Flavobacterium columnare protease knockout shows potential for vaccine development against columnaris disease

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Abstract

Flavobacterium columnare is a gram negative, slender rod bacterium that primarily infects salmonids in hot or cold water systems. Columnaris disease is symptomatically represented by long "straw-like" colonies on mucosal surfaces of fish species. Fish infection trials have been conducted to understand infectivity however, the cause of pathogenesis is still under investigation. These strictly aerobic bacteria require a unique apparatus called the Type Nine Secretion System (T9SS) to infect fish. The T9SS is involved in both secretion and motility. Essential T9SS genes characterized include *porV* and *gldN* genes which have been shown to be essential for virulence of *F. columnare* in fish. The *porV* and *gldN* effects on the T9SS have been studied extensively to understand their contributions pathogenesis of columnaris disease. The aim of the current study is to understand specific cargo of the T9SS and its effect on virulence. Using a zebrafish mortality assay and silver stain gel techniques, we examined the effect of spent media (containing proteins secreted by the bacterium) from both wild type *F. columnare* and strains with knockouts in genes coding for multiple proteases thought to be secreted by the T9SS. Results suggest the strains lacking specific metalloproteases appear to be reduced in virulence, while several other protease knockouts show wild-type virulence. Continued genetic manipulation of *F. columnare* strains and virulence testing in our zebrafish model should shed more light on the mechanisms by which *F. columnare* causes disease in fish and may lead to the identification of vaccine candidates.

Introduction

The fish hatchery industry provided 1.7 million jobs and added 60.8 billion dollars to the GDP in the United States (NOAA 2016). Columnaris disease, whose causative agent is *F. columnare*, substantially hinders the economic output of this industry. Since its discovery and characterization in 1922, columnaris disease has been identified to infect fish in both hot and cold water systems. In 1999, *F. columnare* was divided into three genomovars based on 16s RNA, RFLP(restriction fragment length polymorphism), and DNA-DNA-hybridization (Declercq et al. 2013). The strain of interest, MSFC4 belongs to genomovar one and is found to be more closely related to cold water systems commonly found in WI. Pathologically, columnaris disease affects salmonids and finfish by mechanism of attachment to mucosal membranes of gills and skin. *F. columnare* infections can occur in fish that survive the infection and can spread roughly $5x10^3$ of viable bacteria to other fish in the system(Declercq et al. 2013). In an artificial fish hatchery, this can be detrimental to the fish population because maintenance of tanks and removal of dead/decaying fish may be difficult to accomplish in a timely manner on the infection timeline. The pathogenesis of Flavobacterium can be characterized by its use of a Type Nine Secretion System (T9SS). This mechanism of virulence allows for harmful exotoxin proteins to pass through the layers of membranes and aid in the transmission of fish disease (Li et al. 2017). Several T9SS excretory proteins standout as potential candidates for vaccine synthesis in the prevention of columnaris disease.

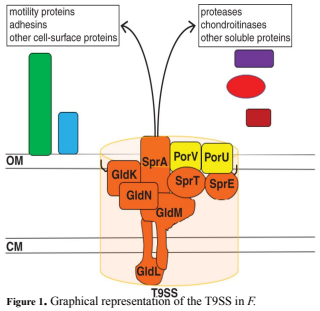

Figure 1. Graphical representation of the T9SS in *F. columnare*. Excretory proteins in the boxes above indicate possible virulent proteins for future research. This graph was generated by Li et al. 2017.

Strain	Resulting Gene Knockout
MSFC4	Wildtype
gldN	Deletion of gliding motility gene in T9SS
FCB 45	Δpeg.762-763 (C6N29_11545 and C6N29_11550) tandem metalloproteases.
FCB 54	Deletion of Δ(C6N29_11545 and C6N29_11550) ΔC6N29_05800 (M4 endopeptidase)
FCB 117	Deletion of 9 genes encoding Non-CTD proteases that still appear to rely on T9SS for secretion.
FCB 123	ΔcslA ΔcslB; chondroitin sulfate lyases
FCB 145	△8 CTD protease genes (lacking 8 T9SS-CTD protease-encoding genes)

Table 1. Table of strains analyzed for virulence testing in zebrafish.

Methods

F. columnare was plated on TYES (Tryptone Yeast Extract Salt) agar and incubated at 28℃ (Gao & Gaunt 2016). After 48 hours of incubation, single colonies were transferred to 5 ml tubes containing TYES broth. These tubes were placed in a shaker to incubate at 180 rpm and 28℃. Once the samples incubated for 24 hr, 1 mL aliquots were deposited in 100 ml of TYES broth in Klett flasks to incubate until an optical density measured by the Klett meter of 120-150 obtained. Previous literature suggested that a Klett reading of 50-60 or "mid-log phase" would produce significant results, however the MSFC4 strain in this study did not yield similar results at that Klett. Once the desired Klett was reached, the samples were transferred to 50mL conical centrifuge tubes and placed in the centrifuge at 6,500 rpm for 10 minutes at 15℃. The centrifuged samples were filtered using a .45 µm PES gravity filter and retained in a 1.0 liter flask on ice until used for zebrafish assays. The zebrafish assay was conducted using 2-4 zebrafish per beaker of strain spent media. The spent media was allowed to heat to ambient temperature before placing fish in the beaker. As indicated in the results below, the spent media from the higher Klett readings was immediately toxic to the fish. In order to maintain the longevity of the fish, the spent media was diluted to a concentration similar to Klett 50-60 measurements. The based on lethality of the strain in comparison to "standardized" survivability of MSFC4 wt and gldN knockouts. These two strains served as positive and negative controls to compare the targeted strains. Sample protein concentration was measured using a BCA assay, however results were inconsistent. As another measure of protein, samples were run on a Bio-Rad THX 4-20% gradient SDS-PAGE gel and stained using the ThermoFisher Silver Stain kit. After the zebrafish trials, all fish ,whether alive or dead, were placed in a 2:100 MS222 solution as per IACUC ethical standards.

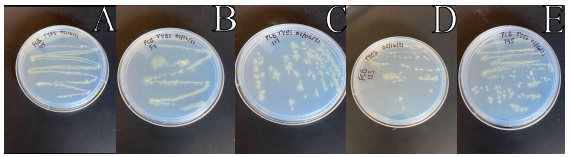

Figure 2. TYES plated strains used in zebrafish assays. A-E. A: FCB 45, B: FCB 54, C: FCB 117, D: FCB 123, E: FCB 145

Results

Strain	MSFC4	FCB 20	FCB 45	FCB 54	FCB 117	FCB 123	FCB 145	gldN
# of fish inoculated	8	12	6	6	6	6	6	8
% survival after 4 hours	12.50%	75%	16.60%	0%	83.30%	16.60%	33.30%	87.50%

Table 2. Quantification of zebrafish survivability with associated strain inoculation. These results are preliminary however they show that strains FCB 20 and FCB 117 have the closest survivability to the gldN knockout. The theoretical survivability for gldN should be 100% however fish physiology as well as ambient temperature and colligative properties of their environment could have led to non-trial related cause of death.

- Fish survivability assay showed that FCB 123, a chondroitinase knockout, was unlikely to be a viable candidate for a vaccine due to significant mortality.
- Excluding external sources of error in the zebrafish assay, FCB 117, a 9 protease non-CTD mutant, and FCB 20, a specific metalloprotease knockout, produced the highest number of surviving fish of the strains tested.
- Oddly, FCB 54, which is a combination knockout of FCB 20 and FCB 45 killed every specimen it inoculated.
- Random external contamination or environmental changes could be the reason for <100% survival for gldN strain inoculation.

Discussion and Future Research

This research was conducted to understand the possible T9SS-secreted proteins involved in the virulence of columnaris disease. One of the key findings of this experiment was the growth conditions of MSFC4 mattered more than for previous strains of *F. columnare*, C#2 and IA-S-4. When grown to a Klett of 50-60, the MSFC4 strain seemed to not produce sufficient concentrations of secreted proteins so show the effects of the various knockouts. Another finding of this research was to show that several protease knockouts did not change the ability of spent media to kill fish. The knockouts of future interest continue to remain associated with proteases and their functions. In order to better understand the molecular properties of the knockouts, reproduction of silver stain assays will need to generate more conclusive data. The purpose of this experiment and research topic is to test strains of MSFC4 on a model organism in order to understand the mechanism in which virulence occurs via the T9SS. In the future, we plan to further quantify the protein concentrations of FCB 20, a knockout isolated to a single gene for a thermolysin protease, based off the results of survivability of FCB 117, a 9 Non-CTD protease knockout. Continuation of this research should shed light on which proteases secreted by the T9SS are important in virulence of *F. columnare* in fish.

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