SHORT SEQUENCE REPORT

Proteomic analysis of head kidney tissue from high and low susceptibility families of channel catfish following challenge with Edwardsiella ictaluri

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Abstract A study was performed to compare proteomic profiles of channel catfish from families with high and low susceptibility to Edwardsiella ictaluri following an immersion challenge. Total protein was isolated from head kidney samples, collected at 2 and 6 h postexposure, and analyzed by 2-D-gel electrophoresis coupled with peptide mass fingerprint analysis by matrix-assisted laser desorption/ionization and time of flight tandem mass spectrometry. Comparisons were made between proteomic profiles from infected and uninfected fish from high and low susceptibility families. Heat shock protein 90-beta, from the high susceptibility infected family, and fructose-1,6-bisphosphatase 1-like protein, from the low susceptibility infected family, were identified at 2 h postexposure. Rab-11-like protein (low susceptibility infected family), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (low susceptibility infected family), were identified at 2 h postexposure. Rab-11-like protein (low susceptibility infected family), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (low susceptibility infected family), glyceraldehyde-3-phosphate dehydrogenase (high susceptibility infected family), and ATP synthase beta subunit (low susceptibility uninfected family) were identified at 6 h postexposure. Four proteins, heat shock protein 90-beta, Rab-11, glyceraldehyde-3-phosphate dehydrogenase (high susceptibility infected family), and ATP synthase beta subunit (low susceptibility uninfected family) have activities involved in macrophage function or cellular stress responses, while the other two have functions associated with cellular energy production and metabolism. These results demonstrate the potential for use of proteomic techniques in channel catfish research.

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The identification of genetic markers for resistance to viral and bacterial diseases for use in selective breeding of channel catfish (Ictalurus punctatus) is of great interest to the catfish aquaculture industry. This research has focused primarily on gene expression studies following bacterial challenge in families of catfish, using primers and probes designed to detect expression of genes that are known to be associated with immune function [1–4]. More recently, microarray analysis has been used to detect changes in gene expression following bacterial challenge of channel catfish [5,6]. While these studies are very useful, they are based on measurements of mRNA expression, which may not accurately reflect protein expression. Proteomic analysis can identify actual proteins that are uniquely expressed or upregulated following bacterial challenge in resistant or susceptible fish and can potentially lead to the identification of novel genes associated with disease resistance.

Unlike species such as Homo sapiens, Mus musculus, or Drosophila melanogaster, whose entire genome has been sequenced, making available 468,000 (H. sapiens), 247,000 (M. musculus), and 111,000 (D. melanogaster) protein sequences, the availability of genomic sequence information from channel catfish is limited to only a little over 1700 protein sequences (National Center for Biotechnology Information [NCBI]), US National Library of Medicine, Bethesda, MD). Peptide identification is limited by this lack of published information and, in most cases, must be determined based on homology to protein sequences from other piscine species, such as Danio rerio and Fugu rubripes, for which the near complete genomic database is available (NCBI). With this in mind, a study was performed to compare proteomic profiles of channel catfish families with high and low susceptibility to Enteric Septicemia of Catfish (ESC) following an immersion challenge with virulent Edwardsiella ictaluri in order to assess the usefulness of applying 2-D gel electrophoresis coupled with peptide mass fingerprint analysis by matrix-assisted laser desorption/ionization and time of flight tandem mass spectrometry (PMF MALDI-TOF-MSMS) to the identification of proteins associated with disease susceptibility.

One week prior to challenge, two families of juvenile USDA303 channel catfish (male and female, average weight 54.6 ± 1.90 g) were stocked in 76 L aquaria with a single-pass flow-rate of 8 L min⁻¹ with aeration, at 26 °C, and on a 14:10 light:dark photoperiod. One family demonstrated high susceptibility to E. ictaluri in previous studies, as determined by cumulative mortality rates of 76.7 ± 16.7% following immersion challenge, and one family of fish that demonstrated low susceptibility in previous studies, with cumulative mortality rates of 3.3 ± 0%. Three tanks per family were stocked for each time point, 2 and 6 h. These time points were chosen for analysis as E. ictaluri is known to invade internal organs as early as 15 min following exposure [7]. Four additional tanks per family were also stocked, two serving as exposed controls and two as non-exposed controls.

Bacterial challenge was performed according to Wolters and Johnson [8] with approximately 1 × 10⁷ CFU mL⁻¹ final concentration of virulent E. ictaluri. At 2 and 6 h postexposure (PE), three fish from each tank were removed and euthanized in a 200 mg L⁻¹ solution of MS-222 (tricaine methanesulphonate) (Western Chemical Inc., Ferndale, WA). Anterior head kidney tissue, a primary organ involved in immune function in fish [9], was removed and flash-frozen in liquid nitrogen. Samples were stored at –80 °C until protein extraction could be performed.

The three head kidney samples from each tank were pooled and protein was extracted using the ReadyPrep™ Protein Extraction Kit (Total Protein) (Bio-Rad Laboratories, Hercules, CA) as per manufacturer’s instructions. Protein concentration was determined using the RC DC Protein Assay Kit I (Bio-Rad Laboratories) as per manufacturer’s instructions. 2-D gel electrophoresis was performed as per manufacturer’s instructions, using ReadyPrep™ 2-D Starter Kit and 11 cm ReadyPrep™ IPG Strips, pH 4.0–7.0 (Bio-Rad Laboratories). A total of 400 μg of protein was loaded onto the strips, which were run according to the manufacturer’s instructions on a Protean IEF Cell (Bio-Rad Laboratories). The second dimension was run on Criterion 12.5% Tris–HCl, 1.0 mm Precast Gels (Bio-Rad Laboratories) as per manufacturer’s instructions in a Dodeca Cell (Bio-Rad Laboratories) at 200 V with cooling provided by an IsoMemp 3013 (Fischer Scientific, Waltham, MA). The gels were fixed with 40% methanesulphonate (tricaine) and stained with SYPRO Ruby Protein Gel Stain (Bio-Rad Laboratories). Gels were scanned on a VersaDoc Imager (Bio-Rad Laboratories) and analyzed with PDQuest™ 2D Analysis Software, version 7.3.1 (Bio-Rad Laboratories). After comparison of gels from resistant and susceptible families, infected and uninfected, at 2 and 6 h post infection, proteins that were uniquely expressed at each time point in all three resistant or susceptible groups were excised and submitted for trypsin digest, PMF MALDI-TOF-MSMS, and protein identification at the Life Sciences and Biotechnology Institute, Mississippi State University.

A total of 56 proteins were excised from the gels and analyzed. Of these only 6 proteins were definitively identified with high similarity to proteins from D. rerio (Table 1). Four of the identified proteins have activities tied to macrophage function or cellular stress responses. Two other proteins have functions associated with cellular energy production and metabolism.

Fructose-1,6-bisphosphatase 1, identified in the low susceptibility family, is a ubiquitous house-keeping protein that catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate, a key component of carbohydrate metabolism [10]. It is unclear what role it might have in disease resistance. Interestingly, the expression of the gene encoding this protein was identified as upregulated in microarray analysis of blue and channel catfish liver following challenge with E. ictaluri [6].

Heat shock protein 90-beta subunit (HSP 90-β) targets chaperoned peptides for antigen presentation, and its activity leads to peptide-independent activities such as secretion of inflammatory cytokines, induction of iNOS, production of nitric oxide, secretion of chemokines, and translocation of NF-κβ into the nucleus, an event that mediates many of the events already listed [11]. Heat shock protein 90 has also been shown to be involved in Toll-like Receptor (TLR) 4/Lipopolysaccharide (LPS) trafficking and targeting of bacterial LPS to the Golgi apparatus [12]. The identification of this protein in the family with high susceptibility suggests that the inflammatory response to bacterial invasion in these fish is contributing to their high level of...
susceptibility to ESC. The classic symptoms of ESC, caused by *E. ictaluri*, such as rapid onset of septicemia, bloody ascites, hemorrhage and necrosis in multiple organs, skin lesions, and lesions of the cranial foramen [13], are characteristic of a strong inflammatory response.

Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, epsilon (14-3-3 polypeptide (D. rerio)) identified from the low susceptibility family, functions as a mediator for apoptosis in response to nitric oxide induced stress [20]. The presence of this protein in the resistant family of catfish suggests an enhanced ability to endure or modulate the stress induced during an intense immune response to *E. ictaluri* challenge, making them more resistant to disease.

Rab-11, identified in the low susceptibility family, is a small GTPase that regulates the removal and recycling of molecules from phagosomes of macrophages [15]. This protein has been reported to enhance macrophage phagocytosis [16], to modulate expression of transferrin receptors from endosomes [17] and MHC class I-related Fc receptors trafficking [18], and to regulate recycling of CD44 from the Salmonella-containing vacuole in HeLa cells [19], all of which are important functions in a successful immune response.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), identified from the high susceptibility family, functions as a mediator for apoptosis in response to nitric oxide induced stress [20]. The presence of this protein in high susceptibility fish may again indicate that extreme stress resulting from the immune response to bacterial invasion in these fish may play a role in their susceptibility to disease.

ATP synthase beta subunit was identified from the low susceptibility uninfected family. This protein is known to be primarily associated with cellular energy production [10] and has been shown to bind enterostatin, a peptide that regulates fat intake in rats [21]. It has also been shown to be involved in regulation of transcription and translation of tyrosine-phosphorylated cytosolic and nuclear proteins involved in the mechanisms of apoptosis during macrophage differentiation [22]. As with Fructose-1,6-bisphosphatase 1, it is unclear what role, if any, this protein might have in disease susceptibility. It is also unclear why both of these were not present in all groups, as they have house-keeping functions and should be ubiquitous. The expression of both of these proteins may be influenced by the eating behavior of the fish, which is highly variable. Studies have shown that one route of entry of *E. ictaluri* into the host is by traversal of the intestinal epithelium [7]. Fish that do not eat well may be less susceptible to ESC infection. A study by Lovell et al. [23] reported that starved fish were more resistant to *E. ictaluri* infection than were fish that were fed regularly.

This is the first published report of proteomic profile analysis of channel catfish using 2-D gel electrophoresis coupled with PMF MALDI-TOF-MSMS. Although these results are limited, they do demonstrate that this technique can be applied successfully to channel catfish and that proteomic analysis of protein expression profiles in response to bacterial challenge has the potential to contribute significantly to the search for genetic markers for disease resistance despite the limited genomic sequence data available. This study will be expanded to include a protein range of 3–10, and a wider range of time points will be analyzed in order to get a more complete picture of the differences in physiological reactions of these two families of fish to challenge with *E. ictaluri*. Gene expression studies of the identified proteins will contribute to the identification of selective markers for use in the selective breeding of channel catfish. Sequencing of the channel catfish genome is progressing rapidly, and as more sequence data becomes available, proteomic profiling coupled with PMF MALDI-TOF-MSMS will be an increasingly powerful research tool in channel catfish studies.

### References


<table>
<thead>
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<th>Table 1</th>
<th>Protein identification showing time sampled in hour post infection (PE), catfish family, protein name, NCBI accession number, protein molecular weight (MW), and isoelectric point (pI).</th>
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