Mutagenesis of virulence genes in an epidemic strain of *Aeromonas hydrophila* ML09-119 for development of an attenuated vaccine strain

by

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Abstract

The epidemic strain of *Aeromonas hydrophila* caused a devastating outbreak of motile Aeromonads septicemia of catfish (MAS) on the fish farms of Southeastern United States in 2009. *A.hydrophila* ML09-119 was reported to cause severe mortality in commercial catfish farms. Research has shown on the virulence of this epidemic strain on channel catfish (*Ictalurus punctatus)* including molecular identification of the specific strain and unique DNA sequences. However, no research has been done so far on the virulence of this specific strain to blue and hybrid catfish. In this study, intraperitoneal injection (IP) challenge method combining cohabitation culture was used to detect the susceptibility of channel, blue *(Ictalurus furcatus)* and hybrid catfish (blue catfish × channel catfish) to *A. hydrophila* ML09-119. Our results showed that the virulence of ML09-119 to channel catfish is significantly higher than it is to blue and hybrid catfish ($p=0.0156 < 0.05$, $a = 0.05$). No significant differences were observed between blue and hybrid catfish ($p=0.0801>0.05$, $a=0.05$). Significant differences in survival were observed for bacterial strains ML09-119, ZC1, and AL06-06 ($P < 0.0001$) and between fish species in the ML09-119 group ($P = 0.0126$). No interaction effect between variables of bacterial strains and fish species was observed ($P_{\text{fish species} \times \text{bacterial strains}}=0.1002$). The difference in survival between channel catfish and grass carp injected with ML09-119 was significant ($P = 0.0069$). No significant differences in survival between channel catfish and grass carp intraperitoneally injected with ZC1 or AL06-06 were observed, but there was a significant difference between strains ZC1 and AL06-06 in both channel catfish ($P = 0.0075$) and grass carp ($P = 0.0089$).

Previously our lab sequenced 11 *A. hydrophila* isolates, 6 of which are epidemic stains, while the others were historical *A. hydrophila* isolates not affiliated with an epidemic outbreak of disease is described as "reference" strains. A comparative genomic analysis indicated that 53 epidemic-associated genetic regions with 313 predicted genes were uniquely present in the epidemic isolates but absent from the reference isolates. Thirty four genes from this region were predicted to be related to the virulence of the epidemic strains. A functional metabolic island that encodes a complete pathway for *myo*-inositol catabolism was identified and demonstrated to be functional based on the ability of epidemic *A. hydrophila* isolate ML09-119 to use *myo*-inositol as a sole carbon source while the reference strain AL06-06 cannot. A novel O-antigen cluster was found in all the epidemic isolates and one reference isolates.

In this study, the gene *iolA* coding for the enzyme aldehyde dehydrogenase for *myo*-inositol catabolism was inactivated by traditional allelic exchange to generate the *A. hydrophila* $\Delta iolA_{tra}$ mutant. The *iolA*-*iolR* genetic region was also mutagenized using a recombineering technique generating $\Delta iolA_{rec}$ mutants. An *in vivo* challenge in channel catfish showed that there was no mortality in the channel catfish that were challenged with $\Delta iolA_{tra}$ mutant, but there was mortality in the channel catfish challenged with $\Delta iolA_{rec}$ mutants similar to wild type ML09-119.

Eight mutants were created by knocking out the upstream of the *iolA* gene in the *iolA*-*iolR* promoter region. Results of the *in vivo* challenge in channel catfish showed that $\Delta iolA_{rec3}$, $\Delta iolA_{rec4}$ exhibited some decrease in mortality, but there were no significant difference in the mortality between the channel catfish challenged with $\Delta iolA_{rec3}$, $\Delta iolA_{rec4}$ and the channel catfish challenged with the wild type ML09-119. ELISA antibody titers of the survivors of the $\Delta iolA_{tra}$

after 21 days showed that $\Delta iolA_{tra}$ can induce strong antibody response against the wild type *A. hydrophila* ML09-119, indicating that this mutant can serve as a promising vaccine candidate against the epidemic *A. hydrophila.*

Lipid A-Core ligase (*waaL*) and O-antigen polymerase (*wzy*) mutants were created by both traditional splicing PCR and conjugation technique and recombineering technique respectively, $\Delta waaL_{tra}$ *or* $\Delta waaL_{Rec}$, Δwzy_{tra} *or* Δwzy_{Rec}. An *in vivo* channel catfish challenge study was committed on channel catfish to study the role of O-antigen in the virulence of the epidemic strain of *A. hydrophila.* The results shows that the channel catfish that were challenged with $\Delta waaL_{tra}$, Δwzy_{tra} had 100% survival rate, but 0% survival rate was observed in the channel catfish that were challenged with $\Delta waaL_{Rec}$, Δwzy_{Rec}.

A *ΔymcA* mutant was created by knocking out the *ymcA* gene by recombineering technique to study the role of *ymcA* gene of the O-antigen in the virulence of *A. hydrophila*. 68.13 ± 16.75% survival rate was observed in the channel catfish that were challenged with *ΔymcA* mutant. Sub-challenge of the survivors of *ΔymcA* treatment group 21 days post first challenge showed that 90.48 ± 8.25% survival rate was observed. Significant difference was observed between the *ΔymcA* treatment group and the positive control group which were naive channel catfish challenged with wild type. ELISA antibody titers of the survivors of the *ΔymcA* treatment group 21 days post first challenge showed that *ΔymcA* induced strong antibody response against the wild type *A. hydrophila* ML09-119 indicating that *ΔymcA* mutant can serve as a promising vaccine candidate.

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Chapter I

Introduction and Literature Review

1. Introduction

Aquaculture is one of the fastest growing industries in the world. It is developing, expending and intensifying in almost all regions of the world due to the vast global population demand of aquatic food products and the leveling-off of the capture fisheries. According to FAO, in 2010 alone, the World aquaculture production reached 60 million tonnes (excluding aquatic plants and non-food products), with an estimated total value of US$119 billion. In 2010, 181 countries and territories was recorded with aquaculture production, with Asia accounted for 89 percent of world aquaculture production by volume, in which 61.4 percent was from China. During the last thirty years, the production of the global food fish protein has expanded by almost 12 times, at a rate of 8.8 percent more per year (FAO 2012). According to FAO, global aquaculture production will need to achieve 80 million tons by 2050 just to maintain the current level of per capita consumption. It is not possible for the capture fisheries itself to meet this big global aquatic food need. Even with the fast growing rate and the significant contribution of the aquaculture industry, it is still a big challenge for the aquaculture to achieve this (FAO 2005).

In United States, the aquaculture industry is dominated by finfish production (FAO 2012). Being the largest sector of the aquaculture industry, Channel catfish *(Ictalurus punctatus)* farming produced more than 400 million dollars which accounts for approximately half of the total aquaculture production in U.S. In 2010, in the top 10 fish and seafood that consumed among Americans rank, farm raised catfish was sixth, about 0.8 pound per person per year (Hanson & Sites, 2012). Most catfish are produced in the southern the United States with Mississippi, Alabama, Arkansas, and Texas being the top four states for catfish production, which accounts for 94 percent of the total sales (USDA, 2014).

Even though U.S. catfish industry is thriving over the years, catfish production if vulnerable to adverse impacts of disease and environmental conditions. Before the 1980's, the strategy of the management practices was 'low-density', which resulted in good pond water quality, lower overall stress on fish populations and less efficient transmission of disease pathogen. However, due to the great competition against the seafood products, especially from the Asian countries. Producers have applied much more intense production strategy, such as multiple batch cropping systems, higher stocking density, more feed put into the culture systems. All these practices lead to the emergence of the infectious diseases which now becomes the primary limiting factor in catfish production. Disease outbreaks in recent years are very common even on efficient and well-built catfish farming facilities. According to Mississippi State University reports, infectious diseases have caused approximately 45% of inventory losses on catfish fingerling farms, and about 60% of the overall catfish losses are attributed to a single or

mixed bacterial infections, 30% due to the parasitic infection, 9% from fungal infections, and 1% result from viral etiology. Economic losses resulting from infectious diseases are believed to cost producers millions of dollars in direct fish losses each year. Furthermore, infectious diseases can also impact the profitability by increasing treatment costs, reducing food consumption by fish due to the flavor change and appearance, increasing feed conversion ratios, and causing harvesting delays.

The major bacterial diseases in catfish that affect the catfish industry are: enteric septicemia of catfish (ESC), caused by *Edwardsiella ictaluri* (Hawke, 1979); motile aeromonad septicemia (MAS), which is caused by *Aeromonas* species (Austin & Adams, 1996) and columnaris disease which is caused by *Flavobacterium columnare (*Wagner, 2002). The economic losses due to the ESC, according to USDA were about 30 to 50 million dollars each year (Shoemaker *et al.*, 2007; USDA, 2010a, 2010b). The yearly losses caused by the Columnaris are estimated to be 30 million dollars (Declercq, 2013). MAS also causes huge amount of economic losses which are not limited to channel catfish but also including tilapia *(Oreochromis niloticus),* catfish, goldfish *(Carassius auratus auratus)*, common carp *(Ctenopharyngodon idella)*, and eel *(Anguilliformes)* (Pridgeon et al., 2011).

Prior to 2009, MAS in channel catfish caused by *A. hydrophila* was not a concerned disease because the catfish aquaculture operations in the southeastern United States have not experienced a major outbreak (Hemstreet, 2010). However In 2009, catfish farmers in west Alabama reported severe disease outbreaks which were then

proved to be caused by a highly virulent strain of *A. hydrophila*, ML09-119, to channel catfish. From 2009-2011, Alabama catfish famers lost more than 7.5 million pounds of catfish that were market-size and estimated to be $3 million due to this epidemic strain of *A. hydrophila* (Pridgeon, 2011; Liles, 2011). It is reported that *A. hydrophila* epidemic strain, ML09-119, is highly virulent to channel catfish, causing severe mortality within 24 h post exposure with certain amount of dose (Pridgeon, 2011). The epidemic MAS outbreaks caused by are so devastating that it is highly essential to investigate the virulence nature of this pathogen, identify the virulence related genes and create live avirulent bacteria mutant vaccine candidates for this bacterial disease. So far there are only a few factors identified for the *A. hydrophila* epidemic strain, and no commercial vaccine or treatment for the epidemic MAS are available right now. Three attenuated *A. hydrophila* vaccines were reported to offered 86–100% protection against their virulent parents at 14 days post vaccination (dpv), when the channel catfish were vaccinated with the mutants at dosage of 4×10^5 CFU/fish. These mutants were developed from the virulent 2009 West Alabama isolates through selection for resistance to both novobiocin and rifampicin (Julia and Klesius, 2011).

This dissertation is focusing on the investigation of the virulence of the *A. hydrophila* ML09-119, virulent factors investigation and vaccine development of the *A. hydrophila* ML09-119.

1.1 Objectives of the study

The overall goal of this research was to ascertain the virulence nature of the *A. hydrophila* ML09-119 on different fish species, channel catfish, blue catfish, hybrid catfish, grass carp, the important farm raised fish species. By knocking out the unique genes of the epidemic strain, gene(s) that are responsible for the virulence of the epidemic strain may be found. We aimed to evaluate the virulence of multiple *A. hydrophila* ML09-119 mutants, and evaluate the efficacy of the avirulent mutants of *A. hydrophila* ML09-119 as live attenuated vaccine candidates.

The specific objectives were to:

1. Evaluate the virulence of ML09-119 to different fish species including channel catfish, blue catfish, hybrid catfish, and grass carp.
2. Investigate the role of the *myo*-inositol pathway in virulence by gene knock-out, virulence tests of the mutants, LD50 tests, and immune responses (ELISA).
3. Investigate the role of the O-antigen in virulence by gene knock-out, virulence tests of the mutants, LD50 tests, and immune responses (ELISA).

2. Literature Review

2.1 Channel catfish industry

2.1.1 Channel catfish industry distribution in the world

Channel catfish are native to the Nearctic. They are mainly distributed in lower Canada and the eastern and northern United States, as well as parts of northern Mexico (Tucker 2004; Page & Burr 1991).Channel catfish have been introduced into Europe, Russian Federation, Cube and portions of Latin America, even though in most of these areas channel catfish are only treated as a recreational fishing species (Figure 1.1. FAO 2006). As a food source, channel catfish are farm raised mainly in Asia, North America and part of Africa region (Morris 1993).

2.1.2 Farmed channel catfish industry in US

The United States catfish industry began in the 1960s. The industry has experienced a huge growth throughout the 1980s, especially the aquaculture of channel catfish *I. punctatus* (Rafinesque). The commercial catfish industry is now the biggest in the United States aquaculture sector, which represents 46 percent of its total value and the channel catfish makes 99% of all the cultured catfish here in U.S. (Li et al. 2008; USDA 2006). In the year of 2005 alone, the catfish production in the U.S. reached 600 million pounds from 165,000 pond water acres. The farm-raised catfish industry is making 450 million dollars profit annually, which makes it the highest economic value of any aquaculture industry in the U.S. followed up by trout.

Channel catfish attracted people's attention in the 1870s when the United States Fish and Fisheries Commission began to collect fish from the wild environment and stock the fish (Stickney 1996). The first case of pond spawning happened at a government hatchery in 1914 followed up by the commercial aquaculture practice in the late 1950. Due to the improvements in the pond management, feed preparation and disease identification and control, the aquaculture of farm-raised catfish developed rapidly in the 1960s and 1970s. Channel catfish since then quickly became the major catfish grown, as it was hardy and easily spawned in earthen ponds (FAO 2006; Lucas 2012).

Most of catfish farms are located in the south United States region. The two major catfish-producing areas of the United States – the alluvial flood plain in Mississippi and Louisiana, and the Blackland Prairie in west Alabama and east Mississippi (Tucker, 2004). Of all the catfish raising regions, Mississippi produces the most amount of catfish (Stickley, 1989). In 2005, it produced 350 million pounds catfish, which makes up 55 percent of all U.S. catfish production (FAO, 2005).

Even though there are approximately 44 valid species of the *Ictaluridae* (Page and Lundberg, 2007), only six have been cultured or have potential for commercial production, because of their growth rate, maximum sizes, life history, etc. The six species are: blue catfish, white catfish (*I. catus*, Linnaeus), black bullhead (*I. melas*, Rafinesque), brown bullhead (*I. nebulosus*, LeSueur), yellow bullhead (*I. natalis*, LeSueur) and flathead catfish (*Pylodictis olivaris*, Rafinesque). (Wellborn, 1988)

2.1.3 Production systems

Most of the channel catfish are grown in ponds, even though they are reared in ponds, cages, raceways and tanks. Most of the time in U.S. catfish farms are monoculture, instead of polyculture with traditional species such as carp in Asian counties. Fingerlings for grow-out are either from the farmers' own hatcheries facilities or bought from commercial fingerling companies (Kinnucan, 1995).

For ponds grow-out techniques, the fingerlings are directly stocked in to ponds to grow out. Intermittent harvesting approach is applied mostly nowadays, in which ponds are partially harvested every several months for the fish that are market size followed by stocking an appropriate number of replacement fingerlings. 50% of estimated fish body weight fish feed will be fed daily as a start when the fingerlings are first stocked into the ponds. The feeding rate may be gradually reduced as the fish grow. A final feeding rate of 4% body weight will be reached once the fish are a few centimeters long. Water quality it the key in pond catfish production (Hargreaves 2002; FAO 2006).

Tanks and raceways grow-out techniques are sometimes used to rear channel catfish, but not commonly applied for commercial successful farms, if there are any (Esquivel 1998). The tanks and raceways grow-out is pretty similar to the pond grow out other than that the flow-through raceway and culture depends on a huge supply of water supply. The temperature is usually controlled around 26-30 ℃ for grow-out. The cages grow-out techniques are also sometimes applied in some streams, lakes or reservoirs.

However, other than culture chambers used, cage culture practices would be similar to the ponds and raceway systems.

2.1.4 Marketing

The fish harvested may be processed depends on the market demand. The fish may be steaked; filleted; or sold headed, gutted, and skinned, but value-added processing practices are avoided by all the catfish product producers as much as they can. The Catfish Institute (TCI) was established in 1986 to advertise and market the channel catfish products nationwide, because it was a big challenge to sell increasing amount of channel catfish produced in the 1980s. The market for the channel catfish is better in the southern United States than the north, because the channel catfish have always been considered as a food fish in this region. However, it was a big challenge to expand the market to nationwide. More than 30 million dollars had been invested in TCI so far to promote the channel catfish to consumers and food service professionals through advertising and other promotion programs. With the rapid increasing of the catfish production and the competition for the Asian counties, the marketing of channel catfish is still a challenge.

2.1.5 Threats: import, water quality, diseases

In spite of the thriving of the channel catfish industry, the sustainability of the industry is still facing major threats including increasing import of catfish products from Asian countries especially China and Vietnam, the water quality of the channel catfish farm and the breakout of the channel catfish diseases.

The catfish industry in Asia is thriving that the increasing import of frozen catfish fillet from Vietnam and China threatened the sustainability of the U.S. channel catfish industry (Hanson 2009). In 2012, around 237 million pounds of frozen catfish imported into U.S., which was increase by 14 percent that the previous year. The 78 percent of all U.S. sales of frozen catfish fillet product is from the imports now (Figure 1.2. Hanson 2012).

According to Boyd and Tucker, the further developing of the channel catfish industry has been mainly limited by the water quality deterioration in the commercial channel catfish ponds (Boyd and Tucker 1998). The intensification of the industry required much more feed amount and feed frequency, which causes increasing the accumulation of toxic ammonia or nitrite concentrations. Ponds management including the application of chemotherapeutants such as copper sulfate, potassium permanganate, and some other therapeutant used for treatments of diseases outbreaks disrupts the microbial communities in ponds, which are important for optimum water quality maintenance (Mischke 2003),. Damaged microbial communities don't function properly to degrade the organic matters in the aquaculture ponds, which might also cause the increase of the toxic ammonia and nitrite concentrations (Boyd 1984; Tucker 1985).

Another threat in the channel catfish industry is the outbreaks of infectious diseases. According to USDA, infectious diseases of catfish fish results in huge economic losses more than 50 million dollars each year, due to the numbers of fish died during the disease outbreaks. Columnaris disease is considered as the major causes of catfish morality in the commercial channel catfish farms (Shoemaker et al. 2011). Other bacterial

diseases such as enteric septicemia of catfish (ESC), motile aeromonads septicemia (MAS) and fish gangrene caused by *E. tarda* also contributes to the losses in channel catfish production (Wagner 2002; Declercq 2013).

2.2 Catfish biology

According to Teichert-Coddington, an ideal finfish species for pond aquaculture should include the following characteristics: controlled reproduction, efficient conversion of natural foods and formulated feeds, tolerance of a broad range of water quality, low susceptibility to disease, tolerance of high density culture conditions and handling, acceptability in the market, and profitable production (Teichert-Coddington, *et al.*, 1997). In that case, channel catfish are a model species for commercial aquaculture.

2.2.1 Spawning:

The spawning procedure is simple for channel catfish, which is just stock male and female brood fish into ponds. Containers that stimulate the nesting cavity by male channel catfish are provided. Once the eggs are fertilized, they will be removed from the containers into troughs for hatching. The trough for hatching channel catfish eggs are provided with mildly flowing water (Hargreaves 2014).

2.2.2 Habitats:

Channel catfish can live in wide range of habitats such as moderately turbid aquatic environment, deeper or lager static waters, moderate gradient or swift flowing

streams or rivers; sand, silt, gravel or boulders bottoms. (Pflieger 1997). Channel catfish are fresh water fish, but they can thrive in brackish water as well. Even, channel catfish can thrive in a variety of habitats, the ideal cover types for channel catfish is woody structures, and they prefer staying at 1 to 2 meters depths (Coon 1991). Channel catfish are relatively tolerant of adverse water-quality conditions (Hargreaves 2014).

2.2.3 Nutrition:

Channel catfish are omnivorous and opportunistic in the natural environment and readily accept prepared diets and perform well on a variety of diet formulations. Factors such as location, climate, and fish size can all determine the diet composition (Flotemersch 1996; Hargreaves 2014). In the wild, insects, crustaceans, fish, plant material and inorganic material will all be consumed by channel catfish (Lawler 1960; Rice 1941; Stevens 1959)

2.2.4 Age and Growth

The optimum temperature for channel catfish is 85°F (29.4°C). The age and growth that channel catfish can reach in the natural environment depends on many factors. In most wild habitats, the fish will not reach a size of 13 inches total length until 8 years old. The life span of channel catfish can reach to almost 40 years as recorded. In commercial catfish production ponds, channel catfish can grow to market size of 0.7 to 0.9 kg from hatch in 15 to 18 months (Figure 1.3. Wellborn 1988).

2.2.5 Water quality:

Water quality can be the limited factor for the growth of channel catfish in both natural environment and commercial catfish farm ponds. The lethal oxygen level for channel catfish is 1 ppm (Wellborn 1988). Reduced growth will be observed when the dissolved oxygen (DO) decreases to 4 ppm. The water temperature extremes are near 0 °C and 38-40 °C. Channel catfish stop feeding when the temperature reaches below 8-10, or higher than 36-38 (Buentello 2000; Tucker 2004). PH ranges for channel catfish are not well defined, however, the optimum pH range is 6.5-9.0 (Bergerhouse, 1990). The upper limit for salinity tolerance of catfish is 12 ppt (Norton and Davis 1977). Lethal concentration (LD50) of channel catfish for ammonia is around 2 mg/L as NH_3-N (Knepp and Arkin 1973; Sheehan and Lewis, 1986; Tomasso, 1980; Colt and Tchobanoglous, 1976; Bader and Grizzle 1992). LD50 of Channel catfish for nitrite is related to the temperature, generally around 22 °C, and LD50 of Channel catfish for nitrite is11.7 mg/l as NO_2-N (Colt and Tchobanoglous 1976).

2.3 Infectious catfish diseases:

Infectious diseases played a minor role in fish losses on the channel catfish farm before 1980, which was the early years of the catfish industry. The reason for that was the less intensive management including less stocking density and less feed etc. That management strategy generally resulted better water quality in the catfish culture ponds with few episodes of oxygen depletion and much less stress on fish populations. The stocking densities were less than 10,000/ha, and the feeding rates were under 50 kg/ha per day (Tucker 1985)

Since the mid-1980s, with the stocking rates (higher than 12,000/ha), feeding rates (larger than 90-120 kg/ha per day) going up, and multiple-batch cropping system being applied, more frequent and devastating bacterial diseases occurred.

2.3.1.1 Protozoan parasites

Ichthyophthirius multifiliis:

Ichthyophthirius multifiliis is a ciliate pathogen that can cause Ichthyophthiriasis ('Ich' for short). It is the main parasitic threat to the freshwater fishes almost all over the world. The pathogen infect and colonize on the epidermis of the fish, and shows its pathogenic effect as 'white spots' at the lesion area, which is actually an encysted parasite (Elser 1955; Jessop, 1995; Traxler et al., 1998). The reproduction of this organism is so fast that once it gets into a large fish far, it is very hard to control the disease. 100 percent mortality can be reached if the disease is not identified and controlled on time. The cost to control this disease can be very high to, due to the fish mortality, chemicals used, and labor (Dickerson 1995; Paperna 1972). The white spots cause damage to the gills and skin. Damage caused on the gills can eventually reduce or even lost the respiratory ability, which makes the fish die from oxygen depletion. The special move this pathogen has can cause most damage while get in and out of the fish tissue, which leads to ulcers and loss of skin. That can cause secondary infection as well (Allison 1963; Matthews 1994; Noga 2010).

Chilodonella:

Chilodonella species are ciliated organisms, most of the Chilodonella species are free living non-parasite (Pádua 2013; Hoffman 1978). Mostly *Chilodonella hexasticha*, *Chilodonella piscicola* and *Chilodonella uncinata* can serve as external parasites and infect a wide range of freshwater fish. They can cause damage to the skin and gills, which leads to hypoxia (lack of oxygen), ulcer and necrosis (Paperna 1991).

Ichthyobodo:

Ichthyobodosis is an important parasitic disease that has caused severe loss among ornamental and farmed fish worldwide *Ichthyobodo necator* is an obligate parasite, which is a small teardrop-shaped, flagellated protozoan (Levine 1980). It can attach to the gills and skin of the fish and cause severe epidermal destruction (Roubal 1987; Urawa 1992). It has cause a lost economical loss and reduced fish welfare (Mitchell & Rodger, 2011), especially it is considered as one of the most damaging parasite among farmed salmon and is the major cause of mortality in salmonids fry (Robertson 1985).

Trichodina:

There are two species that infect the channel catfish: *Trichodina discoidea* and *T. vallata.* They mainly cause damage to the skin and gills of channel catfish. Normally, Trichodina is not a problem unless the fish farm having poor water quality (Padnos

1942). They can affect fish almost all year long, but records show that the lowest levels ovvur in the warmest months (Tucker 1984).

2.3.1.2 Metazoan parasites

Crustaceans:

Most of the crustaceans' parasites in fish are the subclass Copepoda, which infects fish as either direct or intermediate host. Lernaeids which is also referred as the anchor worms, are part of this subclass that commonly cause parasitic problems in aquaculture (Meyer 1966). Lernaeids are not a big problem in pure catfish farms. Most of the infection in catfish happens when catfish are co-cultured with bighead carp, which are typically infected by the anchor warm. This parasite cause damage to the gills of fish, and it can cause mortality in some severe infections (Goodwin 1999).

Cestodes:

Cestodes are, for the most part, parasites of fresh water and marine fish (Robert 1991). Cestodes, also refers to as tapeworms, have complicated life cycles that involves either one or two intermediate hosts. Fish can act as intermediate host, final host or both (Noga 2010) Cestodes have suckers on the head for attachment to the intestine of the fish. Hoffman observed a case that channel catfish were infected by *Corallobothrium fimbriatum* in the catfish intestines (Hoffman 1979). The pathogenesis of tapeworms is causing damage to tissues, mechanical blockage, and nutrient malabsorption. If the fish get massive infection, it can cause much more damage (Chambrier 2008; Hildreth 1985).

Nematodes:

Not a lot nematode parasites present in channel catfish. And even if they do, they don't cause much damage to the channel catfish host (Hoffman 1999). *Eustrongylides spp.* (Baker 1976), *Camallanus oxyephalus* (Plumb, 1979), *Dickelyne robusta* (Anthony 1963) have been reported found in channel catfish.

Trematodes:

Trematodes can be divided into monogenetic Trematodes and digenetic Trematodes. Monogeans are primarily fish parasites and have only one host, which are found on head and flank, fins, branchial arches and surface of the nasal epithelium (Cone 1995). They can cause mortality due to significant tissue damage caused by their attachment and grazing of exposed integument (Noga 2010). Digenetic trematodes have multiple hosts. *Clinostomum marginatum* (yellow grub), *Bolbophorus damnificus* (Overstreet 2002), *Diplostomum spathaceum* (Hoffman 1999), *Hysteromopha triloba* (Hoffman 1999) have been reported to infect channel catfish.

2.3.3 Viral diseases

Channel catfish virus disease:

Channel catfish virus disease (CCVD) is a devastating disease that caused by channel catfish virus, which is and alpha-herpesvirus. It mainly affect young farm raised channel catfish fingerlings usually around 4 cm or less in the first few month after

stocking fry ponds. 100 percent mortality rate can be approached under the proper conditions. This virus has double-stranded DNA genome and a icosahedral capsule. This disease was first reported in 1968 by Fijan (Fijan 1970). Dr. John Plumb at Auburn University did most of the important work on characterization of CCVD (Plumb 1978). Channel catfish virus is a host specific virus that only infected channel catfish. It cannot even infect blue catfish X channel catfish hybrids. The clinical signs of infected channel catfish includes: petechial and ecchymotic hemorrhage in the body, and base of fins, exophthalmia, abdominal distention, and hemorrhagic gills (Noga 2010).

2.3.2 Bacterial diseases

Enteric septicemia of catfish:

E. ictaluri is a gram negative bacterium that causes Enteric septicemia of catfish (ESC), which represents about 30% of all disease cases in the southeastern United States (Hawke 1981). ESC causes huge economic losses to the catfish industry each year, estimated to be 4 to 6 million dollars in 1990. The economic losses are still increasing every year. In 1996, 70 percent of catfish farms suffered from this disease (USDA 1997). ESC was first identified as a new bacterial disease that affecting channel catfish ponds in 1976 at Auburn University (Hawke 1981). *E. ictaluri* is the primary channel catfish pathogen in the southeastern United States, where most of the channel catfish were cultured. ESC has been diagnosed for channel catfish culture facilities in almost all the regions in the southeastern United States including:

Mississippi, Arkansas, Alabama, Louisiana, Georgia, and Florida. Also some occasionally breakout of ESC in Virginia, Idaho, Kentucky, Texas, Indiana, California, Arizona and Maryland (Plumb 2011).

Channel catfish is reported to have the highest susceptibility to the infection of *E. ictaluri*, followed by White catfish *Ameiurus catus,* brown bullhead *Ameiurus nebulosus,* and walking catfish *Clarias batrachus* (Wolters 1994). According to the results of Wolters' fish challenge experiment, juvenile channel catfish have the lowest survival rate (62%) and juvenile blue catfish have the highest (90%), however, channel X blue catfish hybrids have an intermediate survival rate (74%) (Wolters 1996). Other fish species such as rainbow trout *Oncorhynchus mykiss* (Humphrey 1986), blue tilapia *Oreochromis aureus* (Plumb 1983) and Chinook salmon *Oncorhynchus ishawytscha* (Baxa 1990) showed no clinical signs of ESC after experimental infection.

The clinical signs of channel catfish infected with ESC including loss of appetite, tail chasing, lethargic, exophthalmia, abdominal distention, yellowish or bloody Ascites, petechial hemorrhages, small ulcers, necrosis of internal organs such as kidney and liver, 'hole in the head' (Hawke 1981). However, the clinical signs of fish infected with ESC may differ depending on the stage and severity of the infection, stress, water quality. In some acute infections, the disease develops and progresses so fast

that few clinical signs might be observed, before high mortality rates are achieved (Noga 2010).

E. ictaluri can invades the catfish through gut epithelial cell, with the help of actin polymerization and receptor-mediated endocytosis, which will aid the catfish gut epithelial cells to uptake the bacteria *(*Skirpstunas and Baldwin 2002). Once *E. ictaluri* get through intestinal epithelial cell barrier, it will be transported via the blood stream. *Edwardsiella ictaluri* can still survival and replicate even if it is engulfed by macrophages (Miyazaki and Plumb 1985; Booth 2009). Unease and T3SS are required for *E. ictaluri* to be able to survive and replicate within the acidic environment within the phagosome (Booth *et al.,* 2009; Rogge and Thune 2011).

Besides urease, chondroitinase and Lipopolysaccharide, are reported to be the virulence factors for ESC (Cooper 1996; Lawrence 2001). Thune demonstrated another factor might be related to the virulence of E. *ictaluri,* which is a putative adhesion (Thune *et al.*, 2007). The virulent properties of this putative adhesion in catfish was confirmed by Polyak (Polyak 2007). The virulence factors of *E. ictaluri* haven't been fully characterized yet, so the genetic characterization of *E. ictaluri* is still ongoing and methods for identifying and sequencing virulence genes have been developed (Thune *et al.*, 1993; Cooper *et al.*, 1996).

Columnaris disease:

Columnaris disease is caused by *F. columnare,* a gram negative bacteria fish pathogen that is distributed world wild (Declercq *et al.* 2013). Columnaris disease is

historically ranked the second of the important fish diseases in the catfish farming industry in the United States. (Durborow *et al.*, 1998). Columnaris disease or its mixed infections and ESC accounted as leading the most mortality of catfish and causing greatest economic losses by 70 percent on catfish farms in U.S (USDA 1997). Columnaris disease is a worldwide problem in many fish species (Plumb 2011). However, it seems that channel catfish and other *ictalurids* are more susceptible to this disease than others (Meyer 1970).

The clinical signs of Columnaris disease includes: grayish white spots (Noga 2010), decoloration of the skin, 'saddleback' effect which is a yellowish-brown ulcer in the dorsal fin termed (Arias 2012), necrosis and yellowish mucoid material in the oral cavity. Mortality pattern can be both chronic and acute. In some acute cases, mortality happens within 24 hours, so little or without any pathological signs might be shown before fish die (Arias 2012; Noga 2010).

Virulence factors of *F. columnare* are not full addressed yet in channel catfish. An enzyme (Chondroitin AC lyase) produced by *F. columnare* may contribute to pathogenicity in warm water fish (Griffin 1991). Rhamnose-binding lectin in the adhesion of *F. columnare* to the gill in particular is strongly associated with pathogen virulence and host susceptibility (Beck 2012). Out of the three existing genomovars of *F. columnare*, genomovar II is highly pathogenic to channel catfish (Arias *et al.*, 2004; Shoemaker *et al.*, 2008).

Motile Aeromonads Septicemia:

Aeromonas hydrophila, is a causative agent of motile Aeromonads septicemia (MAS). It's a Gram-negative motile bacterium that is widely distributed in aquatic environments all over the world (Harikrishnan et al., 2003). MAS breakouts usually are related to stress of the fish, so it is often induced by some physical or chemical damage or following some other diseases in channel catfish as a secondary pathogen (Tucker). *Aeromonas hydrophila* is involved in single or mixed infections with other bacterial pathogens in 44% of bacterial disease cases for channel catfish farms (Hawke 1992).

The clinical signs of MAS caused by *A.hydrophila* include swelling of tissues, dropsy, red sores, necrosis, ulceration and hemorrhagic septicemia (Karunasagar et al., 1989; Azad et al., 2001). Mortality patterns of MAS can be both acute and chronic. Acute forms of MAS caused by *A.hydrophila* normally can have signs such as abdominal distension caused by acites, hemorrhage in the skin, necrosis, and exophthalmia. Chronic signs of this disease include: ulceration, hemorrhage and necrosis (Thune 1993).

The range and susceptibility of MAS caused by *A.hydrophila* is ubiquitous, and can be found in both freshwater and brackish water environments worldwide (Thune 1993). Most common fish species affected by MAS are tilapia, catfish, goldfish, common carp, and eel (Pridgeon 2011).

As a catfish pathogen, most of the time, *A.hydrophila* is considered as a secondary pathogen that invades into the host following other disease outbreaks such as columnaris disease, which is caused by *F. columnare* and septicemia of catfish (ESC), which is caused by *E. ictaluri* (Olivares-Fuster 2007; Labrie 2004; Noga 2010). In this case, consequently stress is believed as a factor that contributes most in disease outbreaks caused by *Aeromonas hydrophila* (Yin et al., 2009). When it emerges as a primary pathogen, it can also cause significant economic losses to fish farms due to high mortality (Pridgeon, et al., 2011). In 2009, catfish farmers in west Alabama reported severe disease outbreaks which were then proved to be caused by a highly virulent strain of *Aeromonas hydrophila*, ML09-119, to channel catfish. From 2009-2011, Alabama catfish famers lost more than 10 million pounds of catfish that were market-size and estimated to be $3 million due to this epidemic strain of *A. hydrophila* (Pridgeon et al., 2011; Liles et al., 2011). It is reported that *A. hydrophila* epidemic strain, ML09-119, is highly virulent to channel catfish, causing severe mortality within 24 h post exposure with certain amount of dose. The pathogenic strains from 2009 was at least 200-fold more virulent than an *A.hydrophila* stain isolated in 1998 (Pridgeon 2011).

2.4 Therapeutant and antibiotic treatments

In the United States, only a few options available for fish farmers to choose to control fish disease in general. The use of therapeutant mostly antibiotics and vaccination are the major two options for catfish farm to control disease. So far, there are only seven drugs of which four are antibiotics that have been approved by the U.S. Food and Drug

Administration (FDA) for use in aquaculture (Benbrook 2002) and a single anesthetic that were approved by the U.S. Food and Drug Administration (FDA) for use in aquaculture. The use of these drugs was severely restricted by species, life stage, specific pathogen, and use pattern.

Terramycin Aqua® (oxytetracycline hydrochloride) (Antibiotic);

In salmonids, it is used for control of ulcer disease caused by *Hemophilus piscium*, furunculosis caused by *Aeromonas salmonicida*, bacterial hemorrhagic septicemia caused by *A. liquefaciens*, and pseudomonas disease. In catfish, it is used for control of bacterial hemorrhagic septicemia caused by *A. liquefaciens*, *A.hydrophila* and pseudomonas disease. It need 21-day withdrawal period before fish can be released, stocked, or harvested (Vignesh, 2011).

Aquaflor® (florfenicol) (Antibiotic);

Aquaflor® (florfenicol) is approved by the Federal Drug Administration (FDA) in Nov, 2005 to specifically control mortality in catfish due to enteric septicemia (ESC). Although this process may help, but the use of this antibiotics it may also delay treatment, because it requires veterinary feed directive from a licensed veterinarian prior to its use by catfish farmers (Vignesh, 2011).

Sulphadiazine(Antibiotic);

Sulphadiazine Control of furunculosis in rainbow trout, brook trout, and brown trout casued by *Aeromonas salmonicida*. However it is not commercially available yet (Torkildsen, 2000; Vignesh, 2011).

Romet 30® (ormetoprim and sulphadimethoxine) (Antibiotic);

It is applied to in feed for the control of *furunculosis* in salmonids caused *by Aeromonas salmonicida*. In catfish farms, Romet is used to control of enteric septicemia of catfish caused by *E. ictaluri* strains. 42-day withdrawal period before fish can be released, stocked, or harvested is needed for salmonids, 3-day withdrawal period for catfish (Vignesh, 2011; Durborow et al. 1998).

2.5 Vaccines available in fish industry

2.5.1 Application of vaccines in catfish industry

Due to the increasing awareness of the negative effects of using antibiotics, such as increasing resistance of the pathogen to the antibiotics and presence of the antibiotic residues in aquaculture products (Le 2004), in the aquaculture industry, there comes the trend to apply stricter regulations on the use of antibiotics in the sector of aquaculture (Smith 2008), Regulations on the use of antibiotics are strict in some countries especially, in the EU, US and Japan and only a few antibiotics are licensed

for use in aquaculture (FAO 2002). Vaccine development as a better substitute strategy of the antibiotic to control diseases breakout is studied and applied.

2.5.2 Vaccine types:

Inactivated vaccines:

Inactive vaccines are dead bacteria with part of whole organisms as antigens. Normally they are Gram-negative organisms such as *Vibrio ordalii*, *Vibrio anguillarum*, *Vibrio salmonicida* and *Yersinia ruckerii*. Inactivated vaccines are produced by broth cultivation and subsequent formalin inactivation, and some are produced by heat inactivation. The most advantage is inactive vaccines have so few side effects that they can be neglect (Stevenson 1997; Toranzo et al., 1997). Studies showed that for some diseases, such as *Aeromonas salmonicida subsp. salmonicida*, the inactive vaccine can only provide protection when applied with adjuvant and is delivered by injection (Ellis 1997; Midtlyng 1997). So far, most vaccines used in aquaculture are inactivated, bacterial vaccines. However, inactivated virus vaccines against infectious pancreatic necrosis (IPN) in Atlantic salmon and grass carp haemorrhage disease have been reported successful recently (Dixon 1997).

Live vaccines

An attenuated vaccine is a vaccine created by reducing the virulence of a pathogen, but still keeping it live Badgett 2002). There are many advantages applying live attenuated vaccines in aquaculture (Benmansour and de Kinkelin 1997). First of all, the vaccine efficacy would last for a very long time, because the vaccine strain is

processed by the host and the dissemination of the antigens are disseminated effectively. Secondly, live vaccines can stimulate the cellular response of the immune system (Marsden et al., 1996). Thirdly, attenuated vaccines are cost effective, because of the simple delivery and low dose requirements. However, live vaccines might have the risks of virulence reversion under the nature environment (Gudding 1999).

DNA vaccines:

The biggest advantage DNA vaccines have over conventional vaccines is specificity of the vaccine (Lorenzen 2005). And also, since the vaccine contains only the DNA-sequence that encodes just a single viral factor gene, it is impossible for the pathogen to regain the virulence. That is a critical factor in terms of environmental safety in aquaculture. The specific immune responses induced by the DNA vaccine include producing antibodies, activating T-helper cells, as well as cytotoxic cells (Badgett 2002).

Researches on deoxyribonucleic acid (DNA) vaccines are mostly focused on virus disease vaccines. The most successful DNA vaccine is probably the vaccine against viral disease *salmonid rhabdoviruses*, viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), in fish. These vaccines are naked plasmid DNA, which can mediate expression of the viral glycoprotein after uptake by the vaccinated fish. Most of time, researches on DNA vaccine in fish are focusing on the use of gene that encodes for the reporter proteins such as luciferase and green fluorescent protein, and the genes that encode antigens

(Lorenzen 2005). So far, the only licensed recombinant fish vaccine is for protection against IPN (Frost and Ness 1997).

2.5.3 Current live vaccines in fish

So far only three modified live vaccines are licensed for use in the USA. These include the vaccine against bacterial kidney disease (Renogen®1), enteric septicemia of catfish disease (AQUAVAC-ESC®) and columnaris disease (AQUAVAC-COL®) (Shoemaker et al., 2009). In one study, it showed that that RENOGEN may have conferred hidden protection against SRS as well as BKD in salmon. In this study, when challenged with *Piscirickettsia salmonis,* salmon that had received the RENOGEN vaccine exhibited extremely low mortality rates around 88% and 100% relative percent survival (RPS) after 471 dd (degree days), compared k-to only 48.6% in the un-vaccinated *P. salmonis* group (Griffiths 2007).

The live-attenuated vaccine AQUAVAC-ESC® was licensed in 2001-2001. It was registered and marked by Intervet, Inc., for use against ESC (Shoemaker et al., 2002). It is reported to be safe and efficient in reducing mortality in channel catfish fry and fingerlings according to the laboratory studies (Shoemaker et al., 2002, 2007; Wise 2006).

2.6 The Gene knock out technique:

Gene knock out technique is based on horizontal gene transfer (Thomas 2005). It is not a new process for the horizontal gene transfer such as genetic manipulation and mutant creation. As matter of fact, this process happens in nature very common,

especially in bacteria between species/strains and virus *etc*, for the purpose of evolving and surviving (Ochman 2000). All the gene horizontal transfer follows one simple rule that is gene recombination between homologous regions of genes (Koonin 2001). This process commonly generates mutations that might cause one organism to evolve into a new branch or evolutionary path from its parent (Davison 1999; Rocha 2005).

Gene knock out technique generally applied to make one or more genes in a organism inoperative or so called 'knocked out' from the chromosomes of this organism. The generating organism is called a gene knockout mutant or simply a gene knockout. The process of knocking out a gene is a combination of different techniques. First a DNA construct which is normally a plasmid or some other artificial chromosome is designed with homologous arms of the target gene. Then, the construct is introduced into *Escherichia coli (E. coli)*. The *E. coli* is then conjugate with the other bacteria, in which process the gene itself is exchanged the construct. Recombination occurs within the region of the homologous arms, resulting in the insertion or exchange of a foreign sequence to disrupt the gene. Most of the time, the resulting gene may not be translated, but if it does, the translated gene won't be able to produce a functional protein.
(Galli-Taliadoros 1995; Baba 2006; He, 2006; Alexeyev 1999; Alper 2005).

Gene knock out technique has been done very commonly in research. Scientists have applied this technique to study about a sequenced gene whose function has not been known completely (Maiden 1998). Through comparing the difference between the gene knock out mutant and its parent (wild type), researchers learn the possible function of the

knocked out gene (Rocha 2005; Thomas 2005). The knockout techniques are used in the developing of medicine especially antibiotics as a screening method. Through knocking out the targeted gene that is related to a certain biological process, the researchers are able to understand the mechanisms and the function of the genes of this biological process (Alexeyev 1999; Alper 2005; Ochman 2000).

The gene knockout technique has been using for the study of bacterial pathogen vaccine very common for both human beings and fish. For example, in human disease studies, Collins et al studied on the live vaccine candidates include a genetically modified forms of Bacillus Calmette–Guérin (BCG) which is a tuberculosis vaccine. They produced large numbers of mutants through transposon mutagenesis or illegitimate recombination and are screened all the mutants for properties that correlate with attenuation (Collins et al 2000) Wards et al, created an *esat6* knockout mutant of a virulent strain of *Mycobacterium bovis* using a homologous recombination technique. This mutant was proved to be less virulent that its parent, which suggesting that esat6 gene could contribute to the loss of virulence. That will contribute to the development of a live tuberculosis vaccine (Wards 2000). Raupach and Kaufmann used *Salmonella typhimurium* strains with mutation to study the interaction between the bacterial pathogen and host immune response. Their study indicated that *S. typhimurium* strains with mutations in *aroA*, *phoP/phoQ* or *ssrA/ssrB* invoke different immune reaction in the host (Raupach and Kaufman 2001). There are a lot other human diseases study using the gene knockout technique, such as cancer (Hung 1998; Karpf 2006), cholera (Cameron 2008) and diarrhea (Sha 2002), etc. In the fish bacterial disease

studies: Choi, created the double gene knock-out mutant of *Edwardsiella tarda* (*Δalr Δasd E. tarda*) through allelic exchange method, which was a combined vaccine system. Alanine racemase (*alr*) gene and aspartate semialdehyde dehydrogenase (*asd*) gene are two important genes in cell wall biosynthesis. They demonstrated that compared to wild-type *E. tarda,* the virulence of the *E. tarda* mutants was decreased by about 10^6 fold increase of LD_{50} dose. And they also used olive flounder (*Paralichthys olivaceus*) to evaluate the vaccine potential of the mutants. They challenged the fish with 10^7 CFU of the *Δalr Δasd E. tarda* mutants and the fish groups showed no mortality. And the fish challenged with the mutants showed significantly higher serum agglutination activities against formalin-killed *E. tarda* than the control group. Their study showed that their *E. tarda* mutants coupled with a heterologous antigen expression has a great strategic potential to be used as combined vaccines against various fish diseases (Choi 2011).

2.7 *Myo*-inositol pathway and the virulence

Inositol containing molecules are not commonly found in bacteria, but only exist in certain classes of bacteria. The acquisition of inositol has been reported to relate to maintaining the osmotic balance in some Archaea and *Thermotoga sp.* However, most inositol compounds are found in mycobacterium. In bacteria, there are no reports of detectable concentration of *myo*-inositol accumulating. Normally they are converted to soluble phosphate esters, whose roles are commonly related to protecting cells against stress. They are also converted to various phospholipids, which are normally specific to certain bacteria and involved in infectivity of the bacteria (Roberts 2006).

While Gram-positive species such as *Bacillus subtilis* (Miwa and Fujita 2001; Yoshida et al. 1997), *Corynebacterium glutamicum*(Krings, et al. 2006), *Clostridium perfringens* (Kawsar et al. 2004), and *Lactobacillus casei BL23* (Yebra et al. 2007), are known for their ability to utilize *myo*-inositol (MI) as a carbon source, several Gram-negative species such as *Serratia*, *Klebsiella*, and *Pseudomonas* (Berman and Magasanik 1966; Gauchat-Feiss et al. 1985) can also degrade MI. Although the gene clusters in different species show various chromosomal structure and organization, they all share the same negative regulation of the *iol* genes coding for the IolR protein. And also, the intermediate of MI degradation, 2-deoxy-5-keto-D-gluconic acid 6-phosphate (DKP), has been reported to be the inducer for the expression of iol genes by antagonizing the IolR binding (Kröger 2011).

Kröger reported that human pathogen *Salmonella enterica* serovar Typhimurium strain was able to utilize *myo*-inositol as sole carbon source, which is related to the fact that this strain possesses a bistable phenotype (Kröger et al. 2011), which could help this pathogen survive better in different environment than the other strains.

One study showed that *Cryptococcus neoformans (C. neoformans),* the pathogen for the most common human fungal meningitis disease, has a complete pathway to utilize the carbohydrate inositol both in plant and human being. This might contribute to the virulence of this pathogen. In this study, it is indicated that by utilizing inositol *Cryptococcus* can undergo sexual reproduction from plants. Being able to utilize inositol as a carbon source greatly increases the fitting ability of this pathogen both in plants and other niches, which may also contribute to the emergence of *Cryptococcus* virulence in humans. (Xue 2012)

Kawsar *et al.* completed the whole genome sequencing and the results revealed that there are *myo*-inositol operon in two Gram-positive anaerobic pathogens, *Clostridium perfringens* strain 13 and *Clostridium tetani* E88. There are 13 genes in the *myo*-inositol operon of *C. perfringens*, which encoding the proteins needed for the catabolism of *myo*-inositol. (Kawsar *et al.* 2004; Shimizu et al. 2002; Brüggemann *et al.* 2003).

A variety of studies have been done on the relation between the inositol acquisition and the virulence of the pathogens such as fungi, protozoa and certain bacteria (Reynolds 2009). Chen, et al. found that the yeast pathogens *Candida albicans* and *Candida glabrata (C. glabrata)* can acquire inositol through two pathways: 1. *De novo* biosynthesis pathways; 2. Import directly by inositol transporters. The fungal infection didn't get affected by blocking either pathway, but pathogens died when deleting both pathways. This study revealed that the inositol is essential for the survival and full virulence of *Candida.* (Chen *et al.* 2008). Other parasitic microorganisms also have the inositol pathway as a virulence factor. It is worth noticing that sometimes inositol uptake alone is not sufficient to maintain the growth and virulence for the pathogen; thus, inositol synthesis is required for both optimum growth and virulence maintaining. This is supported by the fact that blocking inositol biosynthesis leads to growth defect and virulence attenuation for parasites such as *Trypanosoma brucei* and *Leishmania mexicana* and *mycobacteria* such as *Mycobacterium tuberculosis*, even though they both can synthesize and import inositol (Martin and Smith 2005; Movahedzadeh *et al.* 2004).

2.8 O-antigen and the virulence

Lipopolysaccharide (LPS) is the major component of the Gram-negative bacteria outer membrane. It is composed of three domains: lipid A (endotoxin), an oligosaccharide core and O polysaccharide, also called O0antigen. Even though a lot attention has been drawn to the biological effects of its lipid A portion, a variety of studies indicated that it is O-antigen that plays an important role in the bacterium–host interactivity. (JANSSON, *et al.* 1981; Bengoechea, *et al.* 2004). The lipid A and core are highly conservative within a same genus, but the O-antigen is various in different bacteria species in terms of sugar composition, structure, length, and antigenicity (Zhang *et al.* 1997). O-antigen subunits (monomers) are assembled within the bacteria cell on the inner membrane and then flipped to the outside of the cell membrane. They then will be ligated to a lipid A-core molecule after wzy polymerizes them. (Jiang *et al.* 1991; Reeves *et al.* 1996; Murray *et al.* 2003)

Research has been done on the relations between the O-antigen of the organisms and virulence and antigenicity of pathogens. Bengoechea *et al.* studied the role of the O-antigen in *Yersinia enterocolitica* serotype O:8 pathogenesis. They indicated that the LPS O-antigen mutants they created including (i) LPS with no O-antigen (rough mutant); (ii) LPS with one O unit (semi-rough mutant) and (iii) LPS with random distribution of O antigen chain lengths, were attenuated in virulence. Comparison with the wild type in the co-infection experiments showed the incapability of colonizing of the rough and semi-rough mutants. (Bengoechea *et al.* 2004). This study reveals the O-antigen is related to the virulence of the pathogen *Y. enterocolitica.*

Van Den Bosch *et al.* showed that the *Shigella flexneri*, the causative pathogen of bacillary dysentery in human being, genetically control the modal chain length of the O-antigen polysaccharide chains. And the fact is that this regulation is required for the virulence of this pathogen (Van Den Bosch *et al.* 1997). Thomas, *et al.* Created O-antigen deletion mutants of *Francisella tularensis* subspecies *tularensis* and *Francisella novicida,* which is a pathogen that can cause tularemia, a disease that affects many mammals including humans and rodents, by Allelic replacement. The mutants were proved not being able to produce O-antigen. *In vivo* challenge study showed that they were attenuated mutants. However, the fact that these mutants did not induce a protective immune response indicates that the O-antigen of *F. tularensis* subsp. *tularensis* is important for intracellular survival whereas the O-antigen of *F. novicida* appeared to be critical for serum resistance and less important for intracellular survival (Thomas *et al.* 2007).

Table 1.1. The important diseases of catfish (FAO)

DISEASE	AGENT	TYPE	SYNDROME	MEASURES
Channel catfish virus disease	–	Virus	Reduced feeding activity; erratic swimming behaviour, sometimes spiral; alternating hyperactivity and lethargy; swollen abdomen; distended vent area; bulging eyes; haemorrhaging	No treatment; good management practices
Enteric septicaemia	*Edwardsiella ictaluri*	Bacterium	Haemorrhages external on underside and around mouth; white focal lesions on fish back and sides; occasionally grey lesion on top of head that can erupt to an open lesion	Oxytetracycline; sulfamethoxine; ormetoprin
Columnaris disease	*Flavobacterium columnare*	Bacterium	White spots on mouth, edges of scales and fins; cottony growth around mouth; fins disintegrate at edges; 'saddleback' lesion near dorsal fin; fungal invasion of gills and skin	Oxytetracycline; sulfamethoxine; ormetoprin
Aeromonas septicaemia	*Aeromonas hydrophila*; *A. sobria*	Bacteria	Fraying and reddening of fins; de-pigmentation; ulcers	Oxytetracycline; sulfamethoxine; ormetoprin
Water mould	*Saprolegnia* spp.	Fungi	Grey/white patches on skin or gills resembling cotton-wool, later becoming brown or green; normally small, focal infections spreading rapidly over body or gills	Formalin
Gill and/or external parasites	*Trichodina* sp.; *Trichophora* sp.; *Ambiphrya* sp.; *Ichthyobodo* sp.; *Ichthyophthirius multifiliis*	Protozoans	Small white spots on skin or gills; irritation, flashing, weakness, loss of appetite, and decreased activity; gills pale and very swollen	Formalin
Proliferative gill disease	*Aurantiactinomyxon* sp.; *Dero digitata*	Myxozoans	Swelling and red and white mottling of gills gives raw minced meat appearance	Formalin
Copepod parasites	*Ergasilus* sp.; *Argulus* sp.; *Lernaea cyprinacae*	Copepods	Visible parasites on gills	Formalin
Other parasites	–	Helminths; cestodes; trematodes	–	Formalin

Figure 1.1. Main producer countries of *Ictalurus punctatus* (FAO Fishery Statistics, 2006)

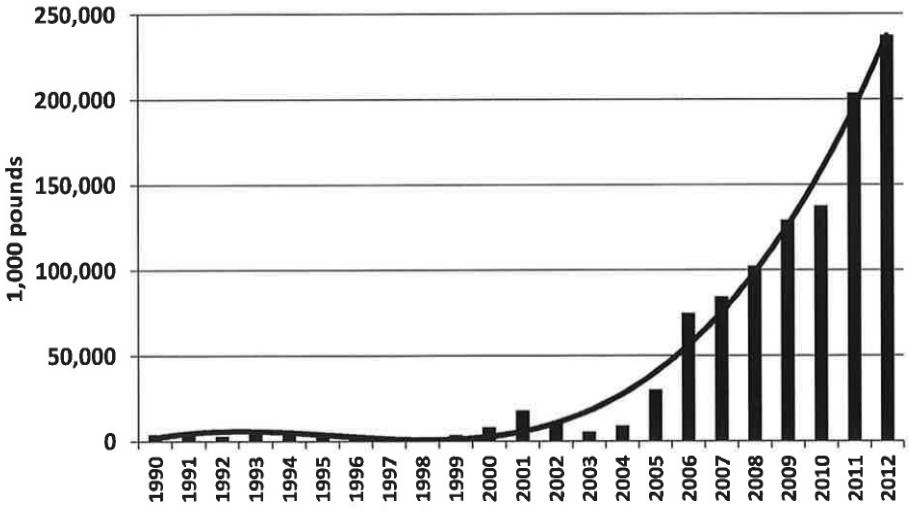

Figure 1.2. Imported catfish, 1999-2012

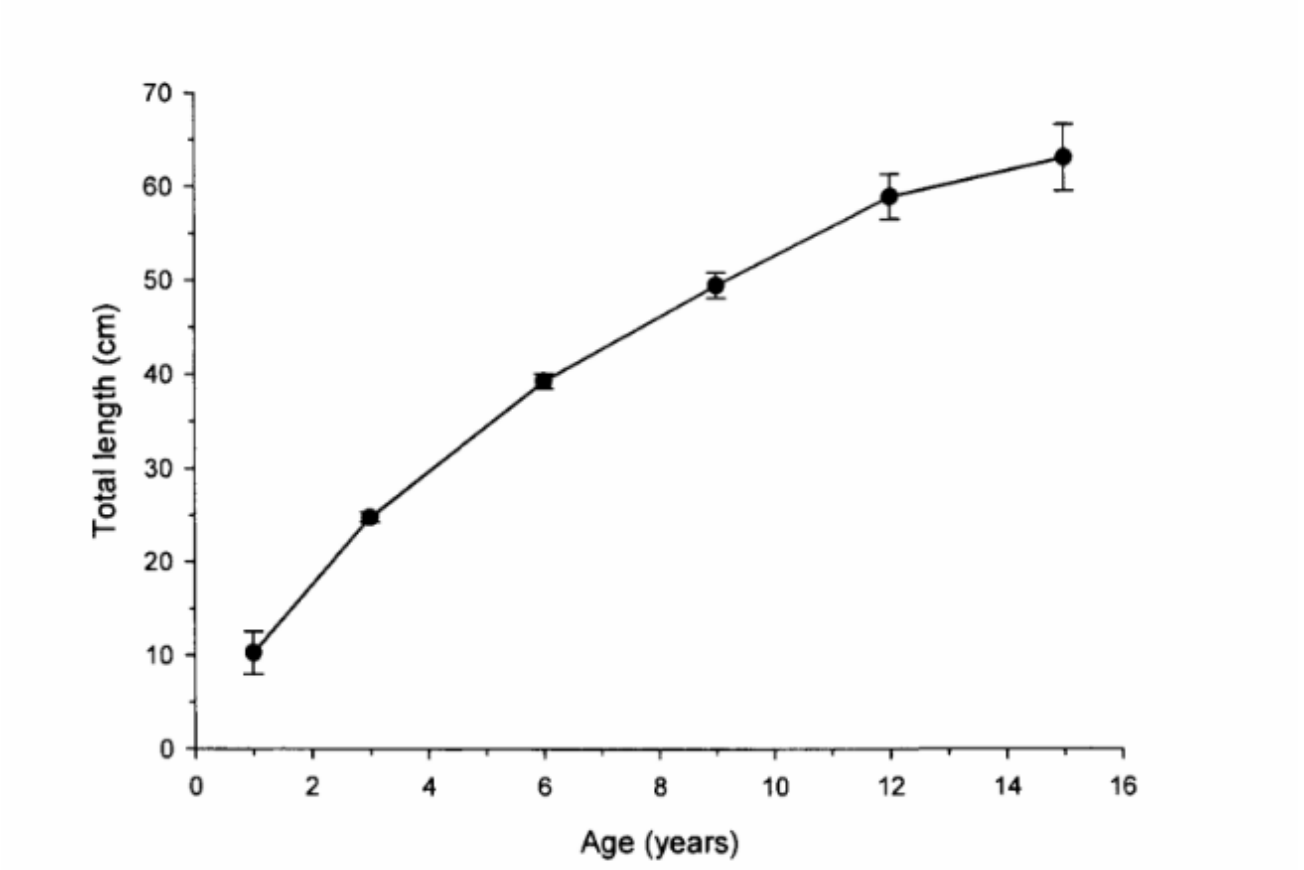

Figure 1.3. Total length at age and 95% confidence internals for wild channel catfish from throughout North America. Number of populations addressed: Age-1, N=8; Age-3 N=94; Age-6, N=86; Age-9, N=46; Age-12, N=20; Age-15, N=8

Chapter II

Experimental cohabitation challenge studies in susceptibility of channel, blue and hybrid catfish to *A.hydrophila* ML09-119

1. Abstract:

The epidemic strain of *Aeromonas hydrophila* caused a devastating outbreak of motile Aeromonads septicemia of catfish (MAS) on the fish farms of Southeastern United States in 2009. *A.hydrophila* ML09-119 was reported to cause severe mortality in commercial catfish farms. Research has shown on the virulence of this epidemic strain on channel catfish (*Ictalurus punctatus)* including molecular identification of the specific strain and unique DNA sequences. However, no research has been done so far on the virulence of this specific strain to blue and hybrid catfish. In this study, intraperitoneal injection (IP) challenge method combining cohabitation culture was used to detect the susceptibility of channel, blue *(Ictalurus furcatus)* and hybrid catfish (blue catfish × channel catfish) to *A. hydrophila* ML09-119. Our results showed that the virulence of ML09-119 to channel catfish is significantly higher than it is to blue and hybrid catfish ($p=0.0156 < 0.05$, $a = 0.05$). No significant differences were observed between blue and hybrid catfish ($p=0.0801>0.05$, $a=0.05$). Significant differences in survival were observed for bacterial strains ML09-119, ZC1, and AL06-06 ($P < 0.0001$) and between fish species in the ML09-119 group ($P = 0.0126$). No interaction effect between variables of bacterial strains and fish species was observed ($P_{\text{fish species} \times \text{bacterial strains}}=0.1002$). The difference in survival between channel catfish and grass carp injected with ML09-119 was significant ($P = 0.0069$). No significant differences in survival between channel catfish and grass

carp intraperitoneally injected with ZC1 or AL06-06 were observed, but there was a significant difference between strains ZC1 and AL06-06 in both channel catfish (P = 0.0075) and grass carp (P = 0.0089).

Key words: A.hydrophila, catfish, cohabitation, challenge, infection, disease, virulence

2. Introduction

Aeromonas hydrophila, Aeromonas spp., is a widely distributed free living, gram-negative rod-shaped bacterium with a single flagellum in aquatic environment (Daskalov, 2006; Bi et al, 2007). This motile bacillus can cause disease in fish known as 'Motile Aeromonads Septicemia' (MAS) (Harikrishnan et al., 2003; Singh et al., 2009). *Aeromonas hydrophila* can invade hosts and cause acute, chronic and latent infections and severity of disease is based on a lot of associating factors which include: the virulence of the particular strain of *A. hydrophila*, the extent of stress the population exposed to, and the immunity and physiological condition of the host (Cipriano et al., 1984). The pathologic conditions caused by *A. hydrophila* includes exophthalmia, reddening of the skin, necrosis, red sores, scale protrusion, dermal ulceration, with focal hemorrhage and inflammation and hemorrhagic septicemia, thus may be referred to as 'Hemorrhagic septicemia' or 'Red-Sore Disease' (Swann et al., 1991; Karunasagar et al., 1989). In terms of the internal organs, liver kidney and spleen of the infected hosts are usually the target organs of *A. hydrophila*, which can cause the kidney and the spleen hemorrhaging, swelling and necrosis, and cause liver turning pale or green (Cipriano et al., 1984). *Aeromonas hydrophila* is a very dynamic species, which has evolved into different kinds of strains. Some strains can produce several potential virulence factors, including adhesins, exoenzymes, hemolysins, and other enterotoxins to enhance its ability to infect hosts and cause disease (Aguilera-Arreola et al., 2005). *A. hydrophila* begins to draw more and more attention because it can invade a host as a primary, secondary or opportunistic pathogen not only in fish, but also in warm-blooded vertebrates including human beings (Gold and Salit, 1993; Merino et al., 1995; Swain, 2010). For example, *A.*

hydrophila can cause septic arthritis, diarrhea, skin and wound infections, Cellulitis, meningitis, and fulminating septicemias (Cipriano et al., 1984; Davis 1978).

As a catfish pathogen, *A. hydrophila* has been historically considered as a secondary pathogen that invades into the host following other disease outbreaks such as columnaris disease caused by *F. columnare* and enteric septicemia of catfish (ESC), which is caused by *E. ictaluri* (Olivares-Fuster 2007; Labrie 2004; Noga 2010). In this case, consequently stress is believed as a factor that contributes most in disease outbreaks caused by *A.hydrophila* (Yin et al., 2009). When it emerges as a primary pathogen, it can also cause significant economic losses to fish farms due to high mortality (Pridgeon et al. 2011). In 2009, catfish farmers in west Alabama reported severe disease outbreaks which were then proved to be caused by a highly virulent strain of *A. hydrophila*, ML09-119, to channel catfish *(Ictalurus punctatus).* From 2009-2011, Alabama catfish famers lost more than 7.5 million pounds of catfish that were market-size and estimated to be $3 million due to this epidemic strain of *A. hydrophila* (Pridgeon et al. 2011; Liles et al. 2011). It is reported that *A. hydrophila* epidemic strain, ML09-119, is highly virulent to channel catfish, causing severe mortality within 24 h post exposure with certain amount of dose (Pridgeon and Klesius 2011). However, it is currently unknown whether *A. hydrophila* epidemic strain, ML09-119, is also highly virulent to Blue catfish (*I. furcatus)* and hybrid catfish *(I. punctatus* × *I. furcatus)*, the other two most important food fish that is economically significant to United States and other countries. In this study channel, blue and hybrid catfish were exposed to *A. hydrophila* epidemic strain, ML09-119. Daily mortality data of these three species of catfish was recorded to provide information on

their susceptibility to *A. hydrophila* ML09-119. Cohabitation method was applied to provide equal environmental conditions to channel, blue and hybrid catfish, to eliminate nuisance factors (covariates) as possible. Determination of the virulence of *A. hydrophila* strains in channel catfish and grass carp was the similar to the channel, blue and hybrid catfish cohabitation study. The virulence of ML09-119, ZC1 and AL06-06 in were determined according to the methods described previously. Statistical significance was determined by the two-way analysis of variance (ANOVA) and Tukey's multiple comparisons to compare individual treatment groups.

3. Materials and methods

3.1 Fish

All the catfish used in this study, channel catfish *(I. punctatus,* Kansas Random Strain*)*, Blue catfish (*Ictalurus furcatus*, D&B Strain*)* and hybrid catfish, were artificially spawned at the hatchery of the Auburn University Fish Genetics Research Unit, prior to transferring to troughs or glass aquaria at the Auburn University Fish Pathology wet lab S-6. Fish were maintained at recirculation systems (temperature around 25 °C and pH 7.5) using well water sources, and constant aeration. Fish were fed daily with commercial feed. Water quality factors including temperature, pH, salt level, total ammonia level, total nitrite level were tested on daily basis to ensure that catfish fingerlings remained unstressed and naive to *A.hydrophila*. Catfish fingerlings were grown out in this system until their body weight (BW) reached 30±5g. Fish used for channel catfish study and grass carp were from s-6 wet lab and E.W shell fish center. Channel catfish (*Ictalurus*

punctatus) (11.8 g ± 1.2 g) were from S-6 wet lab and Grass carp (*Ctenopharyngodon idella*) (11.6 g ± 1.4 g) were from E.W shell fish center.

3.2 Bacteria source, growth conditions and preparation for challenge

The *A.hydrophila* ML09-119 used for the *in vivo* virulence experimental challenge studies were from the bacteria stocks of the fish disease lab in Auburn University. This epidemic strain was originally isolated from the kidneys of channel catfish naturally infected with *A. hydrophila*. The pure culture of epidemic strain was used first in a small test infection of 10 catfish. Moribund catfish that showed clinical signs of *A. hydrophila* ML09-119 was collected for necropsy. *Aeromonas hydrophila* was re-isolated from the moribund fish by taking an inoculum from the kidney using a plastic inoculation loop and streaking onto a BHI agar plate. By doing this, it is expected than the virulence of the epidemic strain stock can be recovered**.** Strain ML09-119 was then confirmed by biochemistry and selective media following the established identification procedures with modifications (Furuwatari *et al.*, 1994; Holt *et al.*, 1994). The identification biochemical tests included: Gram stain, cytochrome oxidase, glucose utilization, 0/129, sucrose, esculin hydrolysis, V-P, DL-lactate utilization and urocanic acid utilization, and then test on selective media M9 minimum media with inositol added.

Bacteria preparation was performed using established procedures (Pridgeon and Klesius 2011) with modifications. A bacterial suspension of exponential phase growth was prepared by overnight culture of 1 colony forming units (CFU) in 80 ml brain heart infusion (BHI) broth medium on shaking bed incubator at 30 °C, post strain identification. Then on the second day, 1 ml of the overnight bacterial culture was used to

inoculate 80 ml fresh BHI broth culture which was incubated on the shaking bed incubator at 30 °C for around 5 hours. The bacterial culture was centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the bacterial pellet was resuspended in fresh BHI media. Optical density (OD) of the bacterial culture was measured by the thermospectronic spectrophotometer (Thermo Spectronic, Rochester, NY, USA) at 540 nm and adjusted to OD=1, which was expected to be $1X10^9$ CFU/ml. Post to OD adjusting, 1:100 serial dilution was performed using fresh BHI broth to get the desired concentration (around $1X10^7$CFU/ml) of *A. hydrophila*. This culture was put on ice and used for challenge within 3 hours. 100 ul of the 10^{-6} serial dilution of *A. hydrophila* was plated onto BHI plates. After 24 hours incubation at 30 °C, the average number of CFU/ml was calculated to get the concentration of the culture that was use in the challenge study.

3.3 Determination of the virulence of *A. hydrophila* ML09-119 in channel, blue and hybrid catfish

All three catfish species, channel, blue, hybrid catfish that were grown out to 30 ± 5g BW at Auburn University Fish Pathology wet lab S-6 were randomly distributed into 7 tanks. MS-222 (30mg/l) was used during the handling of fish to calm the fish down to decrease the stress. Each tank contained all three species to commit cohabitation, 10 fish per species, and 30 fish in each tank in total. Species were differentiated by fin-clipping marking. Cut part of channel catfish right pectoral fin, blue catfish left pectoral fin, and hybrid catfish dorsal fin before put them into cohabitation tanks. Of all the 7 tanks, 2 were randomly picked to be control. The other five tanks were treatment groups.

Recirculating system was applied during the acclimation period, which was lasted for 10 days. Water temperature was originally 25 °C and salt level was kept around 1.8 ppt to decrease the stress caused by environmental changes as well as eliminating the chance of *F. columnare* infection. Water temperature was gradually brought up to 28 ± 2°C, and salt was gradually brought down to 0.8 during the first 3 day of the acclimation time. Every environmental factor was kept stable prior to the challenge. Fish were fed with commercial catfish fed once a day at 4% of their body weight. Water was changed once per day for the recirculating system with constant aeration. At the time of challenge, recirculating system was changed into flow through system, with the temperature at 28 ± 2°C and salt level 0.8 ppt. Fish of each treatment tank were euthanized by immersing in a bucket with MS-222 (30mg/l), before 100 ul of ML09-119 bacterial culture that prepared above was injected intraperitoneally into each fish. Fish were then put back to their cohabitation tanks. Fish of control groups were injected with pure BHI broth medium. Challenged fish were kept the same way as they were during the latter acclimating time.

Post challenge, mortalities were recorded daily for 14 days. Any moribund or dead fish were removed from the system daily for bacteriological identification and tissue sampling. Prior to sampling, moribund or dead fish were inspected externally and internally for any clinical signs. The identification of *A. hydrophila* isolated from anterior kidney of the moribund or dead fish was performed by the biochemistry and selective medium method described previously. Kidney, liver and spleen were sampled from the moribund or moribund fish. Samples were pooled into 10% buffered formalin for histological tissue stain, Haematoxylin and Eosin (H&E) stain.

3.4 Determination of the virulence of *A. hydrophila* strains in channel catfish and grass carp

All the Channel catfish (*I. punctatus*) in this study were grown out to (11.8g ± 1.2 g) at Auburn University Fish Pathology wet lab S-6, and all the Grass carp (*Ctenopharyngodon idella*) were grown out to (11.6 g ± 1.4 g) at the E.W shell fisheries research Unit at Auburn University. Ten channel catfish and ten grass carp were put in each aquarium. Five aquaria were randomly picked for each treatment, so each tank contained all two species to commit cohabitation. MS-222 (30mg/l) was used during the handling of fish to calm the fish down to decrease the stress. Recirculating system was applied during the acclimation period, which was lasted for 10 days. Water temperature was originally 25 ℃ and salt level was kept around 1.8 ppt to decrease the stress caused by environmental changes as well as eliminating the chance of *F. Columnare* infection. Water temperature was gradually brought up to 30±2°C, and salt was gradually brought down to 0.8 during the first 3 day of the acclimation time. Every environmental factor was kept stable prior to the challenge. Fish were fed with commercial catfish fed once a day at 4% of their body weight. Water was changed once per day for the recirculating system with constant aeration.

All the bacterial suspension of exponential phase growth were prepared by overnight culture of 1 colony forming units (CFU) in 5 ml Tryptic Soy Broth (TSB) broth medium on shaking bed incubator at 30 ℃. Then on the second day, 1ml of the overnight bacterial culture was used to inoculate 100 ml fresh TSB broth culture which was incubated on the shaking bed water bath incubator at 30 ℃ until the Optical density (OD) was close to 1. The bacterial culture was centrifuged at 6000 rpm for 8 min. The

supernatant was discarded and the bacterial pellet was resuspended in fresh TSB media. Optical density (OD) of the bacterial culture was measured by the thermospectronic spectrophotometer (Thermo Spectronic, Rochester, NY, USA) at 600 nm and adjusted to OD=1. 10^{-2} serial dilution was performed using fresh TSB. This culture was put on ice and used for challenge within 3 hours.

At the time of challenge, recirculating system was changed into flow through system, with the temperature at 30±2°C and salt level 0.8 using underground water sources with constant aeration at the Auburn University Fish Pathology wet lab S-6. Fish were fed 4% body weight (BW) daily with commercial fish feed. Fish of each treatment tank were euthanized by immersing in a bucket with MS-222 (30mg/l) before 100 ul of ML09-119 bacterial culture that prepared above was injected intraperitoneally into each fish. Fish were then put back to their cohabitation tanks. Fish of control groups were injected with pure TSB broth medium. Challenged fish were kept the same way as they were during the latter acclimating time. Plate count procedure was performed to calculate the actual dosage used in fish. The bacterial dosage used in this study was 2.0×10^{6} CFU/fish. Mortalities were recorded daily for 14 days post challenge; any moribund or dead fish were removed from the system daily for bacteriological identification.

Ten channel catfish and ten grass carp are cohabitated in one aquarium to provide the same culturing condition. At least 5 different dilutions of the OD = 1.0 overnight bacterial culture for each isolate is used to inject fish. The OD = 1.0 bacterial cultures are then stored at 4°C for later plate counting. Soon after the fish are injected by different dilutions of bacteria

(<1 h), serial dilutions (in triplicate) of each A. hydrophila isolate are prepared in BHI broth, and 100 µl of each dilution are plated onto BHI plates. After 24 h incubation at 28°C, the average number of CFU ml^{-1} is calculated for each isolate (Pridgeon & Klesius 2011). Each isolate with each fish species will have 4 replicates.

Recirculating system is applied during the acclimation period, which is lasted for 10 days. Water temperature is originally 25 °C and salt level is kept around 1.8 ppt. Water temperature is gradually brought up to 28 ± 2°C, and salt is gradually brought down to 0.8 during the first 3 day of the acclimation time. Every environmental factor is kept stable prior to the challenge. Fish are fed with commercial fed once a day at 4% of their body weight. Post acclimation, 100 ul of each bacterial culture that prepared above is injected intraperitoneally into each fish species accordingly. Fish are then put back to their cohabitation tanks. Fish of control groups are injected with pure BHI broth medium. Mortality of each tank and each fish species are recorded on daily basis. Blood samples are collected from all the survival fish, for later ELISA experiment.

3.5 Statistical analysis

All statistical analysis of the mortality data was performed with SAS V9.2 software program. One way ANOVA (Analysis Of Variance) was first used to detect if any significant susceptibility differences exist among channel, blue and hybrid catfish groups to A.hydrophila ML09-119. Tukey's multiple range tests for pair-wise differences were then applied to determine which means differed significantly. For channel catfish and grass carp study, Statistical significance was determined by two-way (Fish species × bacterial strain) analysis of variance (ANOVA) and Tukey's multiple comparisons to

compare individual treatment groups. The differences were considered statistically significant when the p-value was less than 0.05. All statistical tests were acceptable, as the residuals were homogeneously and normally distributed (Steel, et al. 1997; SAS Institute Inc 2009).

4. Results

4.1 Fish acclimation and Bacteria identification

All of the catfish in cohabitation appeared normal and healthy after acclimation of 10 days. No signs of any stress or diseases were detected based on the external or internal observation from sampled fish. No pathogen bacteria were isolated from internal organs of the sampled fish. Fish were naive to *A. hydrophila*. Pure culture of *A. hydrophila* ML09-119 was applied to this study. Results of *A. hydrophila* ML09-119 identification were summarized in Table 2.1. The original concentration of the bacterial culture used for the challenge before dilution was 1.03×10^9 CFU/ml (Table 2.2). Post dilution, the final CFU number for the challenge was 1.03×10^6 CFU.

4.2 Virulence of *A. hydrophila* epidemic strain (ML09-119) to channel, blue and hybrid catfish

Of all the catfish species in cohabitation infected with *A. hydrophila* epidemic strain (ML09-119) using dose 10^6 CFU, the average percentage mortalities were highest in channel catfish (90 ± 10 %, Figure 2.1), while the average percentage mortalities were least in blue catfish (50 ± 20 %, Figure 2.1). The mean mortalities for hybrid catfish lied

in the middle of the other two species (63 ± 23 %, Figure 2.1). During the whole experimental challenge process, only 1 blue and 1 channel catfish fin control group were found dead on day 10 (Figure 2.2), but no clinical signs of *A. hydrophila* infection were observed. Bacteria identification and biochemical tests were done for the internal organs including spleen, liver and kidney. No *A. hydrophila* was cultured. For cumulative percentage mortalities in fish exposed to *A. hydrophila* ML09-119, clear trends were observed (Figure 2.2); the highest mortalities were in channel catfish from the beginning of the experimental challenge. Blue catfish and hybrid catfish were laid on lower level to catfish. Cumulative mortalities were lowest in blue catfish (Figure 2.2) through all the experiment time. Most of the mortalities occurred within 48 hours for all the three catfish treatment groups, after which the trend began to drop down and stayed stable around 0 percent with small fluctuations (Figure 2.3). Channel catfish suffered the most severe mortalities, which is almost all of channel catfish mortalities due to the infection of *A. hydrophila* ML09-119 (90 ± 10 %, Figure 2.3). The biggest mortalities for the other two treatment catfish groups occurred within the first two days as well.

Mean mortalities for channel catfish, blue catfish and hybrid catfish showed significant differences in the three groups using one way ANOVA ($p = 0.0156$, $a = 0.05$). Mean mortalities of channel catfish were significantly different from mean mortalities of blue catfish and hybrid catfish based on the results of Tukey's multiple range tests. There were no significant differences between blue catfish and hybrid catfish in terms of the mean mortalities.

Clinical signs of petechial hemorrhages on skin and eyes could be observed on catfish infected with *A. hydrophila* ML09-119. From the results of H&E stained slides of

internal organs including kidney, spleen and liver, white areas of focal necrosis and hemorrhages of different extent could be observed from all three treatment catfish groups (Figure 2.4). Bacterial colonies were able to isolate on BHI medium from all moribund fish samples that were collected during the experiment, except the control fish. Bacteria were identified as *A. hydrophila* ML09-119. Identification of epidemic strain was performed using the biochemical method described previously.

4.3 Comparison of *A. hydrophila* strain virulence between channel catfish and carp

We experimentally challenged channel catfish (*I. punctatus*) and Grass carp (*Ctenopharyngodon idella*) to compare the virulence of ZC1, ML09-119, and AL06-06. We found that ML09-119, ZC1 and AL06-06 are significantly different in their virulence to channel catfish and grass carp ($p < 0.0001$). ML09-119 is significantly more virulent to channel catfish as compare to its virulence to grass carp ($p = 0.0069 < 0.05$) (Figure 2.5), suggesting the evolution of ML09-119 host specificity to channel catfish. In contrast, the virulence of ZC1 was not significantly different to that of channel catfish and grass carp ($p = 0.5119 > 0.05$), suggesting the lack of host specificity of ZC1 strain to channel catfish and grass carp. Our experimental fish challenges evidenced that ML09-119 is significantly more virulent to both the channel catfish ($p = 0.0003 < 0.05$) and grass carp as compare to the ZC1 ($p = 0.0203 < 0.05$) (Figure 2.5). Of all the treatment groups, ML09-119 treatment group has least mean survival rate in both channel catfish (0.02 ± 0.04) and grass carp (0.22 ± 0.13) (Figure 2.5), which reflects the devastating nature of the recent epidemic outbreak of MAS caused by highly virulent *A. hydrophila* strain. We

found that ZC1 strain is significantly more virulent than the reference strain AL06-06 in both channel catfish ($p = 0.0075 < 0.05$) and grass carp ($p = 0.0089 < 0.05$) (Figure 2.5), suggesting that ZC1 strain has the potential to cause epidemic outbreak in fish and could serve as the basis for the emergence of the recent US epidemic catfish isolates.

5. Discussion

A good challenge model is fundamental to study pathogenicity and treatment/control of aquatic animal diseases (Crumlish et al., 2010). In this study we use IP injection to induce pathogen to the fish groups and use cohabitation to provide same environmental conditions to all the fish groups (treatment groups). Most of the time, cohabitation technique used in fish challenge experiment is for cohabitation challenge, one way to induce pathogen to the fish population by cohabitating infected fish with healthy fish (Alcorn et al. 2005; Xu et al 2007; Nordmo *et al.* 1997; Raynard *et al.* 2001). Cohabitation challenge technique is widely used over the world, because it is believed that cohabitation method is a challenge mimic that is most closely to the natural exposure (Xu et al 2007; Haines and Modde 1996). However, cohabitation, as a method to induce pathogen, has its own shortcomings. The infection efficiency is low for cohabitation method (Raynard et al. 2001; Haines and Modde 1996), which is not suitable for some studies such as tissue or molecular change of the infected fish, mortalities of infected fish. The time needed for the mortality peaks is much longer than I.P. method (Raynard *et al.* 2001). In this study, we use cohabitation method as a way to compare the three catfish species within the same experimental unit, thus eliminating the nuisance factors in terms of statistics analysis. I.P. method is applied to induce pathogen to the populations to

ensure the efficiency of infection. Several techniques are used for marking of fish for cohabitation experiments which includes calcein dye marking, percutaneous tags, visible implant tags, injected dyes (Faragher *et al.* 1992; Klesius *et al.* 2006; Hughes *et al*. 2000; Kelly 1967). However, these techniques usually require special equipment or dyes, such as calcein dye and different tags (Klesius *et al.* 2006), which is complicated and expensive to apply. Even though, calcein dye marking technique is the most popular technique that has been used in cohabitation challenge experiment, it is most suitable for large mass fish marking (Klesius *et al.,* 2006). In addition, no research has been done on these dyes such as calcein to test if they can cause negative effect on human health. In this study, fin clipping marking technique was applied. Fin clipping technique is cheap and simply to use and is especially suitable for small scale and short term challenge experiment (Kanno *et al.,* 1989; Haines *et al.*, 1996). Our study showed, post to acclimation, no signs of stress from fin clipping were observed in the catfish populations. The combination of cohabitation and I.P. method is very suitable and works well for this study.

A. hydrophila ML09-119 is highly virulent to channel catfish, which can cause mortalities as early as 6 hours post I.P. challenge (Pridgeon *et al.* 2011). However, it is currently unknown whether *A. hydrophila* ML09-119 is also highly virulent to Blue catfish (*Ictalurus furcatus)* and hybrid catfish, the other two most important food fish that is economically significant to United States and other countries. Our virulence studies showed *A. hydrophila* ML09-119 is not only virulent to channel catfish (*I. punctatus*) (90 ± 10 % mortalities at 1.03×10^6 CFU dose), but also highly virulent to blue catfish (*Ictalurus furcatus)* (50 ± 20 % mortalities at 1.03×10^6 CFU dose) and hybrid catfish

(Channel catfish × blue catfish) (65 ± 23 % mortalities at 1.03×10^6 CFU dose) (Figure. 2.1). Most of the mortalities of all the three catfish groups occurred within 48 hours post to IP challenge (Figure 2.2 & Figure. 2.3). Based on these results, a conclusion can be drawn that *A. hydrophila* ML09-119 can not only cause severe mortalities to channel, blue and hybrid catfish groups, but also, it can cause acute damage to the catfish population rapidly.

No research has been done so far on comparison of susceptibility of channel, blue and hybrid catfish to *A. hydrophila* ML09-119. Our ML09-119 virulence study on cohabitated catfish groups showed that, the mean mortalities of channel catfish is significantly higher that blue and hybrid catfish ($p = 0.0156 < 0.05$, $a = 0.05$), suggesting that virulence of *A. hydrophila* ML09-119 is much higher than it to blue and hybrid catfish. This result is crucial not only to commercial channel catfish farms but also to the entire US aquaculture production, because channel catfish is now one of the most important aquaculture species in the USA, even though its annual production has decreased in the past few years, from a high of 3000000t in 2003 to 255000t in 2007, catfish farming still represents about half the total USA aquaculture production (Tucker, 2003). In addition, even though ML09-119 is also highly virulence to blue and hybrid catfish following channel catfish, no significant difference ($p= 0.0801 > 0.05$, a=0.05) was observed between blue and hybrid catfish groups in this study.

Channel catfish and grass carp were experimentally challenged to compare the virulence of strains ML09-119, ZC1, and AL06-06, with survival rates ranging from 0.02 ± 0.04 to 0.98 ± 0.04. *Aeromonas hydrophila* isolate AL06-06 was included in the challenge experiment in this study as a reference strain that is typical of *A. hydrophila*

strains that have been historically isolated from stressed fish prior to the advent of the MAS epidemic and has shown reduced mortality in channel catfish relative to that observed from ML09-119. In this study, we observed that isolate ML09-119 was significantly more virulent than either ZC1 or AL06-06 and that ZC1 was more virulent than AL06-06 ($P < 0.0001$) in both channel catfish and grass carp. Additionally, overall comparisons between fish species show that channel catfish were more susceptible to A. hydrophila than were grass carp ($P = 0.0126$); however, this observation occurred only for epidemic strain ML09-119. No interaction effect of the independent variables was observed ($P = 0.1002$). These data suggest that ML09-119 has evolved increased virulence and that channel catfish appear to be more susceptible.

Clinical signs of 'Motile Aeromonads Septicemia' (MAS) includes: septicemia, abdominal dropsy, ulcerated skin lesions, depigmentation, fraying and reddening of fins, and hemorrhages (Camus et al. 1998; Roberts et al. 1993; Goswami et al. 2011). In this study, septicemia, depigmentation and hemorrhages were observed in almost all the sampled catfish. However, fraying and reddening of fins were only observed in a few later dead fish, and no abdominal dropsy or ulcerated skin was observed. This is probably due to the fact that most of the fish suffered the acute virulence of the epidemic strain and died within 48 hours, which was too short time for these chronic signs to present.

Table 2.1. Results of *A. hydrophila* ML09-119 identification

Characteristics	Gram stain	Motility	Cytochrome oxidase	Glucose fermenting	0/129 resistance	0% NaCl Growth
Result	-	+	+	+	+	+

Characteristics	Sucrose	Esculin hydrolysis	V-P	Ornithine decarboxylase	DL-lactate utilization	Urocanic acid utilization	*Myo*-inositol utilization
Result	+	+	+	-	+	-	+

Table 2.2. Plate count results for CFU number of the experimental challenge

10^{-6}Dilute replicates	R1	R2	R3
#CFU on BHI medium	90	142	78

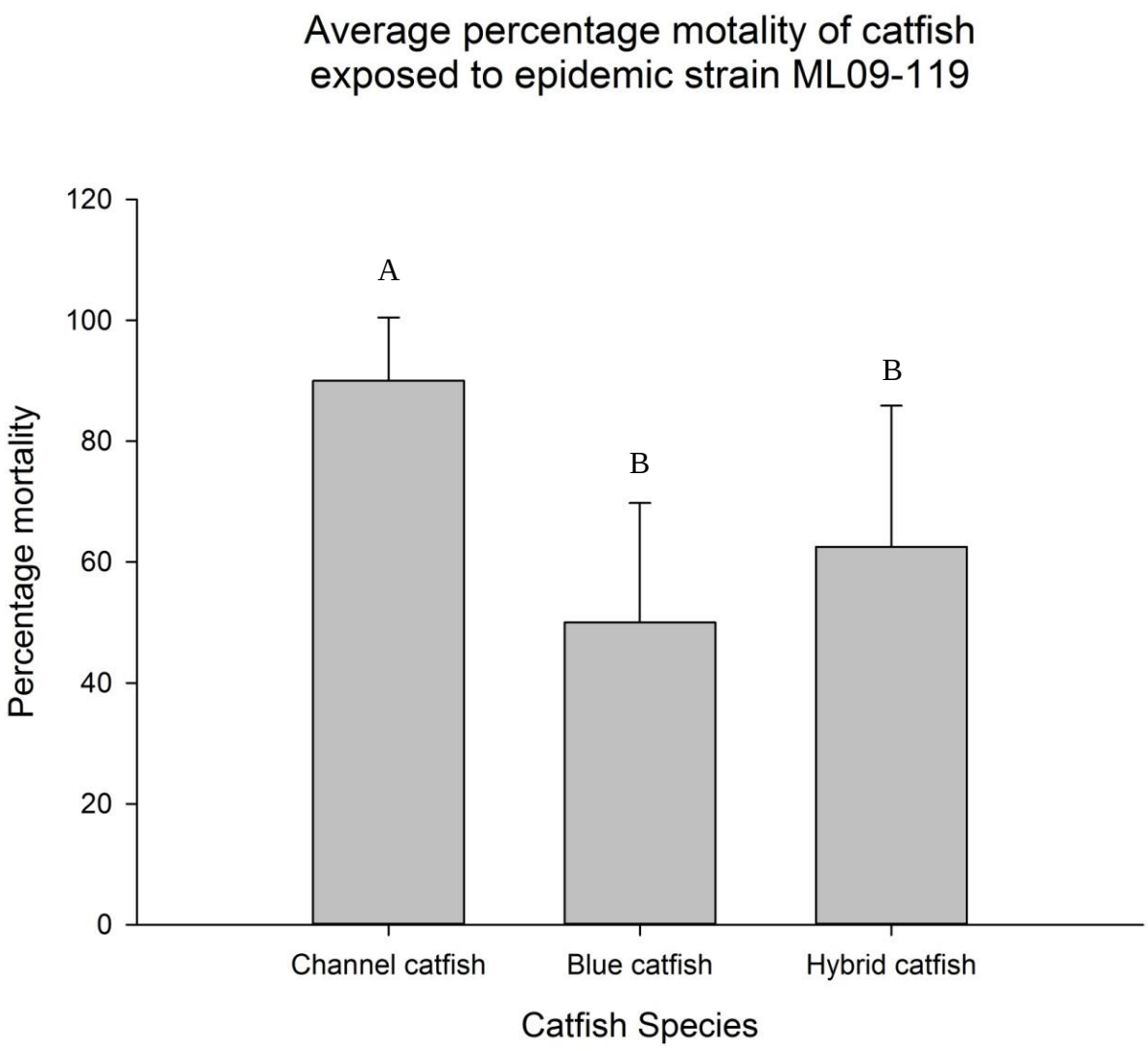

Figure 2.1. The average percentage mortality of channel catfish exposed to ML09-119. The average percentage mortality of channel catfish was highest in channel catfish (90 ± 10) %, while the average percentage mortalities were least in Blue catfish (50 ± 20) %. The mean mortalities for hybrid catfish lied in the middle of the other two species (63 ± 23) %.

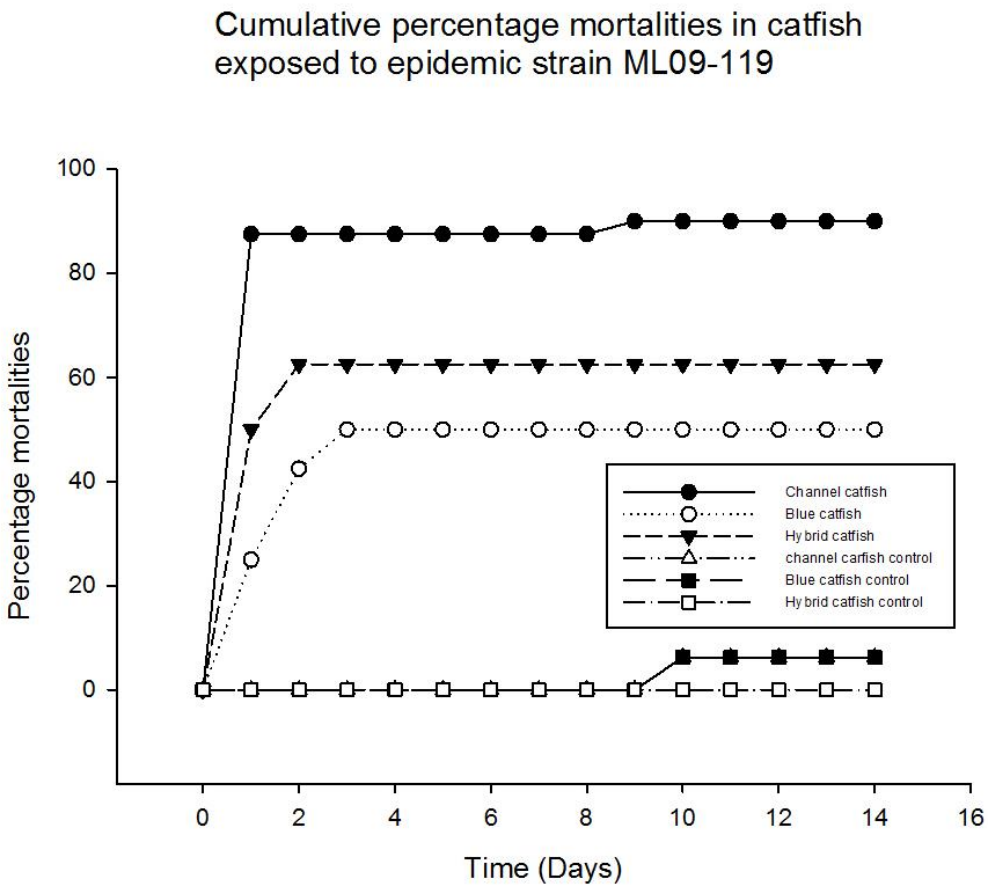

Figure 2.2. Cumulative percentage mortalities in channel catfish exposed to epidemic strain ML09-19. Daily mortalities in catfish were primarily observed within 24-48 hours for all the three catfish groups, after which mortalities decreased. All three groups suffered the highest number of mortalities within 24 hours. Almost all of the channel catfish died within 24 hours due to the infection of *A. hydrophila* ML09-119 (88±9) %.

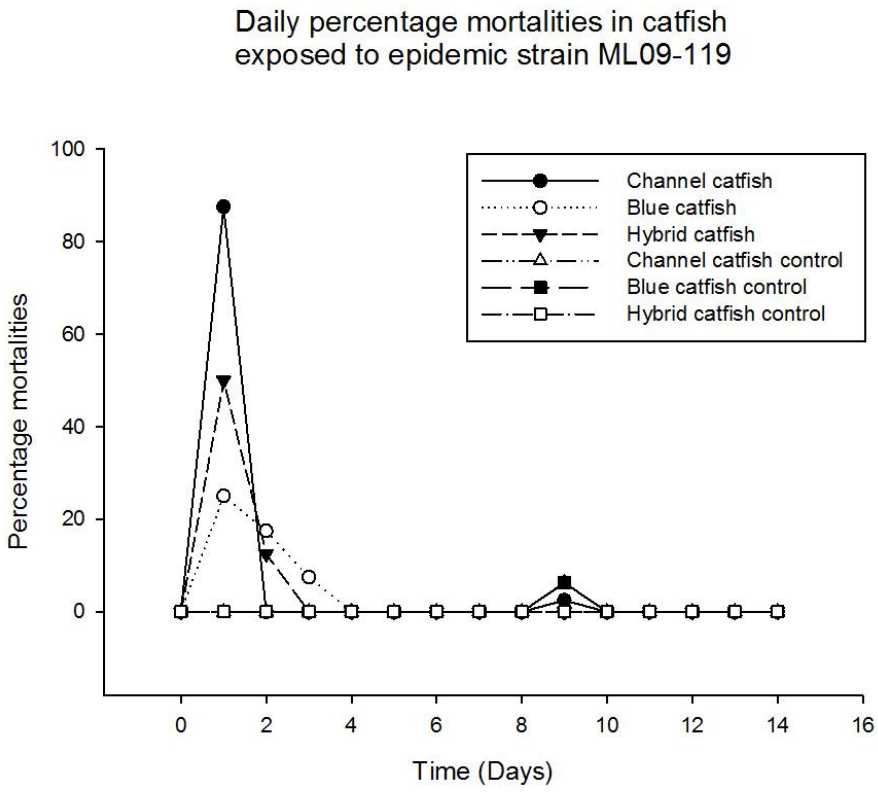

Figure 2.3. Daily percentage mortalities in channel catfish exposed to ML09-119. Daily mortalities in catfish were primarily observed within 24-48 hours for all the three catfish groups, after which mortalities decreased. All three groups suffered the highest number of mortalities within 24 hours. Almost all of the channel catfish died within 24 hours due to the infection of *A. hydrophila* ML09-119 (88±9) %.

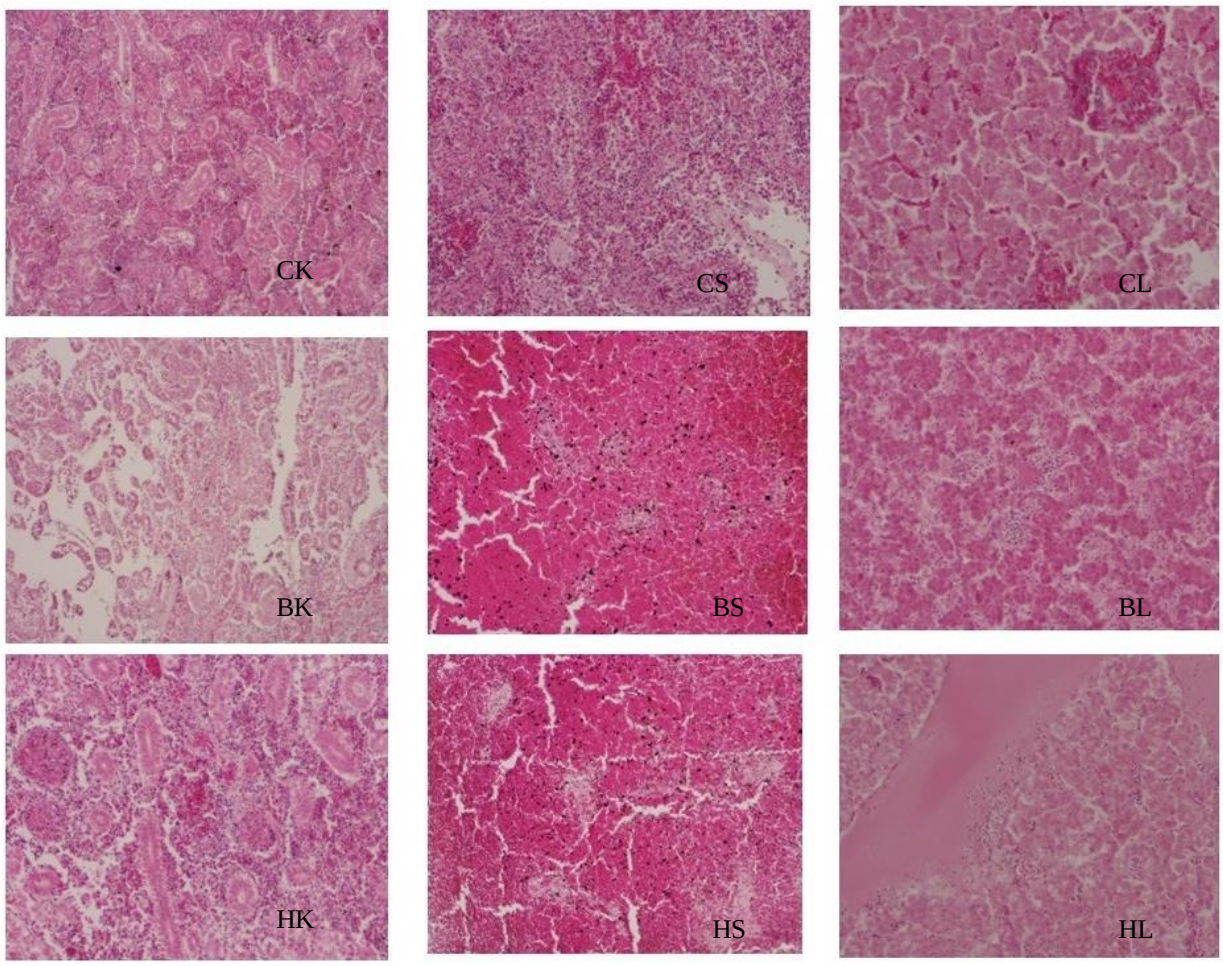

Figure 2.4. H&E stain of kidney (K), spleen(s) and liver (L) of channel catfish(C), Blue catfish (B) and Hybrid catfish (H) that died from *A. hydrophila* ML09-119. White areas of focal necrosis and hemorrhages of different extent could be observed from all three treatment catfish groups.

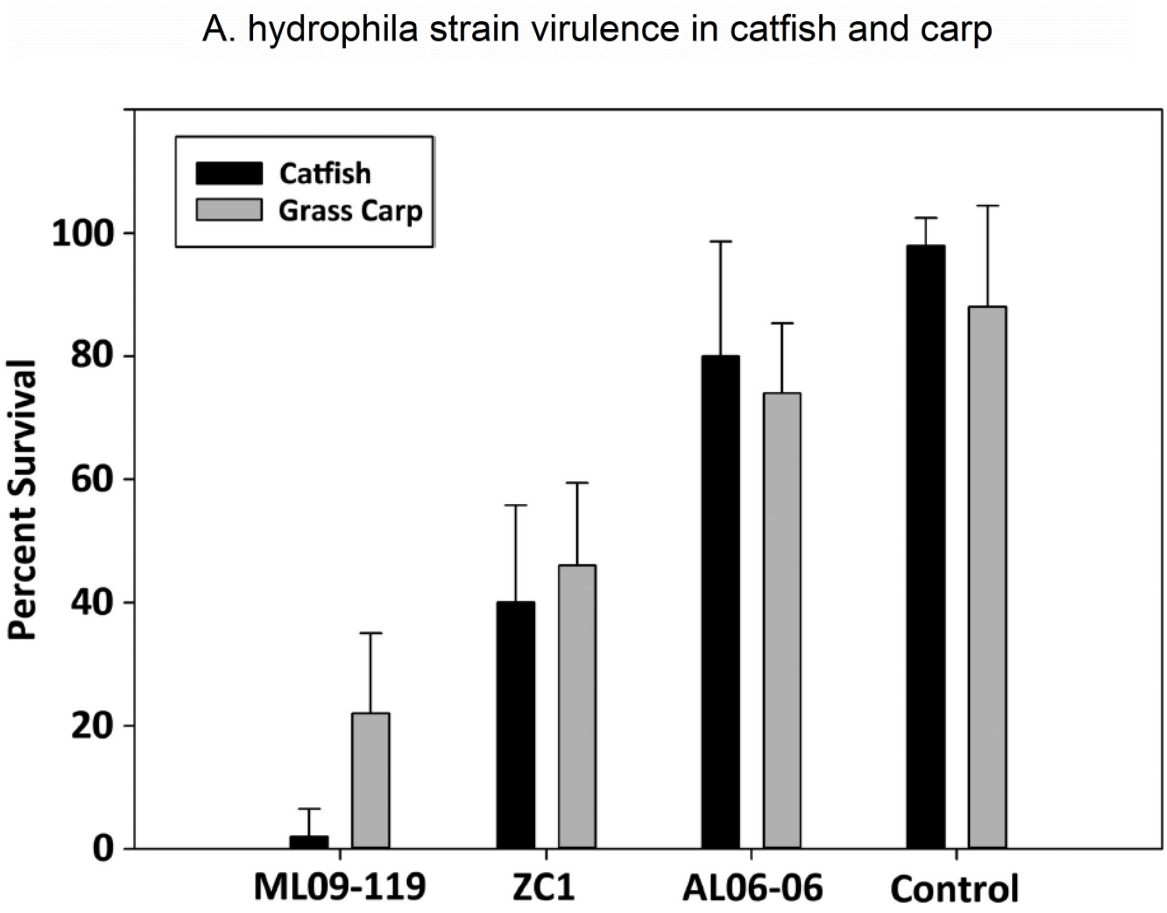

Figure 2.5. Determination of virulence of *A. hydrophila* isolates in Channel catfish and grass carp. The percent survival of channel catfish and grass carp intraperitoneally injected with ML09-119, ZC1 and AL06-06 were presented on the graph. The difference in survival between Channel catfish and grass carp injected with ML09-119 was significant ($p = 0.0069 < 0.05$). The difference in survival between Channel catfish and grass carp injected with ZC1 was not significant ($p = 0.5119 > 0.05$). The difference between ZC1 and AL06-06 in the survival of channel catfish (catfish ($p = 0.0075 < 0.05$) and grass carp ($p = 0.0089 < 0.05$) is also significant.

Chapter III

Determining the role of the *myo*-inositol pathway in *A. hydrophila* ML09-119 virulence

1. Abstract

The epidemic strain caused a devastating outbreak of motile Aeromonads septicemia of catfish (MAS) on the fish farms of Southeastern United States in 2009. Previously our lab sequenced 11 *A. hydrophila* isolates, 6 of which are epidemic stains, while the others were historical *A. hydrophila* isolates not affiliated with an epidemic outbreak of disease that we describe as "reference" strains. A comparative genomic analysis indicated that 53 epidemic-associated genetic regions with 313 predicted genes were uniquely present in the epidemic isolates but absent from the reference isolates. 34 genes from this region were predicted to be related to the virulence of the epidemic strains. A functional metabolic island that encodes a complete pathway for *myo*-inositol catabolism was identified and demonstrated to be functional based on the ability of epidemic *A. hydrophila* isolate ML09-119 to use *myo*-inositol as a sole carbon source while the reference strain AL06-06 cannot.

In this study, the gene *iolA* coding for the enzyme aldehyde dehydrogenase for *myo*-inositol catabolism was inactivated by traditional allelic exchange to generate the *A. hydrophila* $\Delta iolA_{tra}$ mutant. The *iolA*-*iolR* genetic region was also mutagenized using a recombineering technique $\Delta iolA_{rec}$ mutants. An *in vivo* challenge in channel catfish

showed that there was no mortality in the channel catfish that were challenged with $\Delta iolA_{tra}$ mutant, but there was mortality in the channel catfish challenged with $\Delta iolA_{rec}$ mutants similar to wild type ML09-119.

It was hypothesized that the avirulent phenotype of the $\Delta iolA_{tra}$ mutant was due to a polar effect on the upstream and divergently transcribed *iolR* gene, which is known to be a negative transcriptional regulator in other bacteria. Eight mutants were created by knocking out the upstream of the *iolA* gene in the *iolA*-*iolR* promoter region. Results of the *in vivo* challenge in channel catfish showed that $\Delta iolA_{rec3}$, $\Delta iolA_{rec4}$ exhibited some decrease in mortality, but there were no significant difference in the mortality between the channel catfish challenged with $\Delta iolA_{rec3}$, $\Delta iolA_{rec4}$ and the channel catfish challenged with the wild type ML09-119. ELISA titer of the survivors of the $\Delta iolA_{tra}$ after 21 days showed that $\Delta iolA_{tra}$ can induce strong antibody response against the wild type *A. hydrophila* ML09-119, indicating that this mutant can serve as a promising vaccine candidate against the epidemic *A. hydrophila*.

Key words: myo-inositol, *iolA* gene, *Aeromonas hydrophila*

2. Introduction

A. hydrophila can cause Motile Aeromonads septicemia of catfish (MAS) in channel catfish. But this pathogen normally is an opportunistic pathogen following other infections or related to stress caused by fish management or environmental factors (Hemstreet, 2010; Walters and Plumb, 1980). However, in 2009 a highly virulent strain of *A. hydrophila*, ML09-119, caused severe disease outbreaks in channel catfish *(I. punctatus)* on catfish farms in west Alabama. So far, Alabama catfish famers' loss is reported to be more than 10 million pounds of catfish that were market-size and estimated to be more than $3 million due to this epidemic strain of *A. hydrophila* (Pridgeon, 2011; Liles, 2011). This *A. hydrophila* epidemic strain, ML09-119, is reported to be highly virulent to channel catfish. It can cause severe mortality starting at 6 hrs post exposure with certain amount of dose that was IP injected into the catfish (Pridgeon, 2011). The epidemic MAS outbreaks caused by *A. hydrophila* epidemic strain, ML09-119 are so devastating that it is highly essential to investigate the virulence nature of this pathogen, identify the virulence related genes and create live avirulent bacteria mutant vaccine candidates for this bacterial disease. So far there is not any identified virulence factor records of the *A. hydrophila* epidemic strain, and no vaccine or treatment for the MAS caused by the epidemic strain.

Gene knockout and mutant generation is a tool developed from naturally existing mechanisms by which genetic material is exchanged between different bacteria and viruses (Rocha, *et al.* 2005). After the genes are transferred into the host bacteria, these genes are then incorporated onto the host genome by homologous gene recombination (Ishikawa *et al,* 2013; Thomason, *et al.* 2007). Recombineering is a precise technique

for the manipulation of bacterial genes and other organisms (Yu *et al.*, 2000). This technique is very accurate and fast in target gene deletion, insertion, substitution, thus in a very short time mutants for the study of the gene functions can be generated (Datsenko & Wanner, 2000; Datta *et al.*, 2008; Rivero-Müller *et al.*, 2007). A novel recombineering method was developed (Hossain et al., manuscript in preparation) in order to mutagenize genes in *A. hydrophila* to determine their respective roles in virulence.

3. Materials and Methods:

3.1 Bacterial isolates and plasmids

The *A. hydrophila* ML09-119 and reference strain AL06-06 used in this study were picked out from single colony on the plate that were streaked using the -80 ℃ stock. The epidemic strain was from west Alabama MAS outbreak in 2009, was the reference strain. The bacteria were routinely grown on fresh Trypticase Soy Broth (TSB) medium overnight before use. The *A. hydrophila* ML09-119 used for experiment was from the bacterial stocks of the fish disease lab in Auburn University. This epidemic strain was originally isolated from the kidneys of channel catfish naturally infected with *A. hydrophila*. The pure culture of epidemic strain was used first in a small test infection of 10 catfish. Moribund catfish that showed clinical signs of *A. hydrophila* ML09-119 was collected for necropsy. *A. hydrophila* was re-isolated from the moribund fish by taking an inoculum from the kidney using a plastic inoculation loop and streaking onto a BHI agar plate. By doing this, it is expected than the virulence of the epidemic strain stock can be recovered**.** ML09-119 was then confirmed by biochemistry and selective media following the established identification procedures with modifications (Furuwatari,

et al., 1994; Holt, et al., 1994). Briefly, the identification biochemical tests included Gram stain, cytochrome oxidase, glucose utilization, 0/129, sucrose, esculin hydrolysis, V-P, DL-lactate utilization and urocanic acid utilization, and then test on selective media M9 minimum media with inositol added. *E. coli* SM10-λ-pir (Simon *et al.*, 1983) was used for the conjugal transfer of mobilizable plasmid to *A. hydrophila* ML09-119. The list of bacterial strains used in this study is presented in Table 3.1.

3.2 Construction of defined *A. hydrophila ΔiolA*$_{tra}$ mutant by traditional splicing PCR and conjugation technique

To investigate the role of *myo*-inositol utilization pathway in the virulence of epidemic *A. hydrophila* ML09-119 in channel catfish, a *iolA* knockout mutants, *ΔiolA*$_{tra}$ were constructed using plasmid pDMS197, a *sacB* containing suicide plasmid (Edwards, Keller et al. 1998). The primers needed for this study were listed in the table 3.2.

The two pairs of primers, AupF/AupR and AdnF/AdnR, were used to amplify approximately 350bp upstream and downstream sequences of *iolA* gene, respectively using PCR kit (*TaKara Ex Taq*) to construct the *ΔiolA* mutant. The template used in this PCR was the genomic DNA of *A. hydrophila* ML09-119 which was extracted using a E.Z.N.A.® Bacterial DNA Kit (Omega Bio-Tek, USA). The chloramphenicol acetyltranferase gene (*cat*) was amplified from pMHH46 plasmid (Hossain et al 2013) using primers catF and catR. The primers AupR and AdnF, used for the amplification of upstream and downstream sequences of *iolA* gene contained the reverse complemented sequences of catF and catR primers which were added respectively at their 5' ends when the primers were designed. The *Cat*-cassette which was the chloramphenicol resistance

gene (Cm^R) with two arms of the upstream and downstream homologous of *iolA* gene was created by fusing the two arms and the Cm^R gene by splicing through overlap extension PCR (SOE) (Horton, Hunt et al. 1989). The primers for this PCR were Aup-intF and And-intR. The PCR products were purified by agarose gel purification.

The suicide plasmid pDMS197 was digested by restricted digestion enzyme XbaI (New England Biolabs, NEB, USA) following the protocol provided by the manufacturer. A 50 ul reaction was used for the digestion, including 25 ul of the suicide plasmid pDMS197 DNA, 3 ul of the XbaI restricted digestion enzyme, 5ul of the 10 × CutSmart™ Buffer, 1 × BSA and 16ul RNase free H_2O. The reaction system was incubated at 37 °C for one hour. The reaction system was then incubated at 65°C for 20 min to stop the reaction. The digested product was purified by DNA Clean & Concentrator™ (Zymo research), and the concentration was measured by Qubit® dsDNA BR Assay Kit (Life technologies). The product was blunted using end-repair kit DNA terminator (Lucigen, USA) following the producer's instruction. The product was purified by DNA Clean & Concentrator™ (Zymo research) again before ligation.

The purified restriction enzyme XbaI digested and blunted suicide plasmid pDMS197 was ligated with the Gel purified *Cat*-cassette using Quick Ligase (NEB, USA) under the room temperature for 30 minutes. Briefly, 50ng of blunted suicide plasmid pDMS197 and around 3-fold molar excess of the *Cat*-cassette insert was mixed together and the volume was adjusted to 10 ul with RNase free H_2O. 10 ul of the 2× Quick Ligation Buffer and 1µl of Quick T4 DNA Ligase were added into the mixture. The mixture was centrifuged briefly and incubated at room temperature (25°C) for 30

minutes before it was chilled on ice. A SB gel electrophoresis was done to confirm the ligation product (data not shown).

The making of the electrocompetent cells of *E. coli* SM10-λ-pir was following a published protocol (Inoue, *et al.*, 1990) with minor changes. A 0.5 ml of the overnight culture of *E. coli* SM10-λ-pir bacteria was inoculated into 200 ml of Hanahan's Broth *(*SOB Medium*)* with 10 mM $MgCl_2$. The culture was incubated in the 37°C water bathe incubator with shaking bed at 200rpm for around 2.5 hours and the OD_{600}=0.4. The culture was chilled in ice for 10 min before loaded into 200ml centrifuge tubes. The culture was centrifuged at 6000rpm for 8 min at 4°C, the supernatant was discarded and the pellet was washed by resuspended with 10% glycerol and centrifuge again at 6000rpm for 8 min. The wash step was repeated for 3 times before the pellet was gently resuspended in 200ul GYT medium. The whole procedure was performed on ice.

The ligation product was then used in the electroporation (Chassy, et al., 1988; Dower et al, 1988) to create the plasmid pDMS197*iolA*, which contains a deletion of the entire *iolA* gene. 50 ul of the premade electrocompetent cells of the *E. coli* SM10-λ-pir was mixed gently with 2.5ul of the ligation product and chilled on ice for 5 min. The mixture was transferred into ice cold cuvettes (Bulldog bio) before the cuvettes were loaded onto the Eppendorf® Eporator® (Eppendorf). Voltage was set up at 1800V. The mixture was mixed with recovery medium (SOC medium) right after the electronic pulse shock. The culture was transferred to a 2 ml test tube and incubated at 37°C with shaking bed at 200 rpm for 2 hrs. The successful electroporated *E. coli* SM10-λ-pir with the plasmid pDMS197*iolA* was selected on 2XYT agar medium plate with 25ul/ml chloramphenicol, 5ul/ml tetracycline.

The suicide plasmids pDMS197iolA were independently introduced into *A. hydrophila* ML09-119 by conjugation with *E.coli* SM10-λ-pir bearing plasmid pDMS197iolA. A single colony was selected on the selective medium plate for SM10-λ-pir bearing plasmid pDMS197iolA for inoculation of 5ml LB broth medium. The culture was incubated at 37°C with shaking at 200rpm until the OD_{600} was above 1. A single colony of *A. hydrophila* ML09-119 was picked to inoculate 5 ml TSB broth medium. The culture was incubated at 30°C with shaking at 200rpm until the OD_{600} was above 1. A 4 ml ML09-119 culture and 1 ml SM10-λ-pir bearing plasmid were mixed together. The 5 ml culture mixture was filtered through a MicroFunnel 300 SP (MicroFunnel™) by vacuum pressure. 5ml fresh LB broth medium was used for washing the cells onto the membrane. The membrane was transferred to the sheep blood agar medium after 2× wash step. The sheep blood agar medium was incubated at 30°C overnight.

The membrane with the cell culture mixture was vortexed with 3 ml fresh TSB broth medium for selection. Single cross-over mutants were selected on TSA plate supplemented with chloramphenicol, tetracycline and colistin. Double-cross over mutants were obtained by plating onto LB (without NaCl) plates supplemented with 15% sucrose and 12.5 µg/ml chloramphenicol. Mutants grown on this selective plate were subjected to phenotypic and genotypic characterizations. The complete deletion of the *iolA* genes were confirmed by PCR followed by sequencing.

3.3 Construction of defined *A. hydrophila* $\Delta iolA_{Rec}$ mutants by recombineering

A recombineering technique was used to create a precise deletion of the *iolA* gene and generate the $\Delta iolA_{Rec}$ mutant, in order to compare with the $\Delta iolA_{tra}$ created by the traditional technique by splicing through overlap extension PCR (SOE) (Horton, Hunt et al. 1989), as well as to better determine the role of *myo*-inositol utilization pathway in the virulence of epidemic *A. hydrophila* ML09-119 in channel catfish.

The chloramphenicol acetyltranferase (*cat*) gene was amplified from pMHH46 plasmid (Hossain et al 2013) using primers *iolA5Rec*F and *iolA5Rec*R to generate the *cat*-cassette with 50 bp of the upstream and downstream of the targeted *iolA* gene. The primers *iolA5Rec*F contained 50 bp of the upstream of the targeted *iolA* gene and *iolA5Rec*R contained the reverse complemented sequences of 50 bp of the upstream of the targeted *iolA* gene which were added respectively at the 5' ends of each respective primer. The PCR product was validated using gel electrophoresis before another 24× PCR was done using this PCR product to generate more *cat*-cassette insertion.

The PCR product was purified and concentrated using Wizard® DNA Clean-Up system (Promega, USA) following the protocol provided by the manufacturer. Briefly, the 24 different PCRs were pooled together in a 15 ml conical tube, and a Wizard® DNA Clean-Up kit (Promega, Madison, WI) was used to purify the PCR products according to the manufacturer's protocol. The concentration of the final concentrated PCR product was measured using Qubit® dsDNA BR Assay Kit (Life Technologies).

A. hydrophila ML09-119 containing the plasmid pMJH65, which was constructed for the purposes of introducing a recombineering cassette into gram-negative bacteria (Hossain et al, manuscript in preparation), was prepared for electroporation using a standard protocol (Inoue, *et al.*, 1990) with minor changes. 0.5ml of the overnight culture of ML09-119 bacteria was inoculated into 150 ml of Hanahan's Broth *(*SOB Medium*)* with 1.5 ml 1M Arabinose, 300 ul 25mg/ml Tetracycline and 600 2M $MgCl_2$. The culture was incubated in the 30°C water bath incubator with shaking at 200rpm for around 4 hours and the OD_{600} = 0.5. The culture was chilled on ice for 10 min before loaded into 200ml centrifuge tubes. The culture was centrifuged at 6000 rpm for 8 min at 4°C. The supernatant was discarded and the pellet was washed by re-suspending with 10% glycerol and centrifuged again at 6000 rpm for 8 min. The wash step was repeated 4 times before the pellet was gently resuspended in 200ul 10% glycerol. The whole procedure was performed on ice.

The concentrated and purified PCR product was then used in the electroporation (Chassy, et al., 1988; Dower et al, 1988) to create the precise *iolA* gene deletion mutant $\Delta iolA_{Rec}$. 50 ul of the premade electrocompetent cells of *A. hydrophila* ML09-119 (pMJH65) was mixed gently with 3 ug of the concentrated PCR product and chilled on ice for 5 min. The mixture was transferred into ice cold cuvettes (BulldogBio) before the cuvettes were loaded onto the Eppendorf® Eporator® (Eppendorf) with a voltage setting of 1200 V. The mixture was mixed with recovery medium (SOC medium) right after the pulse shock. The culture was transferred to a 2 ml test tube and incubated at 30°C with shaking at 200 rpm overnight.

The successfully electroporated *A. hydrophila* ML09-119 *iolA* deletion mutant was selected on a TSA agar medium plate with 25ul/ml chloramphenicol. A similar strategy was followed for the construction of $\Delta iolA_{Rec2}$ through $\Delta iolA_{Rec8}$ which represent progressively larger deletions of the *iolA*-*iolR* promoter region, with each successive mutant having a deletion of the *iolA* gene and an additional 50 bp upstream of the *iolA*-*iolR* promoter region, respectively (Figure 3.4).

3.4 Evaluating the growth response of *A. hydrophila mutants* using *myo*-inositol as a sole carbon source

A 2 ml TSB culture of the *A. hydrophila* isolate was started by inoculating the medium using a single colony of the bacteria. The culture was grown at 30°C overnight with shaking at 200 rpm. The cell culture next day was centrifuged at 10,000 × *g* for 10 min. The supernatant was poured out, and the pellet was resuspended in M9 minimal medium supplemented with 5.5 mM of *myo*-inositol (M9I). The centrifugation and re-suspension in M9I was repeated twice to remove any TSB residue. At last, the re-suspension of the bacteria cells in M9I was adjusted to an OD_{600} of 0.5. A 1:100 dilution of the suspension was achieved by 10 fold serial dilution from the original M9I suspension. A 100 ul of the dilution was used to inoculate 1.9 ml of M9I. The bacterial cultures were then incubated at 30°C with shaking at 200 rpm for 144 hours and the OD_{600} was recorded at 24 hrs intervals to record the growth condition of the bacteria strains in M9I. The results were used to evaluate the ability of each strain to use *myo*-inositol as a sole carbon source. *A. hydrophila* isolates ML09-119 and AL06-06 were used as positive and negative control, respectively, for the *myo*-inositol utilization assay.

3.5 Virulence study of *A. hydrophila mutants* in channel catfish

All experiments conducted with vertebrate animals (catfish) were approved by the Institutional Animal Care and Use Committee (IACUC) review board at Auburn University in accordance with the animal welfare guidelines specified in the United States.

All the channel catfish *(I. punctatus,* Kansas Random Strain*),* used in this study were spawned at the hatchery of the Auburn University Fish Genetics Research Unit artificially prior to transferring to troughs or glass aquaria at the Auburn University Fish Pathology wet lab S-6. Fish were maintained at recirculation systems (temperature around 25 °C and pH 7.5) using well water sources with constant aeration. Fish were fed daily with commercial feed. Water quality factors including temperature, pH, salt level, total ammonia level, total nitrite level were tested on daily basis to ensure that catfish fingerlings remained unstressed and naive to *A. hydrophila*. Catfish fingerlings were grown out in this system until their body weight (BW) reached 20±5 g.

A bacterial suspension of exponential phase growth was prepared by overnight culture of in 5 ml TSB broth medium with shaking at 200 rpm at 30°C. The next day 1ml of the overnight bacterial culture was used to inoculate 100 ml fresh TSB broth culture which was incubated with shaking at 200rpm at 30°C for 4 hours. The bacterial culture was centrifuged at 6000 rpm for 10 min. The supernatant was discarded and the bacterial pellet was resuspended in fresh TSB media. The optical density of the bacterial culture was measured by the thermospectronic spectrophotometer (Thermo Spectronic, Rochester, NY, USA) at 600 nm and adjusted to an OD = 1, which was expected to be 1×10^9 CFU/ml. After adjusting the bacterial suspension to an appropriate OD, a 1:100

dilution was performed using fresh TSB broth to get the desired concentration (around 1×10^7CFU/ml) of *A. hydrophila*. Another 1:2 dilution was done with fresh TSB. This culture was put on ice and used for challenge within 3 hours. A plate count assay was conducted right after the fish challenge to calculate the accurate CFU/ml concentration used in this study. The bacterial culture used in the fish challenge were serial diluted and 100 ul of each dilution was spread on the TSA plates, with 3 replicates were done for each strain of bacteria.

Channel catfish in Auburn University Fish Pathology wet lab S-6 were randomly distributed into glass aquarium tanks. MS-222 (30mg/l) was used during the handling of fish to calm the fish down to decrease the stress. Each tank contained 10 fish. A recirculating system was applied during the acclimation period, which was lasted for 10 days. Water temperature was originally 25 °C and salt level was kept around 1.8 ppt to decrease the stress caused by environmental changes as well as eliminating the chance of *F. columnare* infection. Water temperature was gradually brought up to 30±1°C, and salt was gradually brought down to 0.8 during the first 3 day of the acclimation time. Every environmental factor was kept stable prior to the challenge. Fish were fed with commercial catfish fed once a day at 4% of their body weight. Water was changed once per day for the recirculating system with constant aeration. At the time of challenge, recirculating system was changed into flow through system, with the temperature at 30±1°C. Fish of each treatment tank were euthanized by immersing in a bucket with MS-222 (30mg/l), before 200 ul of ML09-119 bacterial culture was injected intraperitoneally into each fish. Fish were then put back to their cohabitation tanks. Fish of control groups were injected with pure TSB broth medium. Challenged fish were kept the same way as

they were during the latter acclimating time. Mortalities were recorded daily for 14 days post challenge. Any moribund or dead fish were removed from the system daily for bacteriological identification and tissue sampling. Prior to sampling, moribund or dead fish were inspected externally and internally for any clinical signs. The identification of *A. hydrophila* isolated from anterior kidney of the moribund or dead fish was performed by the biochemistry and selective medium method described previously. Survivors of the challenge were kept for 28 days, before they are challenged again with the wild type ML09-119 to test if any protection effect was provided. The procedure of the re-challenge was similar to the previous challenge. At seven days post re-challenge, blood samples were then drawn from the survivors for the ELISA titer in the later experiment.

3.6 The immunogenicity of the mutants and the Enzyme-linked Immunosorbent Assay (ELISA)

Blood samples collected after the fish challenge were put in the room temperature for 2 hrs then 4°C overnight allowing to clot completely. Serum of each blood sample was collected followed by centrifuging at 5000rpm for 10 min. The supernatant of each sample was collected for Enzyme-linked Immunosorbent assay (ELISA) analysis.

Antibody responses of channel catfish to *A. hydrophila* were quantified by evaluating the presence of specific immunoglobulin to *A. hydrophila wild type* ML09-119 using indirect ELISA. Protein Detector^{TM} ELISA kit was use to conduct the ELISA experiment.

The protocol followed was similar to the product introduction with minor changes. 96-well plastic plates were coated with 100 ul of a solution of 10 ug/ml (10^7 CFU/ml) *A. hydrophila* epidemic strain. *A. hydrophila* were suspended in carbonate-bicarbonate coating solution. The coating solution was prepared by diluting one time coating buffer tablet in 10 times of sterile reagent quality water. The plates with coating buffer and antigen were placed in 4°C pH 9.6 overnight. The plates were washed 4 times with washing buffer provided by the kit the next day, followed by adding 1×BSA blocking buffer to block for 15 min at room temperature. After another wash step, the plates were used to do ELISA analysis. 100ul 1%BSA blocking buffer was added into each well on the *A. hydrophila* ML09-119 coated plate. 200ul of the 1/10 fish blood serum sample diluted with 1%BSA blocking buffer was added to the column A2-A11, A1 and A12 were served as positive and negative control. 100 ul of the solution from A1-A12 was transferred to B1-B12 and mixed carefully by pipetting 3-5 times, and this step was repeated across the plate until E1-E12. The final 100 ul from the wells in the row E after mixing was discarded. The plate was then incubated at room temperature for 1 hour. The plate was emptied, and residual liquid was tapped out. Plate was washed out by the washing buffer that came with the kit for 5 times. 100ul of Rat Anti-catfish monoclonal antibody (Mab) was diluted 32 times and added into each well that contained the primary antibody, after which the plate was incubated at room temperature for 1 hour. After incubation the plated was emptied, and residual liquid was tapped out and the plate was washed out five times using the washing buffer that came with the kit. 50ul of tertiary antibody (goat anti-rat antibody conjugated with horseradish peroxidase) (0.1ug/ml) was added into each wall that contained the secondary antibody. The plate was incubated at

room temperature for 1 hour, after which the plate was washed as above. 50ul of the substrate solution that came with the kit was added into each well that contained the tertiary antibody. The plated was incubated at room temperature for 5-15 min before the reaction as stopped by adding 50 ul of stop solution into each well for full color development and the plate was then read at OD_{405}. A reaction was defined as positive if its OD_{450} value was at least two times the negative control. Ending points were the highest dilution with a positive reaction.

A criss-cross serial dilution analysis was done prior to the ELISA analysis of the samples to optimize the reagent concentration in the immunoassay procedure. 100ul of 1% BSA blocking buffer was added into each well of the *A. hydrophila* ML09-119 coated plate. 200ul of the 1/10 ML09-119 infected survivor fish blood serum sample diluted with 1% BSA blocking buffer was added to the respective columns and serially diluted across the plate to identify the best concentration range for the sample. Prior to adding the Mab, 100ul of 1% BSA blocking buffer was added into each well, followed by 200ul of the secondary Rat anti-channel catfish Mab. This Mab solution was serially diluted across the plate to identify the optimum concentration for the Mab.

3.7 Statistical Analysis

Mortality data of this study was presented as mean ± standard error (SE) and analyzed by one-way analysis of variance and Tukey's multiple range comparison using SAS software (SAS 9.2, SAS Institute Inc., Cary, NC). Significant level was set at 5% ($p < 0.05$). Variances were considered significant when probability (P) values < 0.05 were calculated.

4. Results

4.1 Evaluating the growth response of *A. hydrophila* mutants using *myo*-inositol as a sole carbon Source

The *iolA* gene encodes aldehyde dehydrogenase and terminally located in the inositol catabolic (*iol*) gene cluster of epidemic *A. hydrophila* isolates. It has been demonstrated that the *iolA* gene is required for the conversion of malonate semialdehyde to acetyl-CoA (Hossain et al., 2012). It was predicted that the *iolA* deletion mutants that were created in this study, which were created by replacing the *iolA* gene and 50 bp of upstream of the *iolA* gene with *cat* gene using both traditional technique and recombineering technique, would be unable to utilize *myo*-inositol as a sole carbon source. The growth assay was carried out with M9I for 144 hours, and it was determined that all of the *iolA* mutants were unable to utilize *myo*-inositol as a sole carbon source (Figure 3.1), whereas wild type *A. hydrophila* ML09-119 reached stationary phase after 48 hours of incubation. The results of this *myo*-inositol assay were comparable to the results in previously published research (Hossain et al., 2012). Like *A. hydrophila* ML09-119 *iolA* mutants, wild type *A. hydrophila* AL06-06 that does not carry the *iol* cluster did not show any growth after 144 hours of incubation. The lack of the ability of the *iolA* mutant to utilize *myo*-inositol as a sole carbon source clearly demonstrated that an *iolA* mutation had been constructed and that IolA function is required for *A. hydrophila* utilization of *myo*-inositol as a sole carbon source.

4.2 Virulence study of *A. hydrophila* mutants in channel catfish

4.2.1 Cumulative survival rate of the channel catfish challenged with the *iolA* mutants

For better understanding of the virulence factors of the *A. hydrophila* epidemic strain and to identify possible live vaccine candidates, the *iolA* gene was knocked out by a traditional allelic exchange technique. It has been observed that all of the *A. hydrophila* epidemic strains *A.hydrophila* can utilize *myo*-inositol as a sole carbon source (Hossain, et al, 2013). Since *iolA* gene is required for the conversion of malonate semialdehyde to acetyl-CoA (Kohler, et al., 2011), the hypothesis is that the *iolA* gene can be the key virulence factor and by knocking out the *iolA* gene, the ML09-119 strain may be attenuated and serve as a vaccine candidate.

The results of the *in vivo* channel catfish i.p challenge with $\Delta iolA_{tra}$ showed that this mutant is avirulent. The channel catfish in the $\Delta iolA_{tra}$ treatment group had a 100 ± 0% survival rate, while the wild-type strain-injected group had a 2.5%±0.08 survival rate. The percentage survival rates were transformed by arcsine square root transformation and then analyzed by SAS 9.2, and significant differences were observed between *iolA* and ML09-119 treatment groups ($P < 0.0001$). This indicates that the $\Delta iolA_{tra}$ was an attenuated strain of ML09-119 (Figure 3.2). However, the channel catfish i.p challenged with the $\Delta iolA_{Rec1}$ mutant, which was created by precisely knocking out the *iolA* gene using the recombineering method, was still fully virulent with a 2.5% ± 0.08 survival rate that was the same as the wild type positive control group ($P > 0.05$). This indicated that the $\Delta iolA_{Rec1}$ was not an attenuated strain of ML09-119 (Figure 3.2).

Sub-challenge of the channel catfish survivors in the $\Delta iolA_{tra}$ treatment group with wild type ML09-119 showed a 56.9% ± 0.154 survival rate observed in the $\Delta iolA_{tra}$ group (Figure 3.3). In contrast, there was a 4.4% ± 0.141 survival rate in the positive control group. All survivors of the $\Delta iolA_{tra}$ group were I.P injected with 1 x 10^6 CFU/fish ML09-119 again to determine if any immunogenicity developed. Naive channel catfish were I.P injected the same dosage with ML09-119 as a positive control. Significant differences were observed between the ML09-119 group and *iolA* mutant group ($P < 0.05$) suggesting that a certain extent of immunogenicity to ML09-119 was developed by exposing catfish to the $\Delta iolA_{tra}$ mutant.

4.2.2 The investigation of the virulence of the different *iolA* mutants

The vast difference of the virulence between $\Delta iolA_{tra}$ and $iolA_{Rec1}$ mutants prompted us to remake the $\Delta iolA_{tra}$ mutant using the recombineering method to identify if any secondary mutation was introduce while the $\Delta iolA_{tra}$ mutant was constructed. The hypothesis for the difference between the $\Delta iolA_{tra}$ and *iolARec1* mutants is that there might be a second site mutation that happened during the construction of the $\Delta iolA_{tra}$ mutant that resulted in an attenuated strain.

Furthermore, it was noticed that during the construction of the $\Delta iolA_{tra}$ mutant that a part of the promoter region between the *iolA* and *iolR* genes was deleted as well (Figure 3.4). This promoter region is hypothesized to contain the binding region for the transcriptional regulator protein IolR to regulate its own transcription, as it is expected to do for other genes in the IolR regulon (Kohler, *et al.* 2011). This prompted us to create the $\Delta iolA_{Rec2}$ through $\Delta iolA_{Rec8}$ mutants to determine if the loss of the IolR binding region (as yet undefined) will affect the expression of the *iolR* gene as result in an attenuated

strain. The hypothesis is that by deleting the binding region for the transcriptional repressor IolR that this will increase the transcription of *iolR*, resulting in suppression of other possible virulence factor genes such as aerolysin (Zhang D, *et al*, 2013) that could possibly be co-regulated by IolR and be in the "IolR regulon". Thus the mutant will be attenuated.

A pretrial was carried out to determine the virulence of each mutant as well as to help select specific mutants for vaccine and immunogenicity studies. The result of this pretrial showed that the remake of the $\Delta iolA_{tra}$ mutant using the recombineering method, $\Delta iolA_{rec}$, did not lose its virulence with a 0% survival rate, as did the $\Delta iolA_{Rec2}$, $\Delta iolA_{Rec5}$, $\Delta iolA_{Rec7}$, and $\Delta iolA_{Rec8}$ mutants. In contrast the $\Delta iolA_{tra}$ had a 83% survival rate, and the $\Delta iolA_{Rec3}$, $\Delta iolA_{Rec4}$, and $\Delta iolA_{Rec6}$ treatment groups had 25%, 33%, and 17% survival rates, respectively. (Figure 3.5). These results indicate that there may be some variability in the virulence among these mutants and that there may be some contribution of the IolR regulon to *A. hydrophila virulence*. Even though this pretrial did not give a valid statistical analysis, it did provide a preliminary determination of the virulence of these *iolA* mutants. The $\Delta iolA_{Rec3}$ and $\Delta iolA_{Rec4}$ mutants were picked for the formal vaccine candidate and immunogenicity challenge study.

4.2.3 Vaccine candidate and immunogenicity challenge study.

To determine the virulence of the *iolA* mutants and to evaluate their efficacy as a live vaccine against *A. hydrophila* ML09-119, an *in vivo* channel catfish challenge study was conducted. The results of the *in vivo* channel catfish i.p challenge with $\Delta iolA_{tra}$ again showed that this mutant is attenuated in catfish with a 83.3 ± 11.5 % survival rate, while 0 ± 0% survival rate was observed for the fish in the positive control treatment group($P <$

0.0001) (Figure 3.6). However, channel catfish i.p challenged with $\Delta iolA_{Rec3}$ or $\Delta iolA_{Rec4}$ mutants, that have deletions of the *iolA* gene and 100bp or 500bp upstream of *iolA* gene, respectively, had 12.1 ± 9.1% survival and 17.3 ± 17.2% survival rates, respectively (Figure 3.6). No significant difference was observed between the $iolA_{Rec3}$or $iolA_{Rec4}$ and the wild type treatment group ($P > 0.05$) (Figure 3. 7).

A sub-challenge of the channel catfish survivors was carried out 21 days post challenge. The $\Delta iolA_{tra}$ treatment group surviving fish that were challenged with the wild type ML09-119 showed a 71.4 ± 14.3 % survival rate, in contrast to the 0 ± 0% survival rate observed in the naïve fish challenged with ML09-119 ($P < 0.05$) (Figure 3.8). This suggests that a certain extent of immunity against ML09-119 was developed by exposing catfish to the $\Delta iolA_{tra}$ mutant.

4.3 The Enzyme-linked Immunosorbent Assay (ELISA)

The Enzyme-linked Immunosorbent Assay (ELISA) was carried out to determine the efficacy of protective immunity induced by the $\Delta iolA_{tra}$ mutant immunized channel catfish were i.p injected with 1×10^6 CFU/fish of the mutant. We hypothesized that the $\Delta iolA_{tra}$ mutant expressed epitopes that would retain a similar immunogenicity as the wild type. Thus, a positive reaction should be observed in the titer of the ELISA assay. All of the replicates of the $\Delta iolA_{tra}$ mutant induced a strong antibody reaction to ML09-119 (Figure 3.9). The levels of antibody to ML09-119 were highest in serum from the $\Delta iolA_{tra}$ mutant immunized channel catfish, indicating strong antibody induction by the $\Delta iolA_{tra}$ mutant.

5. Discussion:

This study provided valuable insight into role of the *myo*-inositol pathway in the virulence of *A. hydrophila* ML09-119. One of the *iolA* gene deletion mutants created proved to be attenuated and can provide protection against *A. hydrophila* ML09-119 in an *in vivo* channel catfish challenge study. This mutant may be a promising live vaccine candidate against epidemic *A. hydrophila*.

The recent epidemic outbreak of the MAS, which caused by highly virulent *A. hydrophila* has drawn a lot attention since the catfish farming operations in the southeastern United States have not experienced a large-scale outbreak of MAS before (Hemstreet, 2010). In 2009 and in all subsequent years, catfish farmers in west Alabama have reported severe disease outbreaks which were then proved to be caused by a highly virulent strain of *A. hydrophila*, represented by strain ML09-119, to channel catfish *(I. punctatus)*. From 2009-2011, Alabama catfish famers lost more than 10 million pounds of catfish that were market-size and estimated to be more than $3 million due to this epidemic strain of *A. hydrophila* (Pridgeon et al.,2011; Liles et al., 2011). It is reported that *A. hydrophila* epidemic strain, ML09-119, is highly virulent to channel catfish, causing severe mortality within 24 h post exposure with certain amount of dose. Also, this epidemic *A. hydrophila* has expanded its geographic territory and caused frequent outbreaks in the summer months, resulting in millions of pounds of losses in Alabama, Mississippi and Arkansas. (Pridgeon and Klesius, 2011). Due to its highly virulent nature and huge economic loss so far, it is essential that the virulent factors be studied and an effective vaccine be developed.

A previous study showed that epidemic strains can utilize *myo*-inositol as a sole carbon source. All of the epidemic strains encode the *myo*-inositol catabolic pathway (Hossain *et al.*, 2013). This prompted us to investigate the role of the *myo*-inositol pathway in the virulence of *A. hydrophila* ML09-119.

The $\Delta iolA_{tra}$ mutant was created using a traditional allelic exchange technique, and the *in vivo* channel catfish challenge study showed that this mutant is attenuated compared to its wild-type parent strain ML09-119. However, when we created a precise *iolA* gene deletion mutant $\Delta iolA_{Rec}$ using a more efficient and accurate recombineering technique, we observed that this mutant was still virulent in channel catfish. There are two hypotheses that could explain this difference in virulence between these two *iolA* mutants: 1) the truncation of the IolR binding region causes the over expression of iolR gene, repressing other virulence factors such as aerolysin, and/or 2) the $\Delta iolA_{tra}$ mutant has a secondary mutation responsible for some degree of virulence attenuation

One difference between the $\Delta iolA_{tra}$ and the $\Delta iolA_{Rec}$ mutants is that when the $\Delta iolA_{tra}$ was constructed part of the promoter region between the *iolA* and *iolR* genes was deleted. IolR is a transcriptional repressor for multiple genes in the *myo*-inositol pathway, including *iolR* (Kohler, *et al.* 2011). It is possible that when the $\Delta iolA_{tra}$ was constructed, the deleted promoter region contained a binding region for IolR (Kohler, et al. 2011). Without the binding region for the IolR repressor, the transcription of the *iolR* gene may be increased and the synthesis of more IolR might repress other genes that are related to the virulence of *A. hydrophila* in the IolR regulon such as aerolysin (Zhang et al., 2013; Cordero-Alba et al., 2012). We hypothesize that by deleting the region between the *iolA* and *iolR* gene that the expression of the *iolR* gene might change along with the virulence

of the mutants. The results of RT-PCR using iolR-specific primers showed that there might be differences between the $\Delta iolA_{Rec4}$ and other $\Delta iolA_{Rec}$ mutants (data not shown); however, no quantification of these data has been performed to date. Our *in vivo* channel catfish challenge study showed that there is some attenuation within the $\Delta iolA_{Rec3}$ and $\Delta iolA_{Rec4}$ mutants; however, the statistical analysis did not support a difference at $P < 0.05$, and additional experiments with more animals and groups may be needed in order to observe a statistically significant difference between the $\Delta iolA_{Rec3}$ or $\Delta iolA_{Rec4}$ mutants and wild type ML09-119.

Even though the reason for the attenuation of the $\Delta iolA_{tra}$ has not been completely characterized, the immunogenicity study showed that this mutant can provide around 70% survival rates for channel catfish at doses that result in no survival for naïve fish. The ELISA assay evaluating the antibody induced by the $\Delta iolA_{tra}$ mutant against *A. hydrophila* ML09-119 showed that the $\Delta iolA_{tra}$ mutant could induce strong antibody reaction. This indicates that *ΔiolA5tra* mutant can serve as a promising live vaccine candidate against the recent MAS epidemic outbreak.

This study also raised some interesting studies for the future research including:

1. The reason of the attenuation of the $\Delta iolA_{tra}$ mutant.
2. The role of the *iolR* gene and what are the genes that are included in the IolR regulon.
3. The delivery route for the live vaccine of the channel catfish against the *A. hydrophila* epidemic strain.

Table 3.1. Summary of bacterial strains and plasmids used in this study

Bacterial strains and Plasmid	Relevant features	References
Bacterial strains		
A. hydrophila ML09-119		Hossain et al., 2013
E. coli SM10-λ-pir	*thi-1thr leu tonA lacY supE recA*::RP4-2-TcT::Mu Km^r *λpir*	(Simon, Priefer et al. 1983)
Plasmids		
pDMS197	Suicide vector, *sacB*, Tet^R	(Edwards, Keller et al. 1998)
pDMS197iolA		This study

Table 3.2. The primers used in this study

primer ID	Primer sequence	Primer Application
iolA5RecF	5'-T*G*A*A*ATTTAATTTTCAACAAATTCCGTGATCATCAGCCAAGAGAGAGATC***GTAGACTTCCGTTGAACT***-**3'**	Amplifying the cat-cassette for creating the $\Delta iolA_{Rec}$
iolA5RecR	5'-C*T*G*G*AGAGCGGGATAACCGAGGTGAGTCTGGACGTGGCGCCTTCAGGAGA***GCCTAATGAGTGAGCTAA***-**3'**	Amplifying the cat-cassette for creating the $\Delta iolA_{Rec}$ and serve as the reverse primer for the $\Delta iolA_{Rec2}$- $\Delta iolA_{Rec8}$
iolARecF1	5'-A*A*A*T*TTAATTTTCAACAAATTCCGTGATCATCAGCCAAGAGAGAGATCGA***GTAGACTTCCGTTGAACT***-3'	Amplifying the cat-cassette for c the $\Delta iolA_{Rec1}$ mutant
iolARecR1	5'-CGCCTTCAGGAGAGAGGGGCGACTGTCCCTCTCTTTTGTTACACTGTCCG***GCCTAATGAGTGAGCTAA***-3'	Amplifying the cat-cassette for c the $\Delta iolA_{Rec1}$ mutant
iolARecF2	5'-T*G*C*T*GGATTGATCACAAAAAGGAATTTTTGTTTCACATAAGATTTTTATG***GTAGACTTCCGTTGAACT***-**3'**	Amplifying the cat-cassette for c the $\Delta iolA_{Rec2}$ mutant

iolARecF3	5'- T*G*G*G*GGTTAAACCAGAACCAGGCCAA CATCTGCGGGTATGGTGTCAAGAA***GTAGA*** ***CTTCCGTTGAACT*-3'**	Amplifying the cat-cassette for c the *ΔiolA*$_{Rec3}$ mutant
iolARecF4	5'- A*C*C*A*TTCTTTCATATCATGAAAAACTG TAAACCATTTCGTAACGACAGAC***GTAGACT*** ***TCCGTTGAACT*-3'**	Amplifying the cat-cassette for c the *ΔiolA*$_{Rec4}$ mutant
iolARecF5	5'- G*C*T*T*CATTAACTGCTACAAGATCTCGT TTCCTGTCTGCAGCAATGGAACA***GTAGACT*** ***TCCGTTGAACT*-3'**	Amplifying the cat-cassette for c the *ΔiolA*$_{Rec5}$ mutant
iolARecF6	5'- A*A*T*G*CCAAATCAATTGATATTGTTCAG AAATCTCAACCACTAAACCCCCG***GTAGACT*** ***TCCGTTGAACT*-3'**	Amplifying the cat-cassette for c the *ΔiolA*$_{Rec6}$ mutant
iolARecF7	5'- C*T*G*T*TGCAATCATGTTTACCGGGGCGC CAGCCAGTTACACTTCTTACTTC***GTAGACT*** ***TCCGTTGAACT*-3'**	Amplifying the cat-cassette for c the *ΔiolA*$_{Rec7}$ mutant
iolARecF8	5'- T*C*T*G*CTAGATTCTTCGCCACTGTCATT ATGCACTCCAATCTGTTGCAATC***GTAGACT***	Amplifying the cat-cassette for c the *ΔiolA*$_{Rec8}$ mutant

	***TCCGTTGAACT*-3'**	
Cat F	5'-GTAGACTTCCGTTGAACT-3'	Amplifying the *cat* gene
CatR	5'-GCCTAATGAGTGAGCTAA-3'	Amplifying the *cat* gene
iolA_upF8	5'-TTGCAGAATATGGTGAGGTT-3'	PCR and sequencing to identify the mutant from $\Delta iolA_{Rec6}$ to $\Delta iolA_{Rec8}$
iolA_dnR8	5'-ATATGCCATTCAGTATGCCA-3'	PCR and sequencing to identify the mutant from $\Delta iolA_{Rec1}$- $\Delta iolA_{Rec8}$
Aup_intF	5'-CCAGCCAGTTACACTTCTTA-3'	fusing the two *iolA* gene arms and the *catR* gene by splicing through overlap extension PCR (SOE)
And_intR	5'-TTATCTTCAGGCTTGATGCT-3'	fusing the two *iolA* gene arms and the *catR* gene by splicing through overlap extension PCR (SOE)
AupF	5'- CACTCCAATCTGTTGCAATC-3'	Amplification of upstream sequences of *iolA* gene
AupR	5'-GATCTCTCTCTTGGCTGATG***AGTTCAACGG AAGTCTAC***-3'	Amplification of upstream sequences of *iolA* gene
AdnF	5'-	Amplification of downstream

	TCTCCTGAAGGCGCCA***TTAGCTCACTCATT AGGC***-3'	sequences of *iolA* gene
AdnR	5'-GTAACACCACCGTGAGCAAG-3'	Amplification of downstream sequences of *iolA* gene

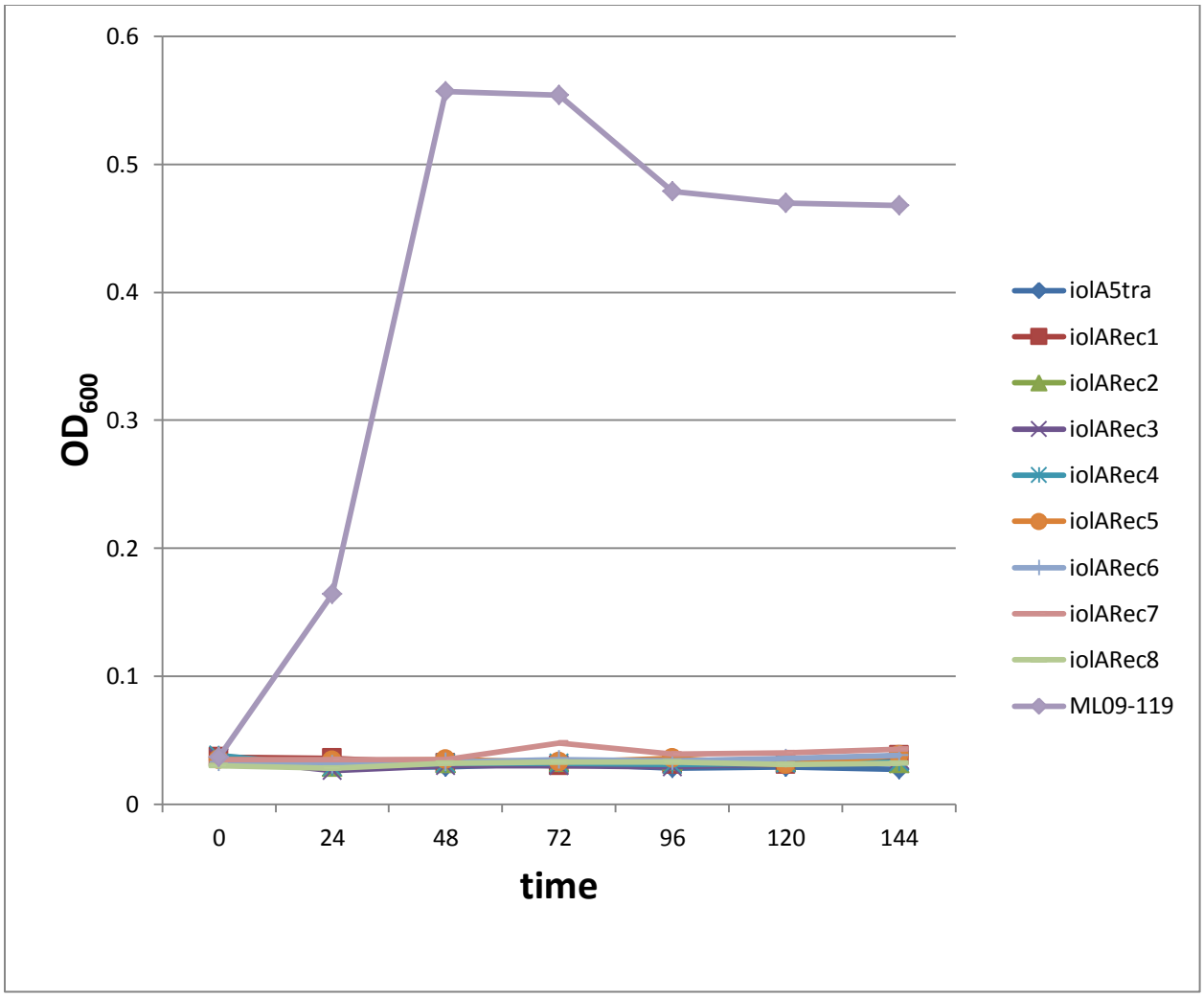

Figure 3.1. *Myo*-inositol assay. Growth of bacterial strains in M9 medium containing *myo*-inositol as the sole carbon source for 144 hours. All the ⧋*iolA* deletion mutants, both created by traditional SOE technique and the recombineering technique, were incapable of growth using *myo*-inositol as a sole carbon source. Wild type *A. hydrophila* ML09-119 reached stationary phase after 48 hours of incubation.

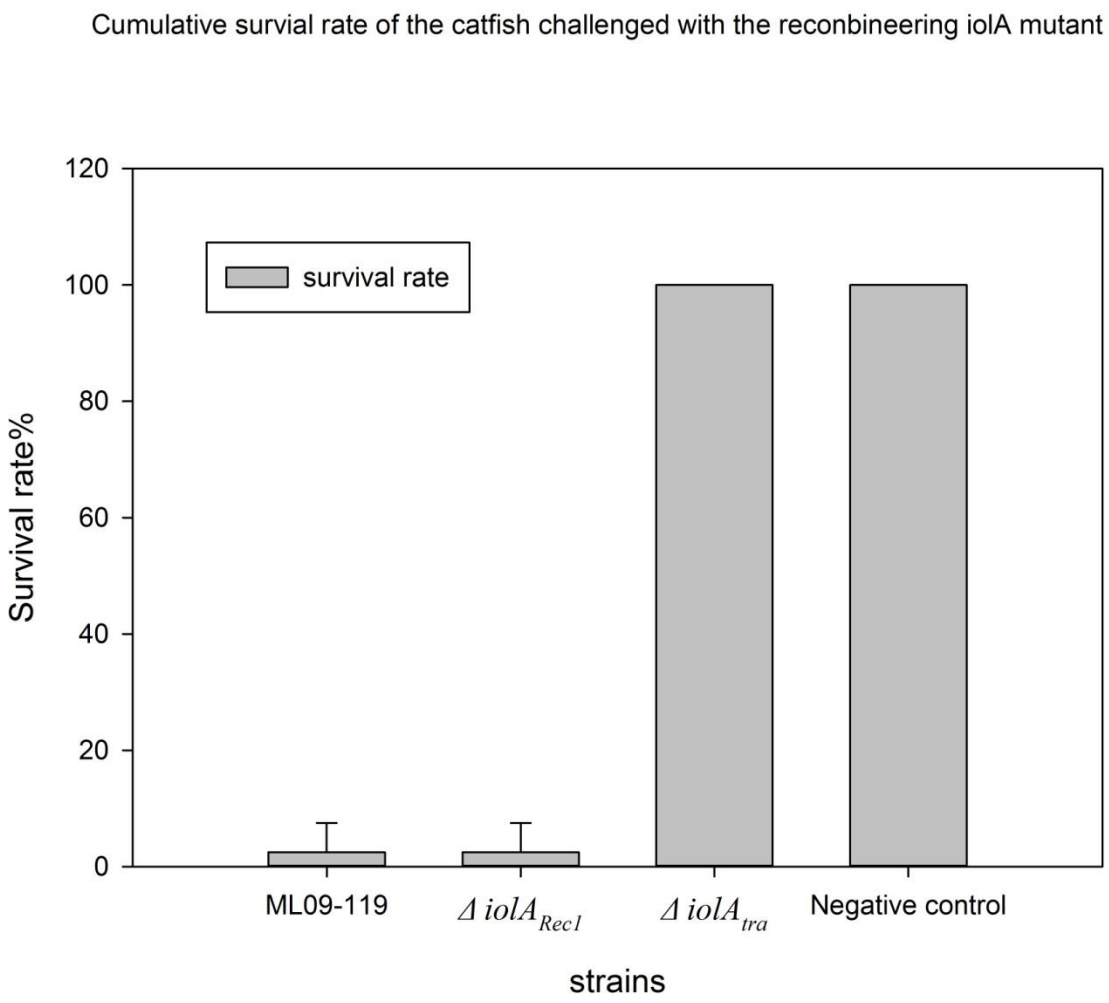

Figure 3.2. Cumulative survival rate of the *ΔiolA*$_{tra}$ and *ΔiolA*$_{Rec1}$ mutants compared to wild type *A. hydrophila* ML09-119. Channel catfish that were i.p injected with 10^6 CFU/fish of the *ΔiolA*$_{tra}$ mutant had 100 ± 0% survival rate, while 2.5% ± 0.08 survival rate was observed in the positive control treatment group in which channel catfish were i.p injected with 10^6 CFU/fish wild type ML09-119($P < 0.0001$). The *iolA*$_{Rec1}$ treatment group had a 2.5% ± 0.08 survival rate which was the same as the wild type positive control group ($P > 0.05$).

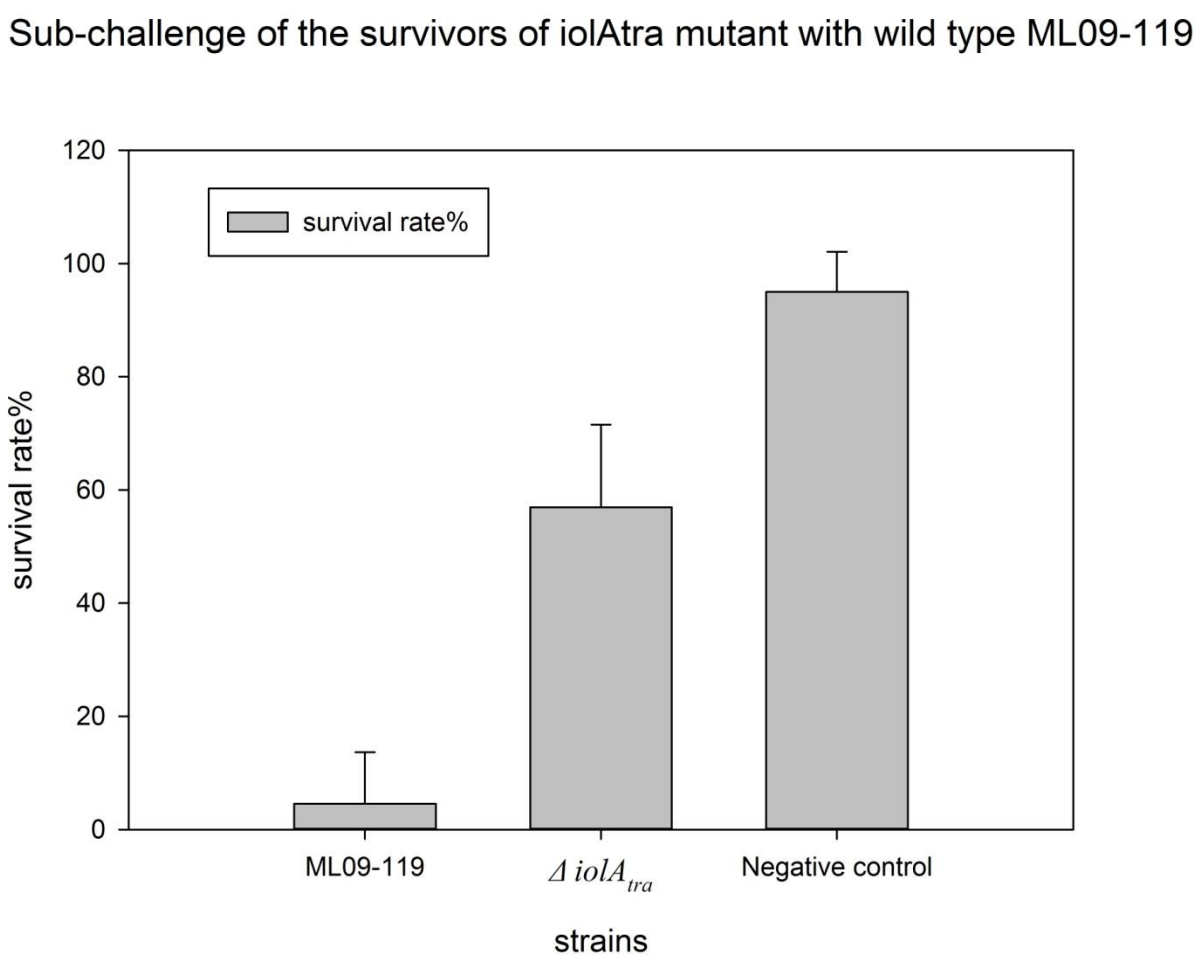

Figure 3.3. Sub-challenge of the survivors in the *ΔiolA*$_{tra}$ group with wild type. 21 days post challenge, all survivors of *ΔiolA*$_{tra}$ group were I.P injected with 1 x 10^6 CFU/fish ML09-119. Naive channel catfish were I.P injected with the same dosage with ML09-119 as a positive control. Significant differences were observed between the ML09-119 group and *ΔiolA*$_{tra}$ mutant group ($P < 0.05$).

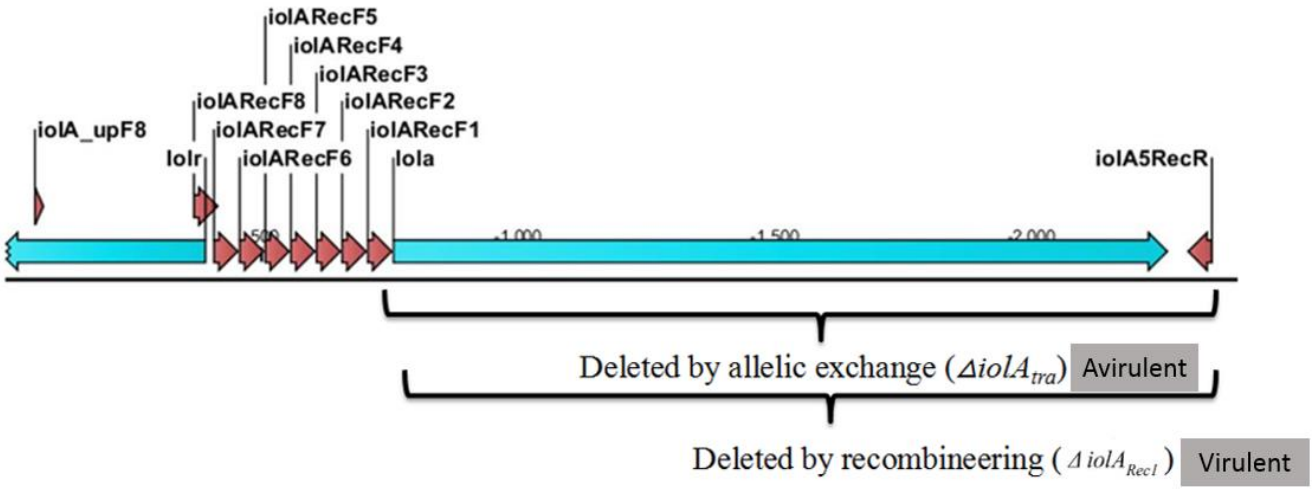

Figure 3.4. The relative positions of the *iolA* and *iolR* gene

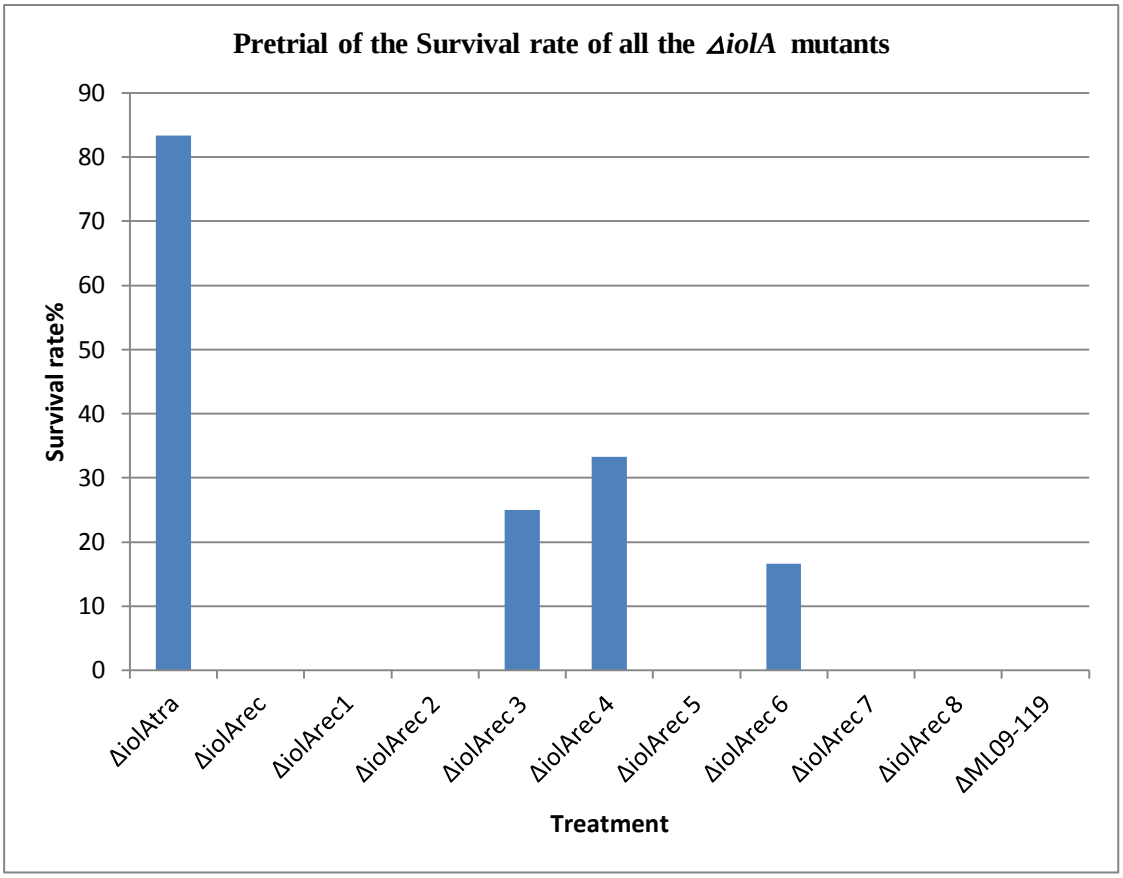

Figure 3.5: Pre-trial challenge of the iolA mutants compared with wild type *A. hydrophila* ML09-119. A 0% survival rate was observed in the channel catfish i.p injected with the $\Delta iolA_{Rec}$ mutant, as did the $\Delta iolA_{Rec2}$, $\Delta iolA_{Rec5}$, $\Delta iolA_{Rec7}$, and $\Delta iolA_{Rec8}$ mutants. In contrast, the $\Delta iolA_{tra}$ mutant had an 83% survival rate, and there was 25%, 33.3%, and 16.7% survival rates observed for the $\Delta iolA_{Rec3}$, $\Delta iolA_{Rec4}$, and $\Delta iolA_{Rec6}$ treatment groups, respectively.

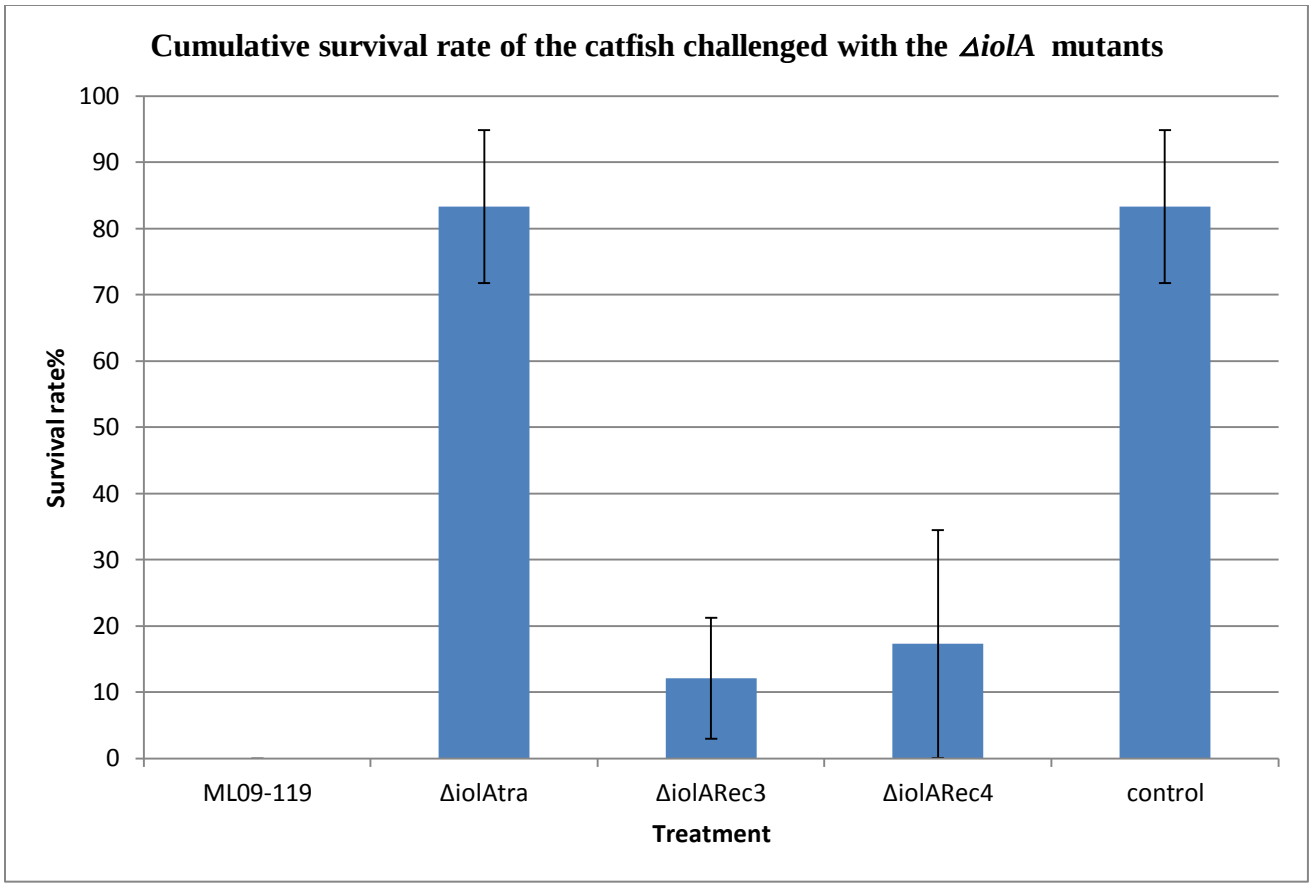

Figure 3.6. Cumulative survival rate of the catfish challenged with the *ΔiolA* mutants. The channel catfish in the $\Delta iolA_{tra}$ treatment group had an 83.3 ± 11.5% survival rate, while a 0 ± 0% survival rate was observed in the ML09-119 treatment group ($P < 0.0001$). Channel catfish challenged with the $\Delta iolA_{Rec3}$ or $\Delta iolA_{Rec4}$ mutants had a 12.1 ± 9.1% and 17.3 ± 17.2% survival rates, respectively.

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**strain**
A	1.00000	4	ML09-119
A			
A	0.87955	4	iolARec3
A			
A	0.82727	4	iolARec4
B			
B	0.27500	4	$\Delta iolA_{tra}$
B			
B	0.16667	3	control

Figure 3.7: SAS Duncan comparisons. Significant differences were observed between $iolA_{tra}$ and ML09-119 treatment groups ($P = 5.23E^{-06}$), indicating that the $\Delta iolA_{tra}$ is an attenuated mutant of ML09-119. No significant differences were observed between the $iolA_{Rec3}$ or $iolA_{Rec4}$ treatment groups and the wild type ($P = 0.09$).

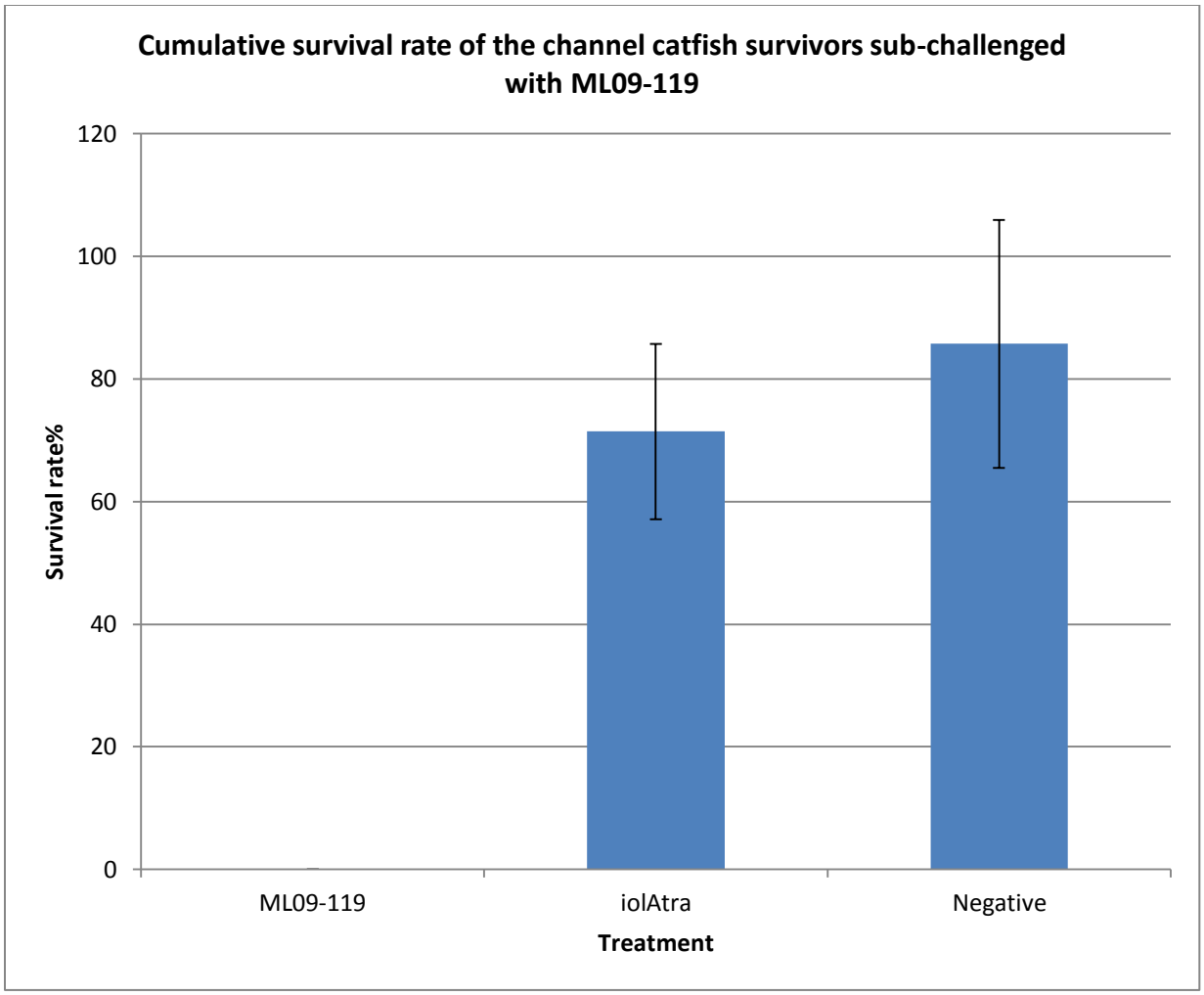

Figure 3.8. Cumulative survival rate of the channel catfish survivors sub-challenged with ML09-119. Sub-challenge of the channel catfish survivors in the $\Delta iolA_{tra}$ treatment group with the wild type ML09-119 showed a 71.4 ± 14.3% survival rate, compared to challenge of naïve fish that resulted in a 0 ± 0% survival rate(P = 0.00098).

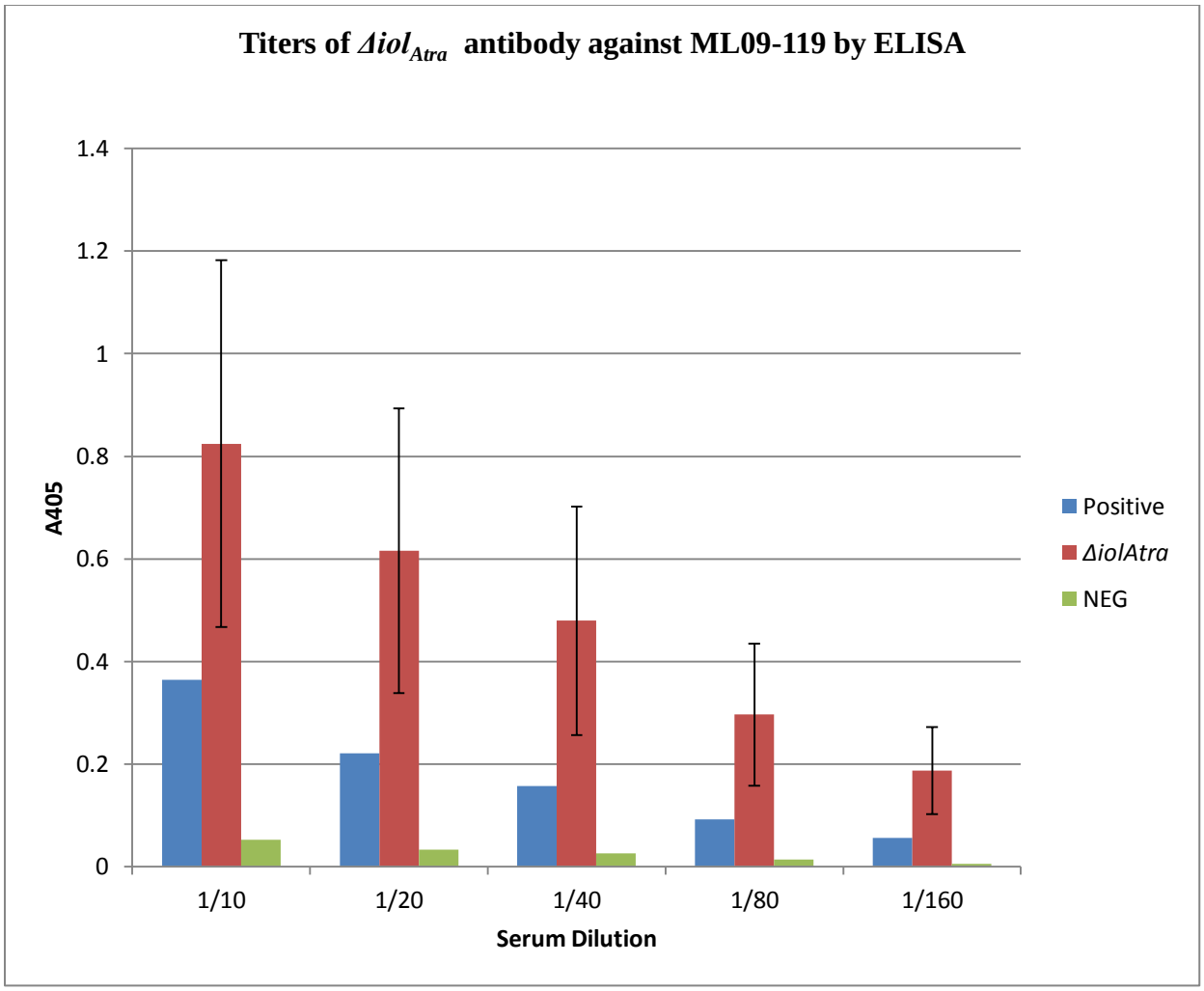

Figure 3.9. Titers of *ΔiolA*$_{tra}$ antibody against ML09-119 by ELISA. All the replicates of the *ΔiolA*$_{tra}$ mutant induced strong antibody reaction to ML09-119. The levels of antibody to ML09-119 were highest in the serum from *ΔiolA*$_{tra}$ mutant immunized channel catfish.

Chapter IV

Determining the role of the O-antigen in *A. hydrophila* ML09-119 virulence

1. Abstract

The epidemic strain of *A. hydrophila* is causing devastating outbreaks of motile Aeromonads septicemia of catfish (MAS) in catfish farms in Southeastern United States, beginning in 2009. Previously we have generated genome sequences for 11 *A. hydrophila* isolates, 6 of which are epidemic stains, while the others are historical *A. hydrophila* isolates not affiliated with an epidemic outbreak of disease that we describe as "reference" strains. A comparative genomic analysis indicated that 53 epidemic-associated genetic regions with 313 predicted genes were present in the epidemic isolates but absent from the reference isolates. 34 genes from this region were predicted to be related to the virulence of the epidemic strains. A novel O-antigen cluster was found in all the epidemic isolates and one reference isolates. In this study, the lipid A-Core ligase (*waaL*) and O-antigen polymerase (*wzy*) knockout mutants, *ΔwaaL* and *Δwzy*, were created by both traditional allelic exchange technique and recombineering technique respectively, resulting in the mutants $\Delta waaL_{tra}$ or $\Delta waaL_{Rec}$, Δwzy_{tra} or Δwzy_{Rec}. An *in vivo* channel catfish challenge study was conducted to study the role of O-antigen in the virulence of the epidemic strain of *A. hydrophila*. The results show that the channel catfish that were challenged with $\Delta waaL_{tra}$ or Δwzy_{tra} had a 100% survival rate, but a 0%

survival rate was observed in the channel catfish that were challenged with $\Delta waaL_{Rec}$ or Δwzy_{Rec} mutants.

It was hypothesized that the avirulent phenotype of the $\Delta waaL_{tra}$ or Δwzy_{tra} mutants was due to a polar effect on the downstream *ymcA* gene, which is reported to be related to the biosynthesis and assembly of the O-antigen (Branda, et al, 2004). A *ΔymcA* mutant was created by deleting the *ymcA* gene using a recombineering method to study the role of *ymcA* in the virulence of *A. hydrophila*. A 68.1 ± 16.8% survival rate was observed in the channel catfish that were challenged with the *ΔymcA* mutant. Sub-challenge of the survivors of *ΔymcA* treatment group 21 days post first challenge showed that 90.48±8.25% survival rate was observed. Significant difference was observed between the *ΔymcA* treatment group and the positive control group which were naive channel catfish challenged with wild type ML09-119. The ELISA titer of the survivors of the *ΔymcA* treatment group 21 days post first challenge showed that *ΔymcA* induced a strong antibody response against the wild type *A. hydrophila* ML09-119 indicating that *ΔymcA* mutant can serve as a promising vaccine candidate.

Key words: A. hydrophila, virulence, O-antigen, Vaccine, ymcA gene

2. Introduction

A. hydrophila is the causative agent of motile *Aeromonas septicemia* (MAS) in channel catfish. The epidemic *A. hydrophila* isolates responsible for current epidemic outbreaks in channel catfish are highly virulent to channel catfish as compared to the reference *A. hydrophila* isolates of diseased fish from previous non-epidemic outbreaks (Pridgeon and Klesius 2011).

The LPS of Gram-negative bacteria are major virulent determinants and are composed of lipid A, an inner core oligosaccharide and repeating O-antigen polysaccharide. The virulent nature of LPS is attributed due to the core oligosaccharide and O-antigen polysaccharide. LPS contributes significantly in bacterial pathogenesis by intestinal colonization (Nevola, Laux et al. 1987; West, Sansonetti et al. 2005), lessening macrophage activation (Lugo, Price et al. 2007), promoting intracellular growth (Nagy, Danino et al. 2006), and serum resistance (DeShazer, Brett et al. 1998).

The truncation or deletion of the components of the LPS, particularly the O-antigen polysaccharide, diminishes the virulence properties of the bacterial pathogen and this attenuation is necessary for development of a live, attenuated vaccine strain. In our previous study, through whole genome comparative genomic analysis, we determined the genetic basis of O-antigen biosynthesis from twelve different *A. hydrophila* isolates obtained from diseased fish (Hossain et al 2013), and observed a unique O-antigen biosynthetic pathway in ML09-119 and other epidemic strains and a total of 5 different O-antigen types among the sequenced strains.

Gene knockout and mutant generation is a tool developed from naturally existing mechanisms by which genetic material is exchanged between different bacteria and

viruses (Rocha, et al. 2005). After the genes are transferred into the host bacteria, these genes are then incorporated onto the host genome by homologous gene recombination (Ishikawa, et al, 2013; Thomason, et al. 2007).

Recombineering is a precise technique for the manipulation of bacterial genes and other organisms (Yu et al., 2000). This technique is very accurate and fast in target gene deletion, insertion, or substitution events; thus, in a very short time mutants for the study of gene functions can be generated (Datsenko & Wanner, 2000; Datta et al., 2008; Rivero-Müller et al., 2007). A novel recombineering method was developed (Hossain et al., manuscript in preparation) in order to introduce a recombineering plasmid into epidemic *A. hydrophila* via conjugation and mutagenize genes to determine their respective roles in virulence.

In this study, the Lipid A-Core ligase gene (*waaL*) and O-antigen polymerase gene (*wzy*) knockout mutants, $\Delta waaL_{tra}$ *or* $\Delta waaL_{Rec}$, Δwzy_{tra} *or* Δwzy_{Rec} were created by both traditional allelic exchange and recombineering techniques. An *in vivo* channel catfish challenge study was conducted to study the role of O-antigen in the virulence of the epidemic strain of *A. hydrophila* ML09-119. A *ΔymcA* mutant was also created by knocking out the *ymcA* gene using the recombineering method to study the role of YmcA in the virulence of *A. hydrophila*.

3. Materials and methods:

3.1 Bacterial isolates and plasmids

The *A. hydrophila* ML09-119 used in this study was picked out from single colony on a TSA plate that was inoculated from a -80°C cryostock. The epidemic strain

was from a west Alabama MAS disease outbreak in 2009. The bacteria were routinely grown on fresh TSB medium overnight before use. The *A. hydrophila* ML09-119 used for experiments was from the bacteria stocks of the fish disease lab in Auburn University. This epidemic strain was originally isolated from the kidneys of channel catfish naturally infected with *A. hydrophila*. The pure culture of the epidemic strain was used first in a small test infection of 10 catfish. Moribund catfish that showed clinical signs of *A. hydrophila* ML09-119 were collected for necropsy. *A. hydrophila* was re-isolated from a dying fish by poking a sterile plastic bacteriology loop into the kidney and inoculating a TSA plate. By doing this, it is expected than the virulence of the epidemic strain stock can be recovered**.** ML09-119 was then confirmed by biochemistry and selective media following the established identification procedures with modifications (Furuwatari, et al., 1994; Holt, et al., 1994). Briefly, the identification biochemical tests included Gram stain, cytochrome oxidase, glucose utilization, 0/129, sucrose, esculin hydrolysis, V-P, DL-lactate utilization and urocanic acid utilization, and then testing on the selective minimal medium M9 with *myo*-inositol added. *E. coli* SM10-λ-pir and *E. coli* CC118-λ-pir (Simon *et al.*, 1983) were used for the conjugal transfer of the mobilizable mutagenesis plasmids to *A. hydrophila ML09*-119. The list of bacterial strains used in this study is presented in Table 4.1.

3.2 Construction of defined *A. hydrophila* Lipid A-Core ligase (*waaL*) and O-antigen polymerase (*wzy*) knockout mutants, *ΔwaaLtra* & Δwzy_{tra}, by traditional splicing PCR and conjugation technique

Lipid A-Core ligase (*waaL*) and O-antigen polymerase (*wzy*) knockout mutants, $\Delta waaL_{tra}$ & Δwzy_{tra} were constructed using suicide plasmid pDMS197 (Edwards, Keller et al. 1998). The primers needed for this study were listed in the Table 4.2.

The two pairs of primers, Li-upF/Li-upR and Li-dnF/Li-dnR, were used to amplify approximately 400bp upstream and downstream sequences of *waaL* gene, respectively using EconoTaq PLUS GREEN 2X Master PCR kit (Lucigen, USA) to construct the *ΔwaaL* mutant. The template used in this PCR was the genomic DNA of *A. hydrophila* ML09-119 which was extracted using an E.Z.N.A. ® Bacterial DNA Kit (Omega Bio-Tek, USA). The chloramphenicol acetyltranferase gene (*cat*) was amplified from pMHH46 plasmid (Hossain et al 2013) using primers catF and catR. The primers Li-upR and Li-dnF were used for the amplification of upstream and downstream sequences of *waaL* gene, and contained the reverse complemented sequences of catF and catR primers which were added respectively at their 5' ends. The *CatR*-cassette which was the chloramphenicol resistance gene (CM^R) with two arms of the upstream and downstream homologous of *waaL* gene was created by fusing the two arms and the CM^R gene by splicing through overlap extension PCR (SOE) (Horton, Hunt et al. 1989). The primers for this PCR were Liup-intF and Lidn-intR. The PCR products were purified by agarose gel purification.

The suicide plasmid pDMS197 was digested by restricted digestion enzyme XbaI (New England Biolabs, NEB) following the protocol provided by the manufacturer. A 50

ul reaction was used for the digestion, including 25ul of the suicide plasmid pDMS197 DNA, 3ul of the XbaI restricted digestion enzyme, 5ul of the 10 × CutSmart™ Buffer, 1 × BSA and 16ul RNase free H_2O. The reaction system was incubated at 37 °C for one hour. The reaction system was then incubated at 65°C for 20 min to stop the reaction. The digested product was purified by DNA Clean & Concentrator™ (Zymo research), and the concentration was measured by Qubit® dsDNA BR Assay Kit (Life technologies). The product was blunted using end-repair kit DNA terminator (Lucigen, USA) following the producer's instruction. The product was purified by DNA Clean & Concentrator™ (Zymo research) again before ligation.

The purified restriction enzyme XbaI digested and blunted suicide plasmid pDMS197 was ligated with the gel purified *CatR*-cassette using Quick Ligase (NEB, USA) incubated for 30 minutes. Briefly, 50ng of blunted suicide plasmid pDMS197 and around 3-fold molar excess of the *CatR*-cassette insert was mixed together and the volume was adjusted to 10 ul with RNase free H_2O. 10 ul of the 2× Quick Ligation Buffer and 1µl of Quick T4 DNA Ligase were added into the mixture. The mixture was centrifuged briefly and incubated at room temperature (25°C) for 30 minutes before it was chilled on ice. A SB gel electrophoresis was done to confirm the ligation product (data not shown).

The making of the electrocompetent cells of *E. coli* CC118-λ-pir and SM10-λ-pir was following a published protocol (Inoue, *et al.*, 1990) with minor changes. A 0.5 ml of the overnight culture of *E. coli* CC118-λ-pir and SM10-λ-pir bacteria was inoculated into 200 ml of Hanahan's Broth *(*SOB Medium*)* respectively with 10 mM $MgCl_2$. The culture was incubated in the 37°C water bath incubator with shaking at 200 rpm for around 2.5

hours and the OD_{600}=0.4. The culture was chilled in ice for 10 min before loaded into 200 ml centrifuge tubes. The culture was centrifuged at 6000 rpm for 8 min at 4°C, the supernatant was discarded and the pellet was washed by resuspended with 10% glycerol and centrifuged again at 6000 rpm for 8 min. The wash step was repeated for 3 times before the pellet was gently resuspended in 200 ul GYT medium. The whole procedure was performed on ice.

The ligation product was then used in the electroporation (Chassy, et al., 1988; Dower et al, 1988) to create the plasmid pDMS197*waaL*, which contains a deletion of the entire *waaL* gene. 50 ul of the premade electrocompetent cells of the *E. coli* CC11810-λ-pir was mixed gently with 2.5 ul of the ligation product and chilled on ice for 5 min. The mixture was transferred into ice cold cuvettes (Bulldog bio) before the cuvettes were loaded onto the Eppendorf® Eporator® (Eppendorf). Voltage was set at 1800V. The mixture was mixed with recovery medium (SOC medium) right after the pulse shock. The culture was transferred to a 2 ml test tube and incubated at 37°C with shaking at 200 rpm for 2 hrs. The successfully electroporated *E. coli* CC118-λ-pir with the plasmid pDMS197*waaL* was selected on 2XYT agar medium plate with 25 ug/ml chloramphenicol and 5 ug/ml tetracycline. A similar strategy was followed for the construction of pDMS197*wzy*, which contains a deletion of the entire *wzy* gene. *E. coli* CC118-λ-pir with the plasmids were grown in fresh LB medium with 25 ug/ml chloramphenicol and 5 ug/ml tetracycline. The plasmid was extracted using E.Z.N.A. ® Plasmid Midi Kit (Omega Bio-Tek, USA) respectively to get more pure suicide plasmids pDMS197waaL and pDMS197wzy.

The suicide plasmids pDMS197waaL and pDMS197wzy were independently introduced into *A. hydrophila* ML09-119 by conjugation with *E. coli* SM10-λ-pir bearing plasmid pDMS197waaL or pDMS197wzy, respectively. A single colony was selected on the selective medium plate for SM10-λ-pir bearing plasmid pDMS197waaL or pDMS197wzy, respectively, for inoculation of 5 ml LB broth medium. The culture was incubated at 37°C with shaking at 200 rpm until the OD_{600} was above 1. A single colony of *A. hydrophila* ML09-119 was picked to inoculate 5 ml TSB broth medium. The culture was incubated at 30°C with shaking at 200 rpm until the OD_{600} was above 1. A 4 ml ML09-119 culture and 1 ml SM10-λ-pir bearing plasmid pDMS197waaL or pDMS197wzy were mixed together, respectively. The 5 ml culture mixture was filtered through a MicroFunnel 300 SP (MicroFunnel™) by vacuum pressure and 5 ml of fresh LB broth medium was used for washing the cells onto the membrane. The membrane was transferred to the sheep blood agar medium after 2 washes. The sheep blood agar medium was incubated at 30°C overnight.

The membrane with the cell culture mixture was vortexed with 3 ml fresh TSB broth medium for selection. Single cross-over mutants were selected on TSA plate supplemented with chloramphenicol, tetracycline and colistin. Double-cross over mutants were obtained by plating onto LB (without NaCl) plates supplemented with 15% sucrose and 12.5 µg/ml chloramphenicol. Mutants grown on this selective plate were subjected to phenotypic and genotypic characterizations. The complete deletion of the *waaL* and *wzy* genes were confirmed by PCR followed by sequencing.

3.3 Construction of defined *A. hydrophila ΔymcA and* Δwzy_{Rec} mutant by Recombineering

A recombineering technique was used to create a precise deletion of the *ymcA* gene and *wzy* gene and generate the *ΔymcA and* Δwzy_{Rec} mutants in order to determine the role of O-antigen in the virulence of epidemic *A. hydrophila* ML09-119 in channel catfish.

The chloramphenicol acetyltranferase (*cat*) gene was amplified from pMHH46 plasmid (Hossain et al 2013) using primers *ymcARec*F and *ymcARec*R to generate the *cat*-cassette with 50 bp of the upstream and downstream of the targeted *ymcA* gene. The primer *ymcARec*F contained 50 bp of the upstream of the targeted *ymcA* gene and the primer *ymcARec*R contained the reverse complemented sequences of 50 bp of the downstream of the targeted *ymcA* gene which were added respectively at the 5' ends of each respective primers. The PCR product was validated using agarose gel electrophoresis before another 24× PCR was done using this PCR product to generate more *cat*-cassette insertion.

The PCR product was purified and concentrated using Wizard® DNA Clean-Up system (Promega, USA) following the protocol provided by the manufacturer. Briefly, the 24 different PCRs were pooled together in a 15 ml conical tube, and a Wizard® DNA Clean-Up kit (Promega, Madison, WI) was used to purify the PCR products according to the manufacturer's protocol. The concentration of the final concentrated PCR product was measured using Qubit® dsDNA BR Assay Kit (Life Technologies).

A. hydrophila ML09-119 containing the plasmid pMJH65, which was constructed for the purposes of introducing a recombineering cassette into gram-negative bacteria

(Hossain et al, manuscript in preparation), was prepared for electroporation using a standard protocol (Inoue, *et al.,* 1990) with minor changes. 0.5 ml of the overnight culture of ML09-119 bacteria was inoculated into 150 ml of Hanahan's Broth *(*SOB Medium*)* with 1.5 ml 1M arabinose, 300 ul 25 mg/ml Tetracycline and 600 ul of 2M $MgCl_2$. The culture was incubated in the 30°C water bath incubator with shaking at 200r pm for around 4 hours and the OD_{600} = 0.5. The culture was chilled on ice for 10 min before loaded into 200 ml centrifuge tubes. The culture was centrifuged at 6000 rpm for 8 min at 4°C. The supernatant was discarded and the pellet was washed by re-suspending with 10% glycerol and centrifuged again at 6000 rpm for 8 min. The wash step was repeated 4 times before the pellet was gently resuspended in 200 ul 10% glycerol. The whole procedure was performed on ice.

The concentrated and purified PCR product was then used in the electroporation (Chassy, et al., 1988; Dower et al, 1988) to create the precise *ymcA* gene deletion mutant *ΔymcA*. 50 ul of the premade electrocompetent cells of *A. hydrophila* strain ML09-119 (pMJH65) was mixed gently with 3 ug of the concentrated PCR product and chilled on ice for 5 min. The mixture was transferred into ice cold cuvettes (BulldogBio) before the cuvettes were loaded onto the Eppendorf® Eporator® (Eppendorf) with a voltage setting of 1200 V. The mixture was mixed with recovery medium (SOC medium) right after the pulse shock. The culture was transferred to a 2 ml test tube and incubated at 30°C with shaking at 200 rpm overnight.

The successfully electroporated *A. hydrophila* ML09-119 *ymcA* gene deletion mutant was selected on a TSA agar medium plate supplied with 25 ug/ml

chloramphenicol. A similar strategy was followed for the construction of $\Delta waaL_{Rec}$ or Δwzy_{Rec}, which contains a deletion of *waaL* or *wzy* genes, respectively.

3.4 Virulence study of *A. hydrophila mutants* in channel catfish

All experiments conducted with vertebrate animals (catfish) were approved by the Institutional Animal Care and Use Committee (IACUC) review board at Auburn University in accordance with the animal welfare guidelines specified in the United States.

All the channel catfish *(I. punctatus,* Kansas Random Strain*)*, used in this study were spawned at the hatchery of the Auburn University Fish Genetics Research Unit artificially, prior to transferring to troughs or glass aquaria at the Auburn University Fish Pathology wet lab S-6. Fish were maintained at recirculation systems (temperature around 25 °C and pH 7.5) using well water sources with constant aeration. Fish were fed daily with commercial feed. Water quality factors including temperature, pH, salt level, total ammonia level, total nitrite level were tested on daily basis to ensure that catfish fingerlings remained unstressed and naive to *A. hydrophila*. Catfish fingerlings were grown out in this system until their body weight (BW) reached 20 ± 5g.

A bacterial suspension of exponential phase growth was prepared by overnight culture in 5 ml TSB broth medium on 200rpm shaking at 200 rpm at 30 °C. The next day 1 ml of the overnight bacterial culture was used to inoculate 100 ml fresh TSB broth culture which was incubated with shaking at 200 rpm at 30°C for 4 hours. The bacterial culture was centrifuged at 6000 rpm for 10 min. The supernatant was discarded and the bacterial pellet was resuspended in fresh TSB media. The optical density of the bacterial

culture was measured by the thermospectronic spectrophotometer (Thermo Spectronic, Rochester, NY, USA) at 600 nm and adjusted to an OD_{600} = 1, which was expected to be 1×10^9 CFU/ml. After adjusting the bacterial suspension to an appropriate OD, A 1:100 dilution was performed using fresh TSB broth to get the desired concentration (around 1×10^7CFU/ml) of *A. hydrophila*. Another 1:2 dilution was done with fresh TSB. This culture was put on ice and used for challenge within 3 hours. A plate count assay was conducted right after the fish challenge to calculate the accurate CFU/ml concentration used in this study. The bacterial cultures used in the fish challenge were serially diluted and 100 ul of each dilution was spread on the TSA plates with 3 replicates for each strain of bacteria.

Channel catfish in Auburn University Fish Pathology wet lab S-6 were randomly distributed into glass aquarium tanks. MS-222 (30mg/l) was used during the handling of fish to calm the fish down to decrease the stress. Each tank contained 10 fish. A recirculating system was applied during the acclimation period, which was lasted for 10 days. Water temperature was originally 25 °C and salt level was kept around 1.8 ppt to decrease the stress caused by environmental changes as well as eliminating the chance of *F. columnare* infection. Water temperature was gradually brought up to 30 ± 1°C, and salt was gradually brought down to 0.8 during the first 3 day of the acclimation time. Every environmental factor was kept stable prior to the challenge. Fish were fed with commercial catfish fed once a day at 4% of their body weight. Water was changed once per day for the recirculating system with constant aeration. At the time of challenge, recirculating system was changed into flow through system, with the temperature at 30 ± 1°C. Fish of each treatment tank were euthanized by immersing in a bucket with MS-222

(30mg/l), before 200 ul of ML09-119 bacterial culture was injected intraperitoneally into each fish. Fish were then put back to their cohabitation tanks. Fish of control groups were injected with pure TSB broth medium. Challenged fish were kept the same way as they were during the latter acclimating time. Mortalities were recorded daily for 14 days post challenge. Any moribund or dead fish were removed from the system daily for bacteriological identification and tissue sampling. Prior to sampling, moribund or dead fish were inspected externally and internally for any clinical signs. The identification of *A. hydrophila* isolated from anterior kidney of the moribund or dead fish was performed by the biochemistry and selective medium method described previously. Survivors of the challenge were kept for 28 days, before they are challenged again with the wild type ML09-119 to test if any protection effect was provided. The procedure of the re-challenge was similar to the preciously challenge. At seven days post re-challenge, blood samples were then drawn from the survivors for the ELISA titer in the later experiment.

3.5 The immunogenicity of the mutants and Enzyme-linked Immunosorbent Assay (ELISA)

Blood samples collected after the fish challenge were put in the room temperature for 2 hrs then 4°C overnight allowing to clot completely. Serum of each blood sample was collected followed by centrifuging at 5000rpm for 10 min. The supernatant of each sample was collected for Enzyme-linked Immunosorbent assay (ELISA) analysis. Antibody responses of channel catfish to *A. hydrophila* were quantified by evaluating the presence of specific immunoglobulin to *A. hydrophila* wild type ML09-119 using indirect ELISA. Protein Detector^{TM} ELISA kit was use to conduct the ELISA experiment.

The protocol followed was similar to the product introduction with minor changes. 96-well plastic plates were coated with 100 ul of a solution of 10 ug/ml (10^7 CFU/ml)) *A. hydrophila* epidemic strain. *A. hydrophila* were suspended in carbonate-bicarbonate coating solution. The coating solution was prepared by diluting one time coating buffer tablet in 10 times of sterile reagent quality water. The plates with coating buffer and antigen were placed in 4°C pH 9.6 overnight. The plates were washed 4 times with washing buffer provided by the kit the next day, followed by adding 1×BSA blocking buffer to block for 15 min at room temperature. After another wash step, the plates were used to do ELISA analysis. 100ul of 1% BSA blocking buffer was added into each well on the *A. hydrophila* ML09-119 coated plate. 200 ul of the 1/10 fish blood serum sample diluted with 1% BSA blocking buffer was added to the column A2-A11, A1 and A12 were served as positive and negative control. 100ul of the solution from A1-A12 was transferred to B1-B12 and mixed carefully by pipetting 3-5 times. This step was repeated across the plate until E1-E12. The final 100 ul from the wells in the row E after mixing was discarded. The plate was then incubated at room temperature for 1 hour. The plated was emptied, and residual liquid was tapped out. The plate was washed out by the washing buffer that came with the kit for 5 times. 100ul of Rat Anti-catfish monoclonal antibody (Mab) was diluted 32 times and added into each well that contained the primary antibody, after which the plate was incubated at room temperature for 1 hour. After incubation the plate was emptied, and residual liquid was tapped out and the plate was washed out five times by the washing buffer that came with the kit. 50ul of tertiary antibody (goat anti-rat antibody conjugated with horseradish peroxidase) (0.1ug/ml) was added into each wall that contained the secondary antibody. The plate was incubated at

room temperature for 1 hour, after which the plate was washed as above. 5 minutes soaking time was given to the last wash. 50ul of the substrate solution that came with the kit was added into each well that contained the tertiary antibody. The plated was incubated at room temperature for 5-15 min before the reaction as stopped by adding 50 ul of stop solution into each well for full color development and the plate was then read at OD $_{405}$. A reaction was defined as positive if its OD_{450} value was at least two times the negative control. Ending points were the highest dilution with a positive reaction.

A criss-cross serial dilution analysis was done prior to the ELISA analysis of the samples to optimize the reagent concentration in the immunoassay procedure.100ul of 1% BSA blocking buffer was added into each well of the *A. hydrophila* ML09-119 coated plate. 200 ul of the 1/10 ML09-119 infected survivor fish blood serum sample diluted with 1% BSA blocking buffer was added to the respective columns and serially diluted across the plate to identify the best concentration range for the sample. Prior to adding the Mab, 100 ul of 1%BSA blocking buffer was added into each well, followed by 200 ul of the secondary rat anti-channel catfish Mab. This Mab solution was serially diluted across the plate to identify the optimum concentration for the Mab.

4. Results

4.1 Cumulative survival rate of the channel catfish challenged with $\Delta waal_{tra}$ or Δwzy_{tra} and $\Delta waal_{Rec}$ or Δwzy_{Rec}

For better understanding of the virulence factors of the *A. hydrophila* epidemic strain and to identify possible live vaccine candidates, the *waal* and *wzy* genes that are expected to be required for O-antigen synthesis and assembly were knocked out by a

traditional allelic exchange technique. The LPS of Gram-negative bacteria are major virulent determinants and are composed of lipid A, an inner core oligosaccharide and repeating O-antigen polysaccharide. The role of LPS in virulence is due to the core oligosaccharide and O-antigen polysaccharide, by contributing to intestinal colonization (Nevola, Laux et al. 1987; West, Sansonetti et al. 2005), lessening macrophage activation (Lugo, Price et al. 2007), promoting intracellular growth (Nagy, Danino et al. 2006), and serum resistance (DeShazer, Brett et al. 1998). Since O-antigen significantly contributes to the virulence of many gram negative bacteria, the hypothesis is that the *waal* and *wzy* genes are virulence factors and by constructing targeted deletions of each of these genes that the resulting mutants of ML09-119 will be attenuated and can serve as promising vaccine candidates.

The results of the *in vivo* channel catfish i.p challenge with $\Delta waal_{tra}$ and Δwzy_{tra} showed that the $\Delta waal_{tra}$ and Δwzy_{tra} are both avirulent. The channel catfish in the $\Delta waal_{tra}$ and Δwzy_{tra} treatment groups had a 100 ± 0% survival rate, while the wild-type strain-injected group had a 5 ± 0.08% survival. The percentage survival rates were transformed by arcsine square root transformation and then analyzed by SAS 9.2, and significant differences were observed between $\Delta waal_{tra}$ or Δwzy_{tra} and ML09-119 treatment groups ($P < 0.0001$). This indicates that the $\Delta waal_{tra}$ and Δwzy_{tra} are both attenuated strains of ML09-119 (Figure 4.1). However, the channel catfish i.p challenged with the $\Delta waal_{Rec}$ and Δwzy_{Rec} mutants, which were created by precisely knocking out the *waal* and *wzy* gene using the recombineering method, were still fully virulent with a survival rate comparable to the wild type ML09-119 (data not shown).

Sub-challenge of the channel catfish survivors in the $\Delta waal_{tra}$ and Δwzy_{tra} mutants treatment groups with wild type ML09-119 was conducted. Unfortunately, due to mistakes in the plate count technique, the exact concentration of ML09-119 used in this experiment was not determined. However, significant differences were still observed between the sham negative control group and the treatment groups ($P < 0.05$). No significant differences were observed between the treatment groups and the positive control group ($P_{\Delta wzy} > 0.05$ and $P_{\Delta waal} > 0.05$). A 35 ± 0.1% survival rate was observed in *Δwaal* group, and a 27 ± 0.3% survival rate was observed in *Δwzy* group, suggesting no immunity developed in either of the O-antigen mutant treatment groups (Figure 4.2)

4.2 The investigation of the virulence of the *ΔymcA* mutant and the vaccine candidate and immunogenicity challenge study

The vast difference in the virulence between the $\Delta waal_{tra}$ or Δwzy_{tra} mutants and $\Delta waal_{Rec}$ or Δwzy_{Rec} prompted us to investigate the molecular difference(s) between the mutations generated in these two groups. It was discovered that when the $\Delta waal_{tra}$ mutant was constructed, a part of the transcription termination site (TTS) of the *ymcA* gene, located downstream of the *waal* gene, was deleted. It is also possible that insertion of the CM^R gene cassette has a polar effect on *ymcA* transcription. This prompted us to create the *△ymcA* mutant to determine if the *ymcA* gene contributes to the virulence of *A. hydrophila* ML09-119. The hypothesis was that by interrupting the *ymcA* gene, the *△ymcA* mutant will be attenuated; therefore, the *△ymcA* mutant was created using the recombineering technique.

To determine the virulence of the *△ymcA* mutant and to evaluate the efficacy of the *△ymcA* mutant as a live vaccine against *A. hydrophila* ML09-119, an *in vivo* channel

catfish challenge study was carried out. The results of the *in vivo* channel catfish IP challenged with ⊿*ymcA* mutant showed that the ⊿*ymcA* mutant was avirulent. The channel catfish in the ⊿*ymcA* treatment group had a 68.1 ± 16.8% survival rate, while a 0 ± 0% survival rate was observed in the positive control treatment group. Note that a 83.3 ± 11.6% survival rate was observed in the negative control treatment group. A significant difference was observed between the ⊿*ymcA* treatment group and the *A. hydrophila* ML09-119 treatment group, $P_{\triangle ymcA} = 0.000186 < 0.05$. This indicates that ⊿*ymcA* is an attenuated mutant of ML09-119 (Figure 4.3).

A sub-challenge of the channel catfish survivors was carried out 21 days post challenge. The ⊿*ymcA* treatment group surviving fish that were challenged with wild type ML09-119 at 1×10^6 CFU/fish showed a 90.5 ± 8.3% survival rate, in contrast to the 0 ± 0% survival rate observed in the naive channel catfish challenged with ML09-119 (Figure 4.4). Significant differences were observed between the ML09-119 group and ⊿*ymcA* mutant group, $P_{\triangle ymcA} = 4.52E^{-05} < 0.05$. This suggested that protective immunity against ML09-119 was developed by exposing catfish to the ⊿*ymcA* mutant.

4.3 The Enzyme-linked Immunosorbent Assay (ELISA)

The Enzyme-linked Immunosorbent Assay (ELISA) was carried out to determine the efficacy of protective immunity induced by the ⊿*ymcA* mutant immunized channel catfish after being i.p injected with 1×10^6 CFU/fish of the mutant. Since LPS contributes significantly to bacterial pathogenesis via multiple mechanisms, and the *ymcA* gene has been reported to be required for the biosynthesis and assembly of the O-antigen (Peleg, *et al.*, 2005), we hypothesized that the *ymcA* gene is a virulence factor in *A. hydrophila*

ML09-119. The deletion of the *ymcA* gene, by removing the O-antigen, might result in an *A. hydrophila* strain that is sensitive to complement, is less invasive, and allows development of antibodies targeting *A. hydrophila* antigens that are present in more typical *A. hydrophila* strains that are opportunistic pathogens in different fish species. All of the replicates of the *ΔymcA* mutant induced a strong antibody reaction to ML09-119 (Figure 4.5). The levels of antibody to ML09-119 were highest in serum from the catfish that were immunized with the *ΔymcA* mutant, indicating strong antibody induction by the *ΔymcA* mutant.

5. Discussion

This study provided valuable insight into role of the O-antigen in the virulence of *A. hydrophila* ML09-119. The *ymcA* gene deletion mutant was observed to be attenuated in its virulence and can provide protection against *A. hydrophila* ML09-119 in an *in vivo* channel catfish challenge study. This mutant may be a promising live vaccine candidate against epidemic *A. hydrophila*.

The recent epidemic outbreak of the MAS caused by highly virulent *A. hydrophila* has drawn a lot of attention since the catfish farming operations in the southeastern United States have not experienced a large-scale outbreak of MAS previously (Hemstreet, 2010). In 2009 and in all subsequent years, catfish farmers in west Alabama have reported severe disease outbreaks which were demonstrated to be caused by a highly virulent strain of *A. hydrophila*, represented by strain ML09-119, in catfish *(I. punctatus)*. From 2009 to the present, Alabama catfish famers lost more than 10 million pounds of catfish that were market-size and estimated to be more than $3 million due to this epidemic strain of *A. hydrophila* (Pridgeon et al., 2011; Liles et al., 2011). The *A.*

hydrophila epidemic strain ML09-119 that has been used in research studies is highly virulent to channel catfish, causing severe mortality within 24 h post exposure at a dose of > 1 x 10^6 CFU by i.p injection. Also, this epidemic *A. hydrophila* strain has expanded its geographic territory and caused frequent outbreaks in the summer months, resulting in millions of pounds of losses in Alabama, Mississippi and Arkansas. (Pridgeon and Klesius, 2011). Due to its highly virulent nature and the resulting huge economic losses, it is essential that the virulent factors expressed by this epidemic *A. hydrophila* be studied and an effective vaccine be developed.

A previous study showed that epidemic strains possess an unique O-antigen cluster compared to reference strains (Hossain et al, 2013). This prompted us to investigate the role of the O-antigen in the virulence of the *A. hydrophila* ML09-119, since the O-antigen is known to contribute significantly in bacterial pathogenesis, such as intestinal colonization (Nevola, Laux et al. 1987; West, Sansonetti et al. 2005), lessening macrophage activation (Lugo, Price et al. 2007), promoting intracellular growth (Nagy, Danino et al. 2006), and serum resistance (DeShazer, Brett et al. 1998).

The $\Delta waal_{tra}$ and Δwzy_{tra} mutants were created using a traditional allelic exchange technique, and the *in vivo* channel catfish challenge study showed that these mutants were attenuated compared to their wild type parent strain ML09-119 strain. However, when we created the precise *waal* and *wzy* gene deletion mutants $\Delta waal_{Rec}$ and Δwzy_{Rec} mutants using a more efficient and accurate recombineering technique, we observed that those mutants were still virulent in channel catfish. It was found that during the construction of the *Δwaal* mutant that a region of the transcription termination site (TTS) between the *waal* gene and the *ymcA* gene was deleted, and the insertion of the gene cassette in the

mutant may have a polar effect on *ymcA* transcription. The *ymcA* gene is reported to be required for the biosynthesis and assembly of the O-antigen (Peleg, et al. 2005). We therefore hypothesized that a *ymcA* mutant would be attenuated in its virulence. The *in vivo* channel catfish challenge study showed that the *ΔymcA* mutant is significantly attenuated in its virulence.

The results of immunogenicity study and the ELISA assay indicates that *ΔymcA* mutant can serve as a promising live vaccine candidate against epidemic *A. hydrophila*. The fact that the ELISA titer of *ΔymcA* mutant is even higher than the positive control is probably due to that the serum for the positive control was collected months ago and stayed in the -20℃ for months, thereby losing some efficacy. We were not able to use fresh ML09-119 serum, since all of the channel catfish in the positive control group were dead and there were no survivors from which to collect blood samples.

Interestingly, there has not been any previous research investigating the contribution of YmcA to the virulence of any bacterial pathogens. There have been studies on the contribution of YmcA in *B. subtilis* on the formation of biofilms in multicellular bacterial assemblages (Branda, et al., 2004; Branda, et al., 2006; Kobayashi,. 2007). However, in these studies the exact function of YmcA is not determined. One study on the human pathogen *Shigella flexneri* mentioned that the *ymcA* gene exists and speculates that it might encode a putative outer membrane lipoprotein that is highly conserved among *Shigella* and *E. coli* (Sun, et al., 2012). Since none of these studies have provided any conclusive evidence for the function of YmcA, this makes the finding in this study is even more valuable, given the evidence that the *ymcA* gene is required for virulence in epidemic *A. hydrophila*.

This study also raised some interesting studies for the future research including:

1. The exact function of YmcA.
2. The complementation of the ⊿*ymcA* mutant and determining if the complemented mutant is restored in its virulence.
3. The delivery route for the live vaccine of the channel catfish against the *A. hydrophila* the epidemic strain.
4. The protective effect of the ⊿*ymcA* mutant against other non-epidemic strains of *A. hydrophila*

Table 4.1. Summary of bacterial strains and plasmids used in this study

Bacterial strains and Plasmid	Relevant features	References
Bacterial strains		
A. hydrophila ML09-119		Hossain et al., 2013
A. hydrophila AL06-06		Hossain et al., 2013
E. coli SM10-λ-pir	*thi-1thr leu tonA lacY supE recA*::RP4-2-TcT::Mu Km^r *λpir*	(Simon, Priefer et al. 1983)
E. coli CC118-λ-pir	*Δ(ara-leu) araD ΔlacX74 galE galKphoA20 thi-1 rpsE rpoB argE(Am) recA1 λpir*	(Herrero, de Lorenzo et al. 1990)
Plasmids		
pDMS197	Suicide vector, *sacB*, Tet^R	(Edwards, Keller et al. 1998)
pDMS197*waaL*		This study
pDMS197*wzy*		This study

Table 4.2. The primers used in this study

Primer ID	Pimer sequence	Primer Application
ymcARecF	5'-T*A*G*A*GATATCAATATTCGTATTGCCAATCTCCTTGCTAATCGAGTACCAGA***GTAGACTTCCGTTGAACT***-3'	Amplifying the cat-cassette for creating the *ΔymcA*
ymcARedR	5'-C*A*A*C*TGCTCGCCCTTTTTGATGAAAAAAGATCGGCTCTATGCAACTTTTGA***GCCTAATGAGTGAGCTAA***-3'	Amplifying the cat-cassette for creating the *ΔymcA*
ymcA_upF	5'-CCGAATGGTAATCCACAGTT-3'	PCR and Sequencing of *ΔymcA* for identification of the mutant
Ymca_dnR	5'-TAGAACAGCTGGTCACGAGA-3'	PCR and Sequencing of *ΔymcA* for identification of the mutant
Cat F	5'-GTAGACTTCCGTTGAACT-3'	Amplifying the *cat* gene
CatR	5'-GCCTAATGAGTGAGCTAA-3'	Amplifying the *cat* gene
Li-upF	5'-ACTTAAGCTCGCCGAACTC	Amplification of upstream sequences of *WaaL* gene
Li-upR	5'-GCTGTCGAGGCCATGTGAGTTCAACGGAAGTCTAC-3'	Amplification of upstream sequences of *WaaL* gene
Li-dnF	5'-	Amplification of downstream

	AAGATCGGCTCTATGCAACTTTAGCTCAC TCATTAGGC-3'	sequences of *waaL* gene
Li-dnR	5'-TGATTATGATGTAATGACTGG	Amplification of downstream sequences of *waaL* gene
Liup-intF	5'-AGAAGCGGTGCTGATAACG	fusing the two *waal* gene arms and the CM^R gene by splicing through overlap extension PCR (SOE)
Lidn-intR	5'-GGCAGTTACCATTCATGAGT	fusing the two *waal* gene arms and the CM^R gene by splicing through overlap extension PCR (SOE)
wzyupF	5'-CCGCGACAACAACTCCTT	Amplification of upstream sequences of *wzy* gene
wzyupR	5'-GCACTTCCTGTATCAAGATTAGTTCAACG GAAGTCTAC-3'	Amplification of upstream sequences of *wzy* gene
wzydnF	5'-CTAGCTGTGGTGCCAGAATATTAGCTCAC TCATTAGGC-3'	Amplification of downstream sequences of *wzy* gene
wzydnR	5'-CATTCAATATAGTGTCTGGA	Amplification of downstream sequences of *wzy* gene
wzyup-inF	5'-GTGACGCCACCGATGATA	fusing the two *wzy* gene arms and the CM^R gene by splicing through overlap extension PCR (SOE)

wzydn-inR	5'-CTGATGTTATTATTGACCAAG	fusing the two *wzy* gene arms and the CM^R gene by splicing through overlap extension PCR (SOE)

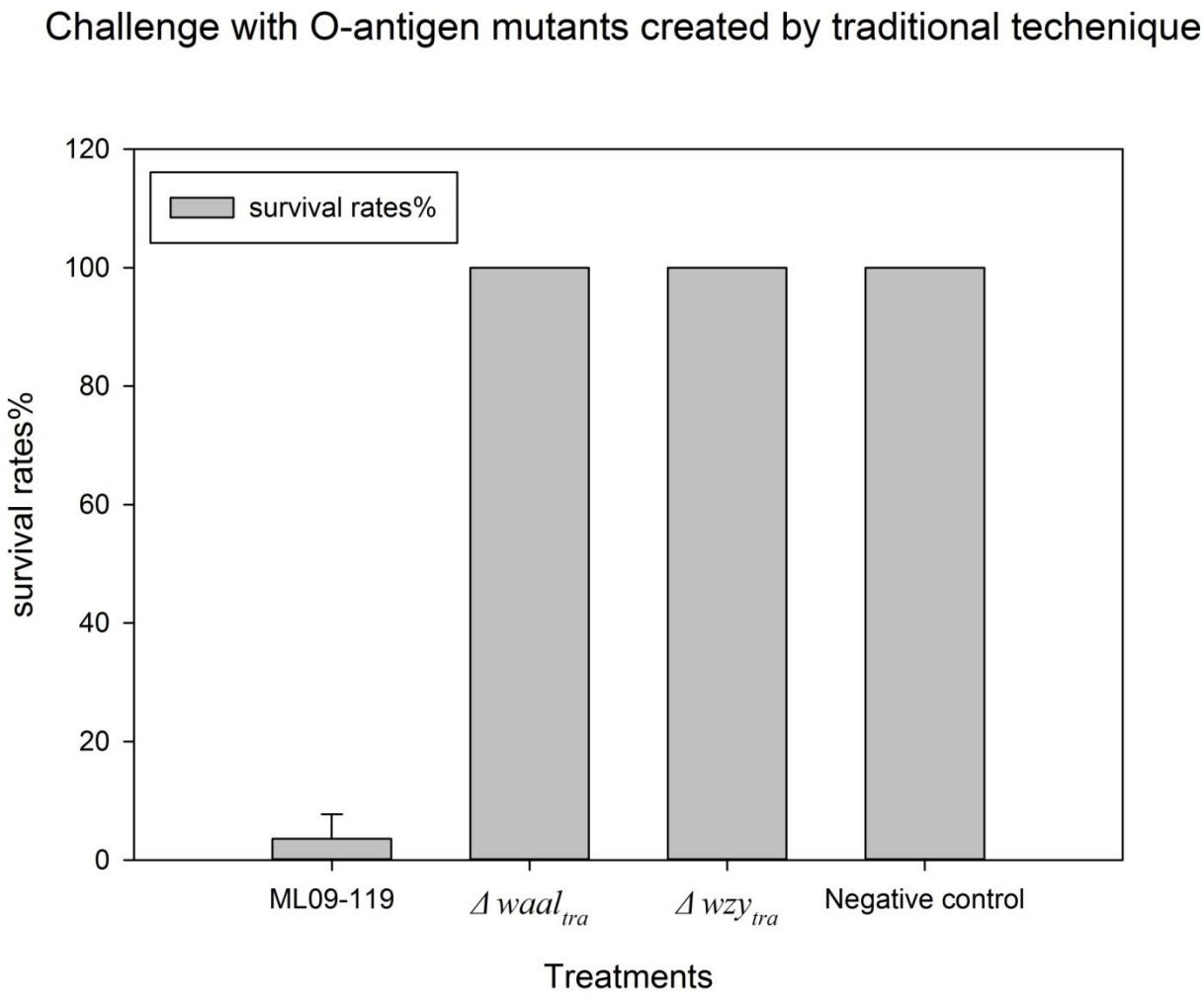

Figure 4.1: Challenge with O-antigen mutants created by traditional Allelic exchange technique. The channel catfish in the $\Delta waal_{tra}$ and Δwzy_{tra} treatment groups had 100 ± 0% survival rate, while a 5 ± 0.08% survival rate was observed in the positive control treatment ML09-119 group. A significant difference was observed between $\Delta waal_{tra}$ or Δwzy_{tra} and ML09-119 treatment groups, $P < 0.0001$.

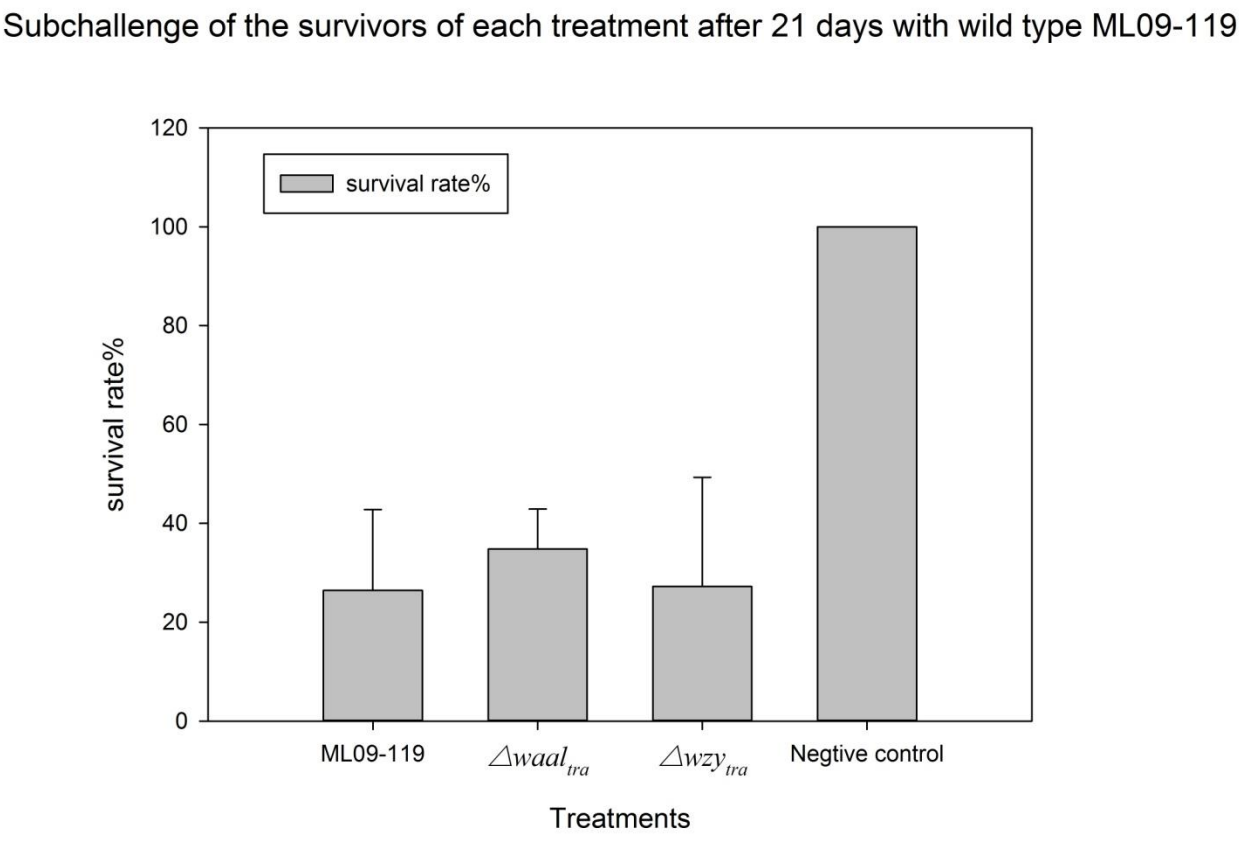

Figure 4.2: Sub-challenge of the survivors of each treatment after 21 days with wild type ML09-119. The exact concentration of ML09-119 used in this experiment was not determined. However, significant differences were observed between the sham negative control group and the treatment groups (P = 0.0044 < 0.05). No significant differences were observed between the treatment groups and the positive control group ($P_{\Delta waaltra}$ > 0.05 and $P_{\Delta waaltra}$ =0.97). A 35 ± 0.18% survival rate was observed in the $\Delta waal_{tra}$ group, and a 27 ± 0.3% survival rate was observed in the Δwzy_{tra} group.

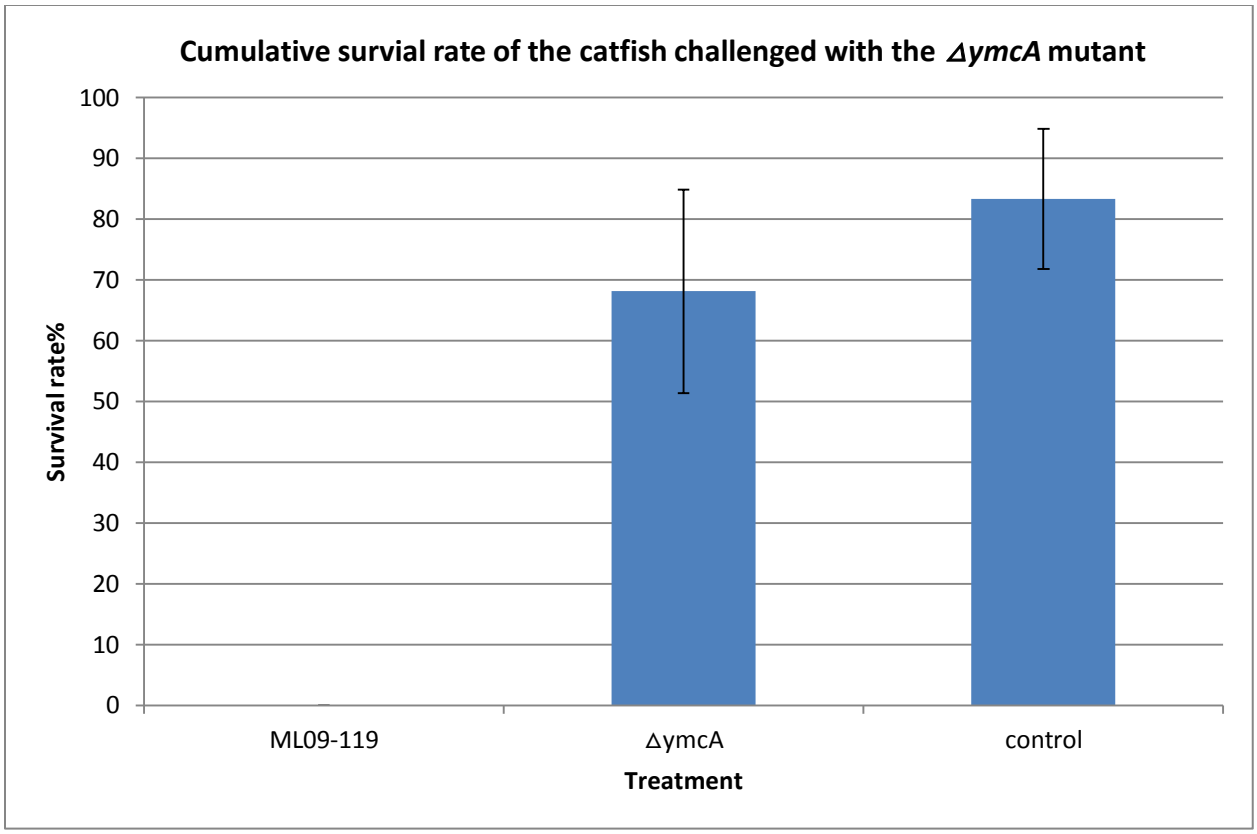

Figure 4.3: Cumulative survial rate of the catfish challenged with the ⊿*ymcA* mutant. The channel catfish in the ⊿*ymcA* treatment group had a 68.1 ± 16.8% survival rate, while a 0 ± 0% survival rate was observed for wild-type ML09-119. Note that a 83.3 ± 11.6% survival rate was observed in the negative control treatment group, with some deaths attributable to non-bacterial causes. A significant difference was observed between the ⊿*ymcA* and ML09-119 treatment groups, $P = 0.000186 < 0.05$.

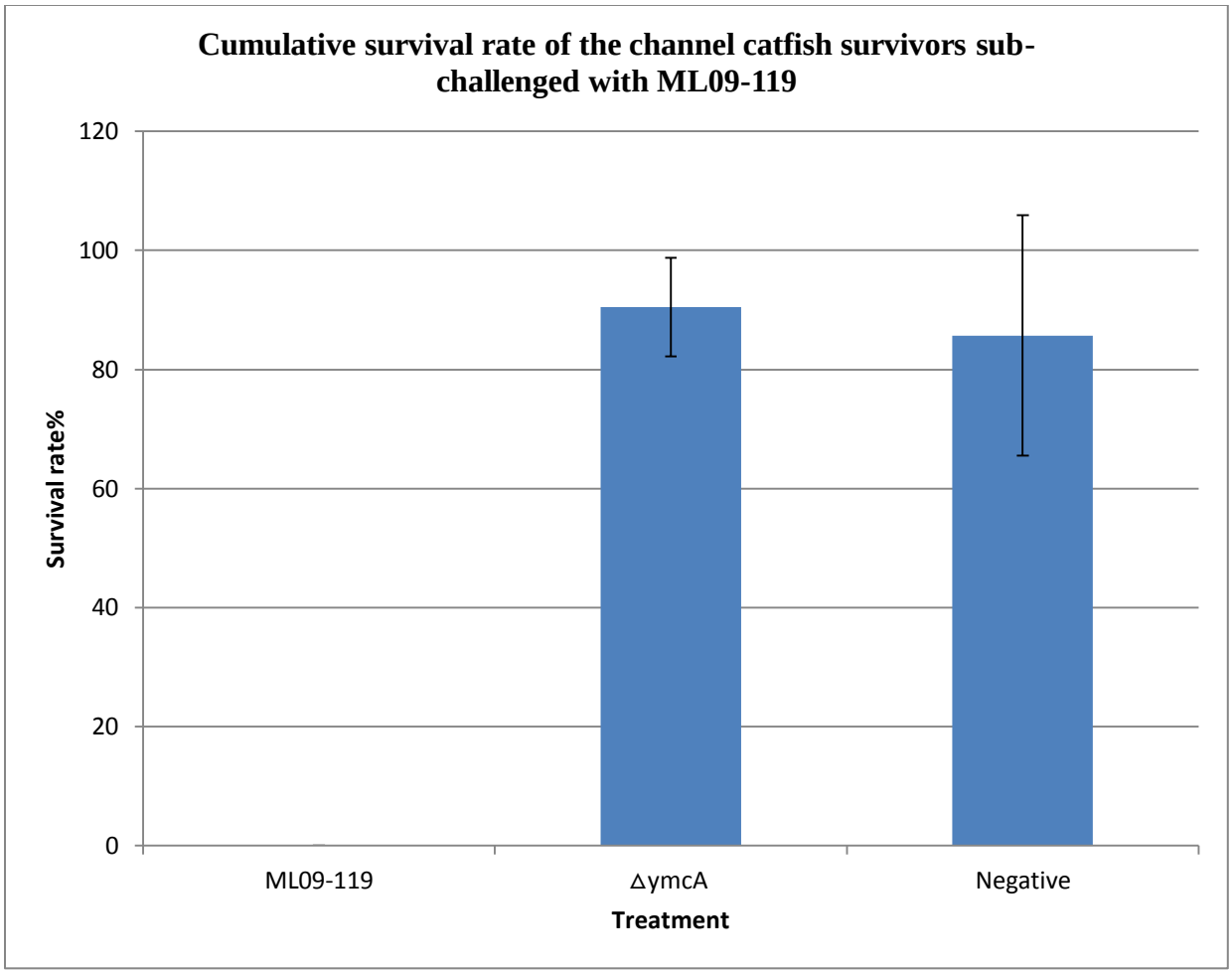

Figure 4.4: Cumulative survival rate of the channel catfish survivors sub-challenged with ML09-119. The ⊿*ymcA* treatment group surviving fish that were challenged with the wild type ML09-119 showed a 90.5 ± 8.3% survival rate, in contrast to the 0 ± 0% survival rate observed in the naive channel catfish challenged with ML09-119. Significant differences were observed between the ML09-119 group and the ⊿*ymcA* mutant group, $P = 4.52E^{-05} < 0.05$.

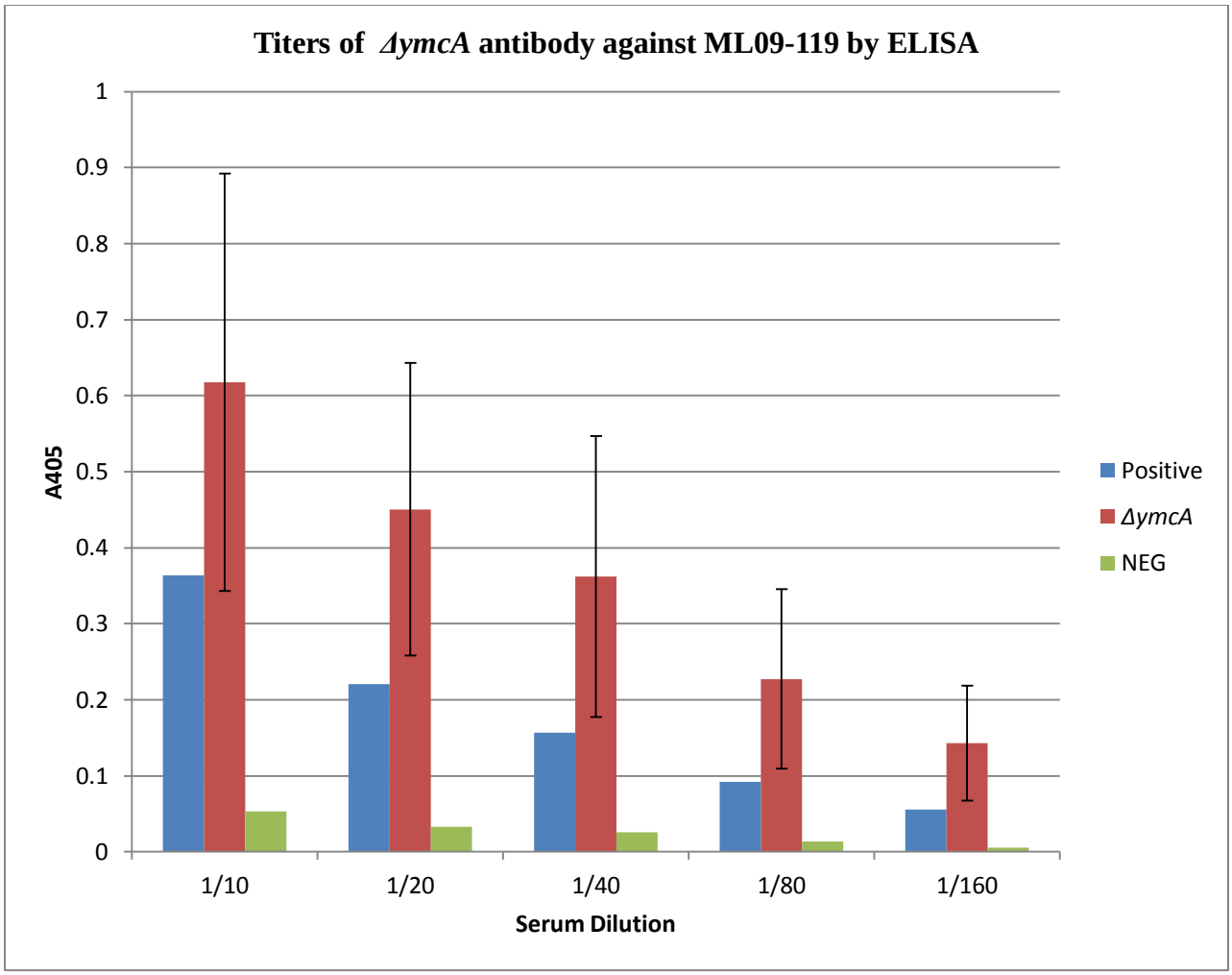

Figure 4.5: Antibody titers offish exposed to *ΔymcA* against ML09-119 by ELISA. All of the replicates of the *ΔymcA* mutant-challenged fish induced a strong antibody reaction to ML09-119. The levels of antibody to ML09-119 were highest in the serum from *ΔymcA* mutant immunized channel catfish.

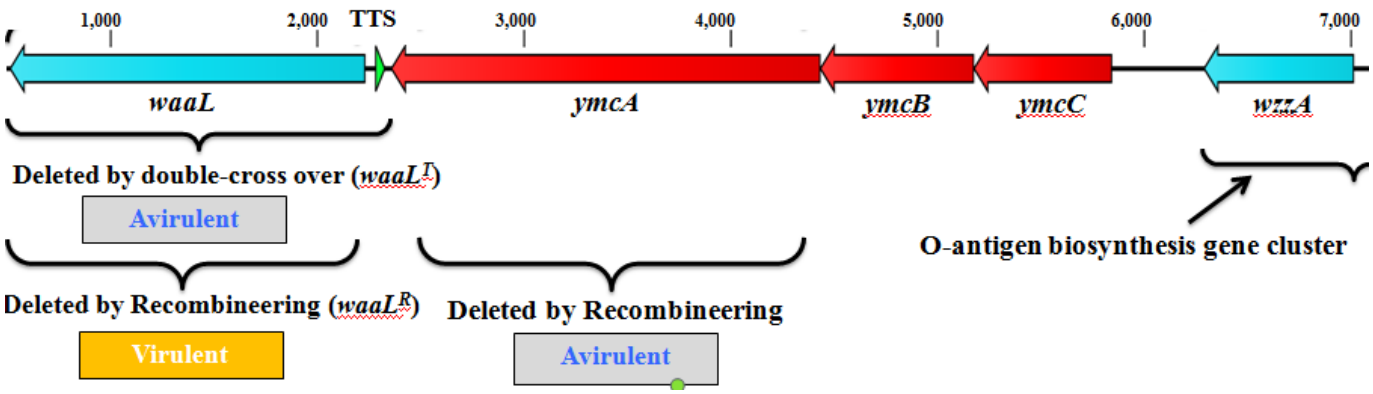

Figure 4.6: Organization of O-antigen Biosynthesis Gene Cluster

Chapter V Conclusions

A. hydrophila ML09-119 has been reported to cause severe mortality in commercial catfish farms. It is important to study the virulence as well as the molecular basis of this epidemic strain to improve the understanding of the genes that are responsible for the virulence of *A. hydrophila* ML09-119. The huge economic damage it cost and its epidemicity make it urgent to create an efficient and safe vaccine to protect the fish farm from this devastating disease.

In this study, IP challenge method combining cohabitation culture was used to detect the susceptibility of channel, blue and hybrid catfish to *A. hydrophila* ML09-119. Our results showed that the virulence of ML09-119 to channel catfish is significantly higher than it is to blue and hybrid catfish. No significant differences were observed between blue and hybrid catfish.

In this study, the gene *iolA* coding for the enzyme aldehyde dehydrogenase for *myo*-inositol catabolism was inactivated by traditional allelic exchange to generate the *A. hydrophila* $\Delta iolA_{tra}$ mutant. An *in vivo* challenge in channel catfish showed that there was no mortality in the channel catfish that were challenged with $\Delta iolA_{tra}$ mutant, but there was mortality in the channel catfish challenged with $\Delta iolA_{rec}$ mutants similar to wild type ML09-119. Results of the *in vivo* challenge in channel catfish showed that $\Delta iolA_{rec3}$, $\Delta iolA_{rec4}$ exhibited some decrease in mortality, but there were no significant difference in

the mortality between the channel catfish challenged with $\Delta iolA_{rec3}$, $\Delta iolA_{rec4}$ and the channel catfish challenged with the wild type ML09-119. ELISA titer of the survivors of the $\Delta iolA_{tra}$ after 21 days showed that $\Delta iolA_{tra}$ can induce strong antibody response against the wild type *A. hydrophila* ML09-119, indicating that this mutant can serve as a promising vaccine candidate against the epidemic *A. hydrophila.*

In this study, Lipid A-Core ligase (*waaL*) and O-antigen polymerase (*wzy*) knockout mutants, ***ΔwaaL, Δwzy*** were created by both traditional splicing PCR and conjugation technique and recombineering technique respectively, $\Delta waaL_{tra}$ *or* $\Delta waaL_{Rec}$, $\boldsymbol{\Delta} wzy_{tra}$ *or* $\boldsymbol{\Delta} wzy_{Rec}$. An *in vivo* channel catfish challenge study was committed on channel catfish to study the role of O-antigen in the virulence of the epidemic strain of *A. hydrophila.* The results show that the channel catfish that were challenged with $\boldsymbol{\Delta} waaL_{tra}$, $\boldsymbol{\Delta} wzy_{tra}$ were avirulent, but $\boldsymbol{\Delta} waaL_{Rec}$, $\boldsymbol{\Delta} wzy_{Rec}$ were virulent.

In this study, a *ΔymcA* mutant was created by knocking out the *ymcA* gene by recombineering technique. The results showed that *ΔymcA* mutant was attenuated. Sub-challenge of the survivors of *ΔymcA* treatment group 21 days post first challenge and ELISA titer of the survivors of the *ΔymcA* treatment showed that this mutant can provide 90.5 ± 8.3% protection against wild type *A. hydrophila* ML09-119 indicating that the *ΔymcA* mutant can serve as a promising vaccine candidate.

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