MOLECULAR CHARACTERIZATION OF *EDWARDSIELLA* SPP. AND

FLAVOBACTERIUM COLUMNARE

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MOLECULAR CHARACTERIZATION OF *EDWARDSIELLA* SPP. AND *FLAVOBACTERIUM COLUMNARE*

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MOLECULAR CHARACTERIZATION OF *EDWARDSIELLA* SPP. AND

FLAVOBACTERIUM COLUMNARE

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VITA

Yinfeng Zhang, daughter of Liren Zhang and Fengling Wang, was born on April 29, 1979, in Shuangcheng, Hei Long Jiang province, China. She graduated from Zhaolin High school of Shuangcheng city, Hei Longjiang Province, China in 1998. In the same year, she attended Dalian Fisheries University, Dalian, Liaoning province, China. In 2002, she received a Bachelor degree in Agricultural Sciences from Dalian Fisheries University. Upon graduation, she entered Auburn University and became a graduate research assistant in the Department of Fisheries and Allied Aquacultures. After studying for two years under the supervision of Dr. Grizzle, she graduated with a Master of Science degree majoring in fish diseases in 2004. She continued her study in the same Department pursuing a Doctor of Philosophy degree. During her PhD, she got married with Mingkang Jiang, a graduate student in the Department of Fisheries and Allied Aquacultures. They have a baby boy Austin N. Jiang.

DISSERTATION ABSTRACT

MOLECULAR CHARACTERIZATION OF *EDWARDSIELLA* SPP. AND *FLAVOBACTERIUM COLUMNARE*

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Bacterial diseases are responsible for large economic losses in aquaculture around the world. *Flavobacterium columnare* and *Edwardsiella* spp. negatively impact the channel catfish industry in southern USA. However, limited biological information is available for *F. columnare* and *Edwardsiella ictaluri*, which has hampered the development of accurate detection methods. In addition, the mechanisms of virulence of these pathogens are poorly understood which has prevented us to propose effective control/treatment methods for them. In the present study, new signature sequences for these pathogens were identified and evaluated as target candidates in PCR-based approaches. Unfortunately, multiplex PCR and real-time PCR tests developed in this

study failed to provide the required specificity and sensitivity and could not be implemented as diagnostic tools. However, an intervening sequence (IVS) was discovered in *E. ictaluri* when sequencing the 23S rRNA gene. IVS are seldom found in bacteria and this is the first time an IVS has been described in the genus *Edwardsiella*. This IVS exhibited 97% similarity to the IVS in *Salmonella typhimurium*. A 23S rRNA gene-based phylogenetic tree was constructed placing *E. ictaluri* and *E. tarda* in context with other enterobacteria. This tree showed that *Edwardsiella* spp. are phylogenetically closer to the genus *Erwinia* than to the core members of the *Enterobacteriaceae* family.

A phenotypic and genotypic comparison among *F. columnare* strains with different degrees of virulence was carried out in order to identify virulence markers. Lipopolysaccharide (LPS) and total protein profiles were characterized to illustrate the phenotypic differences between virulent and avirulent *F. columnare* strains. A *F. columnare* avirulent mutant lacked the high molecular weight bands of the LPS but showed two low molecular weight proteins that were absent in virulent strains.

Four putative virulence genes were identified (*gtf, hemH*, *norB, trx*) by partially sequencing a shotgun genomic library from a virulent *F. columnare* strain. Nucleotide sequences of these genes divided the *F. columnare* strains analyzed into two populations that correlated well with previously described genomovars. Expression of these genes differed among strains under the same conditions. Differential gene expression was also observed when cells were grown under iron-restricted conditions and in the presence of catfish skin explants. These results provide new insights into the understanding of genetics and pathogenesis of *F. columnare*.

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TABLE OF CONTENTS

LISTS OF TABLES

LISTS OF FIGURES

I. INTRODUCTION

Channel catfish (*Ictalurus punctatus* Rafinesque) is the most important fish species commercially cultured in the USA. Total foodsize catfish sales reached $460 million in 2005 (USDA 2005). Alabama, Arkansas, Louisiana, and Mississippi are the major catfish producing states in the USA, accounting for more than 90% of the total catfish sales in 2002 with more than 70% of all catfish operations (USDA 2003a). Channel catfish belongs to the family *Ictaluridae* within the order of Siluriformes (Tucker 1985). This species is characterized by having eight barbels around the mouth; deeply-forked tail; spots on body sides; and soft-rayed fins, although the dorsal and pectoral fins contain sharp, hard spines (Wellborn 1988). Channel catfish possess several desirable attributes for commercial production (Tucker 1985). This species is easy to spawn and usually do not reproduce in culture ponds. Channel catfish is hardy due to its tolerance to low oxygen, crowding, and its well adaptation to the various culture systems. It also presents an efficient feed conversion rate and accepts manufactured feed. All these characteristics make channel catfish a model species for aquaculture production.

The main factor limiting expansion and profitability of the catfish industry is disease control. Currently, the two most prevalent diseases are: enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri* and columnaris disease caused by *Flavobacterium columnare* (USDA 2003a). Other diseases affecting the catfish industry are caused by the protozoan *Ichthyophthirius multifiliis*, the triactinomyxid myxozoan

Aurantiactinomyxon ictaluri (proliferative gill disease (PGD)), trematodes, opportunistic bacterial pathogens, channel catfish virus, channel catfish anemia, and visceral toxicosis of catfish (USDA 2003a). Infectious disease is a main factor in fish losses. Sixty percent of foodsize fish operation experienced ESC and 50% experienced columnaris disease. In fingerling production farms, 50% of losses were caused by ESC and 45% were due to columnaris disease during 2001-2002 (USDA 2003a; USDA 2003b). Currently, *E. ictaluri* and *F. columnare* are considered the two most important bacterial pathogens for the channel catfish industry (Wagner et al. 2002). Although *E. tarda* does not impact channel catfish aquaculture production as much as *E. ictaluri* and *F. columnare*, it was included in this study for comparison purposes. Both *E. tarda* and *E. ictaluri* are thought to be genetically similar (Hawke et al. 1981); however, while *E. ictaluri* is a fairly specific pathogen for catfish, *E. tarda* presents a broader host range. Therefore, a genetic characterization of these two species might shed some light on their host-specificity.

Enteric septicemia of catfish

The genus *Edwardsiella* was first described in 1965, with *E. tarda* as the type species (Ewing et al. 1965). A second species was isolated from reptiles and birds, and was characterized by Grimont et al. (1980) as *E. hoshinae*. *E. ictaluri*, the causal agent of ESC was first isolated in 1976 (Hawke 1979); however, the bacterium was not characterized and classified until 1979 (Hawke et al. 1981).

Characteristics — The physiological and biochemical characteristics of *E. ictaluri* have been described in detail previously (Hawke 1979, 1981; Waltman et al. 1986). *E. ictaluri* is a short, Gram-negative rod with a dimension of about 0.8 X 3 μm. It is cytochrome oxidase negative and ferments glucose. *E. ictaluri* does not produce H_2S

from triple sugar iron agar and is negative for indole production as well. *E. ictaluri* is not able to grow with NaCl concentrations higher than 1.5%. Optimum growth temperature for *E. ictaluri* is between 25 ºC and 30 ºC. Regardless of origin, all *E. ictaluri* isolates contain plasmids (Speyerer and Boyle 1987). *E. ictaluri* is more related to *E. tarda* than to the other members in the family of *Enterobacteriaceae* based on DNA-DNA hybridization (Hawke et al. 1981).

Epidemiology — Enteric septicemia of catfish is a seasonal disease with high prevalence during May and June, and during September and October. *E. ictaluri* has a high infectivity rate in cultured channel catfish as all sizes of fish susceptible to ESC (Plumb 1999). Naturally occurring infections by *E. ictaluri* have been reported in walking catfish (*Clarias batrachus* Linnaeus) (Kasornchandra et al. 1987), white catfish (*Ameiurus catus* Linnaeus) (Hawke et al. 1981) , brown bullhead (*Ameiurus nebulosus* Lesueur) (Hawke et al. 1981), freshwater catfish (*Pangasius hypophthalmus* Sauvage) (Crumlish et al. 2002), green knifefish (*Eigenmannia virescens* Valenciennes) (Kent and Lyons 1982), danio (sind) (*Devario devario* Hamilton) (Waltman et al. 1985), rainbow trout (*Oncorhynchus mykiss* Walbaum) (Keskin et al. 2004), and tadpole madtom (*Noturus gyrinus* Mitchill) (Klesius et al. 2003). Experimental infections by injecting *E. ictaluri* cells intraperitoneally showed that tilapia (*Sarotherodon aureus* Steindachner) was slightly susceptible to *E. ictaluri*, while golden shiner (*Notemigonus crysoleucas* Mitchill), bighead carp (*Aristichthys nolilis* Richardson), largemouth bass (*Micropterus salmoides* Lacepède), and blue catfish (*Ictalurus furcatus* Valenciennes) were resistant to this pathogen (Plumb and Sanchez 1983; Wolters et al. 1996).

Clinical signs and histopathology — There are two forms of ESC: acute and chronic. In the acute form, diseased fish suffering from ESC hang with a head-up-tail-down posture and exhibit spinning swimming behavior. External lesions caused by *E. ictaluri* include petechial hemorrhages on skin, pale gills, exophthalmia, and small cutaneous lesions on the body surface (Hawke 1979). Internally, peritoneal cavity may contain bloody ascitic fluid, and the intestine may exhibit petechial hemorrhages (Hawke 1979). Multifocal necrosis of liver and swollen trunk kidney can be observed (Newton et al. 1989). Microscopically, hepatocytes are swollen and vacuolated and the exocrine pancrease around hepatic vessels is necrotic (Newton et al. 1989). In the chronic form, olfactory epithelia appear degenerated and granulomatous inflammation is found in olfactory lamellae. The brain is swollen and ulcerated (Newton et al. 1989; Morrison and Plumb 1994). An open lesion can develop through the skull giving the disease its common name 'hole in the head' disease.

Diagnosis — Classical fish pathogen identification relies on microbial culture techniques followed by biochemical characterization of the isolates. *E. ictaluri* can be isolated from infected organs by using general culture media such as brain heart infusion agar (BHI), trypticase soy agar (TSA) (Meyer and Bullock 1973), and blood agar. A selective medium (*E. ictaluri* medium or EIM) for the recovery of *E. ictaluri* was developed by Shotts and Waltman (1990). Colonies of *E. ictaluri* are smooth, circular, and slightly convex (Hawke 1979). An *E. ictaluri* colony on EIM appears clear and greenish, which can be distinguished from other Gram-negative bacteria, while the growth of Gram-positive bacteria is inhibited. Motility at 37°C, no indole production in

tryptone broth, and no H_2S production on triple sugar iron differentiate *E. ictaluri* from *E. tarda*.

Besides culture methods, some alternative techniques for direct detection of *E. ictaluri* have been described. Serology methods for detecting *E. ictaluri* have been developed by several groups. Enzyme immunosorbent assay (ELISA) has been used to detect *E. ictaluri* in decomposing channel catfish (Hanson and Rogers 1989). Indirect fluorescent antibody (IFA) test has been developed to diagnose *E. ictaluri* from artificially infected channel catfish fingerlings (Ainsworth et al. 1986). Though IFA had 90.3% correlation with culture methods, some false-negative results have been reported (Ainsworth et al. 1986). However, Panangala et al. (2006) described an efficient IFA test for *E. ictaluri* with no false negative results. In addition, indirect ELISAs have been developed to detect catfish serum antibodies against *E. ictaluri* (Waterstrat et al. 1989; Klesius et al. 1991).

Fluorescent *in situ* hybridization (FISH) has also been used for diagnosis of *E. ictaluri*. A probe targeting an *E. ictaluri* plasmid was radiolabeled for detection of this bacterium in channel catfish. This probe did not hybridize with *Escherichia coli*, *Aeromonas hydrophila* or *E. tarda* (Speyerer and Boyle 1987). Reid and Boyle (1989) found that the same plasmid probe hybridized to all the tested *E. ictaluri* isolates from channel catfish and non-channel catfish except to an isolate from Maryland recovered from white catfish (*Ameiurus catus* Linnaeus).

Polymerase chain reaction (PCR) is another diagnostic method widely used in clinical diagnosis. Since its description by Mullis and Faloona (1987), PCR has been proved to be a very useful tool for pathogen detection. Detection by PCR can be very

specific, sensitive and does not require pathogen isolation from the sample. PCR is a method by which nucleic acid sequences can be exponentially amplified *in vitro* (Mullis and Faloona 1987). This technique enables researchers to generate millions of copies of a single DNA molecule in a short period of time by using of a template DNA, DNA polymerase, nucleotides (A, T, G, C), and primers.

A more advanced PCR technique, named real-time PCR (or quantitative PCR), is a modification of standard PCR. This technique simultaneously amplifies and quantifies target DNAs. Therefore, it will not only determine the presence of a specific DNA sequence but measure the number of copies of DNA. By monitoring fluorescence intensities in a real-time PCR apparatus, quantification of DNA can be accomplished by adding double-stranded DNA dyes such as SYBR Green (Yin et al. 2001), or using fluorescent labelled probes such as TaqMan probes (Heid et al. 1996), hybridization probes (Dietmaier and Hofstadter 2001), and molecular beacons (Tyagi and Kramer 1996).

The main advantage of real-time PCR over standard PCR is that real-time PCR allows quantification of the target nucleic acids. In addition, real-time PCR data are collected during DNA amplification so that contamination risk is reduced. The use of real-time PCR for fish bacterial pathogen detection could be very useful in monitoring changes of pathogen loads in the host or the environment. When bacterial loads are low, accurate quantitative determination of the pathogen can be problematic. The use of real-time PCR might offer a better diagnostic sensitivity than standard PCR methods. Real-time PCR might allow accurate detection of fish pathogens under a preclinical stage.

Pathogen detection prior to the onset of disease will help to implement preventive measures and avoid infectious outbreaks.

To date, no standard PCR protocol has been described for *E. ictaluri*. However, a real-time PCR protocol targeting a transposon was developed to detect *E. ictaluri* in fish samples. This real-time assay was sensitive enough to detect as few as two to three *E. ictaluri* cells from mixtures of noninfected catfish blood and *E. ictaluri* cells (Bilodeau et al. 2003). Interestingly, transposons are variable genetic elements that *a priori* are not considered good targets for PCR detection since they are mobile elements that might not be present in all *E. ictaluri* isolates. Loop-mediated isothermal amplification (LAMP), another nucleic acid amplification method, has been desribed for detection of *E. ictaluri* (Yeh et al. 2005) as well. By targeting a putative antigenic gene, *eip18*, this LAMP assay could detect as few as 20 colony forming units (CFU). The specificity of this method was tested and found to be satisfactory (Yeh et al. 2005).

Pathogenesis — Little is known about the virulence mechanisms of *E. ictaluri*. Possible virulence factors may include bacterial cell surface material, hemolysin, lipopolysaccharide (LPS), and hydrolytic enzymes. Virulent isolates of *E. ictaluri* contained greater amounts of capsular material and surface proteins and showed higher chondroitinase activity than avirulent isolates (Stanley et al. 1994). Futher work is needed to illustrate the role of surface protein and chondroitinase in *E. ictaluri* virulence. Although Williams et al. (2003) revealed that an attenuated *E. ictaluri* strain exhibited decreased hemolysin activity, hemolysin-deficient strains did not exhibit a reduction in virulence (Williams and Lawrence 2005). Arias et al. (2003) reported that a

rifampicin-mutant of *E. ictaluri* (RE-33) used as live vaccine, lacked the high molecular weight bands of the LPS compared with the parent virulent strain; however, RE-33 can still protect channel catfish against ESC. Compared to RE-33, another *E. ictaluri* strain without O-antigen of LPS can not efficiently protect channel catfish against *E. ictaluri* (Lawrence and Banes 2005). A recent study showed that a urease gene may play an important role in the intracellular replication of *E. ictaluri* in the vacuoles of channel catfish macrophages (Booth 2005). Further investigation is needed to clarify the role of urease in *E. ictaluri* pathogenecity.

Treatment and prevention — The first attempt to control ESC was to feed diseased fish with antibiotic medicated food (USDA 2003a). Ormetoprim-sulfadimethoxine, and Aquaflor (Schering-Plough, Kenilworth, NJ) are the approved drugs for treating ESC in catfish in the USA. Ormetoprim-sulfadimethoxine is known to inhibit bacterial growth. The effective ingredient in Aquaflor is florfenicol that inhibits protein synthesis in bacteria (Aquaflor product labeling http://www.aquaflor-usa.com). However, antibiotic therapy should not be considered the best management practice since inappropriate use of antibiotics may generate drug resistant bacterial isolates (Hawke et al. 1998).

Vaccination is one of the best options to prevent infectious diseases. Classical vaccines can be made out of living, attenuated or inactivated microorganisms or purified macromolecular components derived from them (Abbas and Lichtman 2000). Vaccines have the benefit to induce protective immunity against microbial pathogens. Vaccination with inactivated *E. ictaluri* resulted in no strong acquired immunity to protect channel catfish against *E. ictaluri* infection (Thune et al. 1994). As an intracellular pathogen, a

modified-live *E. ictaluri* vaccine may be more effective in inducing acquired immunity in catfish. A modified-live vaccine developed by Klesius and Shoemaker (1999) is now commercially available to farmers (AQUAVAC-ESC, Intervet Inc. Millsboro, DE). This vaccine was developed using a rifampicin-resistant strategy that was previously used to generate the *Brucella abortus* live vaccine (RB51) against cattle brucellosis (Schurig et al. 1991). The rifampicin-resistant mutant was derived by repeated passage on nutrient agar supplemented with rifampicin at varying concentrations. This live *E. ictaluri* vaccine is unable to cause ESC but is capable of protecting catfish against ESC (Klesius and Shoemaker 1999). Channel catfish fry are vaccinated by being immersed in a bath containing the ESC vaccine (Shoemaker et al. 1999). In 2001-2002, 11.4% of fingerling operations vaccinated fry against ESC using AQUAVAC-ESC (USDA 2003b).

Edwardsiella tarda septicemia

The disease caused by *E. tarda* was first discovered in cultured eel (Hoshina 1962). This disease was first called emphysematous putrefactive disease in catfish (Meyer and Bullock 1973). However, it was referred as Edwardsiellosis by Plumb (1999) and appears as *Edwardsiella tarda* septicemia in the AFS-FHS blue book (Hawke 2003).

Characteristics —E. tarda is a small, straight, Gram-negative rod that is 1 μm in diameter and 2-3 μm in length. It is motile, catalase positive, cytochrome oxidase negative, lactose negative, and ferments glucose. *E. tarda* produces indole in tryptone broth and H_2S on triple sugar iron slants.

Epidemiology — E. tarda has a broader host range than *E. ictaluri*, and it can infect both freshwater and marine fish species. Plumb (1993) reported a list of fish species infected by *E. tarda* such as goldfish (*Carassius auratus auratus* Linnaeus),

common carp (*Cyprinus carpio carpio* Linnaeus), grass carp (*Ctenopharyngodon idella* Valenciennes), and largemouth bass (*M. salmoides*). Miyazaki (1985) reported the infection in Japanese flounder (*Paralichthys olivaceus* Temminck & Schlegel), red seabream (*Chrysophrys major* Temminck & Schlegel), nile tilapia (*Oreochromis niloticus niloticus* Linnaeus), and Japanese eel (*Anguilla japonica* Temminck & Schlegel). *E. tarda* can also be isolated from other animals such as frogs (Sharma et al. 1974), reptiles (Sechter et al. 1983), swine (Owens et al. 1974), and humans (Sechter et al. 1983). The most prominent fish species infected by this organism are Japanese eels (*A. japonica*) and channel catfish (*I. punctatus*) (Meyer and Bullock 1973; Plumb 1999).

Clinical signs — E. tarda septicemia causes different clinical signs in different fish species. Diseased Japanese eels display petechial hemorrhages on the ventrum, and the anal region is swollen. Two forms of *E. tarda* septicemia occur in Japanese eels: nephric and hepatic (Miyazaki 1985). The nephric form is characterized by necrotic renal foci that can spread to spleen, liver, gills, stomach, and heart. The hepatic form is associated with microabscesses in the liver and can spread to other organs. *E. tarda* causes small cutaneous lesions, and abscesses can develop as malodorous gas and necrotized tissue-filled cavities within muscle in channel catfish (Meyer and Bullock 1973).

Diagnosis —Bacteriological or serological methods can be used to identify *E. tarda*. Isolation can be achieved on BHI agar or TSA agar. *E. tarda* forms small, green colonies with black center on EIM. Contrary to *E. ictaluri*, *E. tarda* produces indole, hydrogen sulphide and reduces methyl red. Standard PCR protocols for *E. tarda* have been developed, but they lacked the appropriate sensitivity and/or specificity (Aoki and

Hirono 1995; Chen and Lai 1998; Baird et al. 2003). A species-specific fragment cloned from a shotgun DNA genomic library was targeted to detect *E. tarda*; however, the analytical sensitivity of this PCR method was not well described (Aoki and Hirono 1995). Moreover, the authors failed to compare their PCR-based detection results with standard culture methods. Chen and Lai (1998) developed a PCR for *E. tarda* by using the hemolysin gene as the target, but the specificity of the primers was not tested. Finally, Baird et al. (2003) used the small ribosomal subunit as a target to identify *Edwardsiella* spp., but the assay sensitivity was not determined. Therefore, more effective and reliable PCR protocols are desirable for detection of *E. tarda*. Development of PCR protocols for the detection of *E. tarda* need to consider that *E. ictaluri* and *E. tarda* contain almost identical 16S rRNA gene sequences (> 99 %) and share a high similarity of 16S-23S intergenic spacer regions (Panangala et al. 2005). Specific molecular markers are necessary to differentiate *E. tarda* from *E. ictaluri*.

Pathogenesis —several virulence factors are believed to contribute to *E. tarda* pathogenesis. These include exotoxins (Ullah and Arai 1983), hemolysin production (Hirono et al. 1997), ability to resist serum-mediated killing, and invasion of epithelial cells (Janda et al. 1991). Several virulence genes have been identified in *E. tarda* by transposon mutagenesis using a fish infection model. These genes are *fimA* (fimbrial protein precursor), *gadB* (glutamate decarboxylase isozyme), *katB* (catalase precursor), *pstS* (phosphate binding protein), *pstC* (peripheral membrane protein C), *ssrB* (secretory system regulator) (Srinivasa-Rao et al. 2003).

Treatment and prevention— E. tarda infection can be treated with oxytetracycline antibiotic. However, drug-resistant strains of *E. tarda* have already been reported (Aoki

et al. 1987). A live rifampicin resistant vaccine against *E. tarda* in fish has been developed, but it is not commercially available yet (USA Patent 7067122 http://www.freepatentsonline.com/7067122.html).

Columnaris disease

F. columnare belongs to the Cytophaga-Flavobacterium-Bacteroides (CFB) group. Flavobacteria are phylogenetically distant from other Gram-negative and Gram-positive pathogenic bacteria (Figure I.2). Flavobacteria account for 13 % of total bacterial fish pathogens, while most of the fish bacterial pathogens fall within the γ- proteobacteria group. Experimental methods (i. e. bacteria culturing, protein and DNA extraction, and genetic manipulation) used with other common pathogens are not suitable for most *Flavobacterium* spp. It is possible that virulence mechanisms of *Flavobacterium* spp. may also differ from other common fish bacterial pathogens.

The disease caused by *F. columnare* is called columnaris disease and was first described by Davis (1922). However, the first successful isolation of the organism was described about 20 years later (Ordal and Rucker 1944). The causative organism has suffered several taxonomic reclassifications. It has been referred to as *Bacillus columnaris* (Davis 1922), *Chondrococcus columnaris* (Ordal and Rucker 1944), *Cytophaga columnaris* (Garnjobst 1945), *Flexibacter columnaris* (Leadbetter 1974), and again *Cytophaga columnaris* (Reichenbach 1989). Bernardet and Grimont (1989) maintained the name *Flexibacter columnaris* by presenting the DNA relatedness to the type strain of *F. columnare*. This was the first time this bacterial pathogen was officially described as a species. This organism was renamed as *Flavobacterium columnare* in 1996 (Bernardet et al. 1996).

Characteristics — The characteristics of *F. columnare* have been described in detail by Plumb (1999) and Bernardet et al. (1989). *F. columnare* is a Gram-negative rod that is 2-10 μm long and 0.5 μm in diameter, motile by gliding, producing catalase, producing H_2S, hybrolyzing gelatin, and no growth with more than 1% NaCl. Colonies on agar are spreading and more or less rhizoid. Colony colors range from pale yellow, greenish yellow, yellow to golden yellow (Griffin 1992). *F. columnare* presents a G + C content of about 32 %.

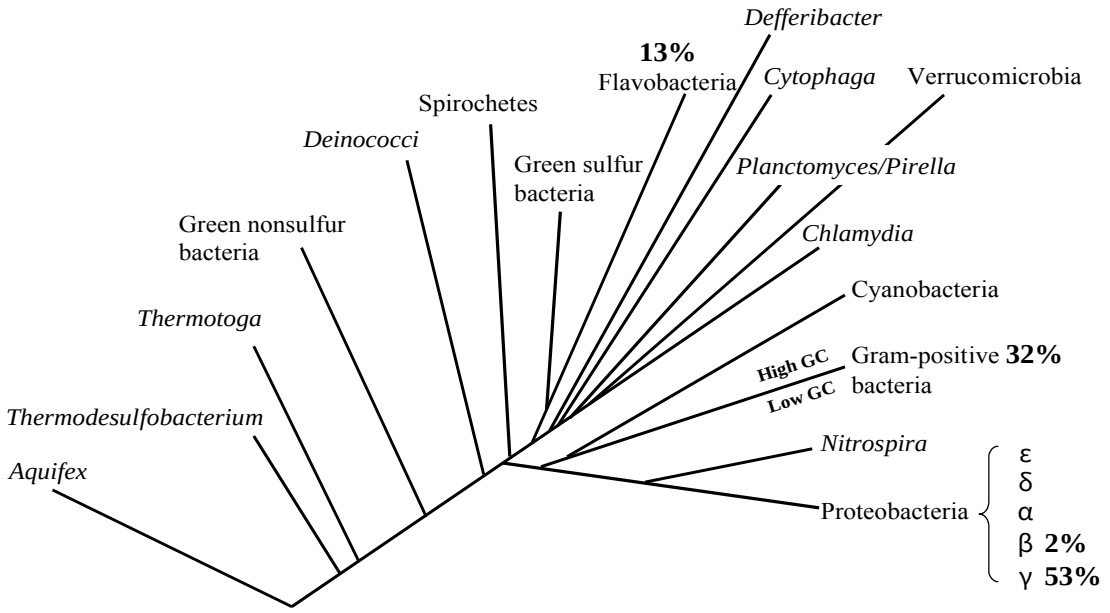


Figure I.1. Modified Eubacteria domain phylogenetic tree based on 16S rDNA sequences (Madigan et al. 2003). Numbers reflect percentage of fish pathogenic bacterial species within each group to total bacterial fish pathogens (calculated based on the fish bacterial disease list by Austin and Austin (1999)).

F. columnare species exhibits variation in serotypes and genotypes. Four serotypes and one miscellaneous group have been described in the species (Anacker and Ordal 1959). Additionally, genotypic differences have been reported based on the analysis of 16S rDNA-RFLP, intergenic spacer region (ISR), amplified fragment length

polymorphism (AFLP), and random amplified polymorphism DNA (RAPD) (Toyama et al. 1996; Triyanto and Wakabayashi 1999; Arias et al. 2004; Thomas-Jinu and Goodwin 2004).

Epidemiology — F. columnare is distributed worldwide in aquatic environments, being capable of infecting most freshwater fish species. In general, fish are considered as the main reservoir for *F. columnare*. However, this bacterium can be isolated from water (Rucker et al. 1953), especially during epizootics (McCarthy 1975). Transmission of this pathogen can occur through water without fish to fish contact (Welker et al. 2005). Several factors are involved in the spread of the disease. Injured or mechanical abraded fish are more likely to be infected by *F. columnare* (Davis 1922; Bader et al. 2003a). Channel catfish deprived of feed are more susceptible to *F. columnare*-induced mortality (Shoemaker et al. 2003a). Water temperature also plays a major role in columnaris disease epidemiology. Although columnaris disease occurs throughout the whole year, warm-weather favors *F. columnare* infection (Davis 1922). Other conditions favoring *F. columnare* infection include crowding (Suomalainen et al. 2005), low oxygen (Chen et al. 1982), high ammonia (Chen et al. 1982), and high nitrite (Hanson and Grizzle 1985).

F. columnare often appears associated with other pathogens as mixed infections. Davis (1922) observed that large quantity of other bacteria present in columnaris lesions besides *F. columnare*. Hawke and Thune (1992) showed that out of 53 *F. columnare* infectionss, 46 involved *E. ictaluri* and/or *Aeromonas* spp. Currently, it is unclear whether *F. columnare* is a primary pathogen or an opportunistic one.

Clinical signs — Columnaris disease usually begins as an external infection of the fins, body surface, or gills. Small lesions can start as areas of pale discoloration at the

base of the dorsal fin or occasionally at the base of the pelvic fin, and lead to deterioration of the fins. Initial skin lesions appear as discrete bluish areas that evolve into depigmented necrotic lesions. Skin lesions can have yellowish mucoid material accompanied by mild inflammation. The lesions can rapidly develop to cover a greater portion of the body (Davis 1922). Lesions on gills are localized as white patches or yellow-orange-brown necrotic areas depending on the presence of debris in the lesion (Davis 1922; Plumb 1999). Although *F. columnare* starts as an external infection, the skin of the fish may be eroded completely to expose the underlying muscle (Pacha and Ordal 1970). *F. columnare* can also become systemic without obvious pathological changes in fish internal organs (Plumb 1999).

Diagnosis — Columnaris is often diagnosed by typical lesions on the body surfaces, and the presence of columns or 'hay stacks' of bacteria in a wet mount. *F. columnare* can be isolated on low nutrient agar plate such as Cytophaga agar (Anacker and Ordal 1959), Hsu-Shotts agar (Bullock et al. 1986), or Shieh agar (Decostere et al. 1997). A simple five-step method to distinguish *F. columnare* from the other yellow pigment producing bacteria was developed by Griffin (1992): (1) ability to grow in the presence of neomycin sulfate and polymyxin B; (2) colonies rhizoid and yellow pigmented; (3) production of gelatin degrading enzymes; (4) colonies absorbing Congo red; and (5) production of an enzyme that degrades chondroitin sulfate A.

Chowdhury and Wakabayashi (1990) described an indirect fluorescent antibody technique to detect *F. columnare* in fish and in water. In their study, immunodetection of *F. columnare* was more sensitive than plate culture methods. Fatty acid methyl ester analysis (FAME) is another rapid and reliable method for identification of *F. columnare*

(Shoemaker et al. 2005a). Specific PCR methods have also been developed to identify putative *F. columnare* isolates (Toyama et al. 1996; Bader and Shotts 1998; Bader et al. 2003b; Darwish et al. 2004). However, all the PCR methods required either two-rounds of PCR amplification or restriction analysis after PCR amplification. A new PCR protocol developed by Welker et al. (2005) exhibited both high specificity and sensitivity. However, this protocol could not be adapted into real-time PCR due to its large amplicon size (about 500 bp). Recently, Yeh et al. (2006) developed a LAMP method to detect *F. columnare*. The assay can detect as low as 30 pg of genomic DNA and was able to detect *F. columnare* from experimentally infected channel catfish.

Pathogenesis — Although columnaris disease has been known for about 80 years (Davis 1922), little work has addressed the mechanisms of this disease in part due to the fastidious nature of this bacterium when cultured under laboratory conditions. Most of the bibliographical references for *F. columnare* are related to its taxonomic status rather than to its biological properties. To date, most *F. columnare* sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) correspond to ribosomal genes. Some non-ribosomal sequences include gliding motility genes (*fjo17, fjo23, gldH, gldJ, and murF*), a prolyl oligopeptidase (*g4*), a major outer membrane protein (*momp*), an alginate-O-acetylation protein (*algI*), and a partial sequence of a metalloprotease-like gene.

Extracellular protease production in *F. columnare* has been described by Bertolini and Rohovec (1992) and Newton et al. (1997). The production of proteases may explain the necrotizing characteristics of *F. columnare* infection. *F. columnare* produces a chondroitin AC lyase, which can break down polysaccharides of connective tissue

(Griffin 1991). A recent study indicated that chondroitin AC lyase activity is related to *F. columnare* virulence (Suomalainen et al. 2006). Bacterial adhesion also appears to be related to virulence of *F. columnare* (Zaldivar 1985; Decostere et al. 1999a). A lectin-like carbohydrate-binding substance may be responsible for attachment of *F. columnare* to gill (Decostere et al. 1999b). Currently, there is limited information available on *F. columnare* genetics and pathogenesis.

Treatment and prevention — Because of the ubiquitous presence of *F. columnare* in aquatic environments, it is unrealistic that eradication from fish farms will occur. Improved water-management has been used to reduce physiological and environmental stress in fish. Increasing salinity to 1‰ in the culture systems may help to reduce fish losses (Altinok and Grizzle, 2001). Antibiotic treatment is not effective due to high costs and drug use restrictions. However, columnaris disease might be prevented through vaccination. A commercially available rifampicin-resistant live vaccine is now approved and has just started to be used by farmers (AQUAVAC-COL, Intervet). Our ability to suggest other health management strategies is limited by the lack of information related to *F. columnare* biology.

The development of molecular detection/quantification methods for fish bacterial pathogens is a basic step needed to study the infection rate and transmission of the pathogens. Early detection of fish bacterial pathogens is critical to treat the disease at preclinical stage, and effective treatment methods have to be based on the knowledge of pathogen's virulence mechanisms. In this dissertation, PCR-based detection methods were not successfully developed. However, an intervening sequence (IVS) was identified from *E. ictaluri* 23S rDNA. It was the first description in *Edwardsiella* genus and may

serve as a good maker for detection of *E. ictaluri*. Regarding *F. columnare* characterization, phenotypic and genotypic differences were identified within the species. Phenotypic differences were illustrated by characterizing the lipopolysaccharide (LPS) and protein profiles in both virulent and avirulent *F. columnare* strains. Four genes (*gtf*, *hemH*, *norB*, *trx*) were, for the first time, identified in *F. columnare*, and their sequences divided *F. columnare* strains into two populations. The dissertation has been arranged according to published manuscripts followed by a section of overall conclusions.

OBJECTIVES

The main objectives of the current study are to develop a multiplex real-time PCR for simultaneous detection/quantification of *E. ictaluri*, *E. tarda*, and *F. columnare* and to identify virulence factors in *F. columnare*. Also, the study aims to evaluate and adapt a real-time PCR developed for *E. ictaluri* in our laboratory. Because molecular sequences are limited for *E. ictaluri* and *F. columnare*, ribosomal genes will be the first targets considered in order to develop a multiplex real-time PCR method. If the available molecular information does not provide enough differentiation, it will be necessary to identify new signature sequences in the three bacterial pathogens.

Currently, there is lack of molecular pathogenesis data for *F. columnare*. The present study will attempt to identify and characterize virulence factors from both phenotypic and genotypic aspects. Lipopolysaccharide (LPS) should be first considered because of its important virulence role in a variety of Gram-negative bacterial species. Fortunately, a shotgun genomic library of *F. columnare* was generated recently, from which some useful information may be extracted. The hypotheses and objectives in the current study are listed as follows:

Hypothesis 1: Ribosomal RNA genes contain specific signature sequences for *E. ictaluri*, *E. tarda*, and *F. columnare* that can be used in PCR based diagnosis

Objective 1: Identify specific DNA sequences within 16S rDNA, 23S rDNA, and 16S-23S ISR and develop a real-time PCR and/or a multiplex real-time PCR (Chapter II and Chapter III)

Hypothesis 2: Differences in virulence among *F. columnare* strains can be correlated with different phenotypic and genotypic markers

Objective 1: Compare protein and lipopolysaccharide profiles among *F. columnare* strains that differ in virulence (Chapter IV)

Objective 2: Identify putative virulence genes from different *F. columnare* strains and to characterize the expression of these genes under different conditions (Chapter V)

II. EVALUATION OF REAL-TIME PCR FOR DETECTION OF *EDWARDSIELLA* SPP. AND *FLAVOBACTERIUM COLUMNARE*

Abstract

The objective of this study was to develop a multiplex real-time PCR for the simultaneous detection and quantification of *Edwardsiella ictaluri*, *E. tarda* and *Flavobacterium columnare*. Current PCR detection methods for *F. columnare* and *E. ictaluri* were tested in our laboratory for comparison purposes. We found that under our conditions, the real-time PCR protocol for *E. ictaluri* described by Bilodeau et al. (2003) did not provide us with the expected sensitivity. In fact, the sensitivity of this protocol was lower than classical culture methods. In addition, we failed to adapt the standard *F. columnare* PCR protocol developed in our laboratory into a real-time PCR method. Based on these preliminary data, additional signature sequences were needed to develop new or improved real-time PCR protocols for the detection of these pathogens. The 16S and the 23S rRNA gene sequences along with the 16S-23S intergenic spacer region (ISR) were used to design new primer sets. The ISR did not provide enough variability to differentiate *E. ictaluri* from *E. tarda*. A real-time PCR method was developed for *F. columnare* by using primers against the 16S rDNA; however, the specificity of these primers was not sufficient to discriminate *F. columnare* from *F. aquatile*. A multiplex real-time PCR protocol was developed to simultaneously detect *E. ictaluri*, *E. tarda*, and

F. columnare by targeting the 23S rDNA gene. Unfortunately, cross-reactivity with non-target sequences will not allow the use of these primers in an effective PCR protocol.

Keywords: *Flavobacterium columnare*, *Edwardsiella ictaluri*, *E. tarda*, PCR, real-time PCR

Introduction

Edwardsiella ictaluri, *E. tarda*, and *F. columnare* are main bacterial pathogens in aquaculture. In fact, *F. columnare* and *E. ictaluri* negatively impact the channel catfish industry in southeastern USA. Detection and identification of these pathogens are critical for disease management (Plumb 1999). Among all the detection and identification methods available to date, polymerase chain reaction (PCR)-based methods have the highest potential of being fast, specific and sensitive.

Currently, there is only one PCR-based protocol for *E. ictaluri* detection. This real-time PCR protocol was developed for *E. ictaluri* quantification in catfish blood samples by Bilodeau et al. (2003). A transposon element was targeted in this protocol as signature sequence, although the transposon type was unknown. As a mobile genetic entity, such transposon may not be present in all *E. ictaluri* isolates, raising the question about the reliability of the protocol. Several PCR protocols have been developed for *E. tarda*. Aoki and Hirono (1995) targeted a species-specific fragment DNA of *E. tarda* with unknown function. However, the encoding information of this fragment was not provided. Labelled as a probe, this fragment hybridized only with *E. tarda* but not with other related fish pathogens. Primers based on such specific fragment provided desirable specificity in PCR; however the analytical sensitivity test was not well described. Additionally, concentrations of PCR reaction components were not given, making it hard to repeat and confirm their studies. Targeting a hemolysin gene of *E. tarda*, the PCR protocol developed by Chen and Lai (1998) amplified the expected amplicon from 40 *E. tarda* strains. Unfortunately, the specificity of the primers with other species was not tested. Baird et al. (2003) described a new PCR protocol using the small ribosomal

subunit gene as target to identify *Edwardsiella* spp. No sensitivity assay was performed in that study, though. Because of the above reasons, more research is needed to validate the already developed methods for *Edwardsiella* spp. detection. Specificity, and analytical and diagnostic sensitivity need to be established and validated before a PCR-based method can be applied for molecular diagnosis (Hiney and Smith 1998).

Several PCR detection protocols targetting the 16S rRNA gene have been described for *F. columnare* detection (Toyama et al. 1996; Bader and Shotts 1998; Bader et al. 2003b; Darwish et al. 2004). The developed protocols either lacked sensitivity tests or needed two-round nested PCR. In addition, a misidentified *F. johnsoniae* isolate was thought to be *F. columnare* and was used in designing *F. columnare* specific primers, making these protocols questionable (Toyama et al. 1996; Bader and Shotts 1998; Bader et al. 2003b). Welker et al. (2005) developed a new protocol to detect *F. columnare* based on the intergenic spacer region (ISR) present between the 16S and the 23S rRNA genes. This new protocol exhibited desirable specificity and sensitivity, being able to detect as few as 100 *F. columnare* cells per sample. Unfortunately, the large size of the amplicon (~500 bp) in this protocol prevented the adaptation of this standard PCR to real-time PCR. Ideally, the size of the amplicon should be between 80-150 bp when using a real-time PCR protocol.

PCR-based detection methods do not require pathogen isolation and recovery from the environment, shortening the time of pathogen detection. Compared to standard PCR, real-time PCR assays are faster and allow target quantification in a single assay. Typically, bacterial pathogens are present in natural environments in low numbers. Therefore, sensitive methods are needed to detect them in their aquatic reservoirs.

Moreover, early detection of fish pathogens in the preclinical stage can be critical in preventing outbreaks. Monitoring the changes of *F. columnare* load on fish body surface, internal organs, or the environment may provide important information about this pathogen's infection rate and transmission. Welker et al. (2005) reported that *F. columnare* can be transmitted through water without direct contact with diseased fish. The authors also found a good survival of *F. columnare* in the biofilm from tank walls four days after inoculating the water with *F. columnare* cells. It will be interesting to determine whether *F. columnare* in biofilm may infect fish and what is the mimimum bacterial load to cause disease. In addition, *F. columnare* bacterial cells have the characteristic of adhering to each other, making it difficult to accurately enumerate by plate counting method. Real-time PCR, at this point, may provide better sensitivity to quantify changes of bacterial cell numbers.

The objectives of this study were: (1) to develop a real-time PCR for *F. columnare*, and (2) to develop a multiplex real-time PCR protocol for simultaneous detection of *E. ictaluri*, *E. tarda*, and *F. columnare*.

Materials and Methods

Bacterial isolates, culture conditions and DNA extraction

The type strains of the species *E. ictaluri* CECT 885, *E. tarda* CECT 849, and *F. columnare* ATCC 23463 as well as other reference strains and clinical and environmental isolates were used in this study (Table II.1). *F. columnare* was cultured in modified Shieh broth (Shoemaker et al. 2005b) at 28 ºC, while *Edwardsiella* strains were cultured in brain heart infusion broth (BHI) (Becton, Dickinson and Company, Sparks, MD) at 28

ºC. Genomic DNA was extracted using the DNeasy tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

Real-time PCR for *E. ictaluri* detection

We adapted the real-time PCR protocol by Bilodeau et al. (2003) for *E. ictaluri* in our laboratory. Probe and primer sequences were identical to those originally described by Bilodeau et al. (2003). Primers and probe were ordered from Applied Biosystems. Probe was labelled with 6-carboxyfluorescein (6-FAM) at 5' end and 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3' end. Some of the reagents and equipment used in our protocol were different from those described by Bilodeau et al. (2003). Instead of using 1 X platinum quantitative PCR superMix-UDG (Invitrogen Life Technologies, Carlsbad, CA) for the real-time PCR reaction mixture, we used Taqman universal PCR master mix (Applied Biosystems, Foster, CA). The real-time PCR thermocyler we used was ABI PRISM 7000 Sequence Detection System (Applied Biosystems) not the iCycler (Bio-Rad Laboratory) used by Bilodeau et al. (2003). Bilodeau et al. (2003) described two methods for DNA isolation: one to extract DNA from erythrocytes and a second one to extract DNA from bacterial cells. We followed their procedure for erythrocyte DNA isolation. In addition, we also tried a commercial kit specific for blood (MO BIO, Carlsbad, CA) and the general DNeasy tissue kit (Qiagen) for purifying erythrocyte DNA. We did not try their method for bacterial DNA isolation, instead, DNeasy tissue kit was used for extraction DNA from bacterial cells, catfish kidney, and brain.

Primers and probe design for real-time PCR of *F. columnare*

F. columnare 16S rRNA gene sequences were downloaded from Genbank. Sequences used were (with accession numbers in brackets): *F. columnare* (AJ491824 AB015480 AY842900 AY747592 AY561521 AY842901 AB016515 AY488506 AY550029 AY488507 AB015481 AY842899 D12659 AY095342 AB023660 AB010952 AB010951 AB078047 AY577821 AY635167 AJ831829 AJ831828 AJ831825 AJ831830 AJ831826 AJ831824 AJ831827), *Flexibacter aurantiacus* (AB078044 AB078045), *Tenacibaculum maritimum* (AB078057), *F. psychrophilum* (AF090991 AY034478 AB078060 AY577822 AY662493 AY662494), *F. aquatile* (M62797), *F. hydatis* (M58764), *F. branchiophilum* (D14017). Vector NTI® Suite 8 was used to align all the sequences. Unique sequence areas were analyzed using the Applied Biosystems Primer Express software (Applied Biosystems) to design real-time PCR primers and probe. The probe labeling was the same as above (Fco-16probe: 6FAM-5'-TTTCTTCGGACGTTTTTCAAGGTGCTGC-TAMRA-3'; primer Fco-16F: 5'-GGAAACGACAGATTTGGAAACAG-3'; primer Fco-16R: 5'-GCACGAGCTGACAACAACCA-3'). The optimized PCR reaction included 1 X Taqman universal PCR master mix (Applied Biosystems), 0.2 μM primers, and 0.25 μM probe with final volume of 25 μL. Quantified data were obtained by using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Amplification profile of PCR was as follows: one cycle of 2 min at 50 ºC, one cycle of 10 min at 95 ºC, and 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC.

Specificity and sensitivity of the primers

Specificity — Real-time PCR specificity was tested with bacterial species listed in Table II.1. For real-time PCR of *E. ictaluri*, tested bacterial species were: *F. columnare* ATCC 49512, *F. johnsoniae* ATCC 43622, and all the listed isolates of *E. ictaluri* and *E. tarda*. For real-time PCR of *F. columnare*, tested bacterial species included *E. ictaluri* CECT 885, *E. tarda* CECT 849, *Yersinia ruckeri* ATCC 29473, and all the listed *Flavobacterium* spp. Bacterial DNA was used as template for PCR amplification. PCR amplification profile was as follows: one cycle of 2 min at 50 ºC, one cycle of 10 min at 95 ºC, and 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC.

Analytical sensitivity — Several DNA preparation methods were used for *E. ictaluri* real-time PCR. Two strains of *E. ictaluri* were used: CECT 885 (type strain) and AL-93-75.

Purified DNA: Purified genomic DNA and plasmid containing the cloned target transposon from *E. ictaluri* were 10-fold diluted, respectively. DNA amount ranged from 100 ng to 100 fg. Sensitivity test was performed in triplicate.

Bacterial cells: Sensitivity was also tested by spiking boiled *E. ictaluri* cells into the PCR reaction. *E. ictaluri* CECT 885 and AL-93-75 were cultured in BHI broth overnight. Ten-fold dilution of the cultures was performed and plate counts were run in parallel. One hundred microliters of each dilution was boiled at 100 ºC for 5 min followed by 2 min centrifugation at 200 *g*. Five microliters supernatant was directly used as template for the real-time PCR. The sensitivity test was performed in triplicate.

DNA from spiked blood: Further sensitivity tests were conducted by spiking known numbers of bacterial cells into catfish blood samples followed by DNA extraction.

Bacterial culture and dilution procedure was the same as above mentioned. Ten microliters of freshly obtained catfish blood was mixed with 25 µL of each dilution followed by DNA extraction, and 50 ng of DNA was subjected to real-time PCR.

Diagnostic sensitivity — Channel catfish were challenged with *E. ictaluri* to establish the diagnostic sensitivity of our modified PCR approach. The challenge experiment was conducted by Craig A. Shoemaker (ARS-USDA, Auburn, AL) following published protocols (Klesius and Shoemaker 1997). Approximate ten-gram channel catfish were used in the study. The experiment setup included 9 unchallenged control fish, and 3 replicates of challenged fish in 3 separate tanks (9 fish per tank). Fish were immersed in 5 X 10^5 CFU/mL of *E. ictaluri* AL-93-75 strain in 57 L tank for 30 min. Water temperature was kept at 25 ºC ± 2 ºC for the duration of the experiment. Blood, brain, and kidney were sampled at 14d post-challenge for later analysis. DNA was extracted from kidney, brain, and 10 µL of blood. Template DNA for real-time PCR included 300 ng of blood DNA, 50 ng of diluted DNA from blood, 50 ng of kidney DNA, and 50 ng of brain DNA. A standard curve was established with DNA from *E. ictaluri* CECT 885. The amount of DNA ranged from 100 ng to 100 fg by serial 10-fold dilution.

As part of a collaborative effort, the above experiment was used to compare the diagnostic sensitivity between the real-time PCR protocol and a new method developed by Panangala et al. (2006) using an indirect fluorescent antibody assay (IFA). Samples for IFA were obtained from experimentally infected fish. Sample smears were made on glass slides and were air dried and heat fixed. Primary antibody was incubated on the slides for 45 min followed by rinsing with PBS and air dried. Fluorochrome-conjugated secondary antibodies were incubated on the slides for 45 min, rinsed, and air dried. Slides

were examined with a Nikon Eclipse 800 M epifluorescence microscope. In addition, samples from infected fish were plated onto *E. ictaluri* selective medium (EIM). Putative colonies were confirmed by fatty acid methyl ester analysis (FAME) (Shoemaker et al. 2005a).

23S rRNA gene and ISR amplification, cloning, and sequencing

Amplification —Internal spacer region within the ribosomal operon of *E. tarda* and *E. ictaluri* were amplified using two pairs of primers G1/L1 and 16S-14F/23S-1R (Arias et al. 1995) (G1:5'-GAAGTCGTAACAAGG-3', L1:5'-CAAGGCATCCACCGT-3'; 16S-14F: 5'-CTTGTACACACCGCCCGTC-3', 23S-1R: 5'-GGGTTTCCCCATTCGGAAATC-3'). The ISR sequences of *F. columnare* had been already sequenced in our laboratory (GenBank assession numbers from AY754360 to AY754388). Welker et al. (2005) developed a standard PCR based on these ISR sequences; however, adaptation of the primers to real-time PCR failed due to the large size of the amplicon (~ 500 bp) (Thomas L. Welker, ARS-USDA, Auburn, Al, personal communication).

The partial 23S rRNA gene of *E. ictaluri*, *E. tarda*, and *F. columnare* were amplified using universal primers 118V and 1037R (118V:5'-CCGAATGGGGAAACCCA-3', 1037R: 5'-CGACAAGGAATTTCGCTAC-3') (Arias et al. 1995). For the 3' end amplification of 23S rRNA gene, another three primers were used (23-3F: 5'-GGCGGCCGTAACTATAACG-3', 23-2R: 5'-AGCCTCACGGTTCATTAGTACC-3', 23-1R: 5'-GACCGAACTGTCTCACGACG-3'). 23-3F was used as the forward primer for *E. ictaluri*, *E. tarda*, and *F. columnare*. 23-2R was the reverse primer for *E. ictaluri* and *E. tarda*. 23-1R was the reverse primer for *F.*

columnare. Bacterial sequences for designing the three primers were obtained from the Comparative RNA Web Site (URL:http://www.rna.icmb.utexas.edu/). Sequences used for comparison were (GenBank accession number in bracket): *Escherichia coli* (AF053965 AJ278710), *Salmonella enterica* (U77919), *S. bongori* (U77927), *Citrobacter freundii* (U77928), *Klebsiella pneumonia* (X87284), *Yersinia pestis* (NC-004088), *Y. enterocolitica* (U77925), *Erwinia carotovara* (BX950851), *Aeromonas hydrophila* (X67946 X87281), *Plesiomonas shigeloides* (X65487), *F. odoratum* (M62807), *Flexibacter flexilis* (M62806), *Chlorobium limicola* (M62805), *S. typhimurium* LT2 (AE008895). The vector NTI® software package (Invitrogen, Carlsbad, CA) was used to design primers.

All reagents unless otherwise stated were purchased from Promega (Madison, WI). Amplification reactions were carried out in a final volume of 50 µL containing 2.5 µM $MgCl_2$, 1 X Taq buffer, 0.2 µM of both primers, 0.2 µM of dNTPs, 1.7 unit of Taq polymerase, and 60 ng of template DNA. The amplification profile was as follows: hot-start for 10 min at 95°C, 30 cycles of 30 s at 94°C, 45 s at 55°C, and 1.5 min at 72°C. PCR products were electrophoresed on 1% agarose gel for 30 min at 100 V. The gel was visualized under ultraviolet light, and the proper bands were cut off and purified using the Geneclean kit (Q-BIO gene company, Irvine, CA). Purified PCR products were ligated into pGEM-T easy vector following manufacturer's instruction.

Cloning and sequencing — Fifty microliters of competent *Escherichia coli* JM109 cells were used for transformation. Cell transformation was carried out as follows: cells were incubated for 20 min on ice, then hot shocked for 45 s at 42 °C, and finally placed on ice for 5 min. The transformed *Escherichia coli* cells were plated on

Luria-Bertani (LB) agar containing ampicillin and IPTG/X-Gal (Invivogen, San Diego, CA). The plates were incubated at 37 °C for 18 h. Transformants were cultured in LB broth at 37 °C for 16-18 h. The aurum plasmid mini kit (Bio-Rad, Nercules, CA) was used to extract plasmids from clones. In order to check the size of the inserted amplicon, plasmids were restriction digested with *Eco*RI and resolved through a 1% agarose gel at 100 V for 30 min. Finally, the plasmid DNA was submitted to Auburn University Genetic Analysis Laboratory (AU-GAL) (http://www.auburn.edu/research/vpr/aurif/sequencing.htm) for sequencing. This laboratory uses ABI 3100 Genetic Analyser (Applied Biosystems) for DNA sequencing.

Multiplex PCR

Primers were designed to detect *E. ictaluri*, *E. tarda*, and *F. columnare* simultaneously. Primers flanking the intervening sequences (IVS) within the 23S rDNA of *E. ictaluri* were designed by aligning 23S rDNA sequences of related species. Comparative RNA Web Site (URL:http://www.rna.icmb.utexas.edu/) was used to obtain 23S rDNA data. These sequences were the same as those used for primer design against the 3' end of 23S rDNA above mentioned. The sequence of the universal forward primer for the three species was 5'-GACAGCYAGGATGYTGGCTT-3'. We based the specificity of the protocol on the reverse primer sequence. Therefore, two reverse primers were needed in the reaction. For *E. ictaluri* and *E. tarda* amplification, we used EicEta-23S-R: 5'-CAGCAGCCCTCACAGGC-3' while the reverse primer for amplifying *F. columnare* was Fco-23S-R: 5'-CCAGAAATCCTCACGGAATC-3'. Due to the high nucleotide similarity between *E. ictaluri* and *E. tarda*, we targeted the intervening

sequence (IVS) since *E. tarda* does not present this sequence. Therefore, the species of *Edwardsiella* can be differentiated based on amplicon size.

Combinations of the universal forward primer with each specific reverse primer were tested in the following bacterial species: *E. ictaluri* CECT 885, *E. tarda* CECT 849, *F. columnare* ATCC 49512, *F. psychrophilum* ATCC 49418, *F. johnsoniae* ATCC 43622, *Yersinia ruckeri* ATCC 29473, *S. typhimurium* (clinical isolate), *F. hydatis* ATCC 29551, and *Escherichia coli* K-12.

Results

The target areas for real-time PCR developed in the current study included the ISR, 16S rDNA, and 23S rDNA. The ISR of *E. ictaluri* shared high similarity with *E. tarda*; therefore, ISR was not suitable for real-time PCR development.

E. ictaluri real-time PCR specificity

The real-time PCR protocol for *E. ictaluri* in our laboratory modified from Bilodeau et al. (2003) amplified all 21 isolates of *E. ictaluri* but did not amplify any of the *E. tarda*, *F. columnare*, and *F. johnsoniae* strains tested.

E. ictaluri real-time PCR analytical sensitivity

By spiking known amount of DNA into real-time PCR reaction, the modified real-time PCR for *E. ictaluri* was able to detect 100 fg by using plasmid DNA containing the transposon, 100 pg of genomic bacterial DNA, and 4 X 10^6 CFU from boiled bacterial cells. The real-time PCR failed to detect any sample containing a mixture of *E. ictaluri* DNA and catfish erythrocyte DNA regardless of the extraction methods used.

E. ictaluri real-time PCR diagnostic sensitivity and comparison with cultural and immunological methods

Table II.2 displayed the comparison of the real-time PCR results with the results from microbial culture and immunological methods. Negative control (i.e. non-challenged fish) samples were negative for *E. ictaluri* by all methods (data not shown). Only 23 challenged channel catfish were tested because 4 fish died before sample collection, and therefore could not be tested by any method. All positive samples by real-time PCR were also positive by culture methods (EIM). By contrast, real-time PCR did not amplify some positive samples identified by IFA and by selective medium (EIM). Real-time PCR failed to detect *E. ictaluri* in 23 blood samples, and only identified *E. ictaluri* from 5 out of 23 kidney samples and 3 out of 23 brain samples. Results obtained by IFA did not match the results obtained from culture method. Some samples that were positive by the culture method were negative by IFA method and vice versa.

Specificity of real-time PCR for *F. columnare* detection

All 32 *F. columnare* isolates tested yielded the expected positive amplification. No amplification was observed from *F. psychrophilum*, *F. hydatis*, *F. johnsoniae*, *E. ictaluri*, and *E. tarda*. However, the specific primers designed did amplify *F. aquatile* DNA under optimized real-time PCR conditions. To ensure *F. aquatile* was not contaminated with *F. columnare* DNA, *F. aquatile* cultures recovered from three different glycerol stock vials were identified by fatty acid methyl ester analysis (FAME) and confirmed as *F. aquatile*. No amplification was observed from *F. aquatile* DNA when the *F. columnare* specific primers by Welker et al. (2005) were used. Since

cross-reactivity could not be eliminated, no further tests, including sensitivity tests, were performed with these primers.

Multiplex PCR

The specificity of the multiplex PCR was first tested using a standard PCR protocol. The combination of forward primer with the *Edwardsiella*-specific reverse primer amplified *E. ictaluri*, *E. tarda* as well as *F. columnare*, *Y. ruckeri*, *S. typhimurium* and *Escherichia coli* DNAs. The combination of forward primer with *F. columnare* specific reverse primer amplified *F. columnare* as well as *F. johnsoniae*, *Y. ruckeri*, and *S. typhimurium*. Even after the annealing temperature was increased from 51ºC to 58 ºC, similar results were obtained (Figure II.1). Therefore, this real-time multiplex PCR was not subjected to further trials.

Discussion

The real-time PCR protocol described by Bilodeau et al. (2003) failed to yield the expected results in our laboratory. Even though the primers and probe sequence were the same, the real-time PCR equipment we had available was different. Bilodeau et al. (2003) used iCycler by Bio-Rad, while we used the ABI PRISM 7000 Sequence Detection System. To my knowledge, both systems should be compatible. Bilodeau et al. (2003) reported a very high sensitivity level, since they were able to detect *E. ictaluri* from all the blood samples spiked with bacterial cells. However, we could not detect *E. ictaluri* from any of the challenged channel catfish blood samples tested, although we tried different methods for DNA extraction besides the one described by Bilodeau et al. (2003), including a commercial kit specifically designed for blood. Only less than 20% of kidney and brain samples were positive by the modified real-time PCR method. Overall, the

sensitivity of the *E. ictaluri*-specific protocol under our conditions was much lower than expected. On the other hand, when we used the same *E. ictaluri* specific primers in a standard PCR reaction, the sensitivity and specificity obtained were within the expected range (Welker et al. 2005).

The combination of primers and probes designed to specifically amplify *F. columnare* also amplified *F. aquatile* DNA. This result was unexpected since the primers and probes used presented 11 mismatches with the *F. aquatile* 23S rDNA sequence. Several reasons may justify this result; e.g., the specific primers amplified was outside the target sequences. However, no conclusion could be inferred unless cloning and sequencing the non-specific band is attempted.

Regarding the multiplex PCR protocol for *F. columnare*, *E. ictaluri*, and *E. tarda*, the designed primers could not differentiate target species from other non-target species. Concurrently with our research, Panangala et al. (2006) developed an indirect fluorescent antibody (IFA) test to simultaneously detect *E. ictaluri* and *F. columnare*. Moreover, the same authors were working on developing a multiplex PCR protocol for *F. columnare*, *E. ictaluri* and *A. hydrophila* (Panangala et al. 2007). At that point, based on our unsuccessful data and considering the ongoing research by Dr. Panangala's group, we decided to end this project and redirect our research efforts.

Table II.1. Isolates used in the PCR detection study.

Species	Isolates	Origin
F. columnare	ALG-00-513, ALG-063, ALG-00-527	Greensboro, AL, USA
	MS 467, MS 463, MS 465, MS 475	Stoneville, MS, USA
	MO-02-23	Lake of the Ozarks, MO, USA
	BZ-05	Brazil
	ATCC 23463	Diseased chinook salmon, *Oncorynchus tshawytscha*, Snake River, WA, USA
	ATCC 49512	Skin lesion of a brown trout fry, *Salmo trutta*, Basse-Normandie, France
	Fco152, Fco96, Fco107, Fco109, Fco145, Fco147, Fco103, Fco63	Channel catfish, Mobile River, AL, USA
	Fco150	Threadfin shad, Mobile River, AL, USA
	Fco115, Fco127, Fco113, Fco136, Fco106, Fco131, Fco142, Fco138	Blue catfish, Mobile River, AL, USA
	Fco97, Fco102, Fco143, Fco94	Freshwater drum, Mobile River, AL, USA
F. psychrophilum	ATCC 49418	Coho salmon kidney, *Oncorhynchus kisutch*, WA, USA
F. johnsoniae	ATCC17061	Soil, England
	ATCC 43622	Salmonid fish?
F. aquatile	ATCC 11947	Deep well in Kent, England
F. hydatis	ATCC 29551	Gills of diseased salmon, MI, USA
Y. ruckeri	ATCC 29473	Rainbow trout, *Onchorhynchus mykiss*, with redmouth disease, USA
E. ictaluri	151,218, 219,195,196, RE33, AL-02-27, AL-93-75, AL-93-58, AL-02-27, ALG-03-275, ALG-03-278, ALG-03-101, ALG-03-190	Catfish, AL, USA
	CECT 885	Catfish, GA, USA

(cont.)

	EILO	Catfish, Thailand
	S94-1051, S94-1034, 1696,1963, 1760,	Catfish, MS, USA
E. tarda	CECT 849	Human feces
	ATCC 15947	Human feces
S. typhimurium	Clinical isolate	
Escherichia coli	K-12	Human feces

CECT, Spanish Type Culture Collection

ATCC, American Type Culture Collection

Table II.2. Comparison of real-time PCR, microbial culture and IFA for detection of *E. ictaluri*. Tank 1-3 contained 9 challenged fish each. Only 5 fish in tank 3 were alive at the 14 d post-challenge. Dead fish were not tested. Non-challenged fish were alive and negative by all detection methods (data not shown).

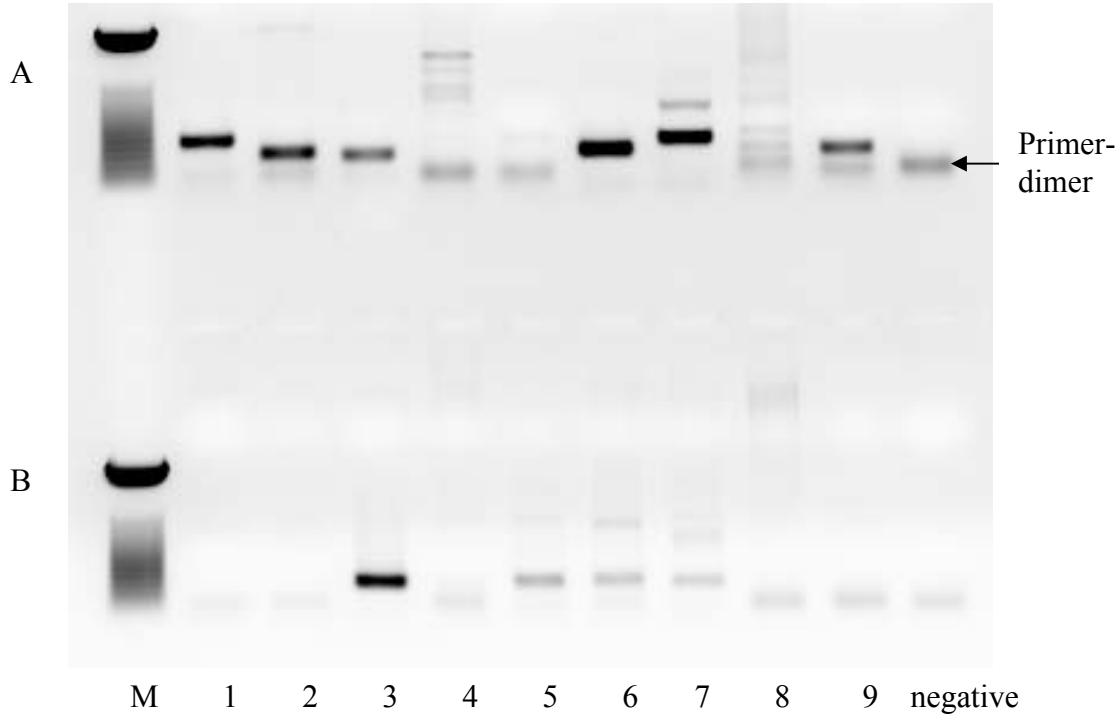
		Blood			Kidney			Brain		
Tank	Fish	Culture	IFA	Real-time	Culture	IFA	Real-time	Culture	IFA	Real-time
1	1	-	-	-	-	-	-	-	-	-
1	2	-	-	-	+	-	-	-	-	-
1	3	-	-	-	-	-	-	-	-	-
1	4	-	-	-	+	-	-	-	+	-
1	5	+	+	-	+	-	+	+	-	-
1	6	+	-	-	+	-	+	+	-	+
1	7	-	-	-	-	-	-	-	-	-
1	8	+	-	-	+	-	-	+	-	+
1	9	-	-	-	-	+	-	-	-	-
2	1	+	-	-	+	-	+	+	+	-
2	2	+	-	-	-	-	-	-	-	-
2	3	+	-	-	+	?	-	+	+	-
2	4	-	+	-	+	?	-	+	?	+
2	5	-	+	-	-	+	-	-	+	-
2	6	+	+	-	+	-	-	+	+	-
2	7	-	+	-	+	+	-	-	+	-
2	8	-	+	-	-	+	-	-	+	-
2	9	+	-	-	+	+	+	-	+	-
3	1	-	-	-	-	-	-	+	-	-
3	2	-	-	-	+	+	-	-	+	-
3	3	-	-	-	-	+	-	-	+	-
3	4	-	-	-	+	-	-	-	+	-
3	5	+	-	-	+	+	+	+	+	-
T		9	6	0	14	8	5	9	12	3

T: total number of positives

IFA: indirect fluorescence antibody test

?: inconclusive result

Figure II.1. Specificity test of the multiplex PCR. M, 50 bp DNA ladder; Lane 1, *E. ictaluri*; lane 2, *E. tarda*; lane 3: *F. columnare*; lane 4, *F. psychrophilum*; lane 5, *F. johnsoniae*,; lane 6, *Y. ruckeri*; lane 7, *S. typhimurium*; lane 8, *F. hydatis*; lane 9, *Escherichia coli*. Primers used for top gel (A) were universal forward primer with EicEta-23S-R. Primers used for bottom gel (B) were universal forward primer with Fco-23S-R.



III. IDENTIFICATION AND CHARACTERIZATION OF AN INTERVENING SEQUENCE WITHIN THE 23S RIBOSOMAL RNA GENE OF *EDWARDSIELLA ICTALURI*

Abstract

Comparison of the 23S rRNA gene sequences of *Edwardsiella tarda* and *E. ictaluri* confirmed a close phylogenetic relationship between these two fish pathogen species and a distant relation with the 'core' members of the *Enterobacteriaceae* family. Analysis of the *rrl* gene for the 23S rRNA in *E. ictaluri* revealed the presence of an intervening sequence (IVS) in helix-45. This new 98 bp IVS shared 97% nucleotide identity with *Salmonella typhimurium* helix-45 IVS. *E. ictaluri* helix-45 IVS was present in all *E. ictaluri* strains analyzed and in at least six *rrl* operons within each cell. Fragmentation of 23S rRNA due to IVS excision by RNase III was observed by methylene blue staining of ribosomal RNA extracted from *E. ictaluri* isolates. This is the first report of an IVS in the 23S rRNA gene of the genus *Edwardsiella.*

Keywords: *Edwardsiella,* intervening sequences (IVS), *rrl* genes

Introduction

The genus *Edwardsiella* was first described in 1965 by Ewing et al. to harbor a collection of 37 strains biochemically distinct from other taxa within the family *Enterobacteriaceae*. The proposed type species for the genus was *Edwardsiella tarda* and at that time comprised isolates of fecal origin from the USA. At about the same time, a Japanese group studying bacterial isolates from reptiles identified a distinct yet homogeneous group, referred to as the Asakusa group, within the *Enterobacteriaceae* family (Sakazaki and Murata 1962) which appeared to be very similar to the newly described *E. tarda*. It was not until 1980 that a second species, *E. hoshinae*, was added to the genus by Grimont et al. (1980) who isolated it from reptiles and birds. Finally, Hawke et al. (1981) added the last species to the genus, *E. ictaluri*, described from isolates recovered from diseased channel catfish (*Ictalurus punctatus*).

The phylogenetic position of the genus *Edwardsiella* within the family *Enterobacteriaceae* is somewhat unclear since few evolutionary studies including this genus have been attempted (Ahmad et al. 1990). Nevertheless, DNA-DNA hybridization data pointed to *Serratia* as the closest genus to *Edwardsiella* at 20% similarity (Krieg and Holt 1984). Other studies based on different signature sequences, such as $tRNA^{Leu}$ gene and 16S rRNA gene, confirmed a distant phylogenetic relationship between *Edwardsiella* and core members of the *Enterobacteriaceae* such as *Escherichia coli* or *Salmonella* (Francino et al. 2003). Phylogenetic comparisons between the three *Edwardsiella* species are also scarce. Panangala et al. (2005) studied the interspecies and intraspecies differences between *E. tarda* and *E. ictaluri* based on the 16S-23S rRNA intergenic spacer region and concluded both species were highly similar (>96% identity). To our

knowledge, no formal ribosomal genes-based trees including *E. tarda* and *E. ictaluri* sequences have been published. At the intraspecific level, it is widely accepted that *E. tarda* presents a higher degree of intrinsic diversity than *E. ictaluri* (Klesius et al. 2003; Panangala et al. 2005). Only by using the high resolution fingerprinting technique AFLP (Amplified Fragment Length Polymorphism) has it been possible to discriminate among *E. ictaluri* isolates (Klesius et al. 2003).

E. ictaluri is the causal agent of enteric septicemia of catfish (ESC) and is responsible for great economic losses to the catfish industry across the world (Plumb 1999; Wagner et al. 2002). Only in the USA, where channel catfish production accounts for more than half of total aquaculture products, ESC costs the catfish industry millions of dollars yearly in direct fish losses (Plumb and Shoemaker 2006). Although a few non-ictalurid species (e.g. tilapia *Oreochromis niloticus*, Chinook salmon *Oncorhynchus tshawytscha*, and rainbow trout *Oncorhynchus mykiss*) are susceptible to experimental infection, *E. ictaluri* natural host range is mainly restricted to members of the *Ictaluridae* family, particularly channel catfish. On the contrary, *E. tarda* has shown a much broader host spectrum with the ability to infect fish from different families as well as reptiles and even humans, though is a minor source of disease in aquaculture. The pathogenic factors that made *E. ictaluri* a specialized catfish pathogen while *E. tarda* retained its broader spectrum are not known, nor are the phylogenetic relationships between these two pathogens.

The aim of this work was to expand our knowledge on the phylogenetic relationships between *E. tarda* and *E. ictaluri* by comparing their 23S rRNA gene sequence and to find new signature sequences for ESC detection. As a result of this study,

a new intervening sequence (IVS) in the 23S rRNA of *E. ictaluri* was identified for the first time.

Materials and Methods

Bacterial strains and cultivation conditions

Twenty nine *E. ictaluri* strains, including the type strain CECT 885, along with five *E. tarda* strains and *Escherichia coli* K-12 strain were used in this study (Table III.1). All strains were grown on brain heart infusion (BHI) (Becton, Dickinson and Company, Sparks, MD) broth or BHI agar (supplemented with 1.5% agar) at 28°C, except for *Escherichia coli* which was incubated at 37°C. Stock cultures were maintained in 15% glycerol at -70°C, and a single colony was isolated prior to use.

23S rRNA gene amplification, cloning and sequencing

Total bacterial DNA was extracted using the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA) following manufacturer's instructions. Two sets of universal primers 118V 5'-CCGAATGGGGAAACCCA-3' (positions 112 to 130 in *Escherichia coli*), 1037R 5'-CGACAAGGAATTTCGCTAC-3'(positions 1930 to 1948), 23_2F (5'-GGCGGCCGTAACTATAACG-3') (positions 1901 to 1919), and 23_2R (5'-AGCCTCACGGTTCATTAGTACC-3') (positions 2874-2895), complementary to highly conserved regions of eubacterial 23S rRNA genes were used to amplify the 23S rRNA gene from *E. ictaluri* CECT 885 and EILO and *E. tarda* CECT 849. PCR conditions were performed according to Arias et al. (1995). The expected size amplicons (both primer pair combinations, 118V-1037R and 23_2F-23_2R, yielded similar products ~1,8 kb) were resolved through standard agarose gel electrophoresis. Amplified products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostic

Corporation, Indianapolis, IN) and cloned into pGEMTEasy (Promega, Madison, WI). To ensure sequence accuracy, two clones from each strain were sequenced at the Auburn University Sequencing Core (Auburn, AL). Internal primers were designed to obtain the full length sequence for each clone. Sequences were trimmed and their similarity to 23S rRNA genes confirmed by BLAST (Basic Local Alignment Search Tool) search . A previously unknown intervening sequence (IVS) in the 23S rDNA of *E. ictaluri* was identified by comparison with other *Enterobacteriaceae* 23S rRNA genes.

Intervening sequences PCR amplification

Primers flanking the newly identified IVS were designed, and all strains used in the study were tested for IVS presence. The IVS primers used were: Eic-IVF-5'-CTAAACCATGCACCGAAGC-3' and Eic-IVR-5'-TTACAGAACGCTCCCCTACC-3'. Fifty nanograms of total DNA was used as template in a PCR reaction containing 0.5 µM primer Eic-IVF, 0.5 µM primer Eic-IVR, 10 µM deoxynucleotides, 1.5 mM $MgCl_2$, one unit of Taq DNA polymerase and 1x buffer (Eppendorf, Hamburg, Germany). PCR conditions were as follows: initial denaturing at 94°C for 5 min; 35 cycles of denaturing at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 45 sec, with a final extension step of 10 min at 72°C. Amplified products were electrophoresed, purified, cloned, and sequenced as described above.

Ribotyping

Analysis of the ribosomal operons present in *E. ictaluri* was performed accordingly to Arias et al. (1997a). Briefly, 5 µg of total DNA were digested with *Bam*HI and *Kpn*I, transferred onto a nylon membrane, and hybridized with a digoxigenin (DIG) labeled probe. Samples were run in duplicate and hybridized with two ribosomal probes.

1037-DIG is a universal probe complementary to a conserved sequence in the 23S rRNA gene of eubacteria . The entire IVS sequence discovered in *E. ictaluri* was PCR amplified using Eic-IVF and Eic-IVR, purified, DIG-labeled with the PCR DIG Probe Synthesis Kit (Roche), and used as probe (IVS-DIG) for ribotyping. While the 1037-DIG-labelled probe highlighted the number of ribosomal operons present in both *E. ictaluri* and *E. tarda*, hybridization with IVS-DIG indicated presence of IVS in a given operon.

RNA isolation, Northern blotting, and methylene blue staining

Cells were grown in BHI broth to mid-log phase and RNA extraction was done as described by Mattatall and Sanderson (1996). Five micrograms of total RNA were electrophoresed under the same denaturing conditions, transferred to a nylon membrane and stained with methylene blue.

Data analysis

The GenBank nr/nt database was searched for similar sequences using the BLASTn algorithm service . Fifty 23S rDNA complete (or near complete) sequences from other *Gammaproteobacteria* were downloaded from GenBank and from the Comparative RNA Web (http://www.rna.icmb.utexas.edu) for comparison with *E. ictaluri* and *E. tarda* 23S rRNA gene sequences. An initial phylogenetic analysis was performed to verify the phylogenetic position of the genus *Edwardsiella* within the *Gammaproteobacteria* class. Sequences used were (with accession numbers in brackets): *Acinetobacter calcoaceticus* (X87280), *Aeromonas hydrophila* (X67946), *A. sobria* (AF508060), *Arsenophonus spp*. (AY587142), *Buchnera aphidicola* (NC_004545), *Citrobacter freundii* (U77928), *E. ictaluri* (DQ211094, DQ314205), *E. tarda* (DQ211093), *Enterobacter aerogenes* (AY116918), *Erwinia carotovora* (BX950851),

Escherichia coli (AJ278710, J01695, AB035926, AB035922, U00096), *Francisella tularensis* (AY146950), *Haemophilus influenza* (U32742), *Halomonas halmophila* (AJ306879), *H. venusta* (AJ306887), *Hamiltonella defense* (AY296733*), Klebsiella pneumoniae* (X87284), *Leucothrix mucor* (X87285), *Morganella morganii* (AY116909), *Photobacterium damselae* (Y18520), *Photorhabdus luminescens* (NC_005126), *Plesiomonas shigeloides* (X65487), *Proteus mirabilis* (AY116929), *P. vulgaris* (AY116927), *Pseudomonas aeruginosa* (Y00432), *P. perfectomarina* (L03788), *P. stutzeri* (X87289), *Regiella insecticolla* (AY296734), *Salmonella bongori* (U77927), *S. enterica* (U77919, U77922, U77926, CP000026), *S. typhimurium* (AE008895, AE008893), *Serratia symbiotica* (AY296732), *Shewanella oneidensis* (AE015456), *Shigella boydii* (CP000036), *S. flexnerii* (NC_004741), *Sodalis glossinidius* (NC_007712), unclassified aphid secondary endosymbiont (AF263561), *Vibrio cholerae* (AE004157), *V. splendidus* (AJ294425), *V. vulnificus* (X87293), *Yersinia enterocolitica* (U77925), *Y. pestis* (AE013984, AE017127), and *Y. pseudotuberculosis* (NC_006155). Results of distance analysis in BioNumerics (see below) indicated that *Edwardsiella* spp. formed a strongly supported clade (100% bootstrap value) within the family *Enterobacteriaceae.* All *Enterobacteriaceae* sequences clustered together presenting a robust clade, consistent with previous phylogenetic studies. Further analysis, aimed at elucidating relationships of *E. ictaluri* and *E. tarda* to other lineages of *Enterobacteriaceae,* were conducted using 34 sequences representing 25 taxa. In this analysis we excluded species outside the *Enterobacteriaceae* family except for *Vibrio choleare,* which was designated as the outgroup. Sequences were aligned using BioNumerics v. 4.0 (Applied Maths, Kortrij, Belgium). Unknown bases were discarded

for the analysis and the multiple alignment was corrected manually. Similarity matrices, distances (using the Jukes and Cantor correction (Jukes and Cantor 1969)), neighbor-joining (NJ) (Saitou and Nei 1987), and maximum parsimony trees were generated. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the trees using 500 bootstrap resamplings of the data. Prediction of *E. ictaluri* 23S rRNA secondary structure was completed by free-energy minimization using online software at http://www.bioinfo.rpi.edu/applications/mfold/old/rna .

Results

23S rRNA gene sequence and analysis

Essentially complete 23S rRNA gene sequences (>2,700 bp) were determined for two *Edwardsiella* species (two *E. ictaluri* isolates and one *E. tarda*). A parsimony tree based on 23S rRNA gene sequences analysis is shown in Figure III.1. Species names and GenBank accession codes are included in the tree. The 23S rRNA gene consensus tree revealed a distant phylogenetic relationship of the genus *Edwardsiella* from the 'core' members (*Salmonella* and *Escherichia)* of the family *Enterobacteriaceae*. The two *E. ictaluri* isolates had very similar (>99%) but not identical 23S rRNA gene sequences. Both *Edwardsiella* species shared a high degree of nucleotide identity (97%) and formed a tight cluster. The phylogenetically closest species to *Edwardsiella* included in the study was *Erwinia carotovora* (94% nucleotide identity).

Identification of IVS in *E. ictaluri*

The alignment of *E. tarda* and *E. ictaluri* 23S rRNA gene sequences revealed an insertion of 98 bp in the 23S rRNA gene of *E. ictaluri*. BLAST analysis of the insert showed high similarity with an IVS present in *Salmonella typhimurium*. The newly found

IVS was present in both *E. ictaluri* isolates but absent in *E. tarda*. *E. ictaluri* IVS was located in helix-45 of the 23S rRNA gene. Available helix-45 IVS sequences from other *Enterobacteriaceae* were downloaded from GenBank and used to construct a neighbor-joining tree (data not shown) that revealed a high degree of sequence diversity between *Enterobacteriaceae* IVSs. *S. typhimurium* IVS was most similar to *E. ictaluri* followed by *Yersinia enterocolitica*. Specific nucleotide identities are displayed in Table III.2. The highest percent of nucleotide identity was found between *E. ictaluri* and *S. typhimurium* at 97%, suggesting that both sequences should be grouped under the same IVS family (Family M) according to Miller et al. (2000). *Yersinia bercovieri* and *Proteus mirabilis* shared 88% nucleotide similarity and could be included under a unique IVS family as well. Sequence similarities among remaining IVSs ranged from 42% to 86% nucleotide identity.

When the secondary structure of both *E. ictaluri* and *S. typhimurium* was generated (Figure III.2) only two differences were found between the two structures. *E. ictaluri* lacked a small loop present in *S. typhimurium* due to a single transversion on position 77, which resulted in a longer stem in this region. The second difference was a transition on position 35 of the IVS that resulted in no structural change.

Characterization of *E. ictaluri* IVS

In order to assess the prevalence of IVS within the species *E. ictaluri,* a collection of *E. ictaluri* isolates was screened for IVS presence. Primers flanking the helix-45 IVS region were designed and used in a PCR assay. Amplified regions containing IVS were 152 bp in length, while 54 bp bands indicated an absence of IVS in helix-45. All twenty nine *E. ictaluri* yielded the expected 152 bp amplified product (Figure III.3) while all *E.*

tarda strains lacked the IVS band and only exhibited the 54 bp amplicon. However, all *E. ictaluri* strains tested also presented a weak band at 54 bp indicating that at least one *rrn* operon in *E. ictaluri* did not contain IVS. All amplified IVSs were cloned and sequenced. No differences in nucleotide sequences between the different *E. ictaluri* isolates tested were observed (data not shown).

Ribotyping analysis was performed in order to further characterize the *rrl* operons in *E. ictaluri*. Restriction analysis by *Kpn*I and *Bam*HI followed by hybridization with the universal probe 1037-DIG revealed at least six *rrl* operons in the *E. ictaluri* genome (data not shown). When the IVS-specific probe was used, the same bands were displayed indicating that all *rrl* displayed by ribotyping contained the IVS. This result contradicts the PCR analysis by which at least one *rrl* operon did not contain IVS. However, bands highlighted by ribotyping were large in size, ranged from 8 kb to 25 kb, and could contain more than one *rrl* operon.

Fragmentation of 23S rRNA due to IVS excision was revealed by methylene blue staining of ribosomal RNA extracted from *E. ictaluri* isolates as well as from controls. If an *rrl* gene contains an IVS in helix-45 and that IVS is removed during posttranscriptional processing (without religation of the rRNA), two smaller fragments of 1.2 kb and 1.7 kb result. Figure III. 4 displays the processing of *rrn* genes in *E. ictaluri*. 16S and 23S rRNAs were represented by a 2.9 kb and a 1.5 kb band respectively, that were displayed by all isolates tested including IVS-negative controls. *E. ictaluri* rRNAs pattern showed two additional bands of 1.2 kb and 1.7 kb demonstrating fragmentation of the 23S rRNA in this species.

Discussion

Intervening sequences were first described by Burgin et al. (1990) as responsible for 23S rRNA fragmentation in *Salmonella*. These intervening sequences, or internal transcribed spacers, are removed (without splicing) after transcription of ribosomal genes by the action of RNaseIII, and the resulting 23S rRNA fragments are held together by the compact structure of ribosomes which remain functional. Since their discovery, IVS have been described in numerous bacteria including *Alphaproteobacteria*, *Gammaproteobacteria*, and *Epsilonproteobacteria* as well as in *Spirochaeta* (Evguenieva-Hackenberg and Klug 2000). Two characteristics of IVS have yet to be explained by microbiologists. First, a seemly random and sporadic distribution of IVS through bacteria has been observed. Second, their function is not known. Presence of IVSs in ribosomal genes seems not to interfere with cell viability nor appear to confer any evolutionary advantage. These unique sequences have been well studied within the family *Enterobacteriaceae*, where six out of 14 tested genera harbored isolates with IVS in their 23S rRNA genes (Pronk and Sanderson 2001). Intervening sequences in *Enterobacteriaceae* appear at two different sites, helix-25 and helix-45, according to the proposed secondary structure of the 23S rRNA, and can be grouped in different families based on nucleotide identity. However, these families (or superfamilies) cannot be correlated to other hierarchal classifications of *Enterobacteriaceae* based on taxonomy or phylogenetics. Horizontal and vertical gene transfer episodes had to be combined in order to justify IVSs distribution in *Enterobacteriaceae* (Pabbaraju et al. 2000).

In this work, we have sequenced and characterized the 23S rRNA genes from two *Enterobacteriaceae* whose genus, *Edwardsiella,* has never been the target of ribosomal

gene-based phylogenetics studies and is rarely included or compared with other members of the family *Enterobacteriaceae*. The analysis of the 23S rRNA of *E. ictaluri* and *E. tarda* confirmed the close relationship between both species which was previously determined by 16S rRNA analysis (data not shown). The 23S rRNA gene derived parsimony tree showed the genus *Edwardsiella* is phylogenetically distant from the 'core' members of the family *Enterobacteriaceae*, such as *Salmonella* and *Escherichia*, as previously reported from DNA:DNA hybridization studies (Krieg and Holt 1984). Interestingly, *E. ictaluri* contains an insertion of 98 bp in helix-45 that has high similarity with IVS described in other *Enterobacteriaceae*. Sequence analysis of this new IVS revealed a 97% nucleotide sequence identity with *S. typhimurium* helix-45 IVS. In fact, only two nucleotide differences in the IVSs of these two species were found (one transition and one transversion). Based on this high similarity between *E. ictaluri* and *S. typhimurium* IVSs, both sequences should be included under the same 45 helix-IVS family M (Miller et al. 2000). Oddly, *S. typhimurium* IVS has a higher similarity (97%) with *E. ictaluri* IVS than with any other helix-45 IVS described to date.

S. typhimurium has seven *rrl* genes, six of which present helix-45 IVSs of identical sequence (Mattatall and Sanderson 1996). The number of *rrl* genes in *E. ictaluri* has not been completely defined but, according to our ribotyping data, at least six *rrl* genes containing helix-45 IVSs are harbored in this species. When the IVSs from all thirty *E. ictaluri* isolates were cloned and sequenced, no differences in sequence between isolates or clones were noted, suggesting all helix-45 IVSs are identical. However, PCR results suggest at least one *rrl* gene in *E. ictaluri* lacks the 45-helix IVS. These results

could be explained by the existence of seven *rrl* genes in the chromosome, six of which would contain helix-45 IVS, as in *S. typhimurium* (Mattatall and Sanderson 1996).

IVSs in members of the seven genera (*Salmonella, Yersinia, Proteus, Providencia, Klebsiella, Citrobacter,* and *Edwardsiella*) of *Enterobacteriaceae* show some degree of sequence similarity with each other, but no significant nucleotide identity between *Enterobacteriaceae* IVSs and any other sequences, including IVSs from other bacteria has been detected by BLAST searches (Pronk and Sanderson 2001). However, the possession of IVS is a rather random phenomenon in this family, since some genera (*i.e. Escherichia* and *Enterobacter*) do not harbor IVSs in their *rrl* genes. Earlier studies on nucleotide sequences supported the idea that IVSs must be exchanged between different genera by lateral transfer. IVSs have terminal inverted repeats flanking the central region, which is characteristic of a mobile genetic element. These mobile elements could be transferred from one species to another by phage-mediated transduction, transformation, or conjugation. However, alternative hypothesis suggests that the progenitor of these species obtained the IVS from a common ancestor but that subsequent variation between different species was simplely due to the loss of IVSs by some strains or species. *Edwardsiella* could have obtained the IVS prior to species divergence and, while *E. ictaluri* still maintains the insertion (in at least six *rrn* operons), *E. tarda* could have lost it.

From an applied point of view, the availability of 16S rRNA gene sequences has facilitated the development of PCR-based methods in molecular diagnosis. However, cases of very closely related species have highlighted the need of using other conserved molecules such as the 23S rDNA (Arias et al. 1995) or the intergenic spacer region (ISR)

(Welker et al. 2005) as targets in a PCR detection methods since the 16S rRNA gene might not provide sufficient genetic variability. This is the case for *E. tarda* and *E. ictaluri*, as both species shared more than 99% identities in their 16S rDNA so that finding species-specific PCR primers has been an obstacle in developing PCR detection methods. To date, the only PCR detection method for *E. ictaluri* (Bilodeau et al. 2003) targets a mobile element present in the *E. ictaluri* genome since non specific signature sequences cannot be found in the 16S rRNA gene. Because of the negative economic impact caused by these two pathogens in the catfish industry, rapid and specific detection methods need to be implemented in fish diagnostic laboratories in order to hasten treatments and control outbreaks. The discovery of a helix-45 IVS sequence in the 23S rRNA gene of *E. ictaluri*, which is not present in *E. tarda*, opens possibilities for using this newly identified sequence in molecular diagnosis of enteric septicemia of catfish (ESC). The potential of the *E. ictaluri* IVS as a PCR-based detection target was described in chapter II.

Table III.1. Strains used in the IVS study.

Species	Strain	Origin
E. ictaluri	CECT 885^{T}	Catfish, Georgia, USA
	195, 196, 151, 218, 219, AL-93-75, AL-02-27, AL-93-58, ALG-03-278, ALG-03-190, ALG-03-161, ALG-03-278, ALG-03-275, ALG-03-277, ALG-03-192	Catfish, Alabama, USA
	S94-728, S94-1034, S94-715, S94-703, S94-827, S94-862, S94-872, S94-1051, S94-707,1696, 1760, 1963	Catfish, Mississippi, USA
	EILO	Catfish, Thailand
E. tarda	CECT 849^{T}	Human feces
	1909, Flb, Flb2, 172,	Catfish, Alabama, USA
Escherichia coli	K-12	Human feces

T, type strain

CECT, Spanish Type Culture Collection

Table III.2. Comparison of *E. ictaluri* helix-45 IVS with other *Enterobacteriaceae* helix-45 IVSs.

Nucleotide sequence identities over 80% are shown in bold.

Strains		% Nucleotide sequence identity with helix-45 IVS of							
		AF 176788	AF 176788	AF 176792	M 35802	AF 177051	U 49925	DQ 314205	M 35814
P. vulgaris	AF176788	100							
Pr. rettgeri	AF176788	**86**	100						
P. vulgaris	AF176792	51	57	100					
Y. bercovieri	M35802	42	48	35	100				
P. mirabilis	AF177051	36	48	38	**88**	100			
S. typhimurium	U49925	**80**	79	52	50	49	100		
E. ictaluri	DQ314205	72	72	52	46	49	**97**	100	
Y. enterocolitica	M35814	**80**	**82**	52	44	51	**85**	**83**	100

Figure III.1. Parsimony tree based on 23S rRNA gene sequences. The tree shown is a consensus bootstrap tree based on 500 resampled parsimony trees. Significant (>50) bootstrap values for each branch are indicated. Species names and GenBank accession numbers are noted as well. Bar shows 5% sequence divergence.

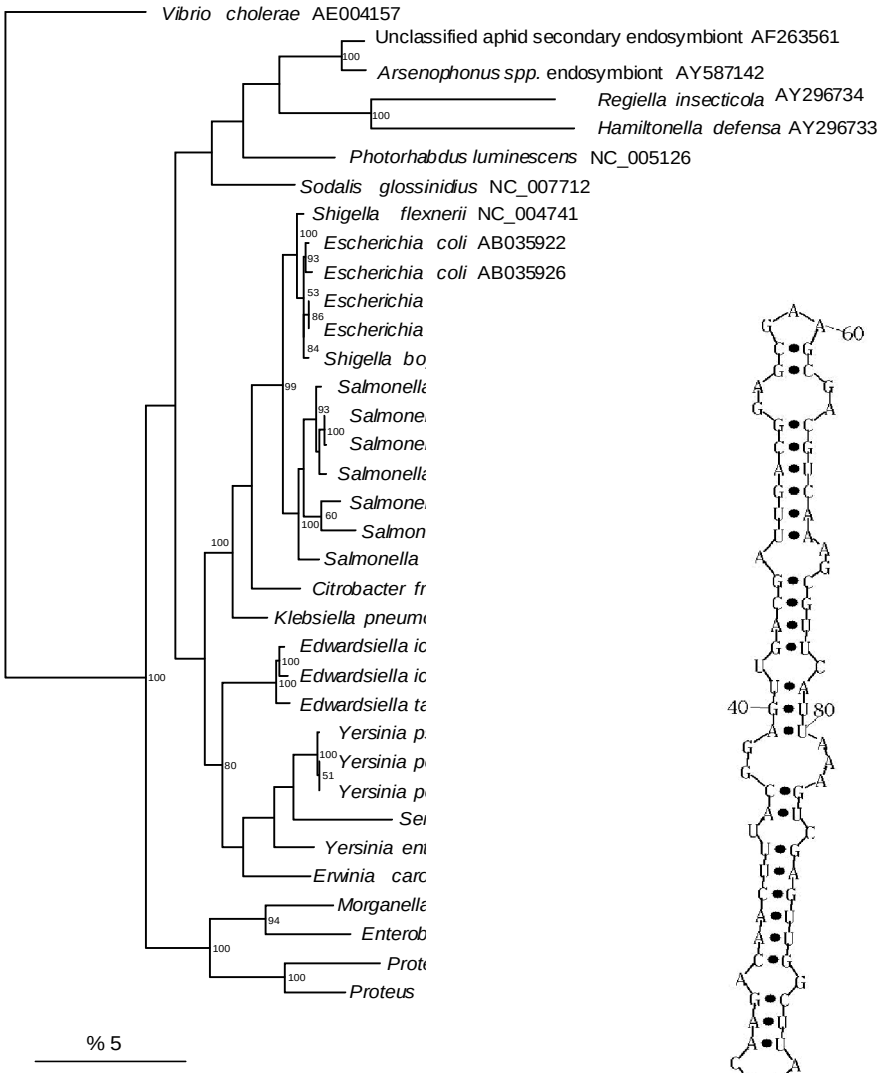


Figure III.2. Proposed secondary structure of IVSs from *E. ictaluri* (A) and *S. typhimurium* (B). Secondary

structure predictions based on free-energy minimization. Nucleotide differences in *E. ictaluri* IVS compared with *S. typhimurium* IVS are marked by asterisks. The proposed conserved site at which helix-45 was replaced by the IVS is indicated by a horizontal line.

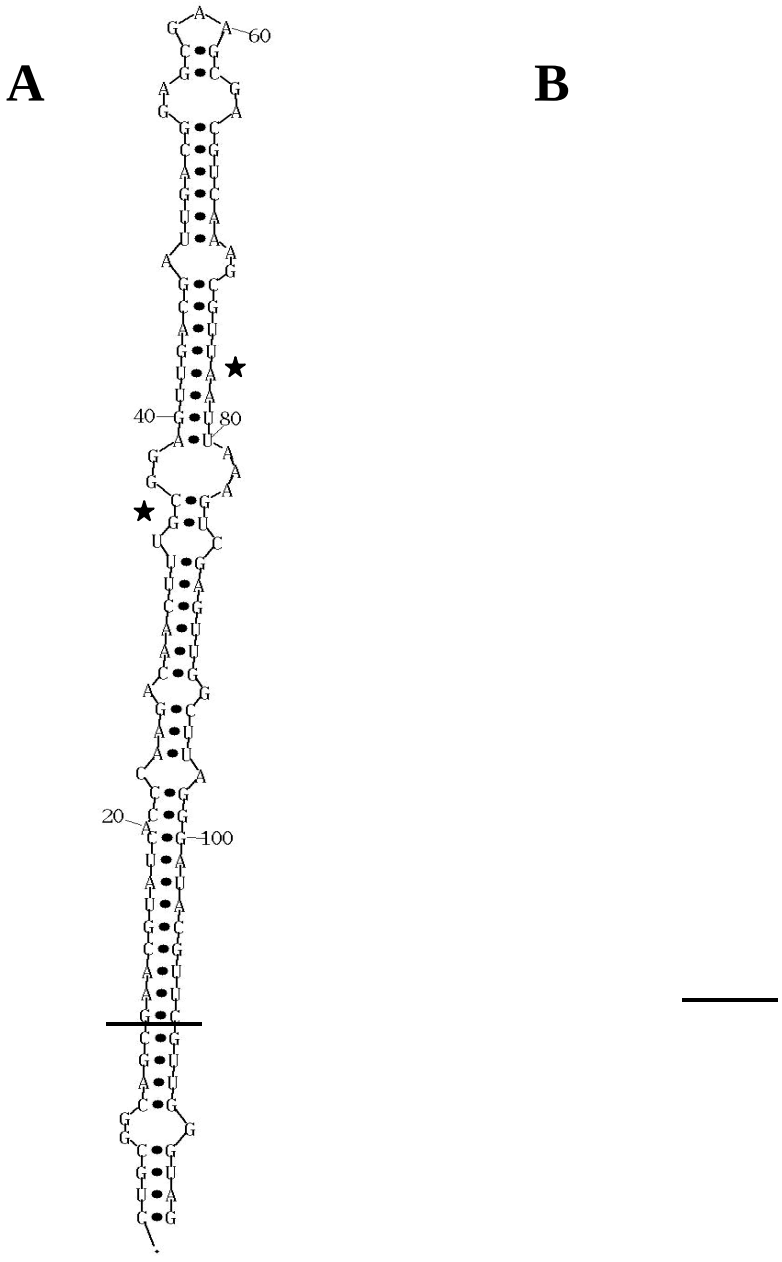


Figure III.3. Agarose gel containing the PCR products from amplification of 23S rRNA gene containing the IVS. Lane M, 50 bp molecular marker. Lane C, no template DNA; lane 1, *Escherichia coli*; lane 2, *E. tarda* CECT 849; lane 3 to lane 9, *E. ictaluri* isolates: CECT 885, EILO, 195, 196, 151, 218 and 219, respectively. *rrl* operons containing IVS in helix-45 yielded a 152 bp amplified product. Operons lacking IVS generated a smaller amplicon of 54 bp. This 54 bp amplified should not be confused with the primer-dimer band observed in the no-template control.

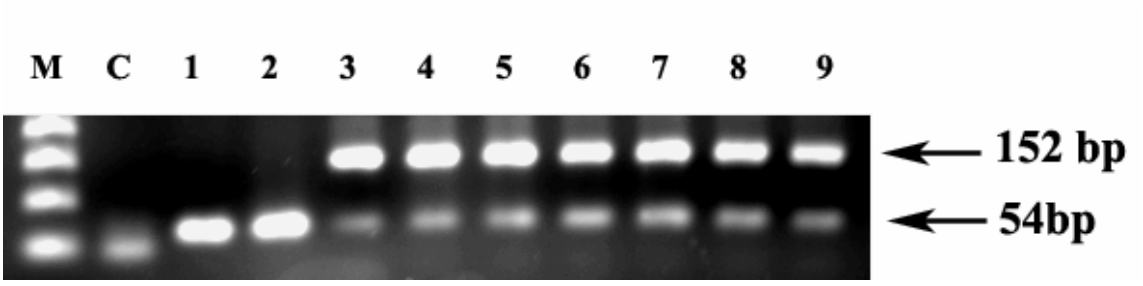
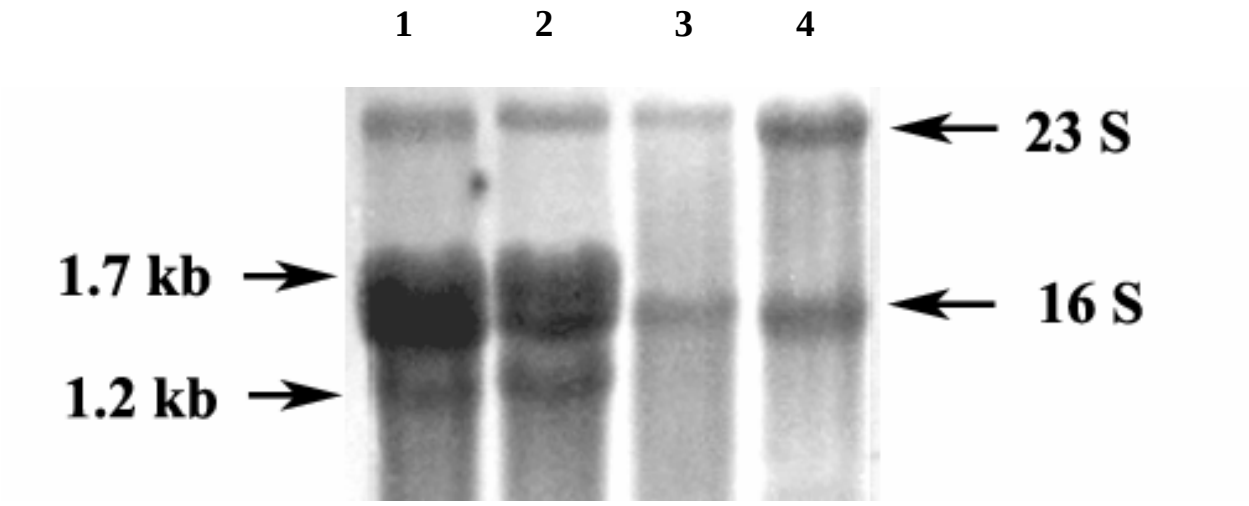


Figure III.4. rRNAs of *E. ictaluri* CECT 885 (1), EILO (2), *E. tarda* CECT 849 (3) and *Escherichia coli* (4). Total RNA was electrophoresed, blotted, and stained with methylene blue as described in Material and Methods**.**



IV. COMPARISON OF LIPOPOLYSACCHARIDE AND PROTEIN PROFILES BETWEEN *FLAVOBACTERIUM COLUMNARE* STRAINS FROM DIFFERENT GENOMOVARS

Abstract

Lipopolysaccharide (LPS) and total protein profiles from four *Flavobacterium columnare* isolates were compared. These strains belonged to genetically different groups and/or presented distinct virulence properties. *F. columnare i*solates ALG-00-530 and ARS-1 are highly virulent strains that belonged to different genomovars while *F. columnare* FC-RR is an attenuated mutant used as a live vaccine against *F. columnare*. Strain ALG-03-063 is included in the same genomovar group as FC-RR and presents a similar genomic fingerprint. Electrophoresis of LPS showed qualitative differences among the four strains. Further analysis of LPS by immunoblotting revealed that the avirulent mutant lacks the higher molecular bands in the LPS. Total protein analysis displayed by immunoblotting showed differences between the strains analyzed although common bands were present in all the isolates. FC-RR lacked two distinct common bands (34 kDa and 33 kDa) shared by the other three isolates. Based on the difference of LPS and total protein profiles, it is possible to discriminate the attenuated mutant FC-RR from other *F. columnare* strains.

Keywords: LPS, whole protein profiles, *F. columnare*, modified live vaccine

Introduction

Flavobacterium columnare is the causal agent of columnaris disease, one of the most important bacterial diseases of freshwater fish species. This bacterium is distributed world wide in aquatic environments, affecting wild and cultured fish as well as ornamental fish in aquaria (Austin and Austin 1999). *F. columnare* is considered the second most important bacterial pathogen in commercial cultured channel catfish, *Ictalurus punctatus*, in the southeastern USA, second only to *Edwardsiella ictaluri* (Wagner et al. 2002). Direct losses due to *F. columnare* are estimated in excess of millions of dollars per year. Mortality rates of catfish populations in ponds can reach 50% to 60% and can be as high as 90% in tank-held catfish fingerlings (USDA 2003a, 2003b).

Columnaris disease usually begins as an external infection of fins, body surface, or gills. The fins become necrotic with grayish to white margins, and initial skin lesions appear as discrete bluish areas that evolve into depigmented necrotic lesions. Skin lesions might have yellowish mucoid material accompanied by mild inflammation. Lesions can develop exclusively on the gills, which usually results in subacute disease and mortality, as is typically the case in young fish (Plumb 1999).

Due to the ubiquitous presence of *F. columnare* in aquatic environments, eradication of the disease in fish farms will not likely occur. Control and treatment of columnaris have primarily been directed toward the use of improved water-management practices to reduce physiological and environmental stress in the fish. Recently, a modified live *F. columnare* vaccine has been developed using a rifampicin-resistant strategy (Shoemaker et al. 2005b). This method had been previously used by Montaraz and Winter (1986) to generate a rough *Brucella abortus* strain, currently employed as the

official vaccine for cattle brucellosis in the USA. The same strategy was used by Klesius and Shoemaker (1999) to create an *E. ictaluri* rifampicin-resistant mutant patented as a modified live vaccine against enteric septicemia of catfish (ESC) (AQUAVAC-ESC, Intervet, Millsboro, DE). Characterization of *B. abortus* and *E. ictaluri* rifampicin-resistant mutants revealed that lipopolysaccharide (LPS), a main virulence factor for Gram-negative bacteria, lacked the high molecular bands observed in virulent isolates (Vemulapalli et al. 1999; Arias et al. 2003).

Flavobacterium columnare belongs to the Cytophaga-Flavobacterium-Bacteroides (CFB) group and is phylogenetically distant from the better studied gammaproteobacteria subclass which contains major human and animal pathogens (including *Brucella* and *Edwardsiella*). To date, virulence factors in *F. columnare* are poorly characterized. Specifically, the role of LPS in columnaris pathogenicity has not been explored. The objective of this study was to investigate if the attenuated *F. columnare* mutant originated through a rifampicin-resistance strategy presented a modified LPS and a different total protein profile by comparison with virulent strains.

Materials and Methods

Bacterial isolates and culturing

Four *F. columnare* strains, ALG-00-530, FC-RR, ARS-1, and ALG-03-063 were used in this study. Isolate ALG-00-530 was obtained from diseased channel catfish at the Alabama Fish Farming Center, Greensboro, Alabama. The ARS-1 isolate was recovered from diseased channel catfish at the Aquatic Animal Health Research Unit, USDA-ARS, Auburn, Alabama. These two isolates have been demonstrated to be virulent for channel catfish (Shoemaker et al. 2003a). *F. columnare* ALG-03-063 was also isolated from

diseased channel catfish. FC-RR strain is a rifampicin-resistant mutant, avirulent for catfish. This attenuated mutant has been licensed as a modified live vaccine by Intervet, Inc. and it is now undergoing field trials (Shoemaker et al. 2005b) (Patent No.: US 6,881,412 B1).

Genetic fingerprinting

A previous study by Arias et al. (2004) divided *F. columnare* species into four well-defined genetic groups. All catfish isolates were grouped into three different genogroups (I through III; genogroup IV included only tilapia isolates). Strains ALG-00-530 and ARS-1 belonged to genogroups I and II, respectively; while ALG-03-063 belonged to genogroup III. The avirulent mutant FC-RR was not included in that former study but was fingerprinted in the present work. Amplified fragment length polymorphism (AFLP) patterns from FC-RR mutant were obtained as described by Arias et al. (2004), added to the existing database and analyzed.

Production of polyclonal anti-serum against *F. columnare*

Twelve channel catfish were randomly divided into three groups of four fish each. Two groups were used to produce antibodies (Ab) against *F. columnare* strains (Ab-ALG-00-530 and Ab-FC-RR) while the third group served as negative control. Antigen preparation was from cells grown overnight in modified Shieh broth (5.0 g tryptone, 2.0 g yeast extract, 1.0 mL of 45.6 mM $CaCl_2$ $2H_2O$, 3 mL of 0.36 mM KH_2PO_4, 10 mL of 0.12 M $MgSO_4$ $7H_2O$, 10 mL of 0.41 mM $FeSO_4$ $7H_2O$, 1 L of distilled water, pH 7.2-7.4) and quantified by plate count (10 X 10^8 CFU/mL). Bacterial cells were sonicated for 30 seconds at speed 4 using a Virsornic 600 sonicator (SP Industries Company, Gardiner, NY). One milliliter of the sonicated suspension was mixed with 1 mL complete Freund's

adjuvant (Sigma, St. Louis, MO). Two hundred microliters of the mixture were intraperitoneally injected into each fish. Control fish were injected with 200 µL of Shieh broth. A booster injection was administered 27 days after initial immunization using 200 µL antigen prepared as above (Freund's incomplete adjuvant (Sigma) was used this time). Prior to the booster immunization, about 1.5 mL of blood was obtained from the caudal vasculature of each fish for titer determination (Shoemaker et al. 2003b). Three weeks after booster immunization, fish were bled again, and this antiserum was used to probe the western blots (see below). Blood was allowed to clot at room temperature for 1 h and serum was carefully removed after centrifugation for 5 min at 1,000 *g*. Sera were stored at -20 ºC until use. In order to determine the relative titer of the sera, enzyme linked immunosorbent assays (ELISA) were conducted according to Shoemaker et al. (2003b). Sonicated cell suspensions were prepared as described above and serial ten-fold dilutions were used to coat the plates; proper positive and negative controls were included. Serum from each individual fish was tested against each antigen dilution.

Lipopolysaccharides (LPS) and total protein extraction

The four isolates were cultured in modified Shieh broth for 24 h at 28 ºC. Three milliliters of broth were centrifuged at 3,000 *g* for 15 min. Pelleted cells were resuspended in lysis buffer and proteins were extracted according to Arias et al. (1997b). Crude LPS was extracted following the phenol-water protocol described by Westphal and Jann (1965). After lyophilization, the LPS extract was diluted in sample buffer at a final concentration of 1mg/mL. Aqueous and phenol LPS phases were stored at -80°C until use.

LPS and total protein analysis

Electrophoresis — LPS from both phases were resuspended in sample treatment buffer and electrophoresed by discontinuous SDS-PAGE on a 4% stacking gel and a 20% separating gel (Laemmli 1970). Gels were run at 15 mAmp for 90 min. Gels were silver stained following the instruction of Bio-Rad Silver Stain Kit (Bio-Rad, Hercules, CA). Total protein electrophoresis followed the procedure described by Arias et al. (1997b). Coomassie staining was used to visualize the protein bands following standard methods (Sambrook and Russell 2001).

Western blotting — Protein and LPS samples were analyzed in duplicate. First, samples were resolved on 12% SDS-PAGE gel, and then transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 100 volts for 1 h. After blotting, one membrane was incubated with anti-ALG-00-530 serum while the second membrane was incubated with anti-FC-RR serum. After blocking for 30 min, 1:500 diluted polyclonal catfish serum was incubated with the membrane overnight followed by a one-hour incubation with a monoclonal antibody (E-8) specific for channel catfish IgM (1:10) (Klesius 1990) , and labeled with conjugated goat anti-mouse Ig (1:5,000), followed by detection with Opti-4CN^{T}M (Bio-Rad).

Results

AFLP analysis

Figure IV.1 shows results of the cluster analysis of the AFLP patterns from the strains used in the study. Attenuated mutant FC-RR displayed a unique AFLP fingerprint although clustered among other genogroup III strains. AFLP profile of strain ALG-03-063 presented the highest percent of similarity with the one displayed by the attenuated

mutant. Based on this result, ALG-03-063 was selected for further comparisons as the most genetically similar strain to FC-RR present in our collection (that currently includes more than 50 *F. columnare* isolates; data not shown).

ELISA analysis

All fish immunized with *F. columnare* yielded positive titers. Control fish presented a very low cross-reactivity with the sonicated cells. All immunized fish produced strong immunity against both antigens. Although antisera from immunized fish reacted with both ALG-00-530 and FC-RR antigens, the corresponding antigen provided the highest titer (data not shown). Individual fish sera showing the highest titer (≥1/800) were chosen for immunoblotting analysis (fish no. 3 anti-ALG-00-530 and fish no.1 anti-FC-RR respectively).

LPS characterization

The results of SDS-PAGE of LPS from *F. columnare* are shown in Figure IV.2. The characteristic ladder-like pattern typical of Gram-negative pathogens was not observed. Only a few bands ranging from 3.5 kDa to 17 kDa were present in all four strains. Phenol phase extracts contained higher amounts of LPS than the aqueous-extracted samples. Strain ALG-00-530 showed a few bands in the phenol phase extract while only one weak band was visualized in the aqueous phase. A similar pattern was observed with strain ARS-1 although band intensities were stronger. Attenuated mutant FC-RR shared a similar LPS pattern with ALG-03-063 although FC-RR bands were slightly smaller.

Bands from *F. columnare* LPS were visualized much more effectively in immunoblots. When catfish polyclonal antiserum was used for immunoblotting,

important differences between the four strains were revealed (Figure III.3). While all strains isolated from diseased channel catfish (ALG-00-530, ARS-1, and ALG-03-063) presented high molecular bands (above 21 kDa) in both phenol and aqueous phases, the FC-RR mutant exhibited bands below that range. ALG-00-530 and ARS-1 again showed a similar LPS pattern regardless of antisera or extraction phase used. ALG-03-063 presented the same size bands in the phenol phase as FC-RR but also exhibited higher molecular weight bands that were absent in the mutant. Interestingly, these results were consistent regardless of antisera used (anti-ALG-00-530 or anti-FC-RR). However, significant differences between aqueous and phenol phase samples were found in both immunoblots.

Total protein analysis

Western blots of total proteins were also analyzed with both sera (Figure IV.4). The immunoblots indicated that the four strains did contain different antigenic proteins but also shared some common bands. ARS-1 showed more distinct antigenic protein bands than any other strain analyzed (Figure IV.4). The strains ALG-00-530, ARS-1 and ALG-03-063 contained two very distinct bands around 35 kDa. FC-RR mutant lacked these bands but instead presented two additional bands in the 30 kDa range. Each strain had very similar banding pattern with anti-ALG-00-530 and anti-FC-RR sera. When ALG-00-530 serum was used, reactivity against low molecular weight bands present in the three clinical strains was observed. FC-RR low molecular bands did not react with anti-ALG-00-530 serum. On the contrary, when anti-FC-RR was used, low molecular weight proteins in FC-RR were revealed.

Discussion

Lipopolysaccharide is a major superstructure of Gram-negative bacteria, contributes greatly to the structural integrity of the bacteria, and protects them from host immune defenses. The LPS has been described and well characterized as a virulence factor in many bacterial species (*Salmonella* sp. (Makela et al. 1973), *Escherichia coli* (Medearis et al. 1968), *Shigella flexneri* (Rajakumar et al. 1994), *Brucella abortus* (Vemulapalli et al. 2000) including fish pathogens such as *Vibrio vulnifucus* (Amaro et al. 1997), *E. ictaluri* (Arias et al. 2003), and *F. psychrophilum* (MacLean et al. 2001). Attempts to characterize the structure of the LPS in *F. columnare* were made by MacLean et al. (2003) and by Vinogradov et al. (2003). Unfortunately, the strain used by these authors (ATCC 43622) had been originally misidentified as *F. columnare* but was actually *F. johnsoniae* (Darwish et al. 2004; Shoemaker et al. 2005a). Therefore, no information on *F. columnare* LPS composition or immunogenic role was available.

In this study, catfish polyclonal antisera against two strains, ALG-00-530 and FC-RR (attenuated mutant), were generated and used to determine LPS immunogenic properties between genetically different *F. columnare* strains. Differences in LPS between four strains of *F. columnare* were evident after electrophoresis and silver staining but even more obvious after western blot analysis. Although a typical LPS ladder has been reported in other *Flavobacterium* species (MacLean et al. 2001), our silver staining of *F. columnare* LPS analysis revealed only a few bands under 7 kDa.

Immunogenic bands present in the LPS were revealed by immunoblots. However, there was a major difference in band molecular weight between strains isolated from diseased channel catfish and the attenuated strain FC-RR. The three strains from diseased

fish (ALG-00-530, ARS-1, and ALG-03-063) presented LPS bands between 21 and 29 kDa while FC-RR exhibited a unique band under 21 kDa. Our results suggest that the induction of rifampicin-resistant mutation(s) in *F. columnare* results in loss of the high molecular weight bands displayed by virulent isolates. Similar results were reported in *B. abortus* and *E. ictaluri* (Arias et al. 2003) when LPS from rifampicin-resistant mutants was analyzed. Rifampicin is a potent and broad-spectrum antibiotic well known as DNA-directed RNA polymerase inhibitor. Mutations in the RNA polymerase gene that conferred resistance against rifampicin have been widely documented (Jin and Gross 1988). On the other hand, the mechanism by which rifampicin induces mutations that affect LPS structure are relatively unknown. It is remarkable how such different pathogens (*Brucella*, *Edwardsiella* and *Flavobacterium*) behaved similarly under the same stressor suggesting that short O-chain LPS will favor cell survival. It has been postulated that rifampicin, like other hydrophobic molecules, enters the cell via simple diffusion through the outer membrane with LPS being the main hurdle (Kirschbaum and Gotte 1993). A structural change in the LPS might enhance the barrier effect protecting the cell against rifampicin. Vemulapalli et al. (1999) suggested that multiple genes involved in LPS biosynthesis are disrupted in *B. abortus* and this results in the attenuated phenotype.

Our results indicate that the LPS in *F. columnare* may play an important role in columnaris pathogenesis because the lack of LPS high molecular weight bands seems to be correlated with total lack of virulence in other bacterial species (Kimura and Hansen 1986; Rajakumar et al. 1994; Amaro et al. 1997; Arias et al. 2003). Although a thorough antigenic characterization in *F. columnare* has not yet been attempted, some serological

diversity has been reported between isolates (Anacker and Ordal 1959; Sanders et al. 1976). This antigenic variation can be justified by the genetic variability observed in this species (Arias et al. 2003; Thomas-Jinu and Goodwin 2004) . In fact, we observed some serological differences in LPS within the virulent strains. While ALG-00-530 and ARS-1 both showed a very similar pattern denoting common antigens, ALG-03-063 presented at least one additional LPS band regardless of extraction phase and antisera used. Interestingly, these three strains belong to different genogroups and present very distinct fingerprinting profiles; however, ALG-00-530 and ARS-1 resulted very similar pattern by LPS immunoblots. These two strains have been used by our group in virulence studies and although both are able to cause columnaris disease in catfish by immersion, ALG-00-530 has consistently exhibited a higher mortality rate than ARS-1 (unpublished information).

When we compared the antigenic variability exhibited by the total protein profiles, all four strains showed a different profile. Differences in protein composition between isolates were expected as they presented differences in their genome. However, there was a large set of common bands shared by all strains and revealed by both antisera. This indicates that the FC-RR mutant presents a number of common epitopes with other *F. columnare* isolates and could explain why the FC-RR strain used as a modified live vaccine is able to confer protection against different *F. columnare* isolates.

In conclusion, we showed that the *F. columnare* FC-RR strain lacks the high molecular weight components of LPS present in other virulent strains. Differences in LPS and immunogenic proteins were observed between genetically different *F. columnare* strains. A secondary outcome of this study was the genetic fingerprinting of FC-RR. The

attenuated mutant presents a unique AFLP profile, not present in our existing database, which can be used to track the modified live vaccine strain (FC-RR) in the fish farm environment.

Figure IV.1. Dendrogram based on AFLP patterns of four strains of *F. columnare* (ALG-00-530, FC-RR, ARS-1, and ALG-03-063). The tree was derived by UPGMA cluster analysis of the AFLP profiles.

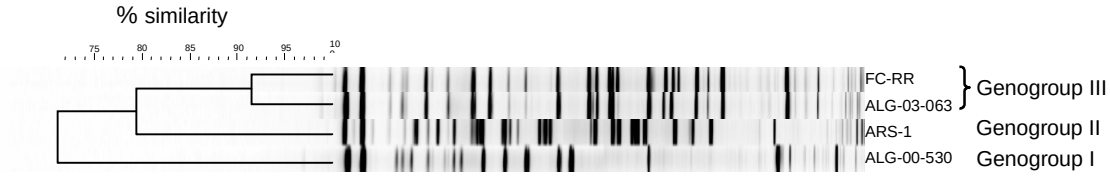


Figure IV.2. Silver stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of the LPS preparations from *F. columnare* strains used in this study. Lane 1,molecular standard; lanes 2 through 5, LPS aqueous phase from ALG-00-530 (2), FC-RR (3), ARS-1 aqueous (4), and ALG-03-063 (5); lanes 6 through 9 show LPS phenol phase from ALG-00-530 (6), FC-RR (7), ARS-1 (8) and ALG-03-063 (9).

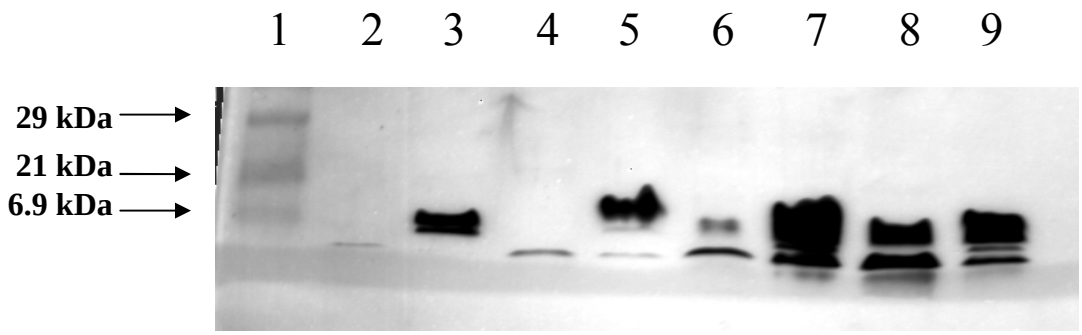


Figure IV.3. Immunoblot using anti-ALG-00-530 serum (A) and anti-FC-RR serum (B) with LPS preparation. Lane 1, molecular standard; lanes 2 through 5, LPS aqueous phase from ALG-00-530 (2), FC-RR (3), ARS-1 aqueous (4), and ALG-03-063 (5); lanes 6 through 9 show LPS phenol phase from ALG-00-530 (6), FC-RR (7), ARS-1 (8) and ALG-03-063 (9).

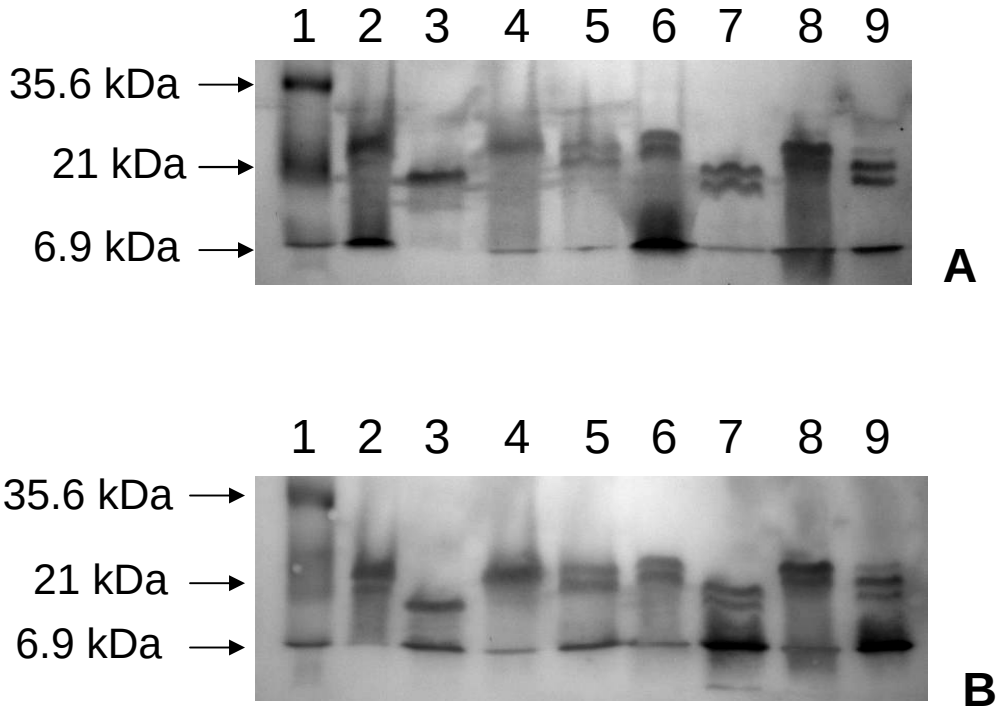
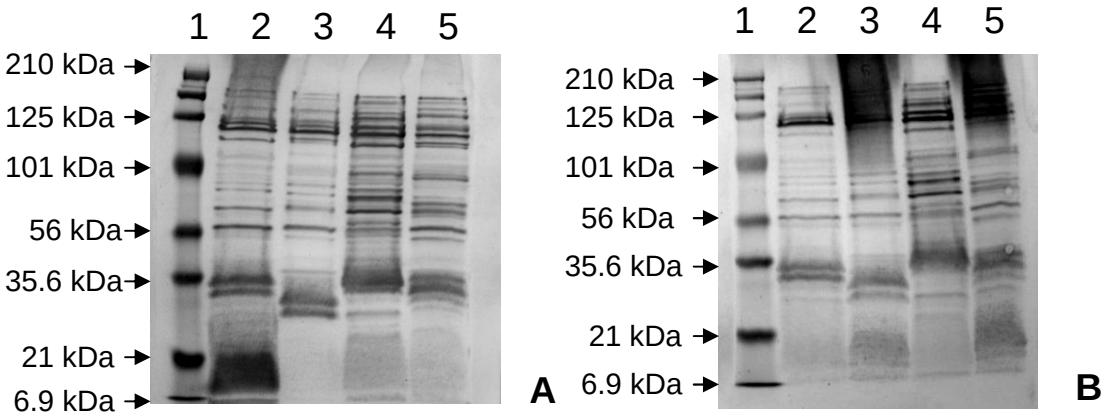


Figure IV.4. Western blot analysis of total protein with anti-ALG-00-530 serum (A) and anti-FC-RR serum (B). Lane 1, molecular standard; lane 2, ALG-00-530; lane 3, FC-RR; lane 4, ARS-1; lane 5, ALG-03-063.



V. IDENTIFICATION, CHARACTERIZATION AND EXPRESSION PATTERNS OF *GTF*, *HEMH*, *NORB*, AND *TRX* GENES IN *FLAVOBACTERIUM COLUMNARE*

Abstract

Four genes from a shotgun genomic library of *F. columnare* virulent strain ALG-00-530 were identified, characterized, and selected for differential expression analysis based on sequence similarity to putative virulence genes from related species. These genes were: glycosyltransferase (*gtf)*, ferrochelatase (*hemH*), nitric oxide reductase (*norB*) and thioredoxin (*trx*). A collection of 30 *F. columnare* strains, including strains from genomovars I and II, were tested for the presence of these genes. Distribution patterns of *gtf*, *hemH*, *norB*, and *trx* across the species were not uniform. Nucleotide sequence variation was observed between genomovars for each gene; however, strains within the same genomovar shared identical gene sequences.

Nine strains of *F. columnare* (from both genomovars I and II) were chosen for gene expression analysis. The expression profile of these genes varied when selected strains were grown *in vitro* under identical conditions. ALG-00-530 was chosen for gene expression comparison under standard growing conditions, iron-limited conditions and in the presence of skin explants from channel catfish (*Ictalurus punctatus*). *gtf*, *hemH*, *norB*, and *trx* gene expression levels varied when ALG-00-530 was incubated under different conditions.

Keywords: *Flavobacterium columnare*, *gtf*, *hemH*, *norB*, and *trx* gene expression, iron-limited, catfish skin explants, pathogenicity

Introduction

Flavobacterium columnare, the etiologic agent of columnaris disease, is a Gram-negative bacterium that can infect most freshwater fish species. Differences in virulence are known to exist among *F. columnare* isolates, resulting in variable mortality of fish (Pacha and Ordal 1970; Suomalainen et al. 2006). Understanding the pathogenesis of *F. columnare* is critical for fish health, but our knowledge of *F. columnare* virulence factors is far from being complete.

Only a few genes from *F. columnare* have been described to date, and most known sequences from this pathogen correspond to ribosomal genes. Among the non-ribosomal sequences identified, the chondroitin AC lyase gene in *F. columnare* has been previously sequenced and characterized (Xie et al. 2005). The enzyme codified by this gene is able to degrade acidic polysaccharides, such as hyaluronic acid and chondroitin sulfates (Griffin 1991; Teska 1993; Stringer-Roth et al. 2002). Differences in chondroitin AC lyase activity have been observed between *F. columnare* isolates (Stringer-Roth et al. 2002). Relation of chondroitin AC lyase activity to virulence in *F. columnare* has been recently reported (Suomalainen et al. 2006). Genes encoding outer membrane proteins, such as zinc metalloprotease and prolyl oligopeptidase, have also been characterized in a virulent *F. columnare* isolate (Xie et al. 2004). However, association of these two genes with *F. columnare* pathogenicity is unclear. In addition, several proteases have been identified in *F. columnare*, but their specific roles in columnaris pathogenicity are still unknown (Bertolini and Rohovec 1992; Newton et al. 1997).

The ability of *F. columnare* to attach to fish tissues is thought to be a factor correlated to virulence (Decostere et al. 1999b). Bader et al. (2005) selected for an

adhesive-defective *F. columnare* strain that exhibited reduced virulence to channel catfish. Unfortunately, no adhesin gene has been identified in *F. columnare*.

Genetic variability of *F. columnare* species has been characterized using different molecular markers (Triyanto and Wakabayashi 1999; Thomas-Jinu and Goodwin 2004; Arias et al. 2004; Darwish and Ismaiel 2005). Restriction fragment length polymorphism analysis of the 16S rRNA gene divided *F. columnare* species into three genomovars (Triyanto and Wakabayashi 1999). The coexistence of three main genomic groups within the species (genomovars I, II, and III) has been corroborated by intergenic spacer region (ISR) sequencing (Darwish and Ismaiel 2005), and single-strand conformation polymorphism (SSCP) analysis (Olivares-Fuster et al. 2007). To date, most of the genetic markers used for *F. columnare* strain typing have relied on ribosomal gene analysis.

The aim of this work was to identify and characterize putative virulence genes in this species and to further investigate the genetic diversity of *F. columnare* using non-ribosomal markers. To achieve this objective, a partially sequenced shotgun genomic library constructed from a virulent *F. columnare* strain was used. Four putative virulence genes were selected for further analysis. Gene presence and expression patterns were analyzed across the species. Two *in vitro* experiments aimed to mimic some of the environmental conditions *F. columnare* encounters during infection (limited iron and fish skin) were used for gene expression analysis.

Materials and Methods

Bacterial strains and culture conditions

Thirty strains of *F. columnare*, representing genomovars I and II, were used in this study (Table V.1). Cells were cultured in modified Shieh broth (Shoemaker et al. 2005a) at 28 ºC.

Putative virulence gene identification

A shotgun genomic library of the *F. columnare* virulent strain ALG-00-530 was constructed. Briefly, *F. columnare* DNA was extracted and purified following standard protocols (Sambrook and Russell 2001). Total DNA was partially digested with *Sau*3A I. Digested DNA ranging from 1 to 1.25 kb was purified by double size selection and ligated to the digested pBluescript II (KS-) vector (Stratagene, Cedar Creek, TX) at a molar ratio of 1:2. The cloning site was *Bam*H I. Library titer was estimated at 35 recombinant clones per microliter. Quality control tests showed 1% or less empty clones and an expected genomic coverage of 20X based on approximate genome size of 4.1 mb. Three thousand clones of this library have been sequenced to date at the USDA-ARS MSA Genomics Laboratory (Stoneville, MS) following standard procedures.

Sequences from the shotgun genomic library were compared with sequences in the GenBank database available at NCBI (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/) using the BLASTx algorithm (Altschul et al. 1990). Sequences with more than 35% identity were recorded. Among the identified genes, four were chosen for further analysis due to their high identity (≥65%) with known genes described as virulence factors in other bacteria. Open reading frames (ORF) of the four sequences were identified using Vector NTI® Suite 8 software package (Invitrogen,

Carlsbad, CA). ORF were translated into amino acid sequences by using Vector NTI® Suite 8 and the GenBank database was searched for protein sequences using the BLASTp tool (Altschul et al. 1990). Protein-protein identity percentage was recorded for each chosen ORF. Specific primers for each gene were designed using Vector NTI® Suite 8. Primer sequences for each gene were shown in Table V.2. These primers were tested on 30 strains of *F. columnare* by PCR. Unless otherwise stated, all PCR reagents were purchased from Promega (Madison, WI). Each 50 µL PCR reaction included 2.5 µM $MgCl_2$, 1X buffer, 0.2 µM of both primers, 0.2 µM of dNTPs, 1.7 unit of Taq polymerase, and 60 ng of DNA template. The PCR amplification profile was 5 min hot start at 95 ºC; 35 cycles of 30 s at 94 ºC, 45 s at 58 ºC, and 1.5 min at 72 ºC; and 10 min at 72 ºC.

DNA, RNA extraction and Reverse transcription PCR (RT-PCR)

Total bacterial DNA was extracted using the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was extracted from 1.5 mL of bacterial culture using RNeasy plus Mini Kit (Qiagen). Turbo DNA-free kit (Ambion, Austin, TX) was used to eliminate DNA contamination in RNA samples. cDNA synthesis and RT-PCR were performed using the Reverse Transcription System (Promega). One microgram of total RNA was used to synthesize the cDNA in 20 µL of reaction. cDNA synthesis reaction was diluted to 100 µL, and 10 µL were used as template for PCR using gene specific primers, as described above.

Cloning and sequencing analysis

PCR products were resolved through standard agarose gel electrophoresis. Amplified products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostic Corporation, Indianapolis, IN) and cloned into pGEMTEasy

(Promega). To ensure sequence accuracy, two clones from each strain were sequenced at the Auburn University Sequencing Core (Auburn, AL). Nucleotide sequence alignments and deduced amino acid sequence alignments were performed by CLUSTAL W multiple sequence alignment algorithm (Chenna et al. 2003).

Gene expression

Expression of *gtf*, *hemH*, *norB*, *trx* was tested under standard conditions, iron-limited conditions, and in the presence of catfish skin explants. Following RNA extraction and cDNA synthesis, genes were amplified from cDNA by the designed primers as mentioned above. A 16S rRNA cDNA internal control was amplified with universal primers UFUL (5'-GCCTAACACATGCAAGTCGA-3') and URUL (5'-CGTATTACCGCGGCTGCTGG-3') (Chen et al. 2004). Positive (genomic DNA) and negative (total RNA) controls were included for each gene amplification. Amplified products were electrophoresed at 100 V for 30 min and visualized under UV light with ethidium bromide added to a 1% gel to a final concentration of 0.5 μg/mL.

Gene expression under standard conditions — Nine strains of *F. columnare*, representing genomovars I and II, were cultured in modified Shieh broth at 28 ºC for 20 h. Bacterial cells were harvested from one and a half milliliters broth culture followed by RNA extraction and RT-PCR.

ALG-00-530 gene expression under iron-limited conditions — Two iron chelators, 2, 2-dipyridyl and transferrin, were used to remove free iron from the growth medium. 2, 2- dipyridyl was added to 50 mL of Shieh broth to a final concentration of 50 μM. Preparation of the transferrin solution followed the protocol described by Biosca et al. (1993). Briefly, human apo-transferrin (Sigma, St. Louis, MO) was dissolved at a

concentration of 1 μM in a solution containing 100 μM Tris, 150 μM NaCl, and 50 μM $NaHCO_3$ (pH 8.0). This solution was sterilized by filtration. Prior to inoculation with *F. columnare*, transferrin was added to 50 mL Shieh medium to achieve a final concentration of 10 μM and incubated at 37ºC for 60 min.

Cultured ALG-00-530 bacterial cells were collected by centrifugation at 2,000 *g* for 20 min. Pellets were washed once in PBS and centrifuged again. Cells were resuspended in approximately 1 mL Shieh broth and then 1/3 mL (of this broth) was transferred into 50 mL Shieh broth containing 2, 2-dipyridyl, or apo-transferrin. Fifty milliliters of Shieh broth was inoculated as control. One and a half milliliters of bacterial cells were collected at time 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h after inoculation. Cells were centrifuged and pellets were resuspended in 100 μL of RNA later (Qiagen) and frozen at -80 ºC until used. Reverse transcription PCR was performed as explained above.

ALG-00-530 gene expression in the presence of catfish skin explants — Skin explants from channel catfish that tested negative for *F. columnare* antibody presence were obtained according to the procedure by Xu and Klesius (2002). One milliliter of Medium 199 (Sigma) supplemented with fetal bovine serum at 2.5% was added into each well of a 24-well culture plate (Costar, Cambridge, MA). One cm^2 of fish skin explant was placed onto the bottom of 12 wells. One and a half milliliters of an overnight culture of ALG-00-530 bacterial cells was collected for the baseline control (C_0); one and half milliliters of the same bacterial culture was added into each of the 24 wells. Wells without fish skin explants were regarded as controls, while wells containing the fish explants were referred to as treatments. Bacterial cells were collected from each

individual well at 30 min, 1 h, 3 h, 6 h, 10 h, and 24 h from both control and treatment wells. After centrifugation, bacterial pellets were suspended in RNA later (Ambion, Austin, TX) and frozen at -80 ºC until used. Total RNA extraction and RT-PCR were performed as described above.

Results

Putative virulence gene identification

Out of 3,000 clones sequenced from the ALG-00-530 shotgun library, 73 sequences displayed a high nucleotide similarity with known genes present in GenBank (based on BLASTx search, data not shown). Within this subset, four sequences were identified from published literature as putative virulence genes and therefore selected for this study: glycosyltransferase gene (*gtf*) (GenBank accession number DQ911521), ferrochelatase gene (*hemH*) (GenBank accession number DQ911518), nitric oxide reductase gene (*norB*) (GenBank accession number DQ911522), and thioredoxin gene (*trx*) (GenBank accession number DQ911523). ORFs were translated into amino acid sequences which were then compared to GenBank sequences using BLASTp. Identity percentages of ORF based on protein-protein alignment were as follows: Gtf was 81% identical to *F. johnsoniae* sequence accession number ZP_01245614, HemH was 63% identical to *F. johnsoniae* sequence accession number ZP_01246582, NorB was 77% identical to an unidentified *Flavobacterium* species sequence accession number ZP_01106280, while Trx was 83% identical to *F. johnsoniae* sequence accession number ZP_01243853. Based on the PCR results, all strains contained *norB* and *trx* genes (Table V.1). However, no PCR amplification for *hemH* was observed in eight strains (GA-02-14,

ARS-1, BM, HS, GZ, BZ-1, BZ-4, BZ-5), while three strains (ARS-1, BM, HS) failed to amplify the *gtf* gene.

Cloning and sequencing analysis

Two *F. columnare* strains from genomovar I (ATCC 49512 and ALG-03-57) and genomovar II (ALG-00-530 and MS-02-475) were selected for multi locus sequence analysis using *gtf*, *hemH*, *norB*, and *trx* genes. Nearly complete sequences were amplified from *gtf*, *norB*, and *trx* and used for comparison. Only partial sequence (250 bp out of 626) of *hemH* was obtained. Alignment of these sequences showed two distinct populations. Both genomovar I strains shared an identical sequence for all three genes as did ALG-00-530 and MS-02-475 genomovar II strains (data not shown). However, nucleotide sequences differed between genomovars (Figure V.1). The variation percentage observed was 6.9% for *gtf*, 4.9% for *hemH*, 3.8% for *norB*, and 4.3% for *trx*. Translation of the nucleotide sequences showed the distinction between genomovars was carried out at the protein level. Amino acid sequence variation derived from each gene was not identical between genomovars except for Trx. Gft displayed a 2.2% variation between both genomovars. HemH protein sequence was 5.1% variable, while NorB presented only 0.88% sequence variation.

Gene expression

Gene expression under standard conditions — The 16S rRNA gene, used as internal control, was expressed at any given time in all experiments. Gene expression patterns of nine strains of *F. columnare* varied at 20 h post inoculation under standard growing conditions and are summarized in Table V.3. Gene expression differed not only between genomovars but also within each genomovar. Genomovar I displayed three

expression patterns. Strains GA-02-14 and HS shared an identical expression profile, showing no expression of both *norB* and *trx* gene. Strains BM and ARS-1 also shared a similar expression profile with no expression of *norB* but positive expression of *trx*; however, the expression of *trx* gene in BM was stronger than in ARS-1. FC-RR (an avirulent mutant) displayed a distinct pattern with expression of *norB* but no expression of the other three genes. Genomovar II strains showed differences in expression patterns, as well. Stronger expression of *norB* and *trx* was observed in ALG-00-530 compared to other strains. LSU strain showed weak expression of *trx* gene, while BZ-1-02 and ALG-00-527 failed to express any of the tested genes.

ALG-00-530 gene expression under iron-limited conditions and in the presence of skin explants — Iron-limited conditions did not interfere with *F. columnare* housekeeping gene expression (Table V.4). The 16S rRNA gene was uniformly expressed throughout the experiment. By contrast, *gtf* did not express at all in the experiment. *hemH* expression was only detected when cells were transferred to standard growing conditions for the first 2 h of incubation and after 2 h of incubation in the presence of 2, 2-dipyridyl (Table V.4, Figure V.2). Overall, transferrin seemed to have little effect on *norB* and *trx* gene expression when compared to standard conditions, although *trx* transcript levels seemed lowered when transferrin was incorporated into the medium. 2, 2-dipyridyl had an immediate repression effect on *norB* and *trx* expression, while the 16S rRNA gene was not affected by this chemical. However, transcript levels for *norB* and *trx* went back to high levels 2 h after transfer to iron-limited conditions, remaining stable until 8 h and 24 h for *norB* and *trx*, respectively.

When *F. columnare* cells were incubated in cell culture medium with or without skin explants, a distinct expression pattern was observed. Little expression of *trx* was detected in both controls and skin explants samples. The *norB* transcript was detected in both samples at different sampling times, although expression levels never reached those observed under standard growing conditions. However, expression levels of the 16S rRNA gene were comparable to those observed under standard conditions. Prior exposure to the culture medium, *F. columnare* cells cultivated overnight displayed the same pattern of expression previously observed under normal growing conditions.

Discussion

F. columnare has been reported to be a morphological (Song et al. 1988; Thomas-Jinu and Goodwin 2004), serological (Anacker and Ordal 1959), and genomically (Toyama et al. 1996; Triyanto and Wakabayashi 1999; Thomas-Jinu and Goodwin 2004; Arias et al. 2004; Darwish and Ismaiel 2005; Schneck and Caslake 2006) diverse species. According to Bernardet and Grimont (1989), only 78% similarity was found among *F. columnare* strains using DNA-DNA hybridization. Triyanto and Wakabayashi (1999) described three different genomovars among the species based on the analysis of the 16S rDNA-RFLP. Genomovar ascription has become routine for *F. columnare* strain characterization (Triyanto and Wakabayashi 1999; Arias et al. 2004; Olivares-Fuster et al. 2007). However, beyond ribosomal variability, no study has been conducted to investigate the intra-species variation of *F. columnare* at the single gene level, mainly due to the lack of genetic information available for this species.

In the current study, the glycosyltransferase gene (*gtf*), ferrochelatase gene (*hemH*), nitric oxide reductase gene (*norB*), and thioredoxin gene (*trx*) have, for the first

time, been identified and characterized in *F. columnare*. These genes presented a high similarity to homologous genes within the *Flavobacterium* genus, showing the highest similarity with *F. johnsoniae* gene sequences. Although strains within the same genomovar showed identical gene sequences, there was a 4 to 7% nucleotide sequence variation observed between genomovars for each gene. *Flavobacterium columnare* strains were clearly divided into two groups, and such division was even expressed at the amino acid level. Lee et al. (1998) suggested that single amino acid substitutions could change the biological activity of proteins. We found some non-conserved amino acid substitutions between the genomovar I ALG-00-530 and the genomovar II ATCC 49512 in Gtf, HemH, and NorB protein sequences. Therefore, the activity of these proteins could differ between *F. columnare* genomovars.

Our data agreed with the genomovar segregation previously reported for *F. columnare*. Genomovars could be identified by sequencing either *norB* or *trx* genes. *gtf* and *hemH* cannot be considered good markers, because they were not present in all *F. columnare* strains. Some examples have been published of such correlation between ribosomal gene-based variability and non-ribosomal gene-based variability i.e. *F. psychrophilum* (Soule et al. 2005). Non-ribosomal variation divided *F. psychrophilum* into two lineages that are associated with different host species. This is not the case for *F. columnare*, since channel catfish is susceptible to both *F. columnare* genomovars. However, preliminary studies showed genomovar II strains were more virulent to channel catfish than genomovar I strains (Craig A. Shoemaker, USDA-ARS, Auburn, AL, personal communication).

gtf, *hemH*, *norB*, and *trx* genes characterized in the present study have been described as virulence factors in other bacterial species (Almiron et al. 2001; Narimatsu et al. 2004; Bjur et al. 2006; Loisel-Meyer et al. 2006). Our expression data showed that *hemH*, *norB,* and *trx* genes are weakly or not expressed when cells reached the end of the log phase approximately 24 h growth; however, they were strongly expressed during the first few hours following inoculation into fresh Shieh broth culture medium. Gene expression was weak when cells were transferred to cell culture medium, regardless of the presence of catfish skin explants. This may be the result of the relative high salinity in this medium. During the catfish skin explants experiment, bacterial cells were cultivated in cell culture medium 199 diluted 1:1 with bacterial broth (final salinity was about 0.45%). This salinity was required to keep the skin cells alive. It has been reported that increased salinity significantly reduced growth and adhesion ability of *F. columnare* (Altinok and Grizzle 2001). These authors reported that the adhesion of *F. columnare* was reduced as salinity increased from 0.1% to 0.3%, and from 0.3% to 0.9%. The general trend observed was a reduction in expression of the four genes analyzed. This might be due to the elevated salinity levels; however, expression levels of the 16S rRNA gene seemed to be unaffected.

Both *norB* and *trx* gene expression in *F. columnare* was not inhibited under iron-limited conditions. Holmes et al. (2005) reported that iron-limited conditions actually increased the transcript level of thioredoxin in *Campylobacter jejuni*. Iron-limited conditions also lead to oxidative stress in *Anabaena* sp.(Latifi et al. 2005). Additionally, both *norB* and *trx* can defend against oxidative and/or NO-mediated stress (Wieles et al. 1997; Jaegera et al. 2004; Philippot 2005; McGee et al. 2006). As a result, it is possible

that *F. columnare* continues to express both *norB* and *trx* in order to reduce oxidative stress due to iron deficiency or limitation.

In conclusion, our data confirmed the genomic diversity of *F. columnare* at the single gene level. Nucleotide sequences of *gtf*, *hemH*, *norB,* and *trx* of *F. columnare* differed between genomovars I and II. Multiple gene expression patterns existed both between and within genomovars. Although the relation between gene expression pattern and virulence is unclear, this study is the first to address *in vitro* gene expression in *F. columnare*. Further studies are ongoing in order to confirm the difference in virulence between genomovars I and II. Nevertheless, and due to the clear genetic division between genomovars, we strongly recommend the inclusion of more than one genomovar in future *F. columnare* studies.

Table V.1. Strains of *F. columnare* used in the study showing genomovar (G) ascription and standard PCR amplification results of *gtf* (glycosyltransferase gene), *hemH* (ferrochelatase gene), *norB* (nitric oxide reductase gene), and *trx* (thioredoxin gene). '+' represents positive PCR amplification of the gene, '-' represents no amplification when DNA was used as template.

Isolates	Fish species	Geographic origin	G	*gtf*	*hemH*	*norB*	*trx*
MS 467	Channel catfish	Mississippi, USA	I	+	+	+	+
MS-02-465	Channel catfish	Mississippi, USA	I	+	+	+	+
27	Channel catfish	Alabama, USA	I	+	+	+	+
IR	Common carp	Israel	I	+	+	+	+
MS 463	Channel catfish	Mississippi, USA	I	+	+	+	+
FC-RR	Channel catfish	Alabama, USA	I	+	+	+	+
ALG-03-063	Channel catfish	Alabama, USA	I	+	+	+	+
ALG-03-57	Channel catfish	Alabama, USA	I	+	+	+	+
ATCC 23463	Chinook salmon	Washington, USA	I	+	+	+	+
ATCC 49512	Brown trout	France	I	+	+	+	+
ALG-03-069	Channel catfish	Alabama, USA	I	+	+	+	+
GA-02-14	Rainbow trout	Georgia, USA	I	-	-	+	+
GZ	Channel catfish	Alabama, USA	I	+	-	+	+
ARS-1	Channel catfish	Alabama, USA	I	-	-	+	+
BM	Channel catfish	Alabama, USA	I	-	-	+	+
HS	Channel catfish	Alabama, USA	I	-	-	+	+
MO-02-23	Largemouth bass	Missouri, USA	II	+	+	+	+
ALG-00-530	Channel catfish	Alabama, USA	II	+	+	+	+
LSU	Channel catfish	Louisiana, USA	II	+	+	+	+
ALG-00-513	Channel catfish	Alabama, USA	II	+	+	+	+
PT-14-151	Channel catfish	Mississippi, USA	II	+	+	+	+
ALG-02-36	Largemouth bass	Alabama, USA	II	+	+	+	+
ALG-00-515	Channel catfish	Alabama, USA	II	+	+	+	+
ALG-00-527	Channel catfish	Alabama, USA	II	+	+	+	+
MS 475	Channel catfish	Mississippi, USA	II	+	+	+	+
ALG-00-522	Channel catfish	Alabama, USA	II	+	+	+	+
ALG-00-521	Channel catfish	Alabama, USA	II	+	+	+	+
BZ-1-02	Nile tilapia	Brazil	II	+	-	+	+
BZ-4-02	Nile tilapia	Brazil	II	+	-	+	+
BZ-5-02	Nile tilapia	Brazil	II	+	-	+	+

Table V.2. Primers for gene detection and gene expression of *F. columnare*.

Genes	Primers
gtf	Glytrans-F: 5'-CCAACATTTGGAGGTAGCGG-3'
	Glytrans-R: 5'-CCACCATCATCAGTTTAGCAGGT-3'
hemH	Ferrochelatase-F: 5'-AAGGAATCATATTGAATACGCGACC-3'
	Ferrochelatase-R: 5'-GCCAAAGCTTCCTTTATTGATGTTC-3'
norB	Nitric-F: 5'-TGCACGGACATCTAGCATTTTG-3'
	Nitric-R: 5'-CCTCATCGTTCGTTTTACCATGTTT-3'
trx	Thioredoxin-F: 5'-TGGCATTAGCAATTACAGATGCTAC-3'
	Thioredoxin-R: 5'-CCCACTACTTCCCCATTTTGAA-3'

Table V.3. RT-PCR results from nine strains of *F. columnare*. G represents genomovar ascription. '–'represents no expression, 'w' represents weak expression, '+' represents expression, '++' represents strong expression. NA, no amplified product was obtained when genomic DNA was used as template.

G	strains	*gtf*	*hemH*	*norB*	*trx*	*16SrRNA*
I	GA-02-14	NA	NA	-	-	+++
I	ARS-1	NA	NA	-	w	+++
I	BM	NA	NA	-	+	+++
I	HS	NA	NA	-	-	+++
I	FC-RR	-	-	+	-	+++
II	BZ-1-02	-	NA	-	-	+++
II	LSU	-	-	-	w	+++
II	ALG-00-530	-	-	++	++	+++
II	ALG-00-527	-	-	-	-	+++

Table V.4. *F. columnare* ALG-00-530 gene expression under standard conditions, iron-limited conditions, and in the presence of catfish skin explants. C_0 is the baseline control for catfish skin explant experiment. '-' represents no expression, relative intensity of the expressed genes is expressed by the number of '+' symbols.

	Time (h)	*hemH*	*norB*	*trx*	16S rRNA gene
In Shieh broth	0	+	++++	++++	+++
(normal	2	++	++++	++++	+++
condition)	4	-	+++	+	+++
	6	-	+++	++	++
	8	-	+	-	+++
	24	-	+++	+++	+++
In the presence	0	-	+	+	+++
of 2,2-dipirydyl	2	+	++++	+++	+++
(iron-limited	4	-	+++	++	++
condition)	6	-	+++	+	++
	8	-	+++	+++	+++
	24	-	-	++++	+++
In the presence	0	-	++++	+	+++
of transferrin	2	-	++++	++	+++
(iron-limited	4	-	+++	++	+++
condition)	6	-	-	-	++
	8	-	+	+	+++
	24	-	+	+	+++
C_0	0	-	+	+	+++
In the presence	0.5	-	++	+	+++
of fish skin	1	-	++	+	+++
explants	3	-	+++	-	+++
	6	-	+	-	+++
	10	-	+	+	+++
	24	-	-	-	+++
Controls for the	0.5	-	-	-	++
fish skin	1	-	+++	-	+++
explants	3	-	++	-	+++
	6	-	+	+	+++
	10	-	+	+	+++
	24	-	-	+	+++

Figure V.1. Nucleotide sequence alignment of *gtf*, *norB*, and *trx* of *F. columnare* from genomovars I and II (GenBank assession numbers in brackets). 530 stands for ALG-00-530 (genomovar II) and 49512 is the abbreviation of ATCC 49512 (genomovar I). Nucleotides that are different between genomovars are indicated by shaded/unshaded box. ***** indicates putative start condon. '**.**' indicates missing nucleotides. Y=C+T, R=A+G, and W=A+T

gtf *****

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530  (DQ911521)  ATGAAAATAG CAATAGTTTG TTATCCAACA TTTGGAGGTA GCGGTGTAGT    50
49512(EF547540)  .......... .......... ....CCAACA TTTGGAGGTA GCGGTGTAGT    50

530  (DQ911521)  AGCAACAGAA TTAGGTTTAG AGTTGGCTCG CCGAGGTCAT GAAATTCATT   100
49512(EF547540)  AGCAACAGAA TTAGGTTTAG AGCTGGCTCG TCGAGGTCAT GAAATTCATT   100

530  (DQ911521)  TTATTACCTA TCGTCAACCT GTGCGTTTGG CACTTTTGAA TCATAATGTA   150
49512(EF547540)  TTATTACCTA TCGTCAACCC GTGCGTTTAG CTCTTTTGAG TCACAATGTA   150

530  (DQ911521)  CATTATCATG AAGTAAATGT GCCTGAATAT CCATTATTTC ATTACCAACC   200
49512(EF547540)  CATTATCATG AAGTAAACGT TCCTGAATAT CCATTATTTC ATTACCAACC   200

530  (DQ911521)  CTATGAATTA GCTCTTTCGA GTAAACTAGT AGATATGGTA AAACTTTATA   250
49512(EF547540)  CTATGAATTA GCTCTTTCGA GTAAATTAGT AGATATGGTA AAACTCTATA   250

530  (DQ911521)  AAATAGATAT ATTACATGTA CATTATGCTA TACCTCATGC TTATGCGGGT   300
49512(EF547540)  AAATAGATGT ATTGCATGTG CATTATGCCA TACCTCATGC TTATGCAGGT   300

530  (DQ911521)  TATATGGCCA AACAGATCTT AAAAGAAGAA GGAATTAATC TGCCAATGGT   350
49512(EF547540)  TATATGGCTA AACAAATTTT AAAAGAAGAG GGAATTAATC TACCAATGGT   350

530  (DQ911521)  TACCACATTG CACGGTACAG ATATAACCTT AGTAGGAAAC CATCCTTACT   400
49512(EF547540)  TACTACATTG CATGGTACAG ATATAACGTT AGTTGGAAAT CATCCTTATT   400

530  (DQ911521)  ATAAACCAGC TGTTACTTTT AGTATCAATA AGTCAGATGT AGTAACATCT   450
49512(EF547540)  ATAAACCAGC AGTTACTTTT AGTATCAATA AGTCAGATGT AGTAACATCT   450

530  (DQ911521)  GTCTCAAAAA GCTTAAAAGA AGATACGTTG AAATATTTTG ATGTAACAAA   500
49512(EF547540)  GTTTCAAAAA GCTTAAAAGA AGATACACTA AAATATTTTG ATGTAACTAA   500

530  (DQ911521)  AGAAATAAAA GTAGTCCCTA ATTTTATAGA AATWGAAGAA AYAGAAGCTC   550
49512(EF547540)  GGAAATAAAA GTAGTTCCTA ATTTTATAGA AATAGAAGAA ACAGAGGCTC   550

530  (DQ911521)  AGAATCTAAT TTGTAAGAGG TCTGTAATGG CTACACCAGA AGAGAAAATT   600
49512(EF547540)  ACAATCTAAT TTGTAAAAGG TCTGTAATGG CTACGCCAGA AGAGAAAATA   600

530  (DQ911521)  ATTACCCATA TTAGTAATTT TAGAAAAGTT AAAAAAATAC CTGATGTTAT   650
49512(EF547540)  ATTACCCATA TTAGTAATTT TAGAAAGGTT AAAAAGATAC CAGATGTAGT   650

530  (DQ911521)  TAAAGTTTTT TATAAAATAC AAGAAAAAAT ACCTGCTAAA CTGATGATGG   700
49512(EF547540)  TAAAGTCTTT TTTAAGATAC AAGAAAAAAT ACCTGCTAAA CTGATGATGG   700
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530  (DQ911521)  TGGGTGATGG ACCTGAAAAA GAAAAGGCAG AATGGCTTTG TAGAGAATTA   750
49512(EF547540)  TGG....... .......... .......... .......... ..........   750

530  (DQ911521)  GGTATTGAAG AAAAAGTTAT TTTTTTTGGG AACCAGTCAT GA 792
49512(EF547540)  .......... .......... .......... .......... .. 792
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norB *

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530  (EQ911522)  ATGCACGGAC ATCTAGCATT TTGGGGAGCA TACGCCATGA TTGTATTAGC   50
49512(EF547542)  .TGCACGGAC ATCTAGCATT TTGGGGAGCA TACGCCATGA TTGTATTAGC   50

530  (EQ911522)  AATTATTAGT TATGCTATAC CTAATCTTAC AGGAAGAAAA AGATATGATT  100
49512(EF547542)  AATTATTAGT TATGCAATAC CTAATCTTAC AGGAAGAAAA AGATACGATT  100

530  (EQ911522)  CAGTTACAGG ACGTATGGCA TTTYTGGTTAT CAAATATTGG TATGTTAGGT  150
49512(EF547542)  CAGTTACAGG ACGTATGGCC TTTTTGGTTAT CAAATATTGG TATGTTAGGT  150

530  (EQ911522)  ATGACCACCG CTTTTGGGGT AGCTGGAGTA GCTCAAGTAT ATTCTTGAAAG  200
49512(EF547542)  ATGACCACCG CTTTTGGGGT AGCTGGAGTC GCTCAAGTAT ACCTTGAAAG  200

530  (EQ911522)  AAAGTTTAAA ATGGAGTTCA TGACCGTTCA GAATGAAATA GCTATTCACT  250
49512(EF547542)  AAAGTTTAAA ATGGAGTTTA TGACCGTTCA AARTGAAATA GCTATTCATT  250

530  (EQ911522)  TTGTAGTATT ACTACTGTGT GCCACATTAT TTACTCTAGG AATYTCTTTA  300
49512(EF547542)  TTGTAGTACT GCTACTATGT GCCACATTAT TCACTATAGG AATTTCTTTA  300

530  (EQ911522)  TACATATACG ATTTTATTAA ACATGGTAAA ACGAACGATG AGGCCATAAT  350
49512(EF547542)  TACATATACG ATTTTATTAA ACATGGTAAA ACGAACGATG AGG.......  350

530  (EQ911522)  TGGCTAA 357
49512(EF547542)  ....... 357
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trx *

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530  (DQ911523)  ATGGCATTAG CAATTACAGA TGCTACATTT GATGAGGTAG TATTACAATC   50
49512(EF547543)  .TGGCATTAG CAATTACAGA TGCTACATTT GATGAGGTAG TATTACAATC   50

530  (DQ911523)  AGACAAACCT GTTTTAGTGG ATTTTTGGGC GGCTTGGTGT GGACCTTGTC  100
49512(EF547543)  AGACAAACCA GTTTTAGTGG ATTTTTGGGC AGCTTGGTGC GGGCCTTGTC  100

530  (DQ911523)  GTATGGTAGG TCCCGTTATT GATGAAATTG CAACAGAATA TGAAGGAAAA  150
49512(EF547543)  GTATGGTAGG TCCCGTTATT GACGAAATTG CAACGGAATA TGAAGGAAAA  150

530  (DQ911523)  GCAGTAATAG GTAAGGTAGA TGTCGATGCC AATCAAGAAT TTGCTGCAAA  200
49512(EF547543)  GCAGTAATTG GTAAGGTAGA TGTCGATGCC AATCAAGAGT TTGCTGCAAA  200

530  (DQ911523)  GTACGGTGTT CGCAATATTC CTACTGTTTT GATTTTTCAA AATGGGGAAG  250
49512(EF547543)  GTATGGCGTA CGTAATATTC CTACTGTTTT GATTTTTCAA AATGGGGAAG  250

530  (DQ911523)  TAGTGGGACG TCAGGTAGGT GTAGCTCCTA AAGATACTTA TGCTAAAGCA   300
49512(EF547543)  TAGTG..... .......... .......... .......... ..........   300

530  (DQ911523)  ATTGATGCCT TATTGTAA 318
49512(EF547543)  .......... ........ 318
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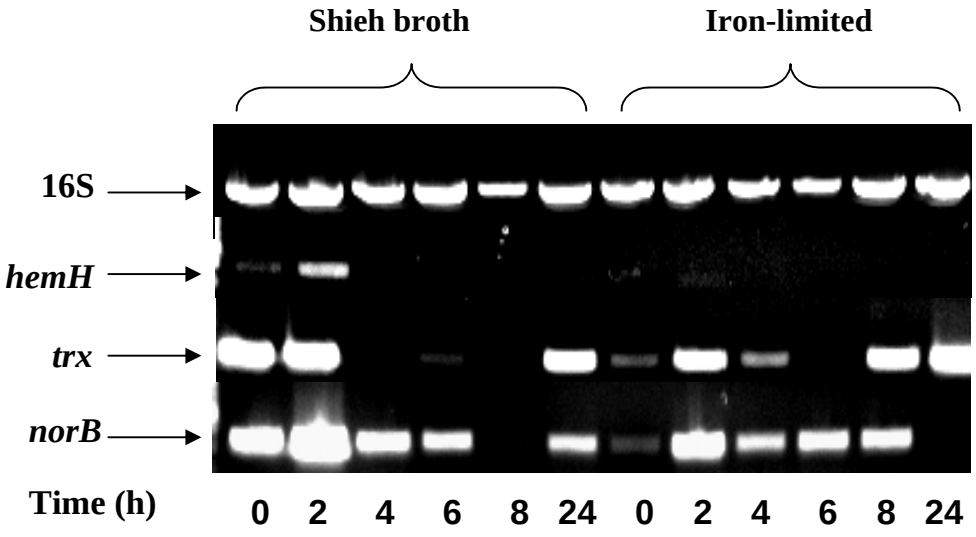
Figure V. 2. *hemH*, *norB*, and *trx* gene expression of *F. columnare* strain ALG-00-530 by reverse transcription PCR (RT-PCR). Expression patterns are shown under iron-limited conditions in the presence of 2, 2-dipyridyl and in Shieh broth at 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h. 16S rRNA gene (16S) was used as an internal control.



VI. CONCLUSIONS

This dissertation focused on molecular characterization of the two main fish pathogens, *E. ictaluri* and *F. columnare*, affecting Alabama aquaculture. The main outcome of my work is the new genetic information that has been identified, characterized and made available to the scientific community. My first objective was to increase the number of known ribosomal sequences from these two pathogens in order to identify new signature sequences for a PCR-based multiplex protocol. Although this objective could not be fulfilled due to technical and practical reasons (see Chapter II), I was able to identify a new IVS in the 23S rRNA gene of *E. ictaluri*. This is the first report (Chapter III) of this type of sequence in the genus *Edwardsiella*. In relation to *F. columnare*, an intraspecies characterization allowed me to analyze the avirulent mutant currently used as a vaccine against columnaris disease. Not only can this mutant now be tracked using fingerprinting methods, but I correlated its lack of virulence to changes in the LPS structure (Chapter IV). In addition, by analyzing sequence data from a shotgun library of *F. columnare*, I identified and characterized 4 putative virulence genes in this species. I showed there is a deep division between genomovars I and II at the genomic level. These results strongly encourage the use of both genomovars in future *F. columnare* studies.

In brief, some of the main results from my dissertation can be summarized as follows:

1) An intervening sequence (IVS) is present in the 23S rRNA gene of *E. ictaluri* but not in *E. tarda*

2) *E. ictaluri* IVS was present in the majority of the *rrl* operons

3) *E. ictaluri* IVS shared 97% nucleotide identity with *S. typhimurium* helix-45 IVS

4) *E. ictaluri* is closely related to *E. tarda* based on 23S rDNA analysis; however, both species are phylogenetically distant from the 'core' members within the *Enterobacteriaceae* family

5) The modified *F. columnare* live vaccine FC-RR lacks the higher molecular weight bands in the lipopolysaccharide (LPS)

6) *gtf*, *hemH, norB*, and *trx* gene sequences divide *F. columnare* strains into two groups that correspond with genomovar ascription

7) Expression profiles of genes *gtf*, *hemH, norB*, and *trx* varied among different *F. columnare* strains under identical culture conditions

Contribution

The work described in this dissertation opens several venues for future studies. New phenotypic and genotypic data were generated for *E. ictaluri* and *F. columnare*. Effectively utilizing the data presented in the dissertation may be relevant to *E. ictaluri* IVS evolution, LPS structure definition in *F. columnare*, iron acquisition system in *F. columnare*, expression of putative virulence factors in mutant strains of *F. columnare*, new virulence genes identification, or evidence collection for defining subspecies in *F. columnare*.

- *E. ictaluri* habors an IVS in 23S rRNA gene and such IVS exhibits 97% similarity to *Salmonella typhimurium*. As is known, *S. typhimurium* can infect many

mammalian species. Did this IVS get transferred between *Salmonella* to *E. ictaluri*? Why does *E. tarda* not harbor this IVS, although *E. tarda* and *E. ictaluri* belong to the same genus, and both can infect fish and display similar/identical sequences in ribosomal gene? The discovery of IVS raises questions about *Edwardsiella* spp. evolution.

- Lipopolysaccharide has been well characterized as a virulence factor in a variety of bacterial pathogens. In this work, LPS profiles are different between virulent and avirulent strains of *F. columnare*. Additionally, *F. columnare* does not display the typical ladder-like pattern of LPS. Further work is needed to show where the difference is located in the genome and the importance of LPS in *F. columnare* pathogenesis. A functional study of LPS will also improve our understanding of *F. columnare* pathogenicity.
- An iron acquisition system has not been identified in *F. columnare*. The results of the dissertation suggested that ferrochelatase, an important iron importing enzyme, was not involved in iron absorption in *F. columnare*. Since iron is such an important element in bacterial growth, survival, and virulence, it is logical that *F. columnare* expresses uptake systems that mediate iron acquisition from the environment and from a catfish.
- Putative virulence gene expression in the mutant strain of FC-RR could contribute to a future research project. FC-RR is a modified live vaccine against *F. columnare*. In the current study, we showed that this strain is phenotypically different from the other virulent strains; however, we did not include this strain in the putative virulence gene expression study. It may be interesting to characterize

gene expression patterns in FC-RR in response to iron-limitation and presence of fish skin.

- Whole genome sequencing of *F. columnare* is ongoing at Mississippi State University. However, the strain selected for this project is a genomovar I isolate. The dissertation discovered that *F. columnare* genomovar I and II differed in gene sequences. It seems necessary to sequence strains from both genomovars. Comparison of whole genomes between the two genomovars should provide some answers for columnaris pathogenesis and guidelines for vaccine development.
- Since a shotgun genomic library of *F. columnare* is already available, further data mining could lead to the identification of new virulence genes.
- The data presented in the dissertation showed that *gtf*, *hemH*, *norB*, and *trx* sequences divided *F. columnare* strains into two populations which correspond to genomovars. Based on my data and other information generated by our group, we see a deep division within the species at the genetic level that is also correlated to host-species-specificity and virulence properties. These findings point towards a division of *F. columnare* into two subspecies. We need more data to support this hypothesis including DNA:DNA hybridization studies.

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