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Sequencing and Analysis of the Flavobacterium Columnare ATCC 49512 Genome

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By

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Sequencing and analysis of the Flavobacterium columnare ATCC 49512 genome

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Flavobacterium columnare is a Gram negative fish pathogen that causes columnaris disease, which infects populations of wild and cultured fish species. However, pathogenic mechanisms of *F. columnare* are largely unknown. The purpose of this research is to obtain the complete sequence of the *F. columnare* ATCC 49512 genome to advance pathogenesis research and increase our understanding of this pathogen. To accomplish this, genome sequencing by using Sanger and 454 sequencing was conducted. The sequences were assembled, gaps were filled, and the circular genome was auto-annotated. The *F. columnare* genome size is 3.2 Mb and AT rich (68.5% AT). It contains 2,882 predicted proteins, 71 tRNA genes and five ribosomal RNA operons. More than half (57.1%) of the open reading frames have assigned function, which included chondroitin AC lyase, proteases, collagenases, and genes involved in biofilm formation, secretion systems, iron acquisition, and gliding motility.

Key words: *Flavobacterium columnare*, genome sequencing, columnaris

DEDICATION

Dedicated to my family…

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LIST OF SYMBOLS/TERMS

MVirDB: microbe virulence database

E. coli: Escherichia coli

F. columnare: Flavobacterium columnare ATCC 49512

F. johnsoniae: Flavobacterium johsoniae UW101

F. psychrophilum: Flavobacterium psychrophilum JIP02/86

F. branchilum: Flavobacterium branchilum FL-15

F. indicum: Flavobacterium indicum GPSTA100-9T

FI: *Flavobacterium indicum GPSTA100-9T*

FP: *Flavobacterium psychrophilum JIP02/86*

FB: *Flavobacterium branchilum FL-15*

FJ: *Flavobacterium johsoniae UW101*

CHAPTER I

INTRODUCTION

Flavobacterium columnare is the causative agent of columnaris disease. It is common throughout the world, infecting populations of wild and cultured fish species. Environmental and host-related factors such as elevated organic loads, high water temperature, crowded conditions, skin and gill damage, and excessive handling play an important role in mortality (Thune, 1991; Wakabayashi, 1991). *F. columnare* infections may be chronic and cause lingering, gradually increasing mortalities in freshwater aquaculture species, but more often, the disease appears suddenly and causes mortalities within a few days (Austin, 1991). The mechanisms of *F. columnare* pathogenesis are not completely understood. The lack of a high quality and annotated genome sequence of *F. columnare* is a critical problem, because until this information becomes available, it will not be possible to conduct functional studies to understand pathogenic mechanisms of this pathogen.

Channel catfish aquaculture is quite important to the U.S. economy (USDA, 2004). At present, columnaris disease is the second-leading cause of channel catfish mortalities in commercial aquaculture. It also affects wild fish populations in the U.S. and worldwide (Durborow, 1999). Thus, possible effects of columnaris disease on the economy are not easy to estimate. Nevertheless, channel catfish are very important in commercial aquaculture in terms of acreage (177,800), dollar value ($1.4 billion dollars),

and production (680 million pounds of catfish per year) (USDA, 2007). Despite their importance, the lack of essential information concerning columnaris disease limits the implementation of methods to manage, treat, and prevent the disease. The completed genome sequence of *F. columnare* will enable future functional genomics research.

The entire genome *F. columnare* will help resolve phylogenetic relationships within the highly heterogenic family Flavobacteriaceae. Due to insufficient knowledge of Bacteroidetes, more genome sequences are needed to develop additional genetic tools provide information to better understand this group of bacteria. Ultimately, the expected outcomes will enhance functional genomics research of *F. columnare*, which will help reduce fish mortalities and economic losses.

CHAPTER II

LITERATURE REVIEW

Flavobacterium columnare

Columnaris disease

Columnaris disease, also named "Saddleback disease" and "Fin rot", has been described and reported as a serious disease of freshwater fish and also commercial aquaculture industry. The etiologic agent of columnaris disease is *Flavobacterium columnare,* which is considered to be ubiquitous in the warm-water environment and affects cultured, wild, and ornamental fish throughout the world (Austin B, 1999; Davis, 1922; Ordal, 1944).

It has been reported that environmental and host-related factors such as poor water quality, high water temperature, excessive handling, skin and gill damage, crowded conditions, organic loads, and stress predispose columnaris disease, and all these factors play an important role in mortalities (Thune, 1991; Triyanto, 1999).

Bacterial characteristics

F. columnare is in the family of Flavobacteriaceae, which is one as the main phyletic lines within the Bacteroidetes group from the domain Bacteria (J. F. Bernardet, P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme, 1996) .

F. columnare is a long slender gram-negative rod (2-10 μm in length) forming yellow-

pigmented and typically rhizoid colonies (J. F. Bernardet, P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme, 1996; Song, 1988a). Colonies with yellow centers adhere tightly to media (J. A. Plumb, Hanson, L. A. , 2010). Three different types of colony morphology have been reported. The most common colony type observed in diagnostic cases is bright yellow, dry, umbonate and spreading with irregular edges. The second colony type bright yellow, moist, and spreading with uneven edges. The third type is pale yellow, dry, and flat, with uneven edges (J. A. Plumb, Hanson, L. A. , 2010). Another important characteristic of *F. columnare* is that bacterial isolates generally require 48 hours to reach optimum expected colony size, and optimum growth temperature is 25-30 °C.

To distinguish *F. columnare* from other yellow pigmented bacteria in the genus *Flavobacterium*, there are several defining characteristics: the shape of the colonies, ability to bind Congo red, sugar utilization, gelatin degradation, production of chondroitinase, salinity tolerance, and optimum growth temperature. There are also highly developed PCR methods (Darwish & Ismaiel, 2005). According to Griffin (Griffin, 1992), there are five main characteristics that help to distinguish *F. columnare* from other yellow pigment producing aquatic bacteria:

(1) capable of growing in the presence of neomycin sulfate and Polymyxin B,

(2) colony shape: thin, rhizoid, yellowish,

(3) ability to degrade gelatin,

(4) ability to bind Congo red,

(5) ability to produce chondroitin lyase.

Detailed characteristics of *F. columnare* are listed in Table 2.1 (J. A. Plumb, Hanson, L. A. , 2010).

Table 2.1 Biophysical and biochemical traits of *F. columnare*

Characteristic	***F. columnare***
Cell morphology	Long, Gram negative rods
Colony morphology	Flat, rhizoid adheres to agar
Cell size (μm)	0.3-0.5 x 3-10
Yellow pigmented colony	+
Motility	Gliding
Flexirubin pigment	+
Binds Congo red	+
Resistant to neomycin sulfate, polymyxin B	+
Chondroitin lyase	+
α- nitrophenyl-β-D-galactopymanoride	-
Growth on peptone	+
Glucose source of carbon	-
Acid from carbohydrates	-
Degradation of Gelation	+
Degradation of Casein	-
Degradation of Starch	-
Tyrosine	-
Urease	?
H_2S production	+
Nitrate reduced	-
Catalase	+
Cytochrome oxidase	+
Optimum growth at (°C)	25-30
Growth tolerance (°C)	37
Growth in 0% SW-HS*	+
33% SW-HS	-
66% SW-HS	-
100% SW-HS	-
G+C content (mol%)	32-37
Habitat	Freshwater (saprohytic)

Like other members of the Flavobacteriaceae, *F. columnare* is capable of gliding motility, the mechanism of which has been partially characterized in the related species *F. johnsoniae* (Hunnicutt, Kempf, & McBride, 2002) (McBride & Braun, 2004; McBride, Braun, & Brust, 2003). The *F. columnare* genome consists of a single circular chromosome with an estimated G+C ratio of 32% (J. F. Bernardet, 1989). The average genome size of bacterial species in the genus *Flavobacterium* has been reported as 4.1 ± 1 Mb (Fogel, 1999).

F. columnare produces several extracellular proteases that are considered to be important virulence factors (J. F. Bernardet, 1989; Bertolini, 1992; Christison, 1971; Griffin, 1991; Newton, 1997; Teska, 1993). Branchial and skin necrosis are associated with columnaris disease, but the role of extracellular proteases has not been studied very well (Pate & Ordal, 1967).

Identification of *F. columnare* genomovars was first made by Triyanto and Wakabayashi (Triyanto, 1999). Later, *F. columnare* strains were divided into three genomovars (Olivares-Fuster & Arias, 2011). The first two genomovars have been identified in European aquaculture, while genomovar I and III have mainly been reported in several Asian countries and the United States (Olivares-Fuster et al., 2007). According to Shoemaker (Shoemaker, Olivares-Fuster, Arias, & Klesius, 2008), genomovar I and genomovar II differ in pathogenicity; genomovar II results in 92-100% mortality in channel catfish experiments, while genomovar I caused 0-46% mortalities in channel catfish. This data suggest that genomovar type II is a more effective pathogen for channel catfish (J. A. Plumb, Hanson, L. A. , 2010).

Taxonomy

Columnaris disease was first described in 1922 by Herbert Spencer Davis, who observed the characteristic masses of bacteria isolated from fish during a major die off in the Mississippi River (Davis, 1922). Due to various technical difficulties, a pure culture was not isolated until 1945. Garnjobst isolated the pure culture in 1945; meanwhile, *F. columnare* was isolated by Ordal and Rucker as well (Ordal, 1944). *F. columnare* heterogeneity caused considerable confusion in classifying this bacterium. When it was first described in 1922, *F. columnare* was named *Bacillus columnaris* (Davis, 1922). Since then, the nomenclature of *F. columnare* changed many times; examples include *Cytophaga columnaris* (Garnjobst, 1945), *Chondrococcus columnaris* (Newton, 1997; Reichenbach, 1989), and the current *F. columnare* (J. F. Bernardet, P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme, 1996).

The species has been classified by morphologically, biochemically, serologically (Anacker, 1959; Arias, 2004; A. Decostere, Haesebrouck, F., and Devriese, L. A., 1998; Michel, 2002; Shamsudin, 1996; Triyanto, 1999) and divided into several groups based on the 16S rRNA (Arias, 2004) and extracellular protease profiles (Newton, 1997). *F. columnare* isolates identification can be accomplished utilizing the method of Griffin (Griffin, 1991) and API NE system from Biomerieux. In one study, this method helped to identify approximately 48 of 49 strains as *F. columnare*. Random amplified polymorphic DNA analysis played an important role in molecular characterization of various *F. columnare* strains (Bader, 2003). Strains have also been confirmed as *F. columnare* by PCR using specific primers published by Bader (Bader, 2003).

Epizootiology

F. columnare is a ubiquitous organism and is generally considered to cause water-borne disease. Columnaris disease is widely distributed, affecting many freshwater fishes. (Becker, 1978; Snieszko, 1976). According to Bullock, columnaris disesase has been generally found in freshwater fishes; myxobacterial disease showed similar symptoms in some marine fishes (G. L. Bullock, D.A. Conroy, and SE Snieszko, 1971). Several commercially important species are affected by columnaris disease such as channel catfish (*Ictalurus punctatus)*, common carp (*Cyprinus carpio)*, goldfish (*Carassius auratus)*, rainbow trout (*Oncorhyncus mykiss)*(Walbaum), Japanese eel (*Anguilla japonica)*(Temminck&Schlegel), and tilapia (*Oreochmis sp)*(Anderson & Conroy, 1969) (Soto, 2008).

Columnaris disease sometimes occurs as a primary infection in the host, but in most of the diagnostic cases, the disease develops as a secondary pathogen. Several environmental factors may contribute to development of columnaris disease such as water temperature (H., 1989 ; Wakabayashi, 1991), salinity (Altinok, 2001), environmental stress including high fish density (Wakabayashi, 1991), nutritional stress (Klesius, 1999), and trauma (J. A. Plumb, Hanson, L. A. , 2010).

Columnaris disease can occur as external and systemic infection (J. A. Plumb, Hanson, L. A. , 2010). Of 53 *F. columnare* cases examined, 17% were only internal, 11% were solely external, and 72% were combination of internal and external infection (Hawke, 1992 ; J. A. Plumb, Hanson, L. A. , 2010).

F. columnare can be transmitted through the environment, by shedding the bacteria into water or by direct contact to healthy fish (G. L. Bullock, Hsu, T.C., and

Shotts , E.B., Jr., 1986; J. A. Plumb, 1999; Welker, Shoemaker, Arias, & Klesius, 2005). PCR confirmed horizontal transmission of *F. columnare* in water, and this method showed that transmission could easily occur without fish-to-fish contact (Welker et al., 2005).

Survival and infectivity of *F. columnare* can be related to the presence of other bacteria. In the presence of *Aeromonas hydrophila* and *Citrobacter freundii*, *F. columnare* failed to invade fish host; however, in the absence of the competitive fish pathogen and non-fish pathogen, the density of *F. columnare* increased exponentially (Chowdhury, 1989).

Pathology

Virulence factors of *F. columnare* have not been studied extensively, but potential virulence factors such as adhesion capabilities and enzyme activities could be responsible for the pathological process. It has been shown that *F. columnare* strains may have different virulence and virulence factors, and those differences may be related to specific fish species (H. M. Kunttu, Jokinen, Valtonen, & Sundberg, 2011; Shoemaker et al., 2008; Suomalainen, Tiirola, & Valtonen, 2006).

Mechanical and physiological injury or environmental stressors play an important role in the initial infection of *F. columnare*; however, infection may occur without stressors. To develop *F. columnare* disease, skin injury is not essentially necessary; however, abrasion of the skin accelerates invasion and dissemination to organs and tissues (Bader, 2003; J. A. Plumb, Hanson, L. A. , 2010).

Degradative enzyme activity may contribute to pathogenicity of *F. columnare,* but more information is needed. For this purpose, Newton (Newton, 1997) partially characterized 23 extracellular proteases that were isolated from channel catfish isolates.

Most of the extensive tissue necrosis caused by *F. columnare* infection was associated with the strong tissue-destroying enzymes produced by the bacteria. Chondroitin AC lyases (ChonAC) and other specific proteases and adherence factors have been determined as virulence factors (A. Decostere, Haesebrouck, Charlier, & Ducatelle, 1999; Newton, 1997). Chondroitin AC lyase degrades components of the extracellular matrix (hyaluronic acid and chondroitin sulphates) contributing to the ability of the pathogen to invade host tissues (Xie, Nie, Chang, Liu, & Yao, 2005).

Capsular polysaccharides, which plan an important role in invasion of the host, are produced by numerous pathogenic bacteria to protect from host defenses and can be induced by stressful conditions (Schiraldi, Cimini, & De Rosa, 2010). A connection has been found between capsular polysaccharide chondroitin sulphate and pathogenicity of *F. columnare* (Suomalainen et al., 2006).

Transmission of *F. columnare* is higher at 23°C compared to 18°C (Suomalainen, Tiirola, & Valtonen, 2005). *F. columnare* can degrade affected tissues more quickly at higher temperatures (H. M. T. Kunttu, Valtonen, Jokinen, & Suomalainen, 2009). However, there is not a precise explanation for disease augmentation and symptoms at high water temperatures (Pulkkinen et al., 2010).

Clinical Signs

Disease generally appears in the dorsal fins and skin. Initially, bluish gray external body lesions appear, followed by necrotic lesions that cause the fish to lose the

metallic sheen of the body surface. As bacteria accumulate, lesions can become yellow and be accompanied by mild inflammation such as ulcers or hemorrhagic necrotic erosions, which are sometimes surrounded by inflamed skin.

Lesions on the gills are common in catfish; these usually begin at the tip of the lamella and develop into the gill arch. Typically, blood vessels of the gills are blocked with dissociation of the surface epithelium of the lamellae from the capillary bed. As a result of this blockage, hemorrhaging may occur in random locations (Pacha & Ordal, 1967). Eventually, the gill filaments are totally destroyed by extensive erosion (Pacha & Ordal, 1970) (Figure 2.1). Lesions caused by erosion on the body may become 3-4 cm in diameter and may cover at least 20 % of the total body surface of the sick fish (Austin B, 1999).

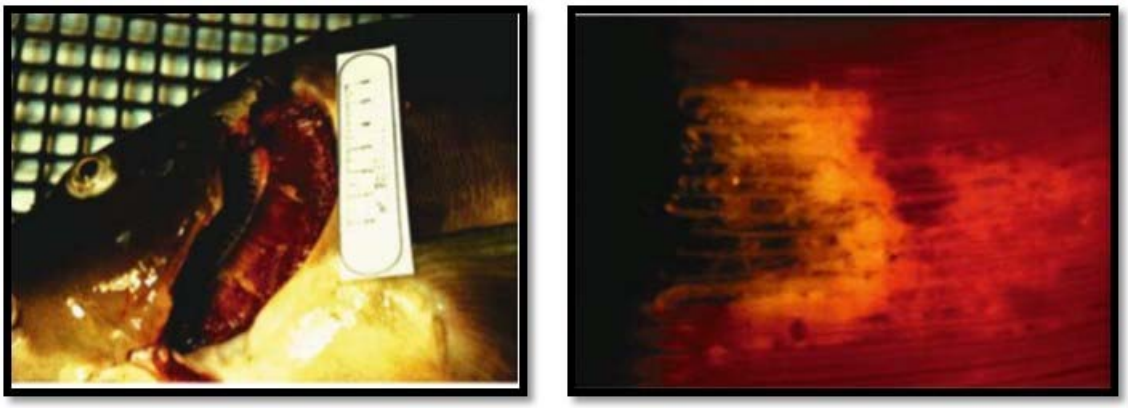

Figure 2.1 Gill necrosis in carp (A). Under the stereomicroscope(x10), destroyed gill tissue by the extracellular enzyme (B).

(J. F. Bernardet & Bowman, 2006)

Additionally, columnaris disease is known to cause an acute to chronic infection of the gills, mouth, and oropharynx (Figure 2.2) (J. A. Plumb, Hanson, L. A. , 2010;

Welker et al., 2005). It is also capable of causing systemic infection and entering the inner organs by the blood stream. Clinical presentation and location of lesions from columnaris disease may vary from outbreak to outbreak (J. A. Plumb, Hanson, L. A. , 2010).

Figure 2.2 Typical gill lesion of columnaris disease*

*(Courtesy of Derek Gibbs)

Diagnosis

Columnaris disease can be presumptively diagnosed by evaluating lesions on the skin, fins, gills, and mouth (J. A. Plumb, Hanson, L. A. , 2010). Microscopic observation of unique characteristic, “haystacks”, formed by the bacteria and found especially in gill tissue, can be confirmatory evidence for columnaris disease (J. A. Plumb, Hanson, L. A. ,

2010). However, highly virulent strains can cause death without macroscopic evidence of tissue damage (Pacha & Ordal, 1967). Additionally, epidermal spongiosis and necrosis may be observed by microscopic examination of tissue lesions, accompanied by ulceration and necrosis that may progress into the dermis. When peripheral lesions are involved, hyperemia can generally be observed (Esteban, 2007).

Isolation and cultivation of the causative agent can be definitive diagnosis, followed by identification methods such as molecular techniques, biochemical methods, or serological methods. Histopathology studies might help to identify and detect severity of the infection (Morrison, Cornick, Shum, & Zwicker, 1981).

Three media are commonly used as a selective method for columnaris disease. First, "cytophaga media" is prepared by using Ordal`s media (Anacker, 1959) and adding 5 µg/ml of neomycin and 200IU/ml polymyxin B; cytophaga media inhibits other bacteria, while promoting the growth of *F. columnare* (Anacker, 1959; Esteban, 2007; J. A. Plumb, Hanson, L. A. , 2010). The second media is "Hsu-Shotts" (Shotts, 1991). "Shieh media" (Shieh, 1980) is also used, and in one study was found to be superior to other media for isolating *F. columnare* (Song, 1988b).

In 1992, Griffin combined all the available studies to develop an identification scheme including five main characteristic of *F. columnare*, and addition of polymyxin B and neomycin sulfate to the selective media was recommended (Griffin, 1992). Tobramycin is an effective alternative that can enhance the selective capacity of Shieh media (A. Decostere, Haesebrouck, & Devriese, 1997).

An immunofluorescent method has been developed using a combination of antibody-conjugated fluorochromes, to help to produce sensitive, rapid, and more

accurate diagnosis (Panangalal et al., 2006). Diagnosis can also be confirmed using specific primers from the ribosomal rDNA region, which is based on 16S-23S intergenic spacer region (Bader, 2003; Welker et al., 2005).

Treatment and prevention of columnaris disease

As previously mentioned, several stress-related factors play an important role in a columnaris infection. For example, water quality, high water temperature, excessive handling, seining, physical injuries including gill and skin damage, and organic loads are predisposing environmental factors for columnaris (J. A. Plumb, 1999; Thune, 1991; Triyanto, 1999).

Potassium permanganate is used for treatment and prevention for columnaris disease. Dose depends on the organic load in the pond. Plumb suggested a combination of permanganate and oxytetracycline-medicated feed (J. A. Plumb, 1999). Combining terramycin with a one hour treatment of potassium permanganate may reduce incidence of posthandling infections; moreover, treatment may stimulate anorexic fish to eat antibiotic medicated feed (Noga, 2010).

Perox-Aid is commonly used as a static and continuous treatment especially for channel catfish and fingerlings with columnaris infection (J. A. Plumb, Hanson, L. A. , 2010). Infected fishes were treated experimentally with Diquat, and this herbicide was found to have some therapeutic efficacy for columnaris disease in channel catfish. Diquat has EPA approval, but it is not FDA approved for fish disease control (Darwish & Mitchell, 2009; J. A. Plumb, Hanson, L. A. , 2010).

In 2007, Shoemaker et al. developed a live *F. columnare* vaccine, Aquavac-Col (Intervet/Schering-Plough Animal Health, Boxmeer, Netherlands), (Shoemaker, Klesius, & Evans, 2007).

Significance

Research on the pathogenesis of columnaris has been very slow due to several factors including: 1) confusion created by heterogeneity between *F. columnare* isolates, 2) difficulties in culturing *F. columnare*, 3) lack of a consistent experimental challenge model, 4) lack of knowledge on gene expression and promoter systems, and 5) lack of fully annotated genome. Thus, there is an urgent need to obtain a fully annotated genome sequence of *F. columnare*.

The genome sequence will enable functional genomics experiments to elucidate mechanisms of pathogenesis as well as comparative genomics to identify putative virulence genes and help resolve systematic classification of *F. columnare*. Therefore, I propose to sequence, assemble, annotate and release the genome of *F. columnare.* Although, the current research is not hypothesis driven, *F. columnare* genome will enable future hypothesis-driven functional genomics research and promote knowledge advancement and collaboration on columnaris disease in the aquatic animal health research community.

CHAPTER III

MATERIAL AND METHODS

Strain selection

F. columnare strain ATCC 49512 was selected for sequencing because this strain is one of the best characterized *F. columnare* strains. It was isolated in 1987 from a lesion of brown trout (*Salmo trutta*) fry in France. ATCC 49512 strain was deposited in the American Type Culture Collection in 1996 by Bernardet and Grimont.

Library construction

At the beginning of the project, we proposed to obtain 48,000 Sanger sequence reads to result in approximately 8.0 fold coverage of the estimated 4.8 Mb *F. columnare* genome based on estimated average read length of 800 base pair.

Small and intermediate insert library construction

Genomic library was prepared and 3-6 kb, 6-10 kb, and 10-12 kb fragments were size-selected by agarose gel electrophoresis. Afterwards, library construction was conducted in pJAZZ-KA (Figure 3.1) linear vector (Godiska et al., 2010; Karsi & Lawrence, 2010).

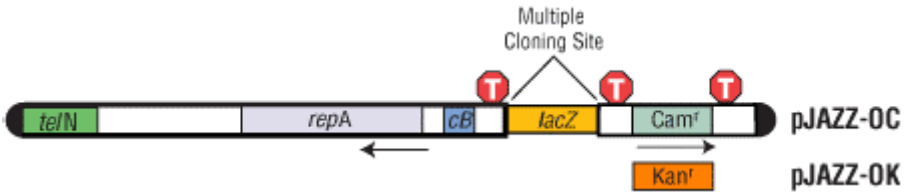

Figure 3.1 PJAZZ Big easy Linear Cloning System

Sequencing

Shotgun sequencing of the *F. columnare* ATCC 49512 genome was conducted using two sequencing platforms: Sanger sequencing and 454 pyrosequencing.

Sanger and Roche 454 Life Science`s pyrosequencing

Shotgun paired-end Sanger sequencing was conducted on the small (3-6 kb), medium (6-10 kb), and large (10-12 kb) insert libraries to obtain 8X coverage of the complete genome at the Laboratory for Genomics and Bioinformatics at the University of Oklahoma Health Sciences Center (Tekedar et al., 2012).

Genomic DNA of *F. columnare* ATCC 49512 was also sequenced using a 454 Life Sciences GS FLX system (Roche, Brandford, Connecticut). A total of 269,256 sequencing reads were obtained with 269.4 bp average read length (Tekedar et al., 2012).

Assembly

We used Phred/Phrap for read trimming and initial assembly (Ewing, 1998). Sequence data was transferred to a Sun V880 server for further processing and assembly. The data was processed by a custom script that does several things:

1) Each of the output files were converted to a filename format convention recognized by Phred/Phrap.

2) Each data file was automatically trimmed to remove vector and low quality sequence, and Phred analyzed each for data quality. Phred makes each basecall, and the resulting output was written as a phd file.

3) These files were then examined by LUCY (Chou & Holmes, 2001) which confirmed that each sequence is of high quality and read length, removed vector sequence, and wrote these to output files that are used by Phrap.

4) Phrap then took the accumulated phd files and assembled them using operator-specified assembly parameters.

The assembler made pair-wise comparisons between sequence reads to find an appropriate sized region between two sequences. Paired read ends were automatically tracked by Phrap during assembly and removed from the assembly if they did not conform to the expected separation of these reads based on the insert size of the library in question.

Consequently, both Sanger and 454 sequencing generated a new assembly, which is approximately 30-fold coverage. Moreover, hybrid assembly resulted in 6 large contigs.

Gap closure

The complete *F. columnare* genome sequence was obtained by running additional sequencing reactions in areas of low quality and by sequencing across the gaps between contigs. PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/). Appropriate pJAZZ-KA clones were used as PCR template to generate amlicons for further sequencing and gap closure reactions. Promega GoTaq DNA Polymerase was used for PCR amplification under the following conditions: an initial denaturation step at

94 °C for 2 min, followed by 30 cycles at 94 °C for 30 second, annealing at 59°C for 30 seconds and extension at 72 °C for 2 minutes, with a final extension of 10 min at 72 °C, followed by 10 °C for preservation. To accomplish this, we employed primer-directed sequencing from small and large insert library templates prepared above. The AUTOFINISH function in the Phred/Phrap suite was used to help design closure experiments (Gordon, Desmarais, & Green, 2001). BioPerl was used to augment paired-read analysis in CONSED for linking contigs and designing closure experiments. Comparisons with publicly available genome sequences of related species Flavobacteria (*Flavobacterium* sp. MED217, *F. johnsoniae* UW101, *F. columnare* 94-081 and *F. psychrophilum* JIP02/86) were done to determine if synteny with these species informed closure experiments.

We also used SPACE walking, which has proven to be very efficient for closure purposes. Single Primer Amplification of Contig Ends (SPACE) is a single primer PCR protocol similar to that described for rapid amplification of transposon ends (RATE)(Karlyshev, Pallen, & Wren, 2000). In brief, two primers were designed for SPACE at the end of each contig after assembly. The first primer was a PCR primer designed approximately 200 bp from the end of the contig and oriented so as to amplify towards the sequence gap. PCR was performed using this single primer on *F. columnare* template DNA using an initial high-stringency step that produces single-strand DNA from the site of the primer towards the gap. In the second low-stringency phase, the SPACE primer anneals at random to several regions DNA. Promega GoTaq DNA Polymerase with the following conditions was used: for the first step, an initial denaturation at 94 °C for 2 min, followed by 25 cycles at 94 °C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72 °C for 3 minutes; for the second step, 30

cycles at 94 °C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72 °C for 2 minutes; and for the third step, 30 cycles at 94 °C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72 °C for 2 minutes; and followed by a final extension for 10 min at 72 °C and preservation at 10 °C. At the conclusion of gap closure, a full genome sequence with no gaps and low quality regions were generated.

Annotation

For annotation, the whole genome DNA sequence of *F. columnare* ATCC 49512 was submitted to the NCBI`s Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html).

Additionally, we used RAST (http://rast.nmpdr.org/) to help identify protein-encoding, rRNA, and tRNA genes and, to predict metabolic pathways and networks (Aziz et al., 2008). We also used MicroScope, which provides a web-based framework for systematic and efficient revision of microbial genome annotation as well as comparative analysis (https://www.genoscope.cns.fr/agc/microscope/home/index.php) (Vallenet et al., 2009).

Genome Analysis Software

Genome Comparison

Genome comparisons with closely related strains were performed using MaGe (https://www.genoscope.cns.fr/agc/microscope/mage/index.php?), which allows graphic visualization enhanced by a synchronized representation of synteny groups.

Pathway analysis

For pathway analyses, Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (http://www.genome.jp/kegg/) was used because it provides integrated genomic, proteomic, metabolic, and systemic functional information (Kanehisa & Goto, 2000; Ogata et al., 1999).

Alignment

Mauve was used to identify potential horizontal transfer loci and genomic rearrangements (Darling, Mau, Blattner, & Perna, 2004).

Genomic islands

To predict genomic islands in the completed *Flavobacterium* genomes, IslandViewer was used. IslandViewer compiles results from three other genomic island prediction methods (IslandPick, IslandPath-DIMOB, and SIGI-HMM) (Dhillon, Chiu, Laird, Langille, & Brinkman, 2013; Langille & Brinkman, 2009).

Virulence factors

MVirDB microbial database (Zhou et al., 2007) was downloaded fom LLNL (Lawrence Livermore National Laboratory) website (http://mvirdb.llnl.gov/) and the protein sequences of *Flavobacterium* strains were compared against MVirDB database with Standalone BLAST using CLC Genomics Workbench v 6.5. An e-value cutoff $1*10^{-10}$ was used, and best hits were analyzed.

Insertion elements

Issaga was used for IS annotation. Issaga performs general prediction and yields information on genome context of individual insertion sequences (Varani, Siguier, Gourbeyre, Charneau, & Chandler, 2011).

CHAPTER IV

RESULTS AND DISCUSSION

Sequencing

To obtain the complete genome sequence of *F. columnare* ATCC 49512, two major shotgun sequencing strategies were applied. In total, 52,580 Sanger sequence reads were assembled and then combined with 269,256 454 sequencing reads (average length 269.4 bp). Figure 4.1 shows the order and orientation of contigs in the progress of assembly.

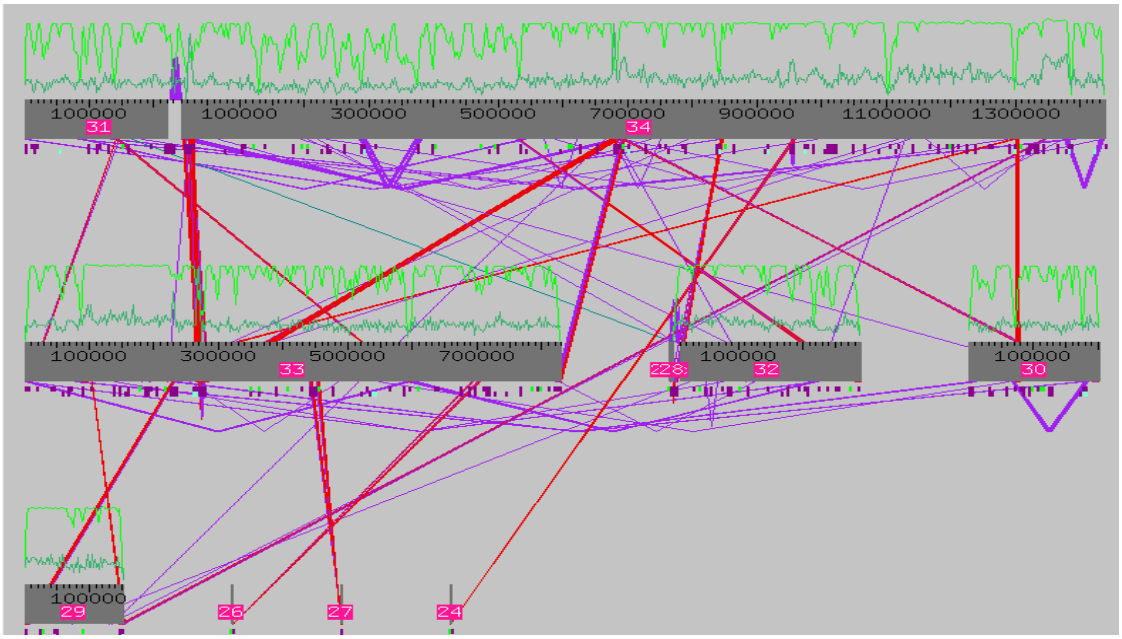

Figure 4.1 Consed assembly view of the *F. columnare* ATCC 49512 genome showing the order and orientation of contigs

General genome features

The *F. columnare* 49512 genome consist of a singular circular 3,162,432 bp chromosome with an average G+C content of 31.5% (Figure 4.2).

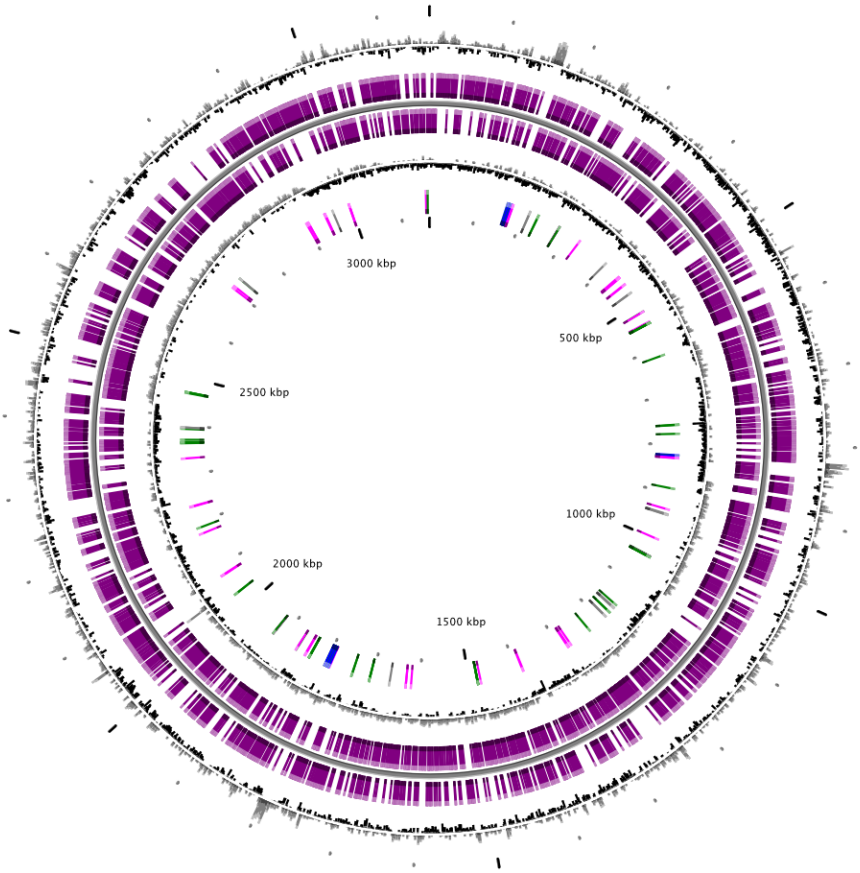

Figure 4.2 Circular representation of the *F. columnare* ATCC 49512 chromosome.

From the outside in, circle 1 shows GC percent deviation (GC window-mean GC) in a 100-bp window. Circle 2 shows predicted coding DNA sequences (CDS`s) transcribed in the clockwise direction. Circle 3 shows predicted CDSs transcribed in the counterclockwise direction. Genes displayed in circle 2 and 3 are colorcoded according to different categories; red and blue: MaGe validated annotations, orange: Microscope automatic annotation with a reference genome, purple: primary/automatic annotations. Circle 4 shows GC skew (G+C/G-C) in a 1000 bp window. Circle 5 shows rRNA (blue), tRNA (green), miscellaneous RNA (orange), transposable elements (pink), and pseudogenes (gray).

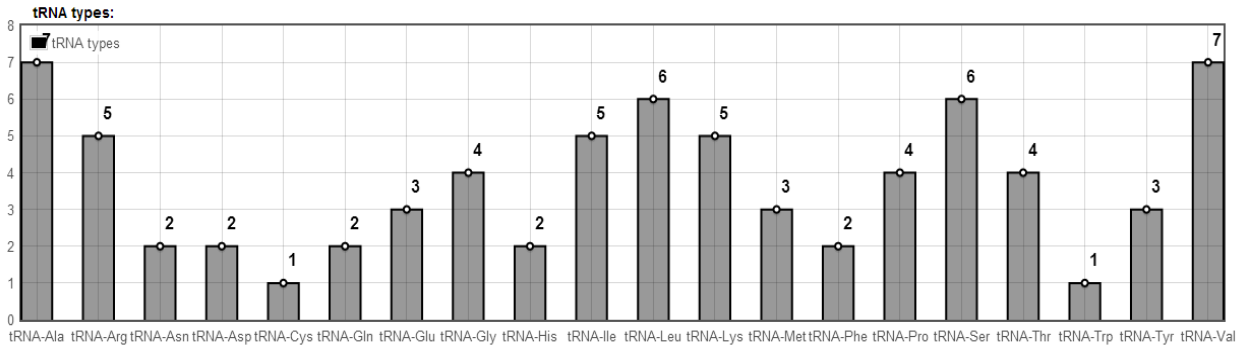

Figure 4.3 *F. columnare* 49512 strain tRNA types

The genome coding regions ratio is 85.2%. Annotation identified 2,731 predicted genes. Of those, 2,642 were protein coding, 1,625 of which have assigned functions. Five rRNAs were identified, which included 15 rRNA genes. Seventy-four tRNA genes were identified, which are categorized in Figure 4.3.

F. columnare ATCC 49512 chromosome features compared against *F. johnsoniae* UW101, *F. psychrophilum* JIP02/86, *F. branchiophilum* FL-15, and *F. indicum* GPSTA100-9T are listed in Table 4.1.

Table 4.1 Chromosome features of *F. columnare* ATCC 49512 compared to other *Flavobacterium* genomes

Chromosome feature	***F. columnare* ATCC 49512**	***F. johnsoniae* UW101**	***F. psychrophilum* JIP02/86**	***F. branchiophilum* FL-15**	***F. indicum* GPSTA100-9T**
Genome size	3,162,432	6,096,872	2,861,988	3,559,884	2,993,089
G+C content (%)	31.5	34.1	32.5	32.9	31.4
Number of rRNA operons	5	6	6	3	4
Number of tRNA genes	74	62	49	44	55
Protein coding genes	2642	5056	2432	2867	2671
Average gene length (bp)	1,021	1,061	1,003	1,030	unknown
Plasmid	No	No	Yes	Yes	No

Genomic islands

Genomic islands are clusters of genes that occur especially in prokaryotic genomes. Often genomic islands are associated with microbial adaptations of medical or environmental interest (Langille, Hsiao, & Brinkman, 2008). Genomic islands can carry genes that encode specific metabolic functions, such as metabolite degradation, secretion, or antibiotic resistance. Genomic islands often carry transmissible genomic elements such as transposons, bacteriophages, or plasmids (Hacker, Blum-Oehler, Muhldorfer, & Tschape, 1997).

Sometimes genomic islands are associated with pathogenicity and contribute to bacterial genome evolution because they result from horizontal gene transfer from pathogenic strains (Dobrindt, Hochhut, Hentschel, & Hacker, 2004). The term "pathogenicity island" refers to these genomic islands that are present in pathogenic strains and are generally absent in nonpathogenic strains. They are large chromosomal (10-200 kb) regions in bacteria (Lee, 1996). This term was first described in human pathogens of the species *Escherichia coli*, and over time it has been used in other organisms because several animal, plant, and human pathogenic species carry them. Often the G+C content of pathogenicity islands differs from that of the rest of the host genome, clearly indicating that those regions originated or adapted from other bacteria by horizontal gene transfer (Dobrindt & Reidl, 2000). The majority of pathogenicity islands may have evolved from either plasmids or integrated phages (Lee, 1996). These regions often encode virulence factors of pathogenic bacteria, including adhesins, toxins, iron uptake systems, and secretion systems (Hacker & Kaper, 2000).

We used IslandViewer (Langille & Brinkman, 2009) to predict genomic islands in the *F. columnare* ATCC 49512 chromosome. IslandViwer combines three different genomic island prediction methods: IslandPick (Langille et al., 2008), SIGI-HMM (Waack et al., 2006), and IslandPath-DIMOB (Hsiao, Wan, Jones, & Brinkman, 2003). Five genomic islands were identified in *F. columnare* ATCC 49512 (Figure 4.4), ranging from 5 to 15 kb.

We compared genomic island results to other closely related strains: *F. psychrophilum* JIP02/86*, F. johnsoniae* UW101*, F. branchiophilum* FL-15*, and F. indicum* GPSTA100-9T.

F. johnsoniae UW101 has 18 predicted genomic islands, *F. psychrophilum* JIP02/86 has 3, *F. branchiophilum* FL-15 has 3, and *F. indicum* GPSTA100-9T has 4 (Figure 4.5). Starting at the origin of replication and progressing clockwise, the first *F. columnare* ATCC 49512 genomic island encodes four phage proteins, nine hypothetical proteins and asparaginase. The second encodes one membrane protein and seven hypothetical proteins. The third island has four different types of genes: integrase, a transcriptional activator, three transposons and four hypothetical proteins. The fourth island encodes three hypothetical proteins, DNA binding proteins, and an integrase. The fifth island encodes nine hypothetical proteins, a helicase, one DNA repair protein, and one DNA binding protein. All identified proteins are listed in Table 4.2 including their location in the genome and whether they are present in other *Flavobacterium* species. Many of the genes encode proteins with unknown function. Three of the islands include genes encoding transposable elements or integrases, suggesting horizontal gene transfer was the source of these genomic islands.

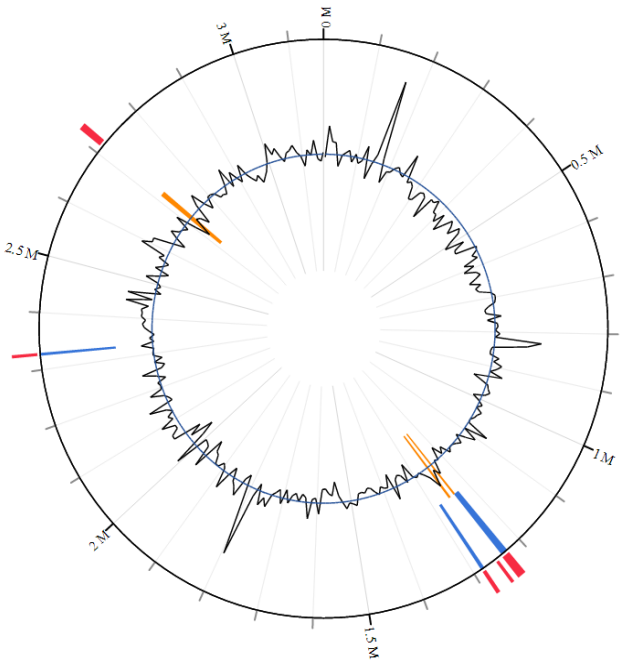

Figure 4.4 Genomic islands in *F. columnare* ATCC 49512.

Predicted genomic islands are plotted to scale in blue for IslandPath-DIMOB, orange for SIGI-HMM, and red for the integrated IslandViewer analyses.

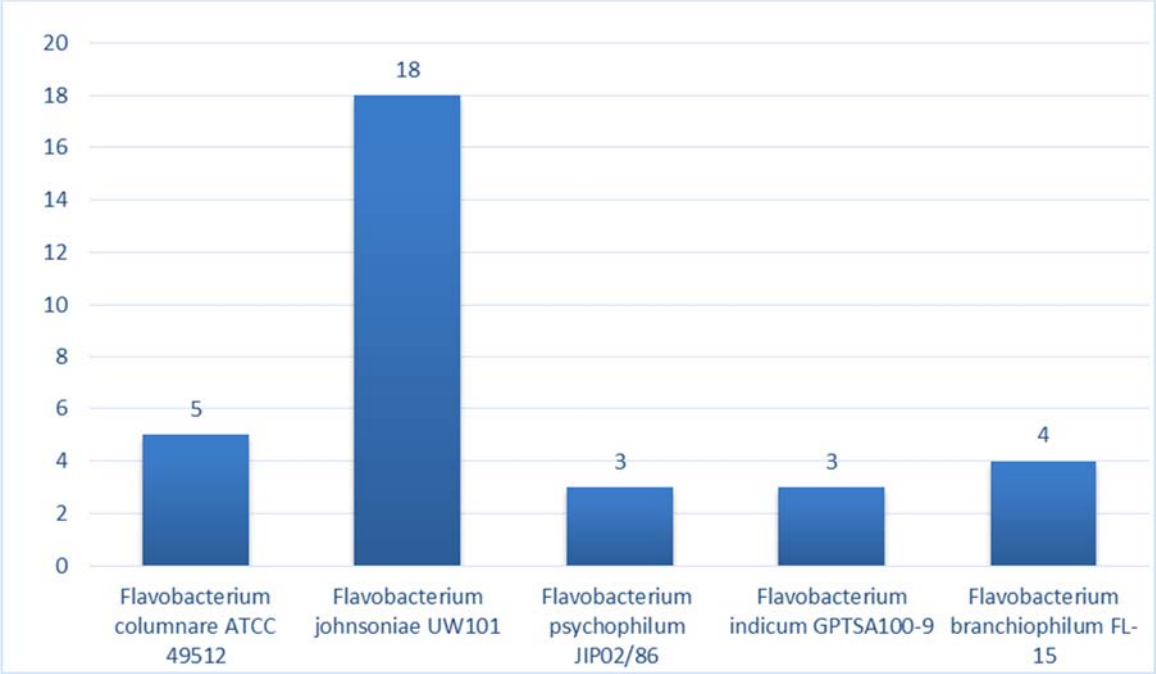

Figure 4.5 Number of genomic islands in *Flavobacterium* strains

Table 4.2 Predicted genomic island proteins encoded in *F. columnare* ATCC 49512

Protein ID	Coordinates	Product	Presence in other *Flavobacterium* strains
AEW85875.1	1229337..1230371(+)	asparaginase 1	
AEW85876.1	1230589..1231755(+)	integrase	
AEW85879.1	1234964..1238071(-)	phage tail tape measure protein, TP901 family	
AEW85885.1	1241010..1241513(-)	Co-activator of prophage gene expression IbrB	
AEW85886.1	1241510..1242790(-)	Co-activator of prophage gene expression IbrA	
AEW85904.1	1255355..1255789(-)	membrane protein	
AEW85931.1	1278960..1280072(+)	integrase catalytic subunit	
AEW85935.1	1282977..1283414(-)	transcription activator effector binding protein	
AEW85936.1	1283552..1283965(+)	transposase IS1182 family protein	
AEW85938.1	1284561..1285355(+)	putative transposase, ISL3 family protein	*F. branchiophilum*
AEW85939.1	1285348..1285614(+)	transposase IS1182 family protein	
AEW86782.1	2325743..2326075(-)	helix-turN-helix domain protein	
AEW86784.1	2327858..2328892(+)	tyrosine recombinase XerD	
AEW86785.1	2328885..2329661(+)	integrase family protein	
AEW87098.1	2714898..2715152(+)	helix-turn-helix domain-containing protein	*F. johnsoniae*
AEW87099.1	2715270..2715722(-)	DNA repair protein RadC	*F. johnsoniae*
AEW87103.1	2718163..2720673(+)	dead-like helicase	
AEW87105.1	2721743..2722609(-)	AAA ATPase	*F. johnsoniae*
AEW87107.1	2723905..2725026(-)	integrase family protein	

Predicted Virulence Factors

To identify possible virulence factors encoded in *F. columnare* ATCC 49512 and other *Flavobacterium* strains, protein sequences were screened for the presence of encoding putative virulence factors. To do this, Microbial virulence Database MvirDB was downloaded from http://mvirdb.llnl.gov/ (Zhou et al., 2007), followed by creating a

local BLAST using CLC Genomic Workbench (version 6.5). To be considered a match, *F. columnare* proteins had to have BLAST result in the MvirDB with an E-value $1*10^{-10}$. Nine putative *F. columnare* ATCC 49512 virulence factors were identified. They are listed along with their presence in other *Flavobacterium* strains in Table 4.3.

Table 4.3 Potential virulence factors identified by MvirDB in *F. columnare* ATCC 49512 and related *Flavobacterium* strains

GI	Protein product	Number of hits	Lowest E-value	Presence in other *Flavobacterium* strains
365960204	Catalase-peroxidase	20	4.51E-32	FI, FJ, FB
365961648	Glutamate dehydrogenase	3	2.62E-16	FI, FP, FB
365959950	ATP-dependent Clp protease proteolytic subunit [*Listeria monocytogenes* EGD-e]	2	8.72E-16	FP, FJ, FB
365960111	Hypothetical protein BT_2310 [*Bacteroides thetaiotaomicron* VPI-5482]	9	1.99E-15	
365960731	SubName: Full=Metalloprotease;	86	1.55E-14	

Table 4.3 (Continued)

365961114	DNA-directed RNA polymerase subunit beta [*Streptococcus agalactiae* NEM316]	6	7.37E-13	FI, FP, FJ
365960252	80 kDa prolyl oligopeptidase [*Trypanosoma cruzi*]	1	7.56E-12	FJ
365961706	Glucosamine--fructose-6-phosphate aminotransferase [*Bradyrhizobium japonicum* USDA 110]	1	4.18E-10	
365961363	Chaperonin GroEL [*Legionella pneumophila* subsp. pneumophila str. Philadelphia1 1]	2	4.23E-10	

Insertion elements

Insertion elements are simple transposable elements, that are widely distributed in bacteria (Kusumoto et al., 2011). They play an important role in prokaryotic genome evolution and organization due to horizontal gene transfer. Insertion sequences can change their location on the same chromosome or they can be transferred to a different chromosome on a plasmid. Their size ranges from 0.7 to 3.5 kbp. They can interrupt a gene on the chromosome and inactive the expression of the coding sequence (Kusumoto et al., 2011).

Issaga was used to identify *F. columnare* insertion elements because it provides general prediction on a genome scale and also gives a graphical overview of insertion elements` distribution (Kusumoto et al., 2011). Using this tool, we identified that *F. columnare* ATCC 49512 genome has 11 different insertion element families and 87 total insertion elements (Figure 4.6). Of these, 87 ORFs related to insertion sequences, 23 are putative complete ORFs, 25 are putative partial ORFs, and 39 uncategorized ORFs (Table 4.4).

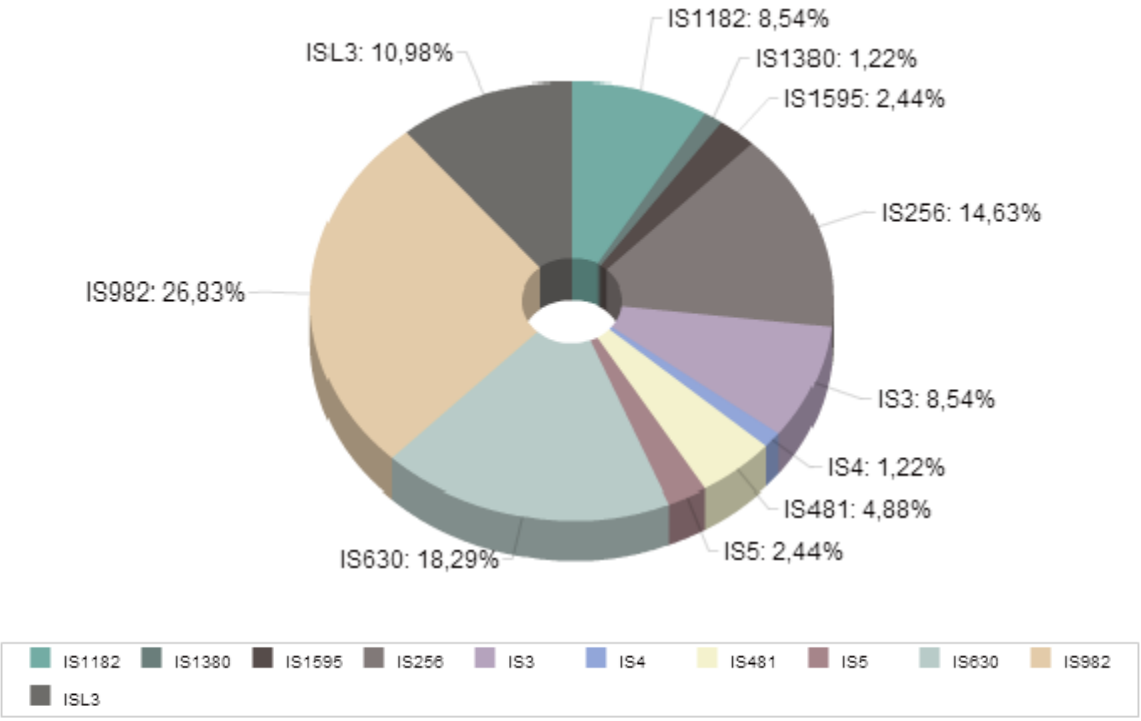

Figure 4.6 Percent distribution of insertion element families in *F. columnare* ATCC 49512

Table 4.4 Predicted insertion elements in the *F. columnare* ATCC 49512 genome by family

Family	ORFs Distribution *	Different IS(s)	Total IS(s)
IS4_ssgr_IS4	[0 / 0 / 0 / 1]	1	1
ISL3	[0 / 1 / 0 / 8]	7	9
IS3_ssgr_IS407	[5 / 2 / 0 / 3]	1	10
IS5_ssgr_IS5	[0 / 0 / 0 / 2]	1	2
IS3_ssgr_IS3	[0 / 0 / 0 / 2]	2	2
IS256	[0 / 4 / 0 / 6]	5	10
IS481	[3 / 1 / 0 / 0]	1	4
IS256_ssgr_IS1249	[0 / 0 / 0 / 2]	2	2
IS1595_ssgr_ISSod11	[1 / 0 / 0 / 0]	1	1
IS1182	[0 / 4 / 0 / 3]	4	7
IS982	[14 / 8 / 0 / 0]	2	22
IS630	[0 / 3 / 0 / 12]	1	15
IS1595	[0 / 1 / 0 / 0]	1	1
IS1380	[0 / 1 / 0 / 0]	1	1

*[Complete / Partial / Pseudogene / unknown]

Other *Flavobacterium* strains were also screened for insertion elements, and findings are listed in Figure 4.7. Several insertion elements were detected in each

Flavobacterium genome. We expected to find a large number of different insertion element families in *F. johnsoniae* due to its size (6,096,872 bp), but interestingly it has fewer families. Therefore, we can conclude that number of insertion elements is not directly related with genome size.

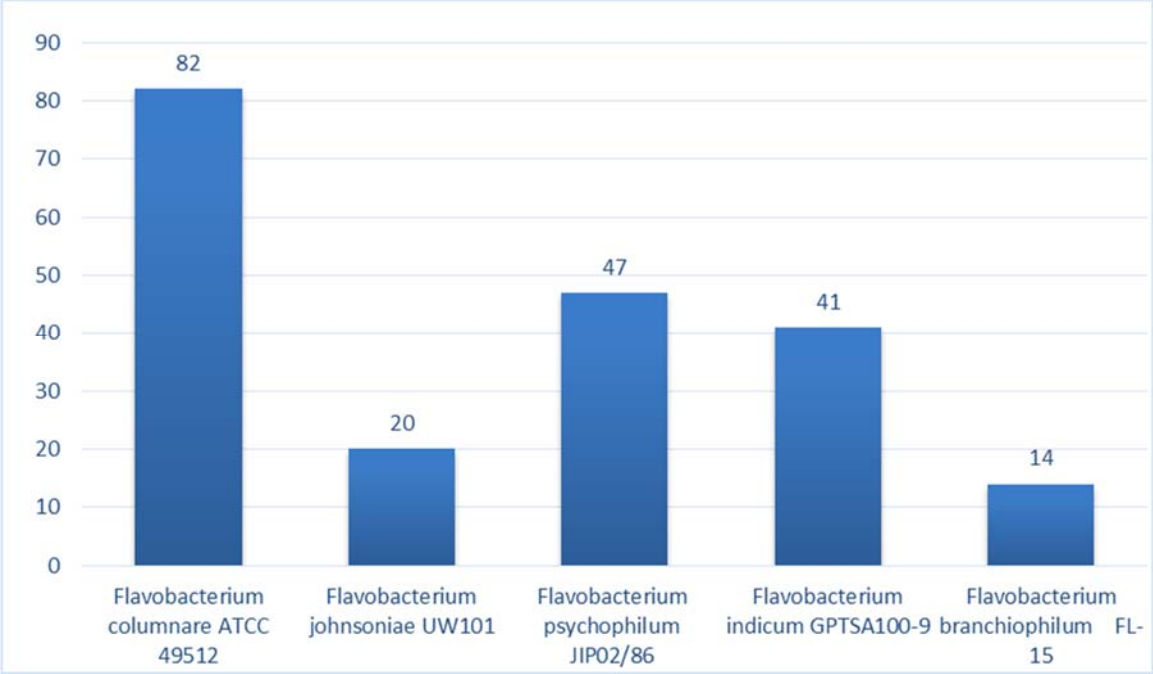

Figure 4.7 Insertion elements in *Flavobacterium* strains

Metabolism

An overview of *F. columnare* ATCC 49512 predicted protein functions by subsystem categories is shown in Figure 4.8.

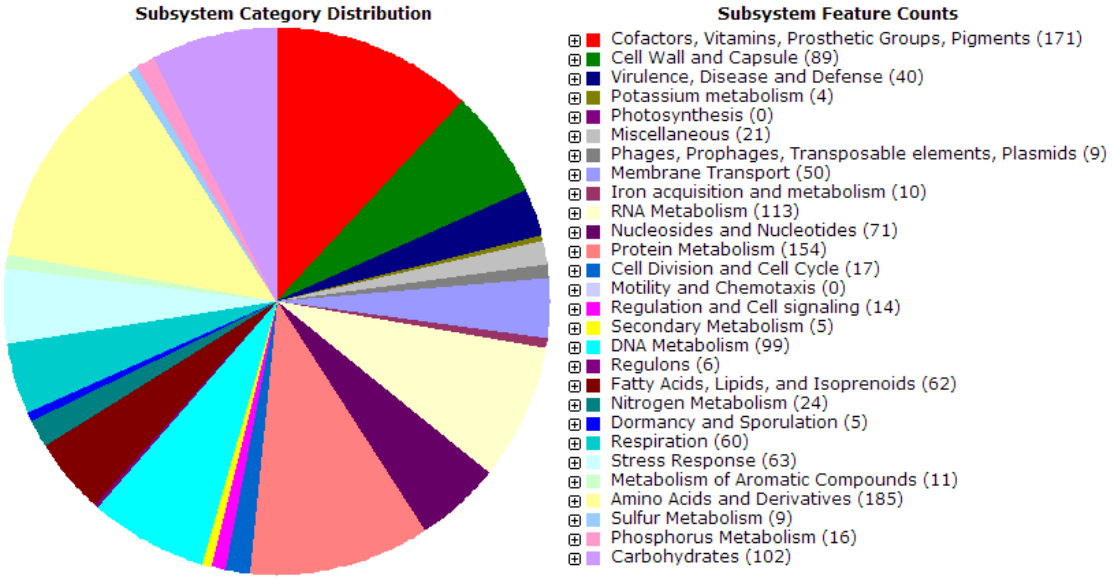

Figure 4.8 Distribution of *F. columnare* ATCC 49512 predicted protein functions by metabolic subsystem categories.

KEGG pathway analyses revealed that *F. columnare* ATCC 49512 genome has a complete TCA cycle and glycolysis pathway. Those findings are similar to other *Flavobacterium* strains. However, some carbohydrate metabolism components are unique to *F. johnsoniae* such as pentose phosphate pathway and pentose and glucuronate interconversions elements. Moreover, pathway comparison showed that starch and sucrose utilization pathways are present only in *F. johnsoniae* and *F. branchiophilum*. Carhydrate metabolism can have a crucial role in the adhesion of bacteria to the phagocytes (Wiklund & Dalsgaard, 2003).

In bacteria, there are four primary types of energy metabolism: 1) oxidative phosphorylation 2) methane metabolism 3) nitrogen metabolism, and 4) sulfur metabolism. *F. columnare* ATCC 49512 has predicted protein functions in two of these categories. Oxidative phosphorylation, is driven by the electron transport chain and

tricarboxylic acid (TCA) cycle (McNeil & Fineran, 2013). Oxidative phosphorylation consists of five different complexes, and our comparison analysis showed that all *Flavobacterium* strains encode oxidative phosphorylation components except for two missing genes. One of the missing genes encodes a protein in complex II, which is an important respiratory enzyme participating in both electron transport chain and TCA cycle. Complex II consists of four subunits: SdhA, SdhB, SdhC, and SdhD. SdhD is a hydrophobic membrane anchor and is missing in all the *Flavobacterium* strains (McNeil & Fineran, 2013). *F. johnsoniae* is also missing one gene encoding a protein from complex IV, cyctochrome c oxidase cbb3-type subunit III. *F. johnsoniae* and *F. branchiophilum*, also encode two cytochrome bd complex proteins (CydA and CydB) that are missing in the other *Flavobacterium* species.

F. columnare and *F. johnsoniae* are unique among the sequenced *Flavobacterium* species in that they also encode proteins in nitrogen metabolism. The other sequenced *Flavobacterium* strains do not carry 80% of the nitrogen metabolism genes carried by *F. columnare* and *F. johnsoniae*.

Fatty acids are crucial components of membranes and play an important role in energy metabolism. To maintain membrane lipid homeostasis, fatty acid biosynthesis and degradation pathways must be controlled coordinately (Fujita, Matsuoka, & Hirooka, 2007). Our metabolic pathway analysis showed that all the sequenced *Flavobacterium* species have fatty acid biosynthesis, whereas fatty acid degradation pathway is present only in the *F. johnsoniae* genome.

Secretion systems

Gram-negative bacteria have several secretion systems to deliver secreted proteins from the cytoplasm into the extracellular environment (Johnson, Abendroth, Hol, & Sandkvist, 2006; Koster, Bitter, & Tommassen, 2000). One of these is the Sec pathway, whose role is transporting newly synthesized proteins to the periplasm in Gram-negative bacteria before they are folded into active form. In this pathway, SecB serves as a chaperone targeting the protein for export and SecA is an ATPase responsible for movement of protein across the cytoplasmic membrane through SecYEG. SecD/F are needed for efficient protein release in the periplasm. The signal recognition particle pathway (SPR) participates in cytoplasmic membrane insertion of proteins (Dalbey & Chen, 2004). In this pathway, Ffh serves as a chaperone for the nascent protein and FtsY serves as a SRP receptor at the cytoplasmic membrane. Either SecYEG or YidC is used for protein integration in the cytoplasmic membrane (Mori & Ito, 2001; Welte et al., 2012). Another secretion system is the twin arginine translocation (Tat) system, which has an important role in virulence and other metabolic activities. Tat transports protein that have already been folded (Rollauer et al., 2012). Three proteins mediate Tat secretion: TatA, TatB, and TatC. TatC recognizes signal peptides substrate proteins and recruits TatA to form the active translocation complex (Rollauer et al., 2012).

Our genome sequencing results revealed that *F. columnare* ATCC 49512 does not carry a complete Type I secretion system, only having one gene encoding a Type I secretion system protein (TolC). However, *F. columnare* ATCC 49512 has two protein secretion systems: Sec-signal recognition particle (Sec-SRP) consisting of SecA, SecD/F, SecE, SecG, SecY, YajC, YidC, FtsY, and Ffh, and twin arginine translocation (Tat)

system consisting of TatA and TatC. Additionally, two signal peptidase genes were identified encoding SpaseI and SpaseII. However, in *F. columnare* secretion systems, genes encoding two Sec-signal recognition particle proteins (SecM and SecB) are not present in the genome. Also, genes encoding TatB and TatE are absent, which are part of the Tat secretion system in *E. coli*.

F. johnsoniea, *F. psychrophilum*, *F. branchilum*, and *F. indicum* share exactly the same secretion systems, including the same missing and present genes as *F. columnare.*

Gliding motility

Bacterial gliding motility is an energy requiring process of translocation of bacteria over a surface. In this process, flagella is not required and cell movement is along the long axis of the cell. Gliding motility results in thin spreading edges on colonies (McBride, 2001), and it is considered one of the characteristics of some species in the phylum *Bacteroidetes*. Many of the motility genes from this phylum are novel and are not found outside the *Bacteroidetes.*

There are two major protein systems responsible for gliding motility. First, Gld proteins are components of the "motor" that moves the cell. Second, Spr proteins are large cell surface proteins responsible for adhesion (McBride et al., 2009). Deletion or disruption of genes encoding Gld proteins results in loss of motility, but spr deletion causes partial loss of gliding motility (Nelson, Bollampalli, & McBride, 2008).

Gliding motility of flavobacteria is well described by several researchers (Bernardet JF, 2006; A. Decostere, Haesebrouck, & Devriese, 1998), but there has not been adequate research conducted on *F. columnare* gliding motility. In the *F. columnare* ATCC 49512 genome, 12 gliding motility genes were identified (*gldA, gldB, gldD, gldF,*

gldG, gldH, gldI, gldJ, gldK, gldL, gldM, and *gldN*). Gene *gldL* is currently annotated as a hypothetical protein. Proteins encoded by *gldA*, *gldF*, and *gldG* are responsible for gliding, and proteins encoded by *gldB*, *gldD*, *gldH*, *gldI*, *gldJ*, *gldK*, *gldL*, *gldM*, and *gldN* are responsible for movement (Nelson et al., 2008). In addition, four spr genes (encoding SprA, SprB, adhesion precursor SprC, and cell surface protein precursor SprD) were identified.

On the other hand, *F. johnsoniae* gliding motility genes and associated proteins are well studied. Fourteen gliding motility genes (*gldA, gldB, gldD, gldF, gldG, gldH, gldI, gldJ, gldK, gldL, gldM, gldN, sprA,* and *sprB*) are in the *F. johnsoniae* genome (McBride et al., 2009).

Using KEGG database, we found that *F. psychrophilum* has thirteen gld genes (*gldA, gldB, gldC, gldD, gldF, gldG, gldH, gldI, gldJ, gldK, gldL, gldM, and gldN*) and six *spr* genes (*sprA, sprB, sprC, sprD, sprE, sprF*). In *F. indicum*, there are twelve *gld* genes (*gldB, gldC, gldD, gldF, gldG, gldH, gldI, gldJ, gldK, gldL, gldM,* and *gldN*) and only two *spr* genes (*sprA and sprE*). In *F. branchilum*, fourteen *gld* genes (*gldA, gldB, gldC, gldD,gldE, gldF, gldG, gldH, gldI, gldJ, gldK, gldL, gldM,*and *gldN*) and only two *spr* genes (*sprA and sprE*) are present (Kanehisa & Goto, 2000; Kanehisa, Goto, Sato, Furumichi, & Tanabe, 2012). All the gld and spr genes in *Flavobacterium* are listed in Table 4.5.

Table 4.5 Gld and Spr genes in *Flavobacterium* species

Gld&Spr Genes	***F. columnare* ATCC 49512**	***F. johnsoniae* UW101**	***F. psychrophilum* JIP02/86**	***F. branchiophilum* FL-15**	***F. indicum* GPSTA100-9T**
GldA	+	+	+	+	-
GldB	+	+	+	+	+
GldC	-	-	+	+	+
GldD	+	+	+	+	+
GldE	-	-	-	+	-
GldF	+	+	+	+	+
GldG	+	+	+	+	+
GldH	+	+	+	+	+
GldI	+	+	+	+	+
GldJ	+	+	+	+	+
GldK	+	+	+	+	+
GldL	+	+	+	+	+
GldM	+	+	+	+	+
GldN	+	+	+	+	+
SprA	+	+	+	+	+
SprB	+	+	+		
SprC	-	-	+		
SprD	-	-	+		
SprE	-	-	+	+	+
SprF	-	-	+		

Iron acquisition

Bacteria require iron as an essential nutrient. Iron acquisition is particularly important for pathogenic bacteria because of limited availability inside the host (Beaz-Hidalgo & Figueras, 2013). Therefore, iron acquisition is linked with bacterial virulence (Guan, Santander, Mellata, Zhang, & Curtiss, 2013). Gram-negative bacteria iron uptake mechanisms have been reviewed (Brown & Holden, 2002).

Recently, two iron acquisition elements were described from *F. columnare* (Guan et al., 2013). One of them was TonB-dependent ferrichrome-iron receptor precursor FhuA and the second was putative ferric uptake regulator Fur.

Our research confirmed that *F. columnare* ATCC 49512 encodes Ton-B dependent outer membrane ferrichrome-iron receptor precursor FhuA, and the genome also revealed three iron ABC transporter genes encoding FhuD, FhuB, and FhuC. Also, our studies confirmed that ferric iron uptake transcription regulator (Fur) is encoded in the *F. columnare* genome.

F. johnsoniae, F. psychrophilum, F. branchilum and *F. indicum* have the same iron acquisition elements.

CHAPTER V

SUMMARY AND CONCLUSIONS

In this study, we aimed to obtain the complete genome sequence of *F. columnare* ATCC 49512 to advance pathogenesis research and increase our understanding of this pathogen. The availability of high quality and annotated genome sequence information to the scientific community will accelerate pathogenesis research and enable development of effective preventive methods.

Our research results showed that the *F. columnare* ATCC 49512 genome consists of a 3,162,432 bp chromosome and 31.5% G+C content. It has 2,731 predicted genes consisting of 2,642 protein coding, 74 tRNA, and 15 rRNA genes. The average length of protein coding genes is 1,021 bp, and 1,625 genes have assigned functions. Our bioinformatic analysis showed that *F. columnare* contains 5 rRNA operons, two of which tandemly arranged. This result was confirmed experimentally by PCR and sequencing. Moreover, our research revealed that *F. columnare* has a complete TCA cycle and glycolysis pathway, and it has oxidative phosphorylation. It also has nitrogen metabolism capabilities not found in most the sequenced *Flavobacteria*. Our genome project showed that fatty acid biosynthesis pathways are encoded in *F. columnare* genome, whereas the fatty acid degradation pathway is missing in *F. columnare* ATCC 49512.

The circularized and completed genome of *F. columnare* ATCC 49512 has several unique characteristic such as five genomic islands and nine potential virulence

factors, four of which are unique to the *F.* columnare genome. Furthermore, 82 insertion elements were detected.

Our genome sequencing results also showed that there are two different types of secretion systems: the Sec secretion pathway and Tat system. However, each of these systems is missing two genes; SecM and SecB are absent in the Sec system, and TatB and TatE are missing from the Tat system. Consequently, further research on secretion systems of *F. columnare* is warranted.

Sequence analysis of *F. columnare* has revealed that sixteen gliding motility protein are encoded in the genome (*gldA, gldB, gldD, gldF, gldG, gldH, gldI, gldJ, gldK, gldL, gldM, gldN, sprA, sprB, sprC*, and *sprD*). In addition, Ton-B dependent outer membrane ferrichrome-iron receptor precursor FhuA, three iron ABC transporters (FhuD, FhuB, and FhuC), and iron uptake transcription regulator Fur are encoded in the genome.

The completed *F. columnare* ATCC 49512 genome will enable functional genomics research on *F. columnare* and allow comparison with other species in the *Flavobacteriaceae* family. Identified features of *F. columnare* ATCC 49512 will also facilitate our understanding of virulence mechanism of flavobacterial diseases.

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