Development, Characterization and Early Evaluation of New Modified Live Vaccines Against Columnaris Disease

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 14th, 2010

Keywords: Columnaris disease; Live vaccine; *Flavobacterium*; Attenuated mutant; Fish protection

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Abstract

Columnaris disease is an acute to chronic bacterial infection that affects commercial aquaculture and the ornamental fish industry. *Flavobacterium columnare*, causal agent of columnaris disease, is a facultative pathogen ubiquitous of fresh water environments. Prevention is the best control measure, and vaccination is a feasible alternative. A commercial vaccine is currently available. Extensive genetic studies of the bacteria have led to the distinction of two genomovars within the species, being genomovar II more virulent to catfish. The commercial vaccine belongs to the less pathogenic genomovar. Thus, efforts to develop new vaccines against the most virulent *F. columnare* type were needed. In this study new rifampicin mutants of *F. columnare* genomovar II have been developed, fully characterized and tested in lab settings against columnaris infection on catfish. Preliminary results proved that some of the newly developed mutants conferred more protection against columnaris diseases than the current commercial vaccine.

Acknowledgments

The studies included in this thesis would not be possible without the presence of a few people and the help from many others. First I need to thank Covadonga for her help and support from the very beginning; without her nothing of this would ever happen. I also want to thank the Department of Fisheries and Allied Aquacultures, from the department head to the staff, students and professors, for giving me the opportunity to develop a new career and care about aquaculture, always with much kindness. Thanks to Dr. Jeff Terhune and Dr. David Rouse for accepting to go along in this atypical journey. And the persons that, each one in their own way, helped me. From the lab my friends Ryan, Matt, Tao, Andrea, Yinfeng and Stacey, who just came aboard but who contributed with work and much needed enthusiasm. Chris Sayles, my eyes and hands at the Disease Lab, always with the best advice. Priscilla Barger and Dr. Joe Newton, for the supply of so many catfish, always with a smile. To the Genetics Unit for the supply of catfish. To Dr. Michael Miller for his lessons on microscopy. To Dr. David Carpenter for his help with the statistical analysis. And to all the persons who indirectly helped my research by sharing their time with me; from playing tennis, soccer or music, to just talking to me with so much care. And special thanks go to María José. Thanks for coming all the way to Auburn, and back…

A mis padres, a mis hermanas y hermanos

Por su respeto y cariño incondicionales

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
BMPs	Best Management Practices
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
ESC	Enteric Septicemia of Catfish
FDA	Food and Drug Administration
ISR	Intergenic Spacer Region
LPS	Lipopolysaccharide
mA	milliamps
MIC	Minimal Inhibitory Concentration
PCR	Polymerase Chain Reaction
ppm	parts per million
ppt	parts per thousand
PVDF	Polyvinylidenedifluoride
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SSCP	Single Strand Conformation Polymorphism
VFD	Veterinary Feed Directive

GENERAL OVERVIEW

Columnaris disease, caused by *Flavobacterium columnare*, has been described as a globally-distributed acute to chronic bacterial infection of freshwater and brackish water fish. Many commercially important species are affected by columnaris disease, including but not limited to the channel catfish (*Ictalurus punctatus* Rafinesque), common carp (*Cyprinus carpio* Linnaeus) and other carps, rainbow trout (*Oncorhynchus mykiss* Walbaum) and other salmonids, Japanese eel (*Anguilla japonica* Temminck & Schlegel), tilapia (*Oreochromis* spp.) and a variety of ornamental species as goldfish (*Carassius auratus* Linnaeus) (Plumb 1994,1999; Austin and Austin 1999). Ictalurids are often the most severely affected, but serious infections have been reported in other species as well (Plumb 1999; Shoemaker et al. 2003a). Historically, columnaris disease has been considered the second most important bacterial infection in channel catfish after *Edwardsiella ictaluri*, the causative agent of enteric septicemia of channel catfish (ESC).

Flavobacterium columnare is a gram negative, strictly aerobic and non-halophilic rod bacteria measuring 2-10 µm in length, with flexing and gliding motility that produces yellow colonies on agar (Bullock et al. 1986). *Flavobacterium columnare* can be distinguished from other fish pathogens by its ability to grow on media containing neomycin and polymixin B or by its rhizoid pattern of growth on a low nutrient and low agar containing medium (Plumb 1999; Griffin 1992). Columnaris disease was first described in 1922 by Herbert Spencer Davis, who observed the characteristic columnar masses of bacterial cells isolated from fish during a major die off in the Mississippi River (Davis 1922). The bacterium was originally named *Bacillus*

columnaris, but the taxonomic classification of this organism has changed over the years. The last emendation resulted in the organism being renamed as *F. columnare* (Bernardet et al. 1996).

In aquaculture settings, disease outbreaks are often related to environmental stress associated to high stocking densities, increased feeding rates, elevated organic loads and high temperatures. Columnaris disease is characterized by an acute to chronic infection of the gills and the integumentary system, including fins. Lesions can occur along the dorsal midline and later extend to the dorsal fin along the lateral flanks, commonly referred to as “saddleback”. Peduncular and peri-oral lesions are common signs of columnaris. The disease is often initiated as an external infection on the fins, body surface or gills, which can then progress to necrotic lesions with yellowish-orange mucoid material. In some cases, columnaris can become systemic with little or no visible pathological signs (Bullock et al. 1986; Plumb 1999; Roberts 2001).

This bacterium has been described as phenotypically homogeneous but genetically diverse. Triyanto and Wakabayashi (1999) described the presence of three genomovar within the species based on DNA:DNA hybridization studies (genomovars I, II and III). Genetic diversity among and between these groups has been further explored using sequence and fingerprinting methods. Molecular analyses of the *16S rDNA* gene or the intergenic spacer region (ISR) are the most commonly used techniques to analyze the genetic diversity of *F. columnare*. Methods used include PCR with restriction fragment length polymorphism (RFLP), PCR followed by sequencing, or PCR follow by single-strand conformation polymorphism (SSCP) (Triyanto and Wakabayashi 1999; Arias et al. 2004; Figueiredo et al. 2005; Darwish and Ismaiel 2005; Schneck and Caslake 2006; Olivares-Fuster et al. 2007a). Other

fingerprinting methods used to analyze the intraspecific diversity of *F. columnare* are Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Pulsed Field Gel Electrophoresis (PFGE) (Arias et al. 2004; Thomas-Jinu and Goodwin 2004b; Soto et al. 2008a). Identification of genetic groups within the species led to pathogenicity studies that proved genomovar II is the most virulent to channel catfish (Shoemaker et al. 2008). Also, a genomovar-specific association to fish hosts in natural settings of the different genomovars have been demonstrated, with genomovar II being mainly associated to channel catfish (Olivares-Fuster et al. 2007a). Another study has shown that skin mucus induced a greater chemotactic response in genomovar II than in genomovar I *F. columnare* isolates (Klesius et al. 2008). Although the role that chemotaxis plays in the virulence of *F. columnare* is not fully defined, the authors suggested a correlation between chemotactic response of genomovar ll isolates and virulence.

Prevention is the best measure against columnaris disease, although treatment options exist and are summarized below in the literature review. Given the ubiquity of *F. columnare* in aquatic environments it is unrealistic to consider eradication as an option. Stress is the most important trigger of the disease therefore reducing stressors (handling, water quality, overcrowding…) will likely have a critical effect on avoiding epizootic episodes. Columnaris disease might also be prevented through vaccination. Aquavac-Col® (Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands), a commercially available rifampicin-resistant live vaccine is now approved and being used by farmers to prevent columnaris disease in channel catfish. Information about the effectiveness of the vaccine on aquaculture settings is lacking, and recent studies have shown that the vaccine was originated from a genomovar I strain of *F. columnare*.

As mentioned above, it is now known that the more virulent strains for channel catfish belong to *F. columnare* genomovar II (Shoemaker et al. 2008).

In order to determine if the genetic origin of the commercial vaccine could affect the vaccine efficacy in protecting channel catfish, the objective of this work was to develop new mutants from strains belonging to the more virulent genomovar II. A complete characterization of the new mutants by comparison with their parents and other related strains has been performed, as well as preliminary tests to determine its avirulence and protective capabilities.

LITERATURE REVIEW

1. THE PATHOGEN

1.1 Bacteriology

The characteristics of *Flavobacterium columnare* have been described in detail by Plumb (1999) and Bernardet (1989). Table 1 resumes its most important biophysical and biochemical traits according to Plumb (1999). *Flavobacterium columnare* is an aerobe, Gram-negative, slender, non-flagellated rod that measures 3 to 10 µm long and around 0.5 µm in diameter; it is motile by gliding, produces yellow colonies on agar and decomposes several polysaccharides (but not cellulose). It has been described that the organism has a wide temperature range (4 to 37°C), with an optimal temperature around 25 to 30°C (Amend 1982; Plumb 1999; Roberts 2001). Most of the isolates appear as spreading, rhizoid, discrete colonies with yellow centers and irregular margins that adhere tightly to the media (Ordal and Rucker 1944). The bacterium forms columnar aggregates on infected tissue that are often referred to as "haystacks". *Flavobacterium columnare* strains are able to grow in Cytophaga broth supplemented with 0.5% NaCl (Bernardet 1989), although have been described as non-halophilic (Pacha and Ordal 1967; Pacha and Porter 1968).

Bernardet and Grimont (1989) described the physiological characteristics of *F. columnare* as follows: strict aerobic growth; no acid produced from carbohydrates; cytochrome oxidase and catalase positive; reduces nitrate to nitrite; hydrogen sulfide is produced; cellulose, chitin, starch, esculin, and agar are not hydrolyzed; gelatin, casein, and tyrosine are hydrolyzed; arginine, lysine, ornithine are not decarboxylated.

Bertolini and Rohovec (1992) reported that 13 *F. columnare* isolates degraded gelatin, casein, hemoglobin, fibrinogen, and elastin. The genus *Flavobacterium*, and especially *F. columnare*, secrete enzymes that can cause rapid DNA hydrolysis (Bernardet and Grimont 1989).

Table 1. Biophysical and biochemical traits of *Flavobacterium columnare* according to Plumb (1999).

Characteristic	***Flavobacterium columnare***
Cell morphology	Long, Gram-negative rod
Cell size (µm)	0.3-0.5 X 3-10
Yellow pigmented colony	+
Motility	Gliding
Flexirubin pigment	+
Binds Congo Red	+
Resistant to neomycin sulfate, polymixin B	+
Chondroitin lyase	+
α- nitrophenyl-β-D-galactopymanoride	-
Growth on peptone	+
Glucose as carbon source	-
Acid from carbohydrates	-
Gelatin degradation	+
Casein degradation	-
Starch degradation	-
Tyrosine degradation	-
Urease activity	?
H_2S production	+
Nitrate reduction	-
Catalase activity	+
Cytochrome oxidase activity	+
Optimum growth at (°C)	25-30
Growth tolerance (°C)	10-37
G + C content (mol%)	32-37
Habitat	Freshwater

Griffin (1992) developed a simple procedure for identifying this bacterium. The procedure, cited by Durborow et al. (1998), takes advantage of five biochemical and cultural characteristics that in combination are believed to be unique to *F. columnare*. These characteristics are: 1) the ability to grow in presence of neomycin sulfate and

polymixin B (Fijan 1968); 2) the production of yellow pigmented rhizoid (root-like in appearance) colonies; 3) the ability to degrade gelatin; 4) the ability to bind congo red dye, which is related to the extracellular production of galactosamine glycan (Johnson and Chilton 1966; McCurdy 1969); and 5) the ability to degrade chondroitin sulfate A. Chondrotin AC lyase, an enzyme produced by *F. columnare*, degrades polysaccharides, particularly those found in cartilaginous connective tissue (Griffin 1992; Teska 1993).

The bacterium produces yellow colored bacterial colonies as a result of flexirubin pigments. Production of flexirubin pigments was demonstrated by Reichenbach and Dworkin (1981) by using the potassium hydroxide method. The color of the colonies and the flexing and gliding movement of individual rods can differ depending on the culture medium utilized (Song et al. 1988; Bader et al. 2005; Kunttu et al. 2009). Colony colors range from pale yellow, greenish yellow, yellow to golden yellow (Griffin 1992).

According to Decostere et al. (1999a), the bacterium produces a capsule and the thickness of the capsule appears to be correlated with virulence. High virulence strains have a thick 120-130 nm capsule, while strains with low virulence have a thinner 80-90 nm capsule, as observed by transmission electron microscopy.

1.2 Taxonomic position

Columnaris disease was first described in 1922 by Herbert Spencer Davis, who observed the characteristic columnar masses of bacterial cells isolated from affected gills and fins during a major die off of fish in the Mississippi River, at Fairport, Iowa

(Davis 1922). The bacterium itself was not isolated, but it was described in wet mounts and named *Bacillus columnaris*.

The nomenclature for this organism has changed over the years due to improvements in phylogenetic classification methodologies. It was initially included in the order Myxobacteriales, and the bacterium was renamed *Chondrococcus columnaris* by Ordal and Rucker (1944). One year later, Garnjobst (1945) placed the organism in the genus *Cytophaga* and suggested *Cytophaga columnaris* as the new name. Buchanan and Gibbons (1974) observed that the columnaris bacterium produced neither fruiting bodies nor microcysts, therefore it was removed from the Myxobacteriales and placed in the order Cytophagales, and renamed *Flexibacter columnaris*. Bernardet and Grimont (1989) presented DNA relatedness data and a complete phenotypic characterization to justify retaining the name *Fl. columnaris*. Later on, Bernardet et al. (1996) published a very detailed study of the *Fl. columnaris*, including more than 100 strains (with 81 of them belonging to the genera *Flavobacterium*, *Cytophaga* or *Flexibacter*). This study showed that the bacterium should be transferred to the *Flavobacterium* genus, with the name *Flavobacterium columnare* as the organism that causes columnaris disease in freshwater fish. The analysis of the 16S rRNA gene revealed a high degree of variability between the different strains, but the percentages of DNA-DNA hybridization recommended to maintain all strains under a unique species. The genus *Flavobacterium*, yellow pigmented bacteria, contains additional fish pathogens such as *F. psychrophilum* (Bernardet et al. 1994).

1.3 Isolation and culture

The primary isolation of most Flavobacteria is problematic, and in many cases has impeded investigations on the pathogenesis of *Flavobacterium* species (Anderson and Norton 1991; Dalsgaard 1993; Shotts and Starliper 1999). It is common that in *F. columnare* cases submitted to diagnosis labs the bacterium is present in a mixed infection with other pathogens such as: *Aeromonas* spp., *Edwardsiella ictaluri* or *E. tarda* (Hawke and Thune 1992). This, altogether with the typically slow growing rate of *F. columnare* in lab conditions, poses a problem in the identification of the primary etiological agent.

Flavobacterium columnare does not grow on standard bacteriological isolation media such as “blood agar”, “brain-heart infusion” and “tryptic soy agar”, or on standard Mueller Hinton agar. It requires specialized media for isolation, culture and antibiotic sensitivity testing. *Flavobacterium columnare* grow on medium with low nutritional content and high moisture. The medium should be prepared for same day use, which ensures the correct moisture level. Media formulated for isolation and culture of the bacteria include Ordal’s (Pacha and Ordal 1967), Hsu-Shotts (Bullock et al. 1986), a modification of Fijan’s media (Fijan 1969) by Hawke and Thune (1992), and the Shieh media (Shieh 1980) or some of its proposed modifications (Song et al. 1988; Shoemaker et al. 2005a).

Following the evaluation of different culture media formulated for *F. columnare*, Farmer (2004) concluded that the low nutrient content of the selective Cytophaga agar (SCA) gave the best results for inhibiting other bacteria and promoting the isolation of *F. columnare* in agar media. The *F. columnare* growing media (FCGM) yield faster

growth, higher number of cells and seemed to prevent cell clumping, thus giving the best results for broth culture. And tryptone yeast extract (TYA) agar with increased moisture was best for maintenance of cultures.

Other studies have shown the growth response of *F. columnare* to be better in Chase, Shieh, and Liewes media containing salts, with the best growth in Shieh medium at 24 hours (Song et al. 1988). Holt was able to routinely culture *F. columnare* with tryptone yeast extract plus salts (TYES) medium at 25°C (Holt 1988). Meanwhile, more enzyme activity per mg bacterial dry weight was achieved in cytophaga broth as compared to TYES, Hsu-Shotts, and Shieh broths (Newton et al. 1997).

In a typical mixed columnaris infection, the dominating *F. columnare* strain can be masked by several saprophytic species of the same genus or other genera, or its growth can be completely inhibited by antagonistic bacteria such as *Pseudomonas* sp. (Tiirola et al. 2002). The authors also reported that the isolation of *F. columnare* was unsuccessful from a number of fish samples (44%) that contained filamentous Gram negative bacteria in microscopic examination. In a competition study, *F. columnare* failed to invade fish in the presence of *Citrobacter freundii* at an initial number approximately 100 times that of *F. columnare* in environmental water (Chowdhury and Wakabayashi 1989a). Fijan (1969) noted that, when trying to isolate *F. columnare*, the spreading growth or predominance of other bacteria sometimes obscures, overgrows, or prevents the formation of discrete isolated colonies.

The minimal inhibitory concentration (MIC) corresponds to the lowest concentration of a drug in a dilution series that inhibits growth of a bacterial strain (Andrews 2001). After determining the MIC for *F. columnare* on polymixin B to be 1000

units/ml and neomycin to be 100 µg/ml, Fijan (1969) recommended adding 5 µg/ml neomyocin and 5 units/ml polymixin B to cytophaga agar to make the medium selective for the isolation of *F. columnare* against other inhibiting bacteria. Hawke and Thune (1992) reported that this selective media did not inhibit many of the bacteria in mixed infections from diseased channel catfish, so the authors improved the formulation by increasing the polymixin B content to 200 units/ml. This medium was effective in inhibiting all bacterial species tested except *Flavobacterium* spp. and *Streptococcus* spp. (Hawke and Thune 1992). Tobramycin at 1 µg/ml has also been reported to be selective for the primary isolation of *F. columnare* from diseased fish in Shieh media (Decostere et al. 1997), inhibiting the growth of *Aeromonas* sp., *Shewanella* sp. and *Pseudomonas* sp.

The MIC of the following drugs was determined for *F. columnare*: amoxicillin 0.06 µg/ml, oxytetracycline 0.06-0.12 µg/ml, oxolinic acid 0.06-0.12 µg/ml, norfloxacin 0.12 µg/ml, and trimethoprim > 64 µg/ml (Soltani et al. 1996). Hawke and Thune (1992) reported the MIC for *F. columnare* isolates for Romet® to be 7-15 µg/ml with disk diffusion zones ranges from 22-28 mm and the MIC for Terramycin® to be 0.195 µg/ml with zones of 38-40 mm.

Flavobacterium columnare isolates show resistance to several antibiotics, characteristic that can be used to isolate the bacteria, but that also reflects the ability of the bacteria to develop resistances. Bernardet and Grimont (1989) reported the bacterium to have 0 mm disk diffusion zones around gentamicin (15 µg), neomycin (30 µg), kanamycin (30 µg), polymixin B (30 µg), trimethoprim (5 µg), and actinomycin D (2.5 µg) disks. Isolates are usually sensitive to oxytetracycline and nifurpirinol, but can become resistant to ormetoprim-sulfadimethoxine (Hawke and Thune 1992). An

important data for the Aquaculture industry was reported by Johnson (1991), who found that out of 207 *F. columnare* isolates obtained from channel catfish in 1990 by the Delta Research and Extension Center (Stoneville, MS) two were resistant to Terramycin® and 60 were resistant to Romet®.

1.4 Strain variability

Several studies have revealed variation between isolates of *F. columnare* cultured from different hosts and geographical regions. The first attempt to discriminate between *F. columnare* isolates was made by Anacker and Ordal (1959). These authors divided the species into four different serotypes and one miscellaneous group. Song et al. (1988) found three types of colony morphology among the species. Warm water and coldwater *F. columnare* isolates can be differentiated based on biochemical test results in the API 20E® system (BioMereux Vitek Hazelwood, MO) (Pyle and Shotts 1980). Several authors have reported slight phenotypic variations within *F. columnare* such as ability to grow at 15 and 37°C (Bernardet 1989) and nitrate reduction (Shamsudin and Plumb 1996). Shamsudin and Plumb (1996) characterized morphological, biochemical and physiologically different isolates of *F. columnare*. They reported that four isolates of *F. columnare* from four different fish species showed uniform biochemical characteristics, although some of the morphological characteristics differed between strains (mainly colony morphology). This study also demonstrated some minor physiological variation between isolates such as the ability to grow at 15°C, on media with 0.5% NaCl, or at pH 6 or less, or pH 10 or higher. Studies by Decostere et al. (1998) also illustrated variations in temperature growth ranges among isolates recovered from tropical and temperate fish species.

Significant differences in the virulence between different strains of *F. columnare* have been reported (Rucker et al. 1953; Bullock et al. 1986; Decostere et al. 1998, 1999a, 1999c; Thomas-Jinu and Goodwin 2004b; Shoemaker et al. 2008). Some of these authors demonstrated differences in virulence among strains of *F. columnare* when infecting different fish species (channel catfish (*Ictalurus punctatus*), black mollies (*Poecilia sphenops* Valenciennes), common carp (*Cyprinus carpio*) or golden shiners (*Notemigonus crysoleucas* Mitchill)) by intramuscular injection or immersion exposure methods. In the studies by Shoemaker et al. (2008), the differences in virulence were correlated to the genomovar adscription of the strain, being genomovar II more virulent to channel catfish than genomovar I. Low virulence strains produced necrotic lesions on the gills and/or body surface, and systemic infections could occur, with a several days elapse before mortality (Rucker et al. 1953). Meanwhile, high virulence strains caused death within 24 to 48 hours post exposure to the pathogen (Pacha and Ordal 1970). Variation in the degree of virulence has also been reported between isolates of *F. columnare* isolated from salmonids (Amend 1982).

Research comparing lipopolysacharide and protein profiles from different strains of *F. columnare* demonstrated correlation to virulence among isolates exhibiting different profiles (Newton et al. 1997; Stringer-Roth et al. 2002; Zhang et al. 2006). Extracellular proteases were used to separate isolates into two groups with apparent molecular masses of 58 and 53.5 kD (group one) and 59.5, 48, and 44.5 kD (group two) (Newton et al. 1997). The proteolytic activity of *F. columnare in vitro* suggests that the production of extracellular proteases is an important virulence mechanism of the bacterium (Song et al. 1988; Bernardet 1989; Griffin 1991; Bertolini and Rohovec 1992; Teska 1993; Plumb 1994).

Flavobacterium columnaris shows a high phenotypic and physiological homogeneity and, therefore, the use of standard biochemical tests or chemotaxonomic markers such as fatty acid methyl ester (FAME) analysis, are not useful for *F. columnare* strain characterization. But development of molecular methods such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), end-label sequencing, DNA-DNA hybridization, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP®) and pulsed filed gel electrophoresis (PFGE) have given researchers valuable tools to identify and analyze the genetic variability of *F. columnare*. The above mentioned tools have provided a better understanding of the intraspecies genotypic diversity of *F. columnare* isolates cultured from a large number of hosts from various geographical regions in an attempt to better understand the epidemiology of the columnaris disease (Bader and Shotts 1998; Triyanto and Wakabayashi 1999; Michel et al. 2002; Thomas-Jinu and Goodwin 2004b; Arias et al. 2004; Figueiredo et al. 2005; Darwish and Ismaiel 2005; Schneck and Caslake 2006; Olivares-Fuster et al. 2007a, 2007b; Soto et al. 2008a).

DNA relatedness studies between *F. columnare* strains revealed homologies as low as 78% (Bernardet and Grimont 1989). Toyama et al. (1996) showed an existing intra-species variation among strains of *F. columnare* based on *16S rDNA* sequences. This work set up the basis for the Triyanto and Wakabayashi (1999) study that led to the description of three genomovars within this species.

The term “genomovar” was introduced to denote phenotypically similar but genotypically distinct groups of bacterial isolates within a species (Rosselló et al. 1991; Ursing et al. 1995). This term is of tremendous interest for the characterization of *F.*

columnare isolates, since the bacterium is highly homogenous phenotypically but shows high genetic diversity. Polymerase chain reaction (PCR) can be used to amplify the highly conserved *5S*, *16S*, and *23S* genes coding for the ribosomal subunits, which can potentially differentiate species and also show intraspecific differences (Triyanto and Wakabayashi 1999; Triyanto et al. 1999; Michel et al. 2002). Nested PCR techniques were developed for this bacterium and can be used to divide phenotypically identical strains of *F. columnare* into three distinct "genomovars" (Triyanto and Wakabayashi 1999). Genomovars were defined based on RFLP analysis of the 16S rDNA gene but were also confirmed by DNA:DNA hybridization. DNA relatedness between strains belonging to the same genomovar was higher (ranging from 83 to 100% homology) than between strains from other genomovars (homologies lower than 69%).

Afterwards, several authors have used the *Hae*III-RFLP analysis of the *16S rDNA* gene as a marker for genomovar ascription in *F. columnare*. All three genomovars have been reported in Asia and in the USA, while only genomovar I has been isolated from farm/wild outbreak in Europe (Decostere et al. 1998). Amplification of the more variable Internal Spacer Region (ISR) of the bacteria has also allowed strain differentiation (Arias et al. 2004), and application of the SSCP technique has revealed a higher level of polymorphisms into these regions of the different *F.* columnare isolates (Olivares-Fuster et al. 2007a, 2007b). Randomly amplified polymorphic DNA analysis has been used to show intraspecific genetic variation among *F. columnare* isolates from warm water fish species (Thomas-Jinu and Goodwin 2004b), and AFLP, a more powerful and reproducible technique, has showed

the polyclonal nature of this species as isolates from distant geographical locations showed surprisingly similar AFLP profiles (Arias et al. 2004).

2. THE DISEASE

2.1 Epidemiology

Flavobacterium columnare is a ubiquitous water-borne bacterium. Natural epizootics of the disease are common. The bacteria is found worldwide and columnaris disease affects practically all species of freshwater fishes and some amphibians (Snieszko and Bullock 1976; Becker and Fujihara 1978). It has been confirmed only in freshwater fishes; however, some marine fish are infected by myxobacterial diseases that are similar to columnaris (Bullock et al. 1971). Columnaris is one of the most common bacterial infections seen in freshwater fish aquaculture. Columnaris disease has been documented from many fish species throughout the world, including many commercially important species such as: channel catfish (*Ictalurus punctatus*); common carp (*Cyprinus carpio*); goldfish (*Carassius auratus*); eels (*Anguilla japonica*, *A. rostrata* Kaup, *A. anguilla* Linnaeus); tilapia (*Oreochromis* spp.); most hatchery-reared salmonids like chinook salmon (*Oncorhynchus tshawytscha* Walbaum), rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta* Linnaeus) and brook trout (*Salvelinus fontinalis* Mitchill); and other cool water species such as the tiger muskellunge (*Esox masquinongy* Mitchill) or the walleye (*Sander vitreus* Mitchill) (Amin et al. 1988; Grabowski et al. 2004; Bootsma and Clerx 1976; Altinok and Grizzle 2001; Decostere et al. 1999b, 1999c; Del Corral 1988; Newman 1993, Austin and Austin 1999; Moyer and Hunnicutt 2007; Anderson and Conroy 1969; Schachte 1983;

Wakabayashi 1991; Rach et al. 2003). The disease also affects important recreational species such as the largemouth bass (*Micropterus salmoides* Lacepede), bluegill (*Lepomis macrochirus* Rafinesque), yellow perch (*Perca flavescens* Mitchill) and crappies (*Pomoxis nigromaculatus* Lesueur, *P. annularis* Rafinesque) (Davis 1949; Bebak et al. 2009; Macfarlane et al. 1986). Reports on ornamental species popular in the aquarium trade like goldfish (*Carassius auratus*), platies (*Xiphophorus maculatus* Gunther), black mollies (*Poecilia latipinna* Lesueur, *P. sphenops*) or zebra fish (*Danio rerio* Hamilton) are also numerous (Wakabayashi 1993; Decostere et al. 1998; Decostere et al. 1999a; Altinok 2004). It is believed that no wild or cultured freshwater fish are totally resistant to columnaris (Noga 2010; Plumb 1999).

Data concerning its natural habitat and prevalence are scarce. It is accepted that fish are the primary reservoir for the bacteria, especially in dense fish populations (Pacha and Ordal 1970; Schachte 1983; Plumb 1999). When fish are under stress due to elevated temperatures, crowding, etc., *F. columnare* may be better fit than the fish and therefore cause disease. Lesions generally develop in 24 to 48 hours after handling, followed by death at 48 to 72 hours if not treated. Infected animals with gill or cutaneous lesions serve as a source of infection. In hatcheries supplied with open water, any species of infected fish in the water supply may serve as a reservoir of infection for the disease. Pacha and Ordal (1970) demonstrated that feral fish, such as catostomids, coregonids and cyprinids, may serve as reservoirs of infection. In a recent study it was possible to establish a correlation between the *F. columnare* genomovars and the predominant wild fish species in the Mobile River (Alabama, USA) (Olivares-Fuster et al. 2007a). In this work, coexistence of *F. columnare* genomovars I and II in the natural environment was proved, as well as a significant association of the strains

belonging to genomovar I with threadfin shad and the ones from genomovar II to catfish, both channel and blue.

Some studies point out the capability of *F. columnare* to survive in water for days. Arias et al. (2006) conducted a culture-independent study of the bacterial community in catfish ponds in Alabama. In that study two main catfish pathogens, *F. columnare* and *Edwardsiella tarda*, were detected in pond water in the absence of infective episodes. *Flavobacterium columnare* represented up to 8% of the total clones sequenced. No correlation could be made between the presence of the pathogens and the composition of the bacterial community or outbreak occurrences in those ponds (Arias et al. 2006), thus supporting the idea of this bacteria acting as a facultative pathogen.

Transmission of *F. columnare* can be indirect via the environment or by cohabitation with carrier fish shedding the bacterium. Direct transmission is also possible as result of contact with infected fish. Although the bacteria can survive in the aquatic environment and mud, fish seem to serve as the primary reservoir (Bullock et al. 1986; Noga 2010; Welker et al. 2005). Moreover, while confirming a newly developed PCR-based detection method for *F. columnare*, Welker et al. (2005) confirmed that the disease can be transmitted horizontally indirectly through the water column without fish-to-fish contact.

The disease may occur as a primary or secondary infection and clinically has been described as having a chronic, acute, or per-acute onset (Roberts 2001). *Flavobacterium columnare* can act both as a primary pathogen or a secondary opportunistic pathogen, although columnaris disease is often thought of as an

opportunistic infection with stress on the fish population being a prerequisite for infection.

Wakabayashi (1991) extensively reviewed the main environmental factors affecting the survival and infectivity of the bacteria. Several factors play an important role in the development of the disease, being temperature one of the main determinants. Although columnaris disease could occur throughout the whole year, warm-weather favors *F. columnare* infection and columnaris is problematic mainly in the warmer periods of the year (Davis 1922). Generally, epizootics occur when the water temperature is in the region of 18-22°C, and the disease is rarely an issue at temperatures below 15°C (Amend and Ross 1970). Thus, most outbreaks of columnaris occur between May and October in the northern hemisphere. Stoskopf (1993) reported that columnaris disease is usually more pathogenic at temperatures above 15° C, and that mortality and acuteness of disease onset typically increase with temperature.

In addition to water temperature, the severity of columnaris is also influenced by stress factors related to water quality such as high mineral content in the water (hardness), low salinity, low oxygen (Chen et al. 1982), high ammonia (Chen et al. 1982), high nitrite (Hanson and Grizzle 1985) and organic pollution (Wakabayashi 1991). *Flavobacterium columnare* can persist in water for up to 32 days when the hardness is 50 ppm or more, but a hardness of 10 ppm reduces viability considerably. The addition of carbon to the system increases the survival of the bacteria in hard water, but this is not the case in soft water (Wakabayashi 1991). Chen et al. (1982) described the highest eel mortality levels to be associated with stagnant water, whereas the lowest losses occurred in running water. Interestingly, with aeration the

total losses fell between these two extremes; the mortality rate was inversely correlated with the level of dissolved oxygen in the water. Moreover, with adequate dissolved oxygen, ammonia levels had a direct correlation with the increase in mortalities. It has been reported that fish age is more important than weight in determining susceptibility to columnaris infection (Austin and Austin 1999).Although epidemiologic data is not available, it is possible that the combination of fish species with specific *F. columnare* genomovars may also be a key determinant of the severity of the epizooty, as suggested by Olivares-Fuster et al. (Olivares-Fuster et al. 2007a).

Additional factors associated with outbreaks include mechanical or physiological injuries due to crowding, starvation, netting, repeated and rough handling, or transporting (Kumar et al. 1986; Stoskopf 1993; Noga 2010; Plumb 1999; Decostere et al. 1999a; Altinok and Grizzle 2001; Shoemaker et al. 2003a; Suomalainen et al. 2005b). Mechanically abraded fish are more likely to be infected by *F. columnare* (Davis 1922; Bader et al. 2003a), and Shoemaker et al. (2003a) demonstrated that deprivation of feed for 7 days reduced innate resistance of catfish to *F. columnare*.

Another important factor to fully understand the epidemiology of this disease is the fact that columnaris is often diagnosed in association with one or more other pathogenic organisms. According to the Aquatic Diagnostic Laboratory at Mississippi State University, in 2007 columnaris diseases singly accounted for only 8.9% of the cases, but in combination with other pathogens, *F. columnare* was present in 37% of all cases (68,4% in 2006), making it the most common disease seen (ADL 2007). ESC and columnaris were diagnosed together in 13.8% of case submissions. Davis (1922) observed that large quantities of other bacteria are present in columnaris lesions

besides *F. columnare*. Hawke and Thune (1992) showed that out of 53 *F. columnare* infections, 46 involved *E. ictaluri* and/or *Aeromonas* spp.

Finally, the effect of competitive bacteria has been also reported. According to Chowdhury and Wakabayashi (1989a) and Plumb (1999), the survival and infectivity of *F. columnare* declines in the presence of other species of bacteria. Chowdhury and Wakabayashi (1989a) showed that survival and infectivity of *F. columnare* declined in the presence *Aeromonas hydrophila* or *Citrobacter freundii*. In fish ponds a similar effect was found. *In vivo* infection experiments showed that columnaris did not occur in the presence of competitive bacteria. Infection did not occur when the density of *F. columnare* was one per cent of the total number of naturally occurring bacteria in pond water, but when the density of *F. columnare* was equal to that of naturally occurring bacteria, all the fish were infected. However, *F. columnare* successfully invaded the fish in the presence of *Streptococous* sp. *F. flavescens* or *F. fuscum*, even when the numbers of these bacteria were 1000 times greater than those of *F. columnare* (Chowdhury and Wakabayashi 1989b).

2.2 Clinical signs

Columnaris is primarily an epithelial disease (Noga 2010). Based on the site of infection and the appearance of the infected tissues, columnaris disease has been commonly known as “saddleback”, “fin rot”, “cotton wool disease”, “cigar mouth disease”, “peduncle disease” or “black patch necrosis”. It is characterized as an acute to chronic infection of the gills, the integument (specially the fins), or the oropharynx (Noga 2010; Plumb 1999; Roberts 2001). The gills are usually the major site of damage (Austin and Austin 1999). The other area of primary infection, often referred to

as a saddleback lesion, can appear along the dorsal fin and extend laterally down both sides of the abdomen (Griffin 1992). Another common clinical sign of the disease is the pronounced erosion and necrosis of external tissues, with the gills and the fins often being a major site of damage (Davis 1922). The severity of the disease is dependent on the virulence of the strain and other stressors, such as water quality and fish handling.

Lesions on the gills are common and specially represent a more direct threat to the health of the young fish. Gill lesions begin at the tip of the lamella and necrosis progresses toward the gill arch. Typically, congestion of the blood vessels supplying the gills occurs, resulting in the dissociation of the surface epithelium of the lamellae from the capillary bed (Austin and Austin 1999; Plumb 1999). Scattered hemorrhaging areas may be present (Pacha and Ordal 1967).

In adults, lesions usually begin externally on body surfaces and gills, but tend to vary with the fish species. In catfish, for example, peduncular and peri-oral lesions are commonly present. Small lesions start as areas of pale discoloration at the base of the dorsal or pelvic fins, leading to deterioration of the fins. These tend to increase in size and may cover as much as 25% of the total surface area of the fish, giving the characteristic saddle appearance. Frequently the skin is completely eroded away, exposing the underlying muscle which also becomes necrotic (Bootsma and Clerx 1976; Austin and Austin 1999). The infected skin loses its natural sheen and lesions usually develop into hemorrhagic or necrotic erosions and ulcers, which may present a whitish or yellowish coloration (due to massive presence of pigmented bacteria), surrounded by a ring of inflamed skin. It is not uncommon for the fish to die within 48 h after the appearance of the skin discoloration (Morrison et al. 1981). Moreover,

bacteria colonization of the skin and the gills predisposes the fish to secondary infections from pathogens as *Aeromonas* sp. or *Saprolegnia* sp. (Bullock et al. 1986; Stoskopf 1993; Noga 2010; Plumb 1999; Roberts 2001). Once the integument is compromised by the bacterium, a systemic infection may follow (Wood 1968; Wolke 1975; Plumb 1999). The bacterium is capable of entering the blood stream and is often isolated from the internal organs, however the pathology of systemic columnaris infections is poorly understood and internal lesions are seldom described and most often lacking (Koski et al. 1993). Only Hawke and Thune (1992) have reported the presence of inflammatory changes (swelling) of the posterior kidney in some cases of columnaris disease.

2.3 Diagnosis

Presumptive diagnosis of columnaris disease is based on the presence of the clinical signs mentioned above and by the typical morphology of *F. columnare* in wet mounts from infected tissues. Highly virulent strains of *F. columnare* have been reported to produce death without macroscopic evidence of tissue damage (Pacha and Ordal 1967), and in young fish there is often negligible pathology before death results (Austin and Austin 1999).

Columnaris is normally diagnosed by recognition of shallow lesions with yellow or brownish discoloration on the body, mouth, fins and gills of diseased fish. Presumptive diagnosis of columnaris disease is obtained by the observation of long, thin (0.5 to 1.0 X 3 to 10 µm), slender and flexing rods from scrapings of lesions on gill, or skin infected tissue in a wet mount at 100 to 400 X magnification. Organisms are most numerous in the spreading margins or expanding lesions. The non-flagellated

bacteria display gliding motility and form column-like masses on isolated tissues placed in wet mounts allowed to stand 5-10 minutes following preparations, which are commonly referred to as “haystacks” (Noga 2010; Plumb 1994). According to Roberts (2001) microscopic examination of tissue lesions can reveal epidermal spongiosis and necrosis, with ulceration and extension of the necrosis into the dermis. Hyperaemia and hemorrhages are often seen at the periphery of the lesions. In some cases, the long thin rods can be better visualized in the tissues with Giemsa stain rather than Gram, Hematoxylin or Eosin stains.

Definitive diagnosis of the disease can be achieved through isolation and culture of the causative bacterium, followed by its identification based on biochemical testing or molecular methods. Moreover, histopathology studies may provide useful information concerning the severity of the infection (Morrison et al. 1981).

As mentioned before, culture of the bacteria is possible and different media have been described. Three media are among the most commonly used: Cytophaga agar, Shu-Shotts or Shieh media. Recommended temperatures for bacteria culture vary from 25-30°C during 48 hours on agar plates or 24 hours for growth in broth with medium agitation (100-125 rpm). Selective media containing antibiotics can help reduce the presence of contaminating bacteria. Griffin (1992) recommended the use of Polimixin B and neomycin sulfate, and Decostere et al. (1997) proposed the addition of tobramycin to selectively isolate and culture *F. columnare* from diseased fish.

A list of the biochemical tests that can be performed for the positive diagnosis of *F. columnare* is given in Table 1 (Plumb 1999). The most common biochemical tests performer for the identification of the bacteria are Gram staining; catalase and oxidade

tests (Shamsudin and Plumb 1996); flexirubin pigment (Reichenbach and Dworkin 1981); congo red adsorption (Johnson and Chilton 1966); Tween-20 hydrolysis (Sierra 1957); or the tests described in MacFaddin (2000) for starch, gelatin, casein, tributyrin, tyrosine and lecithin hydrolysis. But identification using biochemical tests can take one to two weeks to complete (Bernardet et al. 1996), so researchers have developed alternative methods. Some alternatives are based on serological techniques, such as indirect ELISA and indirect fluorescent antibody test (IFA), for the detection of humoral antibody responses against *F. columnare*, which are useful tools to determine natural exposure to the organism and for rapid diagnosis (Shoemaker et al. 2003b; Panangala et al. 2006). Other methods are based on the use of gas chromatography for the analysis of whole-cell fatty acid profiles of the bacteria (Shoemaker et al. 2005a). Moreover, molecular methods have been developed for the genetic identification of *F. columnare*, most of them based on the polymerase chain reaction (PCR). Among them, several studies have focused on the sequence of the *16S rRNA* gene and the 16S-23S rDNA intergenic spacer region (ISR) for the construction of species-specific PCR primers to identify and discern *F. columnare* from other related and common water bacteria (Toyama et al. 1996; Michel et al. 2002; Tiirola et al. 2002; Bader et al. 2003b, Darwish and Ismaiel 2005; Arias et al. 2004; Welker et al. 2005).

2.4 Pathogenesis

It has been suggested that plasmids, adhesion capabilities and enzyme activities could be related to virulence in flavobacterial fish pathogens (Dalsgaard 1993; Madsen and Dalsgaard 2000; Stringer-Roth et al. 2002; Nematollahi et al. 2003), but detailed studies comparing virulence and virulence-related factors of known bacterial strains are still lacking (Suomalainen et al. 2006b). As for *F. columnare*, only very

scarce information is available on the pathogenesis of this bacterial disease, making it difficult to adopt a preventive approach to combat this pathogen (Decostere 2002). Different virulence factors have been described for *F. columnare*, but virulence mechanisms are in generally poorly characterized and more research in this area is needed to achieve a complete understanding of the pathogenesis of the disease.

The occurrence of plasmids in *F. columnare* isolates has not been fully studied; therefore, the possibility of plasmid-mediated virulence is unknown at this point (Suomalainen et al. 2006a).

The Lipopolysaccharide (LPS) is a major suprastructure of Gram-negative bacteria which contributes greatly to the structural integrity of the bacteria, and protects them from host immune defenses (Zhang et al. 2006). The LPS has been described and well characterized as a virulence factor in many bacterial species, e.g. *Salmonella* sp. (Makela et al. 1973) or *Escherichia coli* (Medearis et al. 1968), including fish pathogens such as *Vibrio vulnificus* (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) was originally misidentified as *F. columnare* and actually belongs to the species *F. johnsoniae* (Darwish et al. 2004; Shoemaker et al. 2005a).

LPS along with other capsular polysaccharide have been shown to play important roles in columnaris pathogenesis (Bader et al. 2005; Zhang et al. 2006), but no other studies on *F. columnare* LPS composition or immunogenic role are available.

As for enzymatic activities, Newton et al. (1997) partially characterized 23 proteases produced by isolates of *F. columnare* derived from channel catfish raised in the southeastern United States. The extensive necrosis and tissue destruction associated with *F. columnare* infections suggests that these bacteria produce strong tissue-obliterating enzymes. Chondroitin sulphate (ChS) is a mucopolysaccahride found in animal connective tissues, and the production of ChS-degrading enzymes is suggested to be related to pathogenicity of bacteria (Kitamikado and Lee 1975). The ChS-degrading enzyme, chondroitin AC lyase (ChonAC), has been shown to be produced extracellularly by *F. columnare* and is suggested to contribute to the ability of the pathogen to invade and colonize the host (Griffin 1991; Teska 1993; Stringer-Roth et al. 2002). Chondroitin are components of the extracellular matrix, thus suggesting that the chondroitin AC lyase may play an important role in the severity of the invasion, necrosis and tissue destruction associated with *F. columnare* infection (Newton et al. 1997; Stringer-Roth et al. 2002; Xie et al. 2005). Xie et al. (2005) determined that this protein is located periplasmically, and Suomalainen et al. (2006b) found that the chondroitin AC lyase enzyme was significantly related to strain virulence at 25°C when tested in vivo using rainbow trout.

It is known that bacteria seldom rely upon one single mechanism of adherence, but that both specific and nonspecific mechanisms are often involved (Ofek and Doyle 1994). A correlation between high virulence of some strains and its ability to adhere to the gills of common carp (*Cyprinus carpio*) was found with the gill perfusion model (Decostere et al. 1999c). In this study the AJS 1 strain was a highly virulent strain which had the most affinity for gill tissue, as compared to the AJS 4 strain which showed lower virulence and had a low affinity for gill tissue. The adherence ability of *F.*

psychrophilum and *F. columnare* in gill tissue was correlated with virulence in some studies where a high and low virulence strains were compared (Decostere et al. 1999a; Nematollahi et al. 2003). Interestingly, Kunttu et al. (2009) did not find any correlation between adhesion capabilities and virulence of four *F. columnare* colony types. But it must be pointed that the authors used an *in vitro* model for the adhesion experiments, while all other bibliography relies on *in vivo* studies on challenged fish. Thus, adhesion capability is still considered a major factor of pathogenesis for *F. columnare* until further evidence is found.

2.5 Treatment and control

Control of columnaris disease is a combination of wise management, appropriate use of available chemotherapeutics or antibiotics, and vaccination when feasible. Good management practices (GMPs) are desirable in any aquaculture operation and are the foundation of all disease prevention programs. The GMPs entail proper handling, maintenance of a quality environment and reduction of environmental stressors. Since aquaculture practices are inherently stressful, efforts to minimize the impacts of stress through proper management of water quality parameters, feeding practices, and stocking densities among others must be made. Moreover, ideally, fish should never be handled when in a weakened condition or when environmentally stressed. As previously stated, columnaris disease is a stress-triggered type of infection. Crowding, poor water quality, handling, seining, adverse temperature or physical injuries are predisposing environmental factors to columnaris disease (Plumb 1999). Either when GMPs are not enough or as part of a comprehensive prophylactic approach, specific treatments for each disease should be known.

A common treatment against columnaris disease is fish immersion in a salt bath, or increasing and maintaining the salt level at approximately three parts per thousand (ppt) (Farmer 2004). *In vitro* studies have reported that *F. columnare* growth is inhibited at ten ppt NaCl, but not at five ppt and growth is best at three ppt salinity (Bernardet 1989). In a challenge model, the adhesion of the bacteria was reduced at 3 ppt, which may explain lower mortality rates in higher salinities, and increasing salinity to 1 ppt in the culture systems may help to reduce fish losses (Altinok and Grizzle 2001).

Because *F. columnare* primarily attacks the skin and gills, the most effective treatments for columnaris disease are surface-acting disinfectants such as potassium permanganate, hydrogen peroxide and copper sulfate (Wakabayashi 1991). Potassium permanganate is commonly used at a dose of 2 mg/l (5.4 pounds per acre-foot) or at a higher concentration if the water organic load is high. Copper sulfate (0.5 ppm) is also a common treatment option against columnaris. Use of hydrogen peroxide to control the disease has been studied and recommended (Speare and Arsenault 1997; Rach et al. 2003). However, neither potassium permanganate nor copper sulfate are approved by the FDA, and their effectiveness has been questioned (Durborow et al. 1998; Plumb 1999). The herbicide 6,7-Dihydrodipyrido[1,2-a:2',1'-c]pyrazinediium dibromide (Diquat) was shown to be an effective bath treatment at 5.4 mg/L when compared to potassium permanganate 2 mg/L, chloramine-T 15 mg/L, hydrogen peroxide 75 mg/L, and copper sulfate 1 mg/L (Thomas-Jinu and Goodwin 2004a). Suomalainen et al. (2005a) have reported the use of high concentrations of salt and low pH as another treatment option. These conditions significantly reduced the numbers of viable bacterial cells following in vitro exposures.

According to Plumb (1999), the most effective way to treat columnaris disease is a combination of potassium permanganate and oxytetracycline-medicated feed. Noga (2010) speculates that the potassium permanganate treatment could stimulate anorexic fish to eat the antibiotic feed. Three antibiotics are available for use as medicated feed. Thomas-Jinu and Goodwin (2004a) studied the use of two of them to treat columnaris disease. They found that when Romet® (ormetoprim and sulfadimethoxine) and Terramycin® (commercial oxytetracycline) where administered prior to bacterial challenge, no mortalities were recorded in all groups. These antibiotics, in the form of medicated feeds, can be used to treat outbreaks of the disease at rates of 50 mg/kg body weight for Romet® and 80 mg/kg of body weight for Terramycin® (Hawke and Thune 1992). The withdrawal periods for these antibiotics are 3 days in catfish or 42 days in salmonids for Romet and 21 for Terramycin in any food fish. However, the use of these antibiotics is not approved for treatment of columnaris disease in catfish or other species and fall under the “extra label use”.

In October 2005, the U.S. Food and Drug Administration (FDA) approved the antibiotic Aquaflor® (florfenicol) for treatment of Enteric Septicemia of Catfish (ESC) disease. Since prevalence of columnaris is significantly higher when the pathogen is presented in combination with *Edwardsiella ictaluri*, causal agent of ESC, in 2007 the FDA passed the conditional approval of Aquaflor®-CA1 (same active ingredient), a Type A medicated article for the control of mortality in catfish and freshwater reared salmonids due to columnaris disease. Aquaflor®-CA1 is the first FDA conditionally approved drug for columnaris disease and the first drug that FDA has conditionally approved under the Minor Use and Minor Species Animal Health Act. Like Aquaflor®, Aquaflor-CA1® is a Veterinary Feed Directive (VFD) drug, meaning that users must

receive a signed VFD order from a licensed veterinarian before obtaining the drug through normal feed-distribution channels. The VFD is a category established by FDA in 1996 to help the agency more closely control new therapeutic products, primarily antimicrobials, and their use in food animals.

Nevertheless, treatments of the disease with antibiotics or medicated feeds have several drawbacks. First, they are expensive and only when the cultured product has a fairly high market value the farmers can afford them. Second, diseased fish usually loose appetite reducing antibiotic uptake. Third, emergence of resistant strains to antibiotics are a main public health concern. Finally, antibiotic treatments result in withdrawal periods before the fish can be sold as a food item which negatively affects farmers.

As previously mentioned, prophylactic approaches are more desirable against the disease. In the case of columnaris, infection might be prevented through vaccination. Vaccination against *F. columnare* was promising since Fujihara and Nakatani (1971) administered orally, heat-killed cells of the bacteria to juvenile coho salmon and the fish responded with the production of antibodies. Survivors of infection experiments resisted re-infection, suggesting the presence of a protective immune response (Fujihara and Nakatani 1971). Later, Schachte and Mora (1973) injected channel catfish with a vaccine and high antibody titers were also achieved. Moore et al. (1990) vaccinated fingerling channel catfish by immersion in a formalin killed bacterin of *F. columnare* that resulted in better survival and less need for chemotherapy treatment in vaccinated fish than in controls. Mano et al. (1996) administered formalized cells of *F. columnare* to eel by immersion and injection, resulting in an immune response two weeks later. A previous study by Liewes et al. (1982) found that

common carp could absorb *F. columnare* by immersion and indicated that this could be a means of vaccinating against columnaris.

Shoemaker et al. (2005b) developed a rifampicin resistant mutant of *F. columnare* following the procedures described by Schurig et al. (1991). The mutant showed non virulence when administrated to channel catfish and protective capabilities were described (Shoemaker et al. 2005b). The mutant was patented, licensed and approved for its use in channel catfish against columnaris disease. It became commercially available as a live attenuated vaccine commercialized under the name of Aquavac-Col® (Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands). This live attenuated vaccine is being used by catfish farmers in the US. Genetic studies carried out with the mutant have proved that it was obtained from a parent belonging to the genomovar I of *F. columnare* (Zhang et al. 2006).

CHAPTER 1. DEVELOPMENT AND CHARACTERIZATION OF NEW MODIFIED LIVE VACCINES AGAINST COLUMNARIS DISEASE

INTRODUCTION

Columnaris disease is an acute to chronic bacterial infection caused by *Flavobacterium columnare* that affects commercial aquaculture, the ornamental fish industry, and wild fish populations worldwide (Bullock et al. 1986). Infections have been reported among others from eels (*Anguilla* sp.), salmonids (*Oncorhynchus* sp. and *Salmo* sp.), tilapia (*Oreochromis* sp.), hybrid-striped bass (*Morone saxatilis* × *M. chrysops*), walleye (*Sander vitreus* Mitchill), channel catfish (*Ictalurus punctatus* Rafinesque), cetrachids such as largemouth bass (*Micropterus salmoides* Lacepede), bait minnows (*Pimephales promelas* Rafinesque), goldfish (*Carassius auratus* Linnaeus), zebrafish (*Danio rerio* Hamilton), carp (*Cyprinus carpio* Linnaeus), and aquarium tropical fishes species such as black mollies (*Poecilia sphenops* Valenciennes) or platies (*Xiphophorus maculates* Gunther) (Bullock et al. 1986; Decostere et al. 1998; Wakabayashi 1993; Newman 1993; Austin and Austin 1999; Plumb 1994; Moyer and Hunnicutt 2007). Specifically, columnaris disease is the second most prevalent illness in farm-raised channel catfish, which is the largest aquaculture industry in the U.S. (USDA 2003a, 2003b).

Since the bacterium is ubiquitous in the fresh water environment and acts mainly as a facultative pathogen, eradication of columnaris disease is not likely to occur. Moreover, once the disease develops in a fish population treatments are expensive and losses have to be assumed. Thus, prevention is the most economical and recommended approach to deal with the disease. Alleviating stress on the cultured

fish population is the best strategy, but management procedures on aquaculture settings entail stressful conditions to ensure profitability. Treatments of the disease include therapeutic chemicals legal for use in food fish such as copper sulfate or potassium permanganate. Medicated feeds with the antibiotics Terramycin® (oxytetracycline HCl), Romet® (ormetoprim and sulfadimethoxine) or Aquaflor CA1® (florfenicol) can be effective treatments, but fish in an advanced disease state tend to stop eating (Vinitnantharat et al. 1999) and resistances building up is a concern (Taylor and Johnson 1991; Johnson 1991).

Another prevention strategy is the vaccination of fish prior exposure to the pathogen. Vaccination plays an important role in big-scale commercial fish farming, with vaccines available for salmon, trout, channel catfish, European seabass and seabream, Japanese amberjack, yellowtail, tilapia and Atlantic cod (Sommerset et al. 2005). In general, autologous vaccines based on inactivated bacterial pathogens have proven to be effective in fish. Efficacy data is available for most of the new vaccines, but studies performed by pharmaceutical companies are not always available and salmonids dominate the scientific literature (Sommerset et al. 2005; Shoemaker et al. 2009).

Aquavac-Col® (Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands) contains an avirulent strain of *F. columnare* approved for use in the treatment of columnaris disease in catfish in the US (Shoemaker et al. 2005b). Aquavac-Col® is a live vaccine administered to catfish as a timed bath treatment. The main active ingredient for the vaccine is a rifampicin (3-[4-methylpiperazinyl-iminomethyl] rifamycin SV) (Sigma Chemical Company, St. Louis, Mo.) resistant mutant of *F. columnare*, created by multiple passages of the native isolate on

increasing concentrations of rifampicin. The procedure used to produce the *F. columnare* vaccine was modified from that described by Schurig et al. (1991). A lower initial concentration of rifampicin was used (Cytophaga agar media with 5 µg/ml rifampicin) and more passages (243 instead of 51) were required to achieve the desire rifampicin concentration of 200 µg/ml; the penicillin step was omitted as well (Shoemaker et al. 2005b). Aquavac-Col® has been reported effective in providing acquired immunity in channel catfish to *F. columnare* in tank experiments and has been shown to provide protection from disease and mortality when vaccinated catfish are challenged with common virulent wild-type isolates of *F. columnare* in scientific trials (Shoemaker et al. 2005b). However, efficacy of the commercial vaccine has not been reported from farm settings, and its effectiveness has been questioned in some forums.

The native isolate used for the generation of the Aquavac-Col® mutant was declared to be isolate ARS-1 in the patent document (Shoemaker et al. 2005b). In depth genetic studies on *F. columnare* isolates carried out by Zhang et al (2006) showed that ARS-1 is unlikely to be the parent strain for the avirulent mutant (referred to as FCRR in the scientific literature), Nonetheless, those studies confirmed that, according to the most cited protocol to subdivide this highly heterogeneous species (Triyanto et al. 1999; Triyanto and Wakabayashi 1999), the mutant strain used as commercial vaccine belongs to the genomovar I (Zhang et al. 2006). Interestingly, it has been proved that strains belonging to the genomovar II are more virulent to catfish (Shoemaker et al. 2008) and that they are specifically associated to catfish in the natural environment (Olivares-Fuster et al. 2007a). Attempts from the inventors to

obtain a mutant from strains belonging to the genomovar II were reported, but no rifampicin resistant isolates could be recovered (Shoemaker et al. 2005b).

In this study we report the generation of rifampicin resistant mutants from four different isolates belonging to the more virulent genomovar II of *F. columnare*. Full characterization of some of those mutants by comparison to their parents, to the commercial vaccine and to other isolates the species is also provided.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Table 2 shows the name, origin and collection date of all strains included in this study. Four *F. columnare* isolates previously characterized and ascribed to the genomovar II of the species were selected as parents according to their virulence capacity and genetic composition (data not shown). The selected isolates were recovered from diseased channel catfish in Alabama, and are identified in our collection as AL-CC-11, AL-CC-15, AL-CC-16 and AL-CC-17. Additionally, reference strains were included for the genetic analysis: ATCC 23463 (type strain), ARS-1, JCM 21327, ALG-00-057, AL-00-02, ALG-00-527 and the mutant Aquavac-Col®. Bacteria were stored frozen in 20% glycerol solution at -80°C and cultured on modified Shied media (MS) (Shoemaker et al. 2005a) at 26°C, both as broth and solidified with 10 g/L agar.

Development of F. columnare rifampicin resistant mutants

The process for obtaining the rifampicin mutants was a modification of the original protocol developed by Schurig et al. (1991). All four aforementioned selected isolates of *F. columnare* were initially cultured in rifampicin supplemented MS agar

plates at initial rifampicin concentration of 50 µg/ml. Single colonies that grew on the rifampicin-supplemented medium were then picked and placed onto the next concentration of rifampicin (100 µg/ml). Ulterior transfers were done at concentrations of 150, 200, 250 and 300 µg/ml of rifampicin in the culture media.

Table 2. *Flavobacterium columnare s*trains used in this study.

Strain	Source	Geographic origin	Date
AL-CC-11	Channel catfish	Alabama, USA	2005
AL-CC-15	Channel catfish	Alabama, USA	2000
AL-CC-16	Channel catfish	Alabama, USA	2000
AL-CC-17	Channel catfish	Alabama, USA	2005
Aquavac-Col®	Unknown	Unknown	Unknown
ATCC 23463^{T}	Chinook salmon	Washington, USA	Unknown
ARS-1	Channel catfish	Alabama, USA	1996
JCM 21327	Ayu	Hiroshima, Japan	1997
ALG-00-057	Channel catfish	Alabama, USA	2000
AL-00-02	Channel catfish	Alabama. USA	2000
ALG-00-527	Channel catfish	Alabama, USA	2000

^{T}, type strain

Genetic characterization of mutants

Total DNA was extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA) following manufacturer's instructions. Two specific regions were analyzed, the *16S rRNA* gene and the Internal Spacer Region (ISR) between the 16S and 23S *rRNA* genes. Both regions were amplified by Polymerase Chain Reaction (PCR) and analyzed by Restriction Fragment Length Polymorphism (RFLP) and Single Strand Conformation Polymorphism (SSCP). Amplified Fragment Length Polymorphism (AFLP) was applied to all isolates and mutants in order to define a fingerprint for each one of them.

16S RFLP analysis. Amplification of the *16S rRNA* gene was carried out according to Triyanto & Wakabayashi (1999) with the following modifications. Universal primers 20F (5'-AGAGTTTGATC(AC)TGGCTCAG-3') (positions 8–27 of *Escherichia coli* numbering) and 1500R (5'-CGATCCTACTTGCGTAG-3')(positions 1510–1492 of *E. coli* numbering) were used. Fifty microliter PCR amplifications were used containing: 100 ng of *F. columnare* DNA, 20 pmol of each primer, 15 mL of 2X PCR premix buffer H (Epicentre, Madison, WI), and 3 units of Taq DNA polymerase (Promega, Madison, WI). Thermocycling conditions were as follows: 5 min at 94°C; 40 cycles of 30 s at 94°C (denature), 45 s at 55°C (annealing), and 60 s at 72°C (primer extension); a final extension of 7 min at 72°C was included. Amplified products were digested using the restriction endonuclease *Hae*III (NewEngland Biolabs, NEB, Beverly, MA). Following digestion, restriction fragments were electrophoresed in a 3% agarose-1000 (Invitrogen, Carlsbad, CA) gel for 90 min at 80 V, stained with ethidium bromide, and photographed under UV light.

ISR-RFLP analysis. The universal primers used in the study were 16S-14F (5'-CTTGTACACACCGCCCGTC-3') (positions 1389–1407 of *E. coli* numbering) and 23S-1R (5'-GGGTTTCCCCATTCGGAAATC-3') (positions 124–110 of *E. coli* numbering) which amplify the 16S-23S ISR (Zavaleta et al. 1996). PCR conditions were those previously described (Arias et al. 2004). Amplified ISR fragments were digested using *Taq*$^{\alpha}$I (NEB) endonuclease following the manufacturer's instructions. Restriction fragments were analyzed as described above.

SSCP analysis. One microliter of the restriction products was mixed with 5 µL of denaturing-loading solution (95% formamide, 0.025% bromophenolblue), heated for 5 min at 98°C, and immediately cooled on ice. All 6 µL of denatured products were

separated by electrophoresis on GeneGel SSCP non-denaturing polyacrylamide gels (Amersham Biosciences, Piscataway, NJ) rehydrated with SSCP Buffer A pH 9.0 (Amersham Biosciences). A GenePhor electrophoresis unit (Amerham Biosciences) was used with the following running conditions: 5°C constant temperature, 25 min at 90V, 6 mA, 5W plus 60 min at 500V, 14 mA, 10W. Gels were silver stained with the DNA Silver Staining Kit (Amersham Biosciences) and digitally photographed (Olivares-Fuster et al. 2007b).

AFLP analysis. AFLP fingerprints were determined as previously described by Arias et al. (1997b, 2004). One hundred nanograms of total DNA were digested with 10 units of *Hind*III and *Taq*$^{\alpha}$I (Promega, Madison, WI, USA) in a final volume of 12.5 µl. Following digestions, adapters were added to a final concentration of 0.04 and 0.4 µM for *Hind*III- and *Taq*$^{\alpha}$I adapters respectively, and ligated to the restriction fragments using T4 DNA ligase (Promega). AFLP reactions employed two specific primers, oligonucleotideT000 (5'-CGATGAGTCCTGACCGAA-3') corresponding to the *Taq*$^{\alpha}$I-ends, and H00A corresponding to the *Hind*III-ends (5'-GACTGCGTACCAGCTTA-3'; selective base at the 3'-end is underlined). *Hind*III primerH00A was labeled with an IR700 fluorochrome from LICOR (Lincoln, NE, USA). PCR conditions are described elsewhere (Arias et al. 1997). The PCR products were electrophoresed on the NEN Global Edition IR3 DNA Analyzer (LI-COR) following manufacturer's instructions.

Data analysis. Calculation of similarity values and cluster analysis were performed using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Following conversion, normalization and background subtraction with mathematical algorithms, levels of similarity between fingerprints were calculated with the Dice coefficient for the RFLP and SSCP analyses and with the Pearson product

moment correlation coefficient for the AFLP analysis. Cluster analysis was performed with the unweighted pair-group method using average linkages (UPGMA). Multidimensional scaling analysis was performed using the similarity matrix obtained by combination of the RFLP, SSCP and AFLP data.

Total protein and LPS analysis

Four rifampicin resistant mutants, one per strain, were selected according to their better grow in lab conditions (data not shown). Total protein pattern and LPS composition for these four mutants and their parents were compared. The eight selected isolates were cultured in modified Shieh broth for 24 h at 28°C and 125 rpm. Three milliliters of broth were centrifuged at 3000 *g* for 15 min. Pelleted cells were resuspended in protein lysis buffer and proteins were extracted according to Arias et al. (1997a). Total protein profiles were obtained by SDS-PAGE electrophoresis stained with Gel Code Blue Stain Reagent (Pierce, Waltham, MA). Total protein electrophoresis followed the procedure described by Arias et al. (1997a). Protein concentration was measured using the Quick Start Bradford protein assay (Bio-Rad) and approximately 15 µg of total proteins were loaded per lane on 12% continuous acrilamyde gels. Gels were run at 30 mA during one hour. Coomassie staining was used to visualize the protein bands following standard methods (Sambrook et al. 1989).

Crude lipopolysaccharide (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 approx 1 mg/mL. Aqueous and phenol LPS phases were stored at -80°C until use. LPS from both phases were electrophoresed by discontinuous sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4%-20% gradient separating gel (Laemmli 1970).

Gels were run at 15 mA for 90 min. Gels were silver stained following the manufacturer's instructions for the Bio-Rad SilverStain kit (Bio-Rad, Hercules, CA, USA).

Total protein immunoblot analysis

Western blot analysis of whole-protein profiles of two of the mutants and their parents were conducted in order to detect their immunodominant proteins. Mutants 16-534 and 17-23 were selected based on the LPS profiles obtained. Sera was generated for each one of the mutants and their parents (Zhang et al. 2006).

Catfish culture conditions. Ten grams channel catfish grown at the Pathobiology Laboratory (Veterinary School, Auburn University) were used as experimental animals. Prior to initiation of study, 5 fish were shown to be negative for *F. columnare* by culture and serology as determined by agglutination (Klesius et al. 1999). The fish were housed in 10 gallon glass aquaria in a recirculation system, on a 12:12 hours light:dark photoperiod schedule. Water temperature was kept constant at 26±1°C. Fish were fed daily (2% body weight) with Purina trout chow no. 5104 (Purina Mills, Inc., St. Louis, Missouri).

Inoculums and disease signs. Four channel catfish were stocked per aquaria. Four aquaria were set, one per strain (AL-CC-16, 16-534, AL-CC-17, 17-23). The isolates used to infect the fish were cultured at 28°C for 24 hours in MS broth (Shoemaker et al. 2005a) with shaking (125 rpm). Plate counts on MS agar yielded approx $1 \cdot 10^9$ CFU/mL for both the parents and the mutants. Cultures were sonicated using a 1000 L sonicator equipped with a 40TL needle probe (Ultrasonic Power Inc., Freeport, IL, USA) for 7 cycles of 60 sec. at 60 W and 20 KHz on an ice bath. Before injection, fish were anesthetized by immersion in 100 mg/ml tricaine methane sulfonate

(MS-222, Argent, Redmont, WA). Fish were inoculated by IP injection with approx 50 µL of freshly sonicated *F. columnare* cultures.

Sera collection. Blood samples were collected from the caudal artery and vein of all inoculated channel catfish (5 fish per tank) 28 days following injection. Sera were collected by centrifugation of whole blood at 1,000 X g and stored at -20°C until analyzed.

Immunoblotting. Whole protein profiles from the four selected samples (two parents and two mutants) were obtained as described above. Samples were load as duplicate in two ten wells 12% SDS-PAGE gels (Bio-Rad), one per parent-mutant combination, and resolved as described above. Total proteins were then transferred to two PVDF (polyvinylidenedifluoride) membranes (Bio-Rad) at 100 V for 1 h. Membranes were then divided in two halves. After BSA blocking, one half was incubated with anti-parent serum, and the other with anti-mutant serum. The process was repeated for the second membrane with the correspondent serum. The polyclonal catfish sera were diluted at 1:500 prior use. The membrane-serum complexes were incubated overnight at room temperature. Next day, membranes were washed and incubated for one hour with a 1:500 dilution of a monoclonal antibody (E-8) specific for channel catfish IgM (Klesius 1990). After washing, a 1:5000 dilution of a conjugated goat anti-mouse Ig antibody was added, followed by detection with Opti-4CNTM (Bio-Rad).

Stability of rifampicin-resistant mutants

In order to check for the stability of the mutation(s) induced by rifampicin exposure, four mutants were selected: 11-131, 15-132, 16-534 and 17-23. Mutants were grown overnight on MS media at 28°C and 125 rpm. Out of the broth culture 20%

glycerol stocks were made and kept at -80°C, and all isolates were plate on MS agar media by the triple striking method in order to recover single colonies. Single colonies from the MS agar plates were transferred to fresh MS agar plates every other day for a period of four months (a total of 60 passes). After that period a single colony from each mutant was cultured on MS agar supplemented with 200 µg/ml of rifampicin. Ability to grow on selective media was recorded. From the frozen glycerol stocks, a sample was taken every three weeks for each one of the mutants. Bacteria were then plate on MS agar and on MS agar supplemented with 200 µg/ml of rifampicin. After 48 h bacteria growing on both media were transferred over MS agar supplemented with 200 µg/ml of rifampicin. Culturability of the bacteria on the defined media directly from the frozen stock was recorded.

RESULTS

Flavobacterium columnare rifampicin resistant mutants

A total of 13 new *F. columnare* genomovar II rifampicin resistant mutants were produced. All of them were independently selected and capable of growing on MS media with at least 200 ppm rifampicin. Five of them were originated from the AL-CC-15 isolate, four from AL-CC-11, and two from each of the AL-CC-16 and the AL-CC-17 strains. Mutants were stored at -80°C and replicates were made to ensure long term availability. In order to reduce the number of candidates to analyze, two mutants were selected per strain. Selection of mutants from parents AL-CC-11 and AL-CC-15 was done on the basis of their faster growth on the MS solid media with the higher levels of rifampicin (data not shown).

Genetic characterization

Table 3 lists the sample order used in all the gels for the genetic analyses performed. A total of 19 isolates were included.

Table 3. Order of samples used in all the gels for the genetic analysis.

Sample	Isolate
1	AL-CC-11
2	11-131
3	11-132
4	AL-CC-15
5	15-132
6	15-133
7	AL-CC-17
8	17-13
9	17-23
10	AL-CC-16
11	16-532
12	16-534
13	Aquavac-Col®
14	ATCC 23463
15	ARS-1
16	JCM 21327
17	ALG-00-57
18	AL-00-02
19	ALG-00-527

Figure 1 shows the complete analysis of the *16S rRNA* gene and the ISR region for all the selected samples. Both the RFLP and the SSCP analyses are shown. Results proved the genetic stability of the mutants within the conserved regions analyzed. The restriction analysis did not reflect differences among the selected parent strains and their mutants, as neither did the more discriminatory SSCP analysis.

Figure 1. RFLP and SSCP analyses of all the *F. columnare* isolates used for the genetic study. A) 16S-RFLP with restriction enzyme *Hae*III. B) ISR-RFLP with restriction enzyme *Taq*$^{\alpha}$I. C) 16S-SSCP from 16S-RFLP. D) ISR-SSCP from ISR-RFLP. The order of the samples corresponds to that defined in Table 3. M=100 bp DNA ladder; M1=50 bp DNA ladder.

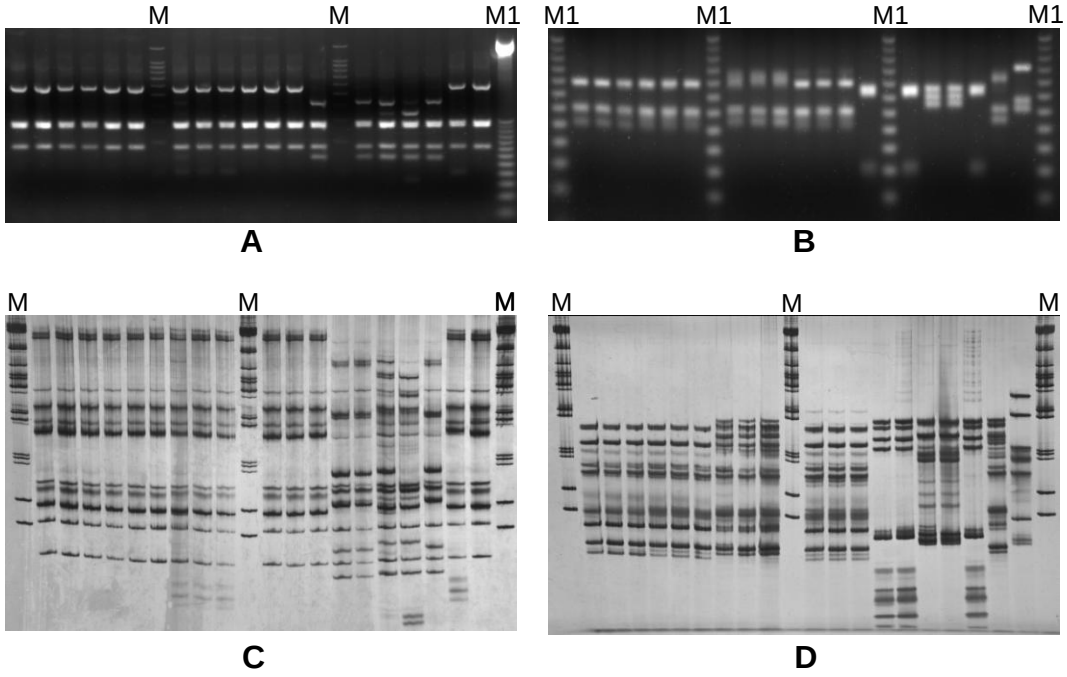


Figure 2 shows the AFLP gel generated for the 19 analyzed isolates. The AFLP analysis screens the whole bacterial and therefore offers a higher resolution level. AFLP has been proved to identify isolates within the species *F. columnare* at the strain level (Arias et al. 2004; Olivares-Fuster et al. 2007a). The AFLP analysis showed some minor differences among the parent and the mutants, as well as between mutants obtained from the same parent (Figure 2).

Figure 2. AFLP analysis of all the strains used in the genetic study. The order of the samples corresponds to that described in the Table 3. M=50-700bp ladder (LiCor).

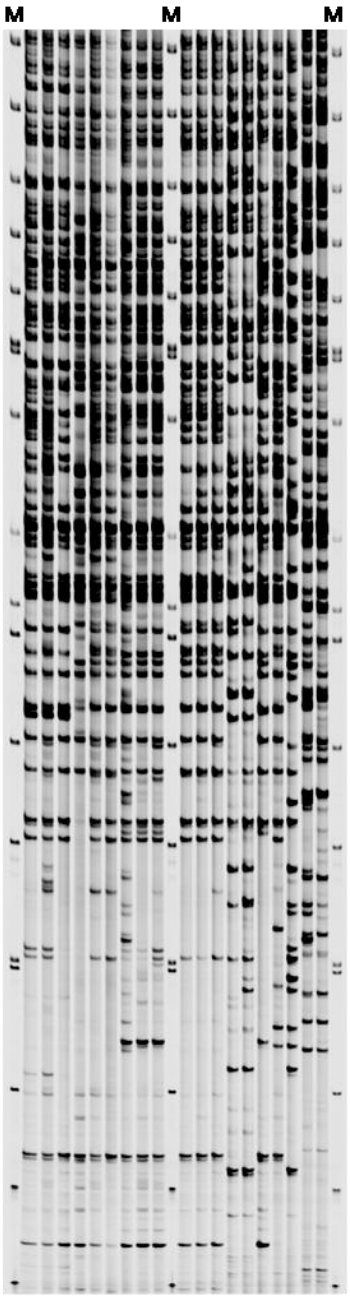
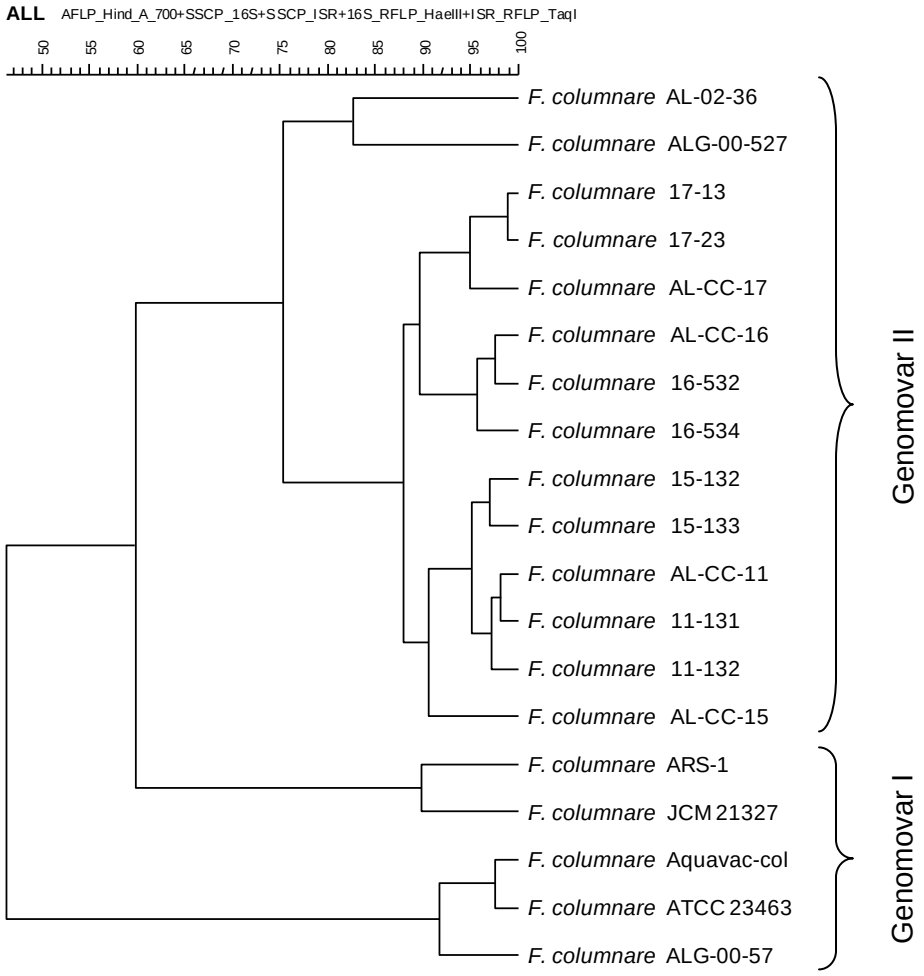


Figure 3 shows the dendrogram corresponding to the combined analysis of the RFLP, the SSCP and the AFLP analysis for all the strains. As we can observe, the mutants grouped with their corresponding parents at high similarity percent. Only in the

case of AL-CC-15 the parent grouped more distant to their mutants, but this result could be explained by the high similarity between strains AL-CC-11 and AL-CC-15.

Figure 3. Dendrogram corresponding to the multidimensional scaling analysis obtained after combination of the RFLP, SSCP and AFLP data. Linkage levels are expressed as percentage similarity based on similarity averages taken from each individual experiment.



Reference strains included in the genetic study were clearly differentiated into genomovar I and II by analysis of the complete *16S rRNA* gene and the ISR by RFLP (Figure 1A and 1B).. As expected, in depth analysis of these two genetic regions by

SSCP analysis did increase the level of resolution (Figure 1C and 1D), but still a clear differentiation among genomovars was achieved. It is interesting to note that genomovar I seems to harbor more variability in the *16S rRNA* gene than in the ISR region, while genomovar II shows a higher level of diversity in the ISR region than in the *16S rRNA* gene. A different 16S-SSCP pattern was scored for each of the four strains analyzed belonging to the genomovar I, while only two at the ISR-SSCP level. Meanwhile, each genomovar II strains showed a different ISR-SSCP pattern, and only three distinct patterns were recorded from the 16S-SSCP analysis. This result has been previously reported (Olivares-Fuster et al. 2007b). The AFLP technique allowed individual characterization of each one of the isolates, but the dendrogram generated after analysis of the AFLP profiles alone provided the expected grouping by genomovars (data nor shown). When all isolates and techniques were combined in a single multidimensional analysis, the genomovar division was still holding (Figure 3).

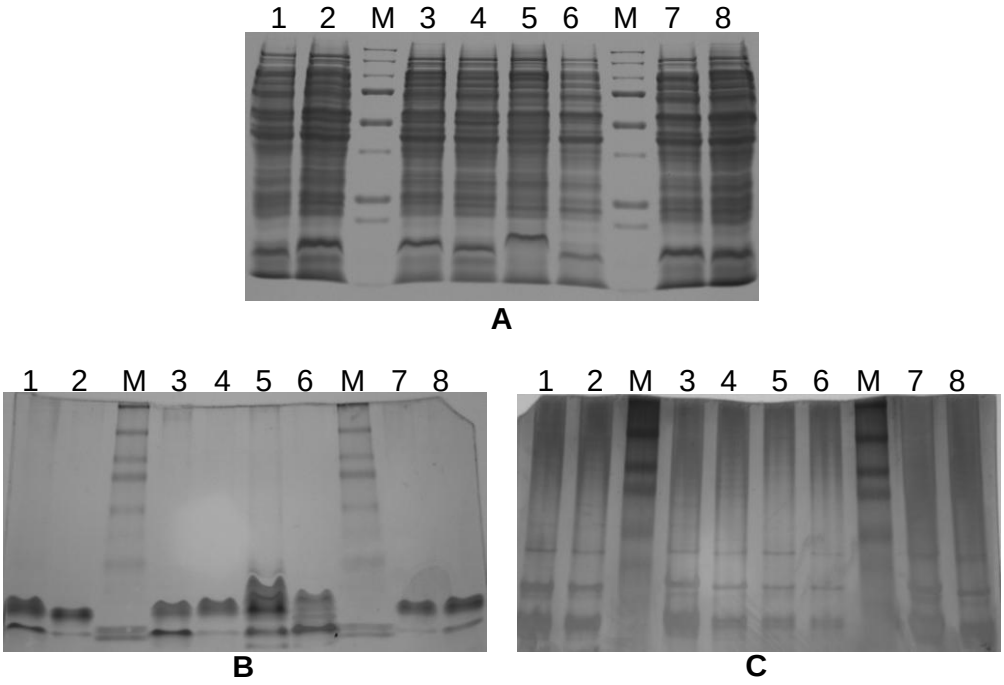
Total protein and LPS characterization

As expected, some differences were detected among the whole protein profiles of the parent strains and their mutants (Figure 4A). Similar results were obtained with the rest of the mutants (data not shown). The mutant that showed more visible differences to the parent at the protein expression level was 16-534, with noticeable changes both in the presence/absence of some bands and the intensity of others. Nonetheless, all mutants showed differences in the lower molecular size area, which presumably correspond to LPS. As it can be observed in Figure 4A, the differences among the parent and its mutant for the smaller bands did not always follow the same pattern.

Table 4. Order of samples followed for the total protein and LPS analyses.

Sample	Isolate
1	AL-CC-15
2	15-132
3	AL-CC-17
4	17-23
5	AL-CC-16
6	16-534
7	AL-CC-11
8	11-131

Figure 4. A) Total protein profiles of four *F. columnare* mutants and their parents. B) Phenol phase of the LPS extraction of four *F. columnare* mutants and their parents. C) Aqueous phase of the LPS extraction of four *F. columnare* mutants and their parents. Order of samples is that described in Table 4. M=broad range protein ladder (BioRad).



Comparison of both fractions composing the LPS of the parent strains and the mutants provided some interesting results (Figure 4B and 4C). While no differences were observed at the aqueous phase (Figure 4C), some variability was scored after analysis of the phenol phase (Figure 4B). The mutants obtained from parents AL-CC-15 and AL-CC-16 showed differences when compared to their parent strains at level of

presence/absence of bands. The bands corresponding to the higher molecular weight LPS are absent in the mutants, and the intensity of some lower bands varies between the parents and the mutants. On the other hand, the mutants obtained from AL-CC-17 and AL-CC-11 showed only differences in the intensity of the lower molecular weight bands, while maintaining the profile of the higher bands.

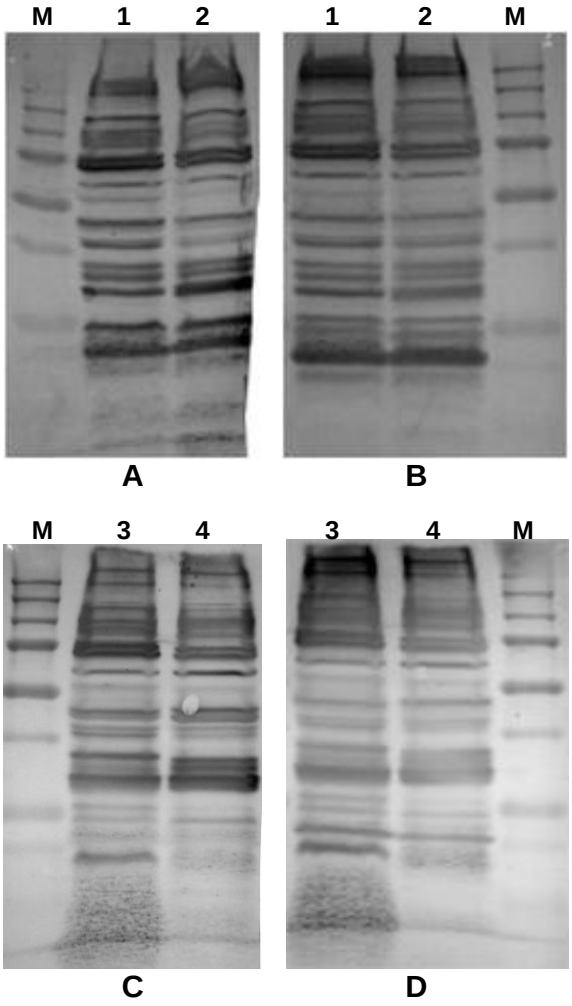
Total protein immnunoblot

Figure 5 shows the results of the Western blots performed with two of the mutants and their parents. The results were different for each one of the parent/mutant combinations. As for the AL-CC-17/17-23 parent/mutant combination, the majority of immunoreactive proteins were shared by both parent and mutant strains. Figure 5A shows that the anti-AL-CC-17 serum was able to recognize almost the exact same profile from the parent and the derived mutant. The same was true for the anti-17-23 serum (Figure 5B). When doing the cross comparison, it can be seen that both sera recognized almost the same epitopes, with some differences noticeable in the area where it could be assumed the LPS is localized (lower portion of the gel).

The membranes corresponding to the AL-CC-16/16-534 parent/mutant combination show differences at the epitope recognition level of each serum, as well as when both sera were compared. The anti-AL-CC-16 serum recognized some different antigens when blotted against the AL-CC-16 total proteins than when blotted against the mutant. It is noticeable that it failed to recognize the lover portion of the 16-534 profile, presumably corresponding to the LPS (Figure 5C). Also, some differences were recorded in the antigenic determinants recognized in the parent and the mutant by the anti 16-534 serum (Figure 5D). Again, the portion of the membrane putatively containing the LPS was not entirely recognized by the serum. When both membranes

were compared (Figures 5C and 5D) differences related to the epitope recognition could be observed.

Figure 5. Immunoblot of the total protein profile of two mutants and their parents. 1) AL-CC-17, 2) mutant 17-23, 3) AL-CC-16, 4) 16-534. Membrane A was blotted with anti-AL-CC-17 serum. Membrane B was blotted with anti-17-23 serum. Membrane C was blotted with anti-AL-CC-16 serum. Membrane D was blotted with anti-16-534 serum. M=broad range protein ladder (Biorad).



Mutation stability studies

After 60 passes on MS agar with no rifampicin, all mutants were able to grow on MS agar supplemented with 200 µg/ml of rifampicin. A slower growth on the

selective plates could be detected after 24 h of incubation, but after the standard incubation period of 48 h all mutants grew to normal density. The mutants were also able to grow directly from frozen stocks. A faster growth was accomplished when bacteria were plate on MS agar with no rifampicin, but growth was also positive on MS agar supplemented with 200 µg/ml of rifampicin. The growth after the second pass onto MS agar supplemented with 200 µg/ml of rifampicin was standard independently on what media the colony was selected from.

DISCUSSION

Modified live vaccines are regularly used in human and animal medicine to prevent disease. Their use in aquaculture is becoming more common and vaccination is now an accepted health management strategy that could have a tremendous impact on the much needed expansion of this sector worldwide. Immune responses to live vaccines are generally of greater magnitude and of longer duration than those produced by killed or subunit vaccines. A single dose of a live-attenuated vaccine can provide better protection against later infection by the wild-type organism, because the attenuated organism persists and metabolizes within the host, and in some cases may replicate in the host for a time (Roberts et al. 1994). Moreover, live vaccines better elicit cell-mediated immune responses, which can have a crucial role in controlling infections by intracellular pathogens. Injectable vaccines are impractical in most commercial fish culture due to extensive pond or cage production techniques, large numbers of individual animals, and low value per individual animal. Live attenuated vaccines can be effectively administrated as a timed bath treatment. The invasion,

persistence, and replication of live-attenuated vaccines have the potential to provide effective, inexpensive vaccines.

In this study we were able to generate a number of rifampicin resistant mutants of *F. columnare* belonging to the genomovar II, known to be more virulent for catfish. The methodology employed was 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. We proved that the mutantion(s) that confer rifampicin resistance to the isolates are stable in lab passing studies and long term freezing storage. Limitations of *F. columnare* to withstand lyophilization are well known, so this methodology was not included in the storage/mutation stability studies.

The genetic analysis of some of the mutants has revealed differences in the genetic composition of them when compared to their parents. No differences were observed when two ribosomal regions were analyzed, but the more exhaustive AFLP analysis highlighted the induced modifications in the genotype (Figure 3). Those genetic changes are expected, since the mutation(s) induced by exposure to rifampicin are stable, as shown in this study and previously for several mutants from different bacterial species. This is the first time though those genetic changes can be shown in a *F. columnare* rifampicin-resistant mutant, since the parent of the previously reported mutant is not longer available (Zhang et al. 2006).

As expected, the analyses of some of these mutants have shown that phenotypic changes were induced as well in the rifampicin-resistant isolates, as recorded by differences in the total protein profiles of the mutants when compared to their parents (Figure 4A). Changes in the LPS were also recorded for those mutants.

Modifications on the LPS, a main virulence factor for Gram-negative bacteria, have been reported on previous rifampicin-resistant mutants (Klesius and Shoemaker 1999; Vemulapalli et al. 1999; Arias et al. 2003), including the commercially available *F. columnare* live attenuated vaccine Aquavac-Col (Zhang et al. 2006). The newly reported mutants have shown different degrees on LPS variability, when compared to their own parents, on silver stained gels. While all four analyzed mutants showed some differences, those were quantitative for one of them (11-131), while both quantitative and qualitative for the other three mutants.

Although a typical LPS ladder has been reported in other *Flavobacterium* species (MacLean et al. 2001), our silver staining of *F. columnare* LPS showed only a few bands, as previously reported (Zhang et al. 2006). In their study, Zhang et al (2006) detected immunogenic bands present in the LPS by immunoblots. They showed differences in band molecular weights between strains isolated from diseased channel catfish and the attenuated strain FC-RR (that corresponds to the commercial vaccine Aquavac-Col). The three isolates 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. The authors suggested that the induction of rifampicin-resistant mutation(s) in *F. columnare* resulted in the loss of the high molecular weight bands displayed by virulent isolates (Zhang et al. 2006). Similar results were reported in *B. abortus* and *E. ictaluri* (Vemulapalli et al. 1999; Arias et al. 2003) when the LPS from rifampicin-resistant mutants were analyzed.

Given the simple composition of the LPS in *F. columnare* (as shown in Figure 4B) and the results obtained in the total protein profiles, in the present study we decided to immunoblot the total protein profiles instead of just the LPS as other authors

did. The nature of the total protein extraction method used allows co-isolation of the LPS, which is also transferred to the blotting membranes after electrophoresis. In those instances, LPS is not identified as discrete bands but as a smear in the lower portion of the membranes. Therefore, I was able to analyze the different antigenic determinants of the parents and mutants as well as the immunogenic properties of their LPS. Some obvious differences were observed both at the total protein and the LPS levels through immunoblotting. As can be seen in Figure 5A, the serum generated against the parent AL-CC-17 detected the LPS in both the parent and the mutant isolates, with some differences, while the serum generated against the corresponding mutant almost failed to detect the LPS of neither the parent nor the mutant (Figure 5B). The anti-AL-CC-16 was able to detect the parent LPS, but not the mutant; same result was recorded when the anti-16-534 serum was used (Figure 5C and 5D).

These results indicate that the two mutants analyzed might have different alterations of their LPS, although both were obtained following the exact same protocol and, together with the disparity in changes on the total protein profiles of the different mutants and the alterations at genetic level detected by AFLP, indicate that every mutant is an independent event, and that mutations induced by rifampicin exposure are not always the same and, in some way, they may be hard to predict. An additional outcome from the immunoblotting study was that mutant 17-23 seems to be more similar to its parent phenotipically and in the immune response generated in channel catfish than mutant 16-534. It will be interesting to study the protective capabilities of those two isolates in order to address if the more similar to the wild type the mutant is, the better protection it would provide after vaccination.

This is the first time that a complete genetic and phenotypic study was performed for rifampicin mutants that have a great potential to act as live attenuated vaccines. The main conclusion of the study is that no major genetic changes are induced by rifampicin selection, but some did occur and became permanent. These mutants could be traced by molecular techniques. At the light of these results it could be possible to follow up in the parent/mutant comparison in order to elucidate the sequences that have been permanently modified in the mutants. The numerous and diverse changes observed in the total protein profiles of some of the mutants, together with the variation in the changes observed in the LPS supports the idea that several mutants, even from the same parent, should be tested as potential live-modified vaccines, since it is fair to expect different immunogenic and protective capabilities of the different isolates.

Columnaris disease is widely spread in the template fresh water environment. It affects a great variety of fish in the natural settings, in aquaculture production systems and in the ornamental aquaria industry, the availability of an effective vaccine targeted against the high virulence group of the pathogen should be of enormous interest for fish health specialists and producers.

CHAPTER 2. EARLY EVALUATION OF NEW MODIFIED LIVE VACCINES AGAINST COLUMNARIS DISEASE

INTRODUCTION

Vaccination is a prophylactic approach used in health management strategies for the control of infectious diseases that has been proved an effective mechanism to prevent infectious disease outbreaks in humans, cattle, poultry and other food animals (Lombard et al. 2007). In aquaculture, the development and use of vaccines is now being fully considered as an effective tool for disease prevention (Shoemaker et al. 2009). The three main components of disease are the host, the pathogen and the environment. However, disease will only occur when all three factors converge. In the case of a pathogen like *Flavobacterium columnare*, who i) is always present in the culture environment, and ii) does not cause infection unless the environment or the host are deficient, prophylactic measures are the best approach to prevent disease. Thus, vaccination protocols along other good management practices are the best control measures against columnaris disease.

The objective of vaccination is to obtain a strong immune response to an administered antigen able to provide acquired long-term protection against a pathogen. To achieve this objective, either a killed or a modified live vaccine should be developed and licensed. The type of immunity needed against a given pathogen widely determines the type of vaccine to develop. Vaccination with killed bacteria (bacterines) produces an antibody-mediated protection, but not cell-mediated immunity. Cellular and/or antibody-mediated immunity can be achieved by immunization with a modified live vaccine. Although *F. columnare* is not considered an intracellular bacteria, cellular

immunity is still desired, since the antibody-mediated immunity induced by killed vaccines provides a relatively short protection, while longer protection is achieved by modified live vaccines. Moreover, killed vaccines are usually administered by intraperitoneal and/or intramuscular injection to individual fish, which entails a high additional economical cost to the aquaculture operation in terms of labor and time. Meanwhile, modified live vaccines might be administered by bath immersion to large numbers of fish, which is a more cost effective method in most aquaculture operations.

Aquavac-Col® (Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands), a commercial vaccine for the protection against columnaris disease in catfish, is now commercially available. However, Klessius and Shoemaker (1997) demonstrated that protective immunity against another bacterial disease, Enteric Septicemia of cafish (ESC), was dependent on the isolate used to immunize the fish, suggesting antigenic heterogeneity. In this study, the authors found that the live attenuated vaccine was protective against only eight out of thirteen isolates.

It has been demonstrated that the *F. columnare* strain commercially named Aquavac-Col belongs to the genomovar I of the species (Zhang et al. 2006). *Flavobacterium columnare* is a phenotypically homogenous but genetically highly diverse species. It can be easily divided into three (or two depending on the authors) genomovars (Triyanto and Wakabayashi 1999; Arias et al. 2004; Soto et al. 2008b). The term genomovar was introduced to denote phenotypically similar but genotypically distinct groups of bacterial isolates within a species (Rosselló et al. 1991; Ursing et al. 1995). This term has become a cornerstone for the genetic characterization and division of *F. columnare* isolates. It has been demonstrated that isolates belonging to the genomovar II are more virulent to catfish, are associated to catfish in natural

populations and that catfish skin mucus induced a greater chemotactic response in genomovar II than in genomovar I *F. columnare* isolates (Olivares-Fuster et al. 2007a; Klesius et al. 2008; Shoemaker et al. 2008). For all these reasons new rifampicin-resistant mutants from *F. columnare* isolates belonging to the genomovar II were produced. A complete genetic characterization of these mutants is presented in the Chapter 1 of this Thesis.

It needs to be understood that, for every new putative vaccine, answers to some basic questions are required before the new live attenuated mutants could even be considered vaccine candidates. Questions about the stability of the mutation were answered and are presented in Chapter 1. Next, the avirulent nature of the mutants needed to be demonstrated. As proved for other bacteria and even for *F. columnare* (Montaraz and Winter 1986; Shoemaker et al. 2005b), resistance to rifampicin could lead to non-virulent mutants, but this end should be thoroughly tested. Once non-virulence has been proved, the next two questions that must be addressed are related to the ability of the mutant to elicit a protective immune response, as tested by challenging vaccinated fish with with a virulent isolate of vaccinated fish.

The objective of this study was to answer some of the first and more basic questions about the newly generated mutants. For that I conducted the following experiments: i) infection of catfish with the mutants to prove vaccine safety, ii) challenge experiments of fish vaccinated with the avirulent mutants using a virulent isolate to test vaccine protection, and iii) comparison of disease resistantce between the fish vaccinated with the commercial vaccine and the new mutants to test for vaccine efficacy.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Out of the new thirteen rifampicin-resistant mutants previously reported (see Chapter 1), eight were selected for the initial vaccination/challenge trials, two from each parent. Thus, genomovar II mutants utilized were: 11-131, 11-133, 15-131, 15-132, 16-532, 16-534, 17-13 and 17-23. Moreover, the commercially available vaccine Aquavac-Col was included in one of the experiments as reference. All challenges were performed with *F. columnare* ALG-00-530, which has been identified as a highly virulent genomovar II strain (Shoemaker et al. 2008; Olivares-Fuster et al. 2010). Bacteria were stored frozen in 20% glycerol solution at -80°C and cultured on modified Shied media (MS) (Shoemaker et al. 2005a) at 26°C, either as broth (for a maximum period of 24 h) or as agar-solidified media (for a maximum period of 48 h).

Bacteria vaccination/challenge studies

Two different laboratory settings were used to evaluate the performance of the mutants and to select for the best vaccine candidates. The first four experiments (I to IV) were carried on a self-made system composed of twelve 10 gallons individual tanks, each one with a build-in independent recirculation system. I will refer to this system as the “AML system”. The last study (V) was performed on a complex system composed of forty two 15 gallons tanks. This system will be referred to as the “S6 system” and it was designed to easily allow switching from a close recirculation configuration to a flow-through water system.

Vaccination/challenge studies in the AML system. All experiments involved all twelve tanks. Four independent experiments were performed, each one comprising two newly generated mutants obtained from the same parent (four tanks per mutant) and

four tanks devoted as non-vaccinated but challenged control. In all four experiments straight channel catfish fingerlings were used. The fish were kindly provided by Dr. Newton from the Department of Pathobiology of the School of Veterinary Medicine (Auburn University). Aeration was constantly and individually provided to each tank. Fish were moved from the facilities at the School of Veterinary into the AML system tanks once water conditions were optimal: non- detectable levels of total ammonia or nitrite, pH 7.2-7.7, no chlorides, alkalinity (measured as total carbonate) at least 40 ppm and hardness (measured as total calcium) at least 40 ppm. Temperature was kept constant at 26°C. Fish were properly acclimated to minimize stress during stocking. No mortalities were recorded for any of the stocking processes. All four experiments were realized with 25 fish per tank, although the average weight of the fish varied from one experiment to another. Thus, a total of 1200 channel catfish were used in experiments I to IV, 300 per experiment. Fish were fed at 2% bodyweight every other day.

Vaccination protocol. Fish starvation for a period of forty hours was consistently performed before each vaccination treatment. All fish from each treatment (100 fish) were pulled together in a bucket with eight liters of water and aeration. Eighty mL of a 24 h culture of the corresponding mutant in MS broth were added. For control tanks same procedure was followed, but 80 mL of MS broth were added to the fish stock. Bath vaccination lasted for thirty minutes. Bacteria density was calculated for each treatment by triplicate plating on MS of a dilution series of the overnight culture. After thirty minutes the fish were equally distributed into the four original tanks. Feeding was resumed 24 h after the treatment. Fish were maintained in the same culture conditions for 28 days.

Challenge protocol. Fish starvation for a period of sixty four hours was consistently performed prior bacterial challenge. Fish from each tank were challenged as a unit. For this, 25 fish were moved into a bucket with 2 L of water and 20 mL of a 24 h culture of *F. columnare* ALG-00-530 (treatment) on MS broth (control) were added. Bacteria density was calculated for each treatment by triplicate plating on MS of a dilution series of the overnight culture. Aeration was provided to each individual bucket. Bacteria challenge lasted for 30 minutes. After that period fish were returned to their original tanks and reared in the same conditions described above. Feeding was resumed 24 hours after the challenge. Mortalities were recorded twice a day during 10 days.

Experiment I. Rifampicin resistant mutants 11-131 and 11-133 were used at a dose of $7 \cdot 10^6$ and $9 \cdot 10^6$ CFU/mL, respectively. A total of three hundred channel catfish were stocked, one hundred per treatment. The average weight was 2.2 g/fish. No mortalities were recorded after stocking or before vaccination. No mortalities were recorded after vaccination or until challenge. During the challenge, ALG-00-530 was used at a dose of $1.6 \cdot 10^7$ CFU/mL.

Experiment II. Rifampicin resistant mutants 15-131 and 11-132 were used at a dose of $2 \cdot 10^7$ and $2.5 \cdot 10^7$ CFU/mL, respectively. A total of three hundred channel catfish were stocked, one hundred per treatment. The average weight was 2.5 g/fish. No mortalities were recorded after stocking or before vaccination. No mortalities were recorded after vaccination or until challenge. During the challenge, ALG-00-530 was used at a dose of $1.7 \cdot 10^7$ CFU/mL.

Experiment III. Rifampicin resistant mutants 16-532 and 16-534 were used at a dose of $2 \cdot 10^7$ and $2.1 \cdot 10^7$ CFU/mL, respectively A total of three hundred channel

catfish were stocked, one hundred per treatment. The average weight was 2.6 g/fish. No mortalities were recorded after stocking or before vaccination. No mortalities were recorded after vaccination or until challenge. During the challenge, ALG-00-530 was used at a dose of $1.7 \cdot 10^7$ CFU/mL.

Experiment IV. Rifampicin resistant mutants 17-13 and 17-23 were used at a dose of $8 \cdot 10^6$ and $1.7 \cdot 10^7$ CFU/mL, respectively. A total of three hundred channel catfish were stocked, one hundred per treatment. The average weight was 2.9 g/fish. No mortalities were recorded after stocking or before vaccination. No mortalities were recorded after vaccination or until challenge. During the challenge, ALG-00-530 was used at a dose of $1.6 \cdot 10^7$ CFU/mL.

Vaccination/challenge studies in the S6 system. In Experiment V a total of one thousand six hundred and eighty fish were used. Fish were provided by the Genetics Unit of the Department of Fisheries and Aquaculture (Auburn University). Fish were originated as backcross of the hybrid catfish (channel catfish (*Ictalurus punctatus* Rafinesque) x blue catfish (*I. furcatus* Valenciennes)) with channel catfish. Fish weighted three grams on average. Twelve hundred fish were vaccinated with five different live attenuated *F. columnare* mutants. The remaining four hundred and eighty fish were set as controls. Fish were distributed into forty two twenty gallon aquaria containing fifteen liters of water. Forty fish were stocked per tank. Constant aeration and a close recirculation water system kept at constant 26°C were provided. Fish were fed a complete diet at a rate of 2% body weight daily. Standard water quality parameters were measured bi-weekly and adjusted as needed. After acclimation, fish were subjected to the following five vaccination experiments: 11-131, Aquavac-Col, 16-534, 17-23 and 15-132. Two controls were established. Six tanks, two hundred and

forty total fish were used per treatment. Channel catfish with an average weight of thre grams per fish were used.

Each one of the four selected mutants was generated in our laboratory from a different strain of *F. columnare*. The fifth vaccination treatment corresponded to the commercial vaccine Aquavac-Col, used as directed by the manufacturer. Six tanks, two hundred and forty fish, were established as the "non-vaccinated but challenged" control. Another set of six tanks were established as the system control corresponding to "non-vaccinated, non-challenged fish".

Vaccination protocol. Vaccination was conducted by collecting all fish assigned to each treatment in one single container with eight liters of the same water used for fish rearing. Aeration was provided. Eighty mL of an overnight culture of the newly generated mutants grown on MS media were added. Thirty minutes later the fish were randomly sorted to the appropriate aquaria at the same rate of 40 fish per tank. The non-vaccinated controls were subjected to the same procedure, but instead eighty mL of MS media with no bacteria were added. No mortalities were recorded from none of the vaccination treatments or the controls.

Challenge protocol. Fish were kept in the same conditions are described above for a period of twenty nine days with the exception of starvation prior to challenge. An individual challenge was performed per tank, for a total of 36 individual treatments. For each one of the tanks belonging to either one of the vaccination procedures or the "non-vaccinated but challenged control", 40 fish were transferred into buckets with two liters of water and 20 milliliters of an overnight culture of *F. columnare* ALG-00-530 at approximately 1.5 10^9 cfu/mL. Aeration was provided during the whole duration of the challenge (30 minutes). After that period fish were transferred back to their

corresponding tanks and the system was kept static, with aeration, for a period of twelve hours, in order to simulate an intensive infection scenario. After that period the aquaria were switched to a flow through water system with constant water temperature of 26°C, which was kept thereafter. Feeding was resumed twenty four hours after the challenge, providing half the usual ration. After 48 hours normal feeding was resumed. Mortalities were recorded twice daily.

Blood sampling and enzyme-linked immunosorbent assay (ELISA)

From Experiment V, serum was collected 28 days-post vaccination to check for antibody titers. Blood samples were taken from the caudal vein of three fish per tank (18 fish per treatments) and allowed to clot at room temperature for 1 h. Samples were centrifuged at 1,000 *g* for 5 min and serum collected and stored at -20°C.

Antibody responses in fish from each treatment were evaluated for the presence of specific immunoglobulin against *F. columnare* using an indirect ELISA (Shoemaker et al. 2003b). *Flavobacterium columnare* cells were harvested from a 24-hour culture on MS broth. Five mL of centrifuged live *F. columnare* cells were suspended in 25 mL of phosphate buffered saline buffer (PBS) and subjected to sonication using a 1000 L sonicator equipped with a 40TL needle probe (Ultrasonic Power Inc., Freeport, IL, USA) for 7 cycles of 30 seconds at 60 W and 20 KHz on an ice bath. Total protein in the supernatant was measured using the bicinchoninic acid assay (Pierce, Rockford, IL) and a standard of bovine serum albumin. Plastic microtiter plates were coated with 100 µL of a solution of 10 µg/mL of *F. columnare* antigen in 0.05 M sodium carbonate buffer pH 9.6 overnight at 4°C. Next day plates were washed five times with washing buffer (PBST: Phosphate-buffered saline at pH 7.4, 0.05% Tween 20), and blocked with 1% BSA in carbonate buffer for 30 min at room

temperature. Plates were washed again. Dilutions (1:10 to 1:20,480) of serum samples made in PBST were added to individual wells (100 μl/well), incubated for 30 min at room temperature and washed five times with PBST. Wells containing PBST only were present in each plate and tested in the same manner. Primary antibody was detected using the mouse monoclonal E-8 anti-IgM channel catfish (Klesius 1990) at a 1:5000 dilution in PBST. After 30 min incubation at room temperature, the plates were washed five times with PBST and a tertiary antibody solution, peroxidase-conjugated AffiniPure rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, PA, USA) was added at a 1:10,000 dilution in PBST. After 15 min incubation at room temperature, plates were washed five times with PBS and 50 μl of Ultra-TMB substrate (Pierce) was added to each well. After 15 min incubation at room temperature, 50 μl of stop solution (2 M sulfuric acid) were added. The absorbance at 450 nm was then recorded in a microplate reader. Absorbance from the negative (non-serum) control wells was subtracted to the absorbance value of each serum sample. The ELISA titer was defined as the reciprocal of the dilution with an OD at least two times the negative control.

Statistical analysis

Mortality data for all experiments was analyzed by one-way analysis of variance (ANOVA) using Tukey's HSD (Honestly Significant Different) test for pairwise comparisons of independent observations to determine significant differences among the means. In Experiment V, the level of variability in among tanks did not allow proper implementation of the ANOVA analysis. Thus, in order to determine the increase in survival associated to each vaccination treatment, generalized estimating equations (GEE) (Liang and Zeger 1986) approach was used. Tanks were treated as individual

clusters in the generalized linear model with none-normally-distributed data. An independent correlation structure was assumed, with correlated observations generated using recorded survival means. Use of logistic regression allowed estimation of parameters and standard deviation. Significant differences among treatments were determined by differences in least square means of estimated parameters. A Type III test was applied to estimate a proportional odds model. Significant differences were considered at $P \leq 0.05$. All statistical analyses were carried out using SAS (version 9.1, SAS Institute, Cary, NC).

For all experiments, relative percent survival (RPS) (Amend 1981) was calculated as indicative of the protection provided for the different mutants.

RESULTS

Bacteria growth

Rifampicin resistant mutants used for the vaccinations grew at high densities in our lab conditions. Table 1 shows bacterial cell counts all isolates used in the vaccination/challenge experiments I to IV. Rifampicin resistant mutants grew at a maximum level of $2 \cdot 10^9$ cfu/mL (mutant 15-132) to a minimum dose of $7 \cdot 10^8$ cfu/mL (11-131 mutant). It has to be noted that the mutants developed from parent AL-CC-11 exhibited a lower growth capacity, trait that was also observed for the parent strain. As defined above, all vaccinations and challenges were done with a density of bacteria two log lower than that of the original culture. Thus, for vaccination with mutant 16-532 in Experiment III, the fish were exposed to a $2 \cdot 10^7$ cfu/mL during vaccination and $1.7 \cdot 10^7$ during challenge with *F. columnare* ALG-00-530.

Table 5. Density of bacteria achieved after overnight culture of the different isolates on MS broth. Growth is expressed as colony forming units (CFU) per mL. The bacteria density during vaccination and challenge was two logs below the scored growth.

	Experiment I			Experiment II			Experiment III			Experiment IV		
	11-131	**11-133**	**530**	**15-131**	**15-132**	**530**	**16-532**	**16-534**	**530**	**17-13**	**17-23**	**530**
Growth CFU/mL (10^9)	0.7	0.9	1.6	2.0	2.5	1.7	2.0	2.1	1.7	0.8	1.7	1.6

Similar growth values were recorded for mutants and the virulent strain when they were used in Experiment V. As for the Aquavac-Col vaccine, the batch used during the experiment contained a $1 \cdot 10^{10}$ cfu/mL culture, which corresponded to a $2.5 \cdot 10^{7}$ cfu/mL bacteria density during the vaccination.

Vaccination/challenge studies in the AML system

One of the most important outcomes of the experiments developed in the AML system was the unexpected homogeneity of the results from tank to tank for the same treatments. As it can be seen in Tables 6 to 9, the standard deviation recorded for all treatments during the four experiments was kept reasonably low given the conditions. As mentioned above, the AML system was composed of twelve totally independent home aquaria, for which every tank could be easily considered as an independent ecological entity. But the fact that no mortalities were recorded since the fish were stocked until the bacterial challenge was performed, added to the relatively low variability encountered in the mortality values for the same treatments, are evidences that the system can be used for small scale experiments, such as preliminary vaccination/challenge trials with new live-vaccines or recombinant vaccines, to determine adhesion properties of different strains, to asses virulence of new isolates…

Table 6 shows the results recorded for the Experiment I, where two mutants obtained from AL-CC-11 were tested for avirulence and the level of protection conferred to channel catfish challenged against a highly virulent *F. columnare* strain. No mortalities were recorded after vaccination and prior challenge.

Table 6. Experiment I. Cumulative percent survival[1] (mean ± standard deviation) for vaccinated and challenged fry channel catfish.

	Cumulative percent survival (mean ± S.D.)	**RPS[2]**
Control-I	47.0 ± 9.5[a]	-
11-131	73.0 ± 6.8[b]	49.1
11-133	61.0 ± 5.0[a]	26.4

1. Different superscript letters indicate a significant difference at $P < 0.01$
2. Relative Percent Survival as defined by Amend (1981)

Table 7 shows the results recorded for the Experiment I, where two mutants obtained from AL-CC-15 were tested for avirulence and the level of protection conferred to channel catfish challenged against a highly virulent *F. columnare* strain. No mortalities were recorded after vaccination and prior challenge.

Table 7. Experiment II. Cumulative percent survival[1] (mean ± standard deviation) for vaccinated and challenged fry channel catfish.

	Cumulative percent survival (mean ± S.D.)	**RPS[2]**
Control-II	45.0 ± 8.9[a]	-
15-131	60.0 ± 8.6[a]	27.3
15-132	72.0 ± 8.6[b]	49.1

1. Different superscript letters indicate a significant difference at $P < 0.01$
2. Relative Percent Survival as defined by Amend (1981)

Table 8 shows the results recorded for the Experiment I, where two mutants obtained from AL-CC-16 were tested for avirulence and the level of protection conferred to channel catfish challenged against a highly virulent *F. columnare* strain. No mortalities were recorded after vaccination and prior challenge.

Table 8. Experiment III. Cumulative percent survival[1] (mean ± standard deviation) for vaccinated and challenged fry channel catfish.

	Cumulative percent survival (mean ± S.D.)	**RPS[2]**
Control-III	50.0 ± 7.7[a]	-
16-532	66.0 ± 13.3[a]	32.0
16-534	70.0 ± 7.7[b]	40.0

1. Different superscript letters indicate a significant difference at $P < 0.05$
2. Relative Percent Survival as defined by Amend (1981)

Table 9 shows the results recorded for the Experiment I, where two mutants obtained from AL-CC-16 were tested for avirulence and the level of protection conferred to channel catfish challenged against a highly virulent *F. columnare* strain. No mortalities were recorded after vaccination and prior challenge.

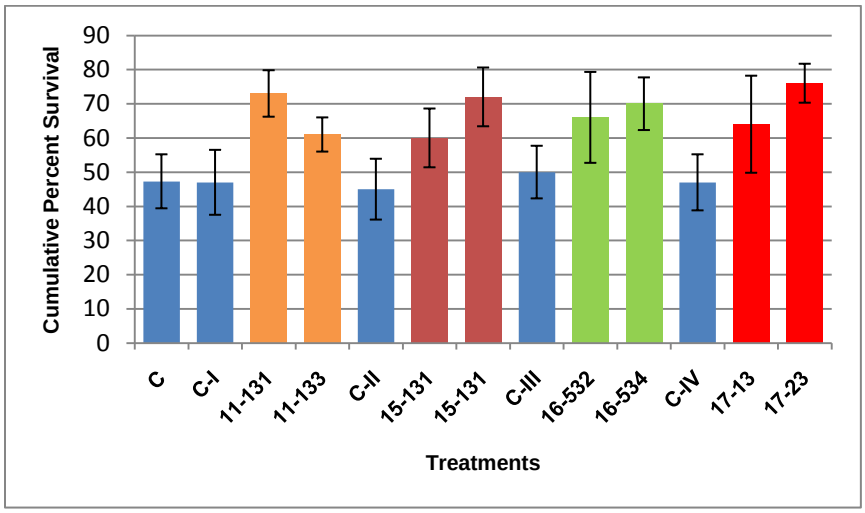
Table 9. Experiment IV. Cumulative percent survival[1] (mean ± standard deviation) for vaccinated and challenged fry channel catfish.

	Cumulative percent survival (mean ± S.D.)[1]	**RPS[2]**
Control-IV	47.0 ± 8.3[a]	-
17-13	64.0 ± 14.2[a]	32.1
17-23	76.0 ± 5.7[b]	54.7

1. Different superscript letters indicate a significant difference at $P < 0.01$
2. Relative Percent Survival as defined by Amend (1981)

A summary of the results obtained after the four vaccination/challenge trials is plotted in Figure 6. The mean obtained after averaging the survival from all controls (non-vaccinated but challenged tanks) is also provided (C).

Figure 6. Graphic representation of the cumulative percent survival (mean with error bar) recorded after four independent vaccination/challenge experiments. C: mean of all four control treatments.



Vaccination/challenge study in the S6 system

In Experiment V, carried out at the S6 system, selection of the strains was done on the basis of the results obtained on Experiments I to IV (Figure 1). The selected mutants were 11-131, 15-132, 16-534 and 17-23. Moreover, the experiment comprised the vaccination with the commercially available vaccine Aquavac-Col, used as reference. Unfortunately one problem was encountered during the trial. Two days after the challenge the water heater failed and the temperature of the water in the tanks, on a flow through system at this time, descended from the desired 26±1°C to around 19°C. Fixing the water heater was not an option at that time and it was decided to change the system to static (to avoid the undesirable mixing of infections that would occur with a recirculation setting), and flush twice a day to avoid water quality problems. Air temperature of the room was increased, but the range of temperature of the water in the tanks varied everyday from 18 to 23°C.

Table 10. Generalized estimating equations analysis of the cumulative percent survival recorded on Experiment V.

Treatment	Cumulative Percent Survival (mean ± standard deviation.)	GEE parameter estimates (parameter ± standard error)[2]	ODDS ratio[3]	RPS[4]
Control	69.2 ± 8.3	0.0000	1.0000	-
11-131	71.0 ± 29.8	0.0935 ± 0.5638[a]	1.0981	5.8
15-132	71.0 ± 10.1	0.0817 ± 0.2306[a]	1.0851	5.8
16-534	80.1 ± 14.4	0.5828 ± 0.3628[a]	1.7910	35.4
17-23	84.5 ± 13.2	0.9145 ± 0.4027[b]	2.4956	49.7
FCRR[1]	74.7 ± 20.6	0.2362 ± 0.4294[a]	1.2664	17.7

1. FCRR corresponds to Aquavac-Col® (Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands)
2. Different superscript letters indicate a significant difference at $P < 0.05$
3. Odds of survival for fish in a given treatment when compared to fish in the control
4. Relative Percent Survival as defined by Amend (1981)

It is very likely that this incident with the water heater affected the mortalities for all the treatments. In fact we recorded high mortalities on several tanks during the first 48 h, but the mortality rate decreased dramatically the third day. We decided to keep the trial and record mortalities for 14 days. As a consequence, the cumulative percent of survival data was highly variable, and when trying to run the ANOVA test with these data the variability inside each treatment masked the results. For this reason we used the generalizing estimated equations, as a better way to manage the high degree of variability. The main outcomes of the analysis are shown in table 6. Only vaccination with 17-23 had a significantly increased survival as compared to controls (p-value=0.023). An estimated odds ratio of 2.50 was scored, meaning that it is estimated that the odds of survival of fish vaccinated with the mutant 17-23 is 2.5 times the odds of survival in the control group (95% CI: (1.58, 4.016)). None of the other results could be considered. No mortalities were recorded on the non-vaccinated/non-challenged control tanks.

Another interesting outcome resulted from the use of Aquavac-Col in this experiment. As it can be seen in Table 6, the commercial vaccine did not significantly protect catfish against columnaris disease.

Finally, the calculation of the relative percent of survival (RPS) according to Amend (1981) showed that only the treatment with the mutant 17-23 had a high enough value to be considered, since it is practically 50% (Table 6).

Enzyme-linked immunosorbent assay (ELISA)

Unfortunately no conclusive results were obtained from the ELISA tests. A total of 126 fish were tested for antibody production against *F. columnare* (18 per each treatment and each control). The fact that many fish from the controls showed significant levels of antibody titters did not allow for any clear conclusion on antibody production of vaccinated fish (data not shown).

DISCUSSION

During the past 20 years, aquaculture has grown into a very significant industry in many parts of the world. According to FAO's The State of World Fisheries and Aquaculture report (SOFIA), aquaculture accounts now for 47 percent of the world's per capita supply of food fish, growing at a rate that offsets the effects of the static capture fishery production and a rising population (FAO 2008). With increased aquaculture, production husbandry practices have evolved from extensive or semi-intensive to intensive or superintensive. Under conditions of high population densities, infectious diseases pose a constant and costly threat to the aquaculture business. Antibiotics provide a useful means to control many bacterial diseases, but often do not provide an acceptable solution and there are problems associated with the development of antibiotic resistances. Due to successful use of vaccines in warm-blooded animals and the decreasing effectiveness of antibiotics in controlling bacterial

fish diseases have led to the development of vaccines for fish (Vinitnantharat et al. 1999).

Discovery of new vaccines against bacterial diseases is a complex process that involves several key steps. The first and probably the most critical step, many times overlooked, is the selection of the best candidate(s). Candidates range from a particular bacterial isolate that could be used as a killed, live attenuated or even genetically modified vaccine, to specific sequence(s) that would be developed into recombinant vaccines. For appropriate selection of the best vaccine candidate(s), an extensive knowledge of the bacteria genetics, pathogenesis and epidemiology is required.

In the case of *F. columnare* and columnaris disease not enough information has been yet collected on these basic aspects, although in recent years a more attention has been paid to this worldwide distributed opportunistic pathogen for which no natural resistance in any fish species is known (Plumb 1999). Although this bacterial species is phenotypically homogenous, studies from several labs around the world have light up the enormous genetic variability harbored in the species (Triyanto and Wakabayashi 1999; Arias et al. 2004; Olivares-Fuster et al. 2007a; Soto et al. 2008a).

The high genetic diversity found has lead to the goal of searching for phenotypic traits that correlate with it. And some important outcomes have been published. Authors have found remarkable phenotypic differences among isolates, and strong correlations with its genomovar adscription. The more important is probably the finding that genomovar II isolates are more virulent to catfish than those belonging to genomovar I (Shoemaker et al. 2008). Moreover, the same authors found a significant distribution of *F. columnare* isolates by fish species in natural populations, with

genomovar's II being primarily associated to catfish, both blue and channel (Olivares-Fuster et al. 2007a). Finally, evidence was found by Klessius et al. (2008) that catfish skin mucus induced a greater chemotactic response in genomovar II than in genomovar I *F. columnare* isolates. Although the role that chemotaxis plays in the virulence of *F. columnare* is not fully defined, the authors suggested a correlation between chemotactic response of genomovar ll isolates and virulence (Klesius et al. 2008).

With this information in hand it was desirable to develop and test a *F. columnare* genomovar II live-attenuated mutant as protective agent against columnaris disease. Especially interesting in this case, for which a live-attenuated vaccine against columnaris disease has been approved and commercialized using an isolate of the least virulence group, was to prove that strain selection indeed is a critical step in vaccine development (Zhang et al. 2006).

In this study we have tested the capacity to protect against a highly virulent strain of *F. columnare* of eight new rifampicin resistant *F. columnare* mutants, which were obtained from four isolates classified as genomovar II. An initial screening allowed the selection of the four best candidates, which provided protection against the infectious disease at different levels, as proved by the RPS scored (Tables 2 to 5). Independently of the RPS values, the original intention was to fully test at least one mutant from each one of the parents, since I was aware of the genetic variability among isolates.

In the second screening I decided to incorporate the commercial vaccine Aquavac-Col as reference. Although not too much data has been published on the

efficacy of this vaccine, it is a reference for the industry, and given the great genetic differences between the vaccine and the new mutants, I found it a desirable comparison. Besides the technical problems encountered during the challenge (see the Results section for a detailed description), two main outcomes were unexpected. First, no clear antibody titters could be measured in the sera of 28 days post-vaccinated catfish. This result is probably related to the difficulty on stocking fully naïve catfish that have been hatched and reared in Alabama, since *F. columnare* is known to be a ubiquitous bacterium in our environment. Nonetheless, the basal exposure to the bacteria was the same for all fish, vaccinated and controls.

The second important outcome was the low performance of the commercial vaccine Aquavac-Col in the study. Out of the five mutants tested in Experiment V, Aquavac-Col was the third less protective, with cumulative survival values that resulted non-significantly different from those of the controls (Table 6). The fact that I challenged all the fish against a highly virulent genomovar II isolate of *F. columnare* could explain the low level of protection registered, given the genetic differences between the genomovars. Nonetheless, these results should raise questions about the effectiveness of the genomovar I vaccine when fish are threaten to face a virulent genomovar II *F. columnare* pathogen.

On the other hand, only one of the newly generated mutants showed a significant level of protection in the largest experiment. Rifampicin-resistant isolate 17-23 scored a RPS value of 50%, and conferred statistically significant protection against columnaris disease in catfish (Table 6). This is only the first screening test performed with this isolate, but these results should lead to more in depth tests to prove if

effective protection can be achieved not only in lab settings, but in farm-like designs and in real aquaculture settings.

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