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Development of Safe and Efficacious Live Attenuated Edwardsiella Ictaluri Vaccines against Enteric Septicemia of Catfish

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Development of safe and efficacious live attenuated *Edwardsiella ictaluri* vaccines against enteric septicemia of catfish

By

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Development of safe and efficacious live attenuated *Edwardsiella ictaluri* vaccines against enteric septicemia of catfish

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Edwardsiella ictaluri is the causative agent of enteric septicemia of catfish (ESC), which is the most economically important disease of farm-raised channel catfish. *E. ictaluri* is considered a facultative intracellular pathogen like other well-known species in the *Enterobacteriaceae*, and it is capable of surviving inside channel catfish neutrophils and macrophages. Its ability to survive inside neutrophils and macrophages has made the development of an effective vaccine against ESC particularly challenging. The goal is to develop a safe, efficacious live attenuated ESC vaccine that is practical and economically beneficial to catfish producers.

In this study, single and combination of mutations in genes encoding TCA cycle enzyme and C-1 metabolism proteins were constructed using in-frame mutagenesis. The virulence, vaccine efficacy, and tissue persistence of the constructed single and combination mutants were determined in channel catfish. The constructed mutants *Ei*delta*sdhC*, *Ei*delta*mdh, Ei*delta*frdA, Ei*delta*glyA, Ei*delta*sdhC*delta*mdh, Ei*delta*sdhC*delta*frdA,* and *Ei*delta*sdhC*delta*gcvP* were significantly attenuated and showed 100% protection against *E. ictaluri* 93-146 infection in juvenile channel catfish.

However, when tested in 15-d old catfish fry, mutant *Ei*delta*sdhC*delta*gcvP* and *Ei*delta*frdA* were found to provide good protection (99% and 60%, respectively) against *E. ictaluri* 93-146 infection. The tissue persistence study indicated higher tissue concentration in mutants *Ei*delta*sdhCdeltagcvP* and *Ei*delta*frdA* relative to the tissue concentration in *Ei*delta*sdhC* and *Ei*delta*sdhC*delta*frdA* mutants.

DEDICATION

To my parents Nirmal Prasad and Nisha Dahal, my parents-in-law, Hom Nath and Kamala Nepal, my husband, Prakash, and my daughter, Himangi for their support and love.

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CHAPTER I

INTRODUCTION

Aquaculture industry in the United States

With over a 1.4 billion dollars of sale values (Table 1.1), the aquaculture industry is an important component of the United States (U.S.) economy (USDA, 2007). U.S. aquaculture production comprises the production of food fish, baitfish, crustaceans, mollusks, ornamental fish, sports/game fish, and other aquaculture products such as reptiles and turtles. The major food fish species grown in the U.S. are catfish, trout, salmon, tilapia, hybrid striped bass, sturgeon, walleye, and yellow perch. Among these food fish, the channel catfish (*Ictalurus punctatus*) industry is the largest, accounting for over 50% ($455 million) of all food fish sales ($854 million) (USDA, 2007). Mississippi, Alabama, Arkansas, and Louisiana are the main catfish producing states with over 286, 132, 95, and 14 million pounds of catfish sales per year (USDA, 2007).

Table 1.1 Total sale value of aquaculture industry in the US, 2007 (USDA, 2007).

Aquaculture type	Sale value (million dollars)
Catfish	455.4
Trout	210.6
Other food fish	187.7
Baitfish	40.3
Crustacean	50.9
Mollusks	243.0
Ornamental fish	61.0
Sports/game fish	80.6
Other aquaculture products	85.8
Total	1415.3

Channel catfish commercial production process

Channel catfish spawn during the late spring when water temperatures reach approximately 24°C. During spawning season, eggs are collected from nesting containers in broodstock ponds and transferred to the hatchery, where they hatch in 5-8 days, depending on water temperature. Fry at this stage are called sac-fry, and they feed on yolk for the first 3-5 days. After that time, they swim to the surface seeking food and are called swim-up fry. After feeding them for 2-7 days, fry are then stocked into nursery ponds. In nursery ponds, fry are grown to fingerling stage (3-8 inches) for 5-10 months, and then they are harvested and stocked into grow-out ponds and grown until they weigh 1.2 to 2.5 pounds (MSU Extension Service, 2010).

Disease affecting channel catfish industry

Channel catfish are susceptible to a wide variety of diseases including viruses, bacteria, fungi, helminths, and parasitic copepods. Some of the most important disease organisms prevalent in catfish are discussed below.

Catfish virus disease (CCVD)

Channel Catfish Virus Disease (CCVD) is caused by *Ictalurid herpesvirus* (Camus, 2004). Clinical signs of CCVD include erratic swimming, exopthalmia, distended abdomen, and haemorrhage at the fin bases (Camus, 2004). Histopathological examination reveals necrosis and extensive haemorrhage (Fig. 1.1) in vital organs, especially in the liver, kidney, and gastrointestinal tract (Camus, 2004). Epizootics usually involve high mortality and occur sporadically on commercial fish farms in the southern U.S. during the summer months (Gray et al., 1999). There is no treatment available for CCVD or other viral infections in catfish. Management practice is the best way of limiting the frequency and severity of CCVD outbreaks in cultured catfish (Camus, 2004).

Figure 1.1 Clinical signs associated with channel catfish virus (*Ictalurid herpesvirus*) disease.

Source: http://ag.ansc.purdue.edu/courses/aq448/images/ccvd.jpg.

Columnaris disease

Columnaris is one of the oldest known diseases of warm water fish, first described in 1922 by Herbert Spencer Davis (Durborow et al. 1998). Historically, the causative bacterium of this disease has been named *Bacillus columnaris, Flexibacter columnaris,*

Cytophaga columnaris, and most recently *Flavobacterium columnare* (Durborow et al., 1998). Fish with columnaris desease usually have brown to yellowish-brown lesions on their gills, skin, and/or fins (Fig. 1.2). At first, bacteria attach to the gill surface, grow in spreading patches, and eventually cover individual gill filaments, which results in cell death (Durborow et al., 1998). Other syndromes associated with columnaris include white spots on mouth or edges of scales and fins, cottony growth around mouth, disintegrated fin edges, and saddleback lesion near the dorsal fin (Durborow et al., 1998). Treatment for external columnaris infection includes treating the culture water with therapeutic chemicals accepted for use on food fish. Potassium permanganate (KMnO4) is a commonly used therapy. Antibiotic treatment for external and internal columnaris infection includes Terramycin® (oxytetracyline HCl) medicated feed (Durborow et al., 1998).

Figure 1.2 Characteristic lessions caused by Flavobacterium columnare.Source: http://microgen.ouhsc.edu/f_columnare/f_columnare_home.htm.

Proliferative gill disease (Hamburger gill disease)

Proliferative gill disease (PGD), also called hamburger gill disease, is one of the common diseases in farm-raised channel catfish. It is capable of killing a few dozen fish

over several days, or up to 100 percent of the fish in less than three days (Mitchelle et al., 1998). However, the disease rarely reoccurs in the same pond (Mitchelle et al., 1998). The causative agent of proliferative gill disease is the myxosporean myxozoan parasite *Henneguya ictaluri* (Pote et al., 2003). Early clinical signs of this infection include anorexia and listlessness; infected fish often try to compensate for damage sustained to their respiratory apparatus by moving toward regions where dissolved oxygen concentration is higher (aeration devices or in shallow areas of the pond) (Pote et al. 2003). Gross lesions are characterized by swelling and fragility of the gills as well as by a mottled red and white appearance (Fig. 1.3), which led to the condition's nickname "hamburger gill disease" (Whitaker et al., 2001). There are no scientifically validated treatments or preventive methods for proliferative gill disease. However, increasing aeration to increase dissolved oxygen concentrations can reduce mortalities during a disease outbreak (Mitchelle et al., 1998).

Figure 1.3 Gill lesions caused by proliferative gill disease (PGD).

Photo courtesy of Dr. Bob Durborow. Source: **https://srac.tamu.edu/index.cfm/event/getFactSheet/whichfactsheet/122/**.

Enteric septicemia of catfish (ESC)

The causative agent of enteric septicemia of catfish (ESC) is the gram negative bacterium *Edwardsiella ictaluri*, which is one of the most important diseases of farm-raised channel catfish (Hawke et al., 1979). Catfish affected with ESC often are seen swimming in tight circles or chasing their tails. This head-chasing- tail, whirling behavior is due to the presence of the *Edwardsiella ictaluri* in the brain. ESC is characterized by external haemorrhages (Fig. 1.4) on the ventral surface and around the mouth, white focal lesions on dorsal surface and sides, and occasionally a grey lesion on top of the head that can erode to an open lesion (Hawke et al. 1998; Plumb 1998). Oxytetracycline, sulfadimethoxine/ormetoprim, and florfenicol are the only treatments currently approved in the U.S. for treatment of ESC (McGinnis et al., 2003), and are delivered orally as medicated feed.

Figure 1.4 Characteristic lesions of ESC caused by *Edwardsiella ictaluri*. Photo courtesy of Drs. A. Karsi, M. Lawrence and L.A. Hanson.

This study focuses on developing live attenuated vaccine against ESC. Further details on the causative agent of ESC, *Edwardsiella ictaluri*, including clinical signs, treatment, pathogenesis, and vaccination, are presented in the following section.

Edwardsiella ictaluri

The causative agent of ESC, *Edwardsiella ictaluri*, belongs to the *Enterobacteriaceae* family and is in the same genus as *Edwardsiella tarda*, a pathogen of humans and fish, and *Edwardsiella hoshinae*, an inhabitant of birds and lizards (Hawke, 1979). *E. ictaluri* is a short, pleomorphic rod, measuring 0.75 × 1.5-2.5 µm (Fig. 1.5); it is weakly motile at 25-30°C, but not at higher temperatures (Hawke et al., 1998). Optimal growth temperature is 25°C (range: 15-35°C), with absence of growth at 37°C (Hawke et al., 1998). Even at optimal temperature, *E. ictaluri* is a slow-growing bacterium, requiring 48 hours for typical growth to occur. Biochemically *E. ictaluri* is lactose negative, catalase-positive, cytochrome oxidase-negative, glucose fermentative, and reduces nitrate to nitrite (Hawke, 1979; Shotts et al., 1989).

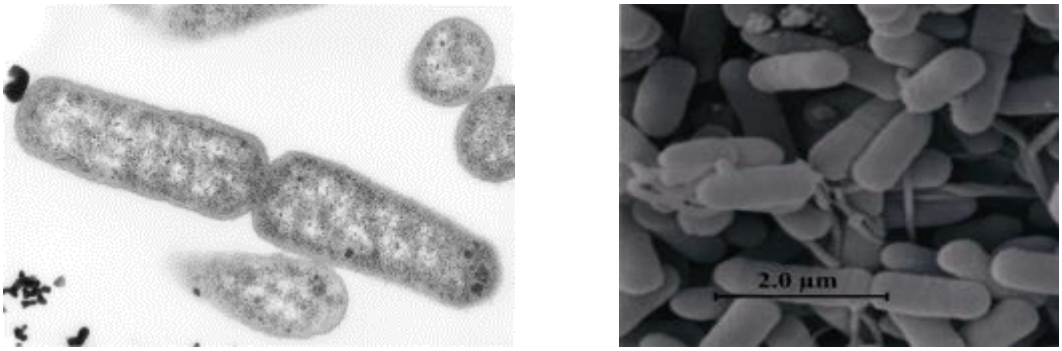

Figure 1.5 Transmission electron micrograph (left) and scanning electron micrograph (right) of *Edwardsiella ictaluri* strain 93-146.

Source: http://microgen.ouhsc.edu/e_ictal/e_ictal_home.htm.

Clinical signs and treatment

ESC affects all sizes and ages of catfish during the late spring and the fall when water temperature is between 22-28°C. There are two forms of ESC: an acute form and a

chronic form. The acute form is characterized by enteritis and septicemia of channel catfish, whereas the chronic form is characterized by meningoencephalitis (MacMillan, 1985; Newton et al., 1989; Shotts et al., 1986). In the acute form, farmers experience economic losses due to rapid mortalities (Shotts et al., 1986). In the chronic form, farmers experience economic losses due to decreased production, with fish manifesting signs three to four weeks after an acute outbreak (Newton et al., 1989).

Sulfadimethoxine/ormetoprim and florfenicol are the only treatments currently approved in the U.S. for the treatment of ESC (McGinnis et al., 2003), and both are delivered orally as medicated feed. However, one of the earliest clinical signs associated with ESC is anorexia; therefore, these antibiotics are only effective in limiting the spread of an outbreak and not in treating the disease. The first "ESC window" is when pond temperatures drop into the 22-28°C and fry are stocked into nursery ponds in the fall. Producers experience greatest losses due to ESC during this period. Therefore, vaccination strategies for ESC are usually aimed at protecting catfish fingerlings during the fall.

Vaccination against ESC

The three routes of vaccine delivery available to catfish producers are injection, bath immersion, and oral (in feed). Injection is not feasible because the individual fish value is not high enough to justify labor costs. Bath immersion is economically feasible, but because of current production practices, it can only be done with fry prior to stocking into nursery ponds. Thus, there is only a maximum of 2 weeks post-hatch available for vaccinating fry by immersion. Once fish are stocked into nursery and grow-out ponds, oral delivery is the only route available.

Live attenuated vaccines can provide effective protection against certain diseases because they can express protective antigens without causing infection in the host (Lan et al., 2007). Identification of genes that are essential for bacterial virulence can provide new gene targets for attenuation by introducing defined non-reverting mutations into chosen target genes. A chondroitinase mutant (Cooper et al., 1996) and auxotrophic mutants (*aroA* and *purA*) (Lawrence, 1997; Thune et al., 1999) are live attenuated vaccine candidates that have been developed for *E. ictaluri.* Although the *aro* mutant is attenuated and highly immunogenic, it is not in commercial production because studies have indicated that they give rise to silent bacteraemia in catfish (Lan et al., 2007). The currently available commercial live attenuated vaccine (RE-33, marketed as Aquavac-ESC by Schering-Plough) was developed by selecting for rifampin resistance. However, the genetic basis for attenuation is undefined (Klesius & Shoemaker, 1997), although it is known that RE-33 expresses shortened LPS O side chains (Arias et al., 2003). Despite availability of the commercial vaccine RE-33, there has been no reduction in the prevalence of ESC in the industry (USDA, 2003; Anonymous 2006). Thus, there is need for new attenuating mutations that will yield a vaccine that retains immunogenicity and results in a safe, efficacious vaccine for ESC.

Pathogenesis of ESC

Potential portals of entry for *E. ictaluri* in catfish include the olfactory sac (Miyazaki & Plumb, 1985; Morrison & Plumb, 1994; Shotts et al., 1986), intestine (Baldwin & Newton, 1993; Newton et al., 1989; Shotts et al., 1986), and gills (Lawrence, 1997; Nusbaum & Morrison, 1996). The mechanism of adherence is yet to be worked out, although genes encoding fimbriae and a type III secretion system have been

identified (Thune et al., 2007). It is known that *E. ictaluri* is capable of rapid penetration of host mucosal membranes; following infection by gastric incubation, it was detected in posterior kidneys 15 minutes post-infection (Baldwin & Newton, 1993). Following immersion exposure, we have found that the organism can be detected 30 minutes post-infection (Lawrence & Banes, 2005). *E. ictaluri* is resistant to normal catfish serum (Karsi et al., 2006; Ourth and Bachinski, 1987), which is at least partially mediated by the O polysaccharide portion of LPS (Lawrence et al., 2003).

E. ictaluri is a facultative intracellular pathogen with the ability to survive inside professional phagocytic cells. Phagocytosed *E. ictaluri* have been observed in several pathology studies (Baldwin & Newton, 1993; Miyazaki & Plumb, 1985; Morrison & Plumb, 1994; Shotts et al., 1986). In vitro studies have shown that *E. ictaluri* is capable not only of surviving in catfish macrophages but also multiplying within spacious vacuoles (Booth et al., 2006). Although *E. ictaluri* is effectively phagocytosed by catfish neutrophils, it is only killed by neutrophils to a limited extent (Ainsworth & Dexiang, 1990; Waterstrat et al., 1991). Previous studies conducted at our lab identified several genes necessary for survival in neutrophils (Karsi et al. 2009), including genes encoding TCA cycle enzymes, glycine cleavage system, a sigmaE regulator, the SoxS oxidative response system, and a plasmid-encoded type III secretion system (TTSS) effector. A TTSS mutant was found to retain its ability to invade catfish cell lines and macrophages, but it was defective in intracellular replication (Thune et al., 2007). It has been suggested that *E. ictaluri*'s ability to survive in phagocytes may be responsible for development of a carrier state for ESC (Klesius, 1992) and that it could be a mechanism for dissemination (Miyazaki & Plumb, 1985; Shotts et al., 1986).

Description of TCA cycle and C-1 metabolism pathway enzymes selected for mutagenesis in *E. ictaluri*

Earlier studies conducted by Karsi et al. (2006) indicated that mutant EiAKMut5, which has an insertion in *sdhC*, and mutants EiAKMut2 and EioAKMut8, both of which have mutations in *gcvP*, were found to be completely avirulent in juvenile catfish by immersion exposure and were very effective in generating protective immunity. The same study also showed good vaccine potential of EiAKMut12, which has an insertion in *mdh*; it caused a low level of mortalities (4.45%) in juvenile catfish by immersion, but it generated 98.96%protection.

Thus, based on earlier findings, five genes (*sdhC, glyA, gcvP, frdA,* and *mdh*) were selected for mutagenesis. Three of these genes encode TCA cycle enzymes (*sdhC, frdA*, and *mdh*) and two encode single carbon metabolism proteins (*glyA* and *gcvP*). Gene *frdA* was chosen for mutation because it encodes fumarate reductase, which catalyzes the same reaction as succinate dehydrogenase but in the opposite direction. Thus, the two proteins can functionally replace each other. The following section describes the roles of these selected genes in virulence.

The sdhC gene encodes one of four subunits of the succinate dehydrogenase complex. It is one of the two subunits that anchors the complex in the cytoplasmic membrane (Nakamura et al., 1996). Succinate dehydrogenase is part of the aerobic respiratory chain and the TCA cycle, oxidizing succinate to fumarate while reducing ubiquinone to ubiquinol. It is closely related to fumarate reductase, which catalyzes the reverse reaction. Succinate dehydrogenase and fumarate reductase can replace each other (Guest, 1981; Maklashina et al., 1998). In *E. coli sdhC* mutants, succinate dehydrogenase activity is located in the cytoplasm, and it utilizes artificial electron

acceptors; in contrast, wild-type *E. coli* has membrane-associated succinate dehydrogenase activity with ubiquinone as the electron acceptor (Nakamura et al., 1996). In *E. ictaluri*, *sdhC* is the first gene in a polycistronic operon that encodes the four components of succinate dehydrogenase; therefore, it is possible that the mutation in *sdhC* has a polar effect on expression of downstream genes. *E. ictaluri* also has genes encoding the fumarate reductase complex in its genome. In *E. coli*, fumarate reductase is expressed under anaerobic conditions with glucose as a carbon source. Although SdhC has similar function, hydrophobicity, and protein size compared to the membrane-binding subunit from fumarate reductase (FrdC), SdhC and FrdC do not share significant sequence identity (Wood et al., 1984).

In *E. coli* and *Salmonella*, succinate dehydrogenase is known to contribute to pathogenicity. The organic acids formate and succinate have a protective effect in stationary phase cells against killing effects of antimicrobial peptide (bactericidal/permeability increasing protein), which appears to disrupt the bacterial respiratory chain (Barker et al., 2000). The maintenance of protective levels of formate and succinate requires the activity of formate dehydrogenase and succinate dehydrogenase, respectively. In *Helicobacter pylori*, fumarate reductase was found to be essential for colonization of mouse gastric mucosa (Ge et al., 2000a).

Recently, it was shown that a full TCA cycle is required for *Salmonella enterica* virulence, and a *sdhDCA* mutant is attenuated in an oral mouse infection model (Tchawa Yimga et al., 2006). The *sdhCDA Salmonella* mutant is not fully avirulent, but when it is combined with a fumarate reductase (*frdABCD*) mutation, the resulting double mutant is fully avirulent and effective as a live attenuated vaccine (Mercado-Lubo et al., 2008).

This study concluded that fumarate reductase was able to partially restore succinate dehydrogenase function in the *sdhCDA* mutant, but when both *sdhCDA* and *frdABCD* were mutated, succinate dehydrogenase function was completely eliminated, resulting in full avirulence. Interestingly, the *Salmonella sdhCDA-frdABCD* mutant replicated normally in Peyer's patches, but it was defective in growth in spleen and liver (Mercado-Lubo et al., 2008). This defect in systemic infection was presumably due to inhibition of ability to replicate in phagocytes, which corroborates findings of Karsi et al. (2006) with the *E. ictaluri sdhC* mutant.

Malate dehydrogenase (MDH) catalyzes the conversion of oxaloacetate and malate utilizing the NAD/NADH coenzyme system. The active site of malate dehydrogenase is a hydrophobic cavity within the protein complex that has specific binding sites for the substrate and its coenzyme, NAD^+. In its active state, MDH undergoes a conformational change that encloses the substrate to minimize solvent exposure and to position key residues in closer proximity to the substrate (Goward and Nicholls, 1994). In *Salmonella*, a *mdh* mutant was avirulent in the mouse oral challenge model (Tchawa Yimga et al., 2006).

The *gcvP* gene encodes a protein that is part of the glycine cleavage system. The glycine cleavage system is a loosely associated four subunit enzyme complex that catalyzes the reversible oxidation of glycine to form 5, 10-methylenetetrahydrofolate, which serves as a one carbon donor. It is one of two sources of 1C units; serine hydroxymethyltransferase is the other (and is considered the more important source). Expression of the glycine cleavage enzyme system is induced by glycine (Meedel and Pizer, 1974; Stauffer et al., 1994, and *gcv* mutants are unable to use glycine as a 1C

source and excrete glycine (Plamann et al., 1983). The glycine cleavage system is also part of the formyltetrahydrofolate biosynthesis system. GcvP is a 104-kDa protein that catalyzes the decarboxylation of glycine. In *E. ictaluri*, *gcvP* is the third gene in a three gene operon; it is located downstream of *gcvH* and *gcvT*, which encode subunits of the glycine cleavage system. *E. ictaluri* also has a gene that encodes serine hydroxymethyltransferase, which plays an important role in cellular one-carbon pathways by catalyzing the reversible, simultaneous conversions of L-serine to glycine (retro-aldol cleavage) and tetrahydrofolate to 5, 10-methylenetetrahydrofolate (hydrolysis) (Appaji et al, 2003).

Objectives

The overall goal of this study is to develop a safe, efficacious live attenuated ESC vaccine that is practical and economically beneficial to catfish producers. The hypothesis is that combinations of mutations in genes encoding TCA cycle enzymes and glycine cleavage system proteins will result in a safe, efficacious vaccine for ESC. The specific objectives of the study include:

1. Constructing new single gene deletion mutations and determining virulence in channel catfish,
2. Constructing combinations of gene deletions and determining virulence in channel catfish,
3. Conducting vaccine safety and efficacy testing, and determining tissue distribution, and
4. Understanding the mechanism of attenuation in a *sdhC* deletion mutant.

To achieve the first and second objective, single and double in-frame deletion mutations were constructed in genes encoding TCA cycle enzymes and C-1 metabolism proteins. The degree of attenuation of mutants was then determined in juvenile catfish. Bioluminescence imaging was utilized to quantify degree of attenuation.

To achieve the third objective, vaccine efficacy was tested under controlled laboratory conditions using juvenile catfish. Efficacy testing was then conducted in 10-14 day old catfish fry. Vaccine efficacy was determined using the standard comparison of percent survival of vaccinated vs. unvaccinated fish. Tissue distribution of selected mutants (based upon from vaccine efficacy results) was determined in juvenile catfish.

To achieve the fourth objective, the effect of *sdhC* deletion and other TCA cycle enzyme deletions on endogenous superoxide production was determined. Enzyme activity was confirmed. The anaerobic and aerobic growth curves for the mutants were compared to wild-type *E. ictaluri*. A viability assay was conducted to determine whether superoxide production would damage the *sdhC* mutant to cause attenuation.

Significance of the study

The economic impact of ESC on the commercial channel catfish industry is substantial. According to USDA Census of Agriculture, 2007, the catfish industry accounted for almost 50 percent of the total estimated value of the aquaculture industry during 2002-2007. However, the channel catfish industry is currently facing severe economic stress from increased feed prices and pressure from foreign competition. *E. ictaluri* is a primary pathogen that affects all sizes classes of fish, and it often predisposes secondary infections with other pathogens such as *Flavobacterium columnare*. The resulting morbidities and mortalities along with decreased production lead to

considerable economic losses to channel catfish producers. By developing an effective vaccine against ESC and consequently reducing catfish losses resulting from ESC, this study aims to improve the long-range sustainability and competitiveness of the U.S. channel catfish aquaculture industry, allowing for increased profitability and competitive edge for catfish farmers in the United States. In addition, studying the effects of deletion mutations in *E. ictaluri* TCA cycle enzymes on superoxide production would provide important information for understanding the relation between superoxide production and pathogenesis at the molecular level. This study will delineate the mechanism of attenuation in *sdhC* mutant.

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CHAPTER II

TRICARBOXYLIC ACID CYCLE AND ONE-CARBON METABOLISM PATHWAYS ARE IMPORTANT IN *EDWARDSIELLA ICTALURI* VIRULENCE

Abstract

Edwardsialla ictaluri is a Gram-negative facultative intracellular pathogen causing enteric septicemia in channel catfish. The disease causes considerable economic losses in the commercial catfish industry in the United States, and an efficient treatment method is not available. Although antibiotics are used as feed additive, vaccination is a better alternative for prevention of the disease. In general, live attenuated vaccines offer the best prospect for a vaccine. Here, we report the development and characterization of novel live attenuated *E. ictaluri* mutants. To accomplish this, in-frame deletions were constructed in tricarboxylic acid cycle (*sdhC, mdh,* and *frdA*), and one-carbon metabolism genes (*gcvP* and *glyA*) in the chromosome of wild-type *E. ictaluri* by allelic exchange. Following bioluminescence tagging of the *E. ictaluri* Δ*sdhC*, Δ*mdh*, Δ*frdA*, Δ*gcvP, and* Δ*glyA* mutants, their dissemination, attenuation, and vaccine efficacy were observed in catfish fingerlings by in-vivo imaging technology. Immunogenicity of each mutant was also determined in catfish fry. Results indicated that all of the *E. ictaluri* mutants were attenuated significantly in catfish compared to the parent strain as evidenced by a 2265.27-fold average reduction in bioluminescence signal from all the

mutants (relative to wild type) at 144 h post-infection. The catfish immunized with *E. ictaluri* Δ*sdhC*, Δ*mdh*, Δ*frdA*, and Δ*glyA* mutants showed high protection (relative percent survival (RPS) = 100%) while *E. ictaluri* Δ*gcvP* vaccinated catfish showed medium protection (RPS = 31.23%) after re-challenge with the wild-type *E. ictaluri* strain.

Introduction

The channel catfish, *Ictalurus punctatus*, industry is the largest aquaculture industry in the United States, and enteric septicemia of catfish (ESC), caused by the Gram-negative *Edwardsiella ictaluri*, is the most prevalent disease affecting this industry. Although Romet® 30, Terramycin®, and Aquaflor® are approved antibiotics to treat infections in aquaculture, their oral delivery in form of medicated feed is not effective as fish develop anorexia at early stages of the infection. Also, antibiotic resistant *E. ictaluri* strains can emerge (Dung et al., 2008). Therefore, vaccination against *E. ictaluri* has been the preferred method for prevention of ESC. Live attenuated vaccines can provide effective protection against certain diseases because they can express protective antigens without causing infection in the host (Lan et al., 2007). Identification of genes that are essential for bacterial virulence can provide new targets for defined non-reverting mutations.

Chondroitinase (Cooper et al., 1996) and auxotrophic (*aroA* and *purA*) (Lawrence, 1997; Thune et al., 1999) mutants of *E. ictaluri* have been developed as live attenuated vaccine candidates. Although the *aroA* mutant is attenuated and highly immunogenic, it is not in commercial production. The commercial vaccine Aquavac-ESC (RE-33) was developed by selecting for rifampin resistance (Klesius and Shoemaker,

1999). However, antibiotic resistance is not a desired trait for a vaccine. In addition, the genetic basis for attenuation in RE-33 is undefined (Klesius and Shoemaker, 1997), although it is known that RE-33 expresses shortened LPS O side chains (Arias et al., 2003). Despite the availability of Aquavac-ESC, ESC is still the most prevalent disease in the catfish industry (Annonymous, 2006; USDA, 2003), which stimulates the search for more effective vaccines.

E. ictaluri is considered a facultative intracellular pathogen, and it is capable of surviving inside channel catfish neutrophils and macrophages (Ainsworth and Dexiang, 1990; Booth et al., 2006). Although *E. ictaluri* is effectively phagocytosed by catfish neutrophils, it is only killed by neutrophils to a limited extent (Ainsworth and Dexiang, 1990; Waterstrat et al., 1988). A recent study by Karsi et al. (Karsi et al., 2009) has shown that genes encoding tricarboxylic acid (TCA) cycle enzymes, glycine cleavage system, a sigmaE regulator, the SoxS oxidative response system, and a plasmid-encoded type III secretion system (TTSS) effector are important for survival in neutrophils (Karsi et al., 2009). The same study discovered that some neutrophil-susceptible *E. ictaluri* strains were highly attenuated and demonstrated very good potential as live attenuated vaccines.

In particular, strains with insertion mutations in TCA cycle enzymes succinate dehydrogenase (SdhC) (*Ei*AKMut5) and malate dehydrogenase (Mdh) (*Ei*AKMut12) generated better protection than the available commercial vaccine when juvenile catfish were vaccinated by immersion (Karsi et al., 2009). Recently, it was shown that a full TCA cycle is required for *Salmonella enterica* virulence, and a *sdhDCA* mutant is attenuated in an oral mouse infection model (Tchawa Yimga et al., 2006). In Salmonella,

mdh mutants were avirulent and highly attenuated in the mouse oral challenge model (Tchawa Yimga et al., 2006). *E. ictaluri* glycine dehydrogenase (encoded by *gcvP*) has also been linked to virulence (Karsi et al., 2009). This enzyme is part of the glycine cleavage system, which participates in one-carbon (C1) metabolism. Therefore, the objective of this research was to introduce in-frame deletions in *E. ictaluri* genes in the TCA cycle and C1 metabolism system and determine their role in *E. ictaluri* virulence.

Materials and methods

Ethics statement

All fish experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University.

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 2.1. *E. ictaluri* was grown at 30 ºC using brain heart infusion (BHI) broth and agar (Difco, Sparks, MD). *Escherichia coli* were grown at 37 ºC using Luria-Bertani (LB) broth and agar (Difco). *E. coli* CC118 λ*pir* and SM10 λ*pir*/S17-1 λ*pir* were used in cloning and transfer of suicide plasmid pMEG-375 and pAK*gfplux*1, respectively. Ampicillin was used at 100 µg/ml to maintain pMEG-375 and pAK*gfplux*1. Colistin was used at 12.5 µg/ml for counterselection against *E. coli* SM10 λ*pir* following conjugation.

Table 2.1 Bacterial strains and plasmids.

Strain	Relevant Characteristics	References
Edwardsiella ictaluri		
93-146	Wild type; pEI1$^+$; pEI2$^+$; Col^r	(Lawrence, 1997)
*Ei*Δ*frdA*	93-146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*frdA*	This study
*Ei*Δ*gcvP*	93-146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*gcvP*	This study
*Ei*Δ*glyA*	93-146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*glyA*	This study
*Ei*Δ*sdhC*	93-146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*sdhC*	This study
*Ei*Δ*mdh*	93-146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*mdh*	This study
Escherichia coli		
CC118 λ*pir*	Δ(*ara-leu*); *araD*; Δ*lacX74*; *galE*; *galK*; *phoA20*; *thi-1*; *rpsE*; *rpoB*; *argE*(Am); *recAl*; λ*pir*R6K	(Herrero et al., 1990)
SM10 λ*pir*	*thi*; *thr*; *leu*; *tonA*; *lacY*; *supE*; *recA*; ::RP4-2-Tc::Mu; Km^r; λ*pir*R6K	(Miller and Mekalanos, 1998)
S17-1 λ*pir*	*RP4-2 (Km::Tn7, Tc::Mu-1), ΔuidA3::pir$^+$, recA1, endA1, thi-1,hsdR17, creC510*	(Mai et al., 2012)
Plasmids		
pMEG-375	8142 bp, Amp^r, Cm^r, *lacZ*, R6K *ori*, *mob incP*, *sacR sacB*	(Dozois et al., 2003)
p*Ei*Δ*frdA*	10242 bp, Δ*frdA*, pMEG-375	This study
p*Ei*Δ*gcvP*	12231 bp, Δ*gcvP*, pMEG-375	This study
p*Ei*Δ*glyA*	14276 bp, Δ*glyA*, pMEG-375	This study
p*Ei*Δ*sdhC*	16295 bp, Δ*sdhC*, pMEG-375	This study
p*Ei*Δ*mdh*	18350 bp, Δ*mdh*, pMEG-375	This study

Construction and bioluminescence tagging of in-frame deletion mutants

The overlap extension PCR method (Horton et al., 1990) was used to generate the in-frame deletion mutations of *E. ictaluri sdhC*, *mdh*, *frdA, gcvP,* and *glyA* genes. Four primers were designed for each gene including forward (lflp), internal-reverse (lfrp), internal forward (rflp), and reverse primers (rfrp) (Table 2.2). Restriction sites were included in forward and reverse primers. Genomic DNA was isolated from *E. ictaluri* using a Wizard Genomic DNA Kit (Promega, Madison, WI) and used as template in PCR. Upper fragments were amplified by forward and internal-reverse primers, while reverse and internal-forward primers were used to amplify lower fragments. The resulting upper and lower PCR products were gel extracted using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), mixed in a 1:1 ratio, and then re-amplified using the forward and reverse primers. The resulting in-frame deleted fragment was purified using a QIAquick

Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR product was digested with respective restriction enzymes (Promega) (Table 2.1) and cleaned using a Wizard SV Gel and PCR Clean-Up Kit (Promega).

The suicide plasmid pMEG-375 was purified from an overnight *E. coli* culture by a QIAprep Spin Miniprep Kit (Qiagen) and cut with restriction enzymes respective to the inserts, producing compatible ends. The purified PCR product with in-frame deletion was ligated into pMEG-375 vector by T4 DNA Ligase (Promega) at 4 ºC overnight, generating p*EiΔsdhC*, p*EiΔmdh*, p*EiΔfrdA*, p*EiΔgcvP*, and p*EiΔglyA* (Table 2.1). Insert in each plasmid was confirmed by enzyme digestion as well as sequencing.

The suicide plasmids with in-frame deleted genes were transferred into *E. coli* SM10 λpir/S17-1 λ*pir* by electroporation and were mobilized into *E. ictaluri* 93-146 by conjugation. The recipient cells were spread on BHI plates containing colistin (12.5 µg/ml) and ampicillin (100 µg/ml) to select the clone with integrated vector by single crossover through allelic exchange. The ampicillin resistant colonies were propagated on BHI plates to allow for the second crossover allelic exchange, and resulting colonies were streaked on LB plates with 5% sucrose, 0.35% mannitol, and colistin (12.5 µg/ml). These plates selected for loss of pMEG-375 with *sacB* gene. Colonies from the selective plates were tested for ampicillin sensitivity to ensure loss of the plasmid. Deleted region was amplified from the resulting ampicillin sensitive colonies and confirmed by sequencing. After confirmation, *Ei*Δ*sdhC, Ei*Δ*mdh*, *Ei*Δ*frdA*, *EiΔgcvP,* and *Ei*Δ*glyA* mutants were labeled with bioluminescence using pAK*gfplux*1 as described in Karsi and Lawrence (Karsi and Lawrence, 2007).

Table 2.2 Primers with restriction enzyme used for the construction of the *E. ictaluri* mutants.

Genes	Primer ID	Primer Sequence ((5'→3')[a]	RE[b]
frdA	*Eifrd*Alflp	AA**GAGCTC**TCGTCCACTTCATTCATCAGAC	*Sac*I
	*Eifrd*Alfrp	GTGGAAGTGGAAATCGAAAGA	
	*Eifrd*Arflp	TCTTTCGATTTCCACTTCCACGAAGCTCAGGAAGCCAAGAAG	
	*Eifrd*Arfrp	AA**TCTAGA**GCAGGGAGATGATATTGAGGAC	*Xba*I
	*Eifrd*A01S	CCTCAACTGAAGATTGCCTTA	
gcvP	*Eigcv*Plflp	AA**TCTAGA**CCTTTGGCGTGGAGATATGC	*Xba*I
	*Eigcv*Plfrp	AGCATCACTGTTTTCAAGCTG	
	*Eigcv*Prflp	CAGCTTGAAAACAGTGATGCTGTAAAGCGCCTGGACGATGT	
	*Eigcv*Prfrp	AA**GAGCTC**CGGACAGAGACATACCACCAA	*Sac*I
	*Eigcvp*01S	GGCCTTTTGGTATGATTTGC	
glyA	*EiglyA*lflp	AA**GAGCTC**GGGCATGGGTCAGTGAATAC	*Sac*I
	*EiglyA*lfrp	CCACAGCTCGGTATCGTAATC	
	*EiglyA*rflp	GATTACGATACCGAGCTGTGGGTGAACGTCTTCCGGTCTATG	
	*EiglyA*rfrp	AA**CCCGGG**GCCTAGACGATGTCTCCTTGA	*Sma*I
	*EiglyA*01S	GGGCCAGATTTACTCAAAACC	
sdhC	*Eisdh*Clflp	AA**GAGCTC**CAGCCTCCTTTGGTACTGCTA	*Sac*I
	*Eisdh*Clfrp	GCAAATCCAGATTGACAGGTCT	
	*Eisdh*Crflp	AGACCTGTCAATCTGGATTTGCGGGTATGGTAAGCAACGCATC	
	*Eisdh*Crfrp	AA**CCCGGG**CCCCATCATGTAGTGACAGGT	*Sma*I
	*Eisdh*C01S	CTCAGTCTCGTGGGATTTGC	
mdh	*Eimdh*lflp	AA**GAGCTC**GGCTTTATAATGGCGTGTGG	*Sac*I
	*Eimdh*lfrp	AGGCAGCTGAGTCTTAAGCAG	
	*Eimdh*rflp	CTGCTTAAGACTCAGCTGCCTCTGGGCGAAGACTTTATCAAT	
	*Eimdh*rfrp	AA**CCCGGG**GGAGCAGGCCCTACAAGACT	*Sma*I
	*Eimdh*01S	CAGCTCGCAATCTGAGTGTT	

[a]RE stands for restriction enzyme added to the 5' end of the primer sequence
[b]Bold letters at the 5' end of the primer sequence represent RE site added. AA nucleotides were added to the end of each primer containing a RE site to increase the efficiency of enzyme cut. Underlined bases in internal primer (rflp) indicate reverse complemented internal primer (lfrp) sequence.

Determination of virulence

Experimental infections were conducted in 40-L challenge tanks supplied with flow-through dechlorinated municipal water. Water temperature was maintained at 25 ºC (±2) throughout the experiments. Each experimental group contained triplicate tanks. In each treatment, 28 specific pathogen free (SPF) catfish fingerlings (14.2± 0.35 cm, 25.45

± 1.82 g) were randomly allocated (four per tank) between *E. ictaluri* mutants (treatment), wild-type *E. ictaluri* (positive control), and phosphate-buffered saline (PBS) (negative control). Fish were anesthetized in water containing 100 mg/L MS222 and injected with approximately 1 x 10^4 colony forming units (CFU) in 100 µl PBS.

Bioluminescent imaging (BLI) was conducted using an IVIS 100 Imaging System (Caliper Corporation, Hopkinton, Massachusetts)to measure number of photons emitted by fish (Karsi et al., 2006). Briefly, catfish were anesthetized in water containing 100 mg/L MS222 and transferred immediately to the photon collection chamber for image capture. Total photon emissions from the whole fish body were collected for one min. Following BLI imaging, fish were returned to well-aerated water for recovery. BLI was conducted at 2, 4, 8, and 24 h post-infection and subsequent daily intervals until168 h post-infection. Bioluminescence was quantified from the fish images using Living Image Software v 2.5 (Caliper Corporation., Hopkinton, Massachusetts) and quantitative data were used in statistical analysis.

Determination of efficacy

Catfish fingerlings vaccinated with the *E. ictaluri* mutants were infected with wild-type *E. ictaluri* strain 93-146, and infections were monitored using BLI. Briefly, after four weeks, the juvenile catfish vaccinated with mutants (described above in the virulence challenge) were immersion challenged with 4.8 x10^7 bioluminescent wild-type *E. ictaluri* parent strain. Photon emissions from fish were collected at 2, 4, 8, 24, 48, 72, and 96 h post-infection using an IVIS 100 as described above, and statistical analysis was performed on the mean photon counts.

Determination of vaccine potential

Approximately 420 eight-month-old SPF channel catfish fingerlings (17.61± 0.63 cm, 47.47± 5.31 g) were stocked in 21 tanks at a rate of 20 fish/tank. Each treatment had three replicate tanks. Vaccination treatments consisted of *Ei*Δ*sdhC*, *Ei*Δ*mdh*, *Ei*Δ*frdA,* *Ei*Δ*glyA*, and *Ei*Δ*gcvP*, and controls consisted of *Ei* wild-type (positive control) and uninoculated BHI (negative control) respectively. Channel catfish were vaccinated by immersion challenge in water containing approximately 4.3 x 10^7 CFU/ml of water for 1 h, followed by gradual removal of bacteria. Mortalities were recorded for 21 days following the vaccination, then both vaccinated and non-vaccinated treatments were immersion exposed to wild-type parent *E. ictaluri* strain 93-146 (approximately 3.06 x 10^7 CFU/ml). Fish mortalities were recorded daily for 14 days. Relative percent survival (RPS) was calculated according to the following formula: RPS= [1-(% mortality of vaccinated fish/% mortality of non-vaccinated fish)] x 100 (Amend, 1981).

Statistical analysis

Photon counts were transformed by taking the base 10 logarithm to improve normality. One-way ANOVA was conducted using SPSS V19 (IBM Corp., Armonk, NY) to compare mean photon counts at each time point ($p < 0.05$). Pairwise comparison of the means was done using Tukey procedure. Data was then retransformed for interpretation.

Results

Construction of the *E. ictaluri* in-frame deletion mutants

Five in-frame mutants (*EiΔsdhC, EiΔmdh*, *EiΔfrdA*, *EiΔgcvP,* and *EiΔglyA*) were obtained successfully (Fig. 2.1) by deleting on average over 90% of each gene (Table 2.3).

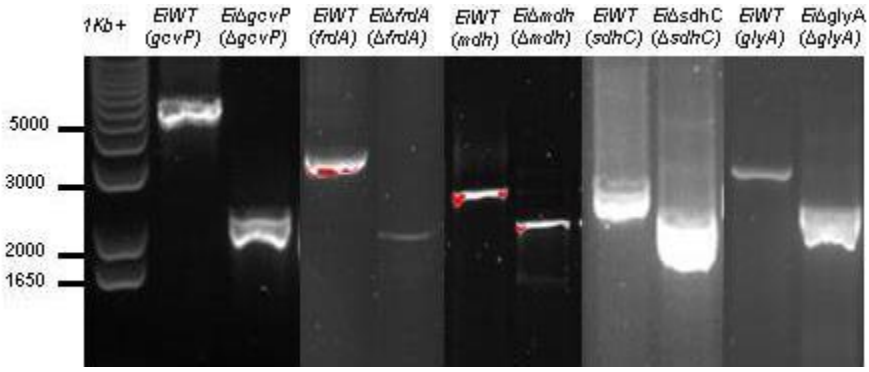

Figure 2.1 Genotypic confirmation of the *E. ictaluri gcvP, frdA, mdh, sdhC*, and *glyA* mutants.

Genomic DNAs was amplified from wild-type *E. ictaluri* and mutants using the two outside primers (lflp and rfrp) and separated on 1% agarose gel.

Table 2.3 Properties of selected *E. ictaluri* TCA cycle and C1 metabolism genes and percentage of gene deleted.

Gene	Locus	Product	ORF (bp/aa)	Remaining (bp/aa)*	% Deletion
sdhC	NT01EI_2872	Succinate dehydrogenase, cytochrome b556 subunit, putative	390/129	57/18	86.05
mdh	NT01EI_0446	Malate dehydrogenase, NAD-dependent, putative	939/312	99/32	89.74
frdA	NT01EI_0392	Fumarate reductase, flavoprotein subunit, putative	1800/899	126/41	95.44
gcvP	NT01EI_3351	Glycine dehydrogenase, putative	2884/960	114/37	96.15
glyA	NT01EI_3190	Serine hydroxymethyltransferase, putative	1254/417	75/24	94.24

*Number of bp/aa remaining from genes following deletion by overlap extension PCR

Determination of virulence in juvenile catfish

BLI results revealed that photon counts from the catfish infected with *Ei*Δ*sdhC*, *Ei*Δ*mdh*, *Ei*Δ*frdA*, *Ei*Δ*gcvP*, and *Ei*Δ*glyA* mutants were low at 2, 6, and 12 h. However, the signal intensities for mutants *Ei*Δ*sdhC*, *Ei*Δ*mdh*, *Ei*Δ*frdA and*, *Ei*Δ*gcvP* increased from 24 h to 72 h and then decreased thereafter. In mutant *Ei*Δ*glyA*, very low signal was detected at all time points. The signal intensity increased in fish infected with wild-type *E. ictaluri* until all fish died (Fig. 2.2). The bioluminescence signal intensity was 14.4 fold higher in the wild-type infected fish than the average signal from the mutant infected fish at 72 h post-infection. At 144 h post-infection, average signal from wild-type infected fish was 2265.27-fold than average signal from the mutant infected fish (Fig. 2.2). The bioluminescence signal intensities were 118.70- and 5329.15-fold less in *Ei*Δ*glyA* compared to wild-type *E. ictaluri* at the same time points (Fig. 2.2). In the wild-type group, two fish died at 144 and 168 h post-infection. Mean signal intensities between all mutants (except *Ei*Δ*frdA*) and the wild-type strain were significantly different ($p < 0.5$) at 24 h post-infection and thereafter.

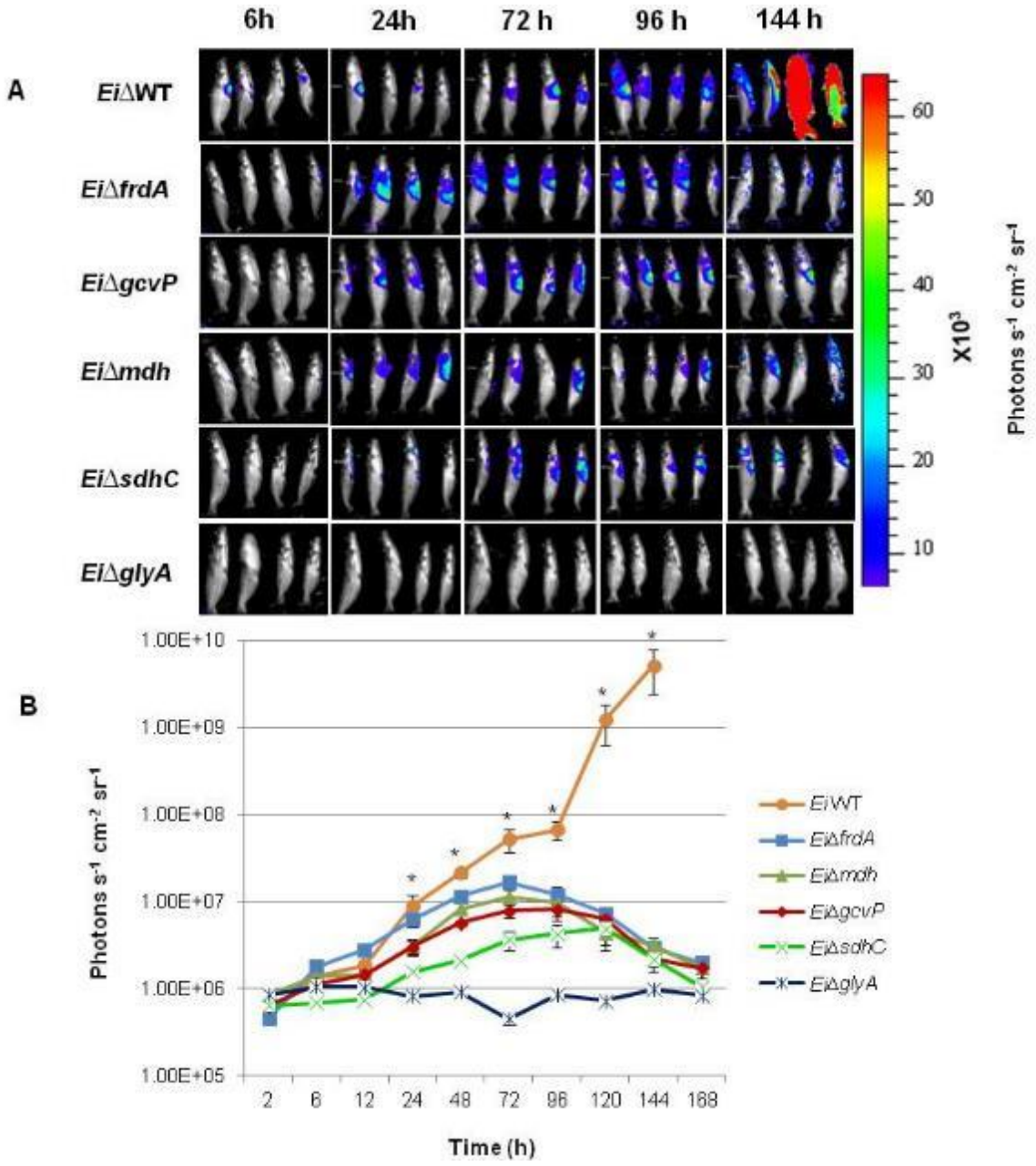

Figure 2.2 Bioluminescence imaging of catfish fingerlings experimentally infected with wild-type or mutant *E. ictaluri* by intraperitoneal injection.

(A) BLI imaging of catfish. (B) Total photon emissions from each fish.Each data point represents the mean photon emissions from four fish. Two of the four channel catfish injected with wild-type died at 144 h post-infection. The remaining two died at 168 h post-infection. Star indicates significant difference between the *E. ictaluri* mutants and wild type.

Protection against *E. ictaluri* infection

Photon counts were significantly lower ($p < 0.5$) at each time point for the vaccinated fish infected with the *E. ictaluri* mutants compared to the sham-vaccinated control (Fig. 2.3). The bioluminescence signal intensity was on average 10-fold higher in sham-vaccinated fish at 6 h, which increased to 20-fold at 96 h. At this time point, signal intensity in *Ei*Δ*sdhC*, *Ei*Δ*mdh*, and *Ei*Δ*frdA* vaccinated fish were in decline while that of in *Ei*Δ*gcvP* and *Ei*Δ*glyA* vaccinated fish were increasing (Fig. 2.3). At 96 h post-infection, all fish in the sham vaccinated group died.

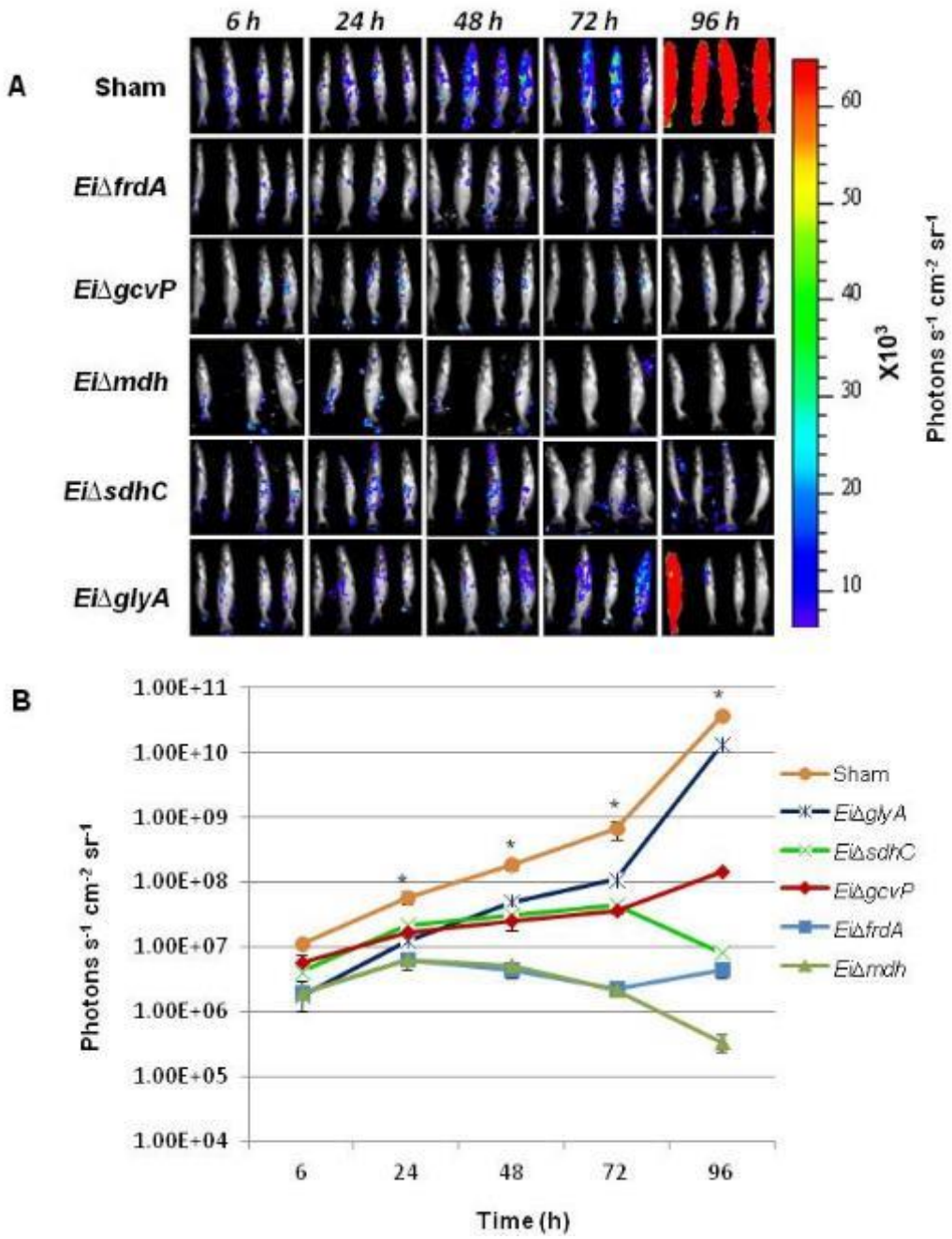

Figure 2.3 Bioluminescence imaging of vaccine efficacy in live catfish after wild-type *E. ictaluri* infection by immersion.

(A) BLI imaging of catfish. (B) Mean photon emissions from fish.Each data point represents the mean photon emissions from four fish. Stars indicate significant difference between mutants and wild-type *E. ictaluri*.

Determination of vaccine potential in catfish

Vaccination of channel catfish with *Ei*Δ*sdhC*, *Ei*Δ*mdh*, *Ei*Δ*frdA,* and *Ei*Δ*glyA* provided complete protection (100% survival) against wild-type *E. ictaluri* infection while the *Ei*Δ*gcvP* mutant showed lower efficacy (68.89% survival) (Fig. 2.4). Survival rate in *Ei*Δ*sdhC*, *Ei*Δ*mdh*, *Ei*Δ*frdA* and *Ei*Δ*glyA* vaccinated groups were 1.96-fold higher than that of the non-vaccinated group when re-challenged with wild-type *E. ictaluri* (100% vs. 51.11%).

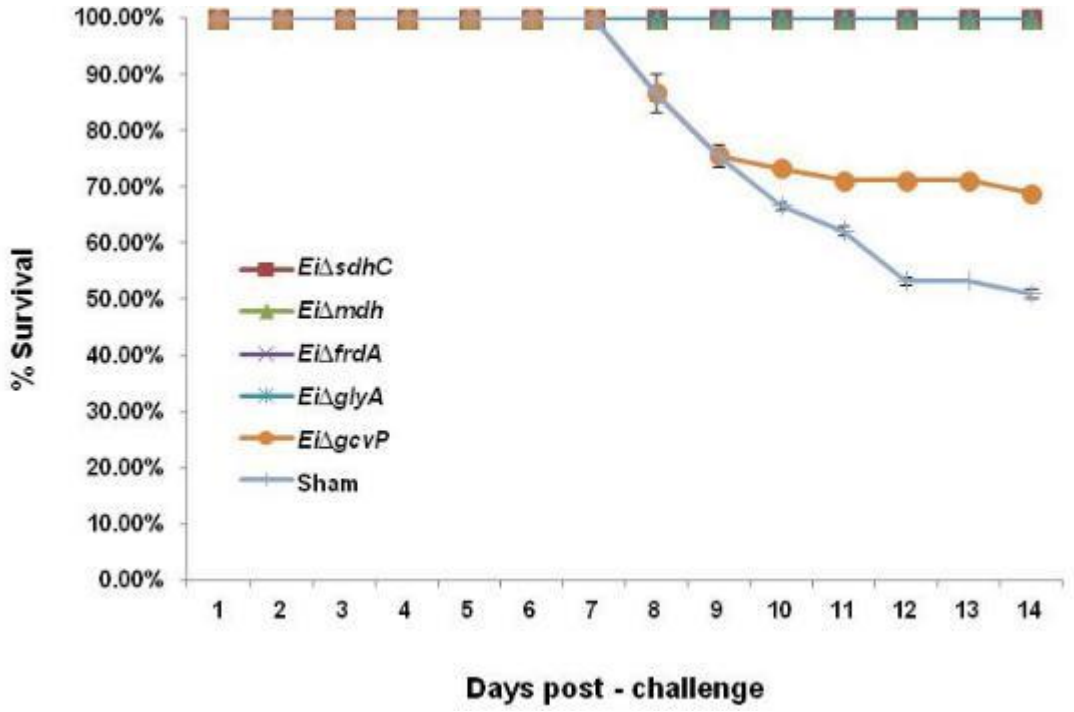

Figure 2.4 Percentage survival of immersion vaccinated catfish.

The catfish fingerlings were vaccinated with *EiΔmdh, EiΔsdhC, EiΔfrdA, EiΔglyA, and EiΔgcvP* mutants and challenged with wild-type *E. ictaluri* strain 93-146. Fish mortalities were used to calculate vaccine efficacies.

Discussion

The primary objective of this study was to construct live attenuated *E. ictaluri* strains based on the mutation of genes *mdh*, *sdhC,* and *frdA*, which encode enzymes in the TCA cycle, and *gcvP* and *glyA*, which encode enzymes in C1 metabolism. Further, we aimed to characterize these mutants in terms of their attenuation in catfish and ability to protect against wild-type *E. ictaluri* infection. In our study, we were able to delete large portion of each gene (on average more than 90% of each gene was deleted) using splicing overlap extension combined with allelic exchange. This work represents one of the few successful applications of this technique in *E. ictaluri* (Abdelhamed et al., 2013; Santander et al., 2010).

Virulence testing was first conducted in juvenile catfish because it represents a more reliable challenge system than catfish fry. In addition, catfish fry are only available once a year in early summer. In juvenile catfish, we utilized bioluminescence imaging to improve our ability to quantify degree of attenuation. In mutant strains *Ei*Δ*sdhC, Ei*Δ*mdh, Ei*Δ*frdA*, and *Ei*Δ*gcvP* bioluminescence signals were detected after 12 h post-infection, clearly indicating they retain the ability to invade channel catfish. However, channel catfish injected with the mutant strains were able to start clearing the bacteria after 72 h post-infection, demonstrating that the mutants were not able to sustain infection and were all attenuated compared to wild-type *E. ictaluri*. Comparison of the current results to an earlier study (Karsi et al., 2006) indicates that bacterial tissue concentration of 1 x 10^9 photons^{-1} cm^{-2} steradian^{-1} seems to be a critical threshold after which fish start dying. The ability of mutants to establish infection for 72 h suggests that the mutants may have the ability to stimulate an immune response and hence protection against wild-

type *E. ictaluri*. Because the *Ei*Δ*glyA* mutant could not persist in host as well as other mutants after injection vaccination, we would expect much less systemic protection from this mutant.

After the virulence and protection trial, vaccine potential was determined in catfish fingerlings. Efficacy result showed that percentage survival was 100% in the mutant strains *Ei*Δ*sdhC, Ei*Δ*mdh, Ei*Δ*frdA,* and *Ei*Δ*glyA*. The percent survival in *Ei*Δ*gcvP* vaccinated group was 68.89% (Fig. 2.4). Similar results were reported in a previous study (Karsi et al., 2006), where insertion mutation in genes encoding *sdhC* and *mdh* were found to give 100% and 98.96% protectionagainst *E. ictaluri* infection, respectively, while 10.85% mortalities were observed in fish vaccinated with a *gcvP* mutant.

Protection seen in fish vaccinated by immersion (fish survival) was very similar to that obtained by injection vaccination (bioluminescence signals), except that *Ei*Δ*glyA* vaccination seemed to provide better protection by immersion vaccination (Fig. 2.4). It is possible that the different protection levels provided by *EiΔglyA* is caused by different vaccination routes (injection or immersion). In addition, the method of detection could cause differences. For example, with injection vaccination, mucosal immune response may not be fully achieved. It is possible that immersion vaccination using *Ei*Δ*glyA* may activate mucosal immunity better, preventing wild-type *E. ictaluri* from establishing infection. This may also indicate that boosting mucosal immunity could be important in fish vaccination. We see an opposite trend in *Ei*Δ*gcvP* mutant, which protects fish better when vaccination is applied by injection rather than immersion. In *Ei*Δ*gcvP* immersion

vaccination, it is possible that systemic immune response may not be triggered completely due to low penetration of the mutant into fish body.

Succinate dehydrogenase (SDH) is part of the aerobic respiratory chain in the TCA cycle, oxidizing succinate to fumarate while reducing ubiquinone to ubiquinol (Maklashina et al., 1998). It is closely related to fumarate reductase, which catalyzes the reverse reaction. Succinate dehydrogenase and fumarate reductase can replace each other (Guest, 1981; Maklashina et al., 1998). Although SdhC has similar function, hydrophobicity, and protein size to the membrane-binding subunit fumarate reductase (FrdC), *sdhC* and *frdC* do not share significant sequence identity (Wood et al., 1984). The organic acids formate and succinate have a protective effect in stationary phase cells against killing effects of antimicrobial peptide BPI, which appears to disrupt the bacterial respiratory chain (Barker et al., 2000). Maintenance of protective levels of formate and succinate requires the activity of formate dehydrogenase and succinate dehydrogenase, respectively.

In *E. coli* and *Salmonella*, succinate dehydrogenase is known to contribute to pathogenicity. Recently, it was shown that a full TCA cycle is required for *Salmonella enterica* virulence, and a *sdhDCA* mutant is attenuated in an oral mouse infection model (Tchawa Yimga et al., 2006), which is similar to our finding. In *Helicobacter pylori*, fumarate reductase was found to be essential for colonization of mouse gastric mucosa (Ge et al., 2000b). Our results indicated that deletion of the *E. ictaluri*, *sdhC* and *frdA* genes resulted in full attenuation in catfish fingerlings. Thus, succinate dehydrogenase and fumarate reductase play an important role in *E. ictaluri* pathogenesis. Malate dehydrogenase is encoded by *mdh* gene, and our results show that *mdh* is important in *E.*

ictaluri virulence. Similarly, an *mdh* mutant was found to be avirulent and highly attenuated in *Salmonella* using the mouse oral challenge model (Tchawa Yimga et al., 2006).

The glycine cleavage system is a loosely associated four subunit enzyme complex that catalyzes the reversible oxidation of glycine to form 5- and 10-methylenetetrahydrofolate, which serves as a one carbon donor. It is one of two sources of C1 units; serine hydroxymethyltransferase is the other source, and it is actually considered the more important source. Expression of the glycine cleavage enzyme system is induced by glycine (Meedel and Pizer, 1974; Stauffer et al., 1994), and *gcv* mutants are unable to use glycine as a C1 source and excrete glycine (Plamann, 1983). Our previous work as well as this current work have shown that knocking out the *E. ictaluri gcvP* gene affects the virulence of *E. ictaluri.*

Although BLI for real-time monitoring of *E. ictaluri* infection in live fish was shown by our group (Karsi et al., 2006), this is the first time we report the use of BLI to quantify the degree of *E. ictaluri* attenuation in channel catfish. It appears that BLI could be used for vaccine evaluation by using very low number of fish (four fish in this work). In addition, use of BLI has provided evidence for the importance of mucosal immunity in catfish.

In summary, our results showed that the *Ei*Δ*sdhC, Ei*Δ*mdh, Ei*Δ*frdA*, *Ei*Δ*gcvP*, and *Ei*Δ*glyA* mutants were significantly attenuated and provided protection against ESC under controlled laboratory conditions. The significant attenuation of *Ei*Δ*sdhC, Ei*Δ*mdh, Ei*Δ*frdA*, and *Ei*Δ*gcvP* mutants indicate that they may be used safely as live attenuated vaccine for vaccinating catfish fingerlings. The *E. ictaluri* Δ*glyA* mutant was found to be

incapable of persisting in catfish when injected, which might be the reason for low level of protection. In contrast, immersion vaccination with the *Ei*Δ*glyA* mutant seems to give better protection.

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CHAPTER III

CONSTRUCTING COMBINATIONS OF MUTATIONS IN TRICARBOXYLIC ACID AND ONE-CARBON METABOLISM PATHWAYS ENZYMES IN *EDWARDSIALLA ICTALURI* AND TESTING THEIR VIRULENCE IN CHANNEL CATFISH

Abstract

Edwardsiella ictaluri is the causative agent of enteric septicemia of Catfish (ESC), the most economically important disease of farm-raised channel catfish. Different approaches have been discovered to develop a live attenuated vaccine but the results were not consistent. Aqavac-ESC is a commercial vaccine on the market, but catfish farmers still suffer from economic loss due to ESC. In this chapter, we report construction of *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdh*C, *Ei*Δ*mdh*Δ*sdhC*, and *Ei*Δ*glyA*Δ*gcvP* mutants. Deletion mutagenesis by allelic exchange was used, and virulence and vaccine efficacy were evaluated using in vivo bioluminescence imaging system (BLI) in catfish fingerlings. All of the mutants were attenuated; there was 6736-fold average reduction in bioluminescence signal from all the mutants at 96 h post-infection compared to wild-type strain 93-146. When catfish fingerlings were vaccinated by injection, all the mutants were effective in protecting channel catfish against subsequent infection with virulent *E. ictaluri* 93-146 strain via immersion route. Vaccination with *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdhC*, and *Ei*Δ*glyA*Δ*gcvP* by immersion provided significant

protection against subsequent immersion challenge with virulent parent strain. In particular, channel catfish fingerlings vaccinated with *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdhC*, and *Ei*Δ*glyA*Δ*gcvP* mutants showed 100% survival following immersion challenge with virulent wild-type *E. ictaluri*.

Introduction

In the United States, the largest aquaculture industry is the channel catfish industry, and the most serious disease affecting this industry is enteric septicemia of catfish (ESC) (Lawrence at al., 1997). Catfish production accounts for 85 to 90 % of total commercial finfish production in the United States, and significant losses due to ESC were reported on over 60% of all farms in operation (Thune et al., 2007). ESC occurs in one of two forms: an acute form, which is characterized by enteritis and septicemia, and a chronic form, which is characterized by meningoencephalitis (MacMillan, 1985; Newton et al., 1989; Shotts et al., 1986). In the acute form, farmers experience economic losses due to rapid mortalities (Shotts et al., 1986). In the chronic form, farmers experience losses due to decreased production, with fish manifesting signs 3 to 4 weeks after an acute outbreak (Newton et al., 1989).

The only treatments currently approved in the U.S. for treatment of ESC are sulfadimethoxine/ormetoprim and florfenicol (McGinnis et al., 2003), which are delivered orally as medicated feed. However, antibiotic therapy is not considered the best management practice for several reasons, including potential development of antibiotic resistant *E. ictaluri* strains (Johnson, M.J., 1991). Moreover, one of the earliest clinical signs associated with ESC is anorexia; therefore, these antibiotics are only effective in limiting the spread of an outbreak and not in treating the disease.

An alternative strategy to preventing ESC is vaccination. Live attenuated vaccines can provide effective protection against certain diseases because they can express protective antigens without causing morbidity in the host (Lan et al., 2007). Commercial vaccine Aquavac-ESC generates protective immunity in fry as young as 7 d post-hatch (Shoemaker et al., 1999). It also provides some protection when used to vaccinate eyed eggs (Shoemaker et al., 2002). However, despite the availability of the commercial vaccine Aquavac-ESC, there has been no reduction in the prevalence of ESC in the industry.

Therefore, this study aims to develop combinations of in-frame mutation in genes encoding TCA cycle and C-1 metabolism for the development of an efficacious live attenuated vaccine. *E. ictaluri* is considered a good candidate for the development of a vaccine because the species is composed of a single serotype (Bertolini et al., 1990; Plumb & Vinitnantharat, 1989). However, there is good evidence that cell-mediated immunity to *E. ictaluri* is important for the development of an effective protective response (Ciembor et al., 1995; Klesius & Sealey, 1995; Thune et al., 1997). Therefore, killed bacterins have only been efficacious under controlled laboratory conditions when given by injection (Saeed & Plumb, 1986), and this route of delivery is not practical for commercial production. When delivered by the immersion or oral route, efficacy of bacterins has been variable (Saeed & Plumb, 1986; Thune et al., 1993).

The currently available commercial live attenuated vaccine (RE-33, marketed as Aquavac-ESC by Schering-Plough) was developed by selecting for rifampin resistance, and the genetic basis for attenuation is undefined (Klesius & Shoemaker, 1997). However, it is known that RE-33 expresses shortened LPS O side chains (Arias et al.,

2003). Lawrence and Banes (2005) found that an isogenic O polysaccharide mutant was only effective when juvenile catfish were vaccinated by IP injection, but not by bath immersion (Lawrence & Banes, 2005). Our group reported vaccine candidates that have insertion mutations in genes encoding TCA cycle enzymes and glycine cleavage protein; these candidates demonstrate potential to substantially improve efficacy compared to RE-33 (Karsi et al., 2009).

In Chapter II, we reported the construction of *EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔgcvP,* and *EiΔglyA* in-frame deletion mutants. These mutants were significantly attenuated when tested in channel catfish compared to the parent *E. ictaluri* strain using bioluminescence. Efficacy studies also showed that the constructed mutants *EiΔsdhC, EiΔfrdA, EiΔmdh*, and *EiΔglyA* were able to produce significant protective immunity against *E. ictaluri*. Mutant *EiΔgcvP* provided a lower level of protection. Mutants *EiΔsdhC, EiΔfrdA, EiΔmdh*, and *EiΔglyA* were found to be good candidates as live attenuated vaccines. In the current study, combinations of in-frame deletions in genes encoding TCA cycle enzymes and C-1 metabolism proteins were constructed to create greater attenuation while retaining antigenicity.

Materials and methods

Bacterial strains, plasmids, media, and growth conditions

Bacterial strains and the plasmids used in this work are listed in Table 3.1. *E. ictaluri* strain 93-146 was cultured in brain heart infusion (BHI) agar and broth (Difco, Sparks, MD) at 30ºC throughout the entire study. *E. coli* strains were cultured on Luria–Bertani (LB) agar and broth from Difco and incubated at 37ºC throughout the entire study. When required, antibiotics and reagents from Sigma (St. Louis, MO, USA) were

added to the culture medium at the following concentrations: ampicillin (Amp: 100 µg/ml), colistin sulfate (Col: 12.5 µg/ml), sucrose (5%), and mannitol (0.35%). *E. coli* CC118 λ*pir* and SM10λ*pir*/ S17-1 λ*pir* were used in cloning and transfer of suicide plasmid pMEG-375 and pAK*gfplux*1, respectively.

Table 3.1 Bacterial strains and plasmids.

Strain	Relevant Characteristics	References
Edwardsiella ictaluri		
93-146	Wild type; pEI1$^+$; pEI2$^+$; Col^r	(Lawrence, 1997)
*Ei*Δ*frdA*Δ*sdhC*	93-146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*frdA*; Δ*sdhC*;	This study
*Ei*Δ*gcvP*Δ*sdhC*	93-146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*gcvP*; Δ*sdhC*;	This study
*Ei*Δ*mdh*Δ*sdhC*	93-146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*mdh*; Δ*sdhC*;	This study
*Ei*Δ*glyA*Δ*gcvP*	93-146 derivative; pEI1$^+$; pEI2$^+$; Col^r; Δ*glyA*; Δ*gcvP*	This study
Escherichia coli		
CC118 λ*pir*	Δ(*ara-leu*); *araD*; Δ*lacX74*; *galE*; *galK*; *phoA20*; *thi-1*; *rpsE*; *rpoB*; *argE*(Am); *recAl*; λ*pir*R6K	(Herrero et al., 1990)
SM10 λ*pir*	*thi*; *thr*; *leu*; *tonA*; *lacY*; *supE*; *recA*; ::RP4-2-Tc::Mu; Km^r; λ*pir*R6K	(Miller and Mekalanos, 1998)
S17-1 λ*pir*	*RP4-2 (Km::Tn7, Tc::Mu-1), ΔuidA3::pir$^+$, recA1, endA1, thi-1,hsdR17, creC510*	(Metcalf et al., 1994)
Plasmids		
pMEG-375	8142 bp, Amp^r, Cm^r, *lacZ*, R6K *ori*, *mob incP*, *sacR sacB*	(Dozois et al., 2003)
p*Ei*Δ*sdhC*	16295 bp, Δ*sdhC*, pMEG-375	This study
p*Ei*Δ*gcvP*	12231 bp, Δ*gcvP*, pMEG-375	This study

Construction of double mutants

E. ictaluri 93-146 *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdhC*, and *Ei*Δ*glyA*Δ*gcvP* mutants were constructed using deletion mutagenesis. Plasmid p*EiΔsdhC* (Chapter II) was separately mobilized from *E. coli* S17-1λ pir into *Ei*Δ*frdA*, *Ei*Δ*gcvP*, and *Ei*Δ*mdh*, (Chapter II) by conjugation. Similarly, p*EiΔgcv*P was mobilized from *E.coli* S17-1 λ pir into *Ei*Δ*glyA*. Recipient bacteria were spread on BHI plates containing the antibiotics colistin (12.5 µg/ml) and ampicillin (200 µg/ml) to select the clone that had integrated the vector by single crossover through allelic exchange. Each clone was

propagated on BHI plates to allow for the second crossover of allelic exchange. The resulting colonies were streaked on LB plates with 5% sucrose, 0.35% mannitol, and colistin (12.5 µg/ml). These plates selected for loss of pMEG-375 vector-encoded *sacB* gene. Colonies growing on these selective plates were tested for ampicillin sensitivity to ensure loss of plasmid by using BHI plates supplemented with ampicillin and colistin. The resulting ampicillin sensitive colonies were confirmed by PCR using outer primers lflp and rfrp (Table 3.2). After mutant confirmation, each constructed mutant strain, *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdh*C, *Ei*Δ*gcv*PΔ*sdh*C, and *Ei*Δ*gly*AΔ*gcv*P were labeled with bioluminescence by transferring pAK*gfplux*1from an *E. coli* donor strain SM10λ pir by conjugation as described in Karsi and Lawrence (2007).

Table 3.2 Primers used for confirmation of the *E. ictaluri* mutants.

Genes	Primer ID	Primer Sequence (5'→3')[a]	RE[b]
gcvP	*EigcvP*lflp	AA**TCTAGA**CCTTTGGCGTGGAGATATGC	*Xba*I
	*EigcvP*rfrp	AA**GAGCTC**CGGACAGAGACATACCACCAA	*Sac*I
sdhC	*EisdhC*lflp	AA**GAGCTC**CAGCCTCCTTTGGTACTGCTA	*Sac*I
	*EisdhC*rfrp	AA**CCCGGG**CCCCATCATGTAGTGACAGGT	*Sma*I

[a]RE stands for restriction enzyme added to the 5' end of the primer sequence
[b]Bold letters at the 5' end of the primer sequence represent RE site added. The additional nucleotides (AA) were added to the 5' end of each primer containing a RE site to increase the efficiency of enzyme cut.

Virulence assays

Six-months-old SPF channel catfish fingerlings (14.2± 0.35 cm, 25.45 ± 1.82 g) were transferred into challenge tanks (4/tank) supplied with flow-through dechlorinated municipal water. Water temperature was maintained at 28°C throughout the experiments. Fish were anesthetized in water containing 100 mg/L MS-222, and 1 x 10^6 CFU of a

specific strain in 100 µl of phosphate-buffer saline (PBS) was injected by sterile syringe into each of four channel catfish fingerlings. There were six treatments in this assay: four vaccination treatments (*Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdh*C, *Ei*Δ*mdh*Δ*sdh*C and *Ei*Δ*gly*AΔ*gcv*P), positive control treatment (wild-type strain 93-146), and negative control treatment (PBS).

Bacterial quantification was conducted using the IVIS Imaging System at 2, 4, 8, and 24 hours post-infection, and subsequent daily intervals. To accomplish this, each fish was anesthetized in water containing 100 mg/L MS222 and transferred immediately to the photon collection chamber for image capture. Total photon emissions from the whole fish body were collected at exposure time of one minute. Following imaging, fish were returned to well-aerated water for recovery. Bioluminescence images were displayed as pseudocolor images, and bioluminescence was quantified from the fish images using Living Image v2.5 software (Caliper Corporation, 2009).

Protection assays

Twenty-one days after experimental infection by injection with *Ei*Δ*mdh*Δ*sdhC*, *Ei*Δ*gcvP*Δs*dhC*, *Ei*Δ*frdA*Δ*sdhC* and *Ei*Δ*glyA*Δ*gcvP*, channel catfish fingerlings were immersion challenged with 4.8 x10^7 CFU of bioluminescent wild-type *E. ictaluri* 93-146. In each experiment, an age-matched set of sham vaccinated channel catfish fingerlings were also challenged with 4.8 x10^7 CFU of bioluminescent wild-type *E. ictaluri* 93-146. Bacterial quantification was conducted using the IVIS Imaging System at 2, 4, 8, 24, 48, 72, and 96 hours post-infection using the methods described in section 2.5. Bioluminescence was quantified from the whole body of each fish, and statistical analyses were performed using the mean bioluminescence for each treatment.

Protection against mortalities in juvenile catfish vaccine trial

Approximately 360 eight-month-old SPF channel catfish fingerlings (17.61± 0.63 cm, 47.47± 5.31 g) were stocked into 18 tanks at a rate of 20 fish/tank. Each treatment had three tank replicates; there were four vaccination treatments (*Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdh*C, *Ei*Δ*mdh*Δ*sdh*C and *Ei*Δ*gly*AΔ*gcv*P), positive control treatment (wild-type strain 93-146), and negative control treatment (uninoculated BHI broth). Channel catfish were vaccinated by immersion challenge in water containing approximately 4.3 x 10^7 CFU/ml of water for 1 h, followed by gradual removal of bacteria. Mortalities were recorded for 21 days following vaccination. After 21 days, fish in both vaccinated and non-vaccinated treatments were immersion exposed to wild-type parent *E. ictaluri* (approximately 3.06 x 10^7 CFU/ml of water), and fish mortalities were recorded daily for 14 days.

Statistical analysis

Photon counts were transformed by taking the base 10 logarithm to improve normality. One-way ANOVA was conducted using SPSS V19 (IBM Corp., Armonk, NY) to compare mean photon counts at each time point ($p < 0.05$). Pairwise comparison of the means was done using Tukey procedure. Data was then retransformed for interpretation.

Results

Construction of *E. ictaluri* double mutants

The four double mutants constructed in this study included *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdhC*, and *Ei*Δ*glyA*Δ*gcvP*. Colony PCR from chromosomal

DNA of the single crossover mutant strains confirmed the presence of two distinct bands on agarose gel. These results proved integration of the entire plasmid into the *E. ictaluri* chromosome and formation of a merodiploid (one intact copy of the gene and one copy of the gene with a large deletion). LB plus sucrose plates selected for colonies that had undergone a second crossover event. Deletion mutants were genotypically confirmed by colony PCR with flanking primers lflp and lflp. Agarose gel analysis of the colony PCR products confirmed the presence of only the single mutant band, which is smaller than the *E. ictaluri* 93-146 band by the amount that was deleted. On average 91.23% of each gene was deleted; the exact number of bases deleted from each gene is shown in Table 3.3.

Table 3.3 Properties of the *E. ictaluri* TCA cycle and C1 metabolism genes and average percent of gene deleted.

Gene	Product	% Deletion
sdhC-mdh	Succinate dehydrogenase, cytochrome b556 subunit, putative - Malate dehydrogenase, NAD-dependent, putative	87.89
sdhC-frdA	Succinate dehydrogenase, cytochrome b556 subunit, putative- Fumarate reductase, flavoprotein subunit, putative	90.74
sdhC-gcvp	Succinate dehydrogenase, cytochrome b556 subunit, putative - Glycine dehydrogenase, putative	91.1
gcvP-glyA	Glycine dehydrogenase, putative- Serine hydroxymethyltransferase, putative	95.19

Determining the degree of attenuation of constructed double mutants

Channel catfish were intraperitoneally injected with $1x10^6$ CFU of wild-type 93-146, *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdh*C, *Ei*Δ*gcvP*Δ*sdh*C, or *Ei*Δ*gly*AΔ*gcvP*. For wild-type 93-146, this dose was fatal for the channel catfish. All four fish died by 168 hours post-infection. Out of four channel catfish that were infected with *Ei*Δ*frdA*Δ*sdhC*, two were dead at 144 hours post-infection. A similar result was observed in channel catfish

infected with *Ei*Δ*mdh*Δ*sdh*C. One mortality was recorded at 144 hours post-infection in channel catfish infected with *Ei*Δ*glyA*Δ*gcvP.* In contrast, all four channel catfish that were infected with *EiΔgcvP*Δ*sdh*C survived at 168 hours post-infection.

BLI results showed that the infection started from the site of IP injection and disseminated throughout the whole body of the channel catfish by 144 hours post-infection in all the mutants and wild-type *E. ictaluri* (Fig. 3.1). At 168 hours post-infection, there was an average 30-fold reduction in signal intensity in the mutants compared with wild type. In wild-type 93-146, the signal intensity was high at 168 hours post-infection, and all the fish were found dead in the following day. Statistical analysis revealed that there were no significant differences between the mutants and the wild-type strain until 144 hours post-infection ($P<0.05$). At 168 hours post-infection, there were significant differences between wild-type 93-146 and *Ei*Δ*glyA*Δ*gcvP* ($P= 0.022$) and *EiΔgcvP*Δ*sdhC* ($P=0.027$).

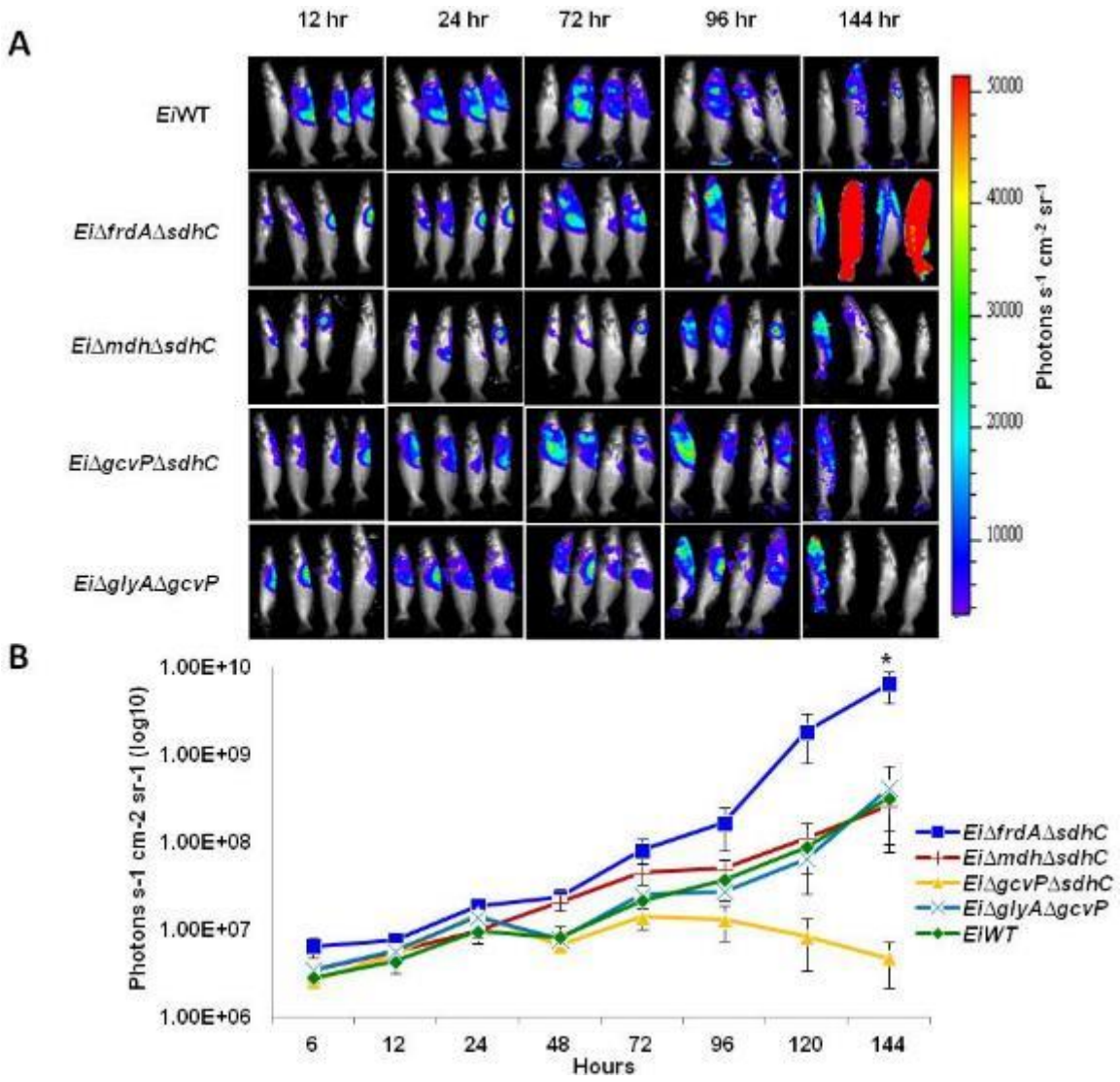

Figure 3.1 Mean photon emissions in IP injected channel catfish ($1X10^6$ CFU/mutant and wild type) at indicated time points post-infection.

Total photon emissions from each fish were quantified using living Image software. Each data point represents the mean photon emissions from four fish. * Two of the four channel catfish injected with *Ei*Δ*frdA*Δ*sdhC* died at 144 h post-infection.

Protection against wild-type *E. ictaluri* 93-146 infection

Results of the protection challenge indicated that the mutants were able to protect channel catfish against subsequent infection with wild-type *E. ictaluri*. When the channel catfish originally infected with $1x10^6$ CFU of *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*,

*Ei*Δ*gcvP*Δ*sdhC*, or *Ei*Δ*glyA*Δ*gcvP* were immersion challenged with 4.8 x10^7 CFU of wild-type 93-146 28 days later, results showed that the mutants provided protection against ESC. BLI imaging revealed that signal intensity in vaccinated channel catfish increased until 48 hours post-infection and then started decreasing from 72 hours post-infection (Fig. 3.2). No mortalities occurred from wild-type *E. ictaluri* infection in all of the mutant-exposed fish. However, in sham–exposed channel catfish, signal intensity increased until 96 hours post-infection, and all the channel catfish died within 120 hours post-infection. Thus, the *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, *Ei*Δ*gcvP*Δ*SdhC*, and *Ei*Δ*glyA*Δ*gcvP* mutants protected against a challenge with wild-type 93-146 and were significantly different from the PBS control at 96 hours post-infection ($P<0.001$).

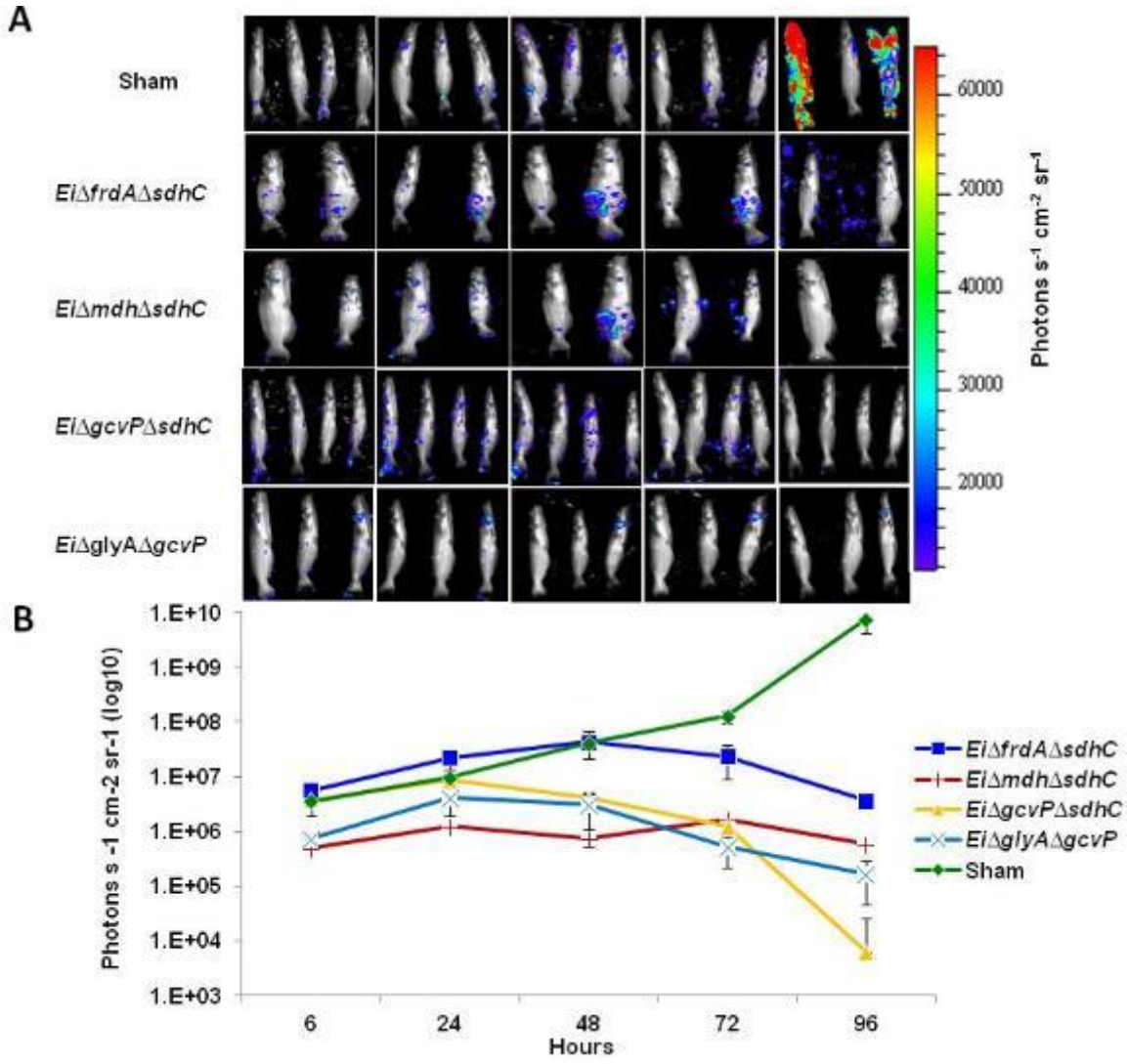

Figure 3.2 Mean photon emissions in immersion challenged channel catfish by 93-146 *E. ictaluri* at indicated time point post-infections.

Channel catfish were immersion challenged in water containing 93-146 *E. ictaluri* (4.8 x10^7 CFU/ml). Total photon emissions from each fish were quantified using living Image software. Each data point represents the mean photon emissions from four fish.

Vaccine trial

Results of the vaccine trial indicated that the constructed mutants *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC, Ei*Δ*gcvP*Δ*sdh*C, and *Ei*Δ*glyA*Δ*gcvP* were efficacious and protected fish against ESC as evidenced by 100% percent survival (Figure 3.3). All the fish that died

following exposure to *E. ictaluri* 93-146 exhibited clinical signs typical of ESC. The survival rate in vaccinated fish was an average of 1.96-fold higher than that of the non-vaccinated group when re-challenged with wild-type *E. ictaluri* (100% vs. 51.11%).

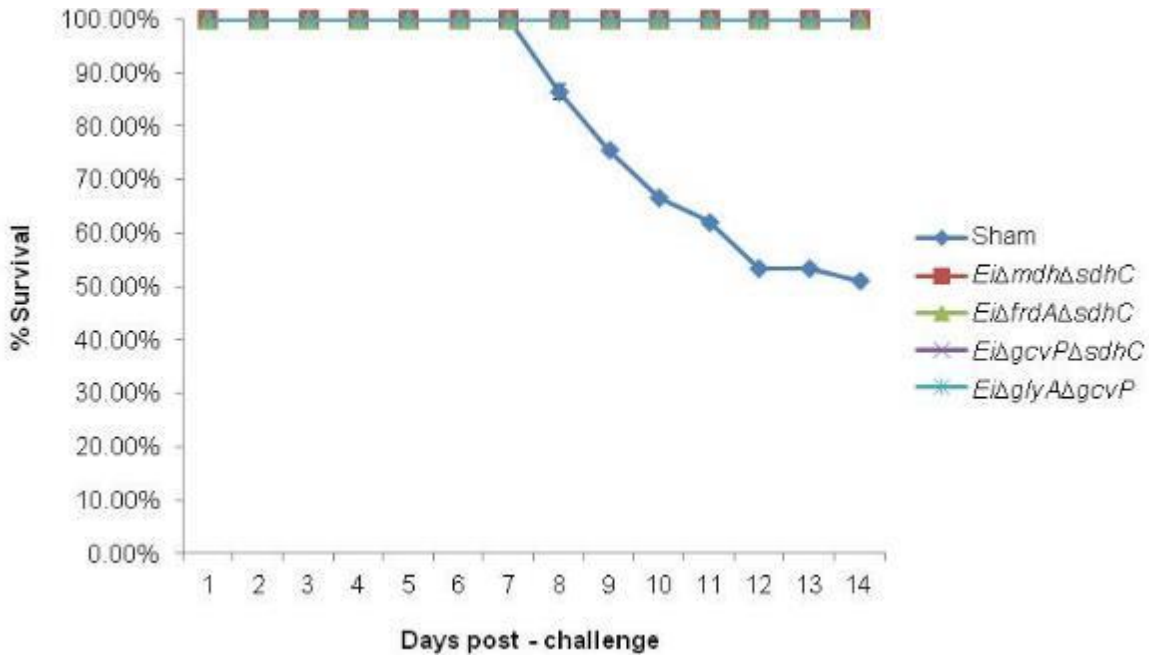

Figure 3.3 Percent survival of channel catfish fingerlings vaccinated with *EiΔfrdAΔsdhC*, *EiΔmdhΔsdhC*, *EiΔgcvPΔsdhC*, and *Ei ΔglyAΔgcvP* following experimental immersion challenge with wild-type *E. ictaluri*.

Discussion

In Chapter II, we showed that the deletion mutants *EiΔfrdA, EiΔsdhC, EiΔmdh, EiΔgcvP,* and *EiΔglyA* were avirulent in channel catfish. Mutants *EiΔfrdA, EiΔsdhC,* and *EiΔmdh* provided protection against subsequent challenge with wild-type 93-146. In the current study, we aimed to improve the safety and attenuation of these mutants by constructing combinations of double mutants in TCA cycle enzymes and 1C metabolism proteins.

Fumarate reductase and succinate dehydrogenase are physiologically reversible isoenzymes in the TCA cycle that are induced under anaerobic and aerobic conditions, respectively (Mercado-Lubo et al., 2007). Malate dehydrogenase is an enzyme that reversibly catalyzes the oxidation of malate to oxaloacetae using the reduction of NAD+ to NADH (Vogel R.F et al., 1987). Mutations in *E. ictaluri* TCA cycle genes caused significant attenuation (Karsi et al., 2009). In particular, the insertion mutants EiAKmut05 and EiAKmut12, which have mutations in *sdhC* and *mdh* genes, were found to be avirulent and provided good protection against ESC in catfish fingerlings. These mutants were highly attenuated by immersion challenge in channel catfish fingerlings; EiAKmut05 caused no mortalities and EiAKmut12 caused 4.45% mortalities compared to wild-type *E. ictaluri*, which caused 93.07% mortality (Karsi et al., 2009). However, when the same mutants were tested in 15 day old catfish fry, the mutants caused mortalities (data not shown). Therefore, our goal in the current study was to construct combination mutations to improve the safety of these vaccine candidates.

The *Ei*Δ*gcvP*Δ*sdhC* mutant has a combination of mutations from two different metabolic pathways: TCA cycle gene *sdhC* and C1 metabolism protein gene *gcvP*. Results from the BLI imaging indicated that the combination of these two pathways resulted in significant attenuation. *Ei*Δ*glyA*Δ*gcvP* has a combination of mutations in genes encoding proteins in two different C1 metabolism pathways. This mutant was also significantly attenuated when compared with wild-type *E. ictaluri*.

The *Ei*Δ*frdA*Δ*sdhC* and *Ei*Δ*mdh*Δ*sdh*C mutants were not fully attenuated. In *Salmonella typhimurium,* mutation in SR-11 Δ*sdhCDA* caused slight attenuation, and the SR-11 Δ*frdABCD* mutant was fully virulent (Mercado-Lubo et al., 2008). However,

mutant SR-11 Δ*sdhCDA* and SR-11 Δ*frdABCD* were combined, the resulting combination mutant was fully attenuated (Mercado-Lubo et al., 2008). In Chapter II, when testing the single deletion mutants for attenuation, we used 10^3 CFU/ml and found that they were significantly attenuated. However, in the current study, channel catfish were injected with 10^6 CFU/ml of each combination mutant, which is 1000-fold more than what we used for the single deletion mutants. This is likely why there were some mortalities caused by *Ei*Δ*frdA*Δ*sdhC* and *Ei*Δ*mdh*Δ*sdhC*. Nevertheless, the subsequent vaccine trial result showed 100% percent survival for *Ei*Δ*frdA*Δ*sdhC* and *Ei*Δ*mdh*Δ*sdhC* vaccinated fish.

The constructed combination mutants *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdh*C, *Ei*Δ*gcvP*Δ*sdhC*, and *Ei*Δ*glyA*Δ*gcvP* were not virulent in catfish fingerlings using the immersion route of exposure. BLI results also showed significant attenuation of these mutants compared to wild-type *E. ictaluri*. Current production practices in commercial catfish production only allow immersion vaccination of channel catfish fry at 10-15 days old. Fry at this age are more susceptible to ESC than fingerlings. Furthermore, to be effective, protective immunity must last through summer and fall. Based on these results in fingerlings, our next goal will be to test these vaccine candidates in 10-14 day old fry. To develop protective immunity in channel catfish, mutants must persist in the tissues for some time. Therefore, the ability of the constructed mutants to persist in catfish tissues will also be examined next.

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CHAPTER IV

TISSUE PERSISTENCE AND VACCINE EFFICACY OF TCA CYCLE AND SINGLE CARBON METABOLISM MUTANT STRAINS OF *EDWARDSIELLA ICTALURI*

Abstract

Studies on the tissue persistence and vaccine efficacy of previously constructed TCA cycle (*Ei*Δ*sdhC, Ei*Δ*frdA,* and *Ei*Δ*mdh*) mutants, single carbon metabolism mutants (*Ei*Δ*gcvP* and *Ei*Δ*glyA*), and combination mutants *(Ei*Δ*frdA*Δ*sdhC, Ei*Δ*gcvP*Δ*sdhC, Ei*Δ*mdh*Δ*sdhC,* and *Ei*Δ*gcvP*Δ*glyA)* of *E. ictaluri* were conducted in channel catfish (*Ictalurus punctatus)*. Mutant strains *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*gcvP*Δ*sdhC* were able to invade the host and persist in the trunk kidney. Numbers of bacteria found in catfish trunk kidney tissue were high for all the mutants until 11 days post-infection, and then they decreased. Vaccination with mutants *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*gcvP, Ei*Δ*glyA, Ei*Δ*frdA*Δ*sdhC, Ei*Δ*gcvP*Δ*sdhC, Ei*Δ*mdh*Δ*sdhC,* and *Ei*Δ*gcvP*Δ*glyA* by immersion challenge in catfish fingerlings provided substantial protection against subsequent immersion challenge with the virulent parental strain. All mutants had 100% percent survival except for the mutants *Ei*Δ*gcvP*Δ*glyA* and *Ei*Δ*gcvP*, for which the percent survival were greater than 87%. Mutant *Ei*Δ*sdhC*Δ*gcvP* was found to be very good at protecting catfish fry, as evidenced by 9.9 fold higher percent survival compared to non-vaccinated fish.

Introduction

Enteric septicemia of catfish (ESC) is still an important disease of farm-raised channel catfish (*Ictalurus punctatus*). In 1999, economic losses due to ESC were estimated to be as high as $60 million (Klesius and Shoemaker, 1999). In 2010, catfish farmers operating in Alabama, Mississippi, Arkansas, and Louisiana reported 36.6% mortality rates due to ESC (U.S. Department of Agriculture 2012). Although considerable progress has been made in developing live attenuated vaccines (Klesius and Shoemaker 1999; Lawrence et al. 1997; Thune et al. 1999; Abdelhamed et al. 2013), ESC is still a major threat to the catfish industry. Until recently, only one vaccine has been commercially approved for use against ESC.

Live attenuated strains of facultative, intracellular bacterial pathogens should maintain their ability to invade the host via oral or immersion route, and they are often effective in developing strong cell mediated immune (CMI) responses (Thune et al. 1999). Karsi et al. (2009) reported that genes encoding TCA cycle enzymes and one-carbon metabolism proteins are required for virulence of *E. ictaluri*. To better understand the role of *E. ictaluri* TCA cycle genes and one-carbon metabolism genes in pathogenesis and immunity, mutants with in-frame deletions in genes encoding TCA cycle enzymes (*sdhC, mdh*, and *frdA*) and genes encoding one-carbon metabolism proteins (*gcvP* and *glyA*) were constructed (Chapter II). To improve safety of vaccine candidates, combinations of mutations (*sdhC-frdA*, *sdhC-mdh*, *sdhC-gcvp* and *gcvP-glyA*) were also constructed (Chapter III).

The *sdhC* gene encodes one of the four subunits of the succinate dehydrogenase (Sdh) complex and oxidizes succinate to fumurate while reducing ubiquinone to

ubiquinol (Nakamura et al. 1996). Fumrate reductase (frd) catalyzes the reverse reaction in aerobic respiration (Nakamura et al. 1996). Sdh is known to contribute pathogenicity in *E. coli* and *Salmonella* (Nakamura et al. 1996 and Mercado-Lubo et al. 2008). Mercado-Lubo et al. (2008) reported slight attenuation of a Δ*sdhCDA* mutant in *S. enterica* serovar Typhimurium. They also showed that a Δ*frdABCD* mutant was fully virulent. However, they achieved complete attenuation by constructing a *sdh* and *frd* double mutation. Malate dehydrogenase (encoded by *mdh*) catalyzes the conversion of oxaloacetate and malate utilizing the NAD/NADH coenzyme system (Minárik et al. 2002). Using a mouse oral challenge model, *Salmonella mdh* mutants were avirulent (Tchawa Yimga et al., 2006). The *gcvP* gene encodes a protein that is part of the glycine cleaveage system, and *glyA* encodes serine hydroxymethyltransferase. To our knowledge, these two genes have not been linked with virulence in other bacterial species, but Karsi at al. (2009) showed that the GcvP protein is critical for both neutrophil and serum resistance in *E. ictaluri*.

Previously, we determined the degree of attenuation of the TCA cycle and C-1 metabolism protein mutants in channel catfish fingerlings using bioluminescence imaging (Chapters II and III). We found that the in-frame deletion mutants *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*gcvP, Ei*Δ*glyA,* and *Ei*Δ*gcvP*Δ*sdhC* were highly attenuated, whereas mutant strains *Ei*Δ*gcvP*Δ*glyA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*mdh*Δ*sdhC* were less attenuated in catfish fingerlings. However, catfish fingerlings were intraperitoneally injected with the mutants, which is generally not a favored route of vaccination. In the current study, vaccine efficacy of mutants was tested in catfish fingerlings and fry by the immersion route, which is considered the natural and the cost-effective route of vaccination. Therefore, the

objectives of this study were to determine the tissue persistence and vaccine potential of TCA cycle and C-1 metabolism mutant strains of *Edwardsiella ictaluri* by using immersion challenge.

Materials and methods

Bacterial strains

E. ictaluri 93-146 and *EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔgcvP, EiΔglyA, EiΔfrdAΔsdhC, EiΔgcvPΔsdhC, EiΔmdhΔsdhC,* and *EiΔgcvPΔglyA* were grown at 30°C on brain-heart infusion (BHI) agar plates. *E. ictaluri* selective medium (EIM) was used for the tissue persistence study to quantify *E. ictaluri* from serially diluted tissue homogenate. For vaccine challenges, *E. ictaluri* 93-146 and *EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔgcvP, EiΔglyA, EiΔfrdAΔsdhC, EiΔgcvPΔsdhC, EiΔmdhΔsdhC,* and *EiΔgcvPΔglyA* were grown in BHI broth for 18 hours at 30°C by shaking at 175 rpm. For the tissue persistence study, *E. ictaluri* 93-146 and *EiΔsdhC, EiΔfrdA, EiΔfrdAΔsdhC,* and *EiΔgcvPΔsdhC* with pAKgfplux1 plasmid (Karsi et al., 2006) were grown in BHI broth with ampicillin (100µg/ml) for 18 hours at 30°C by shaking at 175 rpm.

Tissue persistence

240 six-month-old catfish fingerlings were stocked into ten 40-L tanks (24 fingerlings per tank) supplied with flow-through dechlorinated municipal water and the temperature was maintained at 28 °C throughout the trail. Fish were allowed to acclimate for a week. After a week, two tanks for each mutant group were experimentally exposed to *EiΔsdhC, EiΔfrdA, EiΔfrdAΔsdhC,* and *EiΔgcvPΔsdhC*, and fish in the other two tanks were exposed to wild-type *E. ictaluri* 93-146.

Before exposure, one fish was removed from each tank to establish the baseline negative control. Experimental immersion exposure was conducted by lowering water level in each tank to 10-L with aeration, and fish were exposed to bacterial culture corresponding to 1 x 10^7 colony formation unit (CFU) per milliliter (ml) of water. After one hour, water flow was restored to allow gradual removal of the bacteria.

At each time point, three fish were randomly selected and removed from each tank and euthanatized by transferring to water containing 1 g tricaine methanesulfonate (Sigma-Aldrich, St. Louis, Missouri) per liter of water. Sampling points were at 2 h, 6 h, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d, 9 d, 11 d, 14 d, 17 d, and 21 d post-infection. Trunk kidney was aseptically removed from each fish and weighed. Bioluminescence was quantified from the tissue at each time point using an IVIS Imaging System (Caliper Corporation, Hopkinton, Massachusetts). After imaging, each tissue was suspended in 0.5 ml of sterile water and macerated. The resulting suspension was serially diluted depending upon the bioluminescent signal from IVIS imaging, and 15 µl aliquots were spread on EIM plates for quantification. The colony counts were determined at 48h of incubation. The *E. ictaluri* colonies were identified as 0.5-1.0 millimeter (mm) green, translucent colonies (Shotts and Waltman, 1990). The number of CFU/gm of tissue was calculated for each fish.

For statistical analysis, the calculated CFU/gm was transformed by taking the base 10 logarithm to improve normality. To compare mean bacterial concentrations between strains at each time point, a one-way ANOVA of the transformed data was conducted using SPSS 19 statistical software (SPSS Inc., 2010). Pairwise comparison of

the means was done using Tukey procedure at significance level of 0.05. Data was then retransformed for interpretation.

Vaccine trial

Four-month-old channel catfish were stocked at a rate of 15/tank into 33 40-L tanks and randomly divided into 11 treatment groups with three tanks per treatment. Nine of the 11 treatment groups were vaccinated with *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*gcvP, Ei*Δ*glyA, Ei*Δ*frdA*Δ*sdhC, Ei*Δ*sdhC*Δ*gcvP, Ei*Δ*sdhC*Δ*mdh,* and *Ei*Δ*glyAC*Δ*gcvP*, and one of the 11 treatment groups was exposed to *E. ictaluri* 93-146. One treatment group served as sham-vaccinated control. Immersion challenge was done by lowering the water level from each tank to 10-L, and 100 ml of bacterial culture was added to each tank (on average 3.25 X 10^7 CFU/ml)) and, 100 ml of BHI media was added to Sham-vaccination control. After one hour, water flow was restored to each tank. Mortalities in each tank were recorded for 21 days.

On day 21 post-vaccination, vaccinated and non-vaccinated treatments were experimentally infected with wild-type *E. ictaluri* by immersion exposure with a final bacterial concentration of approximately 3.1 X 10^7 CFU/ml in water. Water flow was stopped for one hour following exposure and then resumed after one hour, which allowed gradual removal of the bacteria. Percent mortalities were determined for each tank after two weeks.

Vaccine safety in catfish fry

Vaccine safety in catfish fry was determined for each five single and four double mutants. Briefly, 14 day old specific pathogen free (SPF) catfish fry were transferred into

42 tanks (approximately 70 fry/tank, three replicates per treatment). Fry were immersion exposed to two doses (10^7 CFU/ml and 10^6 CFU/ml) for combination mutants (*Ei*Δ*frdA*Δ*sdhC, Ei*Δ*gcvP*Δ*sdhC, Ei*Δ*mdh*Δ*sdhC,* and *Ei*Δ*gcvP*Δ*glyAC*) and a single dose of 10^7 CFU/ml for single mutants (*Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*gcvP,* and *Ei*Δ*glyA*). Fry were immersion exposed to 100 ml of BHI media for sham-vaccination control. Immersion challenges were conducted for one hour with subsequent gradual removal of bacteria as described above. Mortalities were recorded daily for 21 days.

On day 21 post-vaccination, all treatments were immersion exposed to parent strain *E. ictaluri* 93-146 at approximately 10^7 CFU/ml using the standard procedure described above.

Results

Tissue persistence

At each time point until 11 days post-infection, *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*sdhC*Δ*gcvP* mutants were isolated on EIM plates from trunk kidney of catfish. The mean CFU/gm of posterior kidney tissue for *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC, Ei*Δ*sdhC*Δ*gcvP* mutants*,* and wild-type *E. ictaluri* are shown in Figure 4.1. Tissue concentration of wild-typestrain was higher than mutants at 96 h, 120 h, 7 d, 9 d, 11 d, and 14 d post-inoculation. From 5 d to 9 d post-infection, tissue concentrations were higher for *Ei*Δ*frdA* and *Ei*Δ*sdhC*Δ*gcvP* compared with *Ei*Δ*sdhC* and *Ei*Δ*frdA*Δ*sdhC.* There was no significant difference between the mean concentration of wild-type *E. ictaluri* and the mutants *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*sdhC*Δ*gcvP* 2 h until 7 days post-infection. However, the tissue concentration of

wild-type *E. ictaluri* was significantly higher than the concentration of *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*sdhC*Δ*gcvP* at 9, 11 and 14 days post-infection.

Bioluminescence detection was less sensitive than CFU/gm determinations to measure bacterial tissue concentrations. No signal or very low signal was detected in the tissue of all the mutants at most of the time points except for 24 h to 96 h post-infection for *EiΔsdhC*, *EiΔfrdA* mutants and wild-type *E. ictaluri*. At these time points, bioluminescence in kidney tissue was high for these two mutants (data not shown).

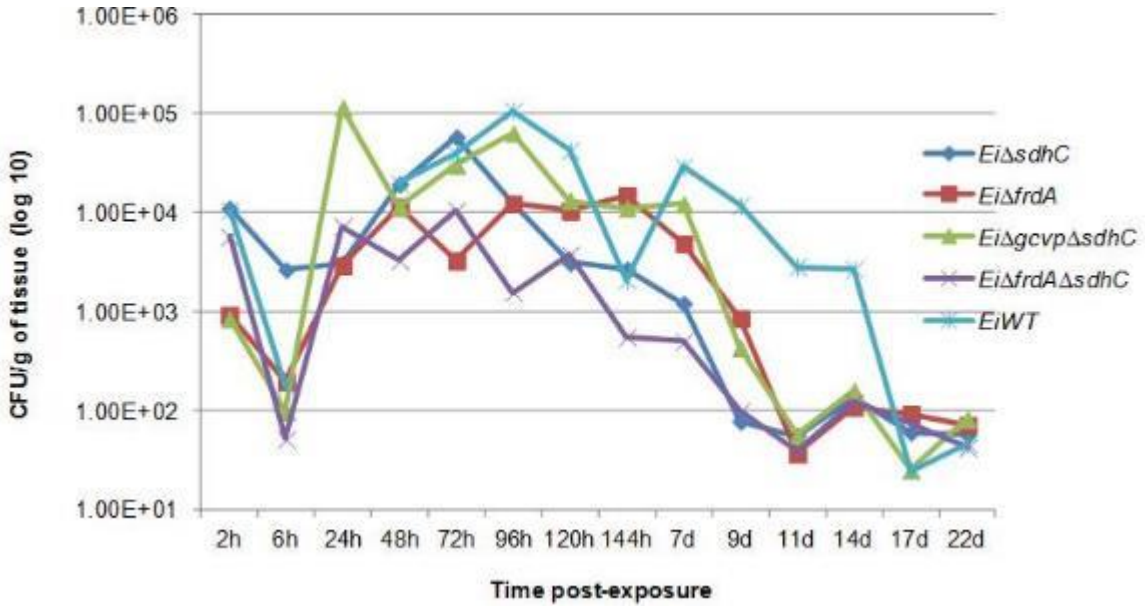

Figure 4.1 Mean tissue concentrations (log_{10} transformed colony forming units per gram (CFU/gm) of the *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*gcvP*Δ*sdhC* mutants and wild-type *E. ictaluri* strain 93-146 in posterior kidneys of channel catfish at various sampling times following immersion exposure.

The mean CFU/gm of posterior kidney is not shown for wild-type *E. ictaluri* at 24 h post-infection due to technical difficulties.

Vaccine trial

To confirm attenuation of the nine mutants (*Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*gcvP, Ei*Δ*glyA, Ei*Δ*frdA*Δ*sdhC, Ei*Δ*sdhC*Δ*gcvP, Ei*Δ*sdhC*Δ*mdh,* and *Ei*Δ*glyA*Δ*gcvP*), an experimental infection by immersion exposure was conducted in catfish fingerlings. Mutants *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*frdA*Δ*sdhC, Ei*Δ*sdhC*Δ*gcvP, and Ei*Δ*sdhC*Δ*mdh* were found to be subsequently attenuated when compared to wild-type *E. ictaluri* (Fig. 4.2A). In fact, no mortalities occurred for these mutants; by comparison, wild-type *E. ictaluri* caused 61.61% mortalities. *Ei*Δ*glyAC*Δ*gcvP, Ei*Δ*gcvP*, and *Ei*Δ*glyA* were the least attenuated mutants, causing 29.63%, 38.54%, and 40.14% fish mortalities, respectively (Fig. 4.2A).

Results of the subsequent efficacy trial are presented Fig. 4.2B. Sham-vaccinated catfish fingerlings had a final percent survival of 47.06%, which is subsequently lower than the percent survival for mutants *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*glyA, Ei*Δ*frdA*Δ*sdhC, Ei*Δ*sdhC*Δ*gcvP,* and *Ei*Δ*sdhC*Δ*mdh*, which had 100% survival. Mutants *Ei*Δ*glyAC*Δ*gcvP* and *Ei*Δ*gcvP* had slightly lower percent survival (94.75%, and 87.98%, respectively) when compared with the other mutants (Fig. 4.2B). All of the mortalities had external and internal lesions that were consistent with ESC.

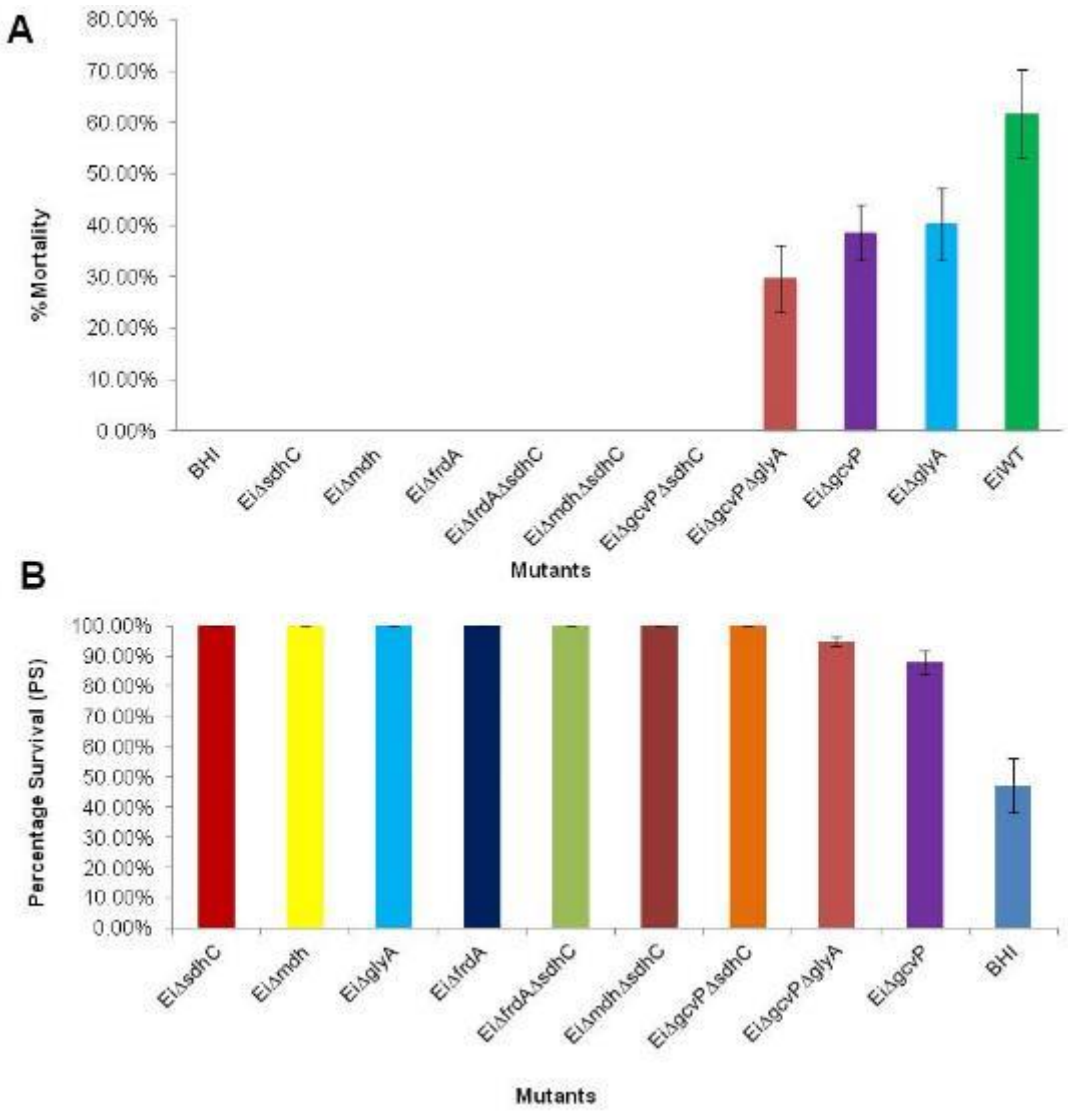

Figure 4.2 Percent mortalities and survival of channel catfish fingerlings.

(A). Percent mortalities of catfish fingerlings challenged with the *EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔgcvP, EiΔglyA, EiΔfrdAΔsdhC, EiΔgcvPΔsdhC, EiΔmdhΔsdhC,* and *EiΔgcvPΔglyAC* mutants and wild-type *E. ictaluri* (B). Percent survival of channel catfish fingerlings vaccinated with the *EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔgcvP, EiΔglyA, EiΔfrdAΔsdhC, EiΔgcvPΔsdhC, EiΔmdhΔsdhC,* and *EiΔgcvPΔglyAC* mutants and challenged with wild-type *E. ictaluri.*

Vaccine safety in catfish fry

Results of the safety trial in catfish fry indicated that combination mutants were less virulent than single knockout mutants were (Fig. 4.3A). Interestingly, there was one

exception: combination mutant *Ei*Δ*frdA*Δ*sdhC* caused higher mortalities than *Ei*Δ*frdA*. *Ei*Δ*frdA* (14% mortalities) was also less virulent than mutant strains *Ei*Δ*mdh* and *Ei*Δ*sdhC* (63% and 76% mortalities, respectively). Mutant strains *Ei*Δ*glyA* and *Ei*Δ*gcvP* were fully virulent in catfish fry, causing 100% mortalities (Fig. 4.3A). In combination mutants, fry challenged with the lower dose (1 x 10^6 CFU/ml) had lower mortalities than fry challenged with the standard vaccine dose (1 x 10^7 CFU/ml). Overall, the safest vaccine candidates at the 1 x 10^7 CFU/ml dose were *Ei*Δ*gcvP*Δ*sdhC* and *Ei*Δ*frdA*.

Preliminary vaccine efficacy of the mutants was determined by challenging vaccinated fry with parent strain 93-146 by immersion. Protection was better in catfish fry vaccinated with the higher dose of combination mutants compared to catfish fry vaccinated with the lower dose (Fig. 4.3B). The best percent survival was in fry vaccinated with *Ei*Δ*gcvP*Δ*sdhC* which gave 99% protection. Mutant *Ei*Δ*frdA* also gave 60% protection (Fig. 4.3B).

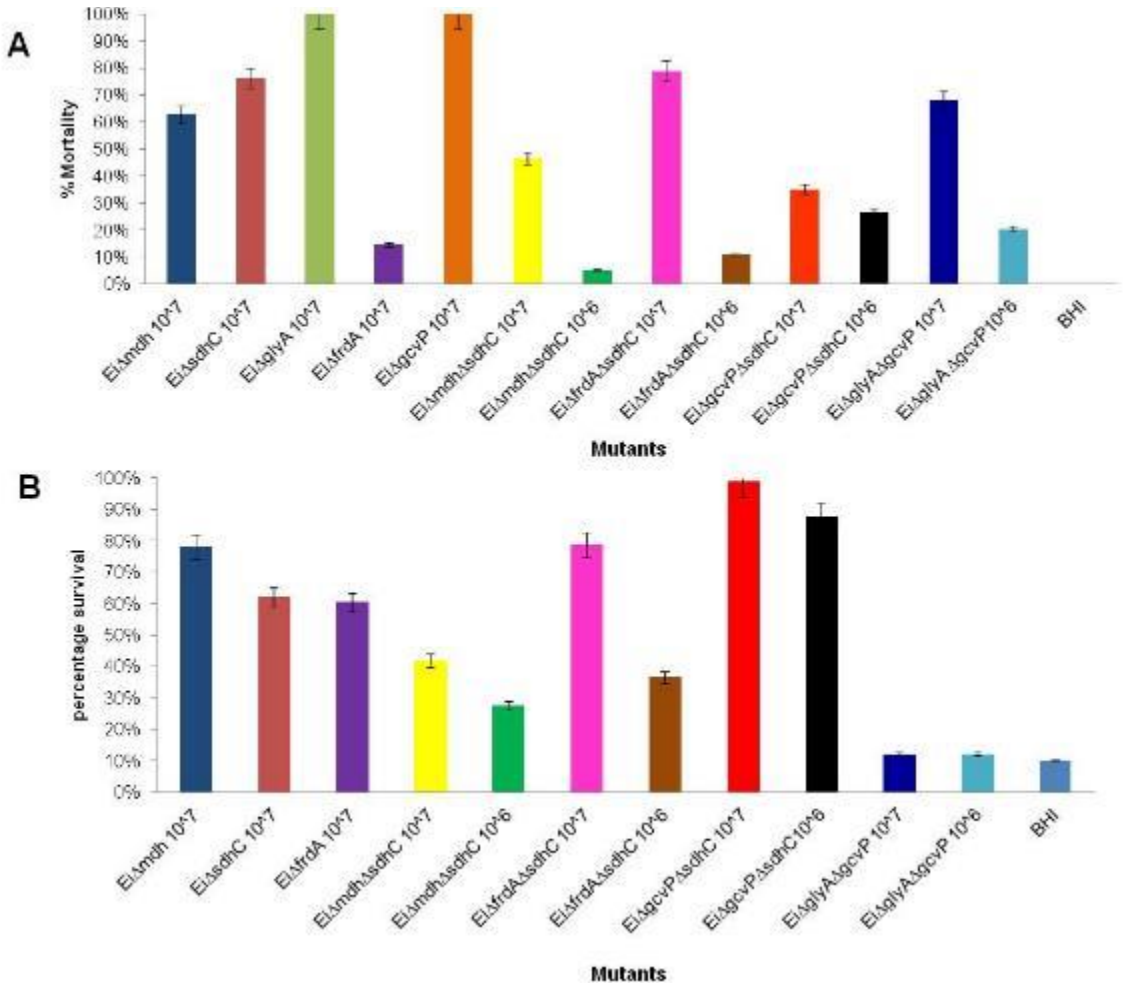

Figure 4.3 Percent mortalities and survivals of channel catfish fry.

(A). Percent mortalities of catfish fry challenged with mutants *EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔgcvP, EiΔglyA, EiΔfrdAΔsdhC, EiΔgcvPΔsdhC, EiΔmdhΔsdhC,* and *EiΔglyACΔgcvP*. (B). Percent survival of catfish fry vaccinated with mutants *EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔgcvP,* and *EiΔglyA*, *EiΔfrdAΔsdhC, EiΔgcvPΔsdhC, EiΔmdhΔsdhC,* and *EiΔgcvPΔglyA* when challenged with the *E. ictaluri* wild-type strain

Discussion

The primary objective of this study was to determine tissue persistence and vaccine potential of TCA pathway and single carbon metabolism mutant strains of *E. ictaluri*. The tissue persistence study was done using posterior kidney because *E. ictaluri* is consistently isolated from this organ (Baldwin and Newton 1993; Lawrence et al. 1997;

Thune et al. 1999; Lawrence and Banes 2005). Results demonstrated that the TCA pathway and single carbon metabolism mutant strains *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*gcvP*Δ*sdhC* retained the ability to penetrate channel catfish mucosa and colonize posterior kidney, indicating that attenuation of the mutant stains in the immersion route of exposure is not due to its inability to invade the host.

All the mutant strains were isolated from the posterior kidney from 2 h to 22 d post-exposure. In fact, tissue concentrations were similar between wild-type *E. ictaluri* and mutant strain *Ei*Δ*sdhC*Δ*gcvP* from 48 h post-infection to 7 d post-infection. *E. ictaluri* auxotrophic mutants (*purA* and *aroA*) (Lawrence et al 1997; Thune et al 1999) were cleared by 72 h post-infection, whereas 93-146 R6 mutant was found to persist for 14 d post-exposure (Lawrence and Banes 2005). Our results indicated that the constructed mutants *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*gcvP*Δ*sdhC* were able to persist as long as wild-type *E. ictaluri* 93-146 persisted. Studies have suggested that *E. ictaluri* can survive and replicate in the macrophage, which might be reason for its longer persistence time (Baldwin and Newton 1993: Miyazaki and Plumb 1985; Shotts et al 1986). In *Salmonella enterica* and *Yersina pestis*, purine and aromatic auxotrophs lost the ability to survive inside macrophages (Fields et al. 1986; Straley and Harmon 1984); perhaps that might be the reason why *E. ictaluri purA* and *aroA* mutants were cleared by 72 h post-infection (Lawrence et al 1997; Thune et al 1999). Recent studies with *Salmonella typhimurium* indicate that mutants with deletion in genes encoding TCA cycle enzymes have the ability to replicate within resting and activated macrophages, suggesting an enhanced ability to survive antimicrobial mechanisms (Bowden et al., 2010). Perhaps *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*sdhC*Δ*gcvP* mutants have the

ability to survive in channel catfish macrophages, which can partially explain the ability to persist longer as observed in this study.

For vaccine efficacy, it is important for the mutant to persist longer and achieve tissue concentration high enough to stimulate protective immunity. The result of the vaccine trials indicated that immersion vaccination of channel catfish fingerlings with *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*gcvP, Ei*Δ*glyA, Ei*Δ*frdA*Δ*sdhC, Ei*Δ*gcvP*Δ*sdhC, Ei*Δ*mdh*Δ*sdhC,* and *Ei*Δ*gcvP*Δ*glyAC* mutants provided significant protection against experimental infection with wild-type *E. ictaluri*. This is similar to the results reported by Karsi et al. (2009), which indicated that insertion mutations in TCA cycle genes and glycine cleavage protein provided significant protection against *E. ictaluri* in channel catfish fingerlings.

Results from this study indicated that *E. ictaluri* mutants with deletions in genes encoding TCA cycle enzymes and C-1 metabolism proteins retained their ability to invade catfish fingerlings from water, persisted in the host, and provided significant protection from ESC by immersion vaccination under controlled laboratory conditions. In 15 d old catfish fry, mutant combinations improved safety of vaccine candidates. The mutant *Ei* Δ*gcvP* Δ*sdhC*was the safest vaccine candidate in catfish fry, and it provided the best protection against *E. ictaluri* infection. This finding suggests that future research should be aimed at adding a *mdh* or *frdA* mutation to *Ei*Δ*gcvP*Δ*sdhC* to improve vaccine safety in channel catfish fry.

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CHAPTER V

UNDERSTANDING THE MECHANISM OF *SDHC* MUTANT ATTENUATION IN SUPEROXIDE PRODUCTION

Abstract

Succinate dehydrogenase (SdhCDAB) catalyzes the oxidation of succinate to fumarate in the TCA cycle, and during the completion of the catalysis process (turnover), it produces superoxide radicals. In *E. coli* and some eukaryotes like *Caenorhabditis elegans* and yeast, mutations in *sdh* gene are associated with increased production of superoxide. The increased production of superoxide may result in metabolic oxidative stress and genomic instability leading ultimately to cell death. To assess extracellular superoxide (O_2^-) production by wild-type *E. ictaluri* and succinate dehydrogenase mutants (*Ei*Δ*sdhC* and *Ei*Δ*sdhA)*, we compared the abilities of these strains to reduce ferriccytochrome c in the presence and absence of superoxide dismutase (SOD). The effect of superoxide production on the viability of TCA cycle mutants was determined by comparing the survivability of *sdh* and *frd* mutants with wild-type *E. ictaluri* in the presence of hydrogen peroxide. No enhanced superoxide production, as indicated by no reduction in ferriccytochrome c, was detected in the *Ei*Δ*sdhC* and *Ei*Δ*sdhA* mutants. The viability of *Ei*Δ*sdhC* mutant decreased by 59.59% in the absence of SOD, and increased by 79.79% in the presence of SOD.

Introduction

Succinate dehydrogenase (Sdh), also known as succinate-coenzyme Q reductase (SQR) or Complex II, is an enzyme complex present in all aerobic cells (Zhao et al. 2006). Sdh is a membrane-bound dehydrogenase linked to the respiratory chain, and it is a part of the tricarboxylic acid (TCA) cycle (Hajjawi 2011). Its activity is modulated by several activators and inhibitors, and it has covalently bound FAD (Hajjawi 2011).

In bacteria, Sdh is encoded by *sdhCDAB* and is a structural homologue of complex II. In aerobic bacteria, it catalyzes the oxidation of succinate to fumarate in the TCA cycle and electron transfer from succinate to the ubiquinone pool in the respiratory chain (Zhao et al., 2006). SdhCDAB consists of 4 subunits: two hydrophilic and two hydrophobic. The first two subunits, a flavoprotein (SdhA) and an iron-sulfur protein (SdhB) are hydrophilic (Nakamura et al. 1996). SdhA contains a covalently attached FAD cofactor and the succinate binding site. SdhB contains three iron-sulfur clusters: [2Fe-2S], [4Fe-4S], and [3Fe-4S]. The second two subunits are hydrophobic membrane anchor subunits, SdhC and SdhD (Page et al., 1999).

Recent studies have suggested that mutations in the gene encoding SdhC cause an increase in O_2^- production, metabolic oxidative stress, and genomic instability in hamster fibroblasts (Slane et al., 2006). Ishii et al. (2005) showed that a point mutation within the coenzyme Q (CoQ) binding site of mev-1 (homologous to SdhC) leads to rapid aging and hypersensitivity to oxygen toxicity in *C. elegans*. Similarly, Zhao et al. (2006) found enhanced superoxide production in site-directed mutations around the heme and at the ubiquinone-binding site (Q site) in *E. coli* succinate dehydrogenase. Based on these studies, we hypothesize that the mutation in succinate dehydrogenase enzyme of *E.*

ictaluri may result in increased production of superoxide, which may cause oxidative stress and DNA damage in the mutant strain *Ei*Δ*sdhC*.

E. ictaluri is facultative anaerobic bacteria. Therefore, the TCA cycle is important for them during aerobic respiration. We have previously constructed mutations in genes encoding TCA cycle enzymes, and it is important to know whether there are growth differences between these mutants and wild-type *E. ictaluri*. We expect that aerobic growth will not be efficient in *E. ictaluri* TCA cycle mutants, but anaerobic growth should be similar between TCA cycle mutants and wild-type *E. ictaluri*. Therefore, we compared aerobic and anaerobic growth kinetics between wild-type strain 93-146 and *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*sdhC*Δ*frdA,* and *Ei*Δ*mdh*Δ*sdhC* mutants.

The effect of *sdhC* deletion on endogenous superoxide production in *E. ictaluri* was compared to wild-type *E. ictaluri* using ferricytochrome c reduction. To determine if effects on superoxide production are specific to the *sdhC* subunit, superoxide production was also compared to a *sdhA* deletion mutant (courtesy of Dr. Attila Karsi). Finally, the effect of superoxide production on viability of TCA cycle mutants was determined by comparing survivability of the *sdh* mutants on hydrogen peroxide (H_2O_2) relative to wild-type *E. ictaluri* both in the presence and in the absence of superoxide dismutase (SOD).

Material and methods

Growth kinetics

*Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*frdA* Δ*sdhC,* and *Ei*Δ*mdh*Δ*sdhC* mutants and wild-type *E. ictaluri* were grown in triplicate in brain heart infusion (BHI) broth at 30°C. Aerobic condition was obtained by growing the cultures at 30°C with rotary shaking. All anaerobic growth assays were conducted using sealed vials containing medium. Vials

were acclimated to anaerobic conditions in an anaerobic chamber and were kept at 30°C throughout the experiment. Bacterial growth was measured at 6 h, 8 h, 12 h, 18 h, 24 h, 36 h, and 48 h post-inoculation using a spectrophotometer at OD_{600}.

Detection and measurement of extracellular superoxide

For the detection and measurement of extracellular superoxide production, we followed the protocols developed by Huycke et al. (1996) and modified by Korshunov and Imlay (2006). All the reagents used in this study were brought from Sigma-Aldrich (St. Louis, MO).

The production of extracellular superoxide was determined in wild-type *E. ictaluri* and *Ei*Δ*sdhC, Ei*Δ*sdhA,* and *Ei*Δ*frdA*Δ*sdhC* mutants by measuring the ability of superoxide to reduce ferricytochrome c. Each treatment included three replicates. To measure the total extracellular superoxide, bacteria from exponential-phase (optical density at 600 nm (OD_{600}) of 0.6) were centrifuged, washed with PBS, and resuspended to an OD_{600} of 0.2 in two 50-ml flasks, each containing 10 ml of pre-warmed PBS supplemented with 0.2% glucose and 20 μM cytochrome c. Superoxide dismutase (SOD) (30 U/ml) was added to one of the two flasks, and they were incubated at 30°C in a shaking water bath. At 20, 40, and 60 min, 1.5 ml of cell suspension was withdrawn from each flask and filtered using syringe filters. The filtrates were kept on ice, and the amount of reduced cytochrome c was determined using the absorbance at 550 nm. Then 0.2 μM potassium ferricyanide was added to the filtrates to oxidize cytochrome c, and then the spectra were recorded again. The amount of reduced cytochrome c was calculated using the ferricyanide-induced absorbance change at 550 nm. The fraction of cytochrome c

reduction mediated by superoxide was then determined by comparing the degrees of reduction in the paired samples with and without SOD.

To quantify superoxide formation by excreted metabolites, cell suspensions were incubated without the addition of cytochrome c. At time points 20, 40, and 60 min, cells were removed by filtration, and cytochrome c was added to the filtrate. Each aliquot was split, and SOD was added to one of each of the paired samples. The samples were incubated at 30°C for 15 minutes, which is sufficient to essentially complete the autoxidation of excreted metabolites. Similar to the methods for extracellular superoxide quantification, the amount of reduced cytochrome c was then determined using the absorbance at 550 nm. Then 0.2 μM potassium ferricyanide was added to the filtrates to oxidize cytochrome c, and then the spectra were recorded again. The fraction of cytochrome c reduction mediated by superoxide was then determined by subtracting the absorbance values from the paired samples with and without SOD.

Enzyme assay

E. ictaluri membranes were isolated using the protocol developed by Maklashina et al. (1998). Briefly, 500 ml broth cultures of *Ei*Δ*sdhC* and *Ei*Δ*sdhA* were grown until OD_{600} reached 0.8. Bacteria were collected by centrifugation at 4,500 × *g* for 10 min, then suspended with 100 ml of 100 mM PBS–5 mM EDTA (pH 7.6), centrifuged again as described above, suspended in 40 ml of the same buffer, and then frozen at −70°C. Unlysed bacteria were removed by centrifugation (15 min at 10,000 × *g*), and the supernatant was centrifuged for 60 min at 100,000 × *g* to collect the membranes. The membrane fraction was suspended in 30 ml of the same buffer, and both centrifugation

steps were repeated. Membranes were suspended in 50 mM PBS–0.2 mM EDTA (pH 7.8) to approximately 15 mg of protein/ml and frozen at −70°C.

Succinate dehydrogenase enzyme activity was measured using the protocol reported by Zhao et al. (2006). Briefly, succinate dehydrogenase activity was assessed by measuring phenazine methosulfate (PMS) mediated reduction of 2,6 dichlorophenolindophenol (DCIP). Electron transfer was initiated by adding 10 mmol/L succinate to the mixture containing three concentrations of membrane fraction (5 μg/ml, 10 μg/ml, and 15 μg/ml), 0.75 mmol/L PMS, 30 umol/L DCIP, and 1 mmol/L KCN in 0.1 mol/L phosphate buffer at pH 7.8 at room temperature. The reduction of DCIP was monitored by measuring absorbance at 600 nm.

Viability assay

The viability assay was conducted to determine whether providing SOD can protect the viability of the TCA cycle mutants in the presence of H_2O_2. Wild-type and mutants were grown to mid-log phase (OD_{600} 0.8-1), pelleted by centrifugation for 10 min at 4200 rpm, and resuspended in 50 ml PBS (0.2 OD_{600}). After resuspension, wild-type and mutants were split into 4 treatments groups: PBS control, PBS saline with 30% hydrogen peroxide (final concentration 10mM), saline with SOD (final concentration 30U/ml), and saline with both hydrogen peroxide and SOD. Viable cells at 60 min (in triplicate) were determined by serial dilution and plate counts.

Results

Growth kinetics

Growth kinetics analysis revealed that there was no difference in the growth of TCA cycle mutants and wild-type *E. ictaluri* during aerobic as well as anaerobic growth conditions. One exception was the *Ei*Δ*frdA* mutant, which grew slower than the other strains (Figure 5.1). During aerobic growth of *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*sdhC*Δ*frdA, Ei*Δ*mdh*Δ*sdhC,* and wild-type *E. ictaluri,* log phase occurred from 6 h to approximately 12 h post-inoculation, and stationary phase occurred from approximately 12 h to 48 h (Fig. 5.1A). However, in anaerobic growth of the mutants and wild-type *E. ictaluri*, cell growth was in lag phase from 6 h to approximately 12 h post-inoculation, in log phase from 12 h to 18 h, and in stationary phase from approximately 18 h to 48 h (Fig. 5.1B).

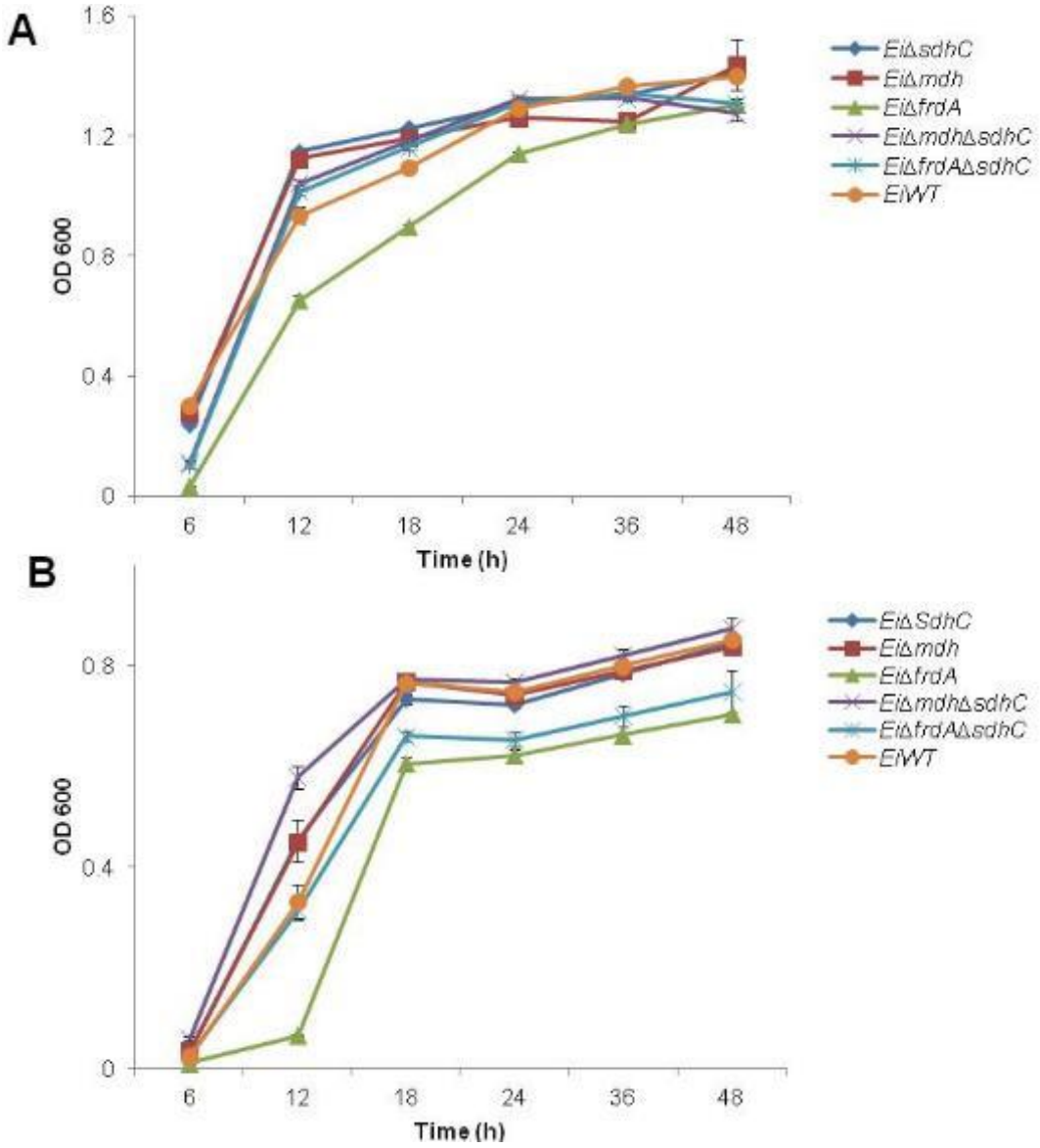

Figure 5.1 Aerobic and anaerobic growth kinetics in wild-type strain 93-146 and *EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔfrdA ΔsdhC, EiΔmdhΔsdhC* mutants.

(A) Aerobic growth curve for EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔfrdA ΔsdhC, EiΔmdhΔsdhC, and wild-type E. ictaluri. (B) Anaerobic growth curve for EiΔsdhC, EiΔfrdA, EiΔmdh, EiΔfrdAΔsdhC, EiΔmdhΔsdhC, and wild-type E. ictaluri.

Determination and measurement of extracellular superoxide

To determine extracellular superoxide production, mutants *EiΔsdhC*, *EiΔsdhA*, *EiΔfrdA ΔsdhC,* and wild-type *E. ictaluri* were assayed for superoxide production using

reduction of ferricytochrome c in the absence and the presence of SOD. Results of spectrophotometric assays representing average absorbance recorded from the three replicates are shown in Figure 5.2A. The difference in reduction of ferricytochrome c in the absence and presence of SOD represents the actual contribution of superoxide to the measured reductive activity and is shown in Figure 5.2B. A low level of superoxide production was detected by wild-type *E. ictaluri* 20 min after the addition of SOD, but superoxide production was negligible at 40 min and 60 min. The same trend was present in *Ei*Δ*sdhC*, except superoxide production was lower than wild-type *E. ictaluri*. No superoxide production was detected for *Ei*Δ*sdhA* and *Ei*Δ*frdA*Δ*sdhC* (data not shown). Therefore, elimination of succinate dehydrogenase genes did not cause increased superoxide production in *E. ictaluri*.

No superoxide production was detected from *E. ictaluri* extracellular metabolites (data not shown). Therefore, the superoxide production shown in Figure 5.2 is the result of periplasmic superoxide production in *E. ictaluri*.

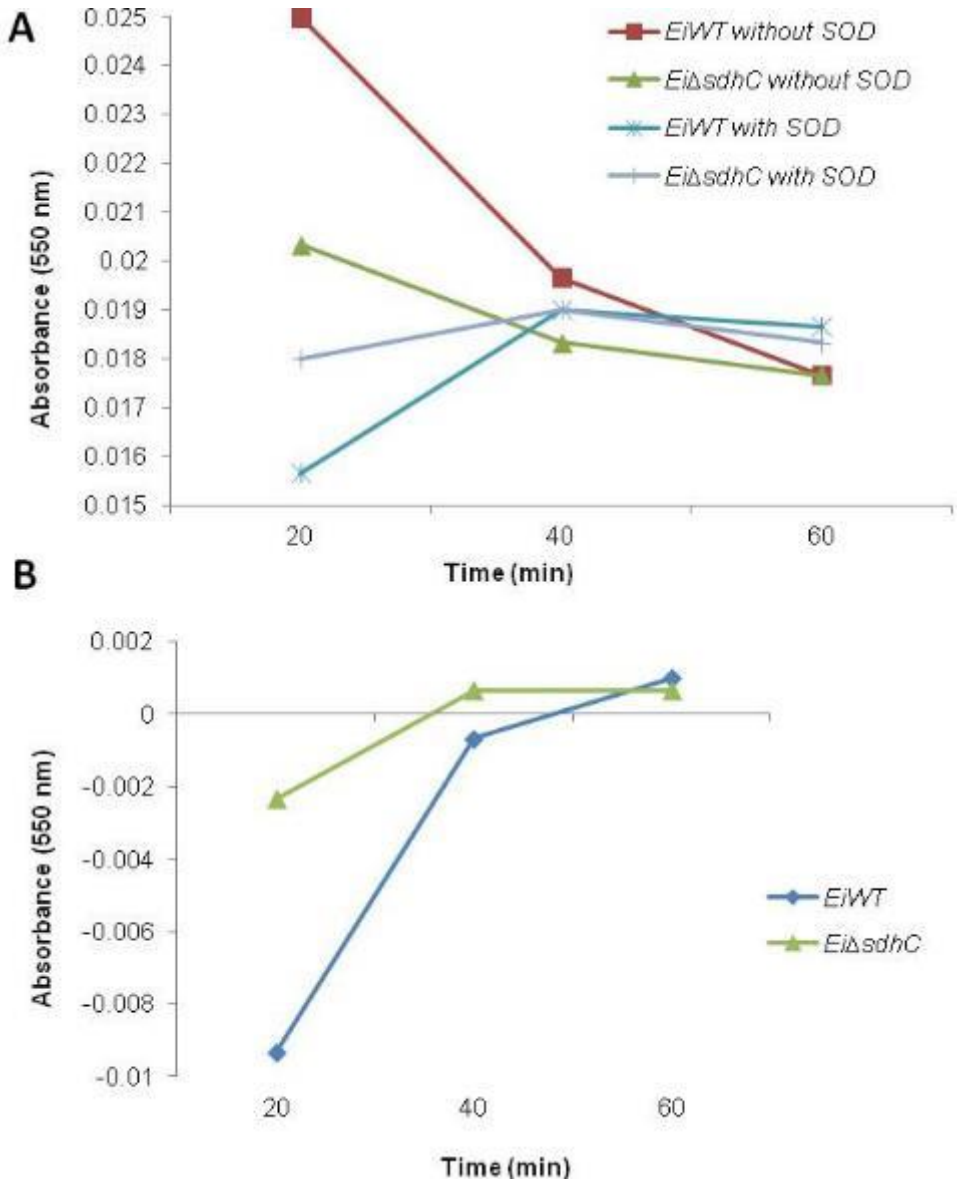

Figure 5.2 Reduction of ferricytochrome c by *Ei*Δ*sdhC* and wild-type *E. ictaluri* with and without superoxide dismutase (SOD).

Each point represents the ferricyanide-induced absorbance change at 550 nm. (A) Time points are measured from the addition of superoxide dismutase. (B) The calculated release of superoxide. For each treatment, three replicates were included; data represents the average absorbance of the replicates.

Enzyme assay

We examined the effects of deletion mutations, *Ei*Δ*sdhC* and *Ei*Δ*sdhA,* on succinate dehydrogenase activity by PMS-mediated reduction of DCIP. Figure 5.3 shows the mean decrease in absorbance a 600 nm resulting from DCIP reduction, which reflects succinate dehydrogenase activity. There was no change in succinate dehydrogenase activity of mutant *Ei*Δ*sdhC* when compared with wild-type *E. ictaluri* except at the lowest membrane concentration (5 µg/ml), where decreased activity was detected. By contrast, *EiΔsdhA* had decreased succinate dehydrogenase activity relative to wild-type *E. ictaluri* at all membrane concentrations (5, 10, and 15 µg/ml) (Fig. 5.3).

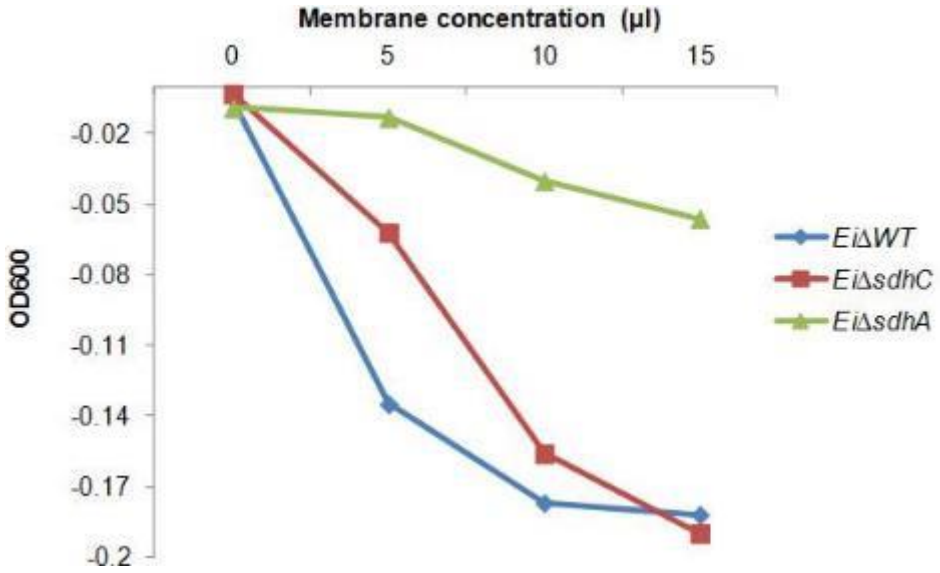

Figure 5.3 Reduction of DCIP by wild-type *E. ictaluri*, *Ei*Δ*sdhC*, and *Ei*Δ*sdhA* at different membrane fraction concentrations.

Each point represents the decrease in absorbance at 600 nm relative to the no membrane control that resulted from DCIP reduction.

Viability assay

A viability assay was conducted to determine whether providing SOD could protect the viability of the *Ei*Δ*sdhC*, *Ei*Δ*sdhA*, *Ei*Δ*frdA*, *Ei*Δ*sdhC*Δ*frdA* mutants when challenged with hydrogen peroxide. Relative to the PBS control, viability of *Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*sdhA*Δ*frdA,* and wild-type *E. ictaluri* decreased by 28% to 67% in the presence of H_2O_2, whereas it increased slightly (6%) for *Ei*Δ*sdhA* (Fig. 5.4). Similarly, the bacterial viability for all mutants decreased by 10% to 75% in the presence of H_2O_2 + SOD relative to the PBS control. However, the bacterial viability increased for *Ei*Δ*frdA* (43%) and wild-type *E. ictaluri* (133%) but decreased for *Ei*Δ*sdhA* (13%), *Ei*Δ*sdhC* (18%), and *Ei*Δ*sdhA*Δ*frdA* (33%) in the presence of SOD only relative to the PBS control.

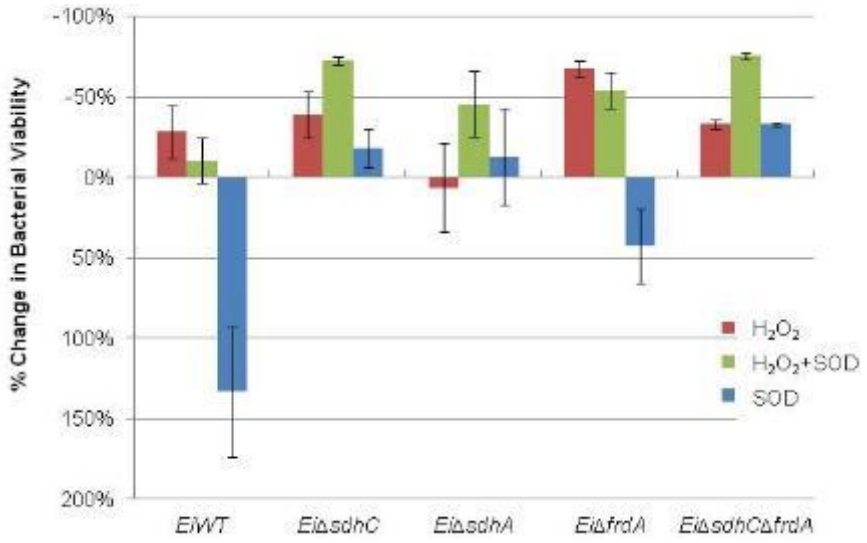

Figure 5.4 Percent change in bacterial viability relative to phosphate buffer solution (PBS) for *Ei*Δ*sdhC*, *Ei*Δ*sdhA*, *Ei*Δ*frdA*, *Ei*Δ*frdA* Δ*sdhC*, and wild-type *E. ictaluri* (control).

Bacterial strains were incubated with phosphate buffer solution, 30 U/ml SOD, 15 mM H_2O_2, or 30 U/ml SOD + 15 mM H_2O_2 for 1h at 25°C, and viable bacteria were enumerated. The data presented are means from three replicates.

Discussion

It has been shown that an *E. ictaluri* succinate dehydrogenase mutant is attenuated and is susceptible to killing by catfish neutrophils (Karsi et al., 2009). We hypothesized that the basis for this attenuation is increased superoxide production in the *E. ictaluri* succinate dehydrogenase mutant. In the current chapter, we aimed to test this hypothesis.

It is also possible that *E. ictaluri* TCA cycle mutants are attenuated simply because their growth rate is slower than wild-type *E. ictaluri.* Therefore, aerobic and anaerobic growth kinetics was compared between wild-type strain 93-146 and *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*mdh, Ei*Δ*sdhC*Δ*frdA, Ei*Δ*mdh*Δ*sdhC* mutants. We expected that aerobic growth will not be efficient in *E. ictaluri* TCA cycle mutants, but anaerobic growth should be similar to wild-type *E. ictaluri.* In contrast to our expectation, results showed no difference between the growth of *Ei*Δ*sdhC, Ei*Δ*mdh, Ei*Δ*sdhC*Δ*frdA, Ei*Δ*mdh*Δ*sdhC*, and wild-type *E. ictaluri*. However, the growth rate for *Ei*Δ*frdA* was slower than wild-type *E. ictaluri* both aerobically and anaerobically. Similar results were reported by (Steinsiek et al , 2011) in *E. coli* MG1655, which has mutations in *frdA* and *sdhC* genes. They analyzed the *frdA* and *sdhC* mutants at different aerobiosis levels and found their growth rate and cell yield were very similar to the parent strain. In *E. coli*, fumarate reductase is proficient in succinate oxidation and is able to functionally replace succinate dehydrogenase in aerobic respiration when this function has been disrupted (Guest et al, 1981). Thus, in *E. ictaluri* it is likely that fumarate reductase can replace succinate dehydrogenase in the TCA cycle, which might be the reason why we found no difference between the growth of *Ei*Δ*sdhC* and wild-type *E. ictaluri*.

E. ictaluri may also have a protein that can functionally replace malate dehydrogenase. In *E. coli*, growth of a double mutant containing *mdh* and *mqo* deletions is slower than growth of a *mdh* mutant (van der Rest et al. 2000). This indicated malate:quinoneoxidoreductase (MQO) is capable of partly taking over the function of malate dehydrogenase in a *mdh* mutant. It is possible that the TCA cycle enzymes work in a similar manner in *E. ictaluri* (MQO replacing Mdh function), which might explain the similar growth between *Ei*Δ*mdh* and wild-type *E. ictaluri*.

To test the hypothesis that an *E. ictaluri* succinate dehydrogenase mutant has increased superoxide production, the effect of *sdhC* deletion on endogenous superoxide production in *E. ictaluri* was compared to wild-type *E. ictaluri*. To determine if effects on superoxide production are specific to the *sdhC* subunit, superoxide production was also compared to a *sdhA* deletion mutant. Results indicated low production of superoxide in wild-type *E. ictaluri* and *Ei*Δ*sdhC*. *Ei*Δ*sdhA* and *Ei*Δ*frdA*Δ*sdhC* mutants had no detectable superoxide production. In *E. coli,* site-directed mutations SdhD-H71L and SdhC-H91L around the heme binding site resulted in enhanced superoxide production (Zhao et al, 2006). Similarly, Slane et al, (2006) suggested that mutations in the genes encoding succinate dehydrogenase caused increased superoxide production, metabolic oxidative stress, and genomic instability in hamster fibroblasts. Our current study did not show superoxide production in *E. ictaluri*. One of the reasons for this difference may be that succinate dehydrogenase mutants in other studies (Zhao et al. 2006; Slane et al. 2006) had site directed mutations changing a single amino acid, while we deleted the entire gene. Some bacterial species like *E. coli* and *Enterococcus faecalis* produce superoxide without any mutations in succinate dehydrogenase (Korshunov and Imlay,

2006; Huycke et al, 1996). However, other bacterial species do not produce superoxide, including many gram-negative species (Huycke et al. 1996). Thus, our findings are not surprising. Since the succinate dehydrogenase mutant of *E. ictaluri* did not have increased production of superoxide, we conclude that the basis for attenuation of mutant strain *Ei*Δ*sdhC* is not due to the deleterious effect of superoxide.

Next, we determined whether the deletion mutations *Ei*Δ*sdhC* and *Ei*Δ*sdhA* had decreased succinate dehydrogenase activity as determined by PMS-mediated reduction of DCIP. Our results showed decreased succinate dehydrogenase activity of the mutant *Ei*Δ*sdhA* compared to *Ei*Δ*sdhC* and wild-type *E. ictaluri*. SdhC serves as a membrane anchor for succinate dehydrogenase, while SdhA contains a catalytic site for the enzyme. Thus, the SdhC mutant may retain succinate dehydrogenase activity, while the SdhA mutant does not.

Next, the effects of superoxide production on *E. ictaluri* viability were determined by comparing the effects of *sdhC*, *sdhA, frdA*, and *sdhC-frdA* mutations on bacterial viability both in presence and absence of hydrogen peroxide and SOD. Hydrogen peroxide caused decreased viability of wild-type *E. ictaluri* and all the mutants except *Ei*Δ*sdhA*. Addition of SOD increased viability in *Ei*Δ*frdA* and wild-type *E. ictaluri* in the presence of hydrogen peroxide. However, SOD did not increase the viability of *Ei*Δ*sdhC*, *Ei*Δ*sdhA*, or *Ei*Δ*frdA*Δ*sdhC* in the presence of hydrogen peroxide. Therefore, the deleterious effect of superoxide production might be minimized by using SOD in *Ei*Δ*frdA* and wild-type *E. ictaluri*, but not in *Ei*Δ*sdhC*, *Ei*Δ*sdhA*, or *Ei*Δ*sdhC*Δ*frdA*.

In conclusion, results from the current study did not confirm our hypothesis that increased superoxide production contributes to the attenuation of *E. ictaluri* succinate

dehydrogenase mutants. Attenuation could not be explained by differences in growth rate either. Further work remains to be done to determine the basis for attenuation of *E. ictaluri* succinate dehydrogenase mutants.

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CHAPTER VI

CONCLUSIONS

The overall goal of this research was to develop a safe and efficacious live attenuated vaccine for channel catfish against enteric septicemia of catfish (ESC). Results shown in chapter II and III showed that we were able to construct nine live attenuated vaccine candidates using in-frame deletion mutagenesis. Among these nine live attenuated vaccines, three had mutations in genes encoding TCA cycle enzymes (*sdhC, mdh*, and *frdA*) , two had mutation in C-1 protein metabolism enzymes (*gcvP* and *glyA*), and other four were combinations between these two pathways (*sdhC-mdh, sdhC-frdA, sdhC-gcvP*, and *gcvP-glyA*). Virulence of the constructed mutants was tested in juvenile channel catfish using bioluminescence. This technique allowed us to detect bacterial dissemination in vivo, and it allowed us to quantify the degree of attenuation of the constructed mutants in catfish fingerlings.

After determining the degree of attenuation of the constructed mutants (chapters II and III), they were tested for vaccine efficacy by immersion in channel catfish fingerlings and fry (15-d old) (Chapter IV). The ability of the mutants to invade and persist in catfish fingerlings was also determined. All the mutants provided 100%protection against *E. ictaluri* 93-146 infection in fingerlings except for the mutants in C-1 metabolism (*glyAgcvP* and *glyA*), which provided lower protection (94.75%, and 87.98%, respectively). However, in catfish fry mortalities were reported for all mutants

except for *Ei*Δ*gcvP*Δ*sdhC*, which provided 99% protection against *E. ictaluri* infection. The mutant strains *Ei*Δ*sdhC, Ei*Δ*frdA, Ei*Δ*frdA*Δ*sdhC,* and *Ei*Δ*sdhC*Δ*gcvP* were able to invade catfish fingerlings and persist in the trunk kidney for at least 17 d post-infection. Moreover, tissue concentrations in *Ei*Δ*gcvP*Δ*sdhC*were as high as wild-type until 7 d post-infection. These results suggested good potential of *Ei*Δ*gcvP*Δ*sdhC* mutant in protecting catfish fry against ESC.

In our last study (Chapter V), the basis for attenuation of *E. ictaluri* succinate dehydrogenase mutants was investigated. Aerobic and anaerobic growth kinetics indicated that attenuation of *E. ictaluri* TCA cycle mutants was not due to a growth defect. Furthermore, no increased production of superoxide was detected in *E. ictaluri sdhC* or *sdhA* mutants. A succinate dehydrogenase enzyme assay showed that there was little effect on enzyme activity due to *sdhC* deletion. However, a *sdhA* mutant did show decreased succinate dehydrogenase activity. Wild-type *E. ictaluri* and all of the mutants except *EiΔsdhA* had decreased viability in the presence of the hydrogen peroxide (H_2O_2), but addition of SOD only increased viability of wild-type strain and *EiΔfrdA*.These findings provided insight into the mechanism of attenuation of the *E. ictaluri sdhC* mutant. It is important to note that this study evaluated superoxide production only in aerobic conditions. An alternative future research strategy could be to examine production of some known *E. ictaluri* virulence factors in wild-type strain and the *sdhC* mutant grown aerobically and anaerobically using RT-PCR.

The overall results of this study provided us with valuable information on the degree of attenuation as well as the safety of new *E. ictaluri* vaccine candidates. The vaccine candidates showed good potential as live attenuated vaccines in catfish

fingerlings. However, in catfish fry, only one vaccine candidate (*Ei*Δ*gcvP*Δ*sdhC*) showed 99%safety and efficacy. It should be noted that we tested vaccine efficacy in 15-day-old catfish fry. It is possible that there is a limit to the effectiveness of vaccinating catfish fry at this age. Testing the vaccine candidates for efficacy in three-week old catfish fry might show improved efficacy; vaccination of older fry can generate protection that lasts up to six months (Mackey, 2002). However, this would require changing current production practices. In the current study, vaccine candidates were only tested in controlled laboratory conditions. Another potential future direction would be to test these vaccines candidates in field conditions for possible commercialization.