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Sequence analysis of the 16s-23s intergenic spacer regions of Flavobacterium columnare

Lorelei Melissa Ford

SEQUENCE ANALYSIS OF THE 16S-23S INTERGENIC SPACER REGIONS OF *FLAVOBACTERIUM COLUMNARE*

By

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The 16S, 23S, and 5S ribosomal RNA (rRNA) genes are highly conserved sequences in bacteria and are often used for phylogenetic classification. Less conserved regions between the structural sequences are intergenic spacer regions (ITS), which can be used to differentiate strains of the same bacterial specie. This study evaluated/compared the 16S-23S ITS of 70 isolates of *Flavobacterium columnare*, an important pathogen of cultured fish. We developed two PCR assays that allow amplification, cloning and sequencing of overlapping regions of one previously identified ITS. We used PFGE to separate I-*Ceu*I restriction fragments from ATCC49512 and sequenced and analyzed the resulting ITSs. We found that the genome of this species harbors at least 6 ITSs that are very similar and contain the same tRNA encoding sequences. Thus, earlier studies that used the ITS for distinguishing between strains of *Flavobacterium columnare* may have misleading data due to comparing sequences from different RNA operons.

DEDICATION

I would like to dedicate this work to my family. My parents, Rob and Twila Ford, always encouraged me to be unique and do what makes me happy. My grandmothers, Helen S. Sheldon and Rose M. Ford, encouraged me to take care of myself. My grandfather, Robert Sedgwick Ford, Sr., once told me that women have no place in science; thankfully, I never listened. I love you all!

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CHAPTER I

INTRODUCTION AND OBJECTIVES

Columnaris disease was first described by Davis after a fish kill in the Mississippi River. The agent was named *Bacillus columnaris* because of the characteristic columnar masses of bacteria (Davis 1922). The nomenclature has since changed several times, going from *Chondrococcus columnaris*, *Cytophaga columnaris* and *Flexibacter columnaris* (Bernardet and Grimont 1989) prior to the current name *Flavobacterium columnare* (Bernardet et al. 1996). The long, thin, gram-negative, bacterium has gliding motility. The organism is ubiquitous in freshwater environments and can infect a wide range of freshwater fish species causing high mortalities under various environmental conditions (Fish and Rucker 1943; Moore et al. 1990; Wakabayashi 1991; Decostere et al. 1998; Shotts and Starliper 1999; Michel et al. 2002). This makes *F. columnare* one of the most common pathogens of ornamental, wild, and cultured fish populations and causes enormous financial losses in the cultured fish industry. Outbreaks generally occur following stress or mechanical injury to tissue. Clinical signs include, but are not limited to, yellowish brown or white lesions on the gills, skin, or fins (Plumb 1999; Altinok and Grizzle 2001). Since *F. columnare* can be found in almost every freshwater environment and infect a wide variety of fish species, it is not surprising that it is the most common bacterial pathogen reported in the Aquatic Diagnostic Laboratory (ADL) at the Thad

Cochran National Warmwater Aquaculture Center. Over the past 11 years, it was reported in 45.87% of the total cases submitted to the ADL (ADL 2005; ADL 2006; ADL 2007). The only other bacterial pathogen considered more important than columnaris is *Edwardsiella ictaluri* (Plumb 1999). Although *F. columnare* is ubiquitous in fresh water environments, it does not always cause fish mortalities (Soto et al. 2007). Recent studies have revealed phenotypic and genotypic differences among different strains.

Flavobacterium columnare strains from different hosts and geographical regions have demonstrated differences in colony morphology, and DNA homology and pathogenicity. Anacker and Ordal (1959) were the first to attempt to discriminate between *F. columnare* isolates. They differentiated the species into four different serotypes and one miscellaneous group. Song *et al.* (1988) reported three types of colony morphology among strains of *F. columnare* isolated from different geographical areas: rhizoid, mucoid, and honeycomb-like. Several authors have reported phenotypic variations of columnaris strains. Bernardet *et al.* (1989) reported differences in the ability of *F. columnare* to grow at 15°C and 37°C. Shamsudin and Plumb (1996) reported variability in their ability to grow at 15°C with 0.5% NaCl or at pH 6 or 10. They also reported variations in colony margin and color intensity that were not related to fish species or tissue of origin.

Of the differences noted, possibly the most important for aquaculture are differences in pathogenicity, virulence, and genetics. Wood (1968) observed virulence differences and classified strains based on outbreak temperature and tissue damage. Amend (1982) classified the virulence of strains based on the time of death of all

salmonids following lethal challenge with an isolate of *F. columnare*. Decostere *et al.* (1999) noted differences in virulence among *F. columnare* strains isolated from salmonids, tilapia, and eel. Decostere *et al.*(1999) reported a positive correlation between virulence and the ability to adhere to gill tissue. Additionally, it has been suggested that one of the virulence factors of *F. columnare* is the extracellular proteases produced by the bacterium (Griffin 1991; Bertolini and Rohovec 1992; Teska 1993). By comparing proteases produced by 23 isolates of *F. columnare*, Newton *et al.* (1997) showed that the isolates were divided into two groups based on the molecular masses of the proteases produced by the bacterium. All of this is important, but what could prove to be most valuable would be a way to accurately determine if an isolate from a diagnostic setting is a highly virulent strain.

Strains of *F. columnare* also demonstrate genetic diversity and can be differentiated into several genetically distinct groups, termed genomovars. 'Genomovars' are phenotypically similar but genotypically distinct groups of strains (Ursing et al. 1995). Bernardet and Grimont's (1989) DNA relatedness studies between strains revealed homologies as low as 78%. This information could possibly be used to identify which strains are highly virulent and would need aggressive treatment. This has led to the necessity to study the genetic relatedness of the columnaris strains.

When studying the genetic relatedness of organisms, one of the most common traits studied is ribosomal gene sequences. These genes are used as a genetic chronometer because their RNA copies are the final products, so the pressure of natural selection directly affects the primary structural RNAs, rather than the proteins that are

encoded by mRNAs. As Turova (2003) points out, there are other reasons to select the ribosomal genes for this choice: ribosomal genes are universal; their primary structure is highly conserved; they are functionally stable, easy to sequence, and have peculiar regions in their primary and secondary structure displaying degrees of sequence variability. Originally ribosomal phylogenetics were used in the study of prokaryote systematics, because each microbial strain is represented by a specific 16S rRNA sequence. However, as this research has advanced, it has become clear that such a principle is not always true. There are usually several ribosomal encoding operons, and sequence polymorphisms of these genes may occur within a single organism's genome (Zahn et al. 2001; Acinas et al. 2004; Candelon et al. 2004; Stevenson and Schmidt 2004; Gonzalez-Escalona et al. 2005).

In addition to studying the 16S rDNA gene, the 16S-23S rDNA intergenic spacer region (ISR) is studied when additional detail is needed to differentiate relationships between more closely related isolates. The 16S-23S rDNA ISR of bacteria are examined, because this region is under less evolutionary pressure to be conserved, and therefore provides greater genetic variation (Zavaleta et al. 1996). Gram-negative bacteria commonly contain tRNA genes in their 16S-23S rDNA ISR: either $tRNA_{Ile}$ and $tRNA_{Ala}$ or only one $tRNA_{Glu}$ (Gurtler and Stanisich 1996; Garcia-Martinez et al. 1999).

Research with other gram-negative bacteria demonstrates that several rRNA operons occur within the genome and each operon can differ in the ISR. This phenomenon inhibits rapid sequence analysis for genotyping studies. In this study, our goal was to design primers that would allow amplification of overlapping fragments from

one operon. The fragment sizes were a convenient size for rapid sequencing and may eventually be used to identify whether or not a strain is virulent.

CHAPTER II

MATERIALS AND METHODS

Bacterial Isolates

Columnaris isolates and available case information were provided by John Hawke (Louisiana State University), Joseph Newton (Auburn University), and the Mississippi State University Diagnostics Lab (Table 1). All but two of the isolates from LSU and Auburn received as *F. columnaris* were presumptively confirmed by the originating diagnostic lab according to Griffin (1992). The Griffin screen is used to identify *F. columnare* based on five distinct biochemical or cultural characteristics that are unique to the bacterium: (1) the ability to grow in the presence of neomycin sulfate and polymyxin B; (2) color and colonial morphology consistent with typical *F. columnaris*; (3) production of a diffusible, gelatin-degrading enzyme; (4) binding of aqueous Congo red dye; and (5) production of a diffusible enzyme that degrades chondroitin sulfate A. The Griffin screen was not performed on the isolates from MSU.

Table 1. Archived *F. columnare* isolates listed by sample ID, isolate name, donating university, fish species from which they were isolated, and state of origin.

Sample ID	Isolate Name	UNIV	Host	Location
C1	1191B	MSU[1]	?	?
C2	1191B	MSU[1]	?	?
C3	PK-LMB #70	MSU[2]	Largemouth Bass	MS
C4	LADL-88-173	LSU	Channel Catfish	LA
C5	LADL-94-060	LSU	Channel Catfish	LA
C6	LADL-94-078	LSU	Channel Catfish	LA
C7	LADL-94-082	LSU	Channel Catfish	LA
C8	LADL-94-104	LSU	Channel Catfish	LA
C9	LADL-94-140	LSU	Channel Catfish	LA
C10	LADL-94-141 #15	LSU	Channel Catfish	TX
C11	LADL-94-147 #39	LSU	Channel Catfish	LA
C12	LADL-94-225	LSU	Channel Catfish	?
C13	LADL-95-132	LSU	Channel Catfish	LA
C14	LADL-97-374	LSU	Channel Catfish	LA
C15	LADL-97-376	LSU	Hybrid Striped Bass	FL
C16	LADL-01-093	LSU	Channel Catfish	LA
C17	LADL-01-100	LSU	White Crappie	LA
C18	LADL-02-063	LSU	White Crappie	LA
C19	LADL-01-089	LSU	Common Carp	LA
C20	LADL-93-002	LSU	Channel Catfish	LA
C21	LADL-94-081	LSU	Channel Catfish	LA
C22	LADL-94-147 #18	LSU	Channel Catfish	?
C23	LADL-92-002	LSU	Channel Catfish	LA
C24	LADL-97-323	LSU	Largemouth Bass	LA
C25	LADL-94-141 #45	LSU	Channel Catfish	?
C26	LADL-02-176	LSU	Common Carp	LA
C27	LADL-02-185	LSU	Yellow Perch	MN
C28	LADL-96-511	LSU	Rainbow Trout	?
C29	LADL-96-513	LSU	Rainbow Trout	?
C30	LADL-03-061	LSU	Channel Catfish	LA
C31	LADL-03-067	LSU	Koi	?
C32	C03118	MSU[3]	?	MS
C33	MS 90-252	AUB	Channel Catfish	MS
C34	143-94	AUB	Channel Catfish	LA
C35	ATCC 49512	AUB	Brown Trout Fry	France
C36	MS 90-507	AUB	Channel Catfish	?
C37	Dickerson I	AUB	Channel Catfish	MS
C38	MS 90-106	AUB	Channel Catfish	MS
C39	MS 90-629	AUB	Channel Catfish	MS
C40	MS 90-640	AUB	Channel Catfish	MS

(Table 1 continued)

Sample ID	Isolate Name	UNIV	Host	Location
C41	MS 064-93	AUB	Channel Catfish	MS
C42	MS 90-659	AUB	Channel Catfish	?
C43	MS 90-503	AUB	Channel Catfish	MS
C44	MS 90-268	AUB	Channel Catfish	?
C45	MS 90-639	AUB	Channel Catfish	?
C46	MS 90-136	AUB	Channel Catfish	?
C47	155-94	AUB	?	?
C48	MS 90-497	AUB	Channel Catfish	MS
C49	LA 88-173	AUB	Channel Catfish	?
C50	MS 91-20	AUB	Channel Catfish	MS
C51	Alg 92-491-C	AUB	Channel Catfish	MS
C52	Evans 2	AUB	Channel Catfish	?
C53	LADL-03-124	LSU	Blue Catfish	LA
C54	LADL-04-046	LSU	Channel Catfish	LA
C55	LADL-04-060	LSU	Tilapia	FL
C56	LADL-04-066	LSU	Largemouth Bass	LA
C57	LADL-04-076	LSU	Bluegill	LA
C58	PB-2	LSU	?	ARK
C59	PB-7	LSU	?	ARK
C60	PB-12199	LSU	Channel Catfish	ARK
C61	PB-10121	LSU	?	ARK
C62	AL-94-203	LSU	Hybrid Catfish	AL
C63	PB-02-12	LSU	Fathead Minnow	ARK
C64	PB-02-41	LSU	Koi	ARK
C65	PB-02-51	LSU	Golden Shiner	ARK
C66	PB-02-97	LSU	Koi	ARK
C67	PB-02-110	LSU	Golden Shiner	ARK
C68	PB-04-02	LSU	Platy	ARK
C69	PB-04-23	LSU	Koi	Washington
C70	LV-359-01	LSU	Channel Catfish	ARK
C71	LV-152-02	LSU	Channel Catfish	ARK
C72	LV-339-01	LSU	Channel Catfish	ARK
C73	LV-345-01	LSU	Channel Catfish	ARK

NOTE: Underlined isolate names indicate samples that did not pass the Griffin screen.
MSU[1]: Michelle Banes
MSU[2]: Steve Rees
MSU[3]: Michele Williams
Largemouth Bass (*Micropterus salmoides*)
Channel Catfish (*Ictalurus punctatus*)
Hybrid Striped Bass (*Morone saxatilis* X *M. chrysops*)
White Crappie (*Pomoxis annularis*)
Common Carp (*Cyprinus carpio*)

(Table 1 continued)

Yellow Perch (*Perca flavescens*)
Rainbow Trout (*Oncorhynchus mykiss*)
Koi (*Cyprinus carpio*)
Brown Trout Fry (*Salmo trutta*)
Blue Catfish (*Ictalurus furcatus*)
Tilapia (*Tilapia mossambique*)
Bluegill (*Lepomis microchirus*)
Hybrid Catfish (*Ictalurus punctatus* X *I. furcatus*)
Fathead Minnow (*Pimephales promelas*)
Golden Shiner (*Notemigonus crysoleucas*)
Platy (*Xiphophorus maculates*)

Each columnaris isolate was grown overnight on either 10% BHI agar plates containing the following: BHI 3.7 g/L (237500-500g, Beckton Dickson, Sparks, Maryland), agar 6.0 g/L (BP1423-500, Fisher Chemicals, Fairlawn, New Jersey) or 9% *Flavobacterium columnare* growth medium (FCGM) agar plates containing the following: Tryptone 8.0 g/L (BP1421-500, Fisher Chemicals, Fairlawn, New Jersey), Yeast Extract 0.8 g/L (0127-17-9, Beckton Dickson, Sparks, Maryland), MgSO4(7H2O) 1.0 g/L (Sigma, M-1880), CaCl2(2H2O) 0.74 g/L (Sigma, C7902), NaCl 5.0 g/L (S271-1, Fisher Chemicals, Fairlawn, New Jersey), Sodium Citrate 1.5 g/L (Sigma, S4641-500g), Agar 9.0 supplemented with 5 mg/L Neomycin Sulfate (Sigma N-6386) and 200 units/ml Polymyxin B Sulfate (Sigma P-1004). Single colonies were isolated and grown overnight in 5 ml FCGM broth, which is chemically identical to the FCGM plates, except the broth does not contain agar. Samples from the overnight broth cultures were archived at -80°C by mixing 1.7 ml of the cultures with 300 µl of sterile glycerol (IB15762 Shelton Scientific-IBI, Peosta, Iowa) and freezing at -80°C.

Preparation of Genomic DNA from Bacteria

The genomic DNA was isolated from the overnight *Flavobacterium columnare* cultures (1 ml) using the Puregene genomic DNA isolation system (158388, Qiagen, Valencia, CA) following the protocol for "DNA Purification from 1 ml Gram-negative Bacteria Culture Medium."

Amplification of the 16S-23S rDNA Spacer

Initially, the 16S-23S rDNA spacer from isolate 1191B was PCR amplified with the previously designed primer pair ProkSpaceL and ProkSpaceU, which targets the 3' end of the 16S sequence and the 5' end of the 23S sequence in the ribosomal RNA operon of prokaryotic DNA. The reactions were performed in a final volume of 25 µl containing 100-250 ng of template (1 µl), 0.2 µM of each primer, 200 µM dNTP (TAK 4030, Takara Bio, Madison, Wisconsin), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ 10×buffer A (2.5 µl), 1.25 units Fisher BioReagents *Taq* DNA Polymerase (FB-6000-15, Fisher Scientific, Houston, Texas). The reaction volume was adjusted to 25 µl with UltraPure water. Amplification mixtures using primer pair ProkSpaceU and ProkSpaceL were subjected to an initial incubation of 94°C for 1 min, followed by 35 cycles of 94°C for 30 seconds, 55°C for 1 min, 72°C for 1 min, with a final extension cycle at 72°C for 2 min, and an indefinite holding time at 4°C. This reaction was performed using an MJ Research thermal cycler (PTC-200, Applied Biosystems). A total of 5 µl of each PCR product was visualized with an electrophoresed 1% agarose gel, 1:10,000 GelStar nucleic acid stain (BioWhittaker Molecular Applications, Rockland, Maine) and UV transillumination (ChemiImager 5500, Alpha Innotech Corporation, San Leandro,

California). We expected to see multiple PCR products of different sizes. We visualized only one product, so the unincorporated PCR reaction mix was removed from the PCR product using Millipore Montage-PCR Filter Unit (UFC7-PC2-50 Millipore, Bedford, Massachusetts) as per manufacturer's instructions. The PCR product was cloned using the TOPO-TA cloning kit (K4575-40 Invitrogen Corporation, Carlsbad, California) following the protocol. A single colony was chosen for sequencing, and the plasmid with insert was isolated using the QIAGEN Plasmid Mini Kit (12125, Qiagen, Valencia, CA). Sequencing was performed as per the manufacturer's instructions on both DNA strands using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with the BigDye® Terminator v1.1 Cycle Sequencing Kit (4337450, Applied Biosystems, Foster City, CA) and sequencing primers T7 and T3, which were supplied with the cloning kit.

Two primer pairs were designed for the 16S-23S rDNA spacer that would create two overlapping PCR products which would span the entire 16S-23S rDNA spacer (Figure 1). Oligo Primer Analysis Software (Molecular Biology Insights) was used to design two primer pairs (Fcol5U20 and Fcol466L19; Fcol257U20 and Fcol783L19) using aligned sequences from GenBank (AB031221, AB031217, AB030748, AB031219, AB031220, AB031218) and our cloned ISR. Primers were ordered from MWG Biotech (High Point, NC), resuspended in 0.5X TE buffer (V6231 Promega, Madison, Wisconsin) to a stock solution concentration of 100 pmol/µl. The working solution concentration of 20 pmol/µl was made by diluting the 100 pmol/µl stock primers with UltraPure DNase/RNase-Free Distilled Water (10977-015, Invitrogen Corporation, Carlsbad, California). The reactions were performed and visualized as previously described with

the following modifications. Amplification mixtures using primer pairs 1 and 2 were submitted to an initial incubation of 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min, with a final extension cycle at 72°C for 5 min, and an indefinite holding time at 4°C. PCR products of approximately 460 base pairs (bp) from Primer set 1, and approximately 525 bp from Primer set 2 were expected. The unincorporated PCR reaction mix was removed from the PCR products using Millipore Montage-PCR Filter Unit (UFC7-PC2-50 Millipore, Bedford, Massachusetts) as per manufacturer's instructions. Direct sequencing was performed on both DNA strands using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with the BigDye® Terminator v1.1 Cycle Sequencing Kit (4337450, Applied Biosystems, Foster City, CA).

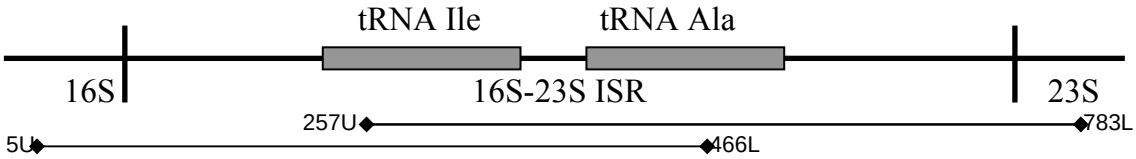

Figure 1 Graphical representation of the two overlapping PCR products using the primer pairs Fcol5U20-Fcol466L19 and Fcol257U20-Fcol783L19. The combined products span the 16S-23S ISR and include both $tRNA_{Ile}$ and $tRNA_{Ala}$.

Cloning and Sequencing of the 16S-23S rDNA Spacer

Each PCR product was cloned using the TOPO-TA cloning kit (K4575-40 Invitrogen Corporation, Carlsbad, California) as per the protocol with the following minor modifications. Three positive colonies per cloning reaction were isolated and inoculated in 500 µl of broth in a deep-well 96-well plate and grown overnight in a 37°C shaking incubator. The following day 170 µl of the overnight broth was mixed with 30 µl

of sterile glycerol in a 96-well Costar Cell Culture Plate (#3595 Corning, Corning, New York) and frozen to -80°C for later sequence analysis. Frozen samples were sent to the USDA facilities in Stoneville, Mississippi for sequencing of both strands using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) with the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and sequencing primers M13F and M13R.

Phylogenetic and Sequence Analysis

Sequencher software v4.8 (GeneCodes Corporation, Ann Arbor, MI) was used to check sequences for accuracy of base calls. Phylogenetic analyses were performed with MegAlign (DNASTAR, Inc., Madison, WI) using the Clustal W method (Thompson 1994). The tRNA sequences were identified with tRNAscan-SE (Lowe and Eddy 1997).

Pulsed Field Gel Electrophoresis and Product Analysis

Pulsed field gel electrophoresis (PFGE) was performed on the ATCC 49512 strain of *F. columnare* using the I-*Ceu*I enzyme (Soto et al. 2007; Soto et al. 2008) whose restriction site is inside the 23S rRNA gene. Bionumerics (Applied Maths, Inc.) version 3 software was used to estimate the DNA fragment sizes relative to those of the lambda ladder and small DNA marker (New England BioLabs). Individual bands were excised from the gel and the DNA was purified from the gel slice using the freeze and squeeze method. PCR was then performed on each purified PFGE band with the primers Fcol5U20 and Fcol783L19 to generate a product of approximately 780 bp. The reactions were performed in a final volume of 25 µl containing 100-250 ng of template (1 µl), 0.2

µM of each primer, 200 µM dNTP (TAK 4030, Takara Bio, Madison, Wisconsin), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ 10×buffer A (2.5 µl), 1.25 units Fisher BioReagents *Taq* DNA Polymerase (FB-6000-15 Fisher Scientific, Houston, Texas). The unincorporated PCR reaction mix was removed from the PCR products using the Millipore Montage-PCR Filter Unit. Each PCR product was then cloned using the TOPO-TA cloning kit. Ten colonies were isolated for each clone, and both DNA strands were sequenced using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit.

Southern Blotting

A 313 bp probe targeting the 16S rDNA of ATCC49512 (AY754373) was designed using Oligo software. The reactions were performed using the primer set ATCC49512U20 and ATCC49512L17 with a final volume of 25-µl containing 100-250 ng of template (1 µl), 0.2 µM of each primer, 200 µM dNTP (TAK 4030, Takara Bio, Madison, Wisconsin), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ 10×buffer A (2.5 µl), and 1.25 units Fisher BioReagents *Taq* DNA Polymerase (FB-6000-15 Fisher Scientific, Houston, Texas). The reaction volume was adjusted to 25 µl with UltraPure water. The amplification mixture, using the 16S probe primer pair, was subjected to initial incubation of 94°C for 2 min, followed by 30 cycles of 94°C for 30 seconds, 59.1°C for 30 seconds, and 72°C for 30 seconds, with a final extension cycle at 72°C for 5 min, and an indefinite hold at 4°C. Sequencher software was used to choose seven 6-base cutting enzymes that have no recognition sites within the 16S-rDNA for *F. columnare* strain ATCC 49512. The following enzymes were used to digest 1 µg of total

DNA: *Eco*RI, *Eco*RIII, *Eco*RV, *Hpa*I, *Sca*I, *Spe*I *and Xba*I according to the manufacturer instructions (New England Biolabs, Beverly, Massachusetts) and electrophoresed on a 1% TAE agarose gel. Gels were blotted onto Hybond N+ (RPN203B, GE Healthcare) nylon membrane using capillary transfer. Southern blotting was performed using the Amersham ECL Direct™ Nucleic Acid Labeling and Detection System (RPN3001, GE Healthcare, Piscataway, NJ), following their protocols. A total of 100 ng of the ATCC probe and 50 ng of the 1 Kb ladder (15615-016, Invitrogen Corporation, Carlsbad, California) was labeled and used for detection. Detection was performed by exposing Hyperfilm ECL (RPN3114K, GE Healthcare) for 20 seconds.

Table 2. PCR primers used for amplification of 16S-23S rDNA and 16S rDNA probe sequences for cloning and Southern hybridizations.

Name	Sequence	Product length
Fcol5U20	TGT ACA CAC CGC CCG TCA AG	461 bp
Fcol466L19	GTG CAA GGC AGG CGC TCT A	
Fcol257U20	AAT GTA GGG GTC GGC AGT TC	526 bp
Fcol783L19*	CGT CCT TCA TCG CCT CTG A	
ATCC49512U20	TAA CGA GCG CAA CCC CTG TT	313 bp
ATCC49512L17	GCT TGA CGG GCG GTG TG	
ProkSpaceL	TTT CGC AGA TTA GCA CGT CCT TCA TCG C	~700 bp
ProkSpaceU	GTC TTG TAC ACA CCG CCC GTC A	
Fcol131U24*	TGG AAC ACC TCC TTT CTA GAG ACA	~660 bp

* Represents the PRC primer pair that makes the ~660 bp product.

CHAPTER III

RESULTS

Targeting the 16S-23S rDNA Spacer

We initially targeted the 16S-23S rDNA spacer using the primers ProkspaceL and ProkspaceU. These primers had been successfully used to amplify the 16S-23S ISR of *Edwardsiella ictaluri* (results unpublished) and *Enterococcus faecium* (Burgess et al. 2006). Knowing that many prokaryotes contain multiple rDNA operons, we expected to visualize multiple PCR products on the gel. However, we saw only one product of approximately 700 bp. After cloning and sequencing the PCR product, the ISR was found to contain both $tRNA_{Ile}$ and $tRNA_{Ala}$ and was similar to previously sequenced ISR elements. This information was used to design the primer sets to make two overlapping PCR products with the goal of using direct sequencing from resulting PCR products. PCR produced the expected single band from all isolates. However, direct sequencing of the products was not effective; the sequencing of PCR products generated with the primers Fcol5U20 and Fcol466L19 would consistently break down at the same point in many of the samples at a long poly-A region. This led us to believe that we had PCR products that were not completely homogenous and that polymerase slippage may have caused the sequencing anomalies. Therefore, we cloned and sequenced our PCR products.

Amplification of the 16S-23S rDNA Spacer, Cloning and Sequencing

Both primer pairs yielded the expected amplified fragments based on the 16S-23S rDNA intergenic spacer region (ISR) of the ribosomal RNA operon from all the isolates included in the study. The final assembled ISR amplicons from *F. columnare* ranged in size from 585 bp to 672 bp. This excluded the 16S and 23S flanking sequences. Several isolates yielded more than one distinct ISR assembly. The isolates that yielded different sequence assemblies had similar sequences, yet they contained regions that were distinctly different, leading us to believe that the *F. columnare* genome did indeed contain multiple copies of the 16S-23S ISR operon. We chose the assemblies that were most common and designed a new primer (Fcol131U24) to be used with Fcol783L19 to target that ISR (Table 2). We then performed PCR using the new primer set on the *F. columnare* samples that did not originally yield this more common ISR sequence. Using the new primer set, we were able to use direct sequencing on our PCR products.

Pulsed Field Gel Electrophoresis and Product Analysis

In order to resolve the issue of the *F. columnare* genome containing more than one ISR with both $tRNA_{Ile}$ and $tRNA_{Ala}$ present we used I-*Ceu*I RFLP. Digestion of the ATCC 49512 type strain of *F. columnare* with the I-*Ceu*I endonuclease resulted in three PFGE fragments: 491.31 kb, 755.09 kb and 1853.59 kb, designated 1, 2, and 3, respectively. The *F. columnare* genome is circular, indicating that it has at least three ribosomal operons. However, after performing PCR which targeted the 16S-23S ISR and cloning and sequencing the resulting PCR products, each PFGE fragment yielded two ISR sequences. Of the six, three were unique: 535 bp, 628 bp and 699 bp. Three

sequences were almost identical; they contained 637 bp, 638 bp and 639 bp. Those three ISR sequences differed by 1-2 single base positions in each sequence. All six ISR products contained both $tRNA_{Ile}$ and $tRNA_{Ala}$. Fragment 1 (491.31 kb) produced sequences that were 637 bp (C) and 638 bp (D) in length. Fragment 2 (755.09 kb) produced sequences that were 639 bp (E) and 698 bp (F). Fragment 3 (1853.59 kb) produced sequences that were 535 bp (A) and 628 bp (B) (Figure 2).

```
                                                                16S rRNA gene
              Primer Fcol5U20 >>>
              TGTACACACCGCCCGTCA
C_637_bp    1 TGTACACACCGCCCGTCAAGCCATGGAAGCTGGGGGTACCTGAAGTCGGTGACCGCAAGG
E_639_bp    1 ............................................................
D_638_bp    1 ............................................................
B_628_bp    1 .....................................G..................G..
A_535_bp    1 .....................................G....................
F_698_bp    1 ........................A........A...T..A........AG........
consensus   1 ************************.********.**..**.********..*****.**

C_637_bp   61 AGCTGCCTAGGGTAAAACTGGTAACTAGGGCTAAGTCGTAACAAGGTAGCCGTACCGGAA
E_639_bp   61 ............................................................
D_638_bp   61 ............................................................
B_628_bp   61 ...................A........................................
A_535_bp   61 ...................A........................................
F_698_bp   61 C..C..............C...G.T.G..................................
consensus  61 .**.**************..**.*.*.********************************

                     Primer Fcol131U24 >>>
                     TGGAACACCTCCTTTCTAGAG                              ACA
C_637_bp  121 GGTGCGGCTGGAACACCTCCTTTCTAGAG----------------------------ACA
E_639_bp  121 ..............................----------------------------...
D_638_bp  121 .............................----------------------------...
B_628_bp  121 ..............................----------------------------.TG
A_535_bp  121 ..............................----------------------------.TG
F_698_bp  121 ........................G...TGTTTTGGCTTGTATTTAGAAGGAAGAT.TT
consensus 121 ************************.***                             *..

C_637_bp  153 AAAAAAAGAAGCTATT-------TTTTAATT-----------TAGATACTAAATTAAAAA
E_639_bp  153 ...............-------........-----------.................
D_638_bp  153 ...............-------........-----------.................
B_628_bp  153 .TTT..CC--.T..GG-------....GG..-----------G.A.---------GG..G
A_535_bp  153 .TTT..CC--.T..GG--------....GG..-----------G.A.---------GG..G
F_698_bp  181 CT.TGG.C.G.T....CTGCTAA....GG..ATCAATTTTAT..A.ACTGG..AC..G..
consensus 181 ..........*.**..       ****..**           .*.*............*.
```

Figure 2. Sequence alignment of our six 16S-23S ISRs of *F. columnare* that we found using the combination of PFGE, PCR, cloning and sequencing. The highlighted bases are positions where the almost identical sequences differ. Primers (underlined) that make our overlapping PCR products and their corresponding sequences are shown. The double underlined sequences show the flanking sequences of the partial 16S and 23S rRNA genes. The $tRNA_{Ala}$ and $tRNA_{Ile}$ are zig-zag underlined.

(Figure 2 continued)

```
C_637_bp  195 AACTTA-----CTCTCGCTG--------------------------TTAATTCAAAAAA
E_639_bp  195 ......-----........-------------------------------.............
D_638_bp  195 ......-----........-------------------------------.............
B_628_bp  184 .TA..------........-------------------------------......T......
A_535_bp  184 .TA..------........-------------------------------......T......
F_698_bp  241 .C.CA.ATGAG.CA.T.G..GGCTTTAAACAATGAGGTAAAGGATTT.AG...T..G.C.
consensus 241 *....*     *..*.*.**                            *..*** **.*.*

C_637_bp  223 AAAGAAA--------AGGCTTAAAAT--------------------------------AC
E_639_bp  223 .......--------.....-------------------------------------..
D_638_bp  223 ..-....--------.....-------------------------------------..
B_628_bp  212 T.....T--------.A..AA.G..A-------------------------------..
A_535_bp  212 T.....T--------.A..AA.G..A-------------------------------..
F_698_bp  301 T....TTTAGGATTT...A......ATCTCAAATCTCGGATCTCAAAATCTCAGATCA.T
consensus 301 .*.**.........*.*...*.**.................................*.
                                                              tRNA_Ile
                                                  Primer Fcol257U20 >>>
                                                  AATGTAGGGGTCGGCAGTTC
C_637_bp  243 AGAGTCTCGTAGCTCAGCT-GGTTAGAGTACTACACTGATAATGTAGGGGTCGGCAGTTC
E_639_bp  243 ..................-.........................................
D_638_bp  242 ..................-.........................................
B_628_bp  232 ..................-.........................................
A_535_bp  232 ..................-.........................................
F_698_bp  361 .T....C.........T.T........C.......................A..G....
consensus 361 *.****.**********.*.********.**********************.**.****

C_637_bp  302 GAGTCTGCCCGGGACTACTTT-TTAAAGCACA-AGGAAATTCTAGAAGCGAATTTTGAAT
E_639_bp  302 ..................-.........-.............................
D_638_bp  301 ..................-.........-.............................
B_628_bp  291 ..................-.........C.............................
A_535_bp  291 ..................-....-..TTG--------.T..A.G.--------A...
F_698_bp  421 A.A..C..T.........AA.GC.....A.G.-------G.T.GAT..----------G.
consensus 421 .*.**.**.*********..* .***.*.... .......*.*....*.........*.*

C_637_bp  360 TATAAATTTTAAATGATGAATT--TGAAAAATAATTCAAAAAAATAATTCAACATCCAAA
E_639_bp  360 ......................--..................................
D_638_bp  359 ......................--..................................
B_628_bp  350 ......................--..................................
A_535_bp  333 .T..G.GG..G.G.A.CCG-..--....GT.CTG..A.CTT.G.ACTG.T...G---..G
F_698_bp  470 .C.TGG....T...AT.TTT..AT.A....C...A.ACT...C.CTGA...CTT.GA...
consensus 481 *.*.....**.*.*......**  *.**.......*.....*.*....*.*......**.
                                                        tRNA_Ala
                                              <<< Primer Fcol466L19
                                                ATCTCGCGGACGGAACGTG
C_637_bp  418 ATAGAAAAAA-GATTGGGGG-ATTAGCTCAGCTGGCTAGAGCGCCTGCCTTGCACGCAGG
E_639_bp  418 ..........-.........G.....................................
D_638_bp  417 ..........-.........-.....................................
B_628_bp  408 ..........-.........-.....................................
A_535_bp  387 .C.CT....C-A.CG....A--.....................................
F_698_bp  530 GCGCGGCTTTTA...T....G.....................A...............
consensus 541 ............*...***...********************.****************

C_637_bp  476 AGGTCATCGGTTCGACTCCGATATTCTCCACCAA--ATTGATTTGAAAATGAATTGATTT
E_639_bp  477 ..................................--.........................
D_638_bp  475 ..................................--.........................
B_628_bp  466 ..................................--.........................
A_535_bp  444 ............................TT.--GC-----------------------
F_698_bp  590 G.....A........A....T.............CA..C........G....G.C....-
consensus 601 .*****.********.****.**********..*  ........................

C_637_bp  534 GAAAATTTGAAAATGATCATCATTTTCAAATTAACGAATCTTAAAATCTTCAAATTGAGT
E_639_bp  535 ............................................................
D_638_bp  533 ............................................................
B_628_bp  524 ............................................................
A_535_bp  480 ---------------GC..CGG....-.....TCGT...GG-------..GC.CC----
F_698_bp  649 ----------------.G..A...A.......GT......A----------------.A.
consensus 661 .................*...**.*..****......**.........**..*......
```

(Figure 2 continued)

```
C_637_bp  594 AAAGTTCATTGACATATTGAGATAAACATACAAACAAGTAGAAAGAACACTTTAGTAATT
E_639_bp  595 ............................................................
D_638_bp  593 ............................................................
B_628_bp  584 ............................................................
A_535_bp  513 --.......................-.....TTT..AA..T.GA..--------...--C
F_698_bp  685 CC.....T.............A.G...CGAG.G.C.TACAG.G.-------------.C
consensus 721 ..*****.**************.*.*.*........*.....*..............

C_637_bp  654 AGTAAAGAGTGAATAGTAATTAGTCTTAGGACTAGTCACTAATTACTTAAAGCTAATCAC
E_639_bp  655 ............................................................
D_638_bp  653 ............................................................
B_628_bp  644 ............................................................
A_535_bp  561 ....G.TGTGT.G..A..CA..T.TA..AAGAA....-...G..----------.T..TTA
F_698_bp  732 T..G....CGCTG..-----.G..CGAA.AC.A...----------.A-------AG..GCG
consensus 781 .**..*.......**......*.....*.....**.....*.**...........**...

                                                          23S rRNA gene
                                                            <<<  Primer
                                                                 AGTCTC
C_637_bp  714 TAAAAAAAAGAAAACAATAAGCAAAATAAGGGCGTATGGGGGATGCCTAGGCTCTCAGAG
E_639_bp  715 ............................................................
D_638_bp  713 ............................................................
B_628_bp  704 ............................................................
A_535_bp  611 .....TT.G.CGGCAC.............................T.............
F_698_bp  776 .....GC.TA.C..A.GA...T..G-C.......C....A........T.........
consensus 841 *****..*..........***.**...*******.****.********.***********

                Fcol783L19
              CGCTACTTCCTGC
C_637_bp  774 GCGATGAAGGACG
E_639_bp  775 .............
D_638_bp  773 .............
B_628_bp  764 .............
A_535_bp  671 .............
F_698_bp  835 .............
consensus 901 *************
```

Southern Blotting

In separate Southern blots, the labeled 1 kb ladder and 16S probe proved to label only the desired target; therefore we were able to use the labeled 16S probe and the labeled 1 kb ladder concurrently on the same blot. Using the restriction enzymes *Eco*RI, *Eco*RIII, *Eco*RV, *Hpa*I, *Sca*I, *Spe*I, and *Xba*I for Southern blotting revealed that there are at least four and as many as six ribosomal operons (Figure 3). In each lane, there is one predominant band that suggests the presence of multiple fragments of the same size. Gene duplication events may have caused this.

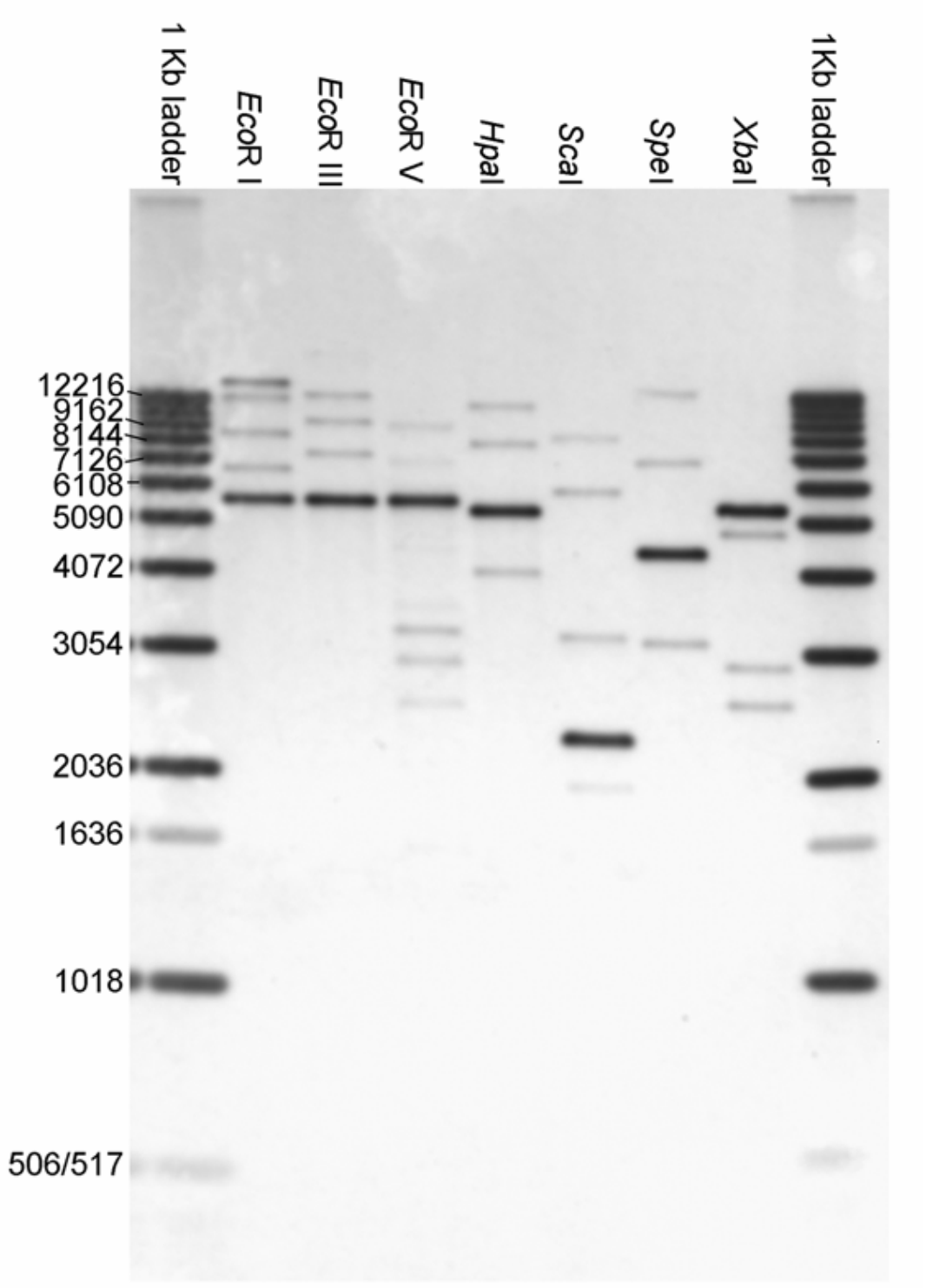

Figure 3. Southern blot of *F. columnare* ATCC49512. Probes targeted a 313 bp target inside the 16S rRNA gene. One µg of total DNA was digested with *Eco*RI, *Eco*RIII, *Eco*RV, *Hpa*I, *Sca*I, *Spe*I and *Xba*I.

Phylogenetic Analysis and Sequence Analysis

tRNAscan-SE revealed that each of the six PFGE ISRs contained both $tRNA_{Ile}$ and $tRNA_{Ala}$ (Figure 4). All of our individual sequences also contained both tRNAs. The phylogenetic analysis for the PFGE ISRs that was done using MegAlign revealed that all six 16S-23S ISRs are related, with ISRs C_637_bp, D_638_bp, E_639_bp and B_628_bp being the most closely related and A_535_bp and F_698_bp being less related (Figure 5).

By removing sequences representing the less related ISR elements from our data set, we were able to do a phylogenetic analysis for genotyping studies (Figure 6). We found three well-defined genotype groupings which correspond to Triyanto and Wakabayashi (1999a) genomovars. Genotype I and II both consisted of two subgroups which corresponded to Darwish and Ismaiel's subgroups (2005).

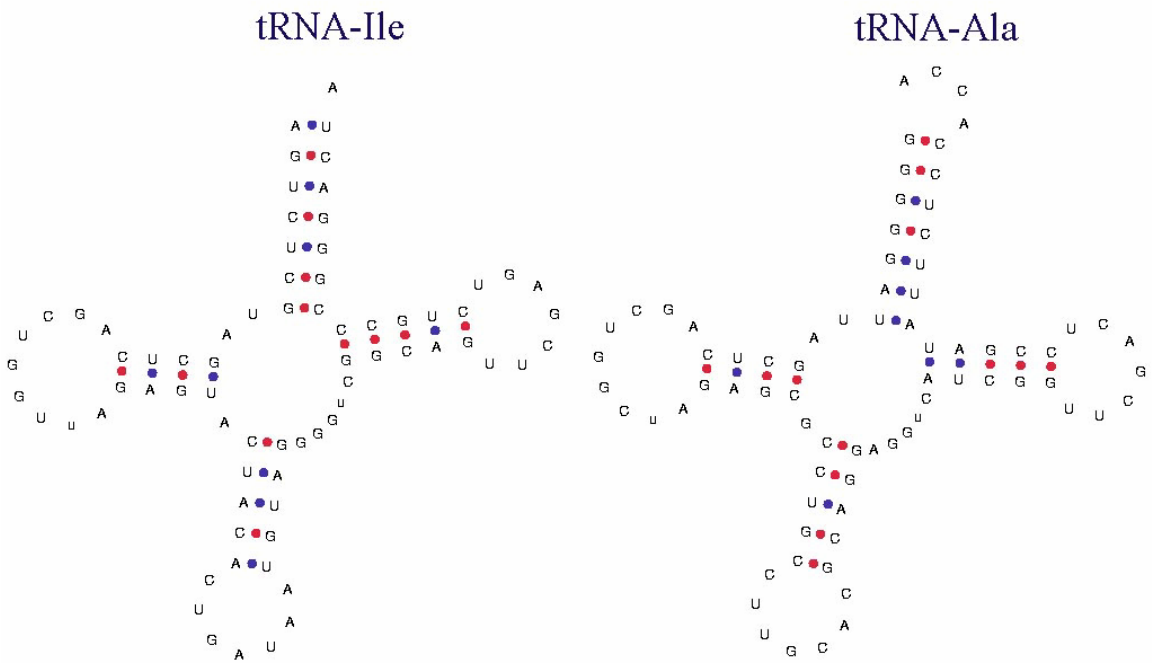

Figure 4. Representatives of $tRNA_{Ile}$ and $tRNA_{Ala}$ present in each 16S-23S ISR of *F. columnare* that were found with tRNAscan-SE.

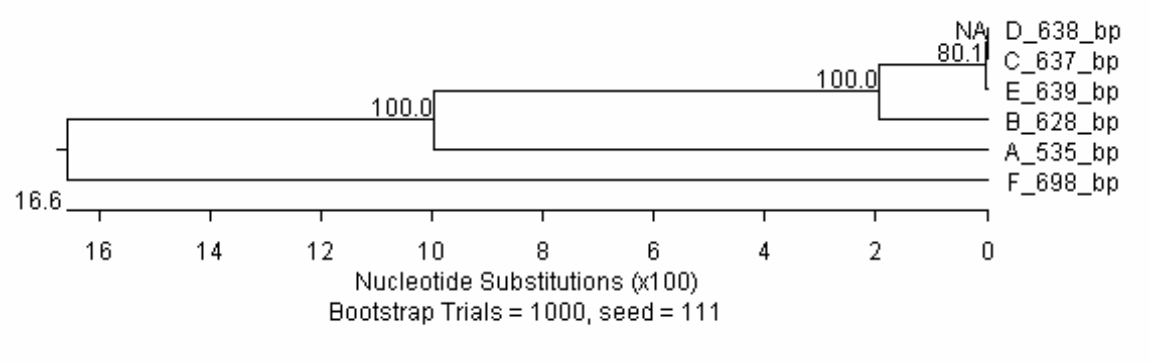

Figure 5. Phylogenetic tree generated with the Clustal W algorithm in MegAlign of all six 16S-23S ISRs of *F. columnare*.

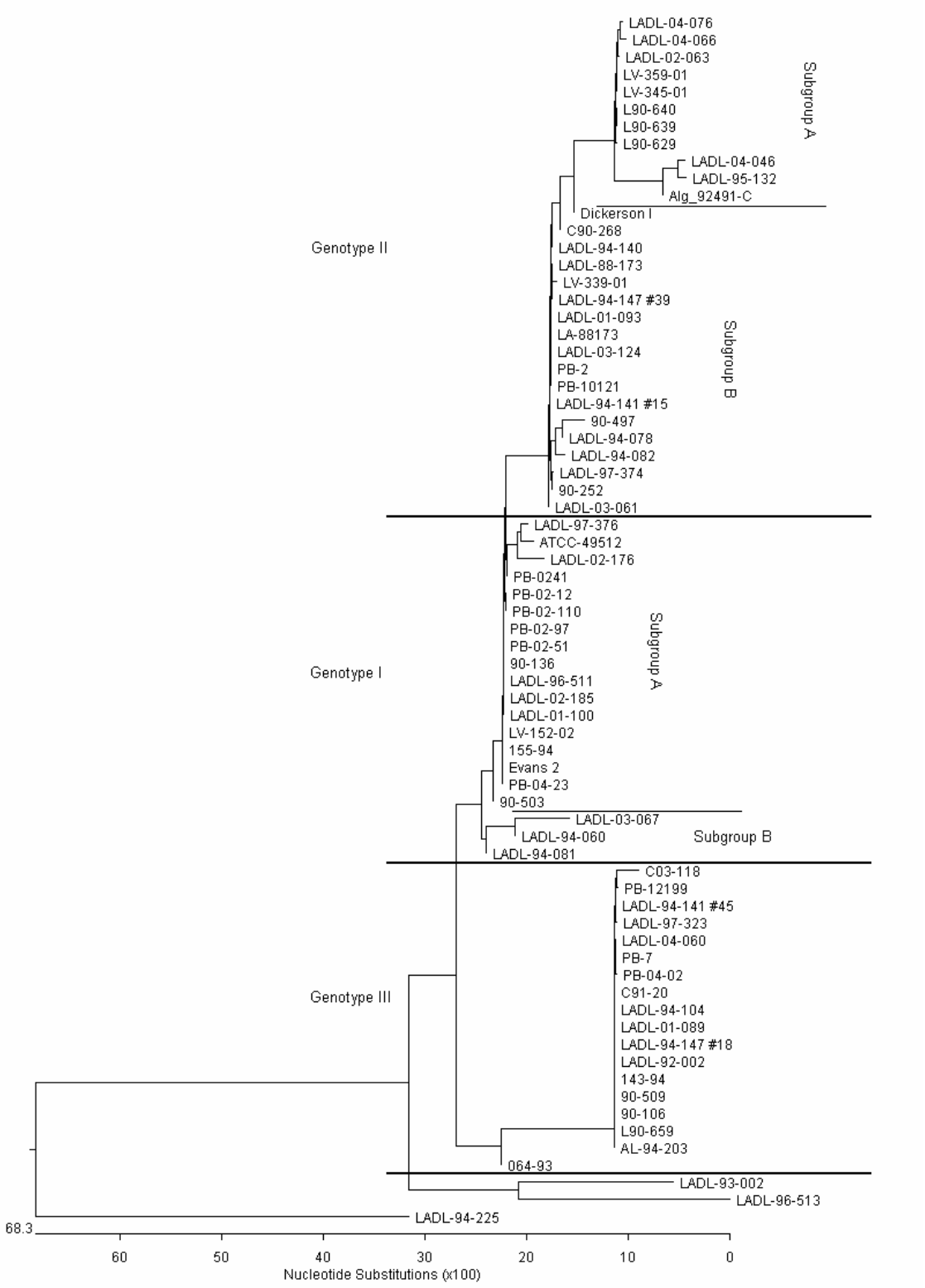

Figure 6. Phylogenetic tree generated with the Clustal W algorithm in MegAlign of all *F. columnare* isolates used in this study showing Genotypes and subgroups.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

This study shows that there are multiple ribosomal operons and that the 16S-23S ISRs for *F. columnare* used in this study all have both $tRNA_{Ile}$ and $tRNA_{Ala}$. Yet there are differences, so to compare different strains or isolates of *F. columnare*, one specific ISR must be targeted. It appears that more than one spacer region could possibly have been used in the previous phylogenetic analyses. tRNAscan-SE prediction revealed that most of the *F. columnare* 16S-23S ISRs that are in GenBank contain both $tRNA_{Ile}$ and $tRNA_{Ala}$, however two contain $tRNA_{Ser}$ (AY754383, AY754376), three contain tRNAs with undetermined/unknown isotypes (AY753073, AY754388, AY842905), and three contain predicted pseudogenes (AY753071, AY754375, AY754363). Recently, relatives of *F. columnare*, *Flavobacterium johnsoniae* (NC_009441) and *Flavobacterium psychrophilum* (NC_009613) have had their complete genomes sequenced. *Flavobacterium johnsoniae* and *F. psychrophilum* both have a circular genome of approximately 6000 kb and 3000 kb, respectively. *Flavobacterium psychrophilum* is roughly the same size that we estimated for *F. columnare*. Both *F. johnsoniae* and *F. psychrophilum* contain six rRNA operons in their completed genome. All six 16S-23S ISR regions of *F. johnsoniae* are identical and all contain $tRNA_{Ile}$ and $tRNA_{Ala}$. Five of the six 16S-23S ISR regions of *F. psychrophilum* are identical and the

sixth spacer differs by only three bases with all spacers containing $tRNA_{Ile}$ and $tRNA_{Ala}$. Darwish and Ismaiel (2005) used the PCR primers ColF3a and ColR3a to amplify the 16S-23S ISR of *F. columnare* and Arias et al. (2004) used the PCR primers 16S-14F and 23S-1R to also amplify the 16S-23S ISR of *F. columnare*. However, both of these primer sets can amplify all six of the 16S-23S ISRs of *F. johnsoniae* and *F. psychrophilum*; therefore, they would probably amplify most if not all of the ISRs of *F. columnare* which would cause phylogenetic chaos. Darwish's primer set will amplify all but one of the 16S-23S ISR that we identified, and will likely amplify the sixth operon. Arias' primer set amplifies a fragment that is larger than our 16S-23S ISR PCR amplicons. The fact that Arias' primer set will amplify the 16S-23S ISR from all six rRNA operons of both *F. johnsoniae* and *F. psychrophilum*, indicates that those primers will probably amplify all of the 16S-23S ISRs of *F. columnare*, thereby making any PCR product heterogeneous. This would inhibit direct sequencing and phylogenetic studies. However, because the 16S-23S ISRs from the different operons of the sequenced clones of *F. johnsoniae* lacked variability, it may not be necessary to target an individual spacer to use for phylogenetic analysis of the 16S-23S ISR. Conversely, because the 16S-23S ISRs of *F. columnare* are not identical, this is not the case. To make sure that any genetic relatedness studies using the 16S-23S ISR of *F. columnare* are most accurate, it is important to know that the same 16S-23S ISR is used for the analysis.

Different methods used in several studies of the genetics of *F. columnare* have shown that there are at least three genotypes or genomovars. Triyanto and Wakabayashi (1999a; 1999b) demonstrated intraspecies variation among strains of *F. columnare* using

RFLPs, 16S rDNA sequencing and DNA-DNA hybridization. With RFLP analysis and 16S rDNA sequencing, they defined three genomovars within the species. They showed that even though the isolates were phenotypically identical, the 16S rDNA sequence alone was different enough to define the genomovars. Following this, Arias *et al.* (2004) explored the diversity of 30 *F. columnare* isolates from different sources of cultured fish using three genotyping methods: 16S rDNA-RFLP, 16S-23S ISR sequence analysis, and Amplified Fragment Length Polymorphism (AFLP). They reported that genomovars I and II are both found in the Southeastern U.S. However they found four isolates for which genomovar ascription was not possible. Those four isolates formed a different group in all three genotyping methods, and Arias *et al.* suggests those isolates may belong to a new genomovar within the species (Genomovar IV). Recently, Darwish and Ismaiel (2005) utilized sequence analysis of the 16S rDNA and RFLP on the 16S-23S rDNA ISR to show three genotypes of *F. columnare* that correspond to Triyanto and Wakabayashi's three genomovars. They also sequenced 16S-23S rDNA ISRs and deposited them in GenBank. However the number of possible different ISR sequences present in each Genomovar group was not defined. We found that their PCR primers for the 16S-23S ISR would not have targeted a single specific ISR in the genome, because their primer sequences are found in all six of our ISR sequences. So, phylogenetic analyses based on sequences from these PCR products may not be as valuable as originally thought. Recently, this has also been observed in another aquatic bacterial pathogen, *Edwardsiella ictaluri* (Williams et al. 2008). Panangala *et al.* (2005) used the 16S-23S ISR to study the interspecies and intraspecies differences of *E. ictaluri* and

Edwardsiella tarda. Following this, Williams *et al.* (2008) found that *E. ictaluri* has eight copies of the *rrn* operon, all of which have similar structure and flanking regions, with two having a unique feature. The previous phylogenetic analysis studies on *F. columnare* with the 16S-23S ISR sequences also did not use specific operon primers, making these early results on *F. columnare* suspect as well. Recently, Suomalainen *et al.* (2006) studied the molecular diversity of *F. columnare* strains from Finland using 16S RFLP, sequence analysis, length heterogeneity analysis of PCR (LH-PCR) products, and ARISA (automated ribosomal intergenic spacer analysis). As suggested by Gurtler and Stanisich (1996), Suomalainen *et al.* (2006) found that ARISA was a very useful typing method when there may be multiple rRNA operons. Even though it may be possible that AFLP and RFLP analyses may not be affected by the presence of multiple 16S-23S ISR PCR products from different *rrn* operons, the possibility exists that one operon could be preferentially amplified in one isolate and a different operon could be preferentially amplified in a different isolate; therefore, it would still be best to choose specific operon primers that would consistently amplify the same ISR.

Construction of physical maps of genomes using pulsed-field gel electrophoresis requires enzymes that cut the genome into an analyzable number of fragments; most produce too many fragments. I-*Ceu*I digestion followed by pulsed-field gel electrophoresis is a powerful tool for determining genome structure and evolution. RFLP analysis of bacterial genomes by PFGE has repeatedly proven to be a useful tool for genotypic analysis of different pathogenic bacteria such as *Bacillus* (Harrell et al. 1995; Leonard et al. 1998; Zhong et al. 2007), *Staphylococcus* (Grady et al. 2001; Osawa et al.

2002), *Escherichia coli* (Osawa et al. 2002), and *Salmonella* (Foley et al. 2006; Bolton et al. 2007). RFLP combined with PFGE has also been used for genotypic studies *Flavobacterium meningosepticum* (Sader et al. 1995), *F. psychrophilum* (Arai et al. 2007) and *F. columnare* (Soto et al. 2007; Soto et al. 2008). The use of type II restriction endonucleases has revolutionized the study of molecular biology; the intron-encoded endonucleases (Colleaux et al. 1986) have a large impact on the field. I-*Ceu*I (Dujon et al. 1989; Gauthier et al. 1991; Marshall et al. 1994) is a representative of this kind of endonuclease. Many others have also been reported, and some are commercially available, including I-*Ppo*I (Muscarella and Vogt 1989), I-*Sce*I (Colleaux et al. 1986), I-*Sce*II (Delahodde et al. 1989), I-*Tev*I and I-*Tev*II (Bell-Pedersen et al. 1990), and I-*Cre*I (Durrenberger and Rochaix 1991). However, I-*Ceu*I is particularly useful, because it is specific for and cuts in a long recognition sequence only found in the *rrl* (23S rRNA) gene. Because rRNA sequences are strongly conserved, this sequence is present in the genomes of many enteric bacteria such as *Salmonella typhimurium* and *Escherichia coli* (Liu et al. 1993a; Liu et al. 1993b; Liu and Sanderson 1995) and in *Rhizobium meliloti* (Honeycutt et al. 1993), as well as in chloroplasts and mitochondria of eukaryotes. Additionally, digestion of DNA of other bacteria (species of *Haemophilus*, *Neisseria*, *Proteus*, and *Pasteurella*) with I-*Ceu*I suggested that only *rrn* genes are cut in all these species (Liu et al. 1993b). Because I-*Ceu*I cleaves only *rrn* genes and because the number and locations of these genes are highly conserved in enteric bacteria (Krawiec and Riley 1990), related wild-type strains should yield similar I-*Ceu*I fingerprints which give valuable genomic information on DNA insertions or rearrangements. Because the

recognition site of I-*Ceu*I should only be found inside the 23S rDNA gene, we used it for PFGE to aid our study of *F. columnare*. The genome of *F. columnare* is circular; therefore, the number of fragments observed should indicate the number of ribosomal operons present in the genome. The restriction profile for the *F. columnare* strain ATCC49512 resulted in three PFGE fragments which would indicate that there should be three copies of the 23S rRNA gene. However, after performing Southern blot analysis with different restriction enzymes, we detected the presence of a minimum of four distinct bands and as many as six. Each digestion product contained a predominant band suggesting the presence of more than one 16S rRNA gene target, suggesting as many as seven copies of the 16S rRNA gene. This, in turn, indicates as many as seven copies of the 16S-23S ISR. One possibility of why we only found six ISRs through sequencing is that like *F. johnsoniae* and *F. psychrophilum*, *F. columnare* could contain some identical 16S-23S ISRs. After cloning the 16S-23S ISR PCR product that was amplified from the I-CeuI digest used for PFGE, we found that each PFGE fragment gave us two different sequences. This could be explained either by mutations in the long restriction enzyme site or by gene orientation. Previous preliminary studies with *F. columnare* and I-*Ceu*I digests used with PFGE revealed that the isolates tested have as few as three fragments (ATCC 49512, 155-94) and as many as four fragments (143-94, LADL 92-002) (Soto 2008).

Flavobacterium columnare infection causes great commercial losses in the aquaculture industry. A method to easily identify virulent strains in the clinical setting is yet to be determined. This study shows that a columnaris isolate type can be identified

by direct sequencing the 16S-23S ISR as long as a specific operon is always targeted. This information could be used in future research and in diagnostic settings to possibly identify extremely virulent strains. Our initial goal was to design primers that would allow amplification of overlapping fragments from one operon. Because all of the 16S-23S ISRs from PFGE 16S-23S ISR cloning contained both $tRNA_{Ala}$ and $tRNA_{Ile}$ this was not possible with the primers we first designed. From the information gathered as a result of PFGE 16S-23S ISR cloning, we were able to redesign one primer set to span almost the entire 16S-23S ISR and target the most similar ISRs. In the future, it would be interesting to see if either of the more dissimilar PFGE ISRs, A_535_bp or F_698_bp, could be used instead. The fragment sizes are a convenient size for rapid sequencing and may eventually be used to identify whether or not a strain is virulent.

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