictalurus punctatus* | 7.00E−29 | 94%        | 438         |
| F08       | JK939361 | Internal transcribed spacer 2 | *Callorhinchus milii* | 6.00E−93 | 96%        | 218         |
| G08       | JK939362 | Heat shock protein 90 | *Danio rerio* | 0       | 86%        | 624         |
| H04       | JK939363 | Isolate IP-P-03 control region | *Ictalurus punctatus* | 0       | 99%        | 481         |
2.11. Statistical analysis

All statistical analyses were performed using SigmaStat 3.5 software (Systat Software, Inc, Point Richmond, CA). Differences in expression levels of gene at one time point, lysozyme activity, and inhibitory effect on cell proliferation were analyzed with Student t-test and the significance level was defined as P < 0.05. Statistical differences of expression levels of up-regulated genes at different time points after first infection compared to that after the second infection were analyzed with Mann–Whitney U significance test, a well-known non-parametric statistical hypothesis test to assess whether one of two samples of independent observations tends to have larger values than the other.

3. Results

3.1. Characteristics of the subtractive cDNA library

A total of 96 clones were obtained from the subtractive library using the secondary infected fish as tester and primary infected fish as driver. All 96 clones were subjected to sequencing. Of the 96 clones, 94 contained inserts. BlastN analysis revealed that 28 unique expressed sequence tags (ESTs) (Table 1) were obtained from the 94 inserts. The insert sizes of the 28 unique ESTs ranged from 124 bp to 1054 bp, with average size of 452 bp (Table 1). Eighteen and seven of the 28 sequences shared high identities with channel catfish (Ictalurus punctatus) and zebra fish (Danio rerio), respectively (Table 1). The 28 ESTs listed in Table 1 were deposited in GenBank dbEST under accession numbers JK993536 to JK993563.

3.2. Expression of the 28 ESTs at 6 h post infection

Of the 28 unique ESTs, 8 were significantly (P < 0.05) more up-regulated by the second exposure to A. hydrophila compared to that by the first exposure at 6 hpi (Fig. 1). Of the 8 ESTs, two (A10: XbaI element 5 and B04: XbaI element 7) were up-regulated greater than 6-fold by the second infection (Fig. 1). The other six ESTs that were also significantly more up-regulated by the second infection were: 1) A08: TLR20-1; 2) CO4: inward rectifier potassium channel 13; 3) C09: immunoglobulin heavy chain gene locus; 4) C11: prolactin; 5) C12: NADH dehydrogenase subunit 2; 6) E05: reverse transcriptase-like protein (Fig. 1).

3.3. Expression kinetics of the 8 ESTs at different time points after first and second infection

Expression kinetics studies at 3, 6, 12, 24, and 48 hpi revealed that the 8 ESTs were significantly (P ≤ 0.016) more up-regulated by the first infection, with a major peak at 3 hpi (Fig. 2). The significantly (P < 0.05) higher expression levels of TLR20-1 induced by first infection compared to that by second infection peaked at 3 hpi, followed by a decreased peak at 24 hpi (Fig. 2A). Similar expression pattern was observed for XbaI element 5 (Fig. 2B), XbaI element 7 (Fig. 2C), inward rectifier potassium channel 13 (Fig. 2D), immunoglobulin heavy chain locus gene (Fig. 2E), and reverse transcriptase-like protein (Fig. 2H). Significantly (P < 0.05) higher expression of prolactin (Fig. 2F) and NADH dehydrogenase subunit 2 (Fig. 2G) induced by first infection compared to that by second infection with the peak at 3 hpi were also observed.

3.4. Screening 96 genes reported in the literature

Based on expression kinetics study results of the 8 ESTs identified by SSH, the time point of 3 hpi was chosen to screen the 96 selected genes. Of the 96 genes selected from literature, 19 were found to be significantly (P < 0.05) induced by A. hydrophila at 3 hpi compared to that by TSB, regardless whether it was the first infection or the re-infection. The 19 genes belonged to the following five main categories: 1) toll-like receptor (TLR2, TLR3, TLR5, TLR21); 2) antimicrobial peptides (AMPs) (NK-lysin type 1, NK-lysin type 2, NK-lysin type 3, cathepsin D, transferrin, hepcidin); 3) cytokine or chemokine (interleukin-1b, interleukin-2, interleukin-10/IL-10, tumor necrosis factor a/TNFz, chemokine CXCL-10); 4) signaling proteins (cadherin EGF LAG seven-pass G-type receptor 1, very large inducible GTPase 1, arginine deiminase type 2, lymphokine-activated killer T-cell originated protein kinase); 5) lysozyme (lysozyme c).

3.5. Expression kinetics of TLRs at different time points after first and second infection

Overall, the expression of the four TLRs identified by the screen at various time points was significantly (P = 0.005) more induced by the first infection compared to that by the second infection. The expression of TLR2 (Fig. 3A) after injection with A. hydrophila once and twice both peaked at 3 hpi, with no statistical difference (P > 0.05) between the two. At 6 hpi, TLR2 was significantly (P < 0.05) more induced by the second infection compared to that by the first infection (Fig. 3A). At 12 and 24 hpi, TLR2 was significantly (P < 0.05) more induced by the first infection compared to that by the second infection (Fig. 3A). Similar pattern was also observed for TLR3 (Fig. 3B) and TLR21 (Fig. 3D). However, the expression pattern of TLR5 (Fig. 3C) after the first infection of A. hydrophila was not statistically different from that after the second infection. The significantly (P < 0.05) higher expression level of TLR5 in fish injected with A. hydrophila compared to that with TSB peaked at 3 hpi (Fig. 3C).

Fig. 1. Relative transcriptional levels of the 8 ESTs in the anterior kidney of channel catfish at 6 h post the first infection or the re-infection with A. hydrophila. The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (Ct) of the sample by that of the 18S rRNA, the calibrator or internal control, as per the formula: \( \Delta\text{Ct} = \text{Ct} (\text{sample}) - \text{Ct} (\text{calibrator}) \). The relative expression level of the specific gene in A. hydrophila infected fish compared to that in TSB control was calculated by the formula 2\( ^{-\Delta\text{Ct}} \) where \( \Delta\text{Ct} = \text{Ct} (\text{infected fish}) - \text{Ct} (\text{TSB fish}) \). A08: TLR20-1; A10: XbaI element 5; B04: XbaI element 7; C04: inward rectifier potassium channel 13; C09: immunoglobulin heavy chain gene locus; C11: prolactin; C12: NADH dehydrogenase subunit 2; E05: reverse transcriptase-like protein. Data are presented as means ± S.D. from three replicates. Differences were considered statistically significant when \( P \) value < 0.05.
Fig. 2. Transcriptional kinetics of the eight genes in the anterior kidney of channel catfish at different time points post exposure to *A. hydrophila* once or twice. A: TLR 20-1; B: XbaI element 5; C: XbaI element 7; D: Inward rectifier potassium channel 13; E: immunoglobulin heavy chain gene locus; F: Prolactin; G: NADH dehydrogenase subunit 2; H: reverse transcriptase-like protein. Data are presented as means ± S.D. from three replicates. Differences were considered statistically significant between treatments when *P* value < 0.05.
3.6. Expression kinetics of AMPs at different time points after first and second infection

Overall, the expression of the six AMPs identified by the screen at various time points was significantly \((P < 0.001)\) more induced by the first infection compared to that by the second infection. Of the 6 AMPs identified by the screen, the expression levels of transferrin (Fig. 4A) and hepcidin (Fig. 4B) induced by \(A.\ hydrophila\) were the highest (>50 fold). The expression of transferrin had a major peak at 12 hpi, regardless whether it was a first infection or a second infection (Fig. 4A). The expression of hepcidin had peaks at 3 and 12 hpi, with significantly \((P < 0.05)\) higher expression at 12 hpi induced by first infection (Fig. 4B). The expression of NK-lysin type 3 was highly and significantly \((P < 0.05)\) induced by the first infection compared to that by the second infection (Fig. 4C). Similar pattern was also observed for NK-lysin type 1 (Fig. 4D) and NK-lysin type 2 (Fig. 4E). The expression of cathepsin D after infection had peak at 12 hpi, with a significantly \((P < 0.05)\) higher expression level induced by the first infection compared to that by the second infection (Fig. 4F).

3.7. Expression kinetics of cytokine or chemokine at different time points

Overall, the expression of the four cytokine or chemokine identified by the screen at various time points post exposure to \(A.\ hydrophila\) once or twice was significantly \((P < 0.001)\) more induced by the first infection compared to that by the second infection. Of the four cytokine or chemokine, the expression level of IL-10 at 12 hpi induced by the first infection was the highest (up to 80 fold) (Fig. 5A). The expression of IL-10 had a peak at 3 hpi, regardless whether it was a first infection or a second infection (Fig. 5A). Its peaked expression at 12 hpi induced by the first infection was significantly \((P < 0.05)\) higher than that by the second infection (Fig. 5A). The expression pattern of IL-1β induced by the first infection was similar to that by the second infection (Fig. 5B). However, the expression levels of IL-1β was induced significantly \((P < 0.05)\) higher by the first infection compared to that by the second infection (Fig. 5B). Chemokine CXCL-10 was induced most at 24 hpi by the first infection (Fig. 5C), which was significantly \((P < 0.05)\) higher than that by the second infection. The expression of TNFα was induced the most at 12 hpi by the first infection, which was significantly higher than that by the second infection (Fig. 5D).

3.8. Expression kinetics of signaling proteins at different time points

Overall, the expression of the four signaling proteins identified by the screen at various time points was significantly \((P < 0.001)\) more induced by the first infection compared to that by the second infection. The expression of cadherin EGF LAG seven-pass G-type receptor 1 peaked at both 3 and 24 hpi, with significantly \((P < 0.05)\) higher expression induced by the first infection compared to that by the second infection after infection had peak at 12 hpi, with a significantly \((P < 0.05)\) higher expression level induced by the first infection compared to that by the second infection (Fig. 6F).
expression of the lymphokine-activated killer T-cell originated protein kinase also peaked at both 3 and 12 hpi after the infection, regardless whether the infection time was once or twice (Fig. 6D). However, its highest expression was observed at 12 hpi after the first infection (Fig. 6D).

3.9. Expression kinetics of lysozyme c at different time points

The expression of lysozyme c identified by the screen at various time points was significantly ($P = 0.026$) more induced by the first infection compared to that by the second infection. Its expression after the first and second infection peaked at 24 and 12 hpi, respectively (Fig. 7), with significantly ($P < 0.05$) higher expression induced by the first infection at both time points.

3.10. Serum lysozyme activity at different time points post infection

Serum lysozyme activity at various time points after the first infection was significantly ($P = 0.005$) higher than after the second infection (Fig. 8). Its activity peaked at 24 hpi after the first infection, whereas its activity peaked at 12 hpi after the second infection. The serum lysozyme activity at 24 hpi after the first infection was significantly ($P < 0.05$) higher that that after the second infection (Fig. 8).
3.11. In vitro effect of serum on cell proliferation of A. hydrophila

At 28 days post infection, serum of channel catfish infected with A. hydrophila exhibited significantly ($P < 0.05$) higher inhibitory effect on the cell proliferation of A. hydrophila compared to the serum of TSB treated control fish (Fig. 9A), regardless whether the fish was infected once or twice. Serum of TSB treated control fish also significantly ($P < 0.05$) inhibited the cell proliferation of A. hydrophila (Fig. 9A). When serum samples of fish were heat-inactivated, no inhibitory effect on cell proliferation was observed (Fig. 9B).

4. Discussion

Using SSH technique, 28 unique ESTs were identified from a total of 96 clones, of which 8 were confirmed to be slightly but significantly up-regulated by the second infection at 6 hpi compared to the first infection. Of the eight ESTs, two Xba elements (XbaI element 5 and XbaI element 7) were up-regulated greater than 6-fold by the second infection. In channel catfish, Xba elements were A/T-rich tandem repetitive non-coding sequences [31]. Non-coding RNA sequences play important roles in regulating transcription of protein-coding genes [32], including immunity-related genes [33]. Transcriptome analysis of the host response to severe acute respiratory syndrome coronavirus (SARS-CoV) infection across four founder mouse strains has revealed differential expression of 500 ncRNAs during infection [34], indicating that differential expression of ncRNAs is widespread in response to infection. Taken together, these results suggest that the overexpression of ncRNAs such as Xba elements might play important roles in immunity.

Expression kinetics studies at 3, 6, 12, 24, and 48 hpi revealed that the 8 ESTs were significantly more up-regulated by the first infection, with a major peak at 3 hpi. Therefore, 3 hpi was chosen to screen the 96 selected genes. Of the 96 genes selected from literature, 19 were found to be significantly induced by A. hydrophila at 3 hpi compared to that by TSB, regardless the infection time (first infection or second infection). The fact that SSH failed to identify these genes could be due to the following reasons. Firstly, we used cDNAs from the second infection as tester in an attempt to identify genes that are more up-regulated in adaptive response, which might have contributed to the failed discovery of other genes. Secondary, we chose 6 hpi instead of 3 hpi as the time point for SSH which might have contributed to the failed discovery of the known immunity-related genes identified by the screen. Nonetheless, SSH identified differentially expressed genes that were previously reported to be up-regulated by bacteria infections, including TLR20 and NADH dehydrogenase, both of which were reported to be up-regulated in channel catfish by Edwardsiella ictaluri infection [24,26]. In addition, SSH also identified genes that were previously not reported to be induced by infection in channel catfish, such as Xba elements, prolactin, and inward rectifier potassium channel. Prolactin, a peptide hormone that shares many properties with cytokines [35], plays an important role in immunity by inducing the expression of the genes encoding the major phagocyte NADPH oxidase components and ROS production in fish macrophages via the JAK2/Stat/IRF-1 signaling pathway [36,37]. Macrophages have two types of potassium channels: inwardly rectifying potassium

Fig. 5. Transcriptional kinetics of the four cytokine or chemokine in the anterior kidney of channel catfish at different time points post exposure to A. hydrophila once or twice. A: IL-10; B: IL-1β; C: chemokine CXCL-10; D: TNFα. Data are presented as means ± S.D. from three replicates. Differences were considered statistically significant between treatments when $P$ value $< 0.05$. 

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[Image]: A, B, C, D graphs showing transcriptional kinetics of different cytokines or chemokines over time.
channel and voltage-gated potassium channel [38,39]. Potassium channel activity is required for the induction of nitric oxide and respiratory burst response in activated macrophages in goldfish (Carassius auratus) [40]. Of the 96 genes selected from literature, 19 were found to be significantly induced by A. hydrophila at 3 hpi, including four TLRs.

Expression kinetics study revealed that the expression pattern of TLR2, TLR3, or TLR21 after the second infection was different from that after the first infection. However, TLR5 exhibited similar expression patterns after the first and the second infections, with significant peaked up-regulation at 3 hpi. TLRs are evolutionarily conserved receptors that function in innate immunity through

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**Fig. 6.** Transcriptional kinetics of the four signaling proteins in the anterior kidney of channel catfish at different time points post exposure to A. hydrophila once or twice. A: Cadherin EGF LAG seven-pass G-type receptor 1; B: Arginine deiminase type II; C: Very large inducible GTPase 1; D: Lymphokine-activated killer T-cell originated protein kinase. Data are presented as means ± S.D. from three replicates. Differences were considered statistically significant between treatments when P value < 0.05.

**Fig. 7.** Transcriptional kinetics of lysozyme c in the anterior kidney of channel catfish at different time points post exposure to A. hydrophila once or twice. Data are presented as means ± S.D. from three replicates. Differences were considered statistically significant between treatments when P value < 0.05.

**Fig. 8.** Lysozyme activity of channel catfish serum at different time points post exposure to A. hydrophila once or twice. Data are presented as means ± S.D. from three replicates. Differences were considered statistically significant between treatments when P value < 0.05.
recognition of the conserved pathogen-associated molecular patterns (PAMPs) of an invading pathogen and eliciting inflammatory and immune responses [41]. The best characterized ligand that TLRs recognize include: (1) lipoproteins by TLR2; (2) dsRNA by TLR3; (3) lipopolysaccharide (LPS) by TLR4; and (4) bacterial flagellins by TLR5 [42]. The recognition of PAMPs by TLRs will trigger an intracellular signaling cascade which activates signaling molecules such as cytokines, chemokines, interferons (IFNs), and co-stimulatory molecules that aid in the development of the immune response [43]. In addition to significant up-regulation of TLR5 at 3 hpi, four signaling proteins were also found to be significantly up-regulated at 3 hpi after the first infection or the second infection, including cadherin EGF LAG seven-pass G-type receptor 1, very large inducible GTPase 1, arginine deiminase type 2, and lymphokine-activated killer T-cell originated protein kinase. At 3 hpi, the expression of the four cytokine or chemokine also peaked, although the expression of IL-1β and chemokine CXCL-10 after the first infection were the highest.

When the expression levels of the total 27 genes at each time point after the first infection were compared to each other, the expression levels of the two XbaI elements were up-regulated the most (~300 fold). At 6 hpi, the expression levels of transferrin and IL-1β were the highest. At 12 hpi, the expression levels of IL-10 and chemokine CXCL-10 were the highest. At 24 hpi, the expression levels of XbaI element 5 and XbaI element 7 were the highest. At 48 hpi, transferrin had the highest expression. IL-1β is an important early response pro-inflammatory cytokine that mediates immune regulation in both innate and adaptive immunity [44]. When IL-1β was over-expressed in common carp (Cyprinus carpio), the macrophage functions such as production of superoxide anion and phagocytosis were significantly stimulated [45]. IL-10 is reported to be an anti-inflammatory cytokine that plays a crucial role in the regulation of inflammation by down-regulating expression of other cytokines such as IL-1β [46]. In consistent with previous report, we also observed the lowest expression level (<10 fold) of IL-1β at 12 hpi when IL-10 was expressed the highest (~60 fold). Significant up-regulation of hepcidin and transferrin has been reported in channel catfish after infection with E. ictaluri or A. hydrophila [22,30]. In consistent with previous reports, this study also revealed that hepcidin and transferrin were induced by infection with A. hydrophila. Taken together, these results suggest that transcriptional regulation of immune genes plays important role in the immunity of channel catfish against bacterial infections.

When the expression levels of the 27 genes at different time points after the first infection were compared to that after the second infection, significantly higher expression levels of these genes were induced by the first infection. Lower transcriptional level of genes in rainbow trout (Oncorhynchus mykiss) re-infected with Yersinia ruckeri O1 at 35 days post the first infection has been previously reported [47]. Taken together, these results suggest that adaptive immunity might play an important role in the defense against the re-infection. Significantly higher bactericidal activity of serum samples at 28 days post infection with A. hydrophila was observed in this study when compared to that of fish serum treated only with TSB. In addition, peaked expression and activity of lysozyme after the first infection was at 24 hpi, whereas that after the second infection was at 12 hpi, further suggesting that adaptive immunity in fish must have developed, therefore enabling faster production of lysozyme c to kill the bacteria.

5. Conclusions

In summary, 96 clones were isolated from the SSH library. Of the 96 clones, 28 ESTs were obtained, of which 8 were confirmed to be slightly but significantly more up-regulated by the twice exposure at 6 hpi. Expression kinetics studies at 3, 6, 12, 24, and 48 hpi revealed that the 8 ESTs were significantly more up-regulated by the first infection, with a major peak at 3 hpi. Of 96 genes that were reported to be up-regulated by bacterial infections in literature, 19 were found to be significantly induced by A. hydrophila at 3 hpi. The 19 genes belonged to the following five main categories: 1) toll-like receptor (TLR2, TLR3, TLR5, TLR21); 2) antimicrobial peptide (NK-lysin type 1, NK-lysin type 2, NK-lysin type 3, cathepsin D, transferrin, hepcidin); 3) cytokine or chemokine (interleukin-1β, interleukin-10, tumor necrosis factor α, chemokine CXCL-10); 4) signaling proteins (cadherin EGF LAG seven-pass G-type receptor 1, very large inducible GTPase 1, arginine deiminase type 2, lymphokine-activated killer T-cell originated protein kinase); 5) lysozyme (lysozyme c). Significantly (P < 0.05) higher bactericidal activity of channel catfish serum at 28 days post the first infection compared to the fish serum treated with tryptic soy broth was observed in this study. Peaked expression of lysozyme c and peaked lysozyme activity after the first infection was at 24 hpi, whereas that after the re-infection was at 12 hpi. Taken together, our results suggest that both innate and adaptive immunity were involved in the defense of catfish against the re-infection of A. hydrophila.

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Appendix A: Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.08.027.

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