Biofilm formation, virulence attenuation and comparative genomics of the fish pathogen *Flavobacterium columnare*

by

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Abstract

Flavobacterium columnare is an important bacterial fish pathogen that causes great economic losses in aquaculture. In this dissertation I aimed at characterizing part of its life cycle including the transition between planktonic and biofilm stages and understanding its pathogenicity mechanisms using an avirulent mutant as model. In the first part, I evaluated biofilm formation on common aquaculture substrates. I found that *F. columnare* efficiently formed biofilm on pond liner, flexible PVC, and nets, while plant material prevented *F. columnare* attachment and inhibited cell growth. Biofilm formation on specific substrates was confirmed using Scanning Electron Microscope (SEM). In addition, I evaluated the role of calcium in biofilm formation using a microtiter plate assay, and the results showed that calcium supplementation greatly enhanced the biofilm formation.

To understand the molecular mechanism involve in the transition between planktonic and biofilm stages as well as to characterize the role of calcium in biofilm formation, I used a transcriptome approach to identify Differential Expressed Genes (DEGs) among different life stages (i.e. planktonic and biofilm cells) and different calcium concentrations. Results showed that oxidative stress and nutrient starvation are predominant drivers in biofilm metabolic pathways, and that aerobic respiration is greatly limited during biofilm development. The DEGs under calcium simulation were also evaluated. I identified 175 DEGs (6.30% of genomic protein-coding sequences), which fall into functional categories including iron acquisition, biofilm signaling, T9SS system, and calcium homeostasis. Together, our data suggested that that biofilm is significantly affected by calcium, which seems to serve as a critical signal in controlling bacterial surface adhesion and biofilm formation in *F. columnare*.

Our group had previously patented a modified-live vaccine (a rifampicin-resistant mutant) against columnaris disease. To understand the molecular basis for attenuation, the mutant and its parent strains were sequenced, and comparative genomic analysis was conducted to identify specific point mutations. Sequence-based genome comparison identified 16 single nucleotide polymorphisms (SNP) unique to the mutant. Genes that contain mutations were involved in rifampicin resistance, gliding motility, DNA transcription, toxin secretion, and protease synthesis. I also compared biofilm production between the mutant and the parent strain, and the results showed that the vaccine strain formed biofilm at a significantly lower level than the parent strain.

F. columnare is a genetically heterogeneous species, which is comprised of several genetic groups (i.e. genomovar). In the last chapter, comparative genomic were conducted to further elucidate the genetic diversity behind this species. Three strains representing 3 different lineages within the species were sequenced and compared with 5 additional strains whose complete genome sequences were available. Results showed that all average nucleotide identity (ANI) values between the genomovars were lower than the recommended cut-off point of 95% for species delineation. The pan- and core-genomes were evaluated, and genes unique to each genomovar were retrieved. Our results revealed an extensively diversity within *F. columnare* species, whose genomic relatedness between the genomovars was below the cut-off threshold for species. Therefore, I propose to consider the species *F. columnare* as a species complex. In summary, my results identified critical genes/pathways related to surface colonization, biofilm formation, and pathogenicity of *F. columnare*. In addition, I confirmed the need for describing at least two cryptic species within the *F. columnare* species complex.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

MS	Modified Shieh
RFLP	Restriction Fragment Length Polymorphism
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
CFU	Colony Forming Unit
OD	Optical Density
LPS	Lipopolysaccharide
PCR	Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
ppt	parts per thousand
ppm	parts per million
EPS	Extracellar Polysaccharides
USDA	United States Department of Agriculture
ANI	Average Nucleotide Identity
DDH	DNA-DNA hybridization
AFLP	Amplified Fragment Length Polymorphism
RAPD	Random Amplified Polymorphic DNA
ISR	Intergenic Spacer Region

Chapter 1. Literature review

1.1 *Flavobacterium columnare*

History and taxonomy

Flavobacterium columnare, the causative agent of columnaris disease, is a Gram-negative bacterium that belongs to Phylum Bacteroidetes, Class Flavobacteria, Order Flavobacteriales and Family Flavobacteriaceae (Bernardet and Bowman 2006). It was first discovered in 1922 in Mississippi River and has since been found worldwide in a large variety of fish species (Davis 1922; Declercq et al. 2013).

The taxonomy of *F. columnare* underwent a series of changes since its initial description. In 1922, David observed the bacteria on the external lesion of numerous fresh-water fishes in Mississippi River, and named it *Bacillus columnaris* (Davis 1922). Later, Ordal and Rucker (1944) isolated this organism from brown bullhead (*Ameiurus nebulosus*) and reclassified it as *Chondroccoccus columnaris* based on the fact that the cells were able to produce microcysts and fruiting bodies. In 1945, Garnjobst (1945) proved that the bacterium was indeed devoid of fruiting bodies and reclassified it as *Cytophaga columnaris*. Later on, further description of the genus *Flexibacter* and *Cytophaga* lead to another name change (*Flexibacter columnaris* (Buchanan and Bibbons 1974). Finally, in 1996, Bernardet et al. (1996) restructured the family Flavobacteriaceae and reclassified the bacterium as *Flavobacterium columnare.* In 1999, *F. columnare* was divided into three genomovars based on differences in the 16S rRNA gene supported by DNA-DNA hybridization (Wakabayashi 1999). Recently, LaFrentz et al. (LaFrentz et al. 2014)systematically analyzed and intragenomic heterogeneity in the 16S rRNA genes of the species by increasing the number of strains in the study and further subdivided *F. columnare* into 6 genomovars.

Phenotypic description

F. columnare is a long, non-flagellated bacillus of approximately 0.3 to 0.5 µm in width and 3.0 to 10.0 µm in length (Buchanan and Bibbons 1974) although under adverse conditions, cells can reach up to 50 µm in length (Hawkes 1974). *F. columnare* cells can be covered with a capsule, which has been suggested as a potential virulence factor (Decostere et al. 1999). Colonies of *F. columnare* are flat and rhizoid and have a typical yellow color due to the pigment flexirubin (Bernardet and Bowman 2006). Typical biochemical characteristics for this species include the ability to absorb Congo red, production of catalase, and lack of acid production from hydrolysis of carbohydrates (Buchanan and Bibbons 1974). This bacterium can be selectively isolated from environmental samples by supplementing the culture medium with neomycin and polymyxin B (Griffin 1992). Different colony morphotypes have been described including spreading (rhizoid), soft and non-spreading (rough) types, but only the spreading type is considered virulent (Penttinen et al. 2018).

1.2 Columnaris disease

Host susceptibility

F. columnare can infect and colonize many freshwater fish species although some appear to be more susceptible to columnaris disease than others. Susceptible host species include: channel catfish *(Ictalurus punctatus*), blue catfish (*I. furcatus*), common carp *(Cyprinus carpio*), tilapia (*Oreochromis spp.*), rainbow trout (*Oncorhynchus mykiss*), black bass (*Micropterus salmoides*), brook trout (*Salvelinus fontinalis*), sunfish (*Lepomis incisor*), bluegill (*Lepomis macrochirus*), and eels (*Anguilla rostrata, A. japonica, A. anguilla*) (Plumb 2018). It is speculated that under favorable conditions nearly all fresh water fishes are susceptible to columnaris disease (Wakabayashi 1993). There are several factors that favor the onset and spread

of the disease. For example, injured or mechanical abraded fish are more vulnerable to infection by *F. columnare* than intact fish (Bader et al. 2003). Similarly, stress induced by starvation significantly increases susceptibility to columnaris disease in experimental fish (Shoemaker et al. 2003). Other factors that favor columnaris disease include high organic loads in water (Chowdhury and Wakabayashi 1988), crowded conditions(Suomalainen et al. 2005), excessive handling (Wakabayashi 2006), low dissolved oxygen, and high ammonia and nitrite in water (Farmer et al. 2011).

Clinical signs

Columnaris disease is typically an external infection in where skin and gills are predominately colonized. Specific lesions tend to vary with fish species and with the severity of the infection. Columnaris disease commonly causes extensive necrosis of the gills, which starts at the margins of the filaments and progresses toward the arches. On the body, small lesions start as areas of pale discoloration. These areas increase in size and may become as large as 3-4 cm in diameter, covering as much as 20-25% of total surface area of fish (Austin and Austin 2007). Skin, gills and fins are the most favored sites for bacteria attachment and colonization, however, on acute infections, *F. columnare* could become systemic without obvious pathological signs on external organs (Plumb 2018). In catfish fingerlings, this disease is often associated with the presence of the so-called "saddleback lesion", a symmetrical area of body discoloration flanking the dorsal fin (Cone et al. 1980; Wakabayashi 1980). Morrison et al. (2006) described the morphology of the saddleback and its histopathological effect on the infected tissues in underyearling Atlantic salmon (*Salmo salar*). They found that *F. columnare* cells were mainly restricted to the dermis and underlying muscle of the saddle area. Bullard et al. (2011) investigated the ultrastructure of "saddleback" lesions of diseased channel catfish (*I. punctatus*)

with scanning election microscopy, and described the saddleback area as characterized by epidermal sloughing intermingled with rod-shaped bacteria and cellular debris on strongly ridged, folded dermal connective tissue.

Columnaris disease is characterized by the appearance of grayish-white or yellow erosive lesions involving the skin and fins, the oral cavity, and the gills. Skin or gill samples from fish with cutaneous columnaris exhibit large number of long, rod-shaped bacteria arranged in columns or "hay stacks" (Plumb 2018). The organism can also be isolated from internal organs in fish with severe cutaneous lesions (Newton et al. 1997).

1.3 Epidemiology

Environmental niche

F. columnare is considered ubiquitous in the natural environment. The natural reservoir for this pathogen is not known yet, but multiple studies have been conducted to investigate its preferable habitat in nature. For practical purposes, fish are considered the main reservoir for *F. columnare* in aquaculture settings (Plumb 1999). However, the presence of the pathogen on the fish does not guarantee infection or disease and asymptomatic carriers are common. In addition, *F. columnare* has been found as part of the bacterial microbiota of fish eggs and the rearing water the fish live in (Barker et al. 1990). Researchers also found that *F. columnare* can survive at least for 5 months in both sterilized distilled and lake water (Kunttu et al. 2009).

The fact that *F. columnare* can be isolated from water and biofilms in environments where fish farming does not occur suggests that biofilm could serve as reservoir for this pathogen in the environment (Kunttu et al. 2012).The resilience of columnaris disease in closed aquaculture system also support this hypothesis. Biofilm formation by *F. columnare* was previously examined in our lab and we found that *F. columnare* can colonize surfaces and form

mature biofilm within 48 h under nutrient-rich medium (Cai et al. 2013). Moreover, microfluidic chamber study revealed that biofilm could form a dense structure at a faster rate under exertion of shear force (Cai et al. 2013). We also found that *F. columnare* has an attachment preference to different inert surfaces that are commonly found in aquaculture farms. Interestingly, *F. columnare* was able to rapidly colonize liner, flexible PVC, and nylon nets. However, natural material, such as bamboo, appeared to inhibit its biofilm formation (Cai and Arias 2017). In fish culture ponds, the handling of fish during harvest season can cause loss of mucus from the fish, which are shed into the surrounding water. Staroscik and Nelson (2008) reported that high mucus concentration in water could promote the biofilm formation.

Genomic analysis showed that *F. columnare* is capable of denitrification, which would enable anaerobic growth in aquatic pond sediments (Kumru et al. 2016). Tekedar et al. (2017) compared *F. columnare* growth under aerobic and anaerobic conditions in the presence and absence of nitrate supplementation, and their results suggest that *F. columnare* is capable of anaerobic growth, and that anaerobic growth is improved in the presence of 5 mM sodium nitrate. This discovery suggests that *F. columnare* could utilize pond sediment as reservoir.

In summary, symptomatic and asymptomatic fish, water, biofilm, and pond sediments can serve as reservoirs for *F. columnare*. However, further studies are needed to further delineate the ecology of *F. columnare* in aquaculture and the freshwater environment.

Transmission

In general, columnaris disease can be water-borne and fish-to-fish contact is not required (Welker et al. 2005). Most state hatcheries utilize untreated river water for the rearing ponds and directly release this water back into the river (Jakaitis 2014), which increases the possibility of transmission through the water supply to both hatchery and wild populations. After a columnaris

outbreak, the survivors may become carriers of the disease, and those fish could continue to shed the pathogen into their surrounding aquatic environment. Fujihara and Nakatani reported that rainbow trout surviving a *F. columnare* infection could release up to 5×10^3 colony forming units/mL/h of viable bacteria into tank water. In addition, survivor fish can release bacteria for up to 140 days (Fujihara and Nakatani 1971). More importantly, bacterium can maintain its virulence in lake water under laboratory condition for at least five months (Kunttu et al. 2009). Arias *et al.* (2012) tested the survival of *F. columnare* in pure water, and they found that the starvation induced morphological changes from a rod shape to a coiled form. In later stages of starvation, an extracellular matrix was observed covering the coiled cells. The cells return back to their original elongated rod shape upon encountering nutrients (Arias et al. 2012). Kunttu *at al.* investigated the saprophytic activity of *F. columnare* by monitoring the transmission of the bacterial post-infection after the dead of fish. The author found that the bacteria were shed from the fish carcasses, and they managed to thrive in both alive and dead fish (Kunttu et al. 2009). These facts indicate that *F. columnare* can persist in the rearing environment even when fish host are not available, and they can readily transmit into new population by propagating in decaying fish.

Multiple factors can affect the transmission process of the pathogen. Suomalainen *et al.* studied the influence of rearing density and water temperature in rainbow trout, and they found that normal rearing densities with high temperatures (23°C) increased both transmission rate of columnaris disease and the mortality in the fish(Suomalainen et al. 2005). Farming practices also affect the speed at which the pathogen is transmitted. During harvest season, air pumps are usually set up at one side of the pond to provide the fish with enough oxygen during seining. The water turbulence facilitates the pathogen dissemination to new niches, and the increased oxygen

and nutrient level promote the bacterial proliferation. The immunological condition of both donor and recipient fish may influence the transmission outcome. In one experiment, Kunttu found that high numbers of *F. columnare* were efficiently shed from the donor fish, but transmission of columnaris disease to the next host was unsuccessful (Kunttu et al. 2009). This suggested the overall immunological status of the fish population is a critical factor for successful columnaris transmission. Last but not least, water itself, as a physical environment, can also aid the transmission of pathogens. The flow of water enables the bacteria to transmit long distances if the infectious source is immobilized, and water can protect the bacteria against dehydration, heat and UV-radiation during the transmission stage (Day 2002).

In summary, *F. columnare* is one of the most resilient fish pathogens found in freshwater aquaculture, due to its resourceful harboring strategies in the environment, versatile virulent factors and keen attachment capacity to the mucosal surfaces. A systemic understanding of the transmission and infect process will definitely be beneficial to columnaris disease control and wellness of aquatic organisms.

1.4 Biofilm

In nature, most microorganisms live and grow in aggregates called biofilms, which are generally defined as a community of microbes attached to a surface and embedded in an organic matrix. Biofilms are ubiquitously distributed in aquatic environments, soil, animals and plants tissues. In these fascinating environments, bacteria grow and interact with each other using life patters that differ from those employed by planktonic cells. Biofilms consist of microcolonies separated by interstitial voids and are heterogenrous in many respects, e.g. structurally, chemically, and physiologically. From an evolutionary point of view, living in a biofilm offers many advantages to the cells including protection against desiccation, oxygen radicals,

ultraviolet radiation, predators and harmful chemicals such as antibiotics (Flemming and Wingender 2010).

Biofilm components

Biofilm are a complicated three-dimensional heterogeneous structure, which consists not only of polysaccharides but also of considerable amounts of protein, nucleic acids, lipids and cations. Extracellular polymeric substances (EPS) fill the space between the cells and account for a considerable proportion of the organic carbon content of biofilm. In most biofilms, the EPS can account for over 90% of the dried mass. EPS forms the scaffold that maintains biofilm architecture (Flemming and Wingender 2010). Polysaccharides, most of which are linear or branched long molecules, constitute a major fraction of the EPS. Those polysaccharides can be homopolysaccharides such as sucrose-derived glucans or heteropolysacchatides such as β-1, 6-linked N-acetylglucosamine (Branda et al. 2005). Extracellular proteins are also common EPS components, which include functional enzymes and structural proteins. Enzymes have a very important role as they can degrade complex biopolymers, such as cellulose or chitin, to generate smaller molecules that bacteria can then internalize. Typical enzymes reported from biofilm include chitinase, glucosidase, esterase, and etc. DNA found in biofilms was initially regarded as residual materials from lysed cells but, it is now recognized as an integral part of the matrix since the addition of DNase during biofilm development inhibited biofilm formation (Watanabe et al. 1998). Harmsen and colleagues studied the role of extracellular DNA (eDNA) during biofilm formation by *Listeria monocytogenes* and found that the eDNA is an essential component during the initial attachment of the cells and early stages of biofilm formation (Harmsen et al. 2010). Finally, lipids are also present in the matrix in where they provide the hydrophobicity required for some of the cell-inert surface interactions. Because EPS may contain anionic groups such as

carboxyl, phosphoryl, and sulphate groups, they offer cations exchange potential. Bacterial EPS typically contains 20-50% of their polysaccharides as uronic acids (Kennedy and Sutherland 1987). The EPS are highly hydrated, and water accounts for up to 98% of the biofilm wet biomass (Flemming et al. 2000). In some biofilm structures, there are channels within the matrix where water and nutrients can circulate and be distributed to all the layers (de Beer et al. 1994). This affinity for water gives a slimy consistency to the biomass and serves as protection against desiccation.

Biofilm development

Biofilm development is dynamic process that consists of 5 main steps: attachment, formation of microcolonies, production of extracellular matrix, maturation, and dispersion. Biofilm development starts when the planktonic bacteria attach to a biotic or abiotic surface. This step is thought to be regulated by the nutritional factors in the environment. The first interactions between the cells and the surface are maintained by weak bonds that can be reversed. However, after a few seconds, those links become permanent and the attachment phase is completed. In many species, the cell morphology changes from planktonic to sessile forms typically due to the loss of flagella. After this initial phase, the cells start to multiply and develop microcolonies. During this phase, attractant molecules are released to recruit more planktonic cells. Once enough cells are attached to the substratum, the production of EPS starts and the biofilm acquires its three-dimensional structure. Microcolonies transform into larger aggregates to form mature biofilms. The cycle is completed when planktonic cells are shed from the biofilm into the aquatic environment (Costerton et al. 1999; An and Friedman 2000; Branda et al. 2005). In this fashion, a biofilm acts as reservoir for bacterial expansion, in which successful colonization in one location subsequently permits surface exploration of numerous regions.

Quorum sensing

Quorum sensing (QS) is the regulatory system for controlling gene expression in response to bacterial population (De Kievit and Iglewski 2000). This complex mechanism involves the production of chemical stimulus to determine the population density of a bacterial community. This special phenomena was discovered and described more than 30 years ago in species of the genus Vibrio (Miller and Bassler 2001). Cell to cell communication between bacteria is generally carried out by chemical signals that can diffuse away from one cell and be received by another. This chemical 'language' facilitates self-organization and regulation of microbial cells and allows bacteria to monitor and respond to their own population densities. High cell densities usually result in high concentrations of signal, which in turns regulates the expression of certain genes in neighboring cells. The physiological activities stimulated by quorum sensing include symbiosis, virulence, competence, conjunction, antibiotic production, motility, and biofilm formation (Miller and Bassler 2001).

Common molecules employed in quorum sensing include N-acylhomoserine lactones (AHL), and indole (Simões et al. 2010). The vast majority of Gram-negative (e.g. *Pseudomonas aeruginosa, Erwinia carotovora, and Brucella melitensis*) bacteria utilize AHLs as signaling molecules (Jones et al. 1993; McClean et al. 1997; De Kievit and Iglewski 2000; Taminiau et al. 2002). The LuxI/LuxR system is a well-studied quorum-sensing system present in many Gram-negative bacteria. LuxI-type proteins synthesize AHL autoinducers, which modulate the activity of LuxR-type transcriptional activators to activate corresponding gene expression. LuxR QS regulators have been identified in diverse Proteobacteria species, and they seem to disseminate across species by horizontal gene transfer (Gray and Garey 2001). In some cases, AHLs with modification in the structure of their N-acyl side chains are used by some Gram-negative bacteria

such as the fish pathogen *Aeromonas hydrophila* (Swift et al. 1997). Previous study showed that *Escherichia coli* and *Salmonella typhimurium* have the potential to respond to acyl homoserine lactone autoinducers generated by other organism through their own LuxR system that acquired horizontally. In *E. coli*, AHLs from other bacteria are sensed through SdiA, so *E.coli* can detect signals that it does not synthesize (Michael et al. 2001). Similarly, Lee et al. (2007) found that exogenous AHLs from *Pseudomonas aeruginosa* reduce *E. coli* biofilm formation. Declercq screened the typical QS molecules, i. e. AHL and autoinducer 2, in both high virulent and low virulent strains of *F. columnare* with different biosensors, and the results proved to be all negative (Declercq et al. 2015). Indole is also a promiscuous signal, which could alter the bacterial phenotypes. For example, indole increases biofilm formation of *P. aeruginosa* and *P. fluorescens* even though these *pseudomonads* do not produce indole (Lee et al. 2007).

Genetic expression during biofilm formation

Several studies have characterized the molecular mechanisms that regulate biofilm formation (Evans 2003). One common trend in those biofilm transcriptome studies is the induction of stress-related genes in biofilms. For example, 42 stress-related genes were identified in a temporal study during *E. coli* K-12 biofilm development on glass wool (Domka et al. 2007) and 5 stress-response genes (*hslST, hha, soxS and ycfR*) were indentified in 7 h *E. coli* biofilm cells (Ren et al. 2004).

RpoS is an alternative sigma factor that plays a role in bacterial stress and virulence factor production (Kan and Hideo 1994). An *rpoS* deletion mutant of *P. aeruginosa* was able to form biofilm similar to the wild type in terms of shape and architecture but the overall structure was significantly thicker in mature biofilms. It is hypothesized that *rpoS* mediates a regulatory cascade that restricts macrocolony growth. Cells in biofilms acquire nutrient by diffusion, which

is inversely proportional to the size of the structure. *RpoS* may help limit macrocolony growth in a mature biofilms to maintain cell viability in the center of the biofilm by allowing sufficient nutrient diffusion (Ghannoum and O'Toole 2004).

An example of downregulated genes in biofilms is rhamnolipids-related genes. For example, the rhamnolipids transcription activator (RhlR) is under the transcriptional regulation of the quorum-sensing regulator *LasR* (Pesci et al. 1997). Purified rhamnolipids exhibit surfactant-like properties and have been shown to disrupt both cell-to-cell and cell-to-surface interactions in *P. aeruginosa*. Overexpression of these compounds by wild-type cells inhibits biofilm development (Davey et al. 2003).

Other factors affect the biofilm formation include motility genes and second messeger, such as c-di-GMP. For example, the mutagenesis strain that failed to produce type I pili were no longer able to attach to surfaces and form biofilm, which indicates gliding genes appear to play a part in biofilm formation (Pratt and Kolter 1998). In another study, whole-transcriptome profiling using microarray indicates that c-di-GMP has been linked to biofilm formation in several strains of *E. coli* (Méndez-Ortiz et al. 2007) and its overproduction increases *E. coli* biofilm formation.

1.5 Host and pathogen interaction

F. columnare causes acute to chronic infections and the clinical signs depend on the virulence of the strain and the immune status of the host. During acute infections, fish can be succumbed to the disease in 12 to 24 h without gross tissue damage while chronic infections are characterized by typical external lesions and gill necrosis (Groff & LaPatra 2000). The following subsections aim at describing the steps of columnaris disease.

Initial attachment

When bacteria encounter a host tissue, specific attachment occurs prior successful infection. Under physiologic conditions, both pathogenic bacteria and the surface of animal cells are negatively charged. In order for bacteria to adhere, the energy barrier (repulsive force) must be overcome. In general, there are four forces involved in this bacterium-substratum interaction at the initial attachment. When two particles are beyond 50 nm distance, the van der Waals' interaction is considered the main force that attracts both particles. The strength of the force is positively related to the size of the cell mass, and negatively related to their distance. If the two particles succeed to get closer (10-20 nm), the electrostatic interaction (repulsive force) chips in. When bacterial cells proceed to get closer, within 5 nm, the hydrophobic interaction (the interaction between nonpolar molecules) and complementary interactions (such as lectin-carbohydrate) participate and finally shapes the irreversible adhesion (Ofek and Doyle 1994). There are specific structures, called adhesins, which are responsible for bacterial attachment, bacterial tissue tropism and pathogenesis.

Straus et al. (2015) reported that increased water hardness can increase the *F. columnare* pathogenesis by facilitating the *in vivo* attachment to fish gills. Our group also found that supplementation of calcium can promote the *F. columnare* attachment to inert surfaces and subsequent biofilm formation (Cai et al. 2013). One of the possible reasons for these two observations is that the presence of bivalent cations can help overcome the repulsive force by neutralizing the anions. Transcriptomic study of catfish gill tissue after experimentally infection with *F. columnare* showed that Rhamnose-Binding Lectin (RBL) was dramatically upregulated after 4 h challenge, indicating that bacterial cell surfaces possibly has adhesins that resemble rhamnose residues (Sun et al. 2012; Thongda et al. 2014).

Colonization

After successful attachment, the bacteria must colonize the mucosal surface of the fish and combat the fish immune system. Gill tissue and skin are considered the primary entry routes for *F. columnare*, but in some cases, less virulent strains can entry through the intestines causing chronic columnaris infection (Groff & LaPatra 2000). In either case, the bacteria would encounter the mucosal surfaces that are covered with mucus secreted by specialized epithelial cells (goblet cell) or glands. Mucosal surfaces typically contain mucus (mucin), commensal bacteria, and underlying mucosa-associated lymphoid tissue, which together regulate immunity and maintain homeostasis in healthy fish (Nataro et al. 2005). There are several defense mechanisms in place at the mucosal level to prevent microorganism invasion. The mucosal blanket of epithelial cells serves as a physical barrier. Chemical defenses include antimicrobial proteins, cytokines, and pattern recognition molecules. The cellular defenses at the mucosal level include M cells, dendritic cells, and phagocytic cells. Scanning electron microscope revealed that bacteria adheres to the surface of gills as aggregates instead of as being evenly distributed across the gill epithelium (Bullard et al. 2011).

F. columnare is capable of excreting extracellular enzymes including chondroitin lyase and proteases (Griffin 1991; Betolini and Rohovec 1992; Suomalainen et al. 2006). Chondroitin lyase acts specifically on a group of acidic mucopolysaccharides found primarily in animal connective tissue. The extracellular exotoxins thereby could degrade skin tissue and produce lesions such as the typical “saddle-back” (Morrison et al. 1981). When *F. columnare* massively multiplies on the gills, yellowish areas of necrotic tissue can be observed which ultimately result in complete gill destruction. The fish are ultimately killed due to insufficient oxygen uptake. In

certain cases, the bacteria colonizes the mouth and causes ulceration of the oral mucosa (mouth rot). These mouth lesions are more lethal than the lesion on the skin since painful oral lesions render the fish anorexic and death ultimately occurs due to starvation. In some acute case, bacteria were able to enter the bloodstream and quickly cause systemic infections (Declercq et al. 2013).

The mucus is known to exhibit antimicrobial activity to a wide range of bacteria, however, *F. columnare* could utilize the mucus as chemotaxis signal to assist the bacterial motility on host tissues (Klesius et al. 2008). Interestingly, supplementation of salmon mucus increased the growth of *F. columnare* (Staroscik and Nelson 2008). This indicates that *F. columnare* developed specialized infection mechanisms that target the aquatic.

***F. columnare* virulent factors**

Gram-negative bacteria use at least nine secretion systems, the type I secretion system to the type IX secretion system (T1SS to T9SS), to transport proteins across their outer membranes. Gliding motility facilitates the movement of bacteria along surfaces in many Bacteroidetes species and results in spreading colonies. *F. columnare* is a motile bacterium that moves by gliding. The proteins required for gliding are secreted through a specialized protein secretion system known as type IX secretion system (T9SS). Genetic analyses suggest that GldK, GldL, GldM, GldN, SprA, SprE, SprT, PorU, and PorV are T9SS components. Proteins secreted by T9SSs have N-terminal signal peptides that facilitate export across the cytoplasmic membrane, and they also typically have conserved C-terminal domains (CTDs) that target them for secretion across the outer membrane by the T9SS. The CTDs are often removed by the protease *PorU* during or after secretion. Some secreted proteins remain attached to the cell surface whereas others are released in soluble form. Genomic study indicated that *F. columnare* encodes three

secretion systems, i.e. T1SS, T6SS and T9SS. A mutant of *F. columnare* defected in a T9SS core protein was deficient in the secretion of several extracellular proteins and lacked gliding motility (Li et al. 2017). It is speculated that *F. columnare* may utilize T9SS for export of macromolecules to directly propel cells as part of the motility mechanism.

Colony types are associated with virulence although in our own experience *F. columnare* can be pleomorphic depending on culture conditions (unpublished data). Kunttu et al. (2011) postulated that rhizoid colony variants were typically high virulence while the smooth colony variants were non-virulent and poorly adherent (Kunttu et al. 2011). Low nutrient level provoked more active gliding motility in individual cells. Gene expression survey using qPCR revealed an increased expression level of *sprA* (a core component of T9SS) and *sprF* (needed for adhesin secretion) under low nutrient conditions, which indicate that the nutrient concentration could be an important factor for the virulence of *F. columnare* (Penttinen et al. 2018).

1.6 Disease control

Because the bacterium is ubiquitous in the freshwater environment (Plumb 2018), eradication of columnaris disease is not likely to occur. Prevention is the most economical and recommended approach to control the disease and minimizing stress in cultured fish seems to be the best strategy to prevent columnaris disease. Unfortunately, under intensive aquaculture practices, stressful conditions for the fish such as high densities or poor water quality are common. Columnaris disease can be treated with therapeutic chemicals such as external disinfectants and antibiotics but success rate varies. In a previous study by our group we showed that potassium permanganate indeed made the fish more susceptible to colonization by *F. columnare* likely due to the significant changes exert on the skin microbiome (Mohammed and Arias 2015). Similarly, medicated feeds, such as florfenicol, are known to induce gut dysbiosis in

mammals and fish (Francisca; paper under review), which render the animals more vulnerable to subsequent bacterial infections.

When disease appears in aquaculture operations, the treatment of choice typically includes both external baths and medicated feed with antimicrobial agent to combat both cutaneous and systemic infections (Gudding et al. 2014). In terms of surface-acting disinfectants, chemical agents such as copper sulfate or potassium permanganate (KMnO4) are typically added into the water body at dose of 0.5 ppt and 2 ppm, respectively. There are three antibiotics available for use as medicated feed in aquaculture, i.e. Romet, oxytetracycline and Aquaflor. Good management practices are the best way to reduce losses due to columnaris disease such as reducing stock densities and maintaining good water quality. In addition, vaccines have been shown to be effective in preventing columnaris disease.

Vaccines in aquaculture

There are several types of vaccines that have been proved effective against many infectious diseases both in humans and in animals. Several types are commercially available for aquaculture species. Inactivated or 'killed' vaccines referred a pathogen that has been cultured in the laboratory (virus or bacteria) and then killed, typically with heat or formalin, so it is no longer infective. Inactivated vaccines account for the majority of commercial vaccines currently used in aquaculture. They have the advantage of being easy to make, eco-safe, and cost-effective (Gudding et al. 2014). A second generation of inactivated vaccines are subunit vaccines. Subunit vaccines are based on specific immunogenic proteins from pathogens that are produced in the laboratory through a recombinant process using expression vectors. Recombinant proteins have to be expressed in large quantities in vitro and then purified. The common used vector is *E. coli*, and subunit vaccines have been made against several fish bacterial pathogens, including

Aeromonas hydrophila (Khushiramani et al. 2012), *Edwardsiella tarda* (Zhang et al. 2012), *Streptococcus iniae* (Cheng et al. 2010), and *A. salmonicida* (Lutwyche et al. 1995). In 2005, Novartis obtained license for the first DNA-based vaccine (3rd generation of inactivated vaccines) that protects farm-raised salmon against infectious haematopoietic necrosis virus (IHN) (Alonso and Leong 2013).

Opposite to use a 'killed' or inert antigen, modified live vaccines are living microbes that are attenuated and although they can colonize the host to some extend, they are not capable of causing disease. Live vaccines mimic the route of entry of bacterial pathogen and stimulate the mucosal immune response, which is particular relevant against pathogens that course as an external infection (Detmer and Glenting 2006). The attenuation strategies used to develop live vaccines include laboratory passage, antigen mimicry, physical and chemical mutagenesis, and genetic modification using molecular techniques (Gudding et al. 2014). In most cases, live vaccines show a significantly higher immunogenicity than inactivated vaccines, since natural infection is imitated almost perfectly by eliciting a wider range of immunologic responses, both humoral and cellular immunity (Kollaritsch and Rendi 2013). In addition, the dissemination of virulent-attenuated bacteria from fish carrier into the environment makes the efficacy of a live vaccine to last longer. There are three modified live bacterial vaccines licensed for use in fish, i.e. vaccines against bacterial kidney disease (licensed in Canada, Chile and US), enteric septicemia of catfish (licensed in US), and columnaris disease (licensed in US) (Shoemaker et al. 2009).

The modified live vaccine available for prevention of columnaris in catfish was licensed to Merck Co. under the brand name AQUAVAC-COL. Successive passages on increasing concentrations of rifampicin is a common strategy to that results in chemical mutagenesis. The

active ingredient in AQUAVAC-COL is an avirulent, rifampicin-resistant mutant of *F. columnare* (Shoemaker et al. 2005). Rifampicin is an antibiotic that inhibits bacterial DNA-dependent RNA polymerase, and bacteria exhibiting resistance to rifampicin typically have reduced virulence (LaFrentz et al. 2008). However, this vaccine derives from a genomovar I strain (low virulent strain to catfish) and only provides limited protection against the most virulent genomovar II. Our group developed several live attenuated vaccines derived from a genomovar II strains and through subsequent experiments we selected a mutant that proved to be protective against genomovar I and II strains in several fish species (Olivares-Fuster and Arias 2011; Mohammed et al. 2013).

Historically, the attenuated derivatives used as live bacterial vaccines were isolated using empirical approaches based on laboratory passage or chemical mutagenesis. Although attenuated derivatives could be obtained in this way, the mechanism of attenuation was often left undefined, resulting in concerns associated with quality control or even the threat of reversion to virulence (MacLennan et al. 2016). The first commercially approved modified live vaccine in the US was against brucellosis in cattle. *Brucella abortus* is a Gram-negative, facultatively intracellular bacterial pathogen that can cause abortion in pregnant cattle and undulant fever in humans (Vemulapalli et al. 2000). *B. abortus* RB51 is a stable attenuated mutant vaccine strain derived from the virulent strain 2381 by using a rifampicin selection method. This strain has been employed as the official vaccine for cattle brucellosis in the United States since February 1996 (Ragan 2002). The vaccine efficacy and stability of *B. abortus* RB51 has been proven in both experimental and field conditions (Cheville et al. 1993; Jensen et al. 1996). RB51 was used for many years before its mechanism of attenuation was characterized. RB51 contains several mutations that prevents the bacteria from making complete O polysaccharide (O antigen)

(Vemulapalli et al. 2000). Similarly, the attenuated *Brucella abortus* strain 104M has been used as a vaccine strain in humans against brucellosis in China. Comparative genomic analysis against the virulent strain revealed a set of mutations in the virulence-related genes including LPS synthesis, secretion and transporter system. A set of genes missing in the vaccine strain were also identified, such as genes encoding superoxide dismutase that detoxify the reactive oxygen intermediates (ROIs), two-component transcription regulator, and bacterial efflux system proteins (Yu et al. 2015).

The live attenuated bacillus Calmette-Guerin (BCG) vaccine was developed against tuberculosis based on a passaged and attenuated variant of the pathogen *Microbacterium bovis*. Recent genome analysis of BCG seed vaccine identified the genomic regions that are likely associated with the attenuated phenotype. Interestingly, comparison of BCG vaccines from different companies found significant variation between different vaccine seed lots used for manufacture, highlighting some of the challenges associated with using live vaccines (Bloom and Lambert 2016).

1.7 Genomics study of *Flavobacterium*

The rapid development of next generation sequencing technology has greatly increased the amount of available microbial genomes. Whole genome sequencing provides a higher resolution in phylogenetic and taxonomic (Yu et al. 2017) and address critical issues in genome function and evolution (Miller et al. 2004). As of September 2018, there are 16 *F. columnare* genomes sequences available, including 5 complete genomes (Benson et al. 2017). Except for one strain sequenced in 2011, all other 15 *F. columnare* strains were sequences after 2016 (Table 1-1).

Table 1-1. Genomic characteristics of *F. columnare*.

TStrain	Genome size (Mb)	GC%	Genes sequence	host	Origin	Genomovar
ATCC49512*^{T}	3.16	31.5	2793	Brown trout	France	I
94-081*	3.32	30.8	2909	Channel catfish	AL,USA	II
C#2*	3.33	31.0	2896	NK	USA	II
Pf1*	3.17	31.6	2805	Yellow catfish	China	I
TC 1691*	3.03	31.6	2664	NK	China	I
CSF-298-10	3.28	31.5	2903	Rainbow trout	ID, USA	I
1215	3.34	30.6	3004	Red tilapia	Thailand	I/II
CF1	3.09	30.8	2766	Striped catfish	Thailand	II
1214	3.38	30.0	3086	Red tilapia	Thailand	II
1362	3.16	30.6	2815	Red tilapia	Thailand	II
NK01	3.39	29.9	3067	Nile tilapia	Thailand	II
1201	3.36	30.1	3080	Red tilapia	Thailand	II
1372	3.18	30.6	2854	Red tilapia	Thailand	II
MS-FC-4	3.45	32.0	3067	Rainbow trout	ID, USA	I
ATCC23463^{T}	3.16	30.5	2818	Chinook salmon	WA,USA	I
CIP109753	3.25	30.7	2807	Ayu	Japan	III

* indicates complete genome; T indicates type strain; NK indicates not known.

F. columnare isolates are genetically diverse and have been assigned to multiple genomovars. The genetic diversity among *F. columnare* isolates has been studied for several decades. Song et al. (1988) were the first ones to report that there were more than one genetic group present in *F. columnare* by using DNA hybridization and DNA homology. Triyanto and Wakabayashi proposed 3 genomovars based on 16S rRNA gene sequences, and the heterogeneity was confirmed with DNA-DNA hybridization (Wakabayashi 1999). Later, LaFrentz et al. (2014) standardized the 16S rRNA-RFLP typing method and increased the species diversity up to 6 genomovars. In addition, he further investigated the genetic diversity of *F. columnare* by conducting the phylogenetic analysis of 16S rRNA and housekeeping gene sequences, and he divided the strains of 6 genomovars into 4 distinct genetic groups. He proposed to use the four genetic groups instead of genomovars for the *F. columnare* assignment (LaFrentz et al. 2018).

Epidemic and pathogenicity studies indicated that genomovars were associated with different virulence and host preferences. Studies showed that genomovar II strains are more virulent than genomovar I strains to channel catfish (*Ictalurus punctatu*s) (Shoemaker et al. 2008), zebrafish (*Danio rerio*) (Olivares-Fuster et al. 2011), Nile tilapia (*Oreochromis niloticus*) (Mohammed et al. 2013) and rainbow trout (*Oncorhynchus mykiss*) (LaFrentz et al. 2012). Host-specific association study conducted with wild fish populations indicated that most genomovar II strains were isolated from catfishes while most genomovar I strains came from freshwater drum and threadfin shad (Olivares-Fuster et al. 2007). In addition, outbreaks in salmonid aquaculture systems have almost invariably been associated with genomovar I strains, whereas epidemics in catfish and other warm-water fish have involved (primarily) genomovars II strains. In Thailand and southern USA, genomovar II has been identified as the predominant group. A recent

epidemiolocal study has suggested that the distribution of bacterial genomovars is likely geographical-dependent (LaFrentz et al. 2014) but movement of aquaculture species worldwide probably masks the original distribution of *F. columnare* genomovars.

Comparative genome analysis revealed extensive sequence diversity within the *F. columnare* species. Kumru (2016) compared the *F. columnare* genomovar I and II genomes and he found that the ATCC49512 and 94-081 strains met the criteria to be considered different species based on the Average Nucleotide Identity (90.71% similar) and DNA-DNA hybridization (42.6% similar). The two genomes have a large number of rearrangement. Both strains carry type I secretion system, type VI secretion system, and type IX secretion system. DNA homology analysis estimated that the digital DNA-DNA hybridization (dDDH) between strains of genomovar I and genomovar II could be as low as 42.6% (Kayansamruaj et al. 2017). If we look at the closely related species *F. psychrophilum*, the genomic diversity of the *F. psychrophilum* species is analyzed using a set of 41 genomes, and the result revealed a limited genomic diversity and the core genome accounts for about 80% of the genes in each genome (Duchaud et al. 2018).

Hypothesis and Objectives

Question background

The overarching goal of this dissertation research was to further elucidate the molecular mechanisms encoded in the genome of *F. columnare* strains from two perspectives: from an applied point of view, I was interested in understanding what factors contribute to biofilm formation with the ultimate goal of preventing it in farms; from a basic point of view, I wanted to understand what genes contribute to attachment and colonization of *F. columnare.*

The specific objectives were as follows:

1. Investigate the environmental factors that affect *F. columnare* attachment and biofilm formation, with a focus on the aquaculture relevant factors (i.e. substrates and hardness).

2. Identify the Differential Expressed Genes (DEGs) during biofilm formation and calcium supplementation with transcriptome analysis.

3. Characterize the molecular basis for the virulent attenuation of a *F. columnare* vaccine candidate strain.

4. Investigate the genetic diversity of the *F. columnare* species complex by comparative genomics.

Dissertation organization

This dissertation is organized into 6 chapters. Chapter 1 includes a literature review of the current knowledge on *F. columnare,* presents the research objectives, and clarifies the dissertation organization. Chapter 2 presents the colonization preferences of representative bacterial catfish pathogens to substrates commonly found in aquaculture farms and their responses to increased calcium concentrations. Chapter 3 describes DEGs in planktonic cells

versus those living in biofilms, and characterizes the molecular mechanisms induced by calcium in biofilm using RNA-seq method. In Chapter 4 the draft genomes of the parent and the vaccine strain (obtained by rifampicin-selection method) are compared. In Chapter 5 the whole genome of 3 strains representing distinct genetic groups of *F. columnare* were sequenced and compared with those *F. columnare* genomes previously available. Finally, Chapter 6 provides a summary of my research, highlights the conclusions, and estates final recommendations.

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Chapter 2. Biofilm formation on aquaculture substrates by selected bacterial fish pathogens

Abstract

The objective of this study was to determine if common bacterial catfish pathogens could attach and colonize surfaces commonly found in aquaculture facilities. In addition, we evaluated the role of calcium in biofilm formation. Attachment to polystyrene plates was used to quantify biofilm formation by five bacterial pathogens (i.e. *Flavobacterium columnare*, *Aeromonas hydrophila*, *Edwardsiella ictaluri*, *E. tarda*, and *E. piscicida*). *Flavobacterium columnare* and *A. hydrophila* formed thick biofilms that were enhanced by calcium supplementation. Biofilm formation was significantly lower in all *Edwardsiella* species tested and calcium had little to no effect on *Edwardsiella* biofilm formation. Attachment to natural and man-made surfaces was quantified by a standard plate count method. Scanning Electron Microscopy (SEM) was used to confirm biofilm formation on the substrates. *Flavobacterium columnare* formed biofilm on liner, flexible PVC, and nets. Bamboo prevented *F. columnare* attachment and inhibited cell growth. *Aeromonas hydrophila* and *E. ictaluri* formed biofilm in all materials tested, although significant differences were found among substrates. While *E. ictaluri* failed to form biofilm on microtiter polystyrene plates, it was able to colonize and multiply on all aquaculture materials tested. Our results demonstrated that common bacterial pathogens had the potential of colonizing surfaces and may use biofilm as reservoirs in fish farms.

Introduction

Aquaculture continues to expand globally as an alternative to capture fisheries. Catfish, including channel (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and their hybrid, are the most important cultured fishes in the US, accounting for over 54% of all domestic aquaculture

production (USDA 2014). Historically, one of the main factors limiting expansion and profitability of the US catfish industry is the lack of disease control (USDA 2010). Direct losses due to disease outbreaks caused by *Flavobacterium columnare*, *Aeromonas hydrophila*, and *Edwardsiella* spp. exceed $150 million per year (USDA 2010). All these pathogens are ubiquitous in freshwater ecosystems where they can survive without a fish host (Plumb and Quinlan 1986; Arias et al. 2012; Zhang et al. 2014). Once they gain entry into an aquaculture facility, they may spread easily from tank to tank (or pond to pond) and are extremely hard to eradicate. While many groups have investigated the transmission strategies of these bacterial pathogens (Welker et al. 2005; Kunttu et al. 2009; Salama and Murray 2011; Declercq et al. 2013), there are still many unanswered questions regarding their natural reservoirs.

Estimates suggest that more than 90% of bacteria exist within biofilms (Chambers and Sauer 2013). Existence in a biofilm offers aquatic bacteria many advantages over a planktonic lifestyle including improved adaptation to nutrient deprivation, and increased resistance to stressors such as desiccation, predation, and antimicrobial compounds. Biofilm is reported as a common cause of persistent infections in both clinical and food industry settings (Costerton et al. 1999). Generally, the formation of biofilms is initiated with surface attachment by planktonic bacteria, followed with an establishment of a complex community characterized by the presence of differentiated structures or microcolonies (O'Toole et al. 2000). The attachment phase is the first, and most crucial, step for successful biofilm establishment. Factors affecting bacterial attachment include physiochemical conditions, such as temperature, pH, hardness and salinity (Cai et al. 2013), and surface properties, such as substrate type, surface roughness, and chemical compositions (AlAbbas et al.). In aquaculture, biofilms are essential for maintaining balanced nitrogen and carbon cycles in the system. Biofilters are reactors, with a high surface-volume

ratio, that are colonized by microorganisms that absorb excess nutrients from water. Among them, nitrifying bacteria are key to maintain water quality by detoxifying the ammonia produced by the fish. In addition, biofilms growing on submerged substrata have been shown to improve shrimp production, and they are considered a good quality protein source (Pandey et al. 2014). However, biofilms could also provide the appropriate environment for fish pathogens to persist in systems and withstand disinfection protocols or even antibiotic treatments (Miller et al. 2015; Almatroudi et al. 2016).

In aquaculture, calcium is added in the form of lime, or limestone, as required to maintain a desired alkalinity and hardness (Lazur et al. 2013). Calcium concentration can fluctuate significantly during the seasons due to management practices, and enrichment on surfaces, either as sedimentary calcium deposits or in association with other organisms (Patrauchan et al. 2005). Hardness is an important index for water quality in aquaculture, and studies indicate that calcium plays a critical role in attachment of *F. columnare* to inert surfaces as well as pathogenesis to channel catfish (Cai et al. 2013; Straus et al. 2015). Despite the extensive documentation of the roles which calcium plays on adhesion bridging and biofilm promotion (Turakhia 1986; Rose 2000; Cruz et al. 2012), little attention has been devoted to the effect of calcium on attachment and biofilm formation of fish pathogens. The objective of this study was to determine the colonization preferences of representative bacterial catfish pathogens to substrates commonly found in aquaculture farms as well as their responses to increased calcium concentrations.

Materials and Methods

Bacterial strains and growth conditions

Nine representative stains belonging to five bacterial species (*F. columnare*, *A. hydrophila*, *E. ictaluri*, *E. tarda*, and *E. piscicida*) were used in this study (Table 2-1). All strains

were isolated from diseased fish. *Aeromonas hydrophila* strain ML 09-119 is referred to in the literature as 'VAh' or virulent *A. hydrophila* (Tekedar et al. 2013); it has been responsible for severe losses in the channel catfish aquaculture industry since 2009. Bacterial strains were maintained as glycerol stocks and stored in -80°C. *Aeromonas hydrophila*, *E. ictaluri*, *E. tarda*, and *E. piscicida* were cultured in Brain-Heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) while modified Shie (MS) broth was used for *F. columnare* (Shoemaker et al. 2005). All stains were incubated at 28 °C under shaking, and passed onto new media every 48 h.

Biofilm quantification

Biofilm formation under different calcium concentrations was tested by supplementing the medium (MS or BHI) with 0, 2.5 mM, 4.5 mM and 6.5 mM $CaCl_2 \cdot 2H_2O$ (Acros Organics, Geel, Belgium). Biofilm formation was assessed as described previously (Alvarez et al. 2006; Cai et al. 2013). Briefly, an over-night inoculum was diluted 100 times in the corresponding medium and 100 µl of each dilution was inoculated into the wells of 96-well microtiter polystyrene plates (Nunc- Immuno MaxiSorp; Nunc, Rochester, NY) in quadruplicate. The microtiter plates were incubated at 28°C for 48 h to allow bacterial attachment and biofilm formation. General bacterial growth was quantified by measuring the OD at 600 nm with a Synergy HT spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). Medium containing unattached cells was discarded, and the wells were washed and stained with 150 µl of 1% (wt/vol) crystal violet for 20 min. Excess crystal violet solution was removed, and the wells were washed three more times. Biofilm formation was quantified by measuring the OD at 600 nm after the remaining dye was dissolved in 96% (vol/vol) ethanol solution.

Substrates materials and bacterial adhesion

Four representative substrates commonly found in catfish farms were used in this study: bamboo pole (locally grown at the E. W. Shell Fisheries Station, Auburn, AL), liner (polyethylene; Reef Industries, Houston, TX), flexible PVC (polyvinyl; Watts Premier, Peoria, AZ), and a nylon dip net (Dynamic Aqua-Supply Ltd., Surrey, Canada). Equally sized substrates (3 cm x 0.5 cm) were washed in ddH_2O and autoclaved before use. Glass tubes, prefilled with 3 ml of medium (BHI or MS) and substrates, were inoculated with 10 µl of 10^9 CFU/ml bacterial culture containing 45.6 µM $CaCl_2 \cdot 2H_2O$. All tubes were incubated at 28 °C for 24 hours under shaking to allow for bacterial attachment and colonization. After incubation, the substrates were rinsed three times with sterile phosphate-buffered saline (PBS) to eliminate non-attached, planktonic cells. Afterwards, 5 ml of sterile PBS were added to the tubes and the samples were vigorously vortexed at maximum speed for 2 minutes (Mo Bio vortex-Genie 2, Carlsbad, CA) to dislodge the cells embedded in the biofilm. Resuspended bacteria were quantified using a standard plate count method. Three replicates were conducted for each substrate test.

Scanning Electron Microscopy

Three strains, i.e. *F. columnare* ALM-00-530, *A. hydrophila* ML-09-119, and *E. ictaluri* SFG-773 were selected for ultrastructural study of biofilm. Substrates samples from the above bacterial adhesion assay were randomly selected and prepared for scanning electron microscope (SEM) examination as described before (Cai et al. 2013). Briefly, substrates samples were carefully rinsed in PBS three times to eliminate planktonic cells, and fixed in 10% neutral formalin solution overnight. The samples were then dehydrated in 30%, 50%, 80% and 100% ethanol for 10 min each. Samples were placed into ethanol-hexamethyldisilazane (HMDS) solution (1:1 v/v) for 10 min and transferred to 100% HMDS for an additional 20 min.

Specimens were air dried under a chemical hood for another 10 min before sputter coating with a gold-palladium alloy in an EMS 550X (Electron Microscopy Science, Hatfield, PA).

Statistical analysis

Quantification of cell attachment to substrate was based on plate counts per cm^2 of material. Colony forming units (CFU) were converted to base 10 logarithms before analysis. All data was analyzed by one-way analysis of variance (ANOVA) with Tukey's test using SAS Software version 9.2 (SAS Institute, Cary, NC). The significant difference was set at $p < 0.05$.

Results

Biofilm quantification under serial [Ca^{2+}]

Higher [Ca^{2+}] promoted biofilm formation in *F. columnare, A. hydrophila,* and in one strain of *E. tarda*, while biofilm formation by *E. ictaluri* and *E. piscicida* was negligible (OD < 0.05) at any given concentration (Figure 2-1).

Increased calcium concentrations promoted biofilm formation on both strains of *F. columnare* (Fig. 2-1A) but the results were remarkably different between strains. *Flavobacterium columnare* ALG-00-530 significantly increased biofilm formation at 2.5 mM [Ca^{2+}] or higher concentration when compared to the control, while ARS-1 required at least 4.5 mM of [Ca^{2+}] to show a significant increase in biofilm. Both strains responded to [Ca^{2+}] but required different concentrations to show a positive effect on biofilm formation. Cell growth in both strains was reduced when [Ca^{2+}] concentrations were higher than 4.5 mM.

Aeromonas hydrophila biofilm formation was significantly affected by [Ca^{2+}] but differences were observed between both strains tested (Figure 2-1 C and 2-1 D). Strain AL01 showed a linear correlation between biofilm formation and [Ca^{2+}].The OD values for *A. hydrophila* stain AL01 were higher than any other strain and bacterial species tested. Strain ML-

09-119 (high virulent strain) significantly produced more biofilm at 2.5 mM [Ca^{2+}] than control but higher concentrations failed to increase biofilm formation.

Interestingly, none of the *Edwardsiella* strains tested formed visible biofilm under our experimental conditions and OD values were never higher than 0.3 (Figure 2-1 E to 2-1 I). Biofilm formation in *E. ictaluri* was very low, and only strain 57 showed a significant increase in biofilm at 4.5 mM [Ca^{2+}]. Calcium had a significant impact on the growth of both strains. At the highest [Ca^{2+}] tested, it had a deleterious impact on cell growth. By contrast, the effect of calcium on *E. tarda* was strain specific. Strain HL213 significantly increased biofilm with higher calcium concentrations in a linear pattern while no significant differences were found in strain 26 (Figure 2-1 G and 2-1 H). *Edwardsiella piscicida* was the only species tested in which increasing calcium concentrations decreased biofilm formation (Figure 2-1 I).

Bacterial colonization of substrates

The quantification of bacterial colonization on the four substrates tested was determined by plate count. The number of bacteria attached per square centimeter of sample is shown in Figure 2-2. Results showed that all three bacterial pathogens were able to attach to and colonize all the substrates tested, but significance differences were observed between species. *Flavobacterium columnare* (ALG-00-530) was able to readily colonize liner, flexible PVC, and net (Figure 2-2 A). Interestingly, *F. columnare* demonstrated the lowest attachment preference to bamboo with less than 10 CFU/cm^2 (Figure 2-2 A). Liner and net had the highest colonization followed by flexible PVC (Figure 2-2 A). By contrast, *A. hydrophila* (ML-09-119) effectively attached and colonized all materials tested (Figure 2-2 B). Colonization of flexible PVC was significantly lower compared to other materials but still higher than 10^7 CFU/cm^2 (Figure 2-2 B). Similarly, *E. ictaluri* (SFG773) also colonized all materials tested but displayed a significantly

higher affinity for bamboo (Figure 2-2 C). When strains were compared within each substrate, *A. hydrophila* showed the highest colonization capabilities to all four materials tested, followed by the *E. ictaluri* and *F. columnare*.

To determine if the cells attached to substrates were indeed forming biofilm, we used SEM to qualitatively describe biofilm formation (Figures 2-3 and 2-4). Figure 2-3 shows the attachment of *A. hydrophila*, *F. columnare*, and *E. ictaluri* to flexible PCV, liner, and net. Overall, SEM images showed that all three pathogens were in the early stages of forming microcolonies on the substrata. *Aeromonas hydrophila* cells formed a monolayer of compact microcolonies that appeared to be covered by extracellular polymeric substances (EPS) (Figure 2-3 D to 2-3 F). Membrane vesicles (MV) were observed on the outer surface of some cells, which are thought to secrete the components of the EPS into the extracellular space (Figure 2-3 D).

The characteristic long and thin bacilli of *F. columnare* also formed microcolonies (Figure 2-3 A and 2-3 B) although cell densities appeared lower than those observed in *A. hydrophila* (Figure2- 3 D to 2-3 F). Cells of *F. columnare* intertwined in the microcolonies create a multilayered structure (Figure 2-3 B) as opposite to the monolayer produced *by A. hydrophila*. Extracellular polymeric substance covering the cells was also observed particularly in the center of the colonies. *Flavobacterium columnare* was unique in that it produced a filamentous matrix that connected net fibers (Figure 2-3 C). *Edwardsiella ictaluri* cells were present in low densities but were actively multiplying (CM; Figure 2-3 G, 2-3 I). Membrane vesicles were also observed (Figure 2-3 G).

Figure 2-4 shows SEM images of *F. columnare*, *A. hydrophila* and *E. ictaluri* on a transverse cut of a bamboo fiber. *Flavobacterium columnare* cells were elongated and

abnormally thin. No replicating cells or microcolonies were observed and there was no EPS covering the cells. Both *A. hydrophila* and *E. ictaluri* attached to the bamboo, and their cell morphology was normal (Plumb 2018). However, we did not observe the formation of microcolonies. It needs to be noted that cell debris (CD; Figure 2-4 A, 2-4 B) from plant tissue, as well as from what appeared to be lysed bacteria (LC; Figure 2-4 A), made the interpretation of these images difficult (Figure 2-4).

Discussion

In this study we examined biofilm formation by five bacterial fish pathogens that affect many warm water aquaculture species worldwide (Austin and Austin 2007). We investigated the role of calcium on biofilm formation as it has been shown to have a positive effect on surface attachment in several bacterial species, including *F. columnare* (Cruz et al. 2012; Martínez-Gil et al. 2012; Cai et al. 2013). Our results showed that, in general, calcium promotes biofilm formation under static conditions although we found significant differences between strains of the same species as well as between species. Both *F. columnare* and *A. hydrophila* responded to higher calcium concentration by significantly increasing the amount of biofilm produced on microtiter plates. In a previous study, we have shown that biofilm formation by *F. columnare* was enhanced using 360 ppm hardness (≈ 2.5 mM [Ca^{2+}]) (Cai et al. 2013). For this study, we speculated that higher concentrations would further enhance attachment and colonization to surfaces. Results obtained in the present study confirmed our previous findings, and further demonstrated that higher concentrations, in some instance, enhanced biofilm production; however, biofilm production by *F. columnare* did not significantly increase beyond 4.5 mM [Ca^{2+}].

Biofilm formation by *A. hydrophila* is regulated by quorum sensing and can be influenced by different physicochemical factors (Lynch et al. 2002; Jahid et al. 2015). Salinity (higher than 0.25%) has been shown to inhibit biofilm formation by *A. hydrophila* on both man-made and natural surfaces (Jahid et al. 2015), but the effect of hardness had not been tested before. *Aeromonas hydrophila* virulent strain ML-09-119 showed a similar response to *F. columnare* strain ALG-00-530, with more biofilm being produced at 2.5 mM [Ca^{2+}] but without a significant increase at higher [Ca^{2+}]. On the contrary, biofilm formation by *A. hydrophila* strain AL01 showed a positive linear correlation with [Ca^{2+}] and achieved the highest levels of biofilm at 6.5 mM [Ca^{2+}].

Calcium had a positive effect on biofilm formation by *E. tarda* strain HL213 but no effect on *E. tarda* strain 26 or on either of the two *E. ictaluri* strains tested. Previous studies have shown little or no biofilm formation by these two species under *in vitro* conditions (Xiao et al. 2009; Martin et al. 2016). However, when *rpoS* (sigma factor; primary regulator of stationary phase genes in *E. coli*) is eliminated by an in-frame deletion, an *E. tarda* mutant was able to form thick biofilm (Xiao et al. 2009). This finding suggests that, under normal conditions, *rpoS* is involved in suppressing biofilm formation but the mechanisms by which biofilm formation is induced are still unknown. Similarly, biofilm formation was observed in *E. ictaluri* when *wabG* (a gene that codifies for a glucoronic acid transferase involved in LPS biosynthesis) was deleted (Martin et al. 2016). Based on previous studies, both species are capable of forming biofilm but they favored the planktonic state under our experimental conditions. Even when higher [Ca^{2+}] negatively affected planktonic growth and cells could be undergoing osmotic stress, we did not observe an increase in biofilm. Interestingly, biofilm formation by *E. piscidida* was significantly inhibited by [Ca^{2+}] although the biofilm produced under control conditions was low (OD<0.1).

Shafiei and co-authors compared the biofilm formation properties of 13 *E. piscicida* strains and except for one strain ATCC15947, all produced thin biofilms (OD ≈ 0.1) (Shafiei et al. 2016).

Biofilms are essential in maintaining water quality in closed aquaculture systems and could enhanced production in intensive systems by providing additional food sources (Pandey et al. 2014). However, biofilms can also harbor pathogens in fish farms and natural ecosystems (King et al. 2004; Hall-Stoodley and Stoodley 2005; Pandey et al. 2014). Recently, *F. columnare* was isolated from biofilms present in Finnish lakes connected to fish farms suggesting biofilm could serve as reservoir for this pathogen in the environment (Kunttu et al. 2012). Few studies have tested the ability of bacterial fish pathogens to attach to and colonize surfaces typically present in aquaculture systems (Pandey et al. 2014). Our results showed that all three species tested could attach to different materials and multiply on the surface. Overall, *A. hydrophila* virulent strain was the best performer and was able to colonize and multiply on all presented surfaces. Interestingly, *E. ictaluri* was also able to attach to surfaces and multiply on them, an unexpected result given the thin biofilm produced on microtiter plates. A possible explanation for this was the different incubation conditions. Microtiter plates were incubated under static conditions while broth cultures containing the different substrates were shaken. It is possible that greater aeration favors the attachment of *E. ictaluri* to solid surfaces. Recently in our laboratory, we have noticed that when *F. columnare* is cultured in flasks with well-sealed caps, biofilm formation around the edge of the flasks is reduced in comparison to when cotton stoppers were used (data not shown). The role of dissolved oxygen in biofilm formation is unknown at this point but warrants further investigation.

Scanning electron microscopy examination of the colonized materials showed two types of biofilm. *Flavobacterium columnare* formed thick, multi-layered biofilms in which cells

attached to the surface, but also to each other. Cells first attached to the surface and then formed microcolonies that were covered by abundant EPS, probably secreted to the extracellular milieu inside membrane vesicles (Cai et al. 2013). Noteworthy was the presence of spider web-like structures (SBS; Figure 2-3 C) produced by *F. columnare* cells attached to net filaments. These structures have been previously observed as bridging structures between cells during the early stages of biofilm formation. We observed the same structures but they were much more abundant in net filaments than on any other material tested in this study (Cai et al. 2013). The second type of biofilm observed was a single-layer produced by *A. hydrophila* and *E. ictaluri.* In that type of biofilm, the interaction between the cell and the surface is more important than the interaction between adjacent cells (Gupta et al. 2016). Both species present peritrichous flagella, which we hypothesize was used to accelerate the transient attachment to the surface. Membrane vesicles were observed on *A. hydrophila* and *E. ictaluri* attached cells but *A. hydrophila* seemed to produce EPS at earlier stages and in larger quantities than *E. ictaluri*.

In this study, we chose bamboo, flexible PVC, liner and nylon net as representative materials typically found in fish farms. Bamboo is an eco-friendly natural material that has versatile usages in aquaculture including its use as a biocarrier in wastewater treatments in shrimp ponds (Khatoon et al. 2007; Wang et al. 2008). We found that *A. hydrophila* and *E. ictaluri* colonized bamboo at a high density but *F. columnare* was greatly inhibited on bamboo substrates. The morphology of *F. columnare* on bamboo was different from that on liners or net materials with noticeable extended cell size. The elongated cells could be the result of filamentation induced by a stressful environment. Filamentation occurs when cell growth continues in the absence of cell division due to mutation or alteration of the stoichiometry of the cell-division components (Justice et al. 2008; Diaz-Visurraga et al. 2010). This morphological

plasticity is the phenomena geared toward bacterial survival. We speculate that the negative effect of bamboo on *F. columnare* cells is due to tannins present in the sample. Tannins have been showed to inhibit attachment and biofilm formation in *Pseudomonas aeruginosa* (Trentin et al. 2013). In developing countries, fish farmers using bamboo in their ponds claimed a lower incidence of columnaris outbreaks but those are anecdotal observations that have not been experimentally tested (personal communication, K. Veverica, Director of the E. W. Shell Fisheries Experiment Station, Auburn University).

It is typical for fish farms to suffer from recurrent epizootic outbreaks caused by the same bacterial pathogen. Biofilms are recognized as permanent or temporary refuge for bacterial pathogens in aquaculture facilities and would explain why some pathogens are hard to eliminate despite the use of disinfection protocols (Wietz et al. 2009). In this study we have shown how effective bacterial fish pathogens are at colonizing and forming biofilm on materials commonly used in aquaculture. Our results, albeit limited by the use of individual bacterial strains, single strain cultures, and laboratory conditions, have clear implications for biosecurity practices in farms and sets the basis for examining the effects of disinfectants not only in water but also on surfaces.

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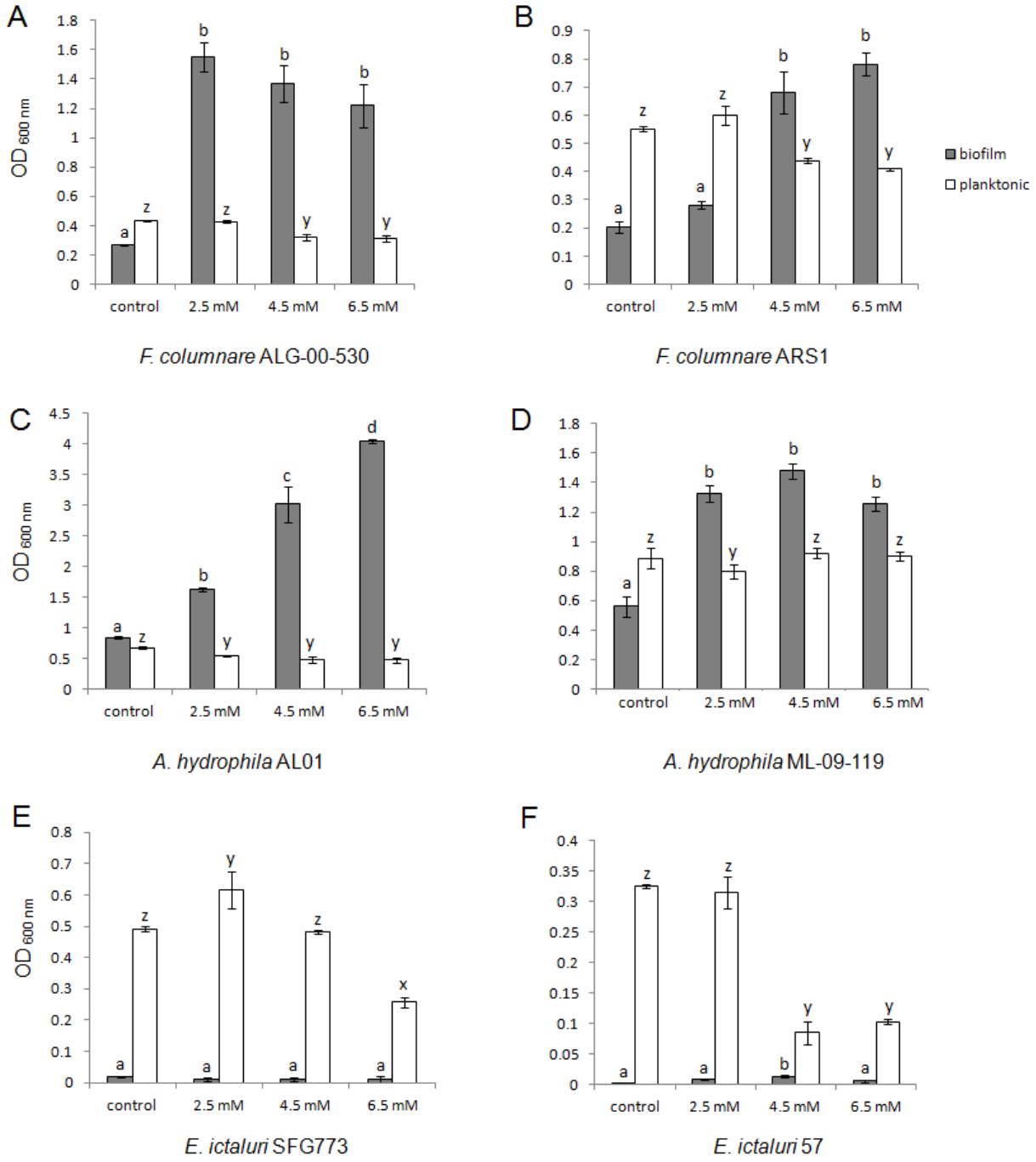
Table 2-1. Bacterial strains used in this study.

Species	Strains[a]	Geographic origin	Source	Date
F. columnare	ALG-00-530[a]	USA	Channel catfish	2000
F. columnare	ARS1[b]	USA	Channel catfish	1996
A. hydrophila	ML-09-119[c]	USA	Channel catfish	2009
A. hydrophila	AL01[a]	USA	Channel catfish	2001
E. ictaluri	SFG773[d]	USA	Channel catfish	2009
E. ictaluri	57[d]	USA	Channel catfish	2009
E. tarda	HL213[d]	Holland	Turbot	2006
E. tarda	26[d]	USA	Channel catfish	2002
E. piscicida	NCIMB 14824[T]	USA	European eel	1989

a, ALG-00-530, AL01 strains were isolated by personnel of the Southeastern Cooperative Fish Parasite and Disease Laboratory at Auburn University
b, ARS-1 strain was isolated by personnel of the USDA-ARS Aquatic Animal Health Laboratory, Auburn.
c, ML-09-119 strain was isolated by personnel of the West Alabama Fish Farm Center, Auburn University
d, SFG773, 57, HL213, and 26 were obtained from N. Bujan, University of Santiago de Compostela, Spain
T, type strain


A
B
C
D
E
F
OD 600 nm
control
2.5 mM
4.5 mM
6.5 mM
biofilm
planktonic
F. columnare ALG-00-530
F. columnare ARS1
A. hydrophila AL01
A. hydrophila ML-09-119
E. ictaluri SFG773
E. ictaluri 57

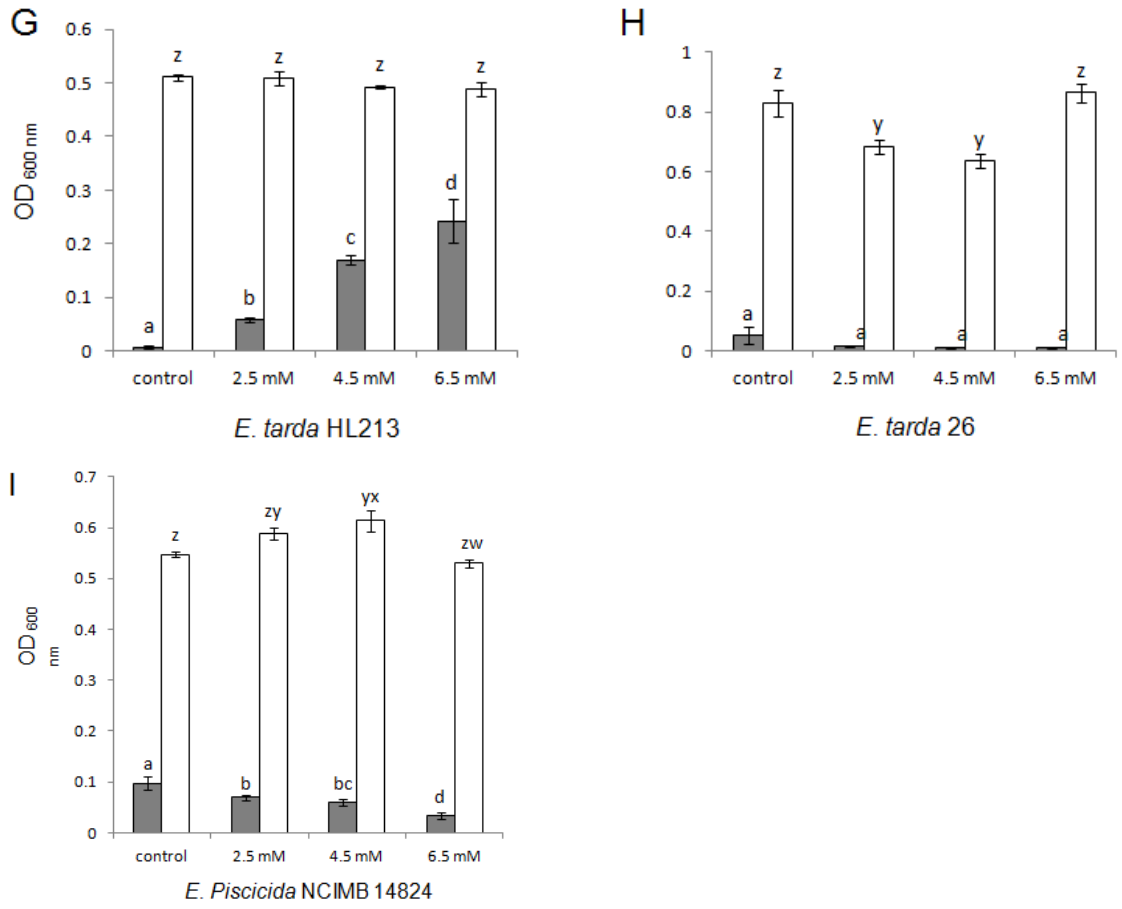
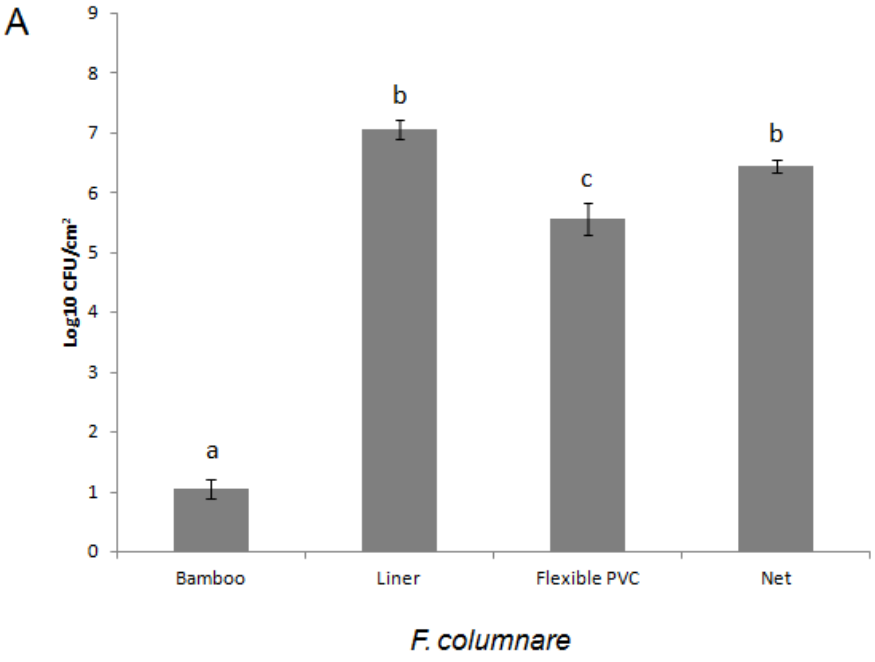
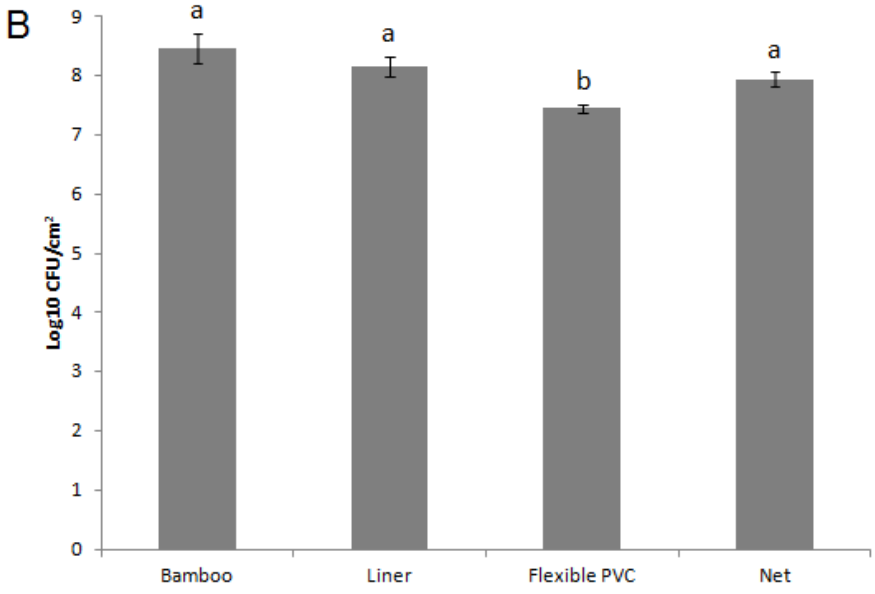


Figure 2-1. Adhesion (mean absorbance ± standard error) of bacterial fish pathogens to microtiter plates. White bars show total cell growth, and gray bars represent attached cells at 48 h post inoculation. Panels A to E show effects of calcium on fish pathogen *F. columnare*, *A. hydrophila*, *E. ictaluri*, *E. tarda*, and *E. piscicida*, respectively. Different letters on top of the bar indicate significant differences ($p < 0.05$).



F. columnare



A. hydrophila

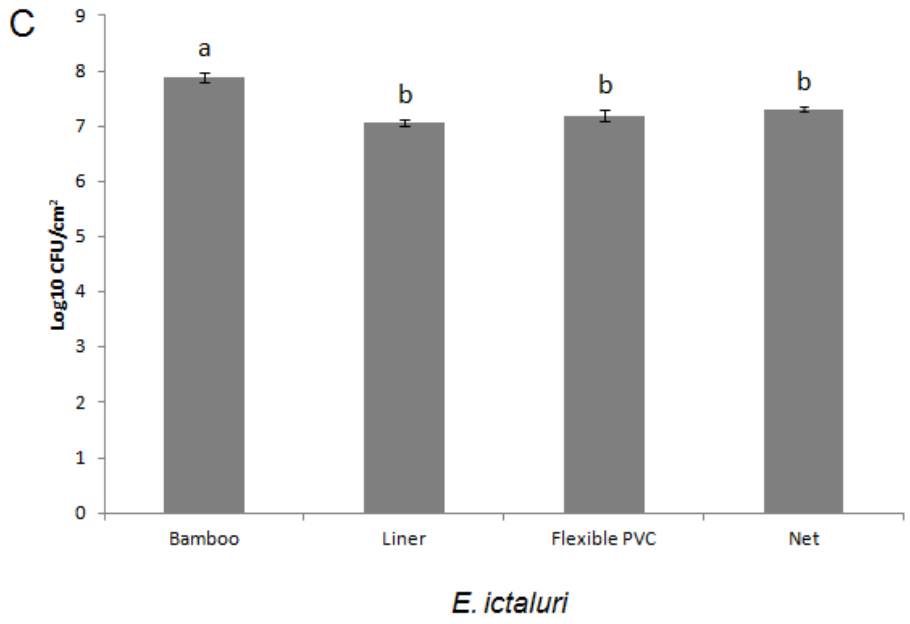


Figure 2-2. Quantification of bacterial attachments (mean attachment ± standard error) on aquaculture substrates. Panel A: *F. columnare*; panel B: *A. hydrophila*; panel C: *E. ictaluri*.

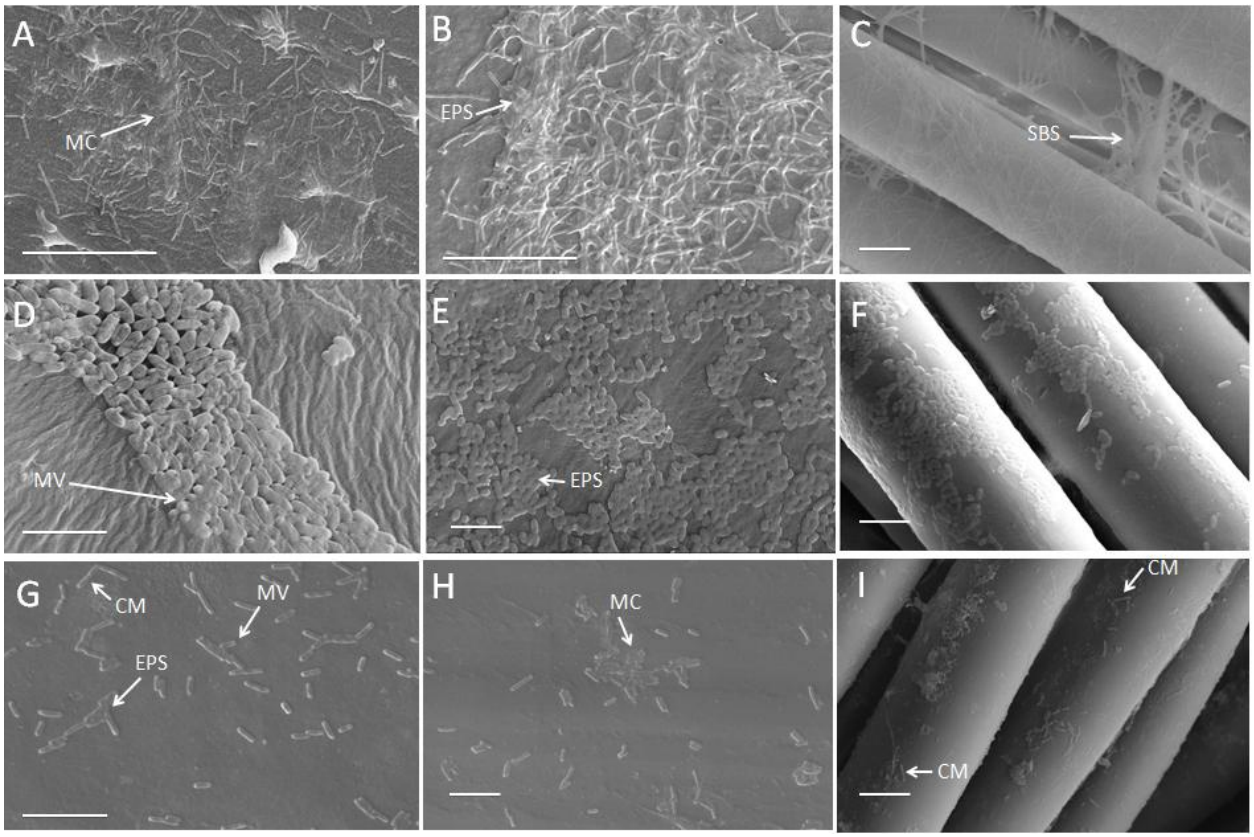


Figure 2-3. Scanning electron micrographs of attachment and biofilm of *A. hydrophila*, *F. columnare* and *E. ictaluri* on different substrates. Panels A, B, and C indicate attachment of *F. columnare* on PVC tube, liner, and net, respectively; panels D, E, and F indicate attachment of *A. hydrophila* on PVC tube, liner, and net, respectively; panels G, H, and I indicate attachment of *E. ictaluri* on PVC tube, liner, and net, respectively. Scale bars: panels A, B, C, E, G, H, and I, 5 µm; panel D, 7 µm; panel F, 10 µm. MV: membrane vesicle. MC: microcolony. CM: cell multiplying. SBS: spider web-like structure. EPS: extracellular polymeric substance.

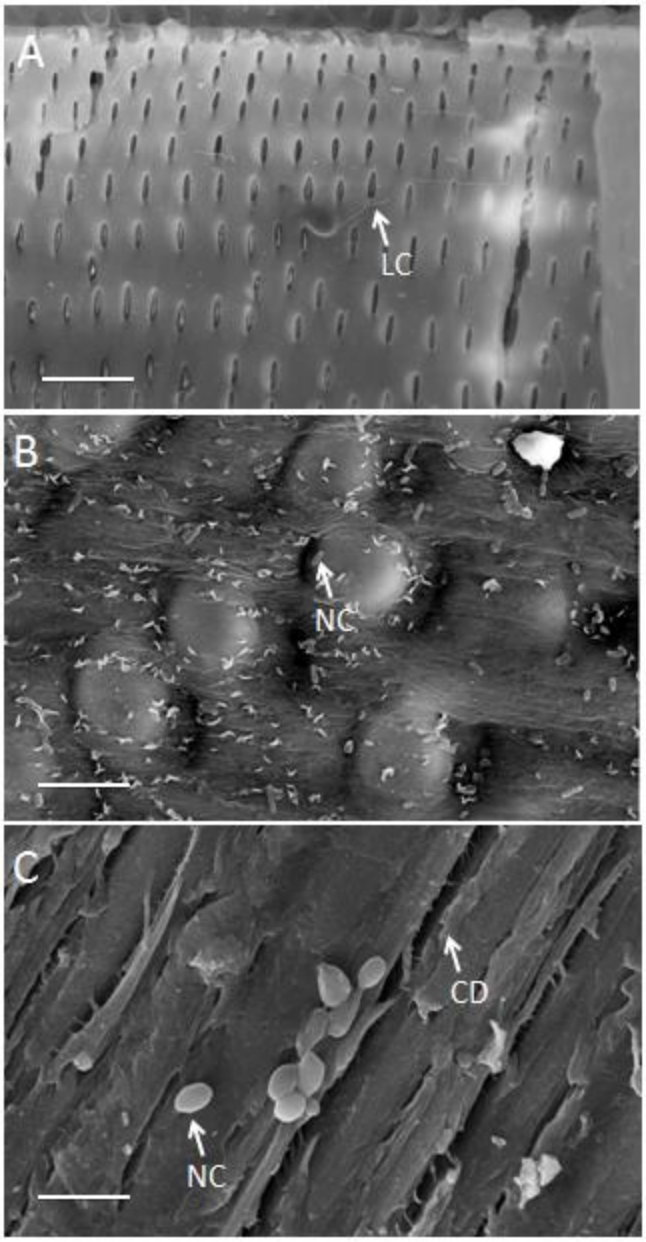


Figure 2-4. Bacteria attachment of *F. columnare* (panel A), *A. hydrophila* (panel B), and *E. ictaluri* (panel C), on bamboo substrates. Scale bars: panel A, 8 µm; panel B, 10 µm; panel C, 8 µm. NC, normal cell; CD, cell debris; LC, lysed cell.

Chapter 3. Transcriptome analysis of the fish pathogen *Flavobacterium columnare*: role of Ca^{2+} in biofilm formation

Abstract

Flavobacterium columnare is the causative agent of columnaris disease that affects cultured freshwater fishes worldwide. *F. columnare* easily colonizes surfaces by forming biofilm, which helps the pathogen resist antibiotic and disinfectant treatments. Previously, we had shown that increasing concentrations of Ca^{2+} promoted biofilm formation by *F. columnare*. The objective of this study was to further characterize the role of calcium on biofilm formation and to compare the transcriptome profiles of planktonic and biofilm cells. RNA-Seq analysis was conducted to identify genes that were differentially expressed between the following states: i) planktonic cells in control medium (P), ii) planktonic cells in calcium-enriched medium (P/Ca), and iii) biofilm cells in calcium-enriched medium (B/Ca). Total RNA was extracted with the TRIzol method and mRNA was reverse transcribed (after rRNA reduction) and sequenced using an Illumina HiSeq platform. The average sequenced reads that were successfully mapped to *F. columnare* strain 94-081 with an average of 15.44 Mb protein-coding sequences per sample. Overall, we identified 441 significant (FDR-adjusted $p < 0.05$, fold change > 2) differentially expressed genes (DEGs) between P and B/Ca samples; 112 significant DEGs between P/Ca and B/Ca samples, and 175 significant DEGs between P/Ca and P samples, corresponding to 15.87%, 4.03% and 6.30% of the total protein-coding sequences, respectively. The significant DEGs fell into different functional categories including polysaccharide synthesis, quorum sensing, iron homeostasis, type IX secretion system, and respiratory metabolism. Our results posit calcium as a critical signal in controlling bacterial surface adhesion and biofilm formation in *F. columnare*.

Introduction

Flavobacterium columnare is a Gram negative bacterium and the causative agent of columnaris disease in fish. This bacterial fish pathogen causes great economic losses in key aquaculture species worldwide such as channel catfish, tilapia, and trout (Austin and Austin 2007). *Flavobacterium columnare* is considered to be ubiquitous in freshwater environments including fish farms. Recurrent columnaris outbreaks are common in farms and eradication is extremely difficult. Columnaris disease is transmitted horizontally by fish-to-fish contact and asymptomatic fish can easily vector the pathogen into a farm. Although columnaris disease can be transmitted through water, cell counts of *F. columnare* in water are typically very low, even during active outbreaks. Once fish are removed from a system, *F. columnare* quickly disappears from the water column suggesting that, in the aquatic environment, the planktonic state of *F. columnare* is transient (Welker et al. 2005). Conversely, *F. columnare* colonizes natural and manmade materials quickly and remains viable, and infective, in biofilms even when fish host are not available (Cai et al. 2013).

Biofilms can be defined as bacterial communities that are attached to solid surface and covered with exopolysaccharides. Living in a biofilm offers aquatic bacteria many advantages over the alternative planktonic stage including a better adaptation to nutrient deprivation, and increased resistance to stressors such as desiccation and antimicrobial compounds (Branda et al. 2005). Biofilm development requires several key steps, i.e., transport and attachment of planktonic bacteria onto a surface, cell proliferation, formation of microcolonies, and dispersion of daughter cells into the water column (Matin et al. 2011). A previous study in our lab showed that the addition of calcium (Ca) strongly promoted biofilm formation in *F. columnare* (Cai et al. 2013). Similar results have been found in *Pseudomonas aeruginosa* (Sarkisova et al. 2005),

Pseudoalteromonas sp. (Patrauchan et al. 2005), *Sinorhizobium meliloti* (Rinaudi et al. 2006), *Xylella fastidiosa* (Cruz et al. 2012), *Enterobacter cloacae* (Zhou et al. 2013), *Aeromonas hydrophila* (Cai and Arias 2017), and *Enterococus faecalis* (Das et al. 2014). On the contrary, the addition of Ca suppresses biofilm formation in *Vibrio cholerae* (Bilecen and Yildiz 2009) and *Staphilococcus aureus* (Shukla and Rao 2013). It is well-known that Ca, a divalent cation, facilitates attachment of cells to surfaces by neutralizing cell surface charges (Williams et al. 2006). In addition, as a universal messenger, Ca regulates a wide range of bacterial functions including motility (Parker et al. 2016), quorum sensing (Werthén and Lundgren 2001) and expression of extracellular proteases (Patrauchan et al. 2007). Growing evidence suggest that Ca can also affect virulence traits. Cruz et al. (2012) showed that Ca increased twitching motility in the plant pathogen *Xylella fastidiosa*. *In vivo* studies by Straus *et al.*(Straus et al. 2015) demonstrated that higher Ca concentrations in rearing water increased *F. columnare* attachment to gills and subsequent host colonization. Columnaris disease is primarily an epithelial disease that causes necrotic skin and gill lesions (Austin and Austin 2007). As *F. columnare* colonizes the host, whitish plaques often appear along the fish body that exhibit yellow borders due to the masses of pigmented *F. columnare*. In a sense, columnaris disease can be considered a biofilm infection that invades the fish from the outside in (Bullard et al. 2011). Previous studies have shown that Ca promotes binding to surfaces and biofilm formation by *F. columnare* (Cai et al. 2013; Cai and Arias 2017) and appears to enhance virulence in fish, however, the mechanisms by which Ca regulates these processes are unknown.

The objectives of this study were: i) to identify differentially expressed genes (DEG) in planktonic cells *versus* cells living in biofilms, and ii) to characterize the metabolic pathways affected by Ca. This transcriptional analysis increased our understanding of the effect of

environmental stimuli (calcium) and subsequent cell responses that drive successful biofilm colonization.

Materials and Methods

Bacteria and culture conditions

Strain ALG-00-530 of *F. columnare* was used in this study. This strain was isolated from channel catfish in Alabama in 2000 (Arias et al. 2012), and it has been used in previous biofilm studies by our group (Cai et al. 2013). Bacterium was stored at -80 °C as glycerol stocks and routinely cultured on modified Shieh (MS) agar or broth with shaking (125 rpm) at 28 °C for 24-48 h (Shoemaker et al. 2005). Calcium concentration in the medium was adjusted to the desired value with $CaCl_2 \cdot 2H_2O$.

Biofilm formation under different Ca concentrations

Biofilm was induced in 250 Erlenmeyer flasks containing 100 ml of MS broth. Calcium concentration was adjusted to 0, 2.5, 4.5 and 6.5 mM with $CaCl_2 \cdot 2H_2O$. Approximately, 10^7 CFU/ml of ALG-00-530 were added to each flask from an overnight culture in MS (OD=0.8). To allow for air exchange, the mouth of the flasks was covered with autoclaved cotton plugs. Cultures were incubated at 28 °C for 48 h under shaking.

RNA extraction

Biofilm was induced in medium containing 4.5 mM [Ca^{2+}] in flasks as described above. Planktonic cells (1 ml) from control (no Ca) and calcium-supplemented media were pelleted by centrifugation at 3,000 rpm for 10 min at 4°C. Biofilm formed at the interphase between air-liquid, around the edges of the flask. Using a sterilized spatula, approximately 0.05 g (wet weight) of biofilm was collected from the calcium-supplemented medium. No observable biofilm was produced in control flasks (no Ca) therefore it was not possible to study gene expression in

biofilm in the absence of Ca. Planktonic cell pellets and biofilm samples were preserved in RNALater (Qiagen, Valencia, CA) at 4°C and frozen at -20°C until processed. RNA was extracted using the TRIzol (Invitrogen, Waltham, MA) method with some modifications required to obtain sufficient RNA from biofilm samples. Briefly, samples were transferred from RNALater into a 2 ml tube (MP Biomedicals, Dayton, OH) containing 0.4 g of acid-washed 500 mm glass beads and 1 ml TRIzol solution. Samples were vortexed for 40 s and chilled down on ice for an additional 30 sec. This process was repeated three times. Afterwards, 200 µl of chloroform was added to each sample and tubes were centrifuged at 12,000 g for 15 min at 4°C. The supernatant was transferred to a new tube and washed again with equal volume of chloroform/isoamyl alcohol (24:1) solution. The aqueous phase was then carefully transferred to a new tube and mixed with equal volume of 100% ethanol. RNA was precipitated at -20°C for at least 30 min and washed twice in 75% ethanol before resuspended it into 60 µl of DEPC-water. RNA concentration and purity was spectrometrically determined using a NanoDrop 1000 (Thermo Scientific, Waltham, MA). RNA concentration and integrity were further assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) before proceeding to RNA sequencing. Three independent replicates were carried out per sample type.

RNA sequencing

RNA-seq library preparation was carried out by HudsonAlpha Genomic Service Lab (Huntsville, AL, USA). RNA integrity numbers (RIN) of the 9 samples were between 7.8-9.8 (average 9.2). Ribosomal RNA was removed from each sample using the RiboZero Gold Epidemiology rRNA Removal kit (Illumina, San Diego, CA). cDNA libraries were prepared through the NEBNext Ultra II synthesis module workflow (New England BioLabs, Ipswich,

MA). The libraries were pooled and sequenced with 2 x 100 bp paired-end reads on an Illumina HiSeq2000 instrument.

RNA-seq data analysis

Adapter removal and quality trimming of FASTQ files was conducted using Trimmomatic (Bolger et al. 2014) by the HudsonAlpha Genomic Service Lab. Data quality was assessed using FastQC version 0.10.1 (Babraham Bioinformatics). Reads were aligned to *F. columnare* strain 94-081 (Kumru et al. 2016) protein-coding sequences (Accession number NZ_CP013992) using the Bowtie2 software (Langmead and Salzberg 2012). The alignment statistics were obtained using Samtools (Li et al. 2009). Differentially expressed genes were identified using EdgeR (Robinson et al. 2010), which calculated the expression in two or more samples and tested the statistical significance of each observed expression changes between them. Genes with an FDR-adjusted p value < 0.05, and fold change > 2 were identified as being differentially expressed. Functional annotation and gene ontology were also conducted using BLAST2Go PRO software (Conesa et al. 2005).

qPCR validation and statistical analysis

Nine genes that were up- or down-regulated by more than twofold were selected for qPCR validation of RNA-Seq data. Genes were selected based on their functional annotations and were involved in iron acquisition, quorum sensing, and protein secretion. The primer pairs used in this study are listed in Supplementary table 1S. Total RNA was reverse transcribed into cDNA with Applied Biosystems Reverse Transcription kit (Life Technologies Corporation, CA) according to the manufacture's protocol. QPCR was carried out using Applied Biosystems 7500 Real-time PCR system with the following cycle conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, and 60°C for 60 sec. Three biological replicates were used for RNA-

seq treatments and subsequent qPCR validation. QPCR results were statistically analyzed using SAS software version 9.2 (SAS Institute, Cary, NC). The significant difference was set at $p \leq 0.05$.

Results and Discussion

Biofilm formation under different Ca concentrations

In previous studies (Cai and Arias 2017), we characterized the dynamics of biofilm formation by *F. columnare* and the effect of Ca under statistic conditions. However, in order to obtain enough biomass for RNA extraction, we needed to determine the optimal calcium concentration for biofilm formation using a broth culture batch under shaking conditions to promote cell growth. Anecdotal observations in our laboratory suggested that biofilm formation by *F. columnare* is oxygen dependent and only by shaking a broth culture we could obtain enough biofilm biomass for RNA extraction. Without calcium supplementation, *F. columnare* cells remained planktonic and there was no observable biofilm in the flask (Figure 3-5). At the other end of the spectrum, at 6.5 mM Ca, cells grew in large clusters that quickly precipitated to the bottom under static conditions without visible biofilm on the glass walls. In addition, at 6.5 mM Ca, there was no turbidity in the medium due to the absence of planktonic cells. Based on those observations, 4.5 mM Ca was deemed as the best concentration to use for RNA-Seq analysis since it produced sufficient biofilm on the walls of the flask while enough planktonic cells remained in culture.

Quantitative analysis of global gene expression

RNA-seq reads were obtained from 9 samples: planktonic cells cultured in control MS medium (45.6 µM $CaCl_2 \cdot 2H_2O$), planktonic cells cultured in 4.5 mM Ca supplemented medium, and biofilm samples induced in 4.5 mM Ca supplemented medium (experiments were carried out

in triplicate for a total of 9 samples). RNA extraction from biofilm samples was challenging and standard RNA extraction kits did not yield sufficient RNA for downstream sequencing (data not shown). The combination of bead beating with the TRIzol method worked the best in our hands. Library sizes ranged from 12.36 Mb to 21.75 Mb, using *F. columnare* 94-081 as reference genome, with an average mapped reads of 15.44 Mb per sample (Figure 3-6) according to EdgerR. Principal component analysis (PCA) was performed on the raw RNA-seq reads that mapped to the reference genome to evaluate repeatability. The PCA plots showed that the biological replicates were well aggregated (Figure 3-1).

Figure 3-2 lists genes exhibiting significant expression changes (fold change ≥ 2; FDR-adjusted $p < 0.05$) among the three pair-wise comparison groups. Overall, we identified 175 DEGs (135 upregulated, 40 downregulated) between planktonic-control (P) and planktonic-4.5 mM Ca (P/Ca) groups (Figure 3-2 A); 112 DEGs (61 upregulated, 51 downregulated) between P/Ca and biofilm-4.5 mM Ca (B/Ca) groups (Figure 3-2 B), and 441 DEGs (286 upregulated, 155 downregulated) between P and B/Ca groups (Figure 3-2 C). These genes represent 6.30, 4.03, and 15.87% of the total protein-coding genes, respectively. A heatmap of the top 50 differentially expressed genes expression profiles was produced using Blast2Go software. Differently regulated genes were compared among the three different treatment groups. Gene expression profiles of P and PCa samples were more similar to each other than those exhibited by BCa samples (Figure 3-3). Gene descriptions were annotated as in GeneBank of NCBI. When appropriate, gene annotations were updated with information from InterPro protein database by InterProScan 5 (Jones et al. 2014) for predicted functional domains.

Differential expression of protein-coding genes in biofilm compared to those expressed by planktonic cells under the same Ca concentration

The top three upregulated DEGs in biofilm were Catalase (WP_060381974; log2 FC = 2.16), DNA starvation protect protein (WP_060382448; log2 FC = 2.08), and hypothetical protein (WP_060382447; log2 FC = 1.75), which contains a Leucine zipper-EF-hand with a transmembrane 1(LETM1) domain that plays a role in potassium and hydrogen ion exchange (Table ST 1). Other upregulated DEGs in biofilm fell into several functional categories including oxidative stress response (4 genes), transcriptional regulators (9 genes), TonB-dependent receptor (2 genes), ABC transporter complex (2 genes), and carbohydrate and protein biosynthesis (7 genes). In addition, two of upregulated genes in biofilm included a Ribosome inactivating protein gene (WP_060383199) that mediates the negative regulation of translation, and an abortive phage infection protein gene (WP_060381920) that leads to programmatic death of cells for self-protection (Dy et al. 2014). Gene ontology analysis indicated upregulated genes were mostly categorized as integral components of membranes (GO: 0016021; Figure 3-7A). The top downregulated DGEs in biofilm were associated with aerobic respiration metabolism (17 genes), T9SS system (2 genes), and carbohydrate and protein biosynthesis (11 genes).

Oxidative stress response in biofilm.

When facing with unfavorable or harsh conditions, microorganisms accumulate reactive oxygen species (ROS), potentially encountering a dangerous condition called oxidative stress (Gambino and Cappitelli 2016). In addition, the consumption of molecular oxygen leads to the production of ROS, including superoxide, hydrogen peroxide, and hydroxyl radicals. ROS is accumulated by a misbalance of ROS production and scavenge molecules and can be induced by both metabolic processes and diverse environmental stress factors, such as exposure of the microorganisms to ionizing conditions, higher or cold temperature and interaction with the host immune system (Gambino and Cappitelli 2016). Interestingly, cells are not only able to face

oxidative stress but also to exploit it using ROS as a signal to prepare for environmental changes. In fact, ROS signaling was regarded as the driving force behind biofilm dominance in many environmental niches (Gambino and Cappitelli 2016). In our current study, a number of antioxidative genes (catalase, AhpC, chromosome c peroxide, and thiol reductase thioredoxin) were identified in response to oxidative stress in biofilm-associated growth (Table 3-1). Catalase was the most upregulated protein-coding gene in biofilm samples when compared to planktonic cells (Log2FC= 2.16). This enzyme breaks down hydrogen peroxide into water and oxygen and its production might increase in response to the accumulation of hydrogen peroxide in biofilm. Alkyl hydroperoxide reductase (AhpC) is another primary scavenger of endogenous hydrogen peroxide in bacterial cells. Oh *et al.* (Oh and Jeon 2014) found that ROS substantially increased biofilm formation in the AhpC mutant of *Campylobacter jejuni* under specific culture conditions. They demonstrated that AhpC played a critical role in biofilm development, although the kind of ROS responsible for enhanced biofilm formation in the AhpC mutant is still unknown.

Oxidative burst has been proposed as a signal that induces bacteria to form biofilms in order to protect themselves against adverse environmental conditions (Karatan and Watnick 2009). For example, *P. aeruginosa* increased the synthesis of the exopolysaccharide alginate when it was exposed to hydrogen peroxide (Mathee et al. 1999). In addition, EPS production pathways are closely connected to environmental stress sensors, and are activated in accordance with external conditions. In the presence of oxidative stress, increased production of polysaccharides was observed in *Azotobacter vinelandii* (Villa et al. 2012) and *Bacillus subtilis* (Gambino et al. 2015) biofilm matrices. We speculate that an increase of Ca concentration lead to the ionization of intracellular water and subsequently to oxidative stress, which triggered the physiological shift from planktonic stage to biofilm. We have previously established that *F.*

columnare favors the production of biofilm at the air/liquid interface on glass slides (Cai et al. 2013) and on other substrates (Cai and Arias 2017) in where oxygen and ROS are expected to be abundant.

Aerobic respiration is greatly limited in biofilm.

A previous study showed that the shift from planktonic to biofilm growth entails adaptation to low oxygen, nutritional limitations, higher cell density, and other stressful conditions (Phillips et al. 2012). In our study, protein-coding genes involved in aerobic respiration were greatly downregulated in biofilm compared to those in planktonic cells (Table 1). Among them were genes involved in electron transfers during oxidative phosphorylation, including NADH-ubiquinone reductase, cytochrome c, and cytochrome oxidase that use O_2 as the final electron acceptor (Table 3-1). In addition, the gene that codifies for the o-succinylbenzoate CoA ligase, an enzyme involved in menaquinone (vitamin K_2) biosynthesis was highly upregulated in biofilm samples. Menaquinone plays an essential role in several anaerobic electron transport systems as it is the major electron carrier during anaerobic growth and can use various electron acceptors (Daruwala et al. 1997). This process of anaerobic respiration allows the bacteria to generate the energy required to survive under oxygen-limited conditions. Although anaerobic respiration in *F. columnare* has not yet been reported, bioinformatics analysis showed that, indeed, the *F. columnare* genome encodes for enzymes that are involved in anaerobic metabolism such as denitrification that could, theoretically, allow the bacterium to remain metabolically active under anaerobic conditions. This *in silico* prediction could explain the high levels of *F. columnare* cells detected in the anaerobic sediments of catfish ponds by metagenomic analysis (Nho et al. 2015). However, aerobic respiration provides the basic energy for bacterial metabolism in aerobic bacteria such as *F. columnare*. The down-

regulation of genes involved in aerobic respiration indicated that cells living in biofilms exhibited a lower metabolic rate, including less protein synthesis, relative to their planktonic counterparts.

Cells in biofilm underwent nutrient starvation.

Biofilm accumulation is determined by the equilibrium between cell attachment, growth, and detachment. Among all the possible environmental cues, nutrient starvation and accumulation of harmful metabolites have been proposed as a trigger for cell detachment (Hunt et al. 2004). We identified a gene that codifies for Dps protein (DNA protecting protein under starved conditions) that was highly upregulated in biofilm. It has been shown that Dps is capable of providing protection to cells during exposure to severe environmental assaults, including oxidative stress and nutritional deprivation (Calhoun and Kwon 2011). *In vitro*, Dps forms non-specific stable complexes with DNA, which becomes DNase resistant (Almiron et al. 1992). Many Dps sequences display an N-terminal extension of variable length that contains two or three positively charged residues that extend into the solvent and are thought to play an important role in the stabilization of the Dps-DNA complex (Ren et al. 2003). Biofilms are known to contain high levels of extracellular DNA (eDNA) (Montanaro et al. 2011), which binds with other biopolymers such as polysaccharides, protein, or metabolites like phenazines, and function as scaffold to provide structural integrity to the EPS matrix (Das et al. 2013). The synthesis and secretion of Dps could be a strategy, not only to protect the cellular DNA in a nutrient starvation context, but also to maintain the biofilm structure by stabilizing eDNA.

In addition, a ribosome-inactivating protein gene that function as a protein synthesis inhibitor during nutrient deprivation was upregulated in biofilm. Similarly, a tyrosine phosphatase protein-coding gene, that has been detected in *Pseudomonas aeruginosa* biofilm,

and in starved cells of *Burkholderia cenocepacia* was found to be up-regulated (Xu et al. 2015; Andrade et al. 2016).

Effect of Ca on planktonic cells: comparison between planktonic cells cultured with no Ca supplementation and at 4.5 mM Ca

We found more upregulated genes than downregulated genes (135 vs 40) in planktonic cells cultured in calcium-supplemented medium, indicating that Ca activated several metabolic pathways in the cells. Upregulated genes fell into several functional groups including calcium homeostasis, iron acquisition, protein secretion system, carbohydrates metabolism and transcriptional regulatory system. Downregulated genes belonged to categories such as respiration electron transfer, and carbohydrates synthesis (Table 3-2).

Calcium induced the synthesis of genes involved in iron acquisition and transport.

Siderophore and TonB-dependent receptors (TBDT) genes were upregulated in cells cultured with Ca. Previous studies found that the level of intracellular iron is a signal for the expression of several virulence genes in *F. psychrophilum* (Møller et al. 2005), and it serves as a signal for biofilm development in *P. aeruginosa* (Banin et al. 2005) and *S. maltophilia* (García et al. 2015). In this study, we identified a number of protein-coding genes involved in iron acquisition and transport that were induced in Ca-supplemented medium. Four siderophore biosynthesis genes (Log2 fold change = 5.6-8.2) and 6 TBDT genes associated with siderophore transportation (Log2 fold change = 1.1-6.1) were upregulated (Table 3-2). In bacterial cells, iron uptake systems usually contain two components, i.e. a siderophore complex and an outmembrane transport proteins. The low-molecular weight siderophore secrete into the outer environment where it chelates ferric iron. The iron-siderophore complex is subsequently mobilized into the cells through outer membrane proteins, e.g. TonB-dependent receptors (TBDT). TBDTs have

transmembrane-transport activity but can also be associated with extracellular or intracellular messengers to initiate a change in cell activity. In addition to the cytoplasmic membrane (CM), which is common to all organisms, Gram-negative bacteria possess an outer membrane (OM), which hinders the uptake of essential nutrients. A previous study showed that TBDTs cannot only positively transport iron complexes and vitamins, but also various carbohydrates (Schauer et al. 2008). TBDTs homologous *SppR* in *Pseudomonas aeruginosa* is regulated by an iron starvation sigma factor, and modulated its swarming motility (Pletzer et al. 2016). Pathogenic bacteria encounter iron-limiting conditions in host tissues (García et al. 2015), and, similarly, aquatic bacteria living in biofilms are exposed to low free iron concentrations. It is very intriguing, from an epidemiological point of view, that *F. columnare* biofilms expressed virulence factors required for host colonization.

It has been proposed that siderophores are not just dedicated to iron shuttling but most likely aid in maintaining homeostasis of other metal ions in microorganism (Brandel et al. 2012). This interaction can affect the bioavailability and consequently the toxicity of metals to microorganism, e.g. by removal of excess metals from effluents (Gilis et al. 1998). Siderophore production showed strain specificity to elevated Ca concentration in *P. aeruginosa*. In one strain, siderophore was increased in high abundance in both biofilm and planktonic samples induced by elevated Ca cocentration, while in the other strain, the siderophore was only observed in the planktonic cells cultured under high Ca levels (Patrauchan et al. 2007). This implied a subtle relationship between the calcium supplementation and iron acquisition system, although the role of Ca in iron-scavenging proteins production is not clear yet. We hypothesize that *F. columnare* perceives an iron starvation cue and expresses multiple iron source utilization systems in the biofilm conditions.

Besides the siderophore-mediated iron acquisition system, several other iron uptake and transporter protein-coding genes were also identified, including iron transporter (WP_060383657), ferrous iron transport protein B (WP_060383656), and NifU family protein containing iron-sulphur cluster (WP_060381373).

Biofilm signaling.

Quorum sensing (QS) is an important component in the biofilm development, however, the quorum sensing mechanism in *F. columnare* has not been elucidated yet. The LuxI/LuxR system is a well-studied quorum-sensing system present in many Gram-negative bacteria. LuxI-type proteins synthesize acylated homoserine lactone (AHL) autoinducers, which modulate the activity of LuxR-type transcriptional activators to activate corresponding gene expression. LuxR QS regulators have been identified in diverse Proteobacteria species, and they seem to disseminate across species by horizontal gene transfer (Gray and Garey 2001). In our study, an upregulation of the LuxR family transcriptional regulator was identified in biofilm (compared to planktonic cells in control medium), as well as in planktonic cells cultured in Ca-supplemented (Table 2 and ST3). Our data showed that calcium-supplemented medium, regardless of the life stage of the cells, increased LuxR expression although the LuxI-type protein gene was not identified in the *F. columnare* strain 94-081genome. Previous study showed that *Escherichia coli* and *Salmonella typhimurium* have the potential to respond to acyl homoserine lactone autoinducers generated by other organism through their own LuxR system that acquired horizontally. Although the QS molecules (such as AHL or autoinducer 2) were not identified in this study, the bacterium was possible to maintain the ability to detect this type of molecules in the surrounding environment through the LuxR system. Declercq et al. screened the typical QS molecules, i. e. AHL and autoinducer 2, in both high virulent and low virulent strains of *F.*

columnare with different biosensors, and the results proved to be all negative (Declercq et al. 2015).

T9SS system.

The type IX secretion system (T9SS), also called por secretion system, is an outmembrane protein secretion system that is widespread among members of the Phylum Bacteroidetes (McBride and Zhu 2013). T9SS protein substrates have conserved C-terminal domains (CTDs) in common, which are important for secretion, posttranslational modification, and cell surface attachment (Slakeski et al. 2011; Wang et al. 2014). A novel C-terminal signal peptidase was also identified to be responsible for the cleavage of CTDs of the T9SS substrates in *P. gingivalis*, indicating the significance of the CTD in signaling as well. Genetic manipulation of RgpB (a surface-associated cysteine proteinase coding gene) to remove the CTD resulted in the loss of posttranslational modification, surface attachment, and function of the enzyme (Veith et al. 2013). In our study, calcium supplementation enhanced the expression of the T9SS C-terminal target domain-containing proteins in transcriptional level of the planktonic samples supplemented with calcium, which may partially explain the calcium function in promoting cell attachment and subsequent biofilm formation (Table 32 and Appendix 2). In calcium-enhanced medium, T9SS CTD genes were upregulated in planktonic cells compared to their biofilm counterparts, indicating the possible role of T9SS in surface recognition and adhesion production. In *F. johnsoniae*, T9SS is required for secretion of the cell-surface motility adhesins *SprB* and *RemA*, the extracellular chitinase *ChiA*, and dozens of other proteins. Mutations in T9SS result in defects in gliding motility (McBride et al. 2015). *F. columnare* T9SS mutants also exhibited decreased virulence towards zebrafish and were defective in secretion of soluble extracellular toxins (McBride et al. 2015). The high abundance of T9SS production in

calcium-supplemented medium (including both planktonic and biofilm cells) may partially explain the elevated virulence of *F. columnare* by facilitating the attachment of the *F. columnare* cells to the channel catfish gill tissue at increased water hardness (higher Ca) (Straus et al. 2015).

Validation of RNA-seq profiles by qPCR

In order to validate the differentially expressed genes identified by RNA-seq, 10 genes from various categories were selected for qPCR confirmation. Fold changes from qPCR were compared with the RNA-seq express analysis results. As shown in Fig. 3, qPCR results were significantly correlated with the RNA-seq results (correlation coefficient 0.88), indicating a consistency with the transcriptional expression analysis.

Conclusion

In summary, our data showed that calcium supplementation induced a transcriptional response that promotes biofilm formation. Transcriptional data suggested that biofilm-embedded bacteria experienced general stresses including nutrient and oxygen limitation, and accumulation of metabolic wastes. The major categories of DEGs include proteins required for iron uptake, oxidative stress response, respiration pathway, and biosynthesis and secretion of extracellular products. Calcium seemed to activate the T9SS protein secretion system and upregulated siderophore expression, both of which are considered main virulence factors in the genus *Flavobacterium*. Our study showed that calcium was able to function as a signal to increase the biofilm formation and virulence of *F. columnare* at the transcriptional level.

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Table 3-1. Selected genes in key functional categories differentially expressed in biofilm compared to planktonic cells (both cultured in 4.5 mM [Ca^{2+}]). Genes were selected based on fold change and their functional relevance.

Functional group	RefSeq	Product	Fold change (log2)	FDR (adjusted p value)
EPS/LPS biosynthesis				
	WP_060381967.1	1,4-polygalactosaminidase	-1.72	1.1E-19
	WP_060381836.1	UDP-N-acetylmuramate--alanine ligase	-1.24	4.1E-05
	WP_060381978.1	SAM-dependent methyltransferase	-1.04	3.8E-07
	WP_060382475.1	Glycosyltransferase	1.99	9.3E-03
Amino acid metabolism				
	WP_060383852.1	Branched-chain amino acid aminotransferase	1.53	3.52E-16
	WP_060382313.1	Anthranilate synthase	1.35	2.71E-10
	WP_060382314.1	Anthranilate phosphoribosyltransferase	1.03	1.18E-06
	WP_060382792.1	Glycyl aminopeptidase M61	-1.11	2.2E-11
	WP_060382658.1	Von willebrand factore type protein	-1.54	5.8E-10
T9SS				
	WP_060383582.1	T9SS C-terminal target domain-containing protein	1.53	3.52E-16
	WP_060383402.1	T9SS C-terminal target domain-containing protein	1.13	3.07E-05
Nutrient limitation				
	WP_060381920.1	Abortive phage infection protein	-1.17	5.0E-08
	WP_060383199.1	Ribosome inactivating protein	-1.30	9.27E-10
	WP_060381977.1	Protein-tryosine-phosphatase	-1.04	1.39E-09
	WP_060382448.1	DNA starvation protect protein under stressful or	-2.08	8.3E-23

		poor nutriention conditions		
Regulatory functions				
	WP_060382614.1	AraC family transcriptional regulator	-1.71	2.9E-11
	WP_060381931.1	Transcriptional regulator	-1.31	5.4E-08
	WP_060381980.1	ArsR family iron regulation transcriptional regulator	-1.54	4.7E-4
Oxidative stress response				
	WP_060381974.1	Catalase	-2.16	8.71E-10
	WP_060381975.1	Alkyl hydroperoxide reductase	-1.50	8.29E-15
	WP_060382235.1	Cytochrome c peroxidase	-1.41	1.31E-10
	WP_014164670.1	Thiol reductase thioredoxin	-1.03	2.35E-09
Respiratory metabolism				
	WP_060381919.1	O-succinylbenzoic acid--CoA ligase	-1.11	7.3E-03
	WP_060382480.1	Cytochrome C oxidase subunit III	2.12	7.45E-06
	WP_060382479.1	Cytochrome c oxidase accessory protein CcoG	2.03	2.9E-06
	WP_060382481.1	Cytochrome C oxidase subunit IV	1.74	2.3E-05
	WP_060382478.1	Cytochrome C oxidase Cbb3	1.74	1.94E-03
	WP_060381577.1	Cytochrome c oxidase subunit I	1.50	1.7E-02
	WP_060381570.1	Cytochrome C	1.39	3.7E-04
	WP_060381576.1	Cytochrome C oxidase subunit II	1.39	2.6E-02
	WP_060381561.1	NADH dehydrogenase	1.27	6.5E-03
	WP_060381560.1	NADH oxidoreductase (quinone) subunit F	1.18	6.1E-03
	WP_060381566.1	NADH-quinone oxidoreductase subunit L	1.07	1.7E-03
	WP_060381562.1	NADH:ubiquinone oxidoreductase subunit H	1.06	1.1E-02
	WP_014164161.1	NADH-quinone oxidoreductase subunit I	1.00	9.1E-03

Table 3-2. Selected genes in key functional categories differentially expressed in planktonic cells cultured in 4.5 mM [Ca2+] compared to planktonic cells cultured in control medium.

Functional group	RefSeq	Product	Fold change (log2)	FDR (adjusted *p* -value)
Siderophore synthesis				
	WP_060383187.1	Siderophore alcaligin biosynthesis protein	8.20	4.1E-41
	WP_060383189.1	Siderophore transcriptional regulator	7.99	3.3E-53
	WP_060383190.1	LucA/lucC family siderophore biosynthesis protein	5.64	1.7E-44
	WP_060383888.1	Siderophore biosynthesis protein	7.21	6.4E-61
Iron transfer				
	WP_060383657.1	Iron transporter	2.45	3.7E-13
	WP_060383656.1	Ferrous iron transport protein B	1.53	3.2E-09
	WP_060381373.1	NifU family protein (iron-sulfur cluster binding)	1.19	4.7E-10
T9SS secretion				
	WP_060382679.1	T9SS C-terminal target domain-containing protein	7.61	8.0E-43
	WP_060383582.1	T9SS C-terminal target domain-containing protein	1.58	2.8E-05
	WP_060383035.1	T9SS C-terminal target domain-containing protein	1.65	6.6E-10
TonB-dependent receptor				
	WP_060383861.1	TonB-dependent receptor	6.03	6.9E-40
	WP_060383182.1	TonB-dependent receptor	6.01	4.4E-49
	WP_060383101.1	TonB-dependent receptor	5.18	3.7E-33
	WP_060382917.1	TonB-dependent receptor	-1.22	5.7E-04
	WP_060382921.1	TonB-dependent receptor	-1.59	4.4E-02

Calcium homeostasis				
	WP_060382447.1	Hypothetical protein with EF-hand motif	1.75	5.8E-24
	WP_060383353.1	ABC transporter permease	1.56	1.9E-11
	WP_060383434.1	Outmembrane efflux protein	1.46	2.3E-08
	WP_060383433.1	Efflux transporter periplasmic adaptor subunit	1.21	1.7E-07
Integral membrane component				
	WP_060381689.1	Flagellar motor protein MotA	1.47	2.9E-06
	WP_060383854.1	Aquaporin	1.69	1.9E-11
Respiration system				
	WP_060381562.1	NADH:ubiquinone oxidoreductase subunit H	-1.01	1.4E-02
	WP_060381556.1	NADH dehydrogenase	-1.02	2.4E-03
	WP_060381560.1	NADH oxidoreductase (quinone) subunit F	-1.12	9.2E-03
	WP_060381555.1	NADH-quinone oxidoreductase subunit A	-1.12	5.3E-03
	WP_060381577.1	Cytochrome c oxidase subunit I	-1.20	5.4E-02
	WP_060381576.1	Cytochrome C oxidase subunit II	-1.23	3.5E-02
	WP_060381574.1	Cytochrome C	-1.30	3.2E-02
	WP_060382919.1	Cytochrome-c peroxidase	-1.31	8.7E-03
	WP_060381571.1	Quinol:cytochrome C oxidoreductase	-1.41	3.2E-02
	WP_060381575.1	Quinol:cytochrome C oxidoreductase	-1.32	2.9E-02
Gliding motility	WP_060383259.1	Gliding motility protein GldN	-1.01	1.5E-02
Quorum sensing	WP_060383185.1	LuxR family transcriptional regulator	3.94	2.8E-29

Table 3-3. Primers used for QPCR validation (5' to 3')

Gene	Forward	Reverse
Siderophore	TCAGAGCGCAGCAGAAGTTT	TGATCTCGTTTGGCTTCTGGT
T9SS	AATGGAGCTTGGAAAGGGAA	TGAGGCTCTTCATCCACAACA
TonB-dependent receptor	TTCATTACAGCCCAGCGGTT	TTAGCCCCATGCGTAACACC
NADH: ubiquinone oxidoreductase subunit H	ATTGCCAA CAGCAACAGG	GGTGACCACCAATAAGTTCAGC
Catalase	TATTGACGGTAACGGCGGAG	AGGTTACCTGGTTGTGTGTAGT
Quorum sensing	ACCTTTCGCACACACAGAAA	GCAATGTCGTTCTTTAGGCTGT
Cytochrome c oxidase accessory protein	ACAGTCTCCCTTGCCTAATAAAGA	AGGACCACTACACAATACAGGT
Protein-tryosine-phosphatase	AGGACATCCCTCATCTGCATT	TCTGGAGGAACAGAAAGTACCAC
Adenylate kinase (reference gene)	TTCTTTTCAACTTCAGATTCCAACA	GGAAAACCTGGAGCGGGAA

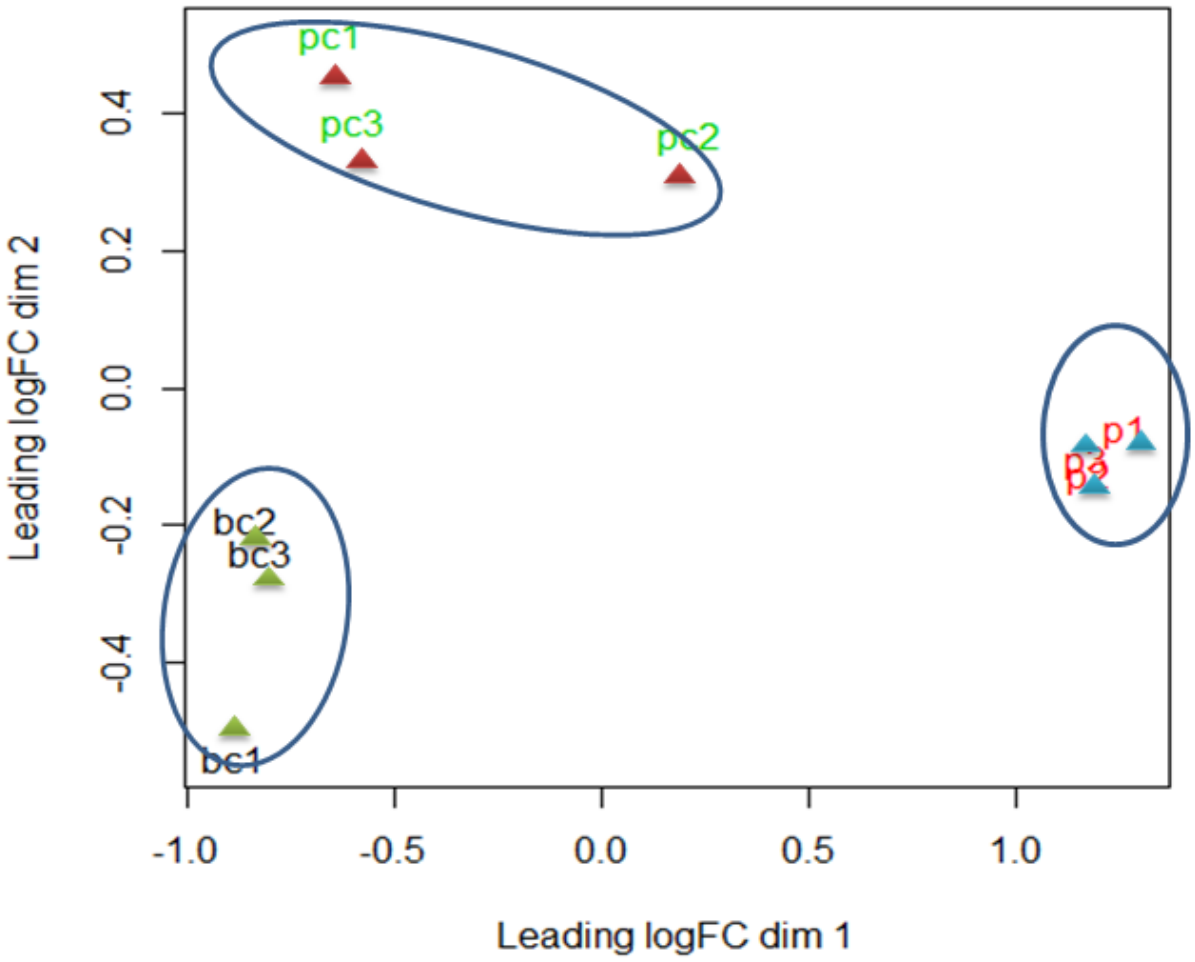


Figure 3-1. MDS plot analysis of the samples after mapping to reference sequence *Flavobacterium columnare* 94-081. P, planktonic samples in control medium; PC, planktonic samples in Ca-supplemented medium (4.5 mM); BC, biofilm samples in Ca-supplemented medium (4.5 mM).

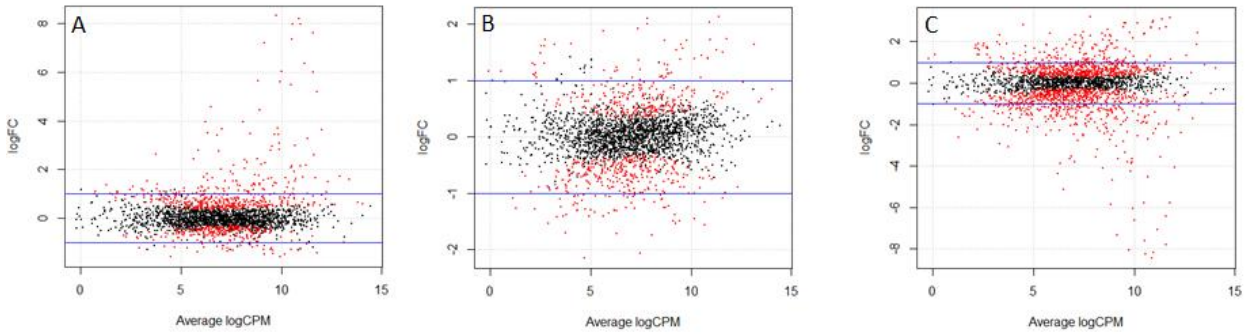


Figure 3-2. PlotSmear plot of the samples. Panel A: Genes expressed in planktonic cells cultured in Ca-supplemented medium (up) vs planktonic cells cultured in control medium (down). Panel B: Genes expressed in planktonic cells cultured in Ca-supplemented medium (up) vs biofilm in Ca-supplemented medium (down). Panel C: Genes expressed in planktonic cells cultured in control medium (up) vs biofilm in Ca-supplemented medium (down). The red dots represent DEGs with $p > 0.05$, and the blue lines set threshold with a fold change > 2.

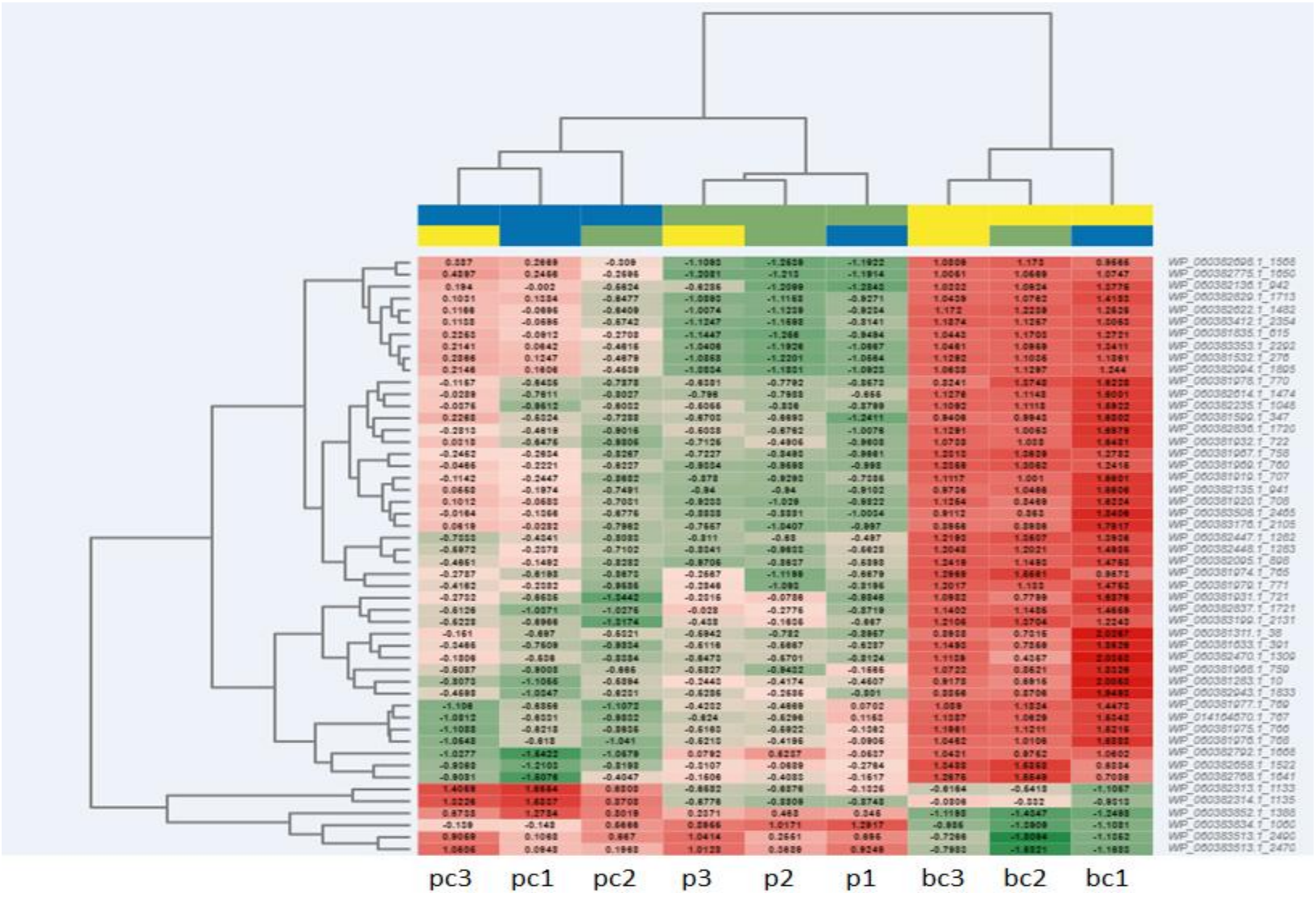


Figure 3-3. Heap-map of the top-50 differentially expressed genes expression profiles among the 9 samples after 48 h incubation. Red and green colors indicate up- and down-regulation as log2 transformed values, respectively.

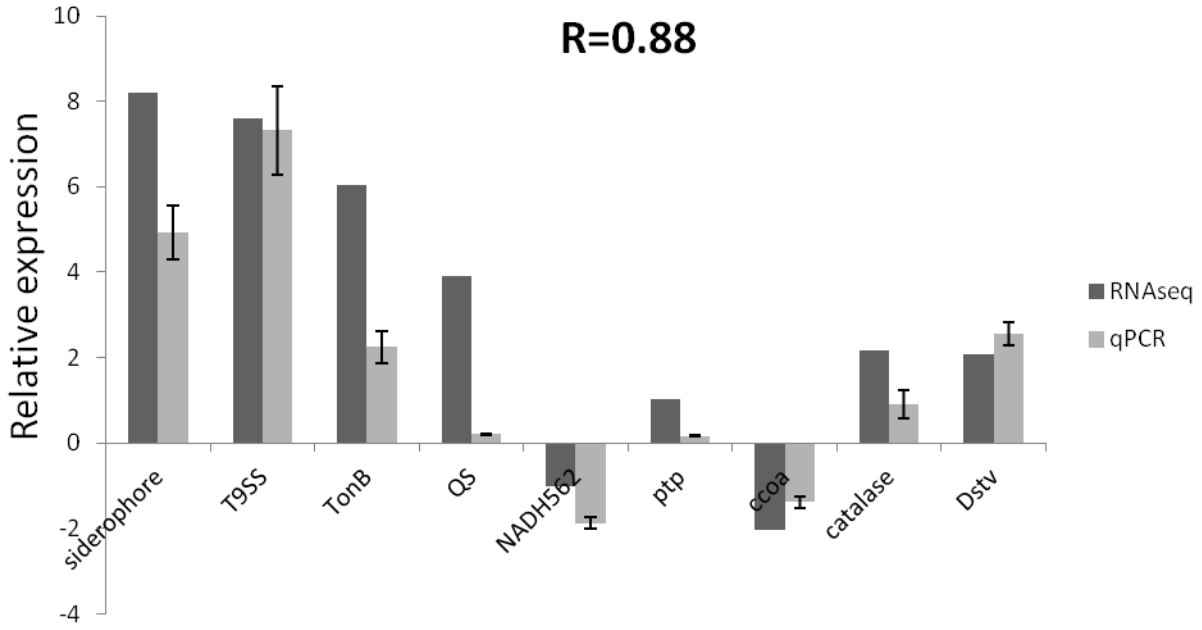


Figure 3-4. Comparison of relative fold change between RNA-seq and qPCR results. Protein coding sequences abbreviations: Siderophore alcaligin biosynthesis protein (WP_060383187), siderophore; T9SS secretion (WP_060382679), T9SS; Ton-B dependent receptor (WP_060383861), TonB; Quorum sensing (WP_060383185), QS; NADH subunit H (WP_060381562), NADH562; Protein-tryosine-phosphate (WP_060381977), ptp; Cytochrome c oxidase accessory (WP_060382479), ccoa; Catalase (WP_060381974), catalase; DNA starvation protect protein (WP_060382448), Dstv.

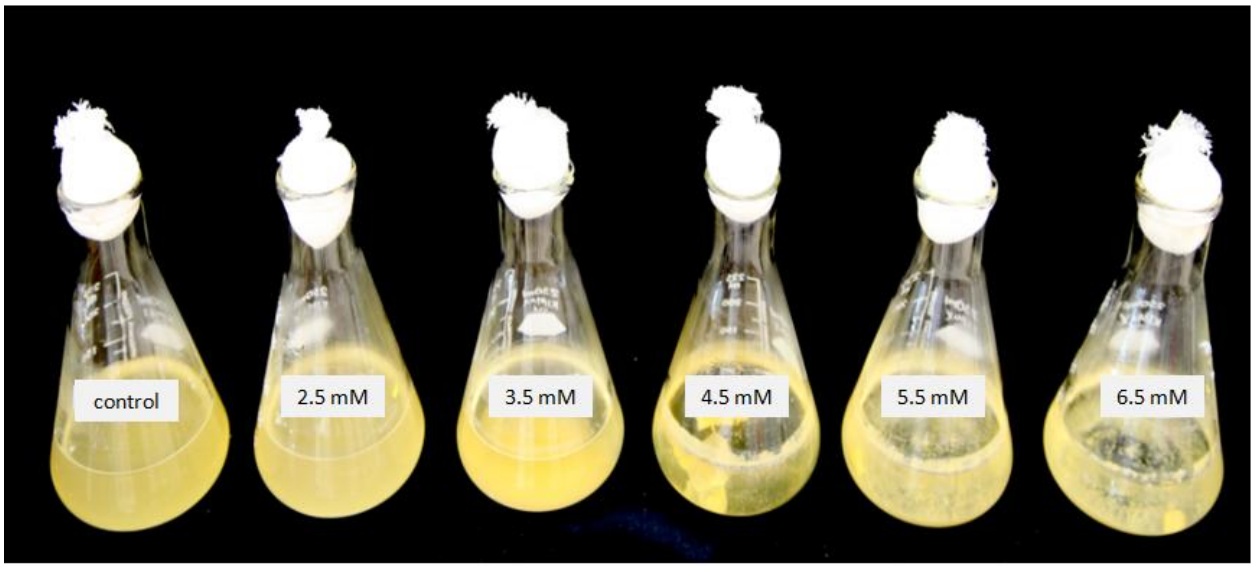


Figure 3-5. Biofilm formation of *F. columnare* in flasks after 48 h inoculation under serial Ca concentration.

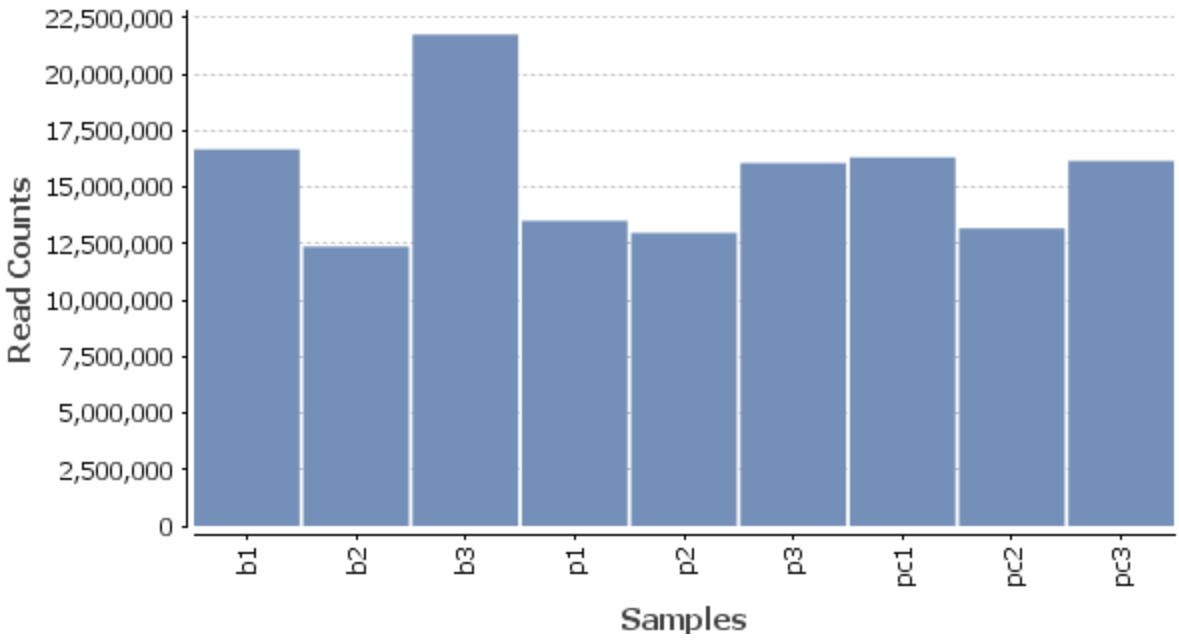
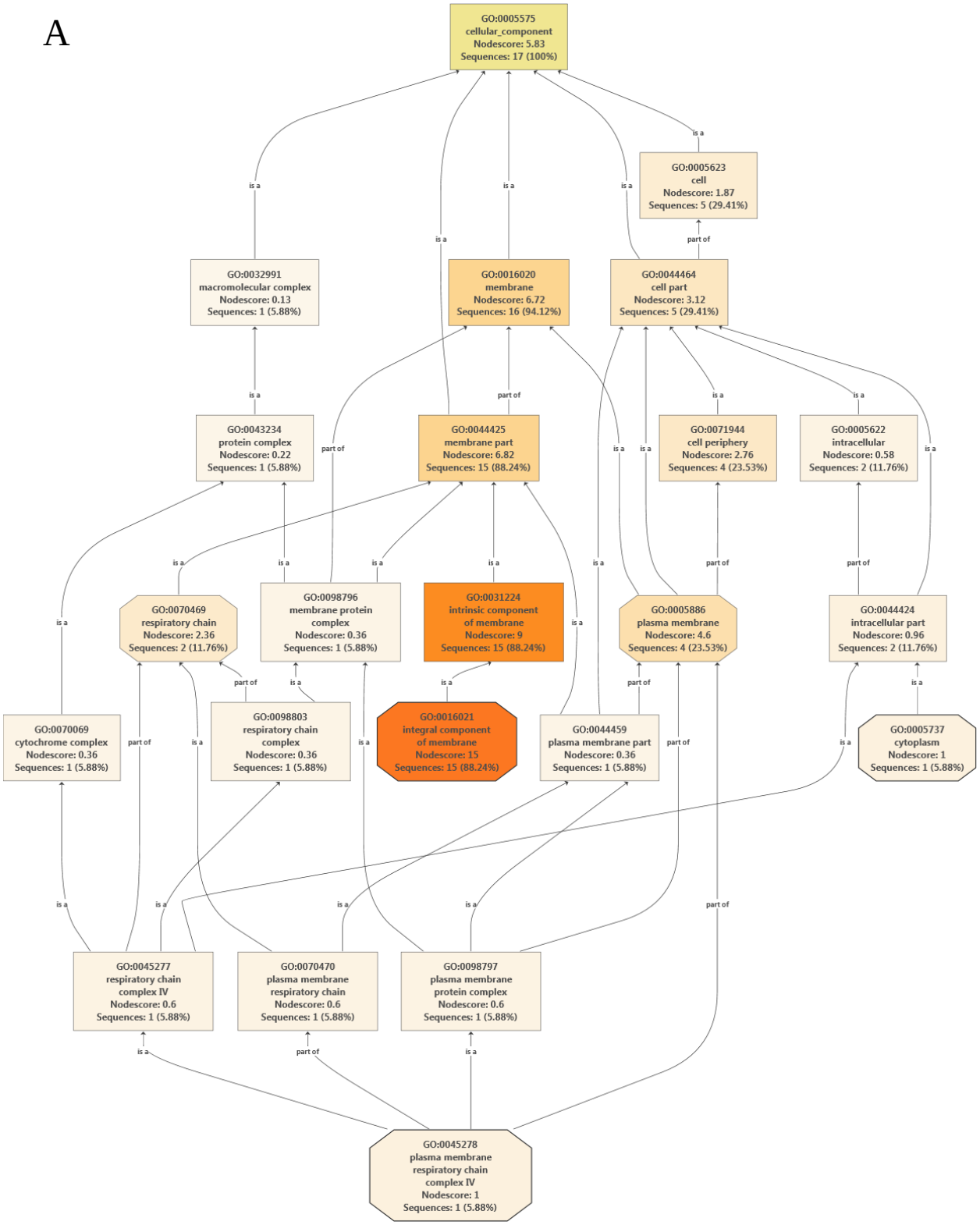


Figure 3-6. Library size that were mapped to the reference coding sequences. b represents biofilm cells in 4.5 mM [Ca2+]; p represents planktonic cells in control medium, pc represents planktonic cells in 4.5 mM [Ca2+].

A


GO:0005575
cellular_component
Nodescore: 5.83
Sequences: 17 (100%)
GO:0005623
cell
Nodescore: 1.87
Sequences: 5 (29.41%)
GO:0032991
macromolecular complex
Nodescore: 0.13
Sequences: 1 (5.88%)
GO:0016020
membrane
Nodescore: 6.72
Sequences: 16 (94.12%)
GO:0044464
cell part
Nodescore: 3.12
Sequences: 5 (29.41%)
GO:0043234
protein complex
Nodescore: 0.22
Sequences: 1 (5.88%)
GO:0044425
membrane part
Nodescore: 6.82
Sequences: 15 (88.24%)
GO:0071944
cell periphery
Nodescore: 2.76
Sequences: 4 (23.53%)
GO:0005622
intracellular
Nodescore: 0.58
Sequences: 2 (11.76%)
GO:0070469
respiratory chain
Nodescore: 2.36
Sequences: 2 (11.76%)
GO:0098796
membrane protein complex
Nodescore: 0.36
Sequences: 1 (5.88%)
GO:0031224
intrinsic component of membrane
Nodescore: 9
Sequences: 15 (88.24%)
GO:0005886
plasma membrane
Nodescore: 4.6
Sequences: 4 (23.53%)
GO:0044424
intracellular part
Nodescore: 0.96
Sequences: 2 (11.76%)
GO:0070069
cytochrome complex
Nodescore: 0.36
Sequences: 1 (5.88%)
GO:0098803
respiratory chain complex
Nodescore: 0.36
Sequences: 1 (5.88%)
GO:0016021
integral component of membrane
Nodescore: 15
Sequences: 15 (88.24%)
GO:0044459
plasma membrane part
Nodescore: 0.36
Sequences: 1 (5.88%)
GO:0005737
cytoplasm
Nodescore: 1
Sequences: 1 (5.88%)
GO:0045277
respiratory chain complex IV
Nodescore: 0.6
Sequences: 1 (5.88%)
GO:0070470
plasma membrane respiratory chain
Nodescore: 0.6
Sequences: 1 (5.88%)
GO:0098797
plasma membrane protein complex
Nodescore: 0.6
Sequences: 1 (5.88%)
GO:0045278
plasma membrane respiratory chain complex IV
Nodescore: 1
Sequences: 1 (5.88%)
is a
part of

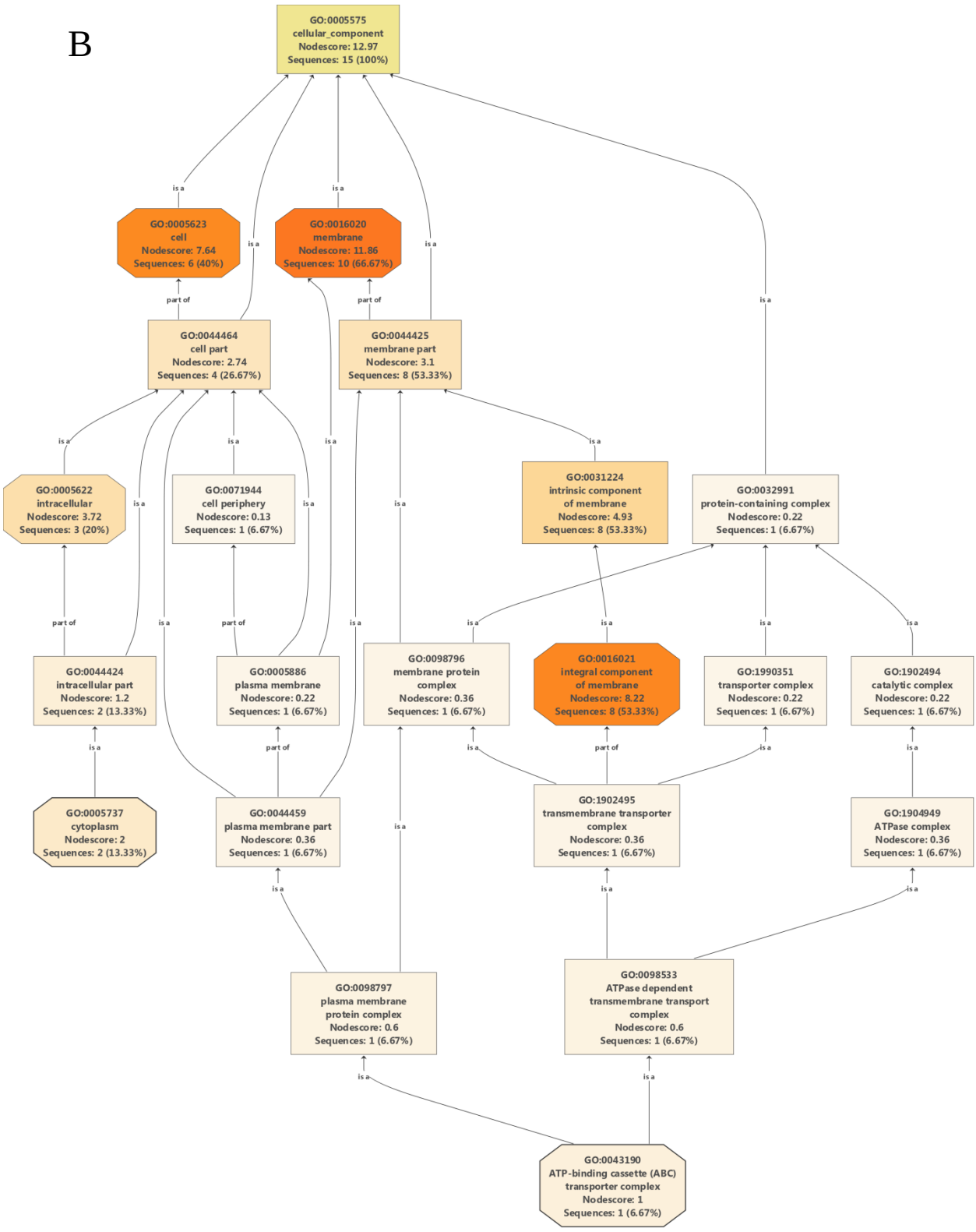


Figure 3-7. GO annotation of the up- (panel A) and down- (panel B) regulated genes between biofilm and planktonic samples in Ca-supplemented medium.

Chapter 4. Deciphering the molecular basis of attenuation for a live vaccine against columnaris disease in fish

Abstract

Bacterial diseases continue to be one of the top problems for the aquaculture industry. Vaccines are widely employed in aquaculture to prevent bacterial infections but their use by the catfish industry is very limited. Our group has patented a modified-live vaccine (a rifampicin-resistant mutant) against columnaris disease. We have proved that our vaccine is stable, safe, and effective but the mechanisms that resulted in attenuation remained uncharacterized. To understand the molecular basis for it, we conducted a comparative genomic analysis to identify the specific point mutations. The mutant (avirulent) vaccine strain 1723 and parent virulent strain B27 were sequenced using PacBio RS long-read sequencing platform, and draft genomes of the two strains were obtained. Sequence-based genome comparison identified 16 single nucleotide polymorphisms (SNP) unique to the mutant. Genes that contained mutations were involved in rifampicin resistance, gliding motility, DNA transcription, toxin secretion, and protease synthesis. We compared biofilm production between the mutant and the parent strain, and our results showed that the vaccine strain formed biofilm at a significantly lower rate than the parent strain. These observations suggested that the rifampicin-resistant phenotype and associated attenuation of the vaccine strain result from altered activity of RNA polymerase and a disrupted protein secretion system, respectively.

Introduction

Flavobacterium columnare is the causative agent of columnaris disease which can infect a broad range of fish hosts including wild, cultured and ornamental species (Austin and Austin 2007). In the United States, *F. columnare* is one of the leading pathogens affecting the catfish industry, with mortality rates among adults and fingerlings up to 60 and 90%, respectively (Decostere et al. 1998; USDA 2003). *F. columnare* is ubiquitous in the natural environment. The ecology of the *F. columnare* is not fully understood yet, but previous reports showed that *F. columnare* could form biofilm (Kunttu, 2012; Cai, 2013) and survive in the water column for up to 6 months (Arias, 2012). In addition, recent metagenomic analysis of the *F. columnare* indicated that this bacterium is capable of denitrification and anaerobic respiration, which suggest pond sediment as an additional reservoir for this pathogen (Tekedar, 2017).

Since eradication of *F. columnare* is unlikely to occur, vaccination presents itself as one of the best strategies to control columnaris disease. Effective vaccination practices mitigate the consequences of infectious diseases in a sustainable manner by reducing the use of antibiotics (Sommerset et al. 2005). Among the several types of vaccines available, live attenuated vaccines can have desirable traits over other type of vaccines because they, in general, elicit a stronger immune response and humoral and cell-mediated protection. Live attenuate vaccines contain multiple immunogens, and require a simple delivery and low dose (Woodrow et al. 2012). In addition, the persistence of a live attenuated bacteria on the host, typically, increases efficacy by increasing exposure time (Duncan et al. 1994).

F. columnare could be dived into three genetic groups, or genomovars, based on the 16S RNA sequence (Tryanto and Wakabayashi 1999). Genomovar II strains present a higher degree

of virulence towards catfish than genomovar I isolates (Arias 2009; Shoemaker 2008). However, the only commercial live attenuated vaccine against columnaris derives from the less virulent Genomovar I group.

Our lab developed several attenuated vaccine from the high virulent genomovar II group and after several experiments we selected the best candidate in terms of protection and ease of culture under laboratory conditions(Olivares-Fuster and Arias 2011). In our of our previous studies, we showed that choosing the right parent strain was critical vaccine efficacy against virulent strains of *F. columnare*. Our vaccine provided protection de protection against both genomovar I and II in channel catfish, zebrafish, and tilapia (Mohammed et al. 2013). We have proved that our vaccine is stable, safe, and effective but the mechanisms that resulted in attenuation remained uncharacterized. In this study, comparative genomics analysis was used to identify the point mutations between the vaccine and parent strain, in order to decipher the molecular basis of attenuation for the live vaccine.

Materials and methods

Bacterial strains and growth conditions

The *F. columnare* strain B27 was isolated from channel catfish in Alabama in 2005 (Olivares-Fuster et al. 2007), and the mutant strain 1723 was obtained in our lab by passing the virulent strain on increasing concentration of rifampicin from the virulent strain B27(Olivares-Fuster and Arias 2011). Bacteria were grown at 28 °C in Modified Shieh (M.S.) medium for 48 h with shaking at 200 rpm (Shoemaker et al. 2005). Stock suspensions of the isolates were stored in M.S. broth with 20% glycerol at -80°C.

Genomic DNA extraction

Bacterial DNA was extracted using a Qiagen DNeasy Blood & Tissue kit (Qiagen, MD, USA) following manufacturer's instructions including the RNase incubation step. All the DNA samples were quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and ran on a 1% agarose gel for integrity check.

Genomic sequencing and assembly

The library preparation and sequencing were conducted at University of Washington PacBio Sequencing Services center (Seattle, WA). The 20 kb library was prepared and sequenced by PacBio long-read sequencing RSII platform with P6/C4 chemistry. Genome assembly of filtered reads was performed using PacBio PBcR HGAP 2.3 pipeline with default settings using *de novo* assembly protocol. A total of 1.55 Gb, 534, 427 subreads with 3064 bp average length were acquired from the parent strain B27, and 1.28 Gb, 418, 658 subreads with average length of 3579 bp were obtained from the mutant strain 1723. The parent and vaccine strain genomes comprised of 6 and 4 contigs, with 184 and 272 X coverage, respectively.

The contigs with mean quality value (QV) of less than 45 were filtered out. We obtained 4 contigs with total length of 3.4 Mb (mean coverage=281 X, mean QV=48.63) for *F. columnare* 1723. We obtained 6 contigs with total length of 3.45 Mb (mean coverage=190 X, mean QV=48.57) for *F. columnare* B27. The QV 45 ensure above 99.997% accuracy (Wei and Zhang 2018).

Gene annotation and SNP identification

The annotation of the contigs was performed using the Rapid Annotation using Subsystem Technology (RAST) for prokaryotic bacteria (Aziz et al. 2008; Overbeek et al. 2013). Pairwise genome alignment was used to identify the single nucleotide polymorphisms (SNPs) with Mauve version 2.4 (Darling et al. 2004). The genes containing the mutations were analyzed using BLAST of NCBI, IGV viewer (Thorvaldsdóttir et al. 2013), and RAST subsystem database (Overbeek et al. 2013). The functional changes of the amino acid were predicted using PROVEAN Protein software (Choi and Chan 2015).

Biofilm quantification test

Biofilm formation was evaluated as described previously (Cai et al. 2013). Briefly, an overnight inoculum was diluted 100 times in the MS broth and 100 µl of the cell suspension was inoculated into the wells of 96-well microtiter polystyrene plates (NuncImmuno MaxiSorp; Nunc, Rochester, NY) in quadruplicate. The microtiter plates were inoculated for 48 h at 28 °C to allow bacterial attachment and biofilm formation. General growth was quantified by measuring the optical density (OD) at 595 nm with MultiSkan FC spectrophotometer (Thermo Scientific). Medium containing unattached cells was discarded, and wells were stained with 1% (w/v) crystal violet for 20 min. Excess crystal violet solution was removed and the wells were rinsed 3 more times. Biofilm formation was quantified by measuring OD_{595} after the remaining dye was dissolved in 96% (v/v) ethanol solution.

Statistic analysis

Four replicates for biofilm test were performed. The results were statistically analyzed using SAS software version 9.2 (SAS Institute, Cary, NC). The significant difference was set at $p \leq 0.05$.

Results

Draft genome of *F. columnare* B27 and 1723

The genomes of *F. columnare* B27 and 1723 were sequenced using PacBio RSII platform. A total of 534,427 subreads with average length of 3,064 bp were acquired for strain B27, and 418,658 subreads with average length of 3,579 bp were obtained for mutant strain 1723. HGAP *de novo* assembly generated 6 and 4 contigs for B27 and 1723, with average coverage of 184 X and 272 X, respectively. Short-length contigs with mean QV < 45 were filtered. The contigs were aligned and reordered using Mauve software with the *F. columnare* C2 (Reference number: NZ_CP015107.1) as reference to build the draft genome. The characteristics of the draft genomes were listed in Table 4-1. The genomes sizes were 3.43 and 3.45 Mb for B27 and 1723, respectively, with an overall GC content of 31.4%. RAST identified 3,008, and 3,061 coding sequences, which were functionally categorized into 324 and 325 subsystems.

Single-nucleotide polymorphisms between the vaccine strain and parent strains

Sequence-based genome comparison by Mauve identified 16 SNP unique to the mutant (Table 4-2). Genes that contained mutations were involved in gliding motility, DNA transcription, toxin secretion, and extracellular protease synthesis.

***rpoB* mutations**

The site of the mutations in *rpoB* gene was analyzed (Figure 4-1). Two point mutations were identified in the *rpoB* gene, which encode the DNA-directed RNA polymerase β subunit. *RpoB* encode 1341 amino acid (aa) in the *F. columnare*. The *rpoB* gene of the vaccine strain has a double mutation with Asn469Lys and His486Tyr substitutions (CDS: AND63316.1 in NCBI). We aligned the amino acid sequences of β-subunit of RNA polymerase for *E. coli* k12 and *F. columnare* B27. The positions 469 and 486 in *F. columnare* correspond to positions 509 and 526 in *E. coli* K12, respectively. There are three clusters defined as rifampicin resistance-determining region (RRDR) in *E. coli*, whose *rpoB* encode 1270 amino acid. The cluster I covers amino acid 507-533; the cluster II covers amino acid 563-572; the cluster III was at amino acid 687 in the β-subunit of RNAP in *E.coli* (Goldstein 2014). The two *rpoB* mutations fall into the RRDR cluster I region. The PROVEAN score was -3.43 and -5.73 for the Asn469Lys and His486Tyr substitution, indicating the mutation were deleterious.

Analysis of single-nucleotide polymorphisms

I identified 14 nonsynonymous amino acid changes and 2 synonymous mutation between the two strains (table 1). Two point mutations generated nonsense mutations, which encode nucleoside permease (NupG) and a phosphoesterase. The C to A transversion on the minus strand in NupG changed the codon GGA (glysine) to TGA (stop codon), resulting in a 240 aa truncated polypeptides from the 417 aa total length (CDS: AND62982.1 in NCBI). The G to T transversion on the positive strand in phosphesterase (annotated in RAST; annotated as hypothetical protein in NCBI with CDS: AND63983.1) changed the codon GAA (glutamic acid) to TAA (stop codon), resulting in a 194 aa truncated polypeptides from the 333 aa total length.

Five point mutations generated missense mutations that resulted in amino acid substitutions, including Phe577Cys in patatin, Ala470Asp in protease IV, Gly386Arg in thiol:disulfide interchange protein, Ile2902Met in thioredoxin reductase, and Phe238Ser in dynein protein. There are two silent mutation were also identified (Figure 4-1).

Mutations effect on biofilm formation

Thioredoxin reductase plays an important role in gliding motility and biofilm formation in *F. psychrophilum* (Alvarez, 2006). Dynein protein has not been well studied in prokaryotes, but we know that it functions as motor protein for gliding motility and intracellular cargo transport. Both proteins are known for its critical role in biofilm formation. The general growth and the biofilm formation of the parent strain and vaccine strain were conducted using the microtiter plate methods. The general growth of the two strains were comparable within the 48 h, however, the biofilm formation of vaccine strain was significantly reduced compared to the parent strain after 48 h (Figure 4-2).

Discussion

Advantages of using live bacterial vaccines include their ability to mimic natural infections, intrinsic adjuvant properties, ease of production, and longer lasting protection, among others. In most cases, live vaccines show a significantly higher immunogenicity than inactivated vaccines since they can elicit a wider range of immunologic responses, both humoral and cellular (Kollaritsch and Rendi 2013). However, live vaccines have safety concerns associated with the risk of reversion to a virulent form and the possibility of causing disease in immune compromised individuals (Detmer and Glenting 2006). In addition, most avirulent mutants used as vaccines are the result of random mutagenesis and the genetic mechanisms for attenuation

remained uncharacterized. These concerns have hindered the use of live vaccines. In recent years, the availability and low cost-next generation sequencing methods increased the number of bacterial genomes available and facilitated the characterization of attenuated mutants (Sammons-Jackson et al. 2008; Nair et al. 2015; Santiago et al. 2015). Nevertheless, the mechanism(s) by which rifampicin induces attenuation has not been fully investigated.

In our mutant 1723, rifampicin resistance was conveyed by two point mutations in the *rpoB* gene. Rifampicin is a potent, broad-spectrum antibiotic that functions by inhibiting the β-subunit of prokaryotic DNA-dependent RNA polymerase (RNAP) (Wehrli et al. 1968). The molecular basis of rifampicin resistance has been extensively studied in *Escherichia coli*, and it has been clearly demonstrated that the drug targets the β subunit of DNA-dependent RNAP, which is encoded by the *rpoB* gene (Ovchinnikov et al. 1983). Under rifampicin selection pressure, mutants with specific genetic changes in the *rpoB* gene are resistant to the effect of rifampicin (Gliniewicz et al. 2015). Comparison of primary structures of *rpoB* from several bacteria led to the identification of six regions of highly conserved sequences. A molecular genetic analysis of the rifampicin-resistant mutants in *E. coli* revealed that majority of these mutations were tightly grouped in cluster I of region II (Jin and Gross 1988). The *rpoB* in *E. coli* K12 encodes a predicted product of 1,342 amino acids (aa), while the *rpoB* in *F. columnare* B27 produce a predicted peptides of 1,270 aa. BLASTp was performed to compare the above two sequences to calculate the similarity between the two peptide sequences. Result showed that mutation positions 468 and 486 in *rpoB* of *F. columnare* B27 are equivalent to the positions 509 and 526 in *rpoB* of *E. coli* K12, respectively. The mutations belong to the cluster I of RIF-resistance-determine region (RRDR) (Goldstein 2014).

Rifampicin-attenuated *B. abortus* RB51, *E. ictaluri* RE33, and *F. columnare* FCRR displayed altered lipopolysaccharide (LPS) structure lacking the high molecular mass of LPS (Klesius and Shoemaker 1999; Vemulapalli et al. 2000; Zhang et al. 2006). On the other hand, attenuated strains of *F. psychrophilum* did not exhibit different LPS profiles as compared to the wild-type strain (LaFrentz et al. 2008). In our case, a previous study showed that the LPS profile of *F. columnare* genomovar II rifampin-resistant mutants was mutant-specific (Olivares-Fuster and Arias 2011). Specifically, two of the mutants (AL-CC-15 and AL-CC-16) lack the higher molecular bands of the LPS when compared to their parental strains but no difference was observed in the LPS profiles of mutant 1723 and its parent strain B27 (denoted as AL-CC-17 in (Olivares-Fuster and Arias 2011)). Interestingly, mutant 1723 exhibited the highest protection efficacy among all the candidate mutants (Mohammed et al. 2013). This suggests that i) the attenuated phenotype was not caused by a shortened LPS and that ii) 1723 displays an intact key antigen that explains the high efficacy of the vaccine in comparison with mutants that contained truncated LPS. Our genetic analysis is consistent with the observed phenotype as we did not find any mutations in genes associated with LPS metabolism.

In *F. columnare*, gliding motility and protein secretion are performed by type IX secretion system (T9SS) (McBride and Zhu 2012). Protein secretion using T9SS includes two steps: First, the cargo proteins are guided by an N-terminal signal peptide to the T9SS machinery in the cytoplasmic membrane. After translocation, the signal peptide is cleaved off by type I signal peptide peptidase, and the cargo is released into the periplasm. Secondly, a C-terminal-sorting peptidase is responsible for the proteolytic removal of the C-terminal conserved domain (CTD) during the cargo's translocation across the outer membrane through T9SS (Lasica et al. 2017). In our study, we found one critical mutation (Ala470Asp) in the gene encoding the signal

peptide peptidase, i.e. protease IV. Virulent factors and adhesins are the common cargo proteins transported by T9SS. The impact of the compromised signal peptide peptidase can be twofold based on its biological importance. First, the secretion of virulent factors, such as chondroitinases and proteases, is inhibited by the accumulation of the cargo protein precursor in the cytomembrane due to failed hydrolytic cleavage by the signal peptide peptidase. Thus, the mutant's ability to secret exoproteins needed to hydrolyze host tissues would be greatly compromised. Second, gliding motility could be compromised by the reduced secretion of adhesins. A previous study showed that the *F. columnare* parent strain B27 displayed the typical rhizoid colonies, while mutant 1723 exhibited smooth colony types (Olivares-Fuster and Arias 2011). This result was consistent with other studies that correlated rhizoid morphology with virulence and smooth colonies with attenuated phenotypes (Kunttu et al. 2011).

Thiol-disulfide exchange protein (DsbA) participates in disulfide exchange reaction with another thiol (Hermanson 2013). Periplasmic DsbA are part of the folding pathway of many secreted proteins (Fabianek et al. 2000). They facilitate the formation of disulfide bridges and are essential for correct folding or assembly of many proteins, including toxins, adhesins, and components of the type III secretion system (Alvarez et al. 2006). For example, periplasmic DsbA is required for the functional maturation of secreted virulence factors in *Vibrio cholera* (Peek and Taylor 1992). Since the cytotoxic potential of most common toxins relies on their subunit disulfide cleavability (Hermanson 2013), the malfunction of the DsbA in mutant 1723 could be results in miss-folded proteins relevant to virulence.

In this study, two mutations were predicted to generate truncated polypeptides. Nucleoside permease is a nucleoside transmembrane transport protein, which transports nucleosides driven by a proton motive force with a high affinity except guanosine and

deoxyguanosine (Munch-Petersen et al. 1979). Nucleobase transport has been studied extensively in the bacteria *Escherichia coli* and *Bacillus species* (de Koning and Diallinas 2000), where they played a key role in nucleic acid and nucleotide metabolism, and they scavenge exogenous preformed bases for nucleotide biosynthesis (Vogels and Van der Drift 1976). The mutation in nucleoside permease possibly compromised the bacterial ability to acquire nutrients under nutrient-poor conditions, such in biofilms where nutrients are limited by covered EPS and high cell density.

Another nonsense mutation occurred in the gene encoding phosphoesterase. This protein has a predicted type II phosphatidate phosphatases (PAP2)-like superfamily. PAP2 are transmembrane enzymes that serve as key regulatory enzymes in lipid metabolism, catalyzing the conversion of phosphatidate to diacylglycerol (Smith et al. 1957). This mutation is predicted to have a detrimental effect on the metabolism of lipid membrane component. We have anecdotal data that vaccine strain 1723 grew poorly in high hardness (high Ca concentration) medium, which can be the result of reduced osmotic resistance associated with membrane lipid.

Another important mutation occurred in the gene encoding a thioredoxin reductase (TrxR). Cell survival depends on efficient DNA replication. One of the critical factors that influence DNA replication is the thioredoxin (Trx) system that consists of Trx, TrxR and NADPH (Liang et al. 2016). The Trx system regulates the activity of ribonucleotide reductase, and the latter is required to provide deoxyribonucleotides for DNA replication (Nordlund and Reichard 2006). TrxR has been found to be essential for growth in *S. aureus* (Uziel et al. 2004). In our study, the growth rates of mutant and parent strain were the same, which indicated that the mutation of amino acid change did not significantly jeopardize the protein function, or the function is regained by other redundant homologous genes.

I found multiple mutations falling into DNA regions that were either poorly annotated or did not have an assigned function in the databases. This is not surprising since the genetic mechanisms of the phylum Bacteroidetes has been largely understudied in comparison with other phyla that contain main human pathogens such as the case of Proteobacteria. In addition, genetic manipulation of *Flavobacterium*, specifically *F. columnare*, has proven very difficult (Staroscik et al. 2008) and our knowledge on the basic metabolisms of this species remains elusive. Therefore, the effect of some of the mutations identified in the study remains undecided and could possibly have a decisive effect on the mutant attenuated phenotype.

Conclusion remarks

Sixteen point mutations were identified in the vaccine strain 1723. Rifampicin resistance was conveyed by two point mutations in cluster I of region II in *rpoB*, which possibly associates with the binding site of rifampicin to RNAP. We identified some key mutations (i.e. protease IV, Thiol-disulfide exchange protein, Nucleoside permease, and phosphoesterase) that are possibly responsible for virulence attenuation. Our vaccine contains mutations in multiple genetic loci that contribute to its stability and would make reversion to a virulent phenotype extremely unlikely.

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Table 4-1. Characteristics of draft genomic structure of *F. columnare* vaccine strain and parent strain

Strains	Number of contigs	Assembly size (Mb)	%GC	CDS	Structural RNAs	Number of subsystems
vaccine	4	3.45	31.4	3061	112	325
parent	6	3.43	31.4	3008	98	324

Table 4-2. Analysis of single nucleotide polymorphisms (SNPs) compared between vaccine strain and parent strain

Reference Position[a]	SNP pattern[b]	Amino acid change[c]	Annotation[d]	Functional group
125027	G > A	Asn469Lys	DNA-directed RNA polymerase beta subunit	Gene transcription
125076	G > T	His486Tyr	DNA-directed RNA polymerase beta subunit	Gene transcription
83013	C > A	Gly240stop	Nucleoside permease	Nucleosides transporter
91216	G > T	Glu194Stop	Phosphoesterase	Lipid metabolism
132995	G > A	Gly386Arg	Thiol-disulfide exchange protein	Protein biosynthesis
114715	C > A	Phe577Cys	Patatin	Glycoprotein biosynthesis
500199	C > A	Ala470Asp	Protease IV	Toxin secretion
534202	C > T	Glu167Glu	Heme O synthase	Protein biosynthesis
99642	T > G	Ile2902Met	Thioredoxin reductase	DNA replication/biofilm formation
41423	T > C	Phe238Ser	Dynein heavy chain	Gliding motility
41416	C > T	Leu236Leu	Dynein heavy chain	Gliding motility
741630	G > A	NA	Hypothetical protein	NA
1361	C > T	NA	Unknown region	NA
1147	A > G	NA	Unknown region	NA
1363	G > A	NA	Unknown region	NA
249370	G > T	NA	Unknown region	NA

[a]Reference position (in bp) are based on B27 draft genome

[b]SNP Pattern represents the nucleotide changes

[c]Predicted amino acid change for the identified SNP

[d]Annotation showed the gene function based on the RAST annotation. NA, Not Applied.

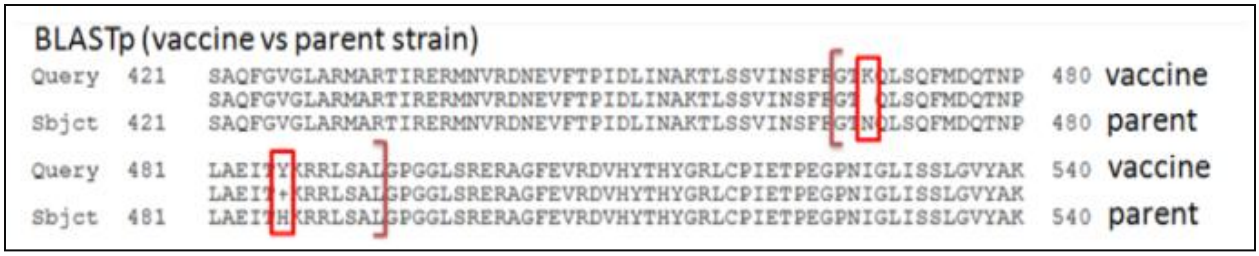
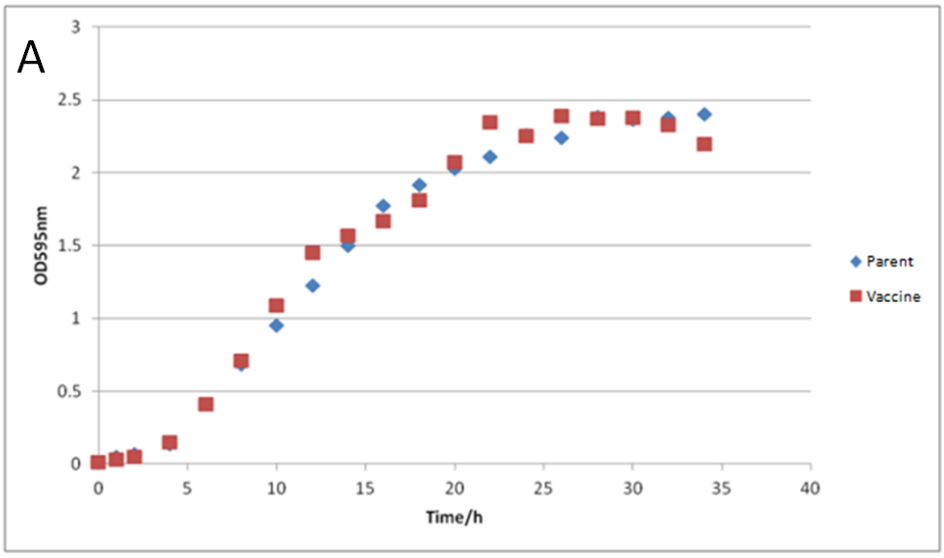


Figure 4-1. Protein blast between vaccine strain and parent strain at the RNA polymerase β-subunit. The rectangular square showed the amino acid substitutions. The square bracket showed the cluster I region of the rifampicin resistance-determining region.



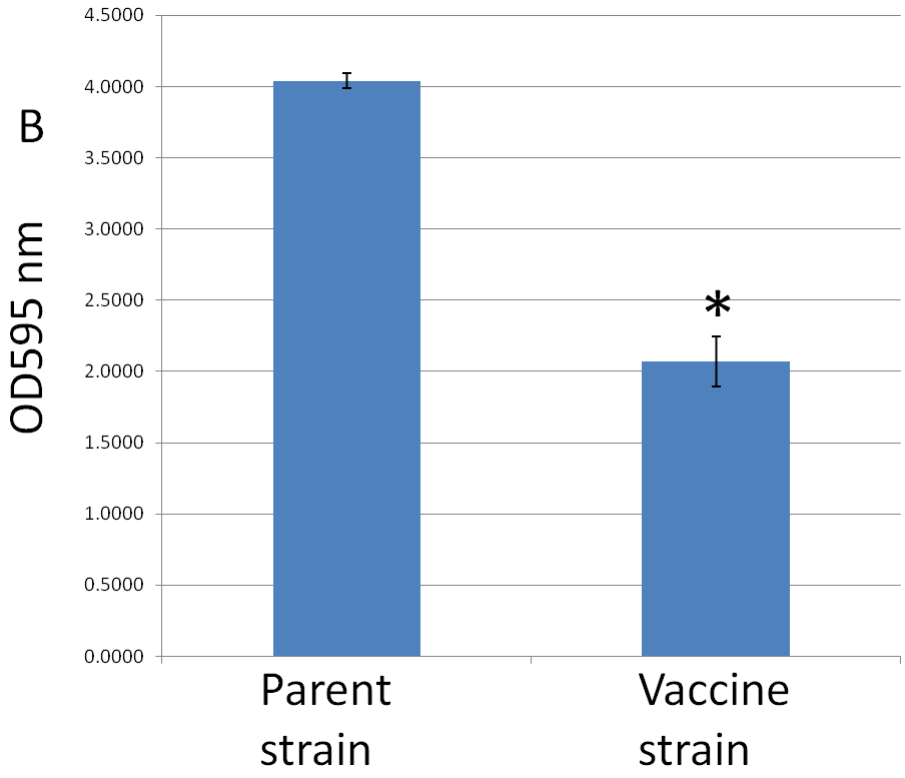


Figure 4-2. (A) Parent strain & vaccine strain growth curves. (B) Biofilm formation of virulent parent strain and vaccine strain after 48 h incubation in microtiter plate. * indicates significant difference.

Chapter 5. Unveiling the genetic diversity behind the species complex *Flavobacterium columnare*

Abstract

Flavobacterium columnare is the causative agent of columnaris disease, which causes significant losses in cultured freshwater finfish species across the world. The intraspecies genetic diversity found in *F. columnare* was first revealed by DNA-DNA hybridization that divided the species into 3 genetic groups or genomovars. Later studies further subtyped the species into 6 genetic groups based on 16S rDNA polymorphisms. Virulence studies in channel catfish and rainbow trout showed that genomovar II strains were more virulent than genomovar I strains, suggesting the presence of more than one patovar within the species. Interestingly, that genetic diversity does not translate well to biotyping as all *F. columnare* strains are biochemically similar. The objective of this study was to elucidate if *F. columnare* was a species complex that harbors more than one cryptic species or if the observed genetic diversity was within the definition of bacterial species. Based on previous polyphasic data obtained by our group, we selected three strains representing 3 different lineages within the species (ATCC 23463 (type strain), ARS-1, and B27) for whole genome sequencing using PacBio RS long-read sequencing platform. *De novo* genome assembly of filtered reads was performed using PacBio PBcR HGAP 2.3 pipeline with default settings, which yield 5 (ATCC 23463), 7 (B27), and 7 (ARS-1) contigs with 214 X, 184 X, and 182 X coverage, respectively. Finally, five additional *F. columnare* strains (ATCC 49512, Pf1, Tc, C2, and 94_081) whose genome sequences were available in public database were included in the analysis.

Average nucleotide identity (ANI) among the three representative strains were 85.55%, 85.69%, and 91.3% for pairwise comparisons ARS1 & ATCC 23463, ARS1 & B27, and B27 & ATCC 23463, respectively. All ANI values were lower than the recommended cut-off point of 95% for species delineation. ANI results validated previous MLST and MALDI-TOFF phylogenetic analyses. Comparative genomic analysis (CGA) identified 1,824 genes in the core genome (shared by all 8 isolates), accounting for 30.5% of the total pan-genome (gene repertoire = 5,976 genes). Phylogenetic analysis based on the core genomic genes identified 3 clusters, similar to those described previously. Strains within genomovar I (represented by ATCC 23463) contained 61 unique genes while genomovar III (represented by ARS-1) harbored up to 459 unique genes. Genomovar II (represented by BGFS-27 and highly virulent for catfish) contained 52 unique genes including several genes encoding for putative virulence factors such as O-antigen polymerase, and streptococcal hemagglutinin protein. Based on our data, three species of *Flavobacterium* can cause columnaris disease in fish: *F. columnare* and (at least) two nomen nudum species that warrant full taxonomic description. Genomovar II strains are genetically homogenous and should be recognized as a new species. Our results have direct implications in control and prevention of columnaris disease in farms.

Introduction

Flavobacterium columnare is a Gram-negative bacterial pathogen that infects a wide range of freshwater species, including wild, aquaculture and ornamental fishes (Plumb 2018). *F. columnare* strains are phenotypically similar but genetically different. Early studies divided the species into three distinct genetic groups or genomovars based on the DNA-DNA hybridization and 16S rDNA sequence (Wakabayashi 1999). Recent studies characterized more strains and increased the number of genomovars up to six (I, II, II-B, I/II, and III) (LaFrentz et al. 2014; García et al. 2018).

The taxonomy of *F. columnare* underwent a series of changes since its initial description. In 1922, David was the first one to observe the bacteria on external lesions of numerous fresh-water fishes in Mississippi River, and named it *Bacillus columnaris* (Davis 1922). Later, Ordal and Rucker (1944) isolated this organism from brown bullhead (*Ameiurus nebulosus*) and reclassified it as *Chondroccoccus columnaris* based on the wrong assumption that the cells were able to produce microcysts and fruiting bodies. In 1945, Garnjobst (1945) proved that the bacterium was indeed devoid of fruiting bodies and reclassified it as *Cytophaga columnaris*. However, further description of the genus *Flexibacter* and *Cytophaga* lead to another name change (*Flexibacter columnaris* (Buchanan and Bibbons 1974). Finally, in 1996, Bernardet et al. (1996) restructured the Flavobacteriaceae family and reclassified the bacterium as *Flavobacterium columnare.*

F. columnare is ubiquitous in freshwater aquaculture farms but few studies have explored its ecology in the wild. Epidemic studies with wild fish population indicated that genomovars were associated with different host species (Olivares-Fuster et al. 2007). In addition, genomovar

II strains are more virulent than genomovar I strains in a wide range of fish species, including channel catfish (*Ictalurus punctatu*s) (Shoemaker et al. 2008), zebrafish (*Danio rerio*) (Olivares-Fuster et al. 2011), Nile tilapia (Mohammed et al. 2013) and rainbow trout (*Oncorhynchus mykiss*) (LaFrentz et al. 2012). From both applied and ecological point of views, I was interested in further exploring the genetic intraspecies diversity within the species *F. columnare*.

In the present study, *F. columnare* intraspecies diversity was revealed by comparative genomics analysis. Three strains (ATCC 23463 (type strain), ARS-1, and B 27) representing different genetic groups were selected according to previous data. We have determined the draft genomes of the three strains, and performed a comparative whole genome analysis within the species.

Materials and Methods

Bacterial culture

Three strains that were previously collected and characterized in our lab were used in this study. *F. columnare* strain B27 and ARS1 were isolated from channel catfish in Alabama USA in 2005 and 1996, respectively (Arias et al. 2004; Olivares-Fuster et al. 2007). Type strain ATCC 23463 was isolated from Chinook salmon in Washington, USA. The isolates were stored at -80 °C in MS broth (Shoemaker et al. 2005) with 20% glycerol. Before use, the *F. columnare* was inoculated in MS broth and incubated at 28 °C for 48 h with agitation. All isolates were tested by PCR and RFLP for the confirmation of the *F. columnare* and genomovar ascriptions.

F. columnare strains ARS1, B27, and ATCC 23463 were selected for whole genome sequencing and compared with the previously sequenced strains ATCC 49512, Pf1, Tc1691,

94_081, and C2. Strain information and accession numbers for *F. columnare* strains are listed in the Table 5-1.

DNA extraction

Bacterial DNA from *F. columnare* was extracted with Qiagen DNeasy Blood & Tissue kit (QiaGen, CA) under manufacture's instruction including the RNAse lysis step. The amount of genomic DNA was measured using NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the integrity and purity was tested on 1% agarose gel.

Sequencing, assembly and annotation

The genomic DNA sequences of *F. columnare* were obtained using PacBio long-read sequencing platform in University of Washington PacBio sequencing center (Seattle, WA). The 20-kb libraries was prepared and sequenced with PacBio long-read sequencing RSII platform with P6/C4 chemistry. Short (< 50 bp) and low-quality (< QV 75) reads were filtered, and the sequences were *de novo* assembled using PacBio PBcR HGAP 2.3 pipeline. The draft genome was obtained with an average coverage of >182 X for each isolate. Annotation of the contigs was performed using Subsystem Technology (RAST) automated service (Aziz et al. 2008; Overbeek et al. 2013).

Whole-genome comparisons

Pan and core genome analysis. To investigate the gene pool of the species complex *F. columnare*, the pan and core genomes were analyzed using the bioinformatics program EDGAR (Blom et al. 2009). Eight isolates, including 4 genomovar I strains (i.e. ATCC 23463, ATCC49512, Pf1 and Tc1691), 3 genomovar II strains (i.e. B27, 94_081 and C2) and 1

genomovar III strain (i.e. ARS1), were selected in this study. The input was GenBank files annotated with either NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) (Tatusova et al. 2016) or RAST annotation tools. The strain information was listed in Table 1. Pan and core genomes were calculated by iterative pairwise comparison of a set of genomes. For orthologous gene calculation, instead of using the absolute bit score provided by the BLAST algorithm, I used the BLAST Score Ratio Value (SRV) developed by EDGAR (Lerat et al. 2003). Two genes were considered orthologs if they had reciprocal best BLAST hits (BBHs), and the SRV value of both single BBHs was above the calculated cutoff (https://www.uni-giessen.de/fbz/fb08/Inst/bioinformatik/software/EDGAR/documentation). Gene islands were predicted using IslandViewer software with integrated approaches (i.e. IslandPick, SIGI-HMM and IslandPath-DIMOB) (Dhillon et al. 2015; Bertelli et al. 2017)

Average Nucleotide Identity (ANI) analysis. Average nucleotide identity (ANI) of conserved genes present in two sequenced strains represents a robust measure of the genetic and evolutionary distance between them. ANI was calculated using the ANI calculator (http://enve-omics.ce.gatech.edu/ani/index) and the ANI heatmap was built using EDGAR software package with the recommended settings (Goris et al. 2007). Pan-genome, core-genome, and singletons calculations were performed using BLAST Score Ratio Values (SRV) (Lerat et al. 2003).

Genomovar-specific genes. To investigate the gene set difference among the three genetic groups, the orthologous genes unique to particular genomovar strains were identified. Unfortunately, genomovar III was only represented by ARS1 strain so it is likely that as more genomovar III strains are sequenced these numbers will change in the future. The gene set was obtained based on the calculation that there has to be a set of orthologous genes in all included

genomes while there must not be any orthology to one of the excluded genomes (Blom et al. 2009).

*Phylogenetic analysi*s. To determine the evolutionary relationships among the 8 *F. columnare* genomes sequenced, a phylogeny tree was constructed based on the whole genomic data, using 1,412 core orthologous gene shared by all 8 isolates. The pipeline calculated the core genome, and every set of orthologous genes found in core genome is separately aligned using the multiple alignment tool MUSCLE (Edgar 2004). A distance matrix was calculated from this alignment and finally a phylogenetic tree is constructed based on this distance matrix using the Neighbor-Joining method in the PHYLIP package (Felsenstein 1993).

Results

General features of *F. columnare* genomes

In this study, three *F. columnare* strains (ARS1, B27, and ATCC23463) representing three distinct genetic groups were sequenced using PacBio RSII platform (Table 5-2). HGAP *de novo* assembly generated 5 (ATCC23463), 7 (B27), and 7 (ARS1) contigs with average coverage of 214 X, 184 X, and 182 X, respectively. RAST annotation identified 3,033 to 3,903 predicted coding sequences, which were functionally categorized into 318 to 324 subsystems (Table 5-2). The 3 genomes were analyzed together with an additional 5 genomes whose sequences were available in the public database (Table 5-1). The genomes of *F. columnare* strains varied in size from 3.03 to 3.61 Mb with a GC content of 30.8-32.1%.

Within the 3 isolates that I sequenced, there were 2,149 genes shared among all isolates, accounting for about 63.9% of the genes in each genome. B27 shared more orthologous genes with type strain ATCC 23463 than with ARS1 (Figure 5-1).

Average nucleotide identity (ANI) analysis

Average nucleotide identity (ANI) is a parameter for genomic sequence similarities. In specific, the ANI values among the three representative strains were 85.55%, 85.69%, and 91.3% for groups ARS1 & ATCC23463, ARS1 & B27, and B27 & ATCC23463, respectively. All ANI values were lower than the recommended cut-off point of 95% for species delineation (Goris et al. 2007). ANI results validated previous MLST and MALDI-TOFF phylogenetic analyses (data not shown; C. R. Arias, personal communication). The results indicated that the *F. columnare* complex harbors 3 lineages that correspond to genomovar ascription. The ANI value within each genomovar was above 99%, while the ANI value between the genomovar I and genomovar II was less than 92%. Strain of genomovar III shared ANI value of around 85% to other genomovars (Figure 5-3).

Pan genome analysis

To investigate the essence and diversity in the *F. columnare* species complex, the pan and core genome were analyzed together with 5 more public available complete genomes. The pan genome (gene repertoire) employed by the 8 strains consisted of 5,976 genes, including 1,824 core genes and 4,152 dispensable genes. This result revealed the extensive sequence diversity within the *F. columnare* complex. Average gene content was 2,813 genes, and the core genome accounted for 64.8% in the *F. columnare* species complex. Singletons are genes that were only observed in one individual isolate. There were 46.6% singletons identified among 8 genomes, indicating a high level of genetic plasticity within *F. columnare*. Heaps' law function analysis indicated that the pan genome was considered to be open with a growth exponent of 0.391 (Figure 5-2 B). As expected, *F. columnare* core genome showed that the number of shared genes

decreased with the addition of each new genome. The core genome was estimated to contain 1,824 genes, which would remain relatively constant even if additional isolates genomes were included.

Genomovar strain-unique genes

To investigate the gene set difference among the three genetic groups, the orthologous genes unique to particular genomovar strains were retrieved using EDGAR. Fifty two genes were exclusively found in genomovar II genomes (include B27, 94_081 and C2; exclude ARS1, ATCC49512, ATCC23463, Pf1 and TC1691), whose products included O-antigen polymerase, glycosyltransferase, streptococcal hemagglutinin protein, type II toxin-antitoxin system *PemK/MazF* family toxin, transposase, and subtilisin-like serine protease. Sixty one orthologous genes were exclusively found in genomovar I strains, including serine endopeptidase, mobile element protein, transposase, and hypothetical proteins. Furthermore, 459 genes were solely found in genomovar III strain ARS1 (include ARS1; exclude B27, 94_081, C2, ATCC49512, ATCC23463, Pf1, and TC1691). The gene set signature provided possible genetic clues for the molecular mechanisms contributing to tailored phenotypic characteristics.

Phylogeny analysis

To determine the evolutionary relationships among the *F. columnare* strains, a phylogeny network was constructed using 1,412 orthologous genes present in the core genome and *P. psychrophilum* JI0286 genome as outgroup. As shown in Figure 5-4, three different clusters can be visualized in the species complex *F. columnare*: cluster I corresponds to the previous genomovar II strains, which include the B27, 94-081, and C2; cluster II including the type strains, Pf1 and Tc1691. Strain ARS1 was equally distant to other *F. columnare* strains than to

the *F. psychrophilum* strain used as outgroup. Within cluster II (=genomovar I strains), the two Chinese isolates (i.e. Pf1 and TC1691) were the most related strains, followed by a French isolate (i.e. ATCC 49512) and the type strain USA (i.e. ATCC23463).

Discussion

Ferninand Cohn is recognized as one of the founding fathers of microbiology (Drews 2000). Although not as famous as Pasteur or Koch, Cohn was the first one to classify bacteria using the binomial nomenclature previously established by Carl Linnaeous. Initially, bacteria were classified based solely on phenotypic markers such as biochemical properties, morphology and pathogenic potential (Ramasamy et al. 2014). With advances in molecular biology during the 70s and 80s, comparison of genetic relatedness among species becomes more common and with the advent of DNA amplification and sequencing techniques, the "gold standard" for species delineation shifted from only phenotypic markers to the required inclusion of DNA-DNA hybridization (DDH) data (Wayne et al. 1987). Later, after DNA amplification and sequencing techniques were developed, 16S rDNA was identified as the best available molecular clock (Woese 1987) and started to drive bacterial taxonomy and systematics. 16S rRNA gene sequence identity of 97 and 95% have been used as the cut-off values to describe novel taxa at the genus and the species levels, respectively (Stackebrandt et al. 2002). With the development of high throughput sequencing platforms that generate high quality data at low cost, average nucleotide identity (ANI) analysis and digital DDH (dDDH) have been widely adopted as a standard rule to replace the more cumbersome DDH. The proposed and generally accepted species boundary for ANI and dDDH are 95% and 70%, respectively (Goris et al. 2007; Richter and Rossello-Mora 2009). Currently, the taxonomy of prokaryotes relies on a polyphasic combination of phenotypic, chemotaxonomic and genotypic characteristics (Ramasamy et al. 2014). In previous studies,

traditional DDH values between genomovars of *F. columnare* were below 53% (Wakabayashi 1999; Verma and Rathore 2013), and dDDH values between genomovars have been reported as low as 43.6% (Kayansamruaj et al. 2017). In this study, ANI values between all 3 genomovar groups were below the 95% cut-off point. There results strongly suggest that *F. columnare* should be considered a species complex with, at least, two cryptic species.

F. columnare displays extensive genomic diversity, which was reflected in the analysis of its pangenome. The pangenome is predicted to be open with a growth exponent of 0.39 with the addition of each new genome. This trend indicates that a very large number of genomes would have to be sequenced in order to fully characterize its entire genome repertoire. Kumru et al. (2017) also reported the vast genetic diversity within *F. columnare* using a comparative genomic analysis, however, only two strains were represented in their study. The pangenome size is determined in part by the frequency of homologous recombination and lateral gene transfer. Therefore, genomic analysis focusing only on one or few genomes per species often underestimates the genetic complexity. The size of the core genome is also an indicator of the relatedness of microbial genomes (Uchiyama 2008). In a closely related species, *F. psychrophilum*, its core genome accounts for ~80% of the genes of each individual genome (Duchaud et al. 2018). However, in our study in where 8 *F. columnare* strains we compared, the core genome only accounted for 64.8% of all genomic coding-sequence genes. The extraordinary genetic heterogeneity found within these strains further challenges the old concept as *F. columnare* as a single species.

The accessory genome is an important driver in bacterial evolution that allows the bacteria to persist in a particular environmental niche. The gene set analysis indicated that genomovar II strains harbored unique genes that could be linked to enhanced virulence in catfish.

For example, within the 61 genomovar II unique genes, several putative virulence factors were annotated, including genes encoding for an O-antigen polymerase and a streptococcal hemaglutinin protein. Lipopolysaccharide (LPS) is a complex glycolipid that comprises the majority of the outer-leaflet of the outer membrane and has three domains including lipid A, core oligosaccharide, and a distal O-antigen (O-Ag). The function of the O-antigen polymerase is to catalyze the addition of newly synthesized O-antigen repeating units to the rest of glycolipid. Both lipid A and the O-antigen polysaccharide act key elements for bacteria virulence. The unique O-antigen polymerase in genomovar II could possible alter the O-Ag component of the LPS by adding longer chains to the O-antigen polysaccharide (Lerouge and Vanderleyden 2002). The main function of the LPS is to modulate membrane permeability and to protect bacteria from harmful attacks, including both chemical and biological, such as from component of the host complement or phagocytic immune cells. Shorter or truncated LPS have been typically linked to attenuated or avirulent phenotypes (Klesius and Shoemaker 1999; Vemulapalli et al. 2000; Zhang et al. 2006). The ability of producing longer O-antigens or the ability of modify those can certainly represent an advantage when invading a host. For example, a unique O-antigen polymerase could protect the pathogen from the host immune system by synthetizing a new O-antigen variant. In *P. aeruginosa*, horizontal-gene transfer has been associated with O-antigen polymerase diversity. The serotype conversion of O5 to O16 in *P. aeruginosa* was caused by an acquired O-antigen polymerase (Wzyβ) through the bacteriophage D3 (Taylor et al. 2016).The phage-derived Wzyβ utilizes a different reaction mechanism in the *P. aeruginosa* host to avoid self-inhibition from the host.

Hemagglutinin are used by bacteria as adhesins and some of them can exhibit proteolytic activity (Madden et al. 1995). The proteolytic activity of the hemagglutinin could benefit the

bacteria during the early stages of host colonization. In our previous study on adhesion dynamics of *F. columnare,* we showed that initial attachment to the host is similar between genomovar II (B27) and genomovar III (ARS1; formerly known as genomovar I) strains, but while B27 successfully persisted in gills and multiply within a few hours post-attachment, ARS1 was cleared from host tissues in a short period of time (Olivares-Fuster et al. 2011). I hypothesize that this hemaglutinin protein could facilitate the early invading process as the result of co-evolution between genomovar II strains and a catfish host.

Large number of transposase genes was found in all genomovars, suggesting a possible role of these mobile elements during bacterial speciation. Mobile genetic elements, such as integrative and conjugative elements, that have limited homology to the host genome use site-specific recombination to integrate at target sequences. Konstantinidis and Tiedje (2005) found that hypothetical, phase-, and transposase-associated genes function as a major driver force to foster speciation, and they found that all these mobile elements comprise 62.4% of the singletons in a study comprised of 70 bacterial miscellaneous strains. In our case, and since plasmid are not present in *F. columnare,* the extensive number of mobile genetic elements could explain the large genetic diversity found within the isolates.

F. columnare is genetically heterogeneous, and it has been divided into 6 genomovars (LaFrentz et al. 2014; García et al. 2018) based on a standardized 16S rDNA-RFLP technique (LaFrentz et al. 2017). LaFrentz et al. (2018) proposed the use of four genetic groups to facilitate a standard nomenclature according to 16S rRNA gene sequences because of its biological relevance, i.e. association between genetic groups to specific fish species or families. The term genomovars was proposed by Rossello et al (1991) to denote genomic groups that have been delineated by DNA-DNA hybridization, but could not be differentiated based on phenotypic

markers. The term genomovar was firstly used in *F. columnare* by Triyanto and Wakabayashi(1999) when they proposed 3 genomovars based on 16S rRNA gene sequences and DNA-DNA hybridization. Later, 16S rDNA-RFLP was used as a proxy method for ascription to genomovars (Arias et al. 2004; Olivares-Fuster et al. 2007; LaFrentz et al. 2014). However, in bacterial taxonomy the lowest recognized level to classify bacteria is the level of species. The correct identification and classification of isolates within the species complex *F. columnare* requires the description of at least one new species. This is important not only for bacterial taxonomists but also for diagnosticians, epidemiologists, and other aquatic animal health experts working towards preventing and controlling columnaris disease. Previous work by our group (unpublished data) using MALDI-TOFF provided the phenotypic evidence required in bacterial systematics to support my conclusions derived from whole genome sequence.

Phylogeny analysis using ANI of all conserved genes provided a better resolution than use of a single gene, such as 16S rRNA gene, for measuring relatedness, because of its robustness in compromising the effect of mutation rate, selection pressure and HGT (Konstantinidis and Tiedje 2005). Genomovar I and II strains formed well-delineated clusters that were more similar to each other than to genomovar III or the outgroup species. However, in this study, the phylogenetic position of the only genomovar III strain was not resolved. More whole genome sequences of genomovar III strains are needed in order to better define the position of genomovar III in relationship with genomovar I and II.

In summary, the comparative genomic data indicated that the *F. columnare* is a species complex, which clearly include more than one cryptic species (at least two). The enhanced virulence of genomovar II to catfish is possibly induced by the unique virulence factors acquired

by horizontal gene transfer. Genomovar II strains are genetically homogenous and should be recognized as a new species.

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Table 5-1. *Flavobacterium columnare* strains used in this study

Strains	Geographic origin	Host origin	Genomovars	sources	Accession number	Gene island[a]	GC%
1	ATCC49512	France	I	Brown trout	CP003222.2	15	31.5
2	ATCC23463	Washington (USA)	I	Salmonids	This study	16	32.1
3	Pf1	China	I	Yellow catfish	CP016277	8	31.6
4	Tc1691	China	I	NA	CP018912	12	31.6
5	ARS1	AL (USA)	III	Channel catfish	This study	10	31.2
6	B27	AL (USA)	II	Channel catfish	This study	24	31.4
7	94081	USA	II	Channel catfish	CP013992	16	30.8
8	C2	USA	II	Warmwater fish[b]	CP015107	10	31.0

a, predicted by SIGI-HMM method using IslandViewer software.

b, the specific fish species was unknown.

Table 5-2. Characteristics of draft genomes of the *F. columnare* ATCC23463, B27 and ARS1.

Strains	Number of contigs	Assembly size (Mb)	%GC	CDS	Structural RNAs	Number of subsystems
ATCC23463	5	3.44	32.1	3033	158	318
B27	7	3.65	31.4	3154	173	324
ARS1	7	3.46	31.2	3903	145	322

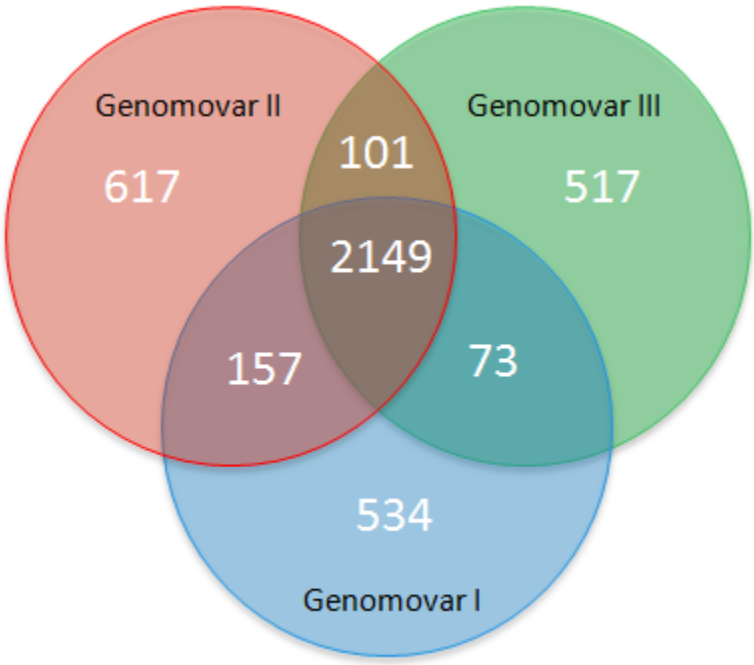
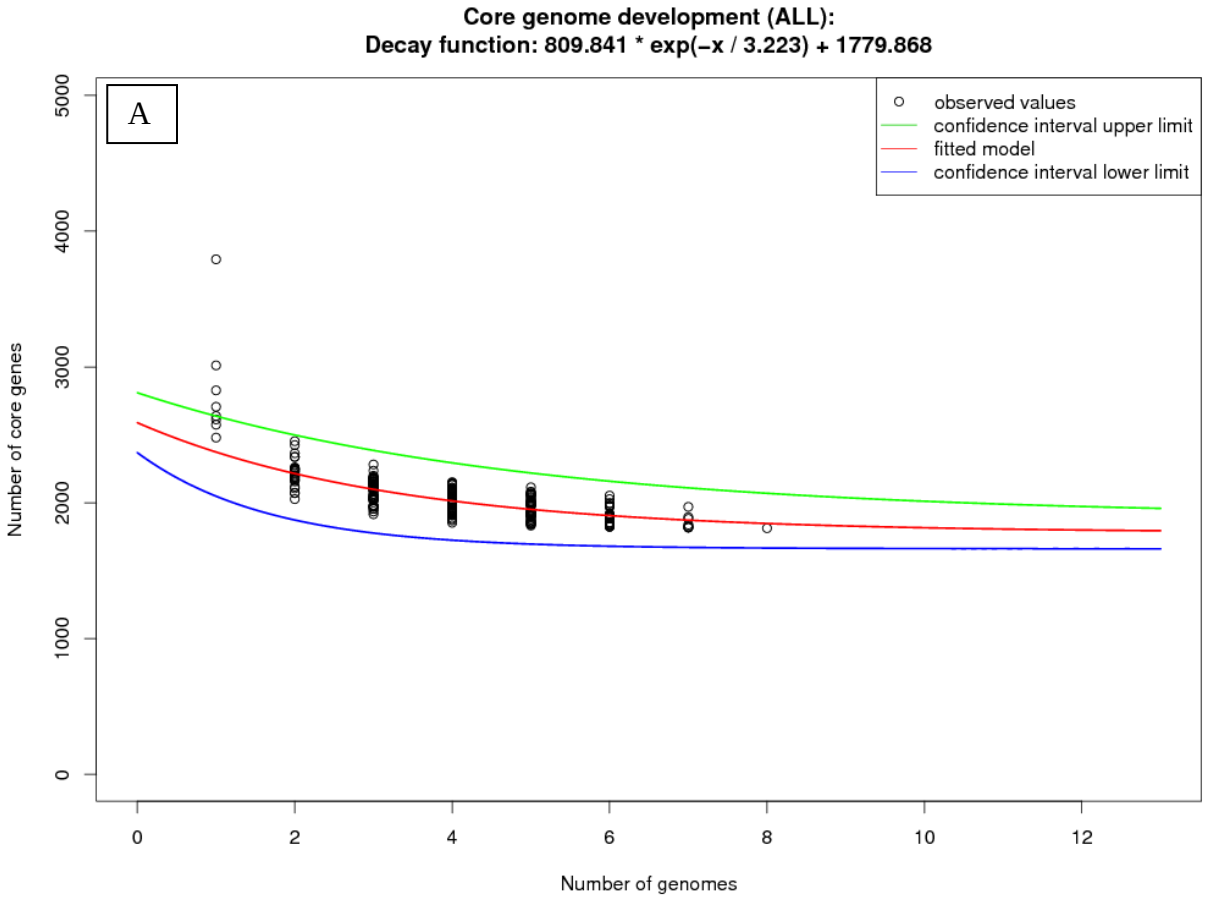


Figure 5-1. Venn diagrams among *F. columnare* strains ATCC23463 (genomovar I), B27 (genomovar II), and ARS1 (genomovar III).


Core genome development (ALL):
Decay function: 809.841 * exp(−x / 3.223) + 1779.868
A
observed values
confidence interval upper limit
fitted model
confidence interval lower limit
Number of core genes
0
1000
2000
3000
4000
5000
0
2
4
6
8
10
12
Number of genomes

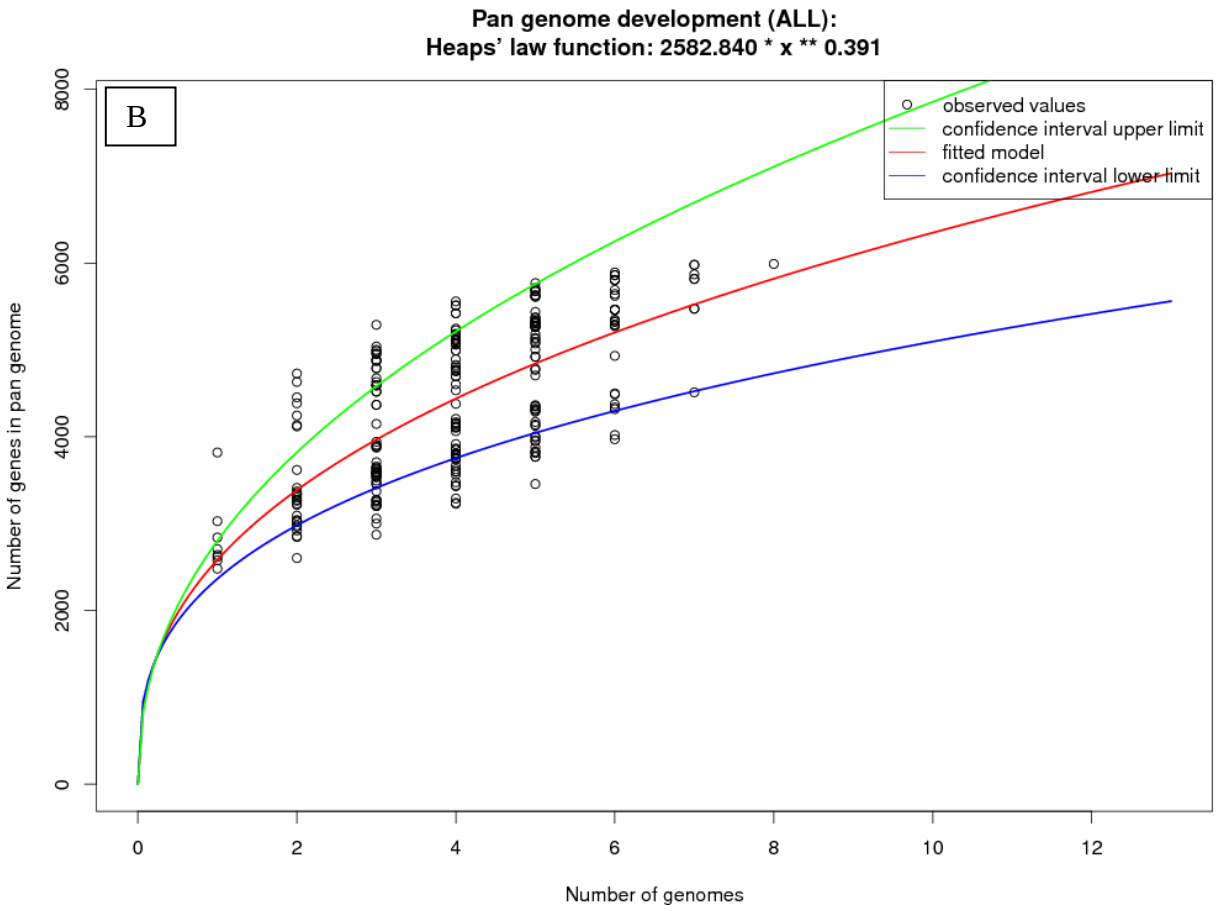


Figure 5-2. Core genome (panel A) and pan genome (panel B) development plots

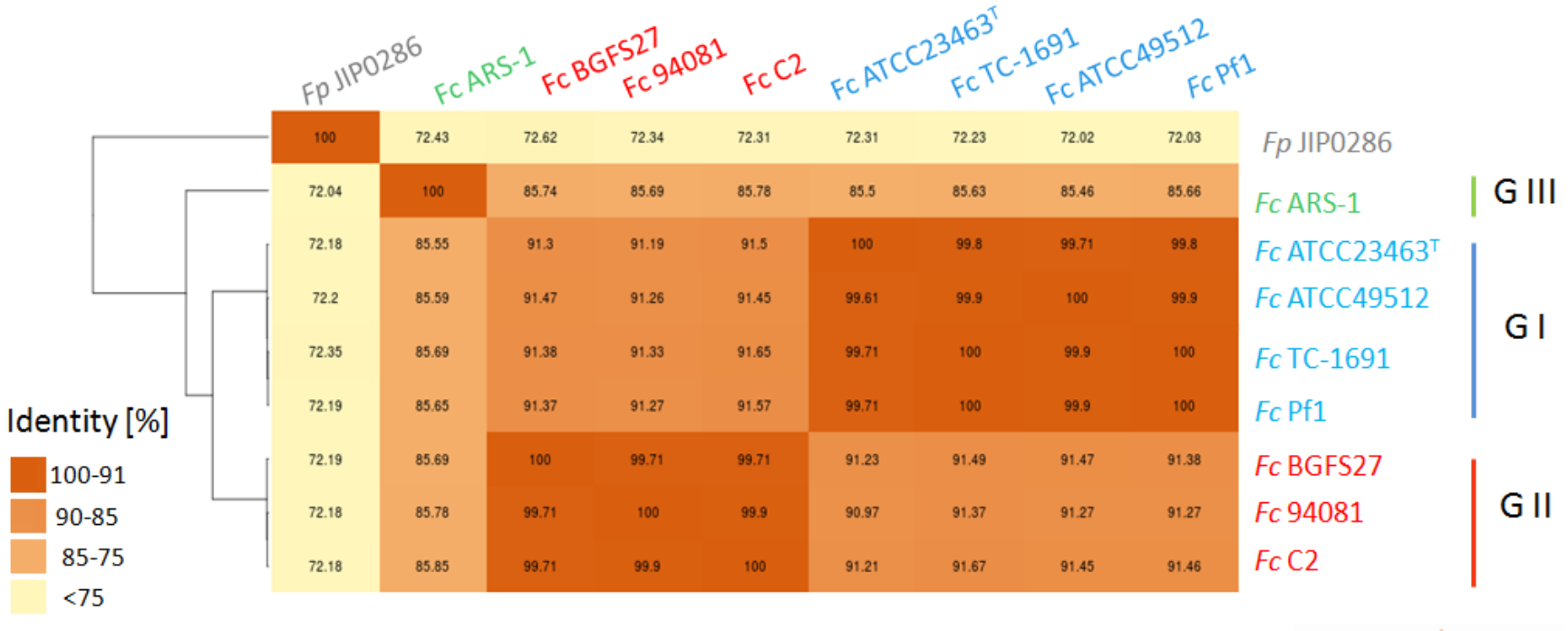


Figure 5-3. ANI matrix of the 8 *F. columnare* strains using *F. psychrophilum* JIP0286 as reference. Fc=*F.columnare*, Fp=*F. psychrophilum*. G= genomovar.

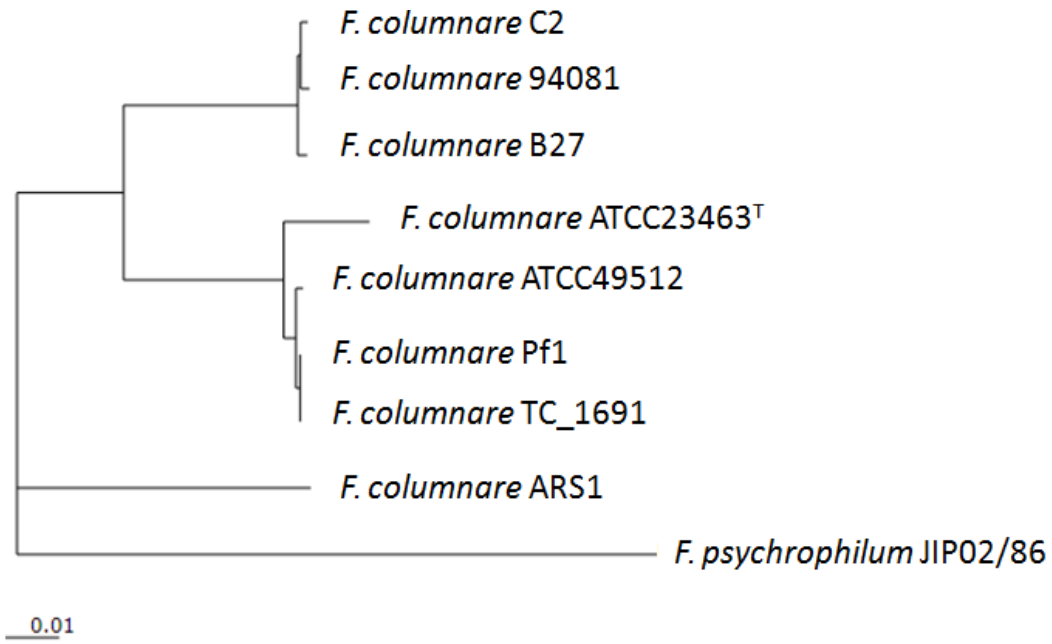


Figure 5-4. *F. columnare* phylogeny tree built based on core genome with *F. psychrophilum* as out-group reference using EDGAR.

Chapter 6. Summary and conclusions

In this dissertation, I explored different life stages of the fish pathogen *F. columnare* using classical and molecular approaches, I investigated the mechanisms for attenuation, and performed a comparative genomic analysis of members of the species. In chapter 2, the colonization of aquaculture substrates by *F. columnare* was examined. I found that *F. columnare* could efficiently form biofilm on pond liner, flexible PVC, and nets, while colonization on plant material was significantly inhibited. In addition, I evaluated the effect of calcium in biofilm formation not only in *F. columnare* but also in other common catfish pathogens. Higher [Ca2+] promoted biofilm formation in most pathogens with the exception of *Edwardsiella ictaluri*. My results demonstrated that common bacterial pathogens had the potential of colonizing surfaces and use biofilm as reservoirs in fish farms. Thus, biosecurity guidelines for hatcheries and production facilities should include disinfection protocols to eliminate biofilms from surfaces.

To further understand the molecular mechanisms of *F. columnare* in biofilm, I used RNA-seq to indentify DEGs in biofilm (B) versus planktonic (P) cells with and without the addition of Ca (chapter 3). I identified 112 significant DEGs between P/Ca and B/Ca samples, corresponding to 4.03% of the total protein-coding sequences. Results showed that cells in biofilm responded to oxidative stress and nutrient starvation in biofilm while aerobic respiration was greatly limited. When DEGs were compared between Ca supplemented medium and control, I found 175 DEGs (6.30%) related to iron acquisition, biofilm signaling, T9SS, and calcium homeostasis. Together, my data suggested that that biofilm is significantly affected by calcium, which seems to serve as a critical signal in controlling bacterial surface adhesion and biofilm formation in *F. columnare*. These results demonstrated that calcium supplementation induced a

transcriptional response that promotes continued biofilm formation. Our results have implication for developing targeted method for biofilm control by intervening the biofilm-specific pathways.

In chapter 4, 16 nucleotide polymorphisms (SNP) unique to vaccine strain 1723 (mutant) were identified. Rifampicin resistance was conveyed by two point mutations in cluster I of region II in *rpoB*, which possibly associates with the binding site of rifampicin to RNAP. Multiple key mutations that are possibly responsible for virulence attenuation were identified, such as a protease IV, a thiol-disulfide exchange protein, a nucleoside permease, and a phosphoesterase. In addition, we compared the biofilm production between the mutant and the parent strains, and our results showed that the vaccine strain formed biofilm at a significantly lower rate compared to the parent strain. My result in chapter 4 indicated that our vaccine contains mutations in multiple genetic loci that contribute to its attenuation and stability, and would make reversion to a virulent phenotype extremely unlikely.

In chapter 5, all ANI values among the different genomovars were lower than the recommended cut-off point of 95% for species delineation. Comparative genomic analysis using ANI and pan/core genome analysis suggested that *F. columnare* is a species complex with, at least, two cryptic species. Gene set analysis indicated that genomovar II strains harbored unique genes that could be linked to enhanced virulence in catfish such as an O-antigen polymerase, and Streptococcal hemagglutinin. The enhanced virulence of genomovar II to catfish possibly resulted by the acquisition of unique virulence factors by horizontal gene transfer. Genomovar II strains are genetically homogenous and should be recognized as a new species. My results have direct implications in control and prevention of columnaris disease in farms since 'columnaris disease' as we know it, it is caused by more than one bacterial species.

Appendix 1. Supplemental DEGs Lists for chapter 3

List 1. DEGs between *F. columnare* 530 planktonic cells in biofilm compared to planktonic cells in 4 mM [Ca2+]. Positive fold change values indicate higher expression in planktonic cells, while negative fold change values indicate higher expression in the biofilms.

Genes (coding sequences)	logFC	logCPM	PValue	FDR	Description
WP_060382480.1	2.122	11.38171	7.45E-06	0.000135984	cytochrome C oxidase subunit III
WP_060381433.1	2.106	7.843856	4.88E-06	9.58423E-05	crystallin J1
WP_060382479.1	2.033	10.9172	2.95E-06	6.28161E-05	cytochrome c oxidase accessory protein CcoG
WP_060382475.1	1.998	7.842193	0.001355	0.009338842	glycosyltransferase
WP_060381434.1	1.919	7.360791	6.55E-05	0.000872263	hypothetical protein
WP_060381432.1	1.875	5.637942	6.87E-06	0.000127991	hypothetical protein
WP_060381571.1	1.738	11.7915	0.0013	0.009091094	quinol:cytochrome C oxidoreductase
WP_060381572.1	1.718	10.94184	0.001774	0.011591856	hydrogenase
WP_060383513.1	1.717	2.727149	2.96E-08	1.23988E-06	hypothetical protein
WP_060382481.1	1.714	8.692903	2.38E-05	0.000371322	cytochrome C oxidase subunit IV
WP_060382478.1	1.714	9.798327	0.001935	0.012484625	cytochrome C oxidase Cbb3
WP_060383027.1	1.652	4.031734	0.004021	0.021299638	hypothetical protein
WP_060381431.1	1.642	3.214371	5.50E-05	0.00075505	hypothetical protein
WP_060382477.1	1.637	9.493366	1.28E-05	0.000216399	hypothetical protein
WP_060382482.1	1.635	13.14644	5.80E-05	0.000784641	cytochrome C oxidase Cbb3
WP_060383512.1	1.606	2.557358	1.75E-06	3.95644E-05	hypothetical protein
WP_060381573.1	1.569	9.590081	0.002927	0.01685227	hypothetical protein
WP_060382476.1	1.546	11.27273	1.48E-05	0.000247361	GTP-binding protein
WP_060383852.1	1.534	9.273206	5.60E-19	3.5199E-16	branched-chain amino acid aminotransferase
WP_060381577.1	1.495	11.58669	0.003062	0.017312822	cytochrome c oxidase subunit I

WP_060381419.1	1.446	10.52043	9.82E-05	0.001170532	cytochrome-c peroxidase
WP_060383027.1	1.410	3.981377	0.013934	0.053272219	hypothetical protein
WP_060381575.1	1.401	10.82421	0.00445	0.022756906	hypothetical protein
WP_060381570.1	1.392	10.16156	0.000367	0.003406465	cytochrome C
WP_060381576.1	1.390	10.83432	0.005217	0.025837142	cytochrome C oxidase subunit II
WP_060381574.1	1.373	9.451262	0.005553	0.026971442	cytochrome C
WP_060382313.1	1.353	7.4134	2.71E-10	1.89626E-08	anthranilate synthase
WP_060383400.1	1.304	2.093476	8.33E-05	0.001049196	transposase
WP_060383120.1	1.288	8.671223	7.58E-05	0.000987788	recombination protein RecR
WP_060381304.1	1.277	2.213413	3.72E-05	0.000544569	transposase
WP_060381561.1	1.271	8.861832	0.000832	0.006497933	NADH dehydrogenase
WP_060383583.1	1.232	6.282526	0.000375	0.00345867	hypothetical protein
WP_060381631.1	1.191	7.472837	0.007194	0.032969489	hypothetical protein
WP_060381560.1	1.185	8.793067	0.000767	0.006141917	NADH oxidoreductase (quinone) subunit F
WP_060383582.1	1.180	6.350154	0.000269	0.002708993	T9SS C-terminal target domain-containing protein
WP_060383834.1	1.171	5.675174	1.77E-08	8.11185E-07	alpha/beta hydrolase
WP_060383838.1	1.169	6.217834	3.65E-06	7.52606E-05	anthranilate synthase subunit II
WP_060383789.1	1.166	-0.10001	0.008158	0.03613531	hypothetical protein
WP_060383628.1	1.156	0.561693	0.00538	0.026438214	transcriptional regulator
WP_060383402.1	1.131	9.053654	1.29E-06	3.06882E-05	T9SS C-terminal target domain-containing protein
WP_060382408.1	1.121	8.942041	0.006089	0.029068003	coproporphyrinogen III oxidase
WP_060382429.1	1.116	8.841991	4.84E-05	0.000680491	MBL fold metallo-hydrolase
WP_060381558.1	1.074	8.570683	0.000893	0.00682904	NADH dehydrogenase
WP_060381435.1	1.068	8.544385	8.72E-03	0.038025178	phosphoribosylpyrophosphate synthetase
WP_060381566.1	1.067	8.766662	0.000156	0.001727037	NADH-quinone oxidoreductase subunit L
WP_060381562.1	1.063	8.467717	0.001731	0.011429833	NADH:ubiquinone oxidoreductase subunit H
WP_060383670.1	1.057	10.48085	0.009496	0.040631335	succinate dehydrogenase
WP_060381563.1	1.055	7.100096	0.004	0.021232805	DNA-binding protein
WP_060383119.1	1.034	8.639823	0.002774	0.016195425	sugar transporter
WP_060382314.1	1.033	6.030786	2.67E-08	1.17725E-06	anthranilate phosphoribosyltransferase

WP_014164161.1	1.003	7.489581	0.001318	0.009162692	NADH-quinone oxidoreductase subunit I
WP_060382096.1	-1.002	9.91266	8.34E-05	0.001049196	band 7 protein
WP_060381444.1	-1.008	10.16969	8.80E-05	0.001075239	Nicotinamide mononucleotide adenylyltransferase
WP_060381969.1	-1.024	6.949804	1.28E-13	2.93744E-11	NAD(P)H-dependent oxidoreductase, Nitroreductase family
WP_060382110.1	-1.028	4.646673	4.53E-05	0.000651384	TetR family transcriptional regulator
WP_014164670.1	-1.03174	7.219294	2.62E-11	2.35315E-09	thiol reductase thioredoxin
WP_060382829.1	-1.036	8.49837	2.75E-08	1.19494E-06	hypothetical protein
WP_060383176.1	-1.044	5.763719	2.44E-07	7.38941E-06	hypothetical protein
WP_060381977.1	-1.045	5.228536	1.33E-11	1.39432E-09	protein-tyrosine-phosphatase
WP_060381978.1	-1.047	4.374454	7.51E-09	3.85644E-07	SAM-dependent methyltransferase
WP_060383765.1	-1.050	6.64372	1.82E-06	4.05597E-05	hypothetical protein
WP_060383508.1	-1.056	7.135941	8.39E-09	4.21969E-07	hypothetical protein
WP_060383025.1	-1.057	4.208032	6.50E-06	0.000122917	XRE-family like transcription factor
WP_060382470.1	-1.063	5.952907	1.33E-07	4.59484E-06	Ribosomal RNA large subunit methyltransferase H
WP_060381599.1	-1.083	6.844685	8.21E-08	3.08147E-06	hypothetical protein
WP_060381777.1	-1.094	1.141965	0.000332	0.003137635	hypothetical protein
WP_060383412.1	-1.094	11.59059	3.91E-08	1.61314E-06	signal peptide peptidase
WP_060382792.1	-1.109	8.565248	8.86E-14	2.23023E-11	peptidase M61 (glycyl aminopeptidase)
WP_060382137.1	-1.111	3.489552	0.000981	0.007298638	RNA polymerase subunit sigma-70, transcriptional regulator
WP_060382994.1	-1.115	8.530702	8.26E-10	5.06974E-08	LemA family protein
WP_060381919.1	-1.118	7.678662	5.54E-12	6.97559E-10	O-succinylbenzoic acid--CoA ligase
WP_060381606.1	-1.131	4.582837	2.58E-06	5.58605E-05	hypothetical protein
WP_060382069.1	-1.154	10.6691	9.92E-07	2.47072E-05	hypothetical protein
WP_060381307.1	-1.161	8.183417	0.000108	0.001269596	hypothetical protein
WP_060381920.1	-1.173	7.05944	7.95E-10	5.00307E-08	abortive phage infection protein.
WP_060382943.1	-1.178	4.225066	9.15E-09	4.34352E-07	hypothetical protein
WP_014165796.1	-1.189	4.357067	1.37E-06	3.18357E-05	hypothetical protein
WP_060382136.1	-1.210	4.140716	1.98E-07	6.14642E-06	hypothetical protein
WP_060381633.1	-1.236	8.897917	2.84E-13	5.48987E-11	hypothetical protein

WP_060381836.1	-1.245	3.540121	1.82E-06	4.05597E-05	UDP-N-acetylmuramate--alanine ligase
WP_060381311.1	-1.255	8.660461	6.16E-09	3.36707E-07	histidine kinase.phosphotransfer
WP_060381932.1	-1.260	8.707618	2.65E-10	1.89626E-08	hypothetical protein,BlaR1 peptidase M56
WP_060381976.1	-1.263	6.765991	7.81E-17	2.80603E-14	hypothetical protein
WP_060382995.1	-1.267	1.310361	6.79E-05	0.000894451	hypothetical protein
WP_060381532.1	-1.271	7.185773	3.74E-10	2.54308E-08	enoyl-CoA hydratase
WP_060382395.1	-1.283	4.718922	4.95E-06	9.66089E-05	hypothetical protein
WP_060382125.1	-1.293	2.253881	1.96E-05	0.000318841	hypothetical protein
WP_060382782.1	-1.297	5.007814	6.16E-06	0.000117556	hypothetical protein
WP_060382836.1	-1.303	6.174188	1.96E-16	6.15065E-14	hypothetical protein
WP_060383199.1	-1.307	4.355146	8.46E-12	9.26542E-10	hypothetical protein, ribosome inactivating protein
WP_060381283.1	-1.313	6.292429	1.80E-12	2.83439E-10	single-stranded DNA-binding protein
WP_060381931.1	-1.314	4.356933	5.39E-08	2.11768E-06	transcriptional regulator
WP_060382837.1	-1.319	6.175612	1.64E-17	8.228E-15	cell division protein ZapA
WP_060382135.1	-1.347	5.208551	5.48E-11	4.75195E-09	hypothetical protein
WP_060381968.1	-1.358	5.890936	1.79E-12	2.83439E-10	TetR family transcriptional regulator
WP_060382698.1	-1.385	9.206716	1.85E-07	5.96342E-06	macrolide ABC transporter ATP-binding protein
WP_060381979.1	-1.388	3.807785	2.03E-10	1.50705E-08	hypothetical protein
WP_060382095.1	-1.413	5.622364	7.87E-16	2.20128E-13	MarR family transcriptional regulator.
WP_060382768.1	-1.413	3.623763	1.89E-07	6.01795E-06	Hypothetical protein
WP_060382235.1	-1.414	4.935591	7.36E-13	1.32291E-10	Cytochrome c peroxidase
WP_060381835.1	-1.427	3.316255	1.44E-11	1.44471E-09	Hypothetical protein
WP_060382622.1	-1.456	10.19297	1.99E-11	1.85347E-09	TonB-dependent receptor
WP_060383353.1	-1.497	9.133181	2.90E-12	4.04796E-10	ABC transporter permease
WP_060381975.1	-1.503	9.415081	1.98E-17	8.28874E-15	alkyl hydroperoxide reductase
WP_060382658.1	-1.540	9.216781	4.40E-12	5.83312E-10	von willebrand factore type protein
WP_060381980.1	-1.544	2.901708	3.11E-05	0.000466474	ArsR family transcriptional regulator.
WP_060382775.1	-1.566	10.66737	2.59E-07	7.76803E-06	TonB-dependent receptor plug domain

WP_060382614.1	-1.706	3.763124	1.41E-13	2.9658E-11	AraC family transcriptional regulator
WP_060381967.1	-1.715	6.314766	1.25E-22	1.0517E-19	Glycoside hydrolase, 1,4-polygalactosaminidase
WP_060382447.1	-1.755	8.059357	2.34E-27	5.89051E-24	Hypothetical protein with EF-hand motif
WP_060382448.1	-2.076	7.458086	6.61E-26	8.31318E-23	DNA starvation protect protein
WP_060381974.1	-2.159	4.697825	7.27E-12	8.71409E-10	Catalase

List 2. DEGs between *F. columnare* 530 planktonic cells in planktonic cells in 4 mM [Ca2+] compared to planktonic cells in control medium. Positive fold change values indicate higher expression in biofilm cells, while negative fold change values indicate higher expression in planktonic cells in 4 mM [Ca2+] medium.

Genes (coding sequences)	logFC	logCPM	P value	FDR	Description
WP_060383188.1	8.320	9.760374	8.48E-46	2.13395E-43	Membrane protein
WP_060383187.1	8.202	10.87413	1.96E-43	4.10694E-41	siderophore alcaligin biosynthesis protein
WP_060383189.1	7.991	10.96539	6.62E-56	3.33363E-53	siderophore transcriptional regulator.
WP_060383186.1	7.974	10.58058	7.84E-58	6.57887E-55	decarboxylase
WP_060382679.1	7.607	11.59612	3.51E-45	8.03004E-43	T9SS C-terminal target domain-containing protein
WP_060382680.1	7.370	10.54294	2.19E-66	5.52094E-63	hypothetical protein
WP_060383888.1	7.207	9.176353	5.09E-64	6.40737E-61	siderophore biosynthesis protein
WP_060382681.1	6.364	11.17095	7.61E-54	2.73526E-51	hypothetical protein
WP_060383861.1	6.030	10.03701	3.60E-42	6.96342E-40	TonB-dependent receptor
WP_060383182.1	6.008	11.59184	1.40E-51	4.41246E-49	TonB-dependent receptor
WP_060383190.1	5.642	8.853831	6.29E-47	1.75954E-44	lucA/lucC family siderophore biosynthesis protein
WP_060381363.1	5.616	9.930441	1.49E-56	9.35274E-54	hypothetical protein
WP_060381364.1	5.483	10.49137	1.74E-55	7.28177E-53	hypothetical protein
WP_060383101.1	5.179	11.78905	2.38E-35	3.73784E-33	TonB-dependent receptor (siderophore transport)
WP_060383184.1	4.585	6.494663	2.94E-37	5.28392E-35	hypothetical protein
WP_060383183.1	4.453	8.909717	1.45E-35	2.43226E-33	peptidase M4
WP_060383191.1	3.944	8.102602	1.76E-30	2.45874E-28	hypothetical protein
WP_060383185.1	3.943	6.20772	1.88E-31	2.7841E-29	LuxR family transcriptional regulator
WP_060382675.1	3.697	9.826139	1.07E-25	1.17568E-23	PadR family transcriptional regulator

WP_060382006.1	3.656	7.052608	4.95E-19	3.88823E-17	MarR family transcriptional regulator
WP_060382676.1	3.630	12.02067	2.17E-26	2.59948E-24	hypothetical protein
WP_060381858.1	3.517	6.351945	4.31E-24	4.16613E-22	hypothetical protein
WP_060383100.1	3.484	8.472096	4.26E-26	4.86861E-24	hypothetical protein
WP_060382677.1	3.451	9.86124	2.02E-26	2.54365E-24	hypothetical protein
WP_060382674.1	3.440	9.665114	5.49E-22	4.93057E-20	hypothetical protein
WP_060382672.1	3.407	6.791994	3.84E-27	5.08558E-25	thioesterase
WP_060382467.1	3.284	11.30332	6.93E-23	6.46079E-21	DUF4856 domain-containing protein
WP_060382673.1	3.098	9.88809	1.57E-15	9.61463E-14	hypothetical protein
WP_060382775.1	2.971	10.66737	1.76E-20	1.53113E-18	TonB-dependent receptor
WP_060381365.1	2.954	9.564651	1.82E-24	1.83593E-22	ArsR family transcriptional regulator
WP_060382096.1	2.816	9.91266	2.03E-25	2.13007E-23	band 7 protein
WP_060382588.1	2.792	9.476391	2.11E-20	1.77107E-18	phosphate transport regulator
WP_060382504.1	2.784	5.862975	3.13E-17	2.07236E-15	hypothetical protein
WP_060383099.1	2.763	6.906051	3.10E-20	2.51538E-18	flagellin biosynthesis protein FlgD
WP_060383004.1	2.616	3.757444	1.03E-10	3.32918E-09	hypothetical protein
WP_060381538.1	2.596	11.62834	1.51E-18	1.08364E-16	hypothetical protein (cholesterol binding)
WP_060383657.1	2.446	5.62707	6.42E-15	3.75496E-13	ion transporter
WP_060382466.1	2.438	8.818583	6.77E-19	5.01185E-17	hypothetical protein
WP_060382034.1	2.415	8.161425	2.00E-18	1.39438E-16	hypothetical protein(integral component of membrane)
WP_060381366.1	2.290	8.277305	2.06E-15	1.237E-13	two-component sensor histidine kinase
WP_060382698.1	2.283	9.206716	7.58E-17	4.89036E-15	macrolide ABC transporter ATP-binding protein

WP_060382550.1	2.208	8.998759	5.57E-19	4.24797E-17	glycerol kinase
WP_060381668.1	2.190	7.619535	1.11E-12	4.71305E-11	2-nitropropane dioxygenase
WP_060383037.1	2.174	11.24629	1.89E-12	7.65027E-11	hypothetical protein
WP_060382587.1	2.139	10.5364	4.25E-14	2.32648E-12	phosphate transporter
WP_060382005.1	2.131	10.94988	1.57E-11	5.55611E-10	3-hydroxyacyl-CoA dehydrogenase
WP_060382870.1	2.063	10.4933	1.24E-17	8.43503E-16	esterase
WP_060381307.1	2.032	8.183417	4.37E-11	1.48429E-09	hypothetical protein
WP_060382465.1	2.019	7.017035	1.28E-12	5.35783E-11	deoxyribonuclease HsdR
WP_060383098.1	1.920	7.473981	3.53E-12	1.38749E-10	hypothetical protein
WP_060383327.1	1.901	10.64187	6.23E-12	2.3745E-10	electron transfer flavoprotein subunit alpha
WP_060382549.1	1.817	7.602577	3.86E-16	2.42706E-14	FAD-dependent oxidoreductase (glycerol related)
WP_060383583.1	1.811	6.282526	2.72E-07	5.65284E-06	hypothetical protein
WP_060383369.1	1.777	8.702484	1.45E-09	4.13406E-08	thioredoxin
WP_060383795.1	1.747	8.877857	3.51E-14	1.96336E-12	RNA-splicing ligase RtcB
WP_060383674.1	1.714	12.90898	2.17E-14	1.24251E-12	X-Pro aminopeptidase
WP_014164868.1	1.701	11.06844	8.26E-12	3.05734E-10	RNA polymerase sigma factor RpoD
WP_060383854.1	1.698	6.147511	3.97E-13	1.91922E-11	aquaporin (water channel protein)
WP_060382871.1	1.669	9.506231	7.24E-14	3.79483E-12	acetyltransferase
WP_060383036.1	1.658	10.21155	8.81E-08	1.97834E-06	cyclase(arylformamidase activity)
WP_060383035.1	1.652	6.557008	1.90E-11	6.63111E-10	T9SS C-terminal target domain-containing protein
WP_060383786.1	1.644	11.71884	7.58E-13	3.33948E-11	endothelin-converting protein
WP_060383326.1	1.639	10.73342	3.09E-09	8.43985E-08	electron transfer flavoprotein subunit alpha
WP_060383401.1	1.613	9.349299	6.22E-13	2.89982E-11	hypothetical protein

WP_060383376.1	1.595	13.38785	3.10E-11	1.06765E-09	hypothetical protein
WP_060383582.1	1.576	6.350154	1.48E-06	2.79601E-05	T9SS C-terminal target domain-containing protein
WP_060383353.1	1.564	9.133181	3.82E-13	1.88387E-11	ABC transporter permease
WP_060382069.1	1.540	10.6691	1.03E-10	3.32918E-09	hypothetical protein
WP_060383656.1	1.532	7.805838	9.73E-11	3.21976E-09	ferrous iron transport protein B
WP_060382464.1	1.528	7.73979	5.41E-12	2.09235E-10	TonB-dependent receptor
WP_060381901.1	1.521	7.581453	7.55E-12	2.83625E-10	hypothetical protein
WP_060381835.1	1.520	3.316255	5.93E-10	1.79762E-08	hypothetical protein
WP_060381532.1	1.519	7.185773	1.74E-13	8.91429E-12	enoyl-CoA hydratase
WP_060382699.1	1.511	12.33655	1.69E-09	4.72496E-08	peptidase M16
WP_060381689.1	1.474	10.37171	1.35E-07	2.93357E-06	flagellar motor protein MotA
WP_060383434.1	1.460	9.679931	7.64E-10	2.28724E-08	outmembrane efflux protein
WP_060381632.1	1.426	8.668103	9.65E-10	2.82206E-08	hypothetical protein
WP_060383675.1	1.421	9.746996	1.12E-11	4.06766E-10	hypothetical protein
WP_060381383.1	1.420	8.912427	3.37E-13	1.69579E-11	hypothetical protein
WP_060381306.1	1.394	8.608299	7.70E-13	3.33948E-11	sugar isomerase
WP_060383550.1	1.386	3.830284	2.25E-05	0.000303011	1-acyl-sn-glycerol-3-phosphate acyltransferase
WP_060382995.1	1.373	1.310361	0.000598	0.004543301	hypothetical protein
WP_060382817.1	1.370	7.837991	1.78E-06	3.29639E-05	hypothetical protein
WP_060382624.1	1.367	8.6717	3.06E-10	9.73656E-09	hypothetical protein
WP_060382678.1	1.339	7.949126	8.97E-10	2.65544E-08	hypothetical protein
WP_060382004.1	1.325	9.591382	7.91E-05	0.000888648	acetyl-CoA acetyltransferase
WP_060382994.1	1.321	8.530702	5.77E-13	2.73746E-11	LemA family protein

WP_060382503.1	1.310	2.607642	4.47E-07	9.136E-06	hypothetical protein
WP_060382314.1	1.308	6.030786	2.84E-12	1.13509E-10	anthranilate phosphoribosyltransferase
WP_060382366.1	1.279	6.787778	3.50E-09	9.47371E-08	hypothetical protein
WP_060381383.1	1.266	10.28724	3.37E-10	1.06122E-08	hypothetical protein
WP_060382586.1	1.258	6.063765	4.44E-10	1.37906E-08	hypothetical protein
WP_060382313.1	1.241	7.4134	6.38E-09	1.63814E-07	anthranilate synthase
WP_060382997.1	1.237	6.389905	4.03E-09	1.0781E-07	peptidase M23
WP_060382993.1	1.234	5.870052	6.74E-13	3.0835E-11	hypothetical protein
WP_060382315.1	1.227	5.160034	1.22E-08	3.01053E-07	indole-3-glycerol phosphate synthase
WP_060383661.1	1.215	6.832323	4.99E-08	1.15215E-06	patatin (lipase or storing function)
WP_060383370.1	1.214	9.068602	6.40E-06	0.000105881	thioredoxin
WP_060382704.1	1.214	3.778093	9.23E-07	1.7857E-05	hypothetical protein
WP_060381785.1	1.213	3.042683	1.23E-05	0.000177248	hypothetical protein
WP_060383433.1	1.208	10.60813	6.78E-09	1.72317E-07	efflux transporter periplasmic adaptor subunit
WP_060381373.1	1.189	7.109908	4.74E-10	1.4553E-08	NifU family protein (iron-sulfur cluster binding)
WP_060381615.1	1.178	8.624158	5.85E-09	1.51711E-07	hypothetical protein
WP_060381372.1	1.169	9.168523	8.87E-09	2.2328E-07	chromosome partitioning protein(ATPase activity)
WP_060381757.1	1.163	8.590069	1.31E-08	3.20997E-07	hypothetical protein
WP_060381679.1	1.155	2.476874	0.001071	0.007127348	hypothetical protein
WP_060381392.1	1.149	3.139791	1.02E-05	0.000154959	hypothetical protein
WP_060382957.1	1.141	4.485988	1.11E-05	0.000166149	hypothetical protein
WP_060383668.1	1.135	7.869841	5.64E-09	1.47895E-07	thioredoxin

WP_060383838.1	1.133	6.217834	7.05E-06	0.000115978	anthranilate synthase subunit II
WP_060383676.1	1.133	8.192526	7.02E-13	3.15472E-11	alpha/beta hydrolase
WP_060383689.1	1.120	6.136414	1.15E-09	3.31403E-08	transcriptional regulator
WP_060382941.1	1.109	7.16821	1.52E-05	0.000214734	hypothetical protein
WP_060382066.1	1.102	3.958931	2.62E-09	7.23208E-08	hypothetical protein
WP_060381305.1	1.101	8.374591	8.77E-11	2.94082E-09	glycosyl transferase family 1
WP_060383910.1	1.095	5.996754	9.83E-08	2.1702E-06	hypothetical protein
WP_060383628.1	1.095	0.740619	0.011824	0.042729572	transcriptional regulator
WP_060383810.1	1.095	1.835	0.000852	0.005980807	hypothetical protein
WP_060383338.1	1.094	5.146889	8.37E-06	0.000132398	hypothetical protein
WP_060382840.1	1.092	8.299183	2.32E-05	0.000309031	hypothetical protein
WP_060382136.1	1.090	4.140716	7.75E-06	0.000125038	hypothetical protein
WP_060382622.1	1.08948	10.19297	4.28E-07	8.83011E-06	TonB-dependent receptor
WP_060381443.1	1.075	12.23509	1.64E-06	3.06135E-05	NADH-dependent alcohol dehydrogenase
WP_060382067.1	1.074	4.920698	9.25E-09	2.3055E-07	hypothetical protein
WP_060383124.1	1.053	8.360609	1.59E-09	4.50235E-08	antibiotic resistance protein MarC
WP_060381635.1	1.039	1.851533	0.002155	0.011711574	hypothetical protein
WP_060383504.1	1.030	4.785884	1.17E-05	0.000170416	methylmalonyl-CoA epimerase
WP_060383579.1	1.029	5.780629	2.40E-08	5.68623E-07	hypothetical protein

WP_060383222.1	1.024	6.678646	1.51E-06	2.83261E-05	3-oxoacyl-ACP synthase
WP_060383545.1	1.018461	11.31238	1.18E-05	0.00017183	TonB-dependent receptor
WP_060383420.1	1.016	6.173865	7.87E-06	0.000126057	hypothetical protein
WP_060383337.1	1.015	4.600515	1.15E-05	0.000169084	hypothetical protein
WP_060381611.1	1.009	3.978543	5.40E-07	1.07902E-05	hypothetical protein
WP_060382985.1	1.009	0.714198	0.005067	0.022846938	hypothetical protein
WP_060383589.1	1.004	9.676746	2.31E-07	4.93155E-06	glutamyl-tRNA reductase
WP_060383259.1	-1.010	13.11652	0.003022	0.01520807	gliding motility protein GldN
WP_060381562.1	-1.013	8.467717	0.002767	0.014210105	NADH:ubiquinone oxidoreductase subunit H
WP_060381556.1	-1.027	7.947913	0.000264	0.002425266	NADH dehydrogenase
WP_060381594.1	-1.033	7.30669	0.000347	0.002989039	hypothetical protein
WP_060381797.1	-1.055	6.051312	0.003222	0.015987033	sulfate transporter
WP_060382476.1	-1.064	11.27273	0.002549	0.013362758	GTP-binding protein
WP_060383671.1	-1.096	8.462906	2.60E-05	0.00034207	succinate dehydrogenase
WP_060381560.1	-1.117	8.793067	0.001494	0.009194884	NADH oxidoreductase (quinone) subunit F
WP_060381555.1	-1.117	7.116917	0.000731	0.005316854	NADH-quinone oxidoreductase subunit A
WP_060382819.1	-1.129	7.985046	1.33E-11	4.78709E-10	carbamoyl phosphate synthase large subunit
WP_060383669.1	-1.129	9.174589	0.002497	0.013145165	fumarate reductase
WP_060381557.1	-1.134	7.07777	0.000133	0.001407973	NADH dehydrogenase
WP_060381795.1	-1.156	6.808492	0.002248	0.012080798	hypothetical protein
WP_060382918.1	-1.168	4.182993	0.001415	0.008789671	hypothetical protein
WP_060381558.1	-1.192	8.570683	0.000233	0.002198883	NADH dehydrogenase
WP_060381577.1	-1.200	11.58669	0.016609	0.054538	cytochrome c oxidase subunit I
WP_060382916.1	-1.202	4.697264	0.000728	0.005307985	hypothetical protein
WP_060382251.1	-1.208	6.135555	9.44E-08	2.10142E-06	lipoyl synthase
WP_060382917.1	-1.220	5.311126	4.78E-05	0.000575157	TonB-dependent receptor
WP_060382250.1	-1.225	7.310715	0.000359	0.003083122	hybrid sensor histidine kinase/response regulator

WP_060382477.1	-1.241	9.493366	0.000824	0.005843194	hypothetical protein
WP_060382302.1	-1.244	2.916828	0.000745	0.005386529	hypothetical protein
WP_060381563.1	-1.263	7.100096	0.000595	0.004538488	DNA-binding protein
WP_060382498.1	-1.279	9.129416	3.80E-06	6.54039E-05	zinc metalloprotease
WP_060381576.1	-1.288	10.83432	0.009439	0.035777542	cytochrome C oxidase subunit II
WP_060381631.1	-1.290	7.472837	0.003652	0.017738317	hypothetical protein
WP_060383670.1	-1.299	10.48085	0.001518	0.009268724	succinate dehydrogenase
WP_060381559.1	-1.306	7.443497	0.000185	0.00180542	NADH dehydrogenase
WP_060381574.1	-1.307	9.451262	0.008156	0.032012578	cytochrome C
WP_060382919.1	-1.314	7.588117	0.001398	0.008725461	cytochrome-c peroxidase
WP_060381575.1	-1.316	10.82421	0.007404	0.029709909	quinol:cytochrome C oxidoreductase
WP_060381573.1	-1.327	9.590081	0.011321	0.041341716	hypothetical protein
WP_060383835.1	-1.347	7.324971	0.000171	0.001712941	type I glyceraldehyde-3-phosphate dehydrogena
WP_060381796.1	-1.400	5.819922	0.001004	0.006735549	NADH dehydrogenase
WP_060381571.1	-1.415	11.7915	0.008237	0.032279367	quinol:cytochrome C oxidoreductase
WP_060382920.1	-1.451	9.592328	0.01141	0.041595661	hypothetical protein
WP_060382922.1	-1.472	9.165178	0.005124	0.023021482	hypothetical protein
WP_060381570.1	-1.482	10.16156	0.000154	0.001575995	cytochrome C
WP_060383214.1	-1.593	4.482674	0.002004	0.011130643	hypothetical protein
WP_060382921.1	-1.596	9.925068	1.22E-02	0.043791953	TonB-dependent receptor