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Missing the target: DNAk is a dominant epitope in the humoral immune response of channel catfish (*Ictalurus punctatus*) to *Flavobacterium columnare*

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ABSTRACT

Vaccination remains a viable alternative for bacterial disease protection in fish; however additional work is required to understand the mechanisms of adaptive immunity in the channel catfish. To assess the humoral immune response to *Flavobacterium columnare*; a group of channel catfish were first immunized with *F. columnare* LV-359-01 cultured in iron-depleted media, before being challenged with wild type *F. columnare* LV-359-01. The immunization protocol did not confer increased protection against *F. columnare*; however both control and immunized responders generated serum and skin IgM antibodies against *F. columnare* proteins. Western blot analyses of individuals from both groups showed that IgM antibodies were generated to the same 70 kDa extracellular protein, which was identified to be the bacterial chaperonin protein DNAk. Antibodies generated were cross reactive to DNAk proteins found in other gram negative bacteria. Our data suggests that DNAk is the dominant epitope in the channel catfish B-cell response to *F. columnare*.

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1. Introduction

Flavobacterium columnare, the etiological agent of columnaris disease, is a ubiquitous and opportunistic fish pathogen that is highly transmissible and causes widespread mortality throughout the aquaculture industry [20,48]. Strategies to combat columnaris infections, going back nearly a century, have included lowering rearing density, salt baths, acid baths, and chemical therapeutants [54]. However, these approaches have failed to reduce columnaris disease incidence, as they are largely reactive measures implemented after the onset of disease [54]. More recently, the effective management of columnaris has been further constrained by ever evolving regulatory burdens associated with new and existing treatment compounds, and emerging concerns over antibiotic resistance [10]. Immunization-based preventative strategies remain a viable and promising alternative for bacterial disease protection in fish; and there has been no shortage of work done to develop and to evaluate immunogens for use in vaccination against columnaris disease [29,30,34–36].

Despite tremendous research effort in this area, findings regarding efficacy have been mixed, and are likely due to a number of factors including the parental strain of the isolate used [29], species and age of the immunized fish, the preparation/engineering of the vaccine candidate, and disparities in vaccination doses and durations. Nevertheless, it is becoming increasingly apparent that the use of live modified or attenuated columnaris vaccines may offer potential for use in aquaculture settings. Generally, and in contrast to killed bacterins, advantages of live attenuated vaccines include the stronger induction of both humoral and cell mediated immunity, require smaller doses to induce a robust and long-lasting immune response, entail minimal to no adjuvants, and feature a more natural means of exposure (i.e., immersion or oral routes); which would be more amenable to the large-scale immunizations required in settings of aquaculture [43].

While our level of understanding is growing, expansive knowledge gaps remain; especially lacking is a comprehensive view of species-specific immune responses to immunogens, and how these responses govern host protection. One such species in

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particular need of further study is the channel catfish, the predominant warmwater aquaculture species in the United States, and a highly susceptible host to columnaris disease. Due to its commercial importance, the channel catfish is one of the better-studied immune models among teleost fish with fundamental discoveries on their immunological form and function dating back more than three decades [1,8,15,22,25–28]. Recently, the rate of discovery has been accelerated in the catfish model system with the rise of next generation sequencing platforms. Transcriptomic studies of target mucosal tissues in the context of experimental disease challenges have helped dissect the initial stages of columnaris pathogenesis [4,31,39,42,47]. Even so, insight into the cellular and humoral effectors, at the level of protein, that modulate vaccine success is needed to make meaningful improvements in columnaris prevention.

Previously, we demonstrated that the *F. columnare* isolate LV-359-01 grew poorly under iron-limited conditions and was significantly less virulent as compared to when cultured under normal conditions [5]. Building upon these previous findings, and borrowing from prior work in *Flavobacterium psychrophilum* where an isolate negatively impacted by iron deprivation showed potential as a vaccine candidate [2], here, we set out to evaluate the utility of iron restriction as a means by which to develop a putative vaccine for columnaris disease. Unexpectedly, we failed to confer protection using an immersion-based immunization protocol with the iron-restricted isolate. However, further investigation revealed a robust and highly specific antibody response, displayed by both serum and skin, to a single *F. columnare* protein identified to be a ~70 kD heat shock protein, orthologous to *Escherichia coli* DNAk. In the following report, we describe the significance of this immunodominant protein and discuss its putative role in hindering appropriate and protective immune responses in the channel catfish host.

2. Materials and methods

2.1. Bacterial culture and fraction preparations

Different bacterial isolates were utilized throughout the study; *F. columnare* LV-359-01 and LSU-066-04, *Escherichia coli* ATCC 25922, *Aeromonas hydrophila* 0702 and *Edwardsiella ictaluri* S97-773. All isolates were retrieved from frozen glycerol stocks that were stored at −80 °C and streaked onto *F. columnare* Growth Medium (FCGM) [9,14]; or tryptic soy agar with 5% sheep's blood (ThermoFisher, Waltham, MA). After 48 h of growth at 28 °C, isolates were dislodged from the agar using a sterile loop and inoculated into 50 mL of FCGM or brain-heart infused medium (Becton Dickinson, Sparks, MD) and incubated in broth at 28 °C for 24 h. The bacterial suspensions were then spun using an Eppendorf 5810R centrifuge at 6320*g* for 20 m. The extracellular portion (ECP) was poured off into a new tube and spun again for an additional 10 m. The bacterial pellets were resuspended in 5 mL of 1× PBS and sonicated on a setting 7 for 5 m in a Powersonic Model (Crest Ultrasonics, Trenton, NJ). The ECP was poured off and concentrated using 3KMWCO Amicon Ultra-15 centrifugal filter units (EMD Millipore, Billerica, MA). All bacterial pellet and ECP fractions had a 5% (v/v) protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) added prior to the total protein concentration was estimated using the Coomassie Plus assay kit (Pierce/ThermoFisher, Waltham, MA) with bovine serum albumin (Sigma Aldrich, St. Louis, MO) as the standard. Absorbance was read at a wavelength of 595 nm with a BioTek Synergy H1 plate reader operating under Gen5 software (Winooski, VT). The pellets and ECPs were dispensed as 0.1–1 mL aliquots and kept at −20 °C and thawed as needed and then at 4 °C for no more than 2 weeks. Genbank accession numbers for chaperone protein DNAk: *Escherichia coli* (P0A6Y8), *Flavobacterium columnare* (WP_014165528), *Aeromonas hydrophila* (KLV44233), *Edwardsiella ictaluri* (C5B7L7).

2.2. Immunization and bacterial challenge

Fingerling channel catfish were reared at the Harry K. Dupree Stuttgart National Aquaculture Research Center in Stuttgart, Arkansas, USA. Two hundred fish each, average weight 5 g, were stocked into two 300 L tanks that received filtered well water and aeration from submerged air stones. Fish were offered pelleted catfish feed (35% protein, 2.5% fat; Delta Western, Indianola, Mississippi). There were four groups of channel catfish, non-challenged and non-immunized (N); immunized and non-challenged (I); non-immunized and challenged (C); immunized and challenged (IC) (see Results). To immunize fish through bath immersion, the water level was lowered to 100 L and the fish were exposed to 1 L of *F. columnare* isolate LV-359-01 cultured in iron-depleted media under static conditions for 30 m with a calculated dose of 1.4×10^8 CFU/mL using a drop plate method [5,18]. After fourteen days, control and immunized groups were challenged through bath immersion with wild type LV-359-01 *F. columnare* with a calculated dose of 2.81×10^8 CFU/mL using a drop plate method [18]. For the challenge three replicates of 50 fish (250 g of biomass/tank) were stocked into 18 L tank containing 10 L of filtered well water. Water was provided through the ultra-low-flow water delivery system at a rate of 30 mL/min [3,5]. Fish were not fed on the day of immunization, or on the first day after bacterial challenge. An additional tank containing 50 fish was not challenged and was used as a negative control. Fish were observed twice daily at which time any moribund fish were promptly removed.

Animal care and experimental protocols were approved by the Harry K. Dupree Stuttgart National Aquaculture Research Center Institutional Animal Care and Use Committee and conformed to Agricultural Research Service Policies and Procedures 130.4 and 635.1.

2.3. Blood and skin explant sampling

Twenty fish from the two challenged groups or the non-challenged controls were maintained (post challenge) for forty days in 18 L recirculating aquaria. Fish were then anaesthetized and blood was first collected using a 21-gauge needle from the caudal vein and allowed to clot overnight at 4 °C. Blood samples were centrifuged at 10000*g* for 10 m using an Eppendorf Minispin; the serum (25–100 μL) was removed and stored at −20 °C until needed. After blood collection we proceeded with the preparation of excised skin for tissue culture as described [50,51]. Briefly we wiped down the surface of the skin on both sides three times with a 70% ethanol solution. Then using sterile instruments we dissected two 1.5 mm^2 skin pieces (along the lateral line), washed them with empty Leibovitz's L-15 medium (ThermoFisher, Waltham, MA), and placed them into 400 μL of complete Leibovitz's L-15 medium (10% FBS, penicillin/streptomycin, amphotericin, gentamicin) in a 48-well plate at 28 °C for 24 h. The next day the skin explant tissue culture medium was removed and stored at −20 °C until needed.

2.4. ELISA

We used an indirect ELISA to measure the serum and skin-based IgM antibodies as described with some modifications [37]. Immulon 2HB 96-well plates (ThermoFisher, Waltham, MA) were coated with 100 μl of 10 μg/mL of sonicated *F. columnare* cell pellet in a sodium bicarbonate buffer. Plates were then rinsed three times with 1× PBS with 0.05% Tween-20 (PBST) and then incubated for