Study of *Edwardsiella ictaluri* Conserved Genes Towards the Development of an Attenuated Recombinant Vaccine for Fish Host

by

Javier Alonso Santander Morales

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Graduate Supervisory Committee:

Roy Curtiss III, Chair
Douglas Chandler
Yung Chang
Yixin Shi

ARIZONA STATE UNIVERSITY

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ABSTRACT

Teleosts have the most primitive adaptive immune system. However, in terms of functionality the teleost immune system is similar to birds and mammals. On the other hand, enteric bacterial pathogens of mammals and birds present conserved regulatory mechanisms that control virulence factors. In this context, deletion of conserved genes that control virulence factors have been successfully used as measure to construct live attenuated bacterial vaccines for mammals and birds. Here, I hypothesize that evolutionary conserved genes, which control virulence factors or are essential for bacterial physiology in Enterobacteriaceae, could be used as universal tools to design live attenuated recombinant bacterial vaccines from fish to mammals. The evolutionary conserved genes that control virulence factors, *crp* and *fur*, and the essential gene for the synthesis of the cell wall, *asd*, were studied in *Edwardsiella ictaluri* to develop a live recombinant vaccine for fish host. The genus *Edwardsiella* is one of the most ancient represent of the Enterobacteriaceae family. *E. ictaluri*, a host restricted pathogen of catfish (*Ictalurus punctatus*), is the causative agent of the enteric septicemia and one of the most important pathogens of this fish aquaculture. Although, crp and fur control different virulence factors in *Edwardsiella*, in comparison to other enterics, individual deletion of these genes triggered protective immune response at

the systemic and mucosal level of the fish. Deletion of *asdA* gene allowed the creation of a balanced-lethal system to syntheses heterologous antigens. I concluded that *crp, fur* and *asd* could be universally used to develop live attenuate recombinant Enterobacteriaceae base vaccines for different hosts.

DEDICATION

Dedicate to my wife, Ignacia, and to my two sons, Raimundo and Ignacio, the lighthouse of my life.

To my parents, Alonso Santander and Sonia Morales

ACKNOWLEDGMENTS

I would like to thank to Roy Curtiss III, my mentor, for give the chance to dive in the ocean of independent ideas, allowing me to create a scientific niche that today is promoting my career. Roy, thank for your trust.

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INTRODUCTION: LITERATURE REVIEW

1. *Aquaculture as global source of food supply*

The worldwide decline of ocean fisheries stocks has provided impetus for rapid growth of fish, crustacean, and shellfish farming, or aquaculture. Currently the aquaculture industry is one of the most important sources of human food and it has the fastest growth-rate of all animal-producing food sectors. The contribution of the aquaculture to the global food supply by weight has increased from 3.9 percent in 1970 to 50 percent in 2008, with a value of US$98.3 billion (26, 27, 97). Asia has the highest production rate accounting for 89 percent by volume and 79 percent by value, with China by far the largest producer (32.7 million tones in 2008) with faster growing fish, like tilapia and carp (8, 26). The rapid growth in this region has been driven by a variety of factors, including pre-existing aquaculture practices, population and economic growth, relaxed regulatory framework, and expanding export opportunities (7, 8). Aquaculture development in Europe and the Americas was rapid during the 1980s–1990s but has since stagnated, probably owing to regulatory restrictions on sites and other competitive factors, although as markets for fish and seafood they have continued growing (7, 8).

In 2008, it was predicted that the annual global consumption of seafood by 2010 would be 110-120 million metric tons. By 2008, the capture fisheries were limited to 60 million metric tons per year, and the predictable scenario by 2010 was a deficit of 36-46 million metric tons for human consumption (29). Thus it was expected that the aquaculture industry double its production to compensate the deficit (29). Currently, the aquaculture industry has tripled its production, with an estimate of 55.1 million metric tones, but the capture fisheries has increased from 60 to 90 million metric tones, accentuating overfishing (7, 26). The need for increased aquaculture output over the next 20 years is widely forecast based on human population projections, anticipated economic development, and concern over the future sustainability of capture fisheries (12). Today the global aquaculture industry has the challenge to increase sustainable production, reduce environmental contamination, and diversify production.

2. *General Status of Fish Vaccinology in the Global Aquaculture Industry*

Economic losses due to infectious diseases in the global aquaculture industry are estimated to be $3 billion annually (28). In all kinds of intensive culture, where single or multiple species are reared in high density, infectious disease agents are easily transmitted between individuals. In those intensive systems, vaccination is one of the

most important enhancers of production yields. Currently, the most used practical method for vaccination in the global aquaculture industry is by intra-coelomic (i.c.) injection. This class of immunization is expensive due to labor intensity and added costs for anesthesia, gas, needles, electricity, and others. Furthermore, i.c. vaccination is cost prohibitive for booster immunizations (98, 106). The injectable vaccines are not applied in the aquaculture of catfish, due to costs and absence of effective vaccines. On the other hand, bath live attenuated bacterial vaccines are commercially available, but not used in the industry due to the lack of efficacy.

The success of any given vaccine is governed by three critical factors, safety, cost, and efficacy. Ideally, vaccines should be free of side effects, lack residual pathogenicity, and have no potential for reversion to virulence. Furthermore, they should pose no threat to the environment (16, 95). Thus, this kind of vaccine could be accepted world-wide for application in the global aquaculture industry. Live attenuated vaccines can be designed to be safe, effective, and massively applied at low cost. However, efforts to design live attenuated vaccines for the aquaculture industry have been relatively modest. *Aeromonas hydrophila* Δ*aroA* (113-116) and *A. salmonisida* Δ*aroA* (64) vaccines are some examples, with their early origin-design based on oral live attenuated *Salmonella* vaccines for mammals (22, 25). Auxotrophic deletions, such as *aroA*, cause silent bacteremia in mammals (62, 72), and in combination with other genetic modifications became hyperattenuated, thus non-

mmuneprotective (14).

The ideal attenuated bacterial vaccine should be totally avirulent yet highly immunogenic. Transposon mutagenesis has been used to search for useful genes to attenuate *Edwardsiella ictaluri*. The transposon-induced *eacF*::Km, chondroitinase::Km, *purA*::Km, *aroA*::Km, and *wibT*::Km, mutations attenuated *E. ictaluri* (5, 53, 54, 82, 105). Some of these mutant strains are prospective vaccine candidates.

The best way to enhance production in the aquaculture industry by vaccination is generating a safe, efficient oral/bath bacterial vaccine vector that can be applied during any stage of fish development, from egg to adults, and providing an accessible and practical means to boost immunization through food.

3. *Edwardsiella ictaluri Epidemiology*

E. ictaluri primarily infects channel catfish, however *E. ictaluri* naturally infects walking catfish (*Clarias batrachus*), blue catfish (*Ictalurus furcatus*), white catfish (*Ameiurus catus*), and brown bullhead (*Ameiurus nebulosus*) (109). In addition, it has been shown that tadpole madtoms (*Noturus gyrinus*) are also susceptible to natural *E. ictaluri* infections (45). Although, there are no reports about *E. ictaluri* outbreaks in blue tilapia (*Oreochromis aureus*), chinook salmon (*Oncorhynchus tshawytscha*), or rainbow trout (*Oncorhynchus mykiss*), these species have been

experimentally infected (3, 80).

The channel catfish industry is concentrated in the southeastern United States, where the epizootics of **e**nteric **s**epticemia of **c**atfish (ESC) commonly occur during late spring and early fall, when temperatures are favorable for *E. ictaluri* growth. The acute form of ESC tends to occur when temperatures are between 22°C and 28°C, while the chronic form tends to occur when temperatures are between 18°C and 22°C or higher than 28°C. Below 18°C or above 30°C, ESC outbreaks are rare (109). Survivors of catfish fingerlings to experimental *E. ictaluri* acute infection, still carry the bacterium in the posterior kidney and brain, but not in the blood (123), demonstrating that chronic infections could be developed in fish that survive an acute ESC infection. Channel catfish ESC survivors serve as carriers of *E. ictaluri*, even after antibiotic treatment (43, 70). Fingerlings known to have suffered ESC and medicated feed with Romet30 still harbor *E. ictaluri* in their internal tissues after 270 days post treatment (44). Perhaps the antibiotic fails to kill *E. ictaluri* within macrophages, leading to establishment of a carrier state.

In the context of environmental persistence, *E. ictaluri* survive in pond water for short periods of time (15 days), and pond mud for longer periods of time (95 days) (81), suggesting that *E. ictaluri* can probably survive outside of the hosts, perhaps associated within invertebrates.

E. ictaluri can be transmitted in a variety of horizontal ways, from infected to uninfected fish (96), from eating infected carcasses (109), and

by the shedding of viable *E. ictaluri* into the water from a moribund fish just prior to death or during decomposition (123).

In the industry, a multiple batch system are a widespread practice among channel catfish producers and it may be contributing to the continued spread of ESC (109). In multiple batch systems, naïve fish are often mixed with *E. ictaluri* carrier fish. In addition, fish farmers may unintentionally facilitate transmission by passing effluent from one pond to another or using equipment, like seines, that had previously been used in ponds with infected animals without disinfection or drying.

4. *Edwardsiella ictaluri Pathogenesis and Vaccine Development*

When the first cases of ESC were investigated, Koch's postulates were used to confirm that the suspect bacterium was causing the disease. Microscopy and biochemical characterization indicated that the causative agent of ESC belonged to the family Enterobacteriaceae (37). Phylogenic analysis indicated that the causative agent of ESC was closely related to *Edwardsiella tarda* (37, 128). The microorganism differed from *E. tarda* in that it was negative for hydrogen sulfide production, absence of tryptophanase activity, and non-motile at 37°C. In 1981 this microorganism was fully characterized, classified, and named *E. ictaluri* (38). *E. ictaluri* present a phenotypically homogenous population with no described serotype variation (76, 89, 121). *E. ictaluri* has fermentative

metabolism, is motile by perotrichous flagella, and has catalase, lysine and ornithine decarboxylase activities (38, 103, 118). Its genome sequence, with a G+C content of 53% mol (38), has been recently annotated. All the isolates of *E. ictaluri* harbor two cryptic plasmids, pEI1 (5.7 kb) and pEI2 (4.9 kb) (30, 61, 70, 99) that seems related to virulence (43). The clinical signs and pathogenesis of *E. ictaluri* infections present two forms, a rapid acute septicemia with high mortality and a chronic form or carrier state confined to the central nervous system (43, 97).

Establishing the means by which *Edwardsiella* infect and colonize fish tissues provides a design strategy to develop effective live vaccines. *E. ictaluri* crosses the intestinal mucosa of channel catfish in 15 min after oral inoculation with 10^9 CFU of *E. ictaluri* (2). Direct inoculation of 10^6 cells into the olfactory organs of channel catfish revealed damage after 1 h (67). An i.c. dose of 10^3 cells is capable of killing the catfish within 10 days (80). Fluorescence microscopy localized the organism on the gills within 5 min, within gill epithelia after 45 min and into the kidney within 4 h, when bacteria were administered by a waterborne route (71, 123). By 72 h, the pathogen is recoverable from blood. Entry, survival and replication in head kidney macrophages of channel catfish have been observed. Opsonisation with normal serum led to greater internalization of *E. ictaluri* (6). It has been established that both gut and olfactory organs are the primary sites of the invasion of *E. ictaluri* in natural outbreaks (96). Certainly, it has been firmly established that channel catfish are

highly susceptible to the facultative intracellular pathogen *E. ictaluri*.

Although there are substantial descriptive data relative to the invasion, spread, and persistence of *E. ictaluri* in channel catfish (3, 68, 108), little is known about the molecular mechanisms of *E. ictaluri* pathogenicity. Extracellular products have been associated with virulence (101, 120). Attenuated strains obtained by lab subculturing passages, lack outer membrane proteins (OMP), exhibit markedly less hemolytic activity and differences in the composition of lipolysaccharide (LPS) (120). LPS, like in most enteric pathogens, is an important virulence factor for *E. ictaluri*. The LPS gene cluster has been identified by transposon mutagenesis (53). Recently, using signature-tagged mutagenesis several genes related to catfish virulence have been found (106). Genes like those in *Salmonella* pathogenesis island 2 (SPI-2) and for the type 3 secretion system (T3SS) have been identified in *E. ictaluri* (106). However, no functional master regulator for virulence has been described.

E. ictaluri has remained confined within the broadly defined geographical limits of the U.S. with catfish as its main host. But recently, *E. ictaluri* outbreaks have been reported in South and East Asia in catfish (42, 89, 108). In contrast, *E. tarda* has a broad geographic spectrum and several mammals and fish hosts (1, 66, 67), including humans, can be infected (34, 98, 126). Indeed, *E. tarda* comprises part of the normal microflora of fish surfaces, including channel catfish (124). Virulence factors of *E. tarda* have been reported, including the ability to

disseminate after infection by invading epithelial cells and other fish tissues (58, 59), resisting serum and phagocyte-mediated killing (41, 59, 100), and producing toxins and exoenzymes such as hemolysins (39) and dermatoxins (110). Molecular virulence factors, such as a two-component regulatory system (104), enzymes for the survival in the host, and putative chaperons for the translocation of T3SS effectors have also been described (86).

The T3SS in both *Edwardsiella* species differ from the SPI-2 class of T3SS in that they encode an AraC-type regulator, EsrC (106). In *E. tarda*, the expression of EsrC is under the control of the EsrA/EsrB two-component system, which regulates the expression of different components of the T3SS (131). Deletion of *esrB* leads to missing or reduced T3SS secretion proteins, such as *eseB*, *eseC* and *eseD*. *E. tarda* Δ*esrB* has markedly increased invasion of the epithelial cells and decreased survival in macrophages. An *E. tarda* Δ*esrB* live attenuated vaccine shows significant protection and immunogenicity (52).

The ferric uptake regulator (Fur) is a metal ion-responsive transcription regulator that controls expression of genes involved in diverse cellular functions. Iron-regulated outer membrane proteins (IROMPs) are considered as potential vaccine candidates against septicemic bacteria (13). Fur has been described in *E. tarda* (39). *E. tarda* Δ*fur* mutants have an altered outer membrane protein profile and have a high LD_{50} in fish (119). It has been speculated that iron is not an

important factor for *E. ictaluri* virulence, however we determined that iron is relevant during fish pathogenesis (92).

5. *Vaccinology in the Catfish Industry*

Commercial catfish production accounts for 85 to 90% of the total finfish aquaculture production in the United States, with almost 300,000 tons produced annually (36). The most serious bacterial pathogens affecting this industry are *E. ictaluri* and *F. columnare*. Losses due to these bacterial pathogens are estimated to be $50-80 million, annually (94).

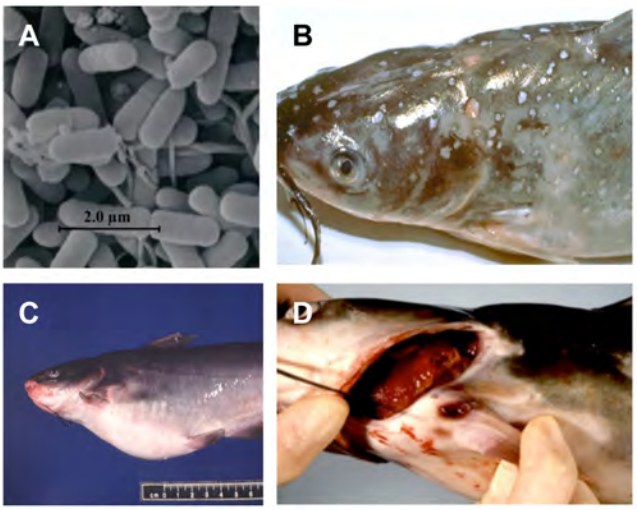

Figure 1. *Edwardsiella ictaluri* and enteric septicemia of catfish symptoms. A. *Edwardsiella ictaluri* scanning electron microscopy; B-D. Clinical signs of the enteric septicemia of catfish (Lawrence, USDASDA/CSREES grant #2004 -35600- 14180).

E. ictaluri, the causative agent of Enteric Septicemia of Catfish (ESC), is the most prevalent disease affecting the catfish industry and causes the biggest economic losses to the industry. In 2002, ESC was reported to have caused losses on 53% of fry/fingerling operations and 61% of food size fish operations (111). Catfish farmers have attempted to control ESC outbreaks by medicated feed or by restricting the amount of feed offered (122). Romet ® (a 5:1 mixture of sulfadimethoxine and ormetoprim) and Aquaflor® (florfenicol) are the only antibiotics approved by the Food and Drug Administration (FDA) for treating ESC (33). However, antibiotic treatment is expensive and fish affected with ESC typically reduce their feeding activity, leading to antibiotic delivery problems (47). In addition, plasmid mediated resistance of several strains of *E. ictaluri* to Romet and Aquaflor has been reported (102). Restricting feed may control ESC, but may lead to a loss of production.

The USDA licensed live *E. ictaluri* AQUAESC® and *F. columnare* AQUACOL® vaccines have been selected by multiple passages in increased concentrations of the antibiotic rifampicin (1, 46, 93). The selected spontaneous mutant strains presented an attenuated phenotype with part of the O-lipopolysacharide lost (95). Because of the unknown molecular mechanisms of the attenuation in these vaccines (which likely are due to point mutations), they are susceptible to in vivo reversion to the virulent wild type. These issues have been poorly addressed for the *E. ictaluri* AQUAESC® vaccine, without strong proof of vaccine

stability (48). Furthermore, these classes of vaccines are environmentally unsafe, due to the possibility of survival in the aquatic environment and transduction of the rifampicin-resistance to natural bacterial flora. All these issues are largely ignored in the field of fish vaccinology, and today live attenuated vaccines for other fish diseases, such as *F. psychophilum*, are being developed using this random method of mutation (50). Indeed, recently it has been proposed to use an *E. ictaluri* novobiocin-resistant vaccine strain as strategy against *E. ictaluri* (84). As mentioned previously, these vaccines are not used in the industry due to poor efficacy in the field and the high cost per dose. However, by using these live vaccines we have learned that they can be easily delivered to young fish and stimulate both humoral and cellular immunity (95). These results and observations provided guidance in the design of effective and safe bath/oral live attenuated vaccines for the aquaculture industry.

6. *Adaptive immune response in Teleost.*

At the cellular level, the immune systems of teleosts and mammals are similar, but the lymphoid system of fish is organized differently, and does not have lymph nodes and Peyer's patches. Fish also lack bone marrow, and functions found in mammalian bone marrow are localized instead in the anterior kidney (head kidney) of the fish, which serves as the site of both lymphopoiesis and hemtopoiesis (107, 127). B cells

comprise 25-50% of all blood lymphocytes (35, 66), a higher percentage than found in mammals (55). Sites equivalent to lymph nodes and Peyer's patches have not been described and it has been proposed that in trout the spleen and kidney serve as sites of B cell differentiation into subpopulations of antibody-secreting cells (ASC) including plasmablasts, short-lived plasma cells and long-lived plasma cells (11, 133). B cell differentiation in fish does not involve class switching (40). In catfish only loci for tetrameric IgM and IgD have been identified (4). IgD represents an ancient immunoglobulin isotype that is found in all vertebrate taxa, except for birds (31, 74, 130). Catfish express two types of IgD+ B cell populations, IgM+/IgD+, and IgD+/IgM-, and a population of circular granular cells that are armed with exogenous IgD via putative IgD-binding receptor (23, 24). Currently the origin and the function of these cells are unknown. Loci for IgT found in trout (35) and zebrafish (19), have not been identified in catfish. To date, only IgM has been shown to be functionally involved in protective immunity in fish, and plasma cells residing in anterior kidney serve as the source of serum IgM antibodies and humoral memory (11, 133).

The skin of fish plays an immune protective role, serving as an anatomical and physiological barrier against the external environment. Cutaneous mucus, secreted by mucous cells present in the epidermis, is considered the first line of defense against infectious agents. Channel catfish skin is compromised of several layers, including a non-

keratinized epidermis 5-10 cells thick, which contains mucus-secreting goblet cells, dermis and hypodermis (Fig. 6) (Santander, unpublished data).

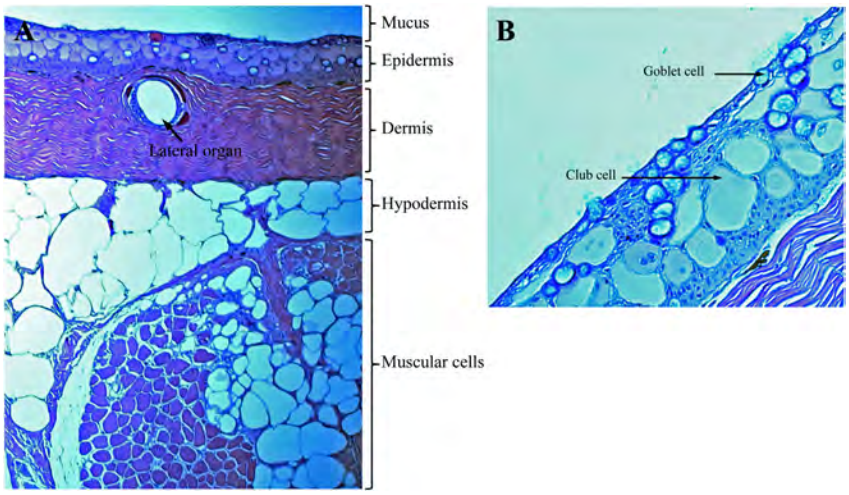

Figure 2. Channel catfish skin sections stained with hematoxylin and eosin. A. Channel catfish skin layers (10X); B. Channel catfish epidermis (40x) (Santander, unpublished data).

Cutaneous mucus contains a low concentration of tetrameric IgM (60, 63, 132), and these antibodies have been shown to play a critical role in host defense (15, 21, 57). ASC, including plasma cells, and B cells reside in the skin of channel catfish with a range of 23-306 ASC per 10^6 skin cells of unvaccinated fish (129). It has been described that after intra coelomic immunization and surface exposition the number of skin ASC increase 20-fold, indicating that the number of skin ASC cells is not fixed and is responsive to immunization (129), but the ontogeny of these cells still unknown.

7. *Zebrafish (Danio rerio) as host of E. ictaluri.*

Zebrafish (*D. rerio*) is an oviparous cyprinid of warm fresh waters common in household aquaria (75). The use of zebrafish as a laboratory animal is well established in developmental biology, genetic research, and carcinogenicity testing and has been gaining popularity for basic immunology research (107). Also, zebrafish has been adopted for the study of host–pathogen interactions. Successful infection of zebrafish has been demonstrated using different fish pathogens including *Mycobacterium marinum*, (20, 32, 85, 112, 117), *Vibrio anguillarum* (73, 88), *Flavobacterium columnare* (68), *Streptococcus iniae* (65, 69), *Aeromonas salmonisida* (56), *Aeromonas hydrphila* (87), *Edwardsiella tarda* (83), and *E. ictaluri* (79, 91).

Research in the channel catfish host is restricted because it takes 3 years to mature and spawn once a year, so the availability of fry or fingerlings for pathogenesis-immunology studies is limited. Additionally, the genome of this species has not been sequenced, and microarray reagents, immunoglobulins, etc., are limited. These factors negatively affect developmental immunology and infectious disease research of this commercially important fish. On the other hand, the development and maturation of the immune systems of zebrafish (51) and channel catfish *Ictalurus punctatus* (77, 78) are very similar. This makes of zebrafish a useful host to study *E. ictaluri* pathogenesis. Although, *E. ictaluri*

cannot infect zebrafish by immersion inoculation, zebrafish inoculated intra-muscular (i.m) with *E. ictaluri* causes similar symptoms as observed in catfish infected by immersion with *E. ictaluri* (79) (Santander, 2007; unpublished data) (Fig. 2). After 6 hours post i.m. infection *E. ictaluri* is already located in the kidney macrophages inside of the *Edwardsiella* containing vesicles (Fig. 3) (Santander, 2007; unpublished data). Although, after six hours of infection the fish does not shown evident symptoms, electron microscopy studies reveal apoptosis-like events, denoted by mitochondrial release from the infected macrophages (Fig. 3) (Santander, unpublished data).

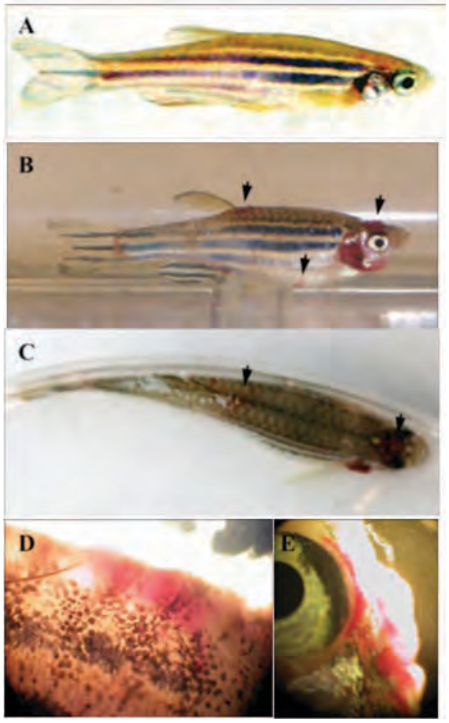

Figure 3. Zebrafish (*Danio rerio*) infection with J100 *E. ictaluri* 2003/c. A. Uninfected Zebrafish (control); **B.** 24 hrs post i.m. infection with 10^8 cfu/ml of J100 *E. ictaluri* 2003/c, lateral view; **C.** 24 hrs post i.m. infection

with 10^8 cfu/ml of J100n *E. ictaluri* 2003/c, dorsal view; **D.** Close-up of external view of swelling kidney; **E.** Close-up of external view of swelling head. The arrows indicate the external lesions.

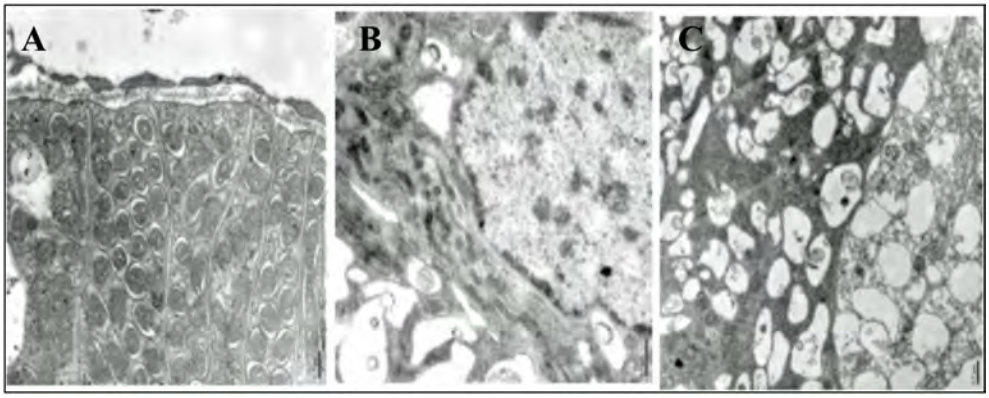

Figure 4. Transmission electron microscopy of uninfected Zebrafish kidney tissues. **A**-**B**. Vessels and connective tissue; **C**. Nefron tubes.

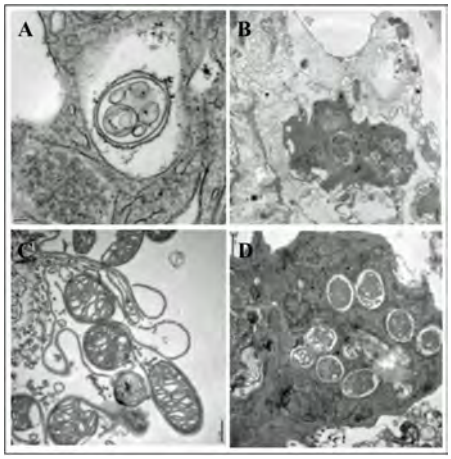

Figure 5. Transmission electron microscopy of zebrafish kidney 6 h post infection with 10^8 cfu/ml of J100 *E. ictaluri* 2003/c. A. Monocyte from uninfected kidney; B. Macrophage; C. Release mitochondria from infected macrophage; D. Intercellular *E. ictaluri* in *Edwardsiella* containing vesicles.

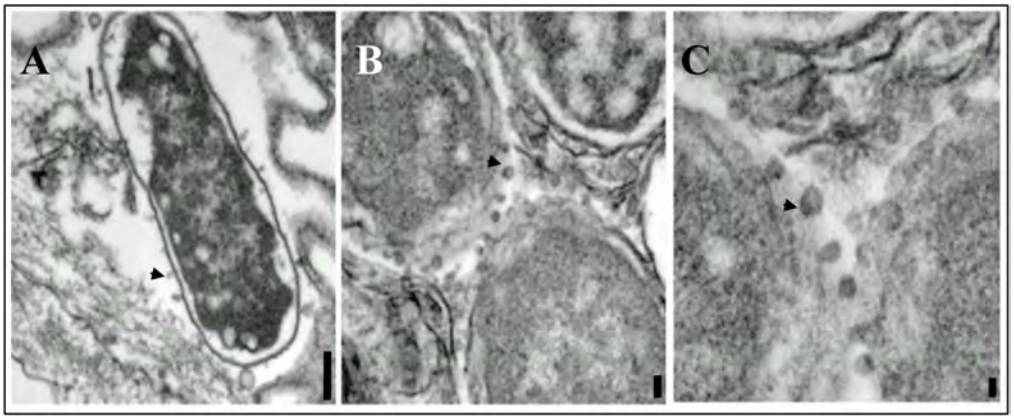

Figure 6. Intercellular *E. ictaluri* in *Edwardsiella* containing vesicles; **A**-**B**. Intracellular bacteria with membrane structures indicated by the arrows; **C**. Detail of the intracellular *E. ictaluri* membrane structures. The bar is equivalent to 0.2 μm.

8. *Genetically Attenuated Bacteria and Immunogenicity Enhancements Towards the Design of a Live Recombinant Vaccine for Teleosts*

Attenuated bacterial vectors that express foreign antigens have been used as live attenuated recombinant vaccines to induce immune responses against both the attenuated bacteria and the protective foreign antigens (9, 17, 49, 125). The design of this kind of oral live recombinant attenuated bacterial vaccine can be separated into 3 phases: (**i**) attenuation, (**ii**) antigen delivery and (**iii**) biocontainment. All these measures must be coordinated to ensure a strong protective immune response, without tissue damage, bacterial persistence, or environmental escape of the vaccine.

The attenuation of bacterial vector vaccines and their use should decrease, if not eliminate, undesirable disease symptoms. The attenuation should be an inherent property of the vaccine and not dependent on fully functional host defenses and immune response capabilities. The attenuation should not be reversible by diet or by host or microbial modification of dietary constituents. The attenuation should not permit development of a persistent carrier state. The attenuated vaccine should be sufficiently invasive and persistent to stimulate both strong primary and lasting memory immune responses. The vaccine should be designed to minimize tissue damage not needed to induce effective immunity. As even attenuated vaccines may cause disease in some individuals or under certain unusual conditions, the vaccine should be susceptible to useful antibiotics. To eliminate the used of plasmid vectors with drug-resistance genes and to stabilize plasmid vectors in vivo, the balanced-lethal host-vector system uses the deletion of essential genes to impose an obligate requirement for the plasmid vector with the wild-type essential gene (18, 90, 91, 125). Lastly, the attenuated vaccine should possess containment features to reduce its shedding and survival in nature. When used as an antigen delivery vector, the recombinant attenuated *Edwardsiella* vaccine (RAEV) must exhibit stable high-level expression of protective antigens in vivo to stimulate induction of long-lasting protective immunity. These features of recombinant and non-recombinant bacterial vaccines have been described in previous publications (18)

9*. Hypothesis*. *E. ictaluri Vaccine Design, First Steps to a Live Attenuate Recombinant Edwardsiella Vaccine (RAEV) for the Aquaculture Industry.*

At this stage, with the *E. ictaluri* genome sequence completed and virulence genes described, it is possible to construct oral live *E. ictaluri* vaccines in the absence of antibiotic resistance. Teleosts are the most primitive bony vertebrates that contain immunoglobulins (31). However, in terms of functionality teleost are still similar to birds and mammals. On the other hand, enteric bacterial pathogens of mammals and birds present evolutionary conserved regulatory mechanisms that control virulence factors, like cyclic adenosine 3',5'-monophosphate receptor protein (Crp), ferric uptake regulatory protein (Fur) and essential genes required for cell growth, like the aspartate-semialdehyde dehydrogenase enzyme (Asd). Here, I hypothesized that conserved genes though Enterobacteriaceae evolution (Fig. 6) that control virulence factors or are essential for bacterial physiology could be used as universal tools to design live attenuated recombinant bacterial vaccines from fish to mammals. Utilizing *E. ictaluri*, one of the most ancient representing genera of the Enterobacteriaceae family and the main pathogen of catfish, the genes *crp*, *fur* and *asdA* were evaluated in fish hosts.

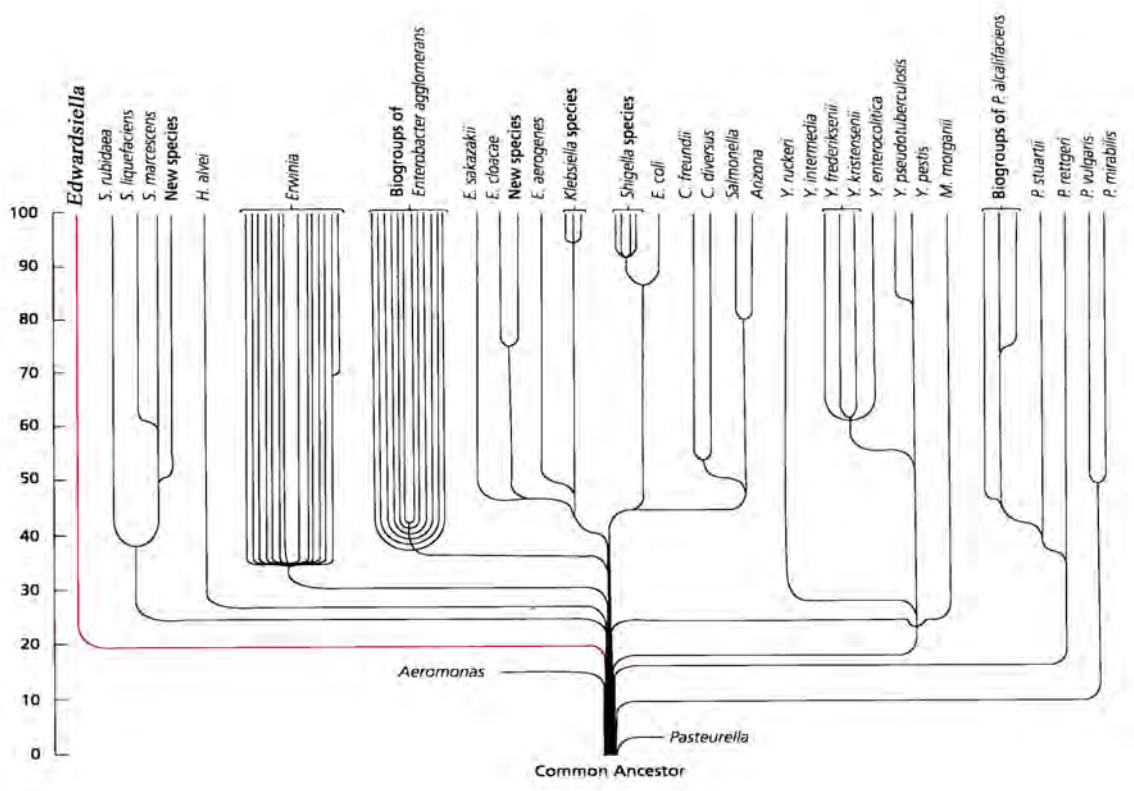

Figure 7. The evolutionary relationships of Enterobacteriaceae based on DNA-DNA hybridizations (10).

10. *Objectives.*

i. Develop a methodology to in frame delete genes in *E. ictaluri*.

ii. Determine the phenotype, virulence and immunogenicity of *E. ictaluri* Δ*crp* mutant strains in fish host

iii. Determine the phenotype, virulence and immunogenicity of *E. ictaluri* Δ*fur* mutant strains in fish host

iv. Develop a balanced-lethal system in *E. ictaluri* mediate the deletion of *asdA* gene.

11. Acknowledgments

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CHAPTER II

PHENOTYPE, VIRULENCE AND IMMUNOGENICITY OF *Edwardsiella ictaluri* CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE RECEPTOR PROTEIN (CRP) MUTANTS IN CATFISH HOST

1. *Abstract*

Edwardsiella ictaluri is an Enterobacteriaceae that causes lethal enteric septicemia in catfish. Being a mucosal facultative intracellular pathogen, this bacterium is an excellent candidate to develop immersion-oral live attenuated vaccines for the catfish aquaculture industry. Deletion of the cyclic 3',5'-adenosine monophosphate (cAMP) receptor protein (*crp*) gene in several Enterobacteriaceae has been utilized in live attenuated vaccines for mammals and birds. Here we characterize the *crp* gene and report the effect of a *crp* deletion in *E. ictaluri*. The *E. ictaluri crp* gene and encoded protein are similar to other Enterobacteriaceae family members, complementing *Salmonella enterica* Δ*crp* mutants in a cAMP-dependent fashion. The *E. ictaluri* Δ*crp-10* in frame deletion mutant demonstrated growth defects, loss of maltose utilization, and lack of flagella synthesis. We found that the *E. ictaluri* Δ*crp-10* mutant was attenuated, colonized lymphoid tissues, and conferred immune protection against *E. ictaluri* infection to zebrafish (*Danio rerio*) and catfish (*Ictalurus punctatus*). Evaluation of the IgM titers indicated that bath immunization

with the *E. ictaluri* Δ*crp-10* mutant triggered systemic and skin immune responses in catfish. We propose that deletion of the *crp* gene in *E. ictaluri* is an effective strategy to develop immersion live attenuated antibiotic-sensitive vaccines for the catfish aquaculture industry.

2. *Introduction*

Edwardsiella ictaluri, a host restricted Gram-negative enteric pathogen, causes lethal enteric septicemia in catfish and considerable economic losses to this food producing industry (33, 66). The natural route of *E. ictaluri* infection in catfish is nasal and oral, but recently the skin-abrasion route of infection has been described [3-6]. *E. ictaluri* is a facultative intracellular fish pathogen that colonizes deep lymphoid tissues, like spleen, liver and kidney (7). These attributes make *E. ictaluri* a promising candidate to be developed as a live attenuated vaccine for the catfish aquaculture industry. Even though several attempts have been made to develop an immersion-oral *E. ictaluri* vaccine, none have induced a high level of protection. The formalin-killed immersion vaccine does not colonize fish lymphoid tissues, stimulates only an antibody response and offers no protection (49, 74). The currently licensed *E. ictaluri* spontaneous rifampicin-resistant (Rif^r) vaccine colonizes deep lymphoid tissues of the fish and confers a modest level of immune protection (42, 65, 77). It has been reported that Rif^r strains of *Escherichia coli* have

genetic modifications in the *ropB* gene, which encodes the *β*-subunit of the RNA polymerase (36-38). It is therefore probable that the *E. ictaluri* Rif^r vaccine strain has mutations in the *ropB* gene. The *E. ictaluri* Rif^r vaccine strain was selected by serial passages in increasing concentrations of rifampicin (33 passages) (43), accumulating another set of unknown genetic alterations due to in vitro selection, like rough LPS, alteration of the outer membrane proteins, fatty acid content, and catabolic pathways (4). This precludes the easy comprehension of the attenuation mechanisms and immunogenicity of the vaccine in the fish host.

Once the cyclic adenosine 3', 5'-monophosphate (cAMP) receptor protein (Crp) is activated by binding to its allosteric effector cAMP, the Crp-cAMP complex regulates aspects of carbon metabolism and may act as a general chromosome organizer (8, 9, 29). The Crp-cAMP complex also regulates transcription of genes related to virulence in many pathogenic bacteria including *Salmonella*, *Vibrio cholerae*, *Yersinia*, and *Mycobacterium tuberculosis* (17, 53, 67, 69). Crp-cAMP regulation occurs either directly, by binding to specific DNA sequences near the target gene, or indirectly, through the action of CyaR, a regulatory RNA (21). In *Salmonella*, several virulence factors are known to be regulated by the Crp-cAMP complex, including *sirA*, which regulates the expression of several key invasion genes encoded in *Salmonella* pathogenicity island 1 (SPI-1) (2, 39). SPI-1 encodes a type III secretion system and effector molecules that drive *Salmonella* invasion of mucosal tissues. In

addition, the Crp-cAMP complex in conjunction with the stress sigma factor RpoS influences the expression of the *spv* genes, required for full invasion of mucosal tissues and expression of several fimbrial operons (23, 25, 50). In *Salmonella* and *Y. enterocolitica*, *crp* deletion mutants are attenuated in mice and stimulate protective immunity against subsequent challenge with the wild-type strain (17, 40, 53, 59). In *Y. pestis*, the Crp-cAMP directly regulates expression of ~37 genes, including *ypkA* and *yopO*, which encode secreted virulence factors, the plasmid *pla* genes, a known virulence factor, and *pst*, encoding the bacteriocin pesticin (53, 80). *Edwardsiella* presents some virulence factors similar to those in other enterics (73), however the mechanisms of virulence are not well understood. On the other hand, the catabolic and virulence regulator Crp is conserved between enteric pathogens, including *E. ictaluri*. We hypothesized that deletion of *crp* could be used as a general means to attenuate enteric pathogens and develop live vaccines, including the catfish pathogen *E. ictaluri*. We found that the putative *crp* gene of *E. ictaluri* complements carbohydrate utilization in *S. enterica* Δ*crp* mutants in a cAMP-dependent fashion. Using the recent described technology (63), we constructed a precise in-frame genetic deletion of the *E. ictaluri crp* gene. The *E. ictaluri* Δ*crp-10* mutant was attenuated and conferred immune protection against *E. ictaluri* challenge to zebrafish (*Danio rerio*) and catfish (*Ictalurus punctatus*). These results indicate that the *E. ictaluri* Δ*crp-10* mutant strain is a potential candidate to further develop as an

immersion/oral vaccine for the catfish aquaculture industry.

3. *Materials and methods*

Ethics statement. All animal work was approved by the Arizona State University Institutional Animal Care and Use Committee, Protocol #09-1042R.

Bacterial strains, plasmids, media, reagents and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteriological media and components are from Difco (Franklin Lakes, NJ). Antibiotics and reagents are from Sigma (St. Louis, MO). Strains were routinely grown in LB broth (tryptone, 10 g; yeast extract 5 g; NaCl, 10 g; glucose 1g; ddH_2O 1L) (6), Bacto-Brain Heart Infusion (BHI), MacConkey agar base, OF-media, and Trypticase Soy Broth (TSB). When required, the media were supplemented with 1.5% agar, 5% sucrose, colistin sulfate (Col; 12.5 μg/ml), ampicillin (Amp; 100 μg/ml), chloramphenicol (Cm; 25 μg/ml), or kanamycin (Km; 50 μg/ml). Swimming medium consisted of BHI with a 0.3% (w/v) agar concentration. Swarming medium was identical but with an agar concentration of 0.5 % (w/v). Oligonucleotides were from IDT (Coralville, IA). Restriction endonucleases were from New England Biolabs. Taq DNA polymerase (New England Biolabs) was used in routine PCR tests. Qiagen products (Hilden,

Germany) were used to isolate plasmid DNA, gel-purified DNA fragments and purified PCR products. T4 ligase, T4 DNA polymerase and shrimp alkaline phosphatase (SAP) were from Promega.

Table 1

Bacterial strains and plasmids

Strain	Relevant characteristics	Source or reference
Escherichia coli		
χ6212	ϕ80d *lacZ* ΔM15 *deoR* Δ(*lacZYA-argF*)U169 *supE44* λ^- *gyr96 recA1 relA1 endA1* Δ*asdA4* Δ*zhf-2*::Tn10 *hsdR17* ($r^- m^{+}$); F^- Rec^- (UV^s) DAP^- Lac^- Nal^r Tet^s	(18)
χ7213	*thr-1 leuB6 fhuA21 lacY1 glnV44 recA1* Δ*asdA4* Δ(*zhf-2*::Tn*10*) *thi-1* RP4-2-Tc::Mu [λ*pir*]; Km^r Tet^s Amp^s DAP^-	(57)
χ7232	*endA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) *U169* λ*pir deoR* (ϕ*80dlac*Δ(*lacZ*)*M15*); Nal^r UV^s Thi^- Lac^-	(57)
Edwardsiella ictaluri		
J100	Wild-type; $pEI1^+$; $pEI2^+$ API20E 40040057; smooth LPS; Col^r	(54)
J100 (pEZ104)	J100 derivative; Δ*crp-10*::pEZ104; $pEI1^+$; $pEI2^+$; API20E 40040057; smooth LPS; Col^r; Amp^r	This study
J113	J100 derivative; Δ*crp-10*; $pEI1^+$; $pEI2^+$ API20E 40040057; smooth LPS; Col^r; Amp^s	This study
Salmonella enterica		
χ3761	*S*. Typhimurium UK-1	(32)
χ8132	*S*. Typhimurium UK-1; Δ*cya-27* Δ*crp-27*	(17)
χ3751	*S*. Choleraesuis *crp-773*::Tn*10* Tet^r	(40)
Plasmids		
pMEG-375	8,142 bp, Cm^r, Amp^r, *lacZ*, R6K *ori, mob incP, sacR sacB*	(62)
pACYC184	4,245 bp, Tet^r, Cm^r, p15A *ori*	(14)
pEZ104	Δ*crp-10*, pMEG-375	This study
pEZ135	P_{crp}-*crp*, Cm^r, Tet^r, pACYC184	This study
pEZ151	4,065 bp, pSC101 *ori*, Gm^r	This study
pEZ163	P_{crp}-*crp*, Gm^r, pEZ163	This study

Sequence analysis. Nucleotide Basic Local Alignment Search Tool (BLAST) was performed based on the sequence of the putative *crp* gene present in the genome sequence of *E. ictaluri* 93-146 (NC_012779). Crp sequences used were obtained from NCBI's Entrez Protein database. Amino acid sequence alignments were performed using the CLC Free Workbench software tool (v. 6.1 CLC bio A/S, Aarhus, Denmark). Protein structural-based alignments were performed using the web-based interface for ESPript v.2.2 located at http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi (28). The *E. ictaluri* Crp 3D structure was predicted using position specific iterative (PSI)-BLAST alignment and HHpred (68).

Construction and characterization of *crp* mutants. The recombinant suicide vector pEZ104 (Table 1) harboring the linked flanking regions (5' 333 bp and 3' 345 bp) to generate an in-frame deletion of the *crp* gene was constructed as described earlier (62, 63). The Δ*crp-10* defined deletion mutation encompasses a 630 bp region including the ATG start codon, but not including the TAG stop codon. Primers (primer 1) 5'-ACATGCATGCTTGCATGAAAGCGTCAATAT-'3 and (primer 2) 5'-CCGCTCGAGCCGGTGCGCCAAACGCATCC -'3 were designed to amplify the upstream *crp* flanking region (333 bp). A *Sph*I site was included in primer 1 (underlined) and a *Xho*I site was included in primer 2 (underlined). The downstream *crp* flanking region (445 bp) was amplified by primers (primer 3) 5 –

CCGCTCGAGTGATTTTGCGCCCGCGCCGC - 3 and (primer 4) 5'-TCGTCTAGACCTACAATGCTGAGGGTAA -'3. A *Xho*I site was included in primer 3 (underlined) and *Xba*I site was included in primer 4 (underlined). The flanking regions were amplified from *E. ictaluri* J100, ligated and cloned into pMEG-375 digested with *Sph*I and *Xba*I. The resulting plasmid was designated pEZ104. To construct the *E. ictaluri* Δ*crp-10* mutant, the suicide plasmid was transferred from *Escherichia coli* χ7213 (57) to *E. ictaluri* wild-type strain J100 by conjugation (63). Strains containing single-crossover plasmid insertions (*E. ictaluri crp*::pEZ104) were isolated on BHI agar plates containing Col, and Amp. Loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected by using the *sacB*-based sucrose-sensitivity counter-selection system (22). The colonies were tested for Amp^s and Col^r and screened by PCR using primers 1 and 4. Biochemical profiles of *E. ictaluri* strains were determined using the API 20E system (bioMériux, Marcy l'Etoile, France).

Complementation of *crp* gene. The *crp* gene of *E. ictaluri*, with its own promoter, was cloned into the pAYCY184 vector (14) at the *Xba*I and *Hin*dIII restriction sites and into pEZ151 at the *Adh*I restriction site (Table 1). The upstream and downstream primers used to amplify *crp* were 5' – TCGTCTAGAGCCGATATGCACCTTTAATG – 3' and 5' –

CCCAAGCTTTCAACGCGTCCCGTAGACGA – 3'. *Xba*I and *Hin*dIII sites were included in these primers, respectively (underlined). The resulting plasmid, pEZ135 was used to complement different Δ*crp* mutant strains.

Sample preparation and transmission electron microscopy (TEM). To increase flagella synthesis, the bacterial samples were collected from motility agar plates away from point of inoculation (52). Negative staining was performed as described by Chandler and Robson (12).

SDS-PAGE and western blot. To evaluate the synthesis of Crp, the strains were grown in 3 ml of BHI or LB broth at 28°C with aeration (180 r.p.m.). The samples were harvested when the culture reached an absorbance of 1.0 ($O.D_{600}$ 1.0 ~$1x10^8$ CFU/ml). One ml of culture was collected and prepared for western blot analysis (61). The total proteins were normalized using a nanodrop spectrophotometer (ND-1000, NanoDrop) at 25 μg/μl and separated by 10% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (61). Fat-free milk powder solution (5%, wt/vol) in PBS supplemented with 0.05% Tween 20 (PBS-T) was used for blocking. The membrane was incubated individually with a primary rabbit polyclonal anti-GroEL antibody (Sigma) (1:10,000) or rabbit polyclonal anti-Crp antibody (1:10,000) for 1 h at room temperature, washed three times with PBS-T, and then incubated with a 1:10,000 dilution of

alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) (Sigma). Color was developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Amaresco), chromogenic substrates for alkaline phosphatase.

Infection and immunization of zebrafish (*D. rerio*). The zebrafish challenges were performed by the methodology described earlier (54), with modifications. The water temperature was 26 ± 1 °C and the fish were acclimated for 2 weeks prior to the experiment. Groups of twelve adult zebrafish (average weight, 0.5 g) were sedated in buffered tricaine methanesulfonate (pH 7.5) (100 mg/L; MS-222, Sigma) and injected intramuscularly (i.m.) with 10 µl of the bacterial suspension per fish. Two sets of controls were used: fish that were not injected and fish that were injected with 10 µl of sterile phosphate-buffered saline containing 0.01% gelatin (BSG) (18). Moribund fish demonstrating clinical signs were euthanized, necropsied, and plated for enumeration of bacterial loads in various organs (54). Survivors of each dose at 4 weeks post i.m. inoculation were challenged with 10^5 CFU of *E. ictaluri* (100 LD_{50}). The fish were fed twice daily with TetraMin Tropical Fish Flake Feed. During the experiments, the fish were observed daily, and every other day water quality was monitored for pH, NO_2, and NO_3 with standard kits (Lifegard Aquatics® Water Testing Strips). The LD_{50} was calculated by the method of Reed-Muench (3).

Intracoelomic infection and immunization of catfish (*I. punctatus*). Specific-pathogen-free channel catfish fingerlings were used with a mean weight of 18.5 ± 1.3 g. The animals were randomly assigned to treatment groups of 10-25 fish each in 100-liter tanks. Each tank was equipped with a re-circulating, biofiltered, mechanical filtered, and U.V. water treated system with 12 h light cycle per day (Fig. 1). The water temperature was set at 28 ± 1 °C during the first two weeks of acclimatization and during the course of the experiments. The fish were fed daily with commercial Aquamax grower 400 (Purina Mills Inc., St. Louis, MO). During the experiments, the fish were observed daily, and every other day water quality was monitored for pH, NO_2, and NO_3 with standards kits (Lifegard Aquatics® Water Testing Strips). Catfish were infected by the intracoelomic (i.c.) route with 10^8 to 10^5 CFU of *E. ictaluri* strains (fish were not fed until 1 h after infection). The fish were anesthetized with buffered MS-222 (pH 7.5) (100 mg/L) prior to handling. The LD_{50} was calculated by the method of Reed-Muench (3). Moribund animals were euthanized and then necropsied to evaluate presence of *E. ictaluri* in kidney, spleen and liver.

Figure 1. Catfish tanks (100 L) equipped with a self-contained, recirculating, biofiltered, mechanical filtered, and U.V. water treated system.

Bath infection and immunization of fish host. Adult zebrafish and catfish fingerlings were immersed into a solution of *E. ictaluri* (wild-type or Δ*crp-10*) of 10^7 CFU/ml for 30 min. Six weeks post-immunization, fish were challenged either i.m. or i.c. with 10^7 CFU of *E. ictaluri* (100 LD_{50}) or by bath with 10^7 CFU/ml of *E. ictaluri* (10 LD_{50}) for 30 min. The animals were fasted 24 h prior to oral inoculation and 1 h post inoculation. Non-

immunized animals were used as controls. During the experiments, the fish were observed daily. The LD_{50} was calculated by the method of Reed-Muench (3).

Oral immunization of catfish. Catfish were fasted for 24 h before oral inoculation with the respective *E. ictaluri* strains. Fish were anesthetized before handling. The animals were orally inoculated with 100 μl of the corresponding bacterial suspension (see below). BSG was used as a control. The fish were not fed until 1 h after inoculation. The LD_{50} was calculated by the method of Reed-Muench (3).

Colonization of zebrafish tissues by *E. ictaluri*. Colonization of spleen, kidney and gills by *E. ictaluri* was evaluated as follows. Following euthanasia, selected organs from infected and uninfected fish were removed by dissection with the aid of a stereomicroscope. Dissected organs were placed in a 1.5-ml microcentrifuge tube containing 200 μl of BSG and homogenized with pellet pestle (Pellet Pestle, catalog no. K749520-0090; Fisher Scientific). Serial dilutions of homogenates were prepared in BSG, and numbers of CFU were determined by plating on BHI Col agar plates.

Bacterial inoculum preparation. Bacterial strains were grown overnight as standing cultures that were diluted 1:20 into pre-warmed BHI

broth and grown with mild aeration (180 r.p.m.) at 28°C to an OD_{600} of 0.8 to 0.9 (~10^8 CFU/ml). Bacteria were sedimented 10 min by centrifugation (5,865 g) at room temperature and resuspended in BSG to densities appropriate for fish inoculation.

Purification of outer membrane proteins (OMP) and lipopolysaccharide (LPS) from *E. ictaluri*. *E. ictaluri* wild-type strain J100 was grown in 50 ml of BHI broth at 28°C with aeration (180 r.p.m.). The bacterial cells were collected when the culture reached an absorbance of 1.0 (A_{600} 1.0 ~$1x10^8$ CFU/ml) and centrifuged at 10,000 g for 10 min at 4°C. *E. ictaluri* cells resuspended in Tris-OH/EDTA buffer pH 7.4 (20 mM Tris-OH; 1 mM EDTA) were lysed by passing the culture twice through a French press (Thermo Electron Corporation) at 10,000 p.s.i. (6.9 MPa; 40K cell). The lysed cell preparation was centrifuged at 7,000 g for 10 min at 4°C to remove cell debris and unlysed cells. Outer membrane proteins (OMPs) were prepared as described previously (56), except 0.5% (wt/vol) Sarkosyl was used instead of Triton X-100. Lipopolysaccharide (LPS) extraction was performed by using TRI-regent (Sigma) as described previously (79). The LPS profile was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by silver staining (34, 76).

Production of rabbit anti-catfish IgM polyclonal antibody. IgM was purified from pooled channel catfish (*I. punctatus*) as described previously (47). A New Zealand rabbit (*Oryctolagus cuniculus*) was injected intradermally three times over the course of 2 months (0, 4 and 6 weeks) with 1.0 μg of purified catfish IgM dissolved in 1.0 ml of phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.2)) mixed 1:1 in TiterMax (Thermo). Blood was periodically collected from the jugular vein by using a sterile needle and syringe and stored at 4°C. Serum was stored at –20°C in small aliquots. Rabbit IgG was purified from sera with a Protein A/G column (Pierce). The rabbit IgG antibody was biotinylated with a Sulfo-NHS-Biotinylation kit (Pierce). Percent biotinylation was calculated by a competitive binding assay incorporating 2,4'-hydroxy azonbenzene benzoic acid and avidin, according to the instructions of the manufacturer.

Determination of IgM titers in serum and skin mucus. Cutaneous mucus and blood samples from the caudal sinus were collected both at two weeks prior to immunization and four weeks post immunization. Ten i.c. immunized fish and ten immersion-immunized fish were sampled individually. The experiment was repeated twice independently. Mucus was collected before blood collection to prevent possible cross contamination of samples. *E. ictaluri* outer membrane proteins and purified *E. ictaluri* LPS were independently applied to polyvinyl chloride 96-well

plates (100 ng per well). The plates were incubated overnight at 4°C, washed once with 200 μl of PBS–0.05% Tween (PBST) per well, and blocked with 1% sea buffer (Thermo, Rockford, IL) diluted in PBS (1 h at room temp). Catfish serum samples diluted in PBS (1:10) and undiluted mucus samples were plated in triplicate wells at 100 μl/well. Triplicate control wells on each plate contained diluted sera from immunized and non-immunized catfish. Mucus assays also included wells of pooled mucus from non-exposed fish. The plates were incubated overnight at 4°C and washed five times with PBST. The biotinylated rabbit anti-catfish Ig antibody was applied at 0.25 to 0.50 μg/well. The plates were incubated for 1 h at room temp and were washed five times with PBST. Application of the primary antibody was followed by application of streptavidin-alkaline phosphatase conjugate (Southern Biotech, Birmingham, AL) diluted in PBS (1:4,000). Enzyme substrate *p*-nitrophenyl phosphate diethanolamine (100 μl; Sigma) was added and incubated for 30 min at room temperature. The reaction was stopped with 50 μl of 3M NaOH. The absorbance (A_{405}) values were determined on a kinetic microplate reader (model V-max; Molecular Devices Corp., Sunnyvale, Calif.) at 30 min and 1 h. The immunized fish were challenged 6 weeks post immunization as described previously.

Statistics. Data are presented as the standard deviation in all assays. An ANOVA (SPSS Software) analysis, followed by LSD (Least Significant

Difference) method, was used to evaluate differences in antibody titers discerned to 95% confidence intervals. The Kaplan-Meier method (SPSS Software) was applied to obtain the survival fractions following challenges. $P<0.05$ was considered statistically significant.

4. *Results*

Sequence analysis. The catabolic and virulence regulator *crp* gene is wide spread not only between enteric pathogens, including *E. ictaluri*, but also within the bacteria domain (Fig. 2). Particularly, the *crp* gene from *E. ictaluri* (gene ID 238917983) presented similar organization to other *crp* genes found in Enterobacteriaceae (Fig. 3). The guanine plus cytosine (G+C) content found in the *E. ictaluri crp* gene was 51%, similar to the *Escherichia coli* and *Salmonella crp* genes.

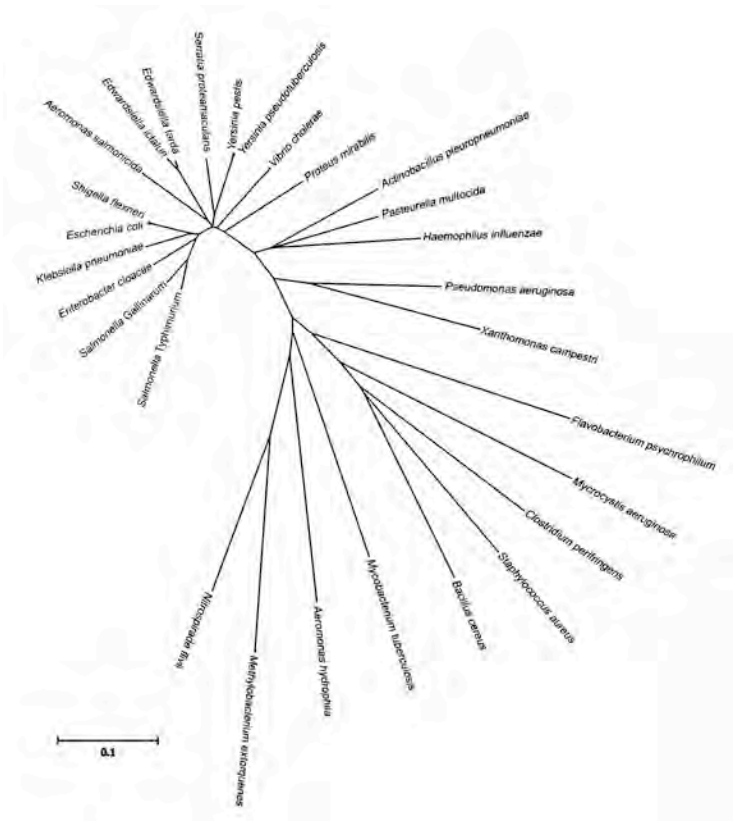

Figure 2. Evolutionary relationships of *crp* gene. The evolutionary history was inferred using the Neighbor-Joining method (60). The optimal tree with the sum of branch length = 4.69296866 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the *p*-distance method (48) and are in the units of the number of base differences per site. The analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 279 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (72).

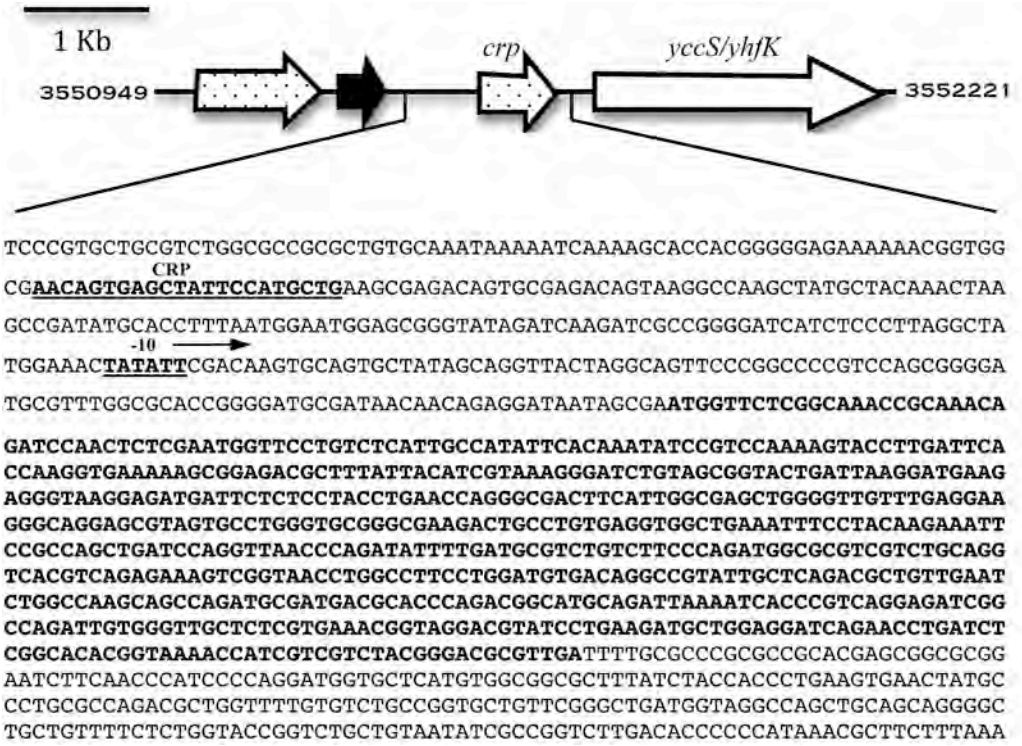

Figure 3. *E. ictaluri crp* gene. In bold is the gene sequence of the *E. ictaluri crp* gene. The predicted –10 region of the *crp* promoter is indicated. A predicted Crp binding region in the negative DNA strand is indicated in bold. The arrows at the DNA sequence indicate the direction of the transcription.

Sequence and structural alignment between functional representative bacterial Crp proteins revealed that 185 amino acid residues (~88%) are strictly conserved out of 210 residues in *E. ictaluri* Crp (Fig. 3). *E. ictaluri* Crp has 99%, 99.5%, 100%, and 100%

amino acid similarity to the Crp of *Y. pestis*, *Escherichia coli, S. enterica*, and *E. tarda,* respectively. Crp conformation and activity are dependent of cAMP binding. In the presence of cAMP, Crp adopts a conformation that promotes its interaction with DNA and RNA polymerase. These interactions are key to establishing active transcription complexes at Crp-dependent promoters (31). *E. ictaluri* Crp organization is similar to other Gram-negative Crp-family members, presenting the conserved cAMP binding amino acid residues: Tyr63, Glu72, Arg82, Ser83, Arg123, Thr127, and Ser128 (Fig. 4). The overall *E. ictaluri* Crp 3-D predicted structure is similar to *E. coli* Crp, presenting the C-helix and the F-helix that interacts with the DNA and the flexible hinge required for Crp dimerization (31) (Fig. 4). This indicates that Crp function is conserved through the evolution of the Enterobacteriaceae family, including enteric fish pathogens.

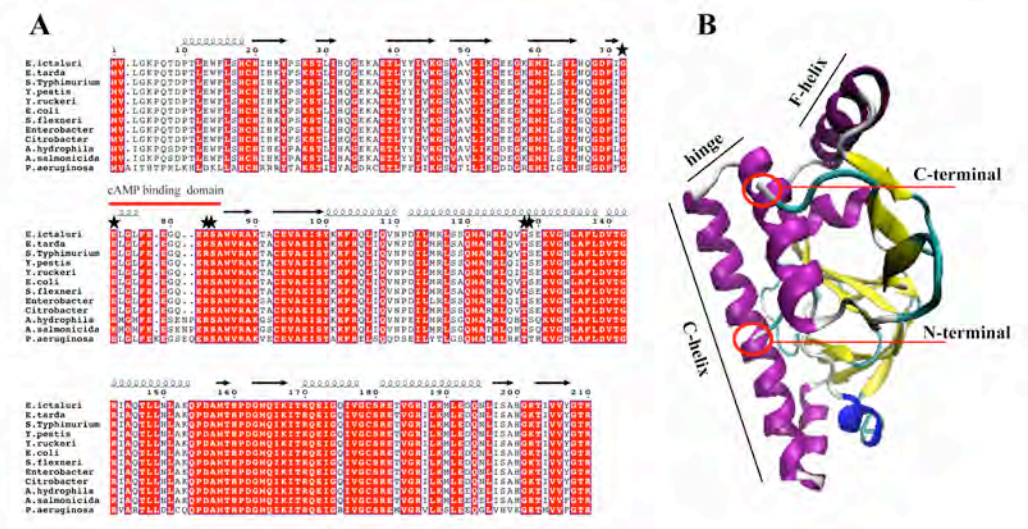

Figure 4**.** *E. ictaluri crp* gene and Crp protein. A. Secondary structure of *E. ictaluri* Crp and alignment between representative Crp

proteins. The secondary structure at the top of the alignment corresponds to the *E. ictaluri* Crp (spirals represent α-helix; arrows represent β-sheet). Conserved amino acids residues are indicated in red. The star indicate the amino acid residues required for cAMP binding (Gly 71, Glu72, Arg82, Ser83, Thr127, and Ser128); B. 3-D *E. ictaluri* Crp monomer structure. The helixes required for DNA binding and dimmer Crp formation are indicated (C-helix, F-helix, and hinge). The N- and C-termini are indicated in a red circle.

Construction and characterization of *crp* mutants. The construction of the *E. ictaluri* Δ*crp-10* mutant was performed using pEZ104, a pMEG-375 (Cm, Amp) based suicide vector (Table 1). *E. ictaluri* Δ*crp-10* mutants were recovered from TSA sucrose agar selection plates. The genotype was verified by PCR (Fig. 5A-5B), and the phenotype by growth on MacConkey agar supplemented with maltose (1%) (Fig. 4C). The biochemical profile, evaluated by API20E, did not show differences (400400057) between the wild-type and Δ*crp-10* mutant strains. This is mainly due to the fact that *E. ictaluri* uses/ferments fewer types of carbohydrates in contrast to other members of the Enterobacteriaceae family (Table 2). In particular, *E. ictaluri* Δ*crp-10* does not utilize maltose (Fig. 5C; Table 2). These results indicate that Crp positively regulates the genes related to maltose utilization. Galactose utilization was partially affected in *E. ictaluri* Δ*crp-10*. The same result was observed with

mannose (Table 2).

The Crp regulatory complex not only is involved in positive regulation of catabolic functions but also is required for flagella synthesis (8). We evaluated the motility of the Δ*crp-10* mutant in BHI supplemented with 0.3% and 0.5% agar and flagella synthesis by TEM. We found that the Δ*crp-10* mutant loses motility (Fig. 5D) due to the lack of flagella synthesis (Fig. 5E), indicating that Crp positively regulates flagella synthesis in *E. ictaluri*.

Table 2

Crp effect on sugar utilization; the strains were grown in BHI broth at 28°C overnight and inoculated into OF-media supplemented with 1% of the corresponding carbohydrate. The results were read after 48 h of incubation at 28°C.

Sugar	*E. ictaluri* Wild-type	*E. ictaluri* Δ*crp-10*	*S.* Choleraesuis Wild-type	*S.* Choleraesuis *crp*::Tn10	*S.* Choleraesuis *crp*::Tn10 (pEZ135)
None (control)	–	–	–	–	–
L-arabinose	–	–	–	–	–
Fructose	+	+	+	+	+
Galactose	+	±	+	+	+
D-glucose	+	+	+	+	+
Glycerol	+	+	+	+	+
Maltose	+	–	+	–	+
Mannitol	–	–	+	–	+
D-mannose	+	±	+	+	+
D-lactose	–	–	–	–	–
Sorbitol	–	–	+	–	+

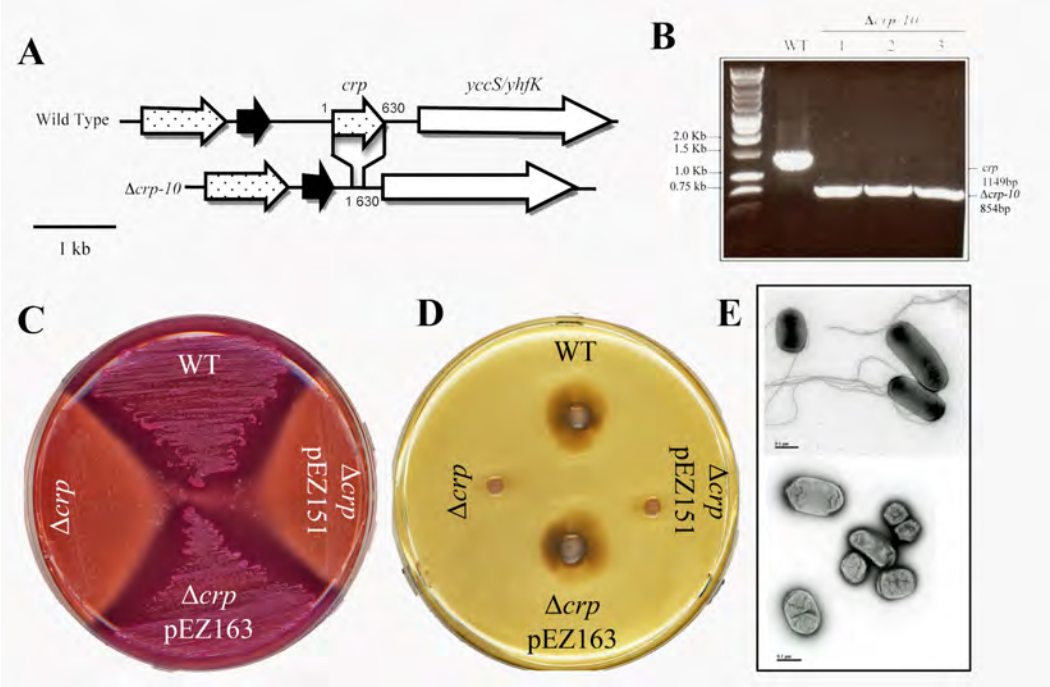

Figure 5. *E. ictaluri* J113 Δ*crp-10* genotype and phenotype. A. Deletion map of *E. ictaluri* J113 Δ*crp-10*; B. Genotype verification of *E. ictaluri* J113 Δ*crp-10* by PCR; C. Phenotype verification on MacConkey agar plates supplemented with 1% maltose; WT: *E. ictaluri* J100 wild type; Δ*crp-10*: *E. ictaluri* J113 Δ*crp-10*; Δ*crp-10* (pEZ163): *E. ictaluri* J113 Δ*crp-10* complemented with *crp* gene wild-type; D. Swimming zones through 0.3% BHI agar; E. Negative staining-transmission electron microscopy of *E. ictaluri* J100 wild-type and *E. ictaluri* J113 Δ*crp-10* mutants. Formvar-coated copper grids (Electron Microscopy Sciences, Hat¢eld, PA, USA) were coated with *E. ictaluri* samples and negatively stained with 1% uranyl acetate (pH 7.0). Grids were viewed with a Philips 301 electron microscope (Philips International, Eindhoven, the Netherlands) at a 60 kV acceleration voltage.

The *malT*/*malP* and *malE*/*malK* promoter regions present several Crp binding sites (Fig. 6), as well as do the promoters for the *galETK* (galactose utilization), *galABC* (galactose transport), *manXY* (mannose transport) and *flhDC* (master flagella regulator) operons (data not shown). These last observations correlate with the phenotype of *E. ictaluri* Δ*crp-10* mutants.

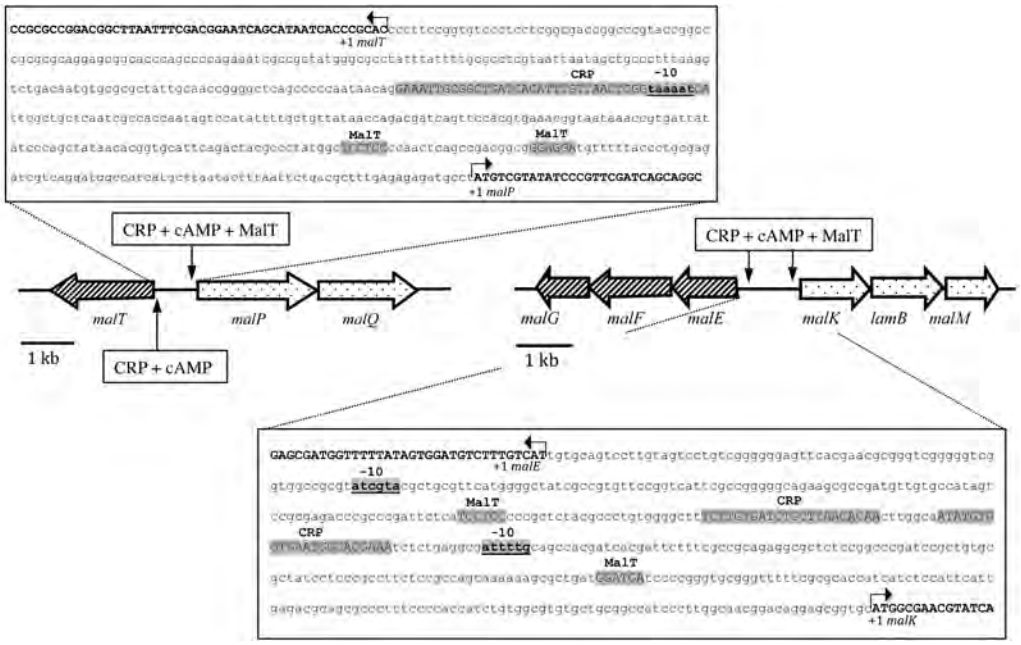

Figure 6. Putative binding site for Crp at the *malT/malP* and *malQ/malK* promoters. Expression of *malT* requires Crp + cAMP complex binding at the promoter region. Expression of the rest of the genes requited for maltose transport and utilization required Crp + cAMP + MalT binding at their respective promoter regions. The boxes show the promoter sequences at the *malT/malP* and *malQ/malK* promoters.

Complementation of the *E. ictaluri crp* gene and *E. ictaluri* Δ*crp-10* mutants. The structural analysis of *E. ictaluri* Crp indicated that the overall domain organization is similar to other Crp-family members. It has the same set of key active-site functional groups and probably the same mechanism as Crp in other organisms (Fig. 4). To evaluate the likely broad functionality of *E. ictaluri* Crp, *crp* mutants of *Salmonella enterica* were complemented with the *E. ictaluri crp* gene. *S*. *enterica* serovar Choleraesuis χ3751 *crp-773*::Tn10 was utilized for complementation assays with pEZ135.

As mentioned previously, Crp requires cAMP to activate Crp-dependent promoters. However, mutant alleles encoding forms of Crp exist, collectively known as Crp*, that activate Crp-dependent promoters in the absence of cAMP (31). *cya* mutant strains do not synthesizes cAMP due to the lack of the adenyl cyclase enzyme and fail to express Crp-dependent operons. The Δ*cya* Δ*crp* mutants allow the detection of Crp* alleles by complementation with *crp*. Therefore, *S. enterica* serovar Typhimurium χ8132 Δ*cya-27* Δ*crp-27* was used to evaluate the dependence on cAMP by the *E. ictaluri* Crp.

The MalT regulator, which controls maltose import and utilization, is positively regulated by Crp (15, 16) (Fig. 6). Consequently, *crp* mutants lack the capacity to utilize maltose as a carbon source. We evaluated whether *E. ictaluri crp* reverts this phenotype in *S. enterica crp* mutants. The *E. ictaluri crp* gene complemented *S*. *enterica* Δ*crp*

mutants, allowing the utilization of maltose and other carbon sources regulated by Crp-cAMP (Table 2; Fig. 7A-7C). We also determined that *E. ictaluri crp* is dependent on cAMP, not complementing *S. enterica* Δ*cya-27* Δ*crp-10* mutants (Fig. 7A-C). Typically, the growth rate of Δ*crp* mutants is much slower than wild type (19, 59), including *E. ictaluri* Δ*crp-10* mutants (Fig. 7D). This phenotype was reversed in *S. enterica crp* mutants complemented with the *E. ictaluri crp* gene (Fig. 7E).

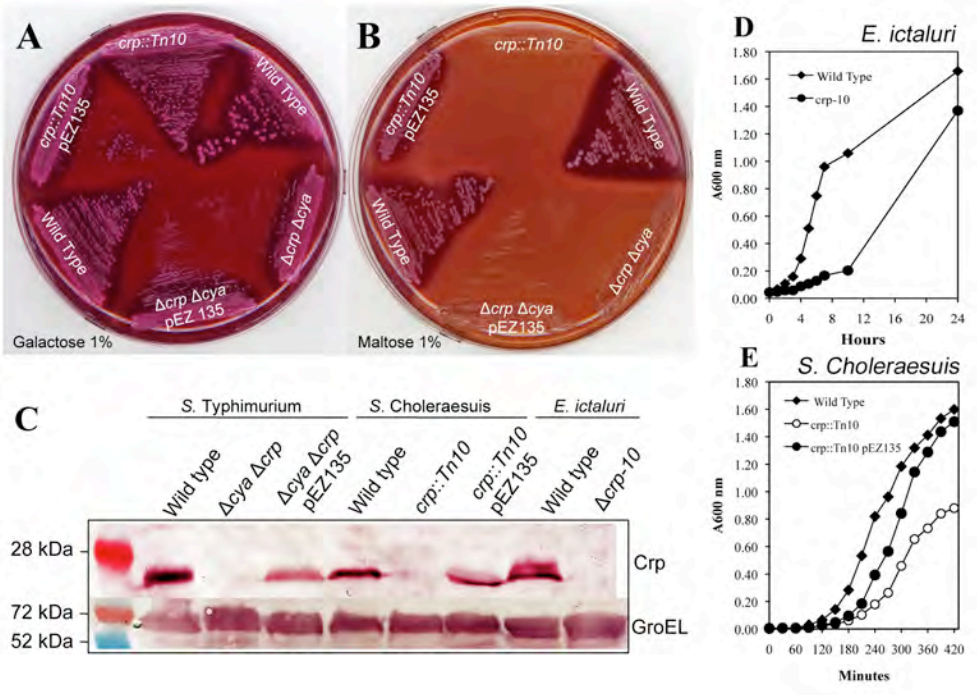

Figure 7. Complementation of *S. enterica crp* mutants by *E. ictaluri crp* gene cloned in pEZ135. A. Phenotype verification of functionality of Crp on MacConkey agar plates supplemented with galactose (positive control); B. Phenotype verification of functionality of Crp on MacConkey agar plates supplemented with maltose; C. Synthesis of Crp verified by western

blot analysis. GroEL was used as control; D. Growth of *E. ictaluri* J100 and J113 Δ*crp-10* in BHI broth at 28°C with aeration (180 r.p.m.); E. Growth of *S.* Choleraesuis χ3751 *crp::*Tn*10* and χ3751 *crp::*Tn*10* (pEZ135) in LB broth at 37°C with aeration (180 r.p.m.).

Virulence and immune protection of *E. ictaluri* Δ*crp-10* in zebrafish. The ideal live attenuated bacterial vaccine should be totally attenuated and immunogenic. Here we evaluated J113 *E. ictaluri* Δ*crp-10* mutants in the zebrafish host. Zebrafish have been developed as an easy and powerful model to test pathogenesis of *E. ictaluri* (35, 54, 63) and other bacterial fish pathogens (45, 51, 55). We found that *E. ictaluri* Δ*crp-10* was attenuated with an LD_{50} of 10^6 CFU, a thousand-fold increase over wild type (Figs. 8A-5B). Fish that survived the inoculation of 10^6 and 10^4 CFU of *E. ictaluri* Δ*crp-10* were challenged 4 weeks post immunization with 10^5 CFU *E. ictaluri* J100 wild type (100 LD_{50}). The *E. ictaluri* Δ*crp-10* mutant was immune protective in the zebrafish host, with 100% survival after immunization with 10^6 CFU and 90% survival after immunization with 10^4 CFU doses by i.m. immunization, respectively (Fig. 8C).

Establishing the means by which *Edwardsiella* infect and colonize fish tissues provides a design strategy to develop effective live vaccines. We evaluated the colonization of spleen, kidney, and gills 3 days post i.m. infection with *E. ictaluri* J113 Δ*crp-10* in comparison with the wild type. We found that *E. ictaluri* J113 Δ*crp-10* colonized spleen, kidney and gills

but at lower levels than the wild type (Fig. 8D). This indicated that *E. ictaluri* Δ*crp-10* reached lymphoid tissues after i.m. immunization, thus trigging a protective immune response (Figs. 8C-8D).

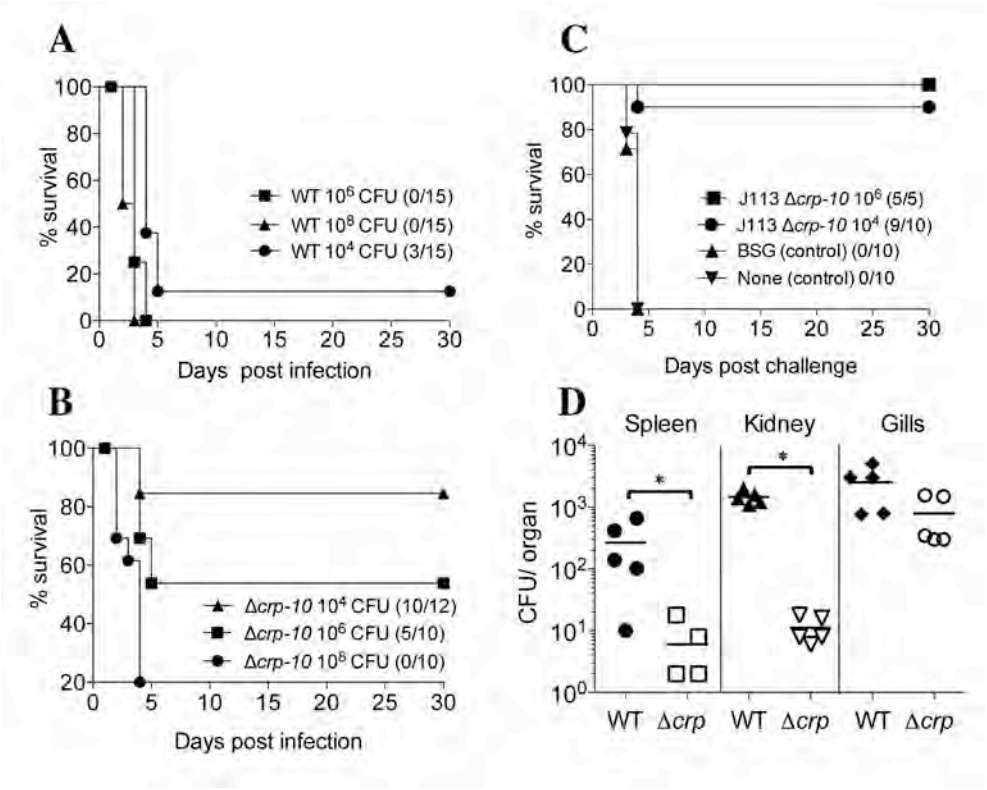

Figure 8. Zebrafish survival post i.m. infection with (A) J113 *E. ictaluri* Δ*crp-10* (n=15 per dose); B. J100 *E. ictaluri* 2003/C wild type (n=10-12 per dose). The experiments were done two times independently for J113 and ten times independently for the wild type. Challenge and colonization of zebrafish i.m. inoculated with *E. ictaluri* strains. C. Challenge of zebrafish immunized with *E. ictaluri* Δ*crp-10* four weeks post-immunization. Zebrafish were challenged with 100 times LD_{50} of J100 *E. ictaluri* wild type ($1.0x10^5$ CFU). The experiments were done two times independently

for each strain; D. Colonization of zebrafish tissues by *E. ictaluri* wild type and *E. ictaluri* Δ*crp-10* after three days of i.m. inoculation with 10^4 CFU of the respective strain. Each point represents one fish. **P*<0.001

We determined that *E. ictaluri* Δ*crp-10* inoculated by immersion ($3.2x10^7$ CFU/ml during 30 min) colonized zebrafish spleen, kidney and gills at low levels (Fig. 9A). Therefore, we evaluated the immune protection after 4 weeks post bath immunization with *E. ictaluri* Δ*crp-10*. *E. ictaluri* Δ*crp-10* applied by immersion conferred 93% protection in zebrafish (Fig. 9B), indicating that *E. ictaluri* Δ*crp-10* is a promising candidate to develop bath vaccines for the catfish aquaculture industry.

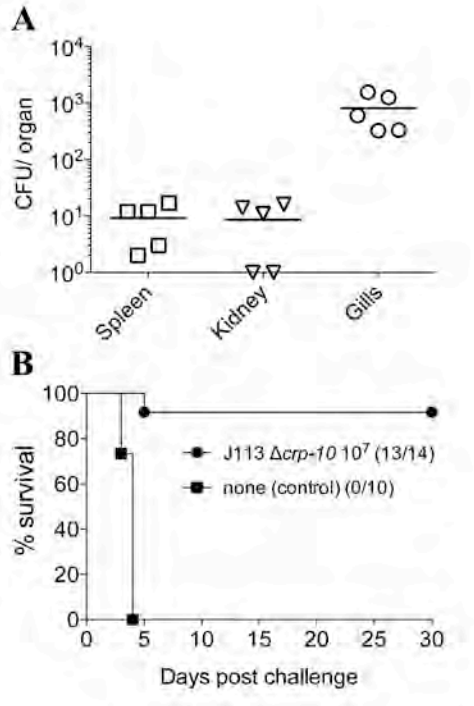

Figure 9. Colonization and challenge of zebrafish inoculated by immersion with *E. ictaluri* J113 Δ*crp-10*. A. Challenge of zebrafish bath

immunized with *E. ictaluri* J113 Δ*crp-10* four weeks post-immunization. Zebrafish were challenged with 100 times LD_{50} of *E. ictaluri* J100 wild type ($1.0x10^5$ CFU). The experiments were done two times independently for each strain. B. Colonization of zebrafish tissues by *E. ictaluri* Δ*crp-10* three days post immersion inoculation with 10^7 CFU/ml of *E. ictaluri* J113 Δ*crp-10*. Each point represents one fish.

Virulence and immune protection of the *E. ictaluri* Δ*crp-10* mutant in catfish. The *E. ictaluri* Δ*crp-10* mutant was evaluated in the catfish host. We found that *E. ictaluri* Δ*crp-10* applied by the i.c. route was attenuated with an estimated 1000-times LD_{50} increase or more over the wild type (Fig. 10A). This result indicates that *E. ictaluri* Δ*crp-10* does not cause systemic symptoms and that the host is able to control the infection. Fish that survived the i.c. inoculation with *E. ictaluri* Δ*crp-10* were challenged i.c. with 10^7 CFU *E. ictaluri* J100 wild type (100 LD_{50}) 6 weeks post immunization. *E. ictaluri* Δ*crp-10* applied by the i.c. route was immune protective in the catfish host, with 100% survival (Fig. 10B).

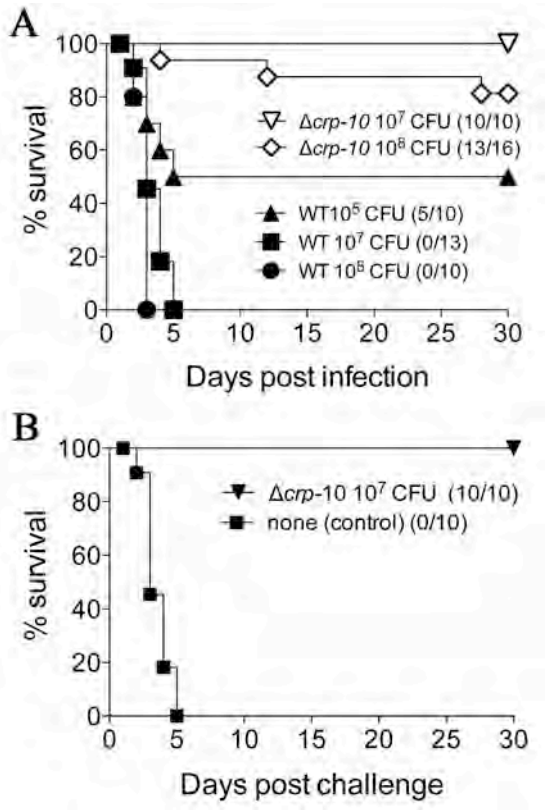

Figure 10. Catfish survival post i.c. infection with (A) J113 *E. ictaluri* Δ*crp-10* (n=15 per group); B. J100 *E. ictaluri* 2003/C wild type (n=10 per group) and challenge. The experiments were repeated two times independently for J113 and ten times independently for the wild type.

We evaluated the immune protection after 6 weeks post bath immunization with *E. ictaluri* Δ*crp-10* (10^7 CFU/ml during 30 min). Vaccinated catfish were challenged i.c. with *E. ictaluri* wild type to evaluate systemic immune protection conferred by the single bath immunization. We found 20% survival at the 10^7 CFU/dose

challenge and 70% survival at the 10^6 CFU/dose challenge (Fig. 11A). Catfish that were independently immersion immunized and independently orally immunized presented 92% and 100% protection against the wild type immersion challenge, respectively (Fig. 11B).

We observed that catfish i.c. infected with *E. ictaluri* wild type developed a systemic infection characterized by red skin, typically covering the whole animal (Fig. 11D). Catfish that were bath or orally infected with *E. ictaluri* wild type developed the typical symptoms of enteric septicemia, skin lesions and distended abdomen (Fig. 11E). Immersion vaccinated catfish that succumbed to the *E. ictaluri* i.c. challenge presented enteric septicemia symptoms, with skin lesions and distended abdomen, instead of hemorrhagic symptoms (Fig. 11D).

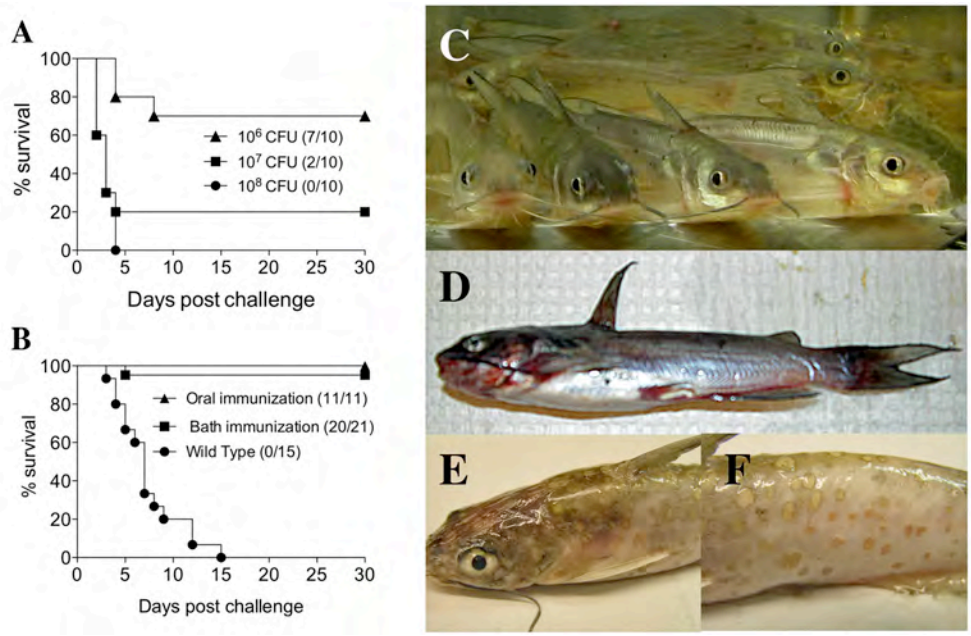

Figure 11. Catfish survival post immersion and oral vaccination with

E. ictaluri Δ*crp-10.* A. *E. ictaluri* wild type i.c. challenged 6 weeks post immersion vaccination with *E. ictaluri* Δ*crp-10* (10^7 CFU/dose); B. *E. ictaluri* wild type immersion challenged 4 weeks post immersion and oral vaccination with *E. ictaluri* Δ*crp-10* (10^7 CFU/dose); C. Catfish fingerling non-infected; D. Catfish i.c. infected with *E. ictaluri* wild-type; E. Catfish bath infected with *E. ictaluri* wild-type.

The skin mucosal immune system is an important defense mechanism in fish. It is also known that skin IgM is produced independently of the systemic IgM (10, 24, 30, 46, 47, 58). Evaluation of the IgM titers against *E. ictaluri* LPS and OMPs from i.c. or immersion vaccinated catfish indicated that *E. ictaluri* Δ*crp-10* triggers systemic and skin antibody responses (Fig. 12). IgM titers of immersion-vaccinated catfish were lower compared to i.c.-vaccinated catfish as expected. However, in both cases skin IgM titers were significantly higher than naïve non-vaccinated fish (Fig. 12). *E. ictaluri* Δ*crp-10* triggers a systemic and skin antibody response, making it a promising live immersion vaccines for catfish.

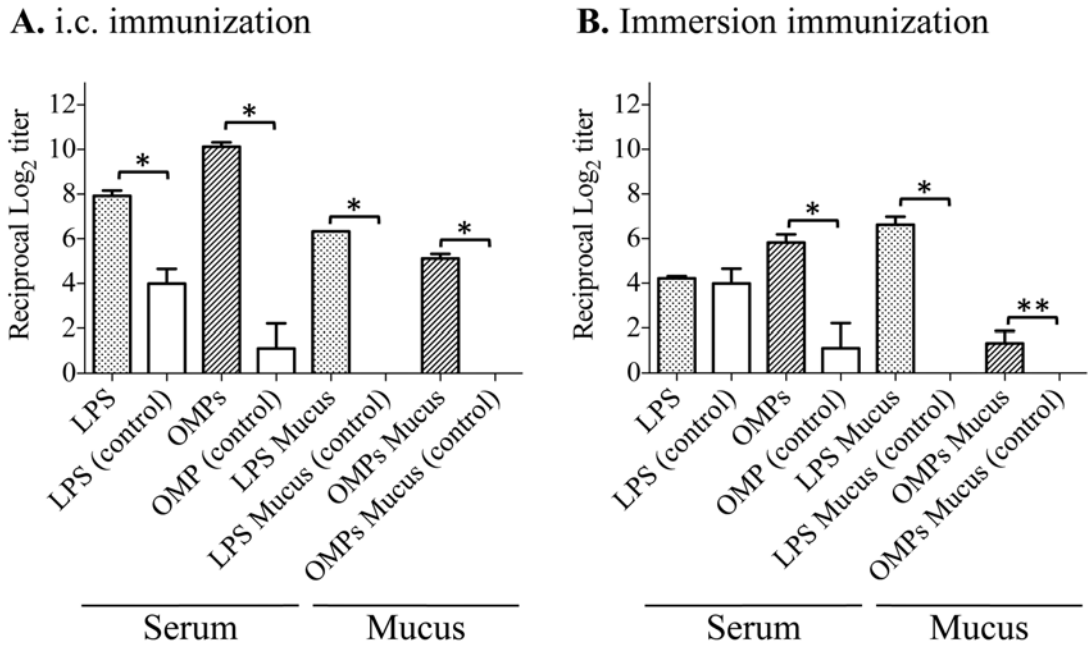

Figure 12. Immune response of catfish vaccinated with *E. ictaluri* Δ*crp-10.* A. Catfish immunized i.c. with *E. ictaluri* Δ*crp-10* (10^7 CFU/dose) (n=20); B. Catfish bath immunized with *E. ictaluri* Δ*crp-10* (10^7 CFU/ml, 30 min) (n=20). The samples were taken 30 days post immunization. The samples correspond to two independent experiments with 10 animals each. **P*<0.001; ***P*<0.05

5. *Discussion*

The cAMP and cAMP-binding domain are conserved from bacteria to humans as an ancient ubiquitous signaling mechanism to translate extracellular stress signals into appropriate biological responses (5). cAMP stimulates protein kinases and regulatory proteins that bind directly to DNA. Proteins harboring a cAMP-binding domain that covalently links to the DNA binding domain are conserved in prokaryotes, like Crp, which is broadly distributed among the bacteria (Fig.2). This suggests that Crp regulates a similar family of genes in both non-pathogenic and pathogenic bacteria. In most kinds of bacteria where Crp is present, it regulates transcription of several operons related to carbohydrate metabolism (8, 44, 75), development of competence for transformation (11, 13), and growth phase-dependent regulation of gene expression (1). In pathogenic bacteria, Crp also regulates genes related to virulence (17, 20, 41, 59, 67, 70, 78). As an example of this last point, we showed that *E. ictaluri* Crp regulates the transcription of genes related to carbohydrate utilization in a cAMP-dependent fashion (Figs. 5 and 7), and regulates genes related to pathogenesis (Figs. 8 and 9). On the other hand, deletion of *crp* has been successfully used in different bacterial pathogens to develop live attenuated bacterial vaccines for mice (17), pigs (41), horses (64), and birds (59). We thus proposed that deletion of *crp* can be universally used to develop live attenuated bacterial vaccines. Deletion of the *crp* gene

to develop live attenuated vaccines has not been tested in teleost fish, the most primitive bony vertebrates that contain immunoglobulins (26). In this study, *E. ictaluri* Δ*crp* evaluated in the fish host confirm our prediction. We determined that *E. ictaluri* Δ*crp-10* was attenuated and immune protective in zebrafish and catfish (Figs.8-11).

Traditional vaccines like the formalin killed *E. ictaluri* vaccine applied by immersion does not colonize lymphoid tissues, and results in poor immune protection (74). Live attenuated bacterial vaccines mimic the route of natural infection, possess intrinsic adjuvant properties, and can be administrated mucosally. *E. ictaluri* Δ*crp-10* applied by immersion colonized gills, spleen, and kidney, conferring immune protection to zebrafish (Fig. 9). Transient colonization of gills, spleen, and kidney, is important to trigger a complete immune response and confer protection in fish. However, other mucosal tissues like skin, the olfactory organ, gut, and eyes may also be important to trigger a complete immune response, or may engage a response at the skin mucosal immunity level, which could be reflected in the outcome of the symptoms of immersion vaccinated fish challenged by the i.c. route (Fig. 11). Gut mucosal immunity certainly plays an important role in fish adaptive immunity, as has been elegantly demonstrated in trout (81). Analysis of gut mucosal immunity, skin immunity and systemic immunity during *E. ictaluri* vaccination could explain the outcome of the symptoms after challenge.

As mentioned previously, deletion of the evolutionary

conserved *crp* gene has been used to developed live attenuated bacterial vaccines. Although this strategy has often been successful, its use in the human host restricted pathogen *S*. Typhi was not satisfactory (27, 71). The results shown here and present literature about *crp* mutant vaccine strains raise the question of which combination of evolutionary conserved genes are regulated in pathogenic bacteria that make *crp* mutants such an excellent vaccine candidate for different hosts. The answer to this question could allow us to optimize the trade-off between attenuation and immune protection in order to develop a universal family of live attenuated bacterial vaccines.

In summary, we conclude that deletion of *crp* can be successfully used as a means to attenuate *E. ictaluri* to develop effective immersion live attenuated antibiotic-sensitive vaccines for the aquaculture industry.

6. *Acknowledgments*

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CHAPTER III

THE FUR REGULATED IRON UPTAKE SYSTEM OF *Edwardsiella ictaluri* AND ITS INFLUENCE ON PATHOGENESIS AND IMMUNOGENICITY IN THE CATFISH HOST

1. *Abstract*

The ability of bacterial pathogens to uptake iron from the host during infection is necessary for their multiplication within the host. However, host high-affinity iron-binding proteins limit levels of free iron in fluids and tissues. To overcome this deficiency of iron during infection bacterial pathogens have developed iron uptake systems that are up-regulated in the absence of iron, typically tightly controlled by the ferric uptake regulator (Fur) protein. The iron uptake system of *Edwardsiella ictaluri*, a host-restricted pathogen of channel catfish (*Ictalurus punctatus*) and the main pathogen of this fish aquaculture, is unknown. Here we describe the *E. ictaluri* Fur protein, the iron uptake machinery controlled by Fur, and the effects of *fur* gene deletion on virulence and immunogenicity in the fish host. Analysis of the *E. ictaluri* Fur protein shows that it lacks the N-terminal region found in the majority of pathogen encoded Fur proteins. However, it is fully functional in regulated genes encoding iron uptake proteins. *E. ictaluri* grown under iron limited conditions up-regulates an outer membrane protein (HemR) that shows heme-hemoglobin

transport activity and is tightly regulated by Fur. In vivo studies showed that an *E. ictaluri* Δ*fur* mutant is attenuated and immune protective in zebrafish (*Danio rerio*) and catfish (*Ictalurus punctatus*), triggering systemic and skin mucosal immunity. We conclude that an *E. ictaluri* Δ*fur* mutant could be an effective component of an immersion-oral vaccine for the catfish industry.

2. *Introduction*

Vertebrates sequester iron from invading pathogens as a means of nutritional immunity, using high-affinity iron-binding proteins to limit levels of free iron in biological fluids and tissues in order to deprive pathogens of this key nutritional component. Invading bacterial pathogens sense this iron depletion as a signal that they are within a host and induce the expression of genes that allow iron uptake in order to overcome the host defenses. To obtain this host sequestered iron, most pathogenic bacteria have developed iron uptake systems that usually are siderophore mediated or that directly uptake iron from host proteins (50). Siderophore-mediated systems typically involve low molecular weight siderophores released by the bacteria that chelate iron and subsequently transfer it to iron-regulated outer membrane proteins (IROMPs) that function as receptors of the iron-siderophore complexes (39, 50). These siderophore mechanisms of iron acquisition have been linked to the virulence of

different fish bacterial pathogens such as *Vibrio anguillarum* (*Listonella anguillarum*) (38, 71), *Aeromonas salmonicida* (24, 27), *Photobacterium damselae* subsp. Piscicida (13), *Edwardsiella tarda* (25) and *Tenacibaculum maritimum* (2). Direct iron uptake systems from host proteins rely on the interaction between specific microbial receptors and host transferrin or heme-containing compounds (31, 41), and often involve bacterial hemolytic or proteolytic activity (10). Usually, both siderophore-mediated and direct iron uptake systems are controlled by the ferric uptake regulator (Fur) protein (10).

Fur is a dimeric metal ion-dependent transcription regulator that controls the expression of genes involved in a diversity of cellular functions, including iron uptake. Fur monomers typically contain two structural domains, the N-terminal DNA binding domain and the C-terminal dimerization domain (26, 28, 29). When Fur monomers are bound to Fe^{2+} they form a dimer that binds to promoter DNA regions (Fur box), repressing gene expression (29). During oral-gastric infection, it is thought that the small intestine conditions are anaerobic and therefore replete with free Fe^{2+}, leading to an active Fur protein that represses genes involved in iron uptake. The iron uptake system is induced upon invasion, when iron is presumably sequestered by host iron-binding proteins (26, 28).

Channel catfish (*Ictalurus punctatus*) is the most important aquaculture species in the United States, accounting for more than 60% of all U.S. aquaculture production (20), and *E. ictaluri* is one of the

most important pathogens in this industry (61). The most highly up-regulated group of catfish genes following *E. ictaluri* infection are the genes involved in iron homeostasis, including intelectin, haptoglobin, haemopexin (*Wap65*), ceruloplasmin, transferrin and ferritin (34, 35, 45, 46, 63). This indicates that there is a "tug of war" for the iron between the catfish host and the bacterial pathogen *E. ictaluri*. While the iron acquisition system of *E. ictaluri* has not been previously characterized, we observed that *E. ictaluri* grown in the absence of iron up-regulates synthesis of a specific outer membrane protein. This observation prompted us to investigate the iron uptake system of *E. ictaluri*.

In this study, we characterized the *E. ictaluri* Fur protein, the iron uptake system controlled by Fur, and the effects of the *fur* gene on virulence and immunogenicity in the fish host. We determined that fish isolates of *Edwardsiella* have a smaller *fur* gene compared to other *fur* family members, where its evolutionary pathway may have undergone genome degradation. We also established that *E. ictaluri* does not secrete detectable siderophores, but does contain a heme-hemoglobin uptake system regulated by Fur.

The Fur protein not only regulates iron uptake related genes, but also genes important to virulence. *Salmonella* Δ*fur* mutants are attenuated in mammals when administered orally (51) or intraperitoneally (16), but are not very immunogenic (11). We also evaluate the potential utilization of *E. ictaluri* Δ*fur* mutants as a live attenuated vaccine. *E. ictaluri* Δ*fur*

mutants were attenuated in zebrafish and catfish hosts. When *E. ictaluri* Δ*fur* mutants were administered by immersion or orally they conferred immune protection, triggering systemic and skin immune responses.

3*. Materials and methods*

Bacterial strains, plasmids, media, and reagents. The bacterial strains and plasmids are listed in Table 1. Bacteriological media and components are from Difco (Franklin Lakes, NJ). Antibiotics and reagents are from Sigma (St. Louis, MO). LB broth (tryptone, 10 g; yeast extract 5 g; NaCl, 10 g; glucose 1g; ddH_2O, 1L) (5), Bacto-Brain Heart Infusion (BHI) broth, CAS broth (58), and Trypticase Soy Broth (TSB), were used routinely. When required, media were supplemented with 1.5% agar, 5% sucrose, colistin sulphate (Col; 12.5 µg/ml), ampicillin (Amp; 100 µg/ml), chloramphenicol (Cm; 25 µg/ml), kanamycin (Km; 50 µg/ ml), $FeSO_4$ (150 µM; Sigma) or 2,2'-dipyridyl (150 µM; Sigma). Bacterial growth was monitored spectrophotometrically and/or by plating. Oligonucleotides were from IDT (Coralville, IA). Restriction endonucleases were from New England Biolabs. Taq DNA polymerase (New England Biolabs) was used in all PCR tests. Qiagen products (Hilden, Germany) were used to isolate plasmid DNA, gel-purify fragments or purify PCR products. T4 ligase, T4 DNA polymerase and shrimp alkaline phosphatase (SAP) were from Promega.

Table 1

Bacterial strains and plasmids

Strain	Relevant characteristics	Source or reference
Escherichia coli		
χ6212	φ*80d lacZ* Δ*M15 deoR* Δ(*lacZYA-argF*)-U169 *glnV44* λ$^-$ *gyrA96 recA1 relA1 endA1* Δ*asdA4* Δ*zhf-2*::Tn*10 hsdR17* (r$^-$ m$^+$); F$^-$ Rec$^-$(UV^s) DAP$^-$ Lac$^-$ Nal^r Tet^s	(12)
χ7213	*thi-1 thr-1 leuB6 glnV44 fhuA21 lacY1 recA1* RP4-2-Tc::Mu [λ*pir*] Δ*asdA4* Δ(*zhf-2*::Tn10); Km^r Tet^s Amp^s DAP$^-$	(52)
χ7232	*endA1 hsdR17* (r_K^- m_K+) *glnV44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)-U169 λ*pir deoR* (φ*80dlac* Δ(*lacZ*)*M15*); Nal^r UV^s Thi$^-$ Lac$^-$	(52)
χ7122	*E. coli* O78	(49)
Edwardsiella ictaluri		
J100	Wild-type; pEI1$^+$; pEI2$^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	(47, 57)
J135	J100 derivative; Δ*fur-35*; pEI1$^+$; pEI2$^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	This study
J146	J100 derivative; Δ*hmuR36*; pEI1$^+$; pEI2$^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	This study
J147	J135 derivative; Δ*fur-35* Δ*hmuR36*; pEI1$^+$; pEI2$^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	This study
J135	J100 derivative; Δ*fur-35*; pEI1$^+$; pEI2$^+$ API20E 40040057; smooth LPS; Col^r; H_2S^-	This study
Salmonella enterica		
χ3761	*S*. Typhimurium UK-1	(21, 36)
χ11143	*S*. Typhimurium UK-1; Δ*fur-44*	This study
χ11143(pEZ136)	*S*. Typhimurium UK-1; Δ*fur-44*; pEZ136; Cm^r	This study
Plasmids		
pMEG-375	8,142 bp, Cm, Amp, *lacZ*, R6K *ori, mob incP, sacR sacB*	(56)
pRE112	5173 bp, Cm, R6K *ori*, *oriT*, *oriV*, *sacR, sacB*	(14)
pYA4807	Δ*fur-44*, Cm, pR112	This study
pACYC184	4,245 bp, Tet, Cm, p15A *ori*	(7)
pEZ123	Δ*fur-35*, pMEG-375	This study
pEZ116	P_{fur}-*fur*, Cm, pACYC184	This study
pEZ156	Δ*hmuR36*, pMEG-375	This study

Sequence analysis. Nucleotide Basic Local Alignment Search Tool (BLAST) was performed based on the sequences of the putative *fur* and heme uptake genes present in the genome sequence of *E. ictaluri* 93-146 accessed from NCBI's Entrez Genome database (NC_012779). *fur* sequences were obtained from NCBI's Entrez Protein database for *Edwardsiella ictaluri* 93-146 (YP_002934295.1), *E. tarda* PPD 130/90 (AEO72442.1), *E. tarda* EIB202 (YP_003296656.1), *E. tarda* FL6-60 (ADM42454.1), *E. tarda* ATCC 23685 (ZP_06715756.1), *Escherichia coli* O157:H7 EDL933 (NP_286398.1), *S. enterica* serovar Typhi Ty2 (NP_455254.1), *Yersinia pestis* KIM 10 (NP_668533.1), *Vibrio cholerae* (AAA27519.1), *A. salmonisida* A449 (YP_001143048.1), *Pseudomonas putida* (YP_001269900.1), and *P. aeruginosa* (NP_253452.1).

Amino acid sequence alignments were performed using the CLC Free Workbench software tool (v. 6.1 CLC bio A/S, Aarhus, Denmark). Protein structural-based alignments were performed by using the web-base interface for ESPript v.2.2 located at http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi (18). The 3D structure of *E. ictaluri* Fur, HemP, HemR, HemT, HemU, HemV, and HemS proteins were predicted using position specific iterative - BLAST (PSI-BLAST) alignment and HHpred (62).

Construction of *E. ictaluri* mutants. The recombinant pEZ suicide vectors (Table 1) carrying the linked flanking regions to generate

in-frame deletion of *fur* or *hmuR* gene were constructed as described previously (55-57). The defined deletion mutations encompass a deletion including the ATG start codon, but not including the TAG stop codon. The primers used to construct the suicide vectors are listed in Table 2. Primers 1 and 2 were designed to amplify the upstream gene-flanking regions. The down stream gene-flanking regions were amplified by primers 3 and 4. The flanking regions were ligated and cloned into pMEG-375 digested with *SphI* and *XbaI*. To construct *E. ictaluri* mutants, the suicide plasmid was conjugationally transferred from *Escherichia coli* χ7213 (52) to *E. ictaluri* strains. Strains containing single-crossover plasmid insertions were isolated on BHI agar plates containing Col and Amp. Loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected by using the *sacB*-based sucrose sensitivity counter-selection system (14) adapted to *E. ictaluri* (55, 57). The colonies were selected for Amp^s, Col^r and screening by PCR using primers 1 and 4. Biochemical profiles of *E. ictaluri* strains were determined using the API 20E system (bioMériux, Marcy l'Etoile, France).

Table 2

Primer used in this study

Primer identification	Primer sequence
Δ*fur-35* (*SphI*) F1	5'ACAT**GCATGC**TGGGTTAATGTCTGCCGCC 3'
Δ*fur-35* (*XhoI*) R1	5'CCG**CTCGAG**ATCGATTAGCTTCTTGTACA 3'
Δ*fur-35* (*XhoI*) F2	5'CCG**CTCGAG**TGAGTGACCTGGCGCCCACA 3'
Δ*fur-35* (*XbaI*) R2	5'TCG**TCTAGA**TAAATACGGTCTATGTCATA 3'
Δ*hmuR* (*SphI*) F1	5'ACAT**GCATGC**AAACTGCTTGCCGTCATCGG 3'
Δ*hmuR* (*XhoI*) R1	5'CCG**CTCGAG**GGCTCAACACTCCAAATGTA 3'
Δ*hmuR* (*XhoI*) F2	5'CCG**CTCGAG**CGCGATTTTCACCCCGGGGC 3'
Δ*hmuR* (*XbaI*) R2	5'TCG**TCTAGA**AAAGGCGGCGTGCCAGTGCT 3'
Δ*fur-44* (*BglII*) F1	5'GGA**AGATCTT**GTGTAAATCTTTCGAAGAGCCAACCG 3'
Δ*fur-44* (*XmaI*) R2	5'TCC**CCCCGGG**TATACCCAGTATGGAGGCGGTACTGG 3'
hmuR-F	5'GACATCAACGCGGACAAATGGTCA 3'
hmuR-R	5'GTTGGGACGCCAGTAGTTGACAAA 3'
mntH-F	5'TATCTGCATTCGTCGCTGACCCAA 3'
mntH-R	5'TGATCCCACTGTGCCCGTTAAAGT 3'
hemF-F	5'ACCGCCTCATTGTTCAACTGCGTA 3'
hemF-R	5'ATCAAAGGTCTGGATGCCGATGGA 3'
mgtB-F	5'AGTCGACGAGCTTCCGTTTGACTT 3'
mgtB-R	5'CGTGATCTTCAATGTGGCTGGCAA 3'
fadR-F	5'AGCTGATTGGCGTTACCCGAACTA 3'
fadR-R	5'AATATTCAGCCCGGAGGTTTCCCA 3'
gmpA-F	5'ATCGCCTACACCTCCGTGCTTAAA 3'
gmpA-R	5'ATAGCTTGACCTGTTCATCGCCGT 3'
rrn (16S)-F	5'TCGACATCGTTTACAGCGTGGACT 3'
rrn (16S)-R	5'TGCATCCAAGACTGGCAAGCTAGA 3'

Construction of *Salmonella* Typhimurium Δ*fur-44*. The *fur* gene of χ3761 *Salmonella* Typhimurium UK-1 (Table 1) was deleted in frame mediated the previous suicide vector methodology (55, 57). The primers used to construct the suicide vectors are listed in Table 2. The flanking regions were ligated and cloned into pR112 (14) digested with *BglII* and *XmaI*. *S.* Typhimurium Δ*fur-44* mutation consisted in a deletion of 706 bp including 453 bp of the *fur* gene and 253 bp of the *fur* promoter region, with the Crp binding and the OxyR binding sites.

Complementation of the *fur* gene. The *fur* gene of *E. ictaluri*, with its own promoter, was cloned into the pAYCY184 vector (7) at the *Xba*I and *Hind*III restriction sites. The primers used to amplify *fur* were 5' – TCGTCTAGATGTCTGCCGCCTGCCGGCGC – 3' (upstream) and 5' – CCCAAGCTTTCAGGCCTTTTCATCGTGCA – 3' (downstream). *XbaI* and *HindIII* sites were included in these primers, respectively (underlined). The resulting plasmid, pEZ136, was used to complement *Salmonella* Δ*fur-44* mutant strains.

SDS-PAGE and western blot. To evaluate the synthesis of Fur, the strains were grown in 3 ml of BHI broth or LB broth at 28°C with aeration (180 rpm). The samples were collected when the culture reached the absorbance of 0.85 ($O.D_{600}$ 1.0~1.0x10^8 cfu/ml). One ml of culture was collected and prepared for western blot analysis (54). The total proteins

were normalized using a nanodrop spectrophotometer (ND-1000, NanoDrop) at 25 μg/μl and separated by 10% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (54). Fat-free milk powder solution (5%, wt/vol) in PBS supplemented with 0.05% of Tween 20 (PBS-T) was used for blocking. The membrane was incubated individually with a primary rabbit polyclonal anti-GroEL antibody (Sigma) (1:10,000) or rabbit polyclonal anti-Fur antibody (1:10,000) (60) for 1 h at room temperature, washed three times with PBS-T, and then incubated with a 1:10,000 dilution of alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) (Sigma). Color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate mixture (NBT-BCIP) (Amaresco), chromogenic substrates for alkaline phosphatase.

Outer membrane protein isolation and protein identification. Sarkosyl-insoluble outer membrane proteins (OMPs) were obtained as previously described (55). OMP proteins were isolated from *E. ictaluri* grown in iron-replete conditions (BHI broth and BHI broth supplemented with 150 μm $FeSO_4$). Iron-regulated outer membrane proteins (IROMPs) were isolated from *E. ictaluri* grown in BHI broth supplemented with 2'2' -dipyridyl (150 μM) (iron-depleted conditions). The total proteins were normalized to 25 μg/μl by using the nanodrop spectrophotometer (ND-1000, NanoDrop) and separated by 10% (wt/vol) sodium dodecyl

sulfate (SDS)-polyacrylamide gel electrophoresis. Coomassie blue staining was performed to visualize proteins.

Protein Identification. The single protein band of ~72 kDa synthesized in absence of iron or in *E. ictaluri* Δ*fur* mutants was excised from the SDS-PAGE gel for peptide sequencing analysis (ProtTech Inc, Norristown, PA, USA) by using NanoLC-MS/MS peptide sequencing technology. The protein gel band was destained, cleaned, and digested in-gel with sequencing grade modified trypsin (Promega, Madison, WI). The resulting peptide mixture was analyzed by an LC-MS/MS system, in which a high performance liquid chromatography (HPLC) with a 75 μm inner diameter reverse phase C18 column coupled to an ion trap mass spectrometer (Thermo, Palo Alto, CA).

The mass spectrometric data was utilized to search the non-redundant protein database at the National Center for Biotechnology Information (NCBI).

Detection of secreted siderophores. Production of compounds with siderophore activity was tested by the chemical assays of Schwyn and Neilands (58) in solid and liquid media. The assays were performed by spotting 10 μl of each bacterial culture grown in iron-limiting conditions onto a modified chromoazurol S (CAS; Sigma) agar. The siderophore levels produced by the strains on plates were visualized by a yellow-

orange halo around the bacterial colony after 18-72 h of incubation. For siderophore detection in liquid, supernatants from bacterial cultures grown in BHI broth or LB broth supplemented with 2'2' -dipyridyl (150 μM) were mixed with CAS solution and the absorbance mixture were measured at 630 nm.

Iron-free siderophores were obtained by the following method. Bacterial culture grown in 5 ml of LB broth supplemented with 2'2' -dipyridyl (150 μM) or $FeSO_4$ (150μM) for 18-24 h at 28°C (OD_{600} of 1.0) were harvested by centrifugation at 5,000 g for 10 min. The supernatants were filtered through a 0.2-μm (pore-size) membrane filter to completely remove the cells and acidified with 25 μL of 10N HCl and extracted twice with a total of 4 mL of ethyl acetate for catechols and benzyl alcohol for hydroxamates (44). The aqueous phase was dried with gaseous N_2 and resuspended in 40 μl of methanol and 10 μl were spotted onto 250-μM layer-flexible (20 × 20 cm) PE SIL G/UV254 plates (Whatman). Plates were developed with benzene:glacial acetic acid:water (125:72:3 vol/vol/vol) in a closed chamber. Plates were then removed from the chamber and allowed to dry, then immersed briefly in 0.1% $FeCl_3$ to visualize Fe-binding compounds. Avian pathogenic χ7122 *Escherichia coli,* χ3761 *S.* Typhimurium UK-1 (36, 52) and χ11143 *S*. Typhimurium Δ*fur-44* were used as controls (Table 1).

Utilization of heme and hemoglobin by *E. ictaluri*. Sterile filter paper disks were placed onto a BHI agar plate supplemented with 2'2' dipyridyl (150 μM) and swab inoculated with the respective *E. ictaluri* strain. The disks were inoculated with 5 μl of water (control), heme (Sigma), hemoglobin (Sigma) and siderophore preparations from *E. coli* or *S*. Typhimurium resuspended in PBS. The plates were incubated for 48 h at 28°C. Growth around the paper disk indicated positive utilization of the iron source.

Semi-quantitative RT-PCR. Expression of putative Fur-regulated genes were evaluated by RT-PCR. Total RNA extraction was performed by RNeasy QIAgene kit from *E. ictaluri* wild-type grown in presence and absence of iron and from J135 *E. ictaluri* Δ*fur-35* grown in BHI. The cells were grown until late exponential phase (OD_{600nm} 0.85 ~ $1x10^8$ CFU/ml). The cDNA synthesis was performed by SuperScript™ III First-Strand Synthesis System (Invitrogene) using random hexamer primers. Semi-quantitative PCR was performed using the specific primers listed in Table 2. 16S (*rrn*) was used as control.

Bacteria inoculate preparation. Bacterial strains were grown overnight in standing cultures that were diluted 1:20 in pre-warmed BHI broth and with mild aeration (180 rpm) at 28°C to an OD_{600} of 0.85 (~10^8 CFU/ml). Bacteria were sedimented 10 min by centrifugation (5,865 g) at room

temperature and resuspended in BSG (12) to densities appropriate for the inoculation.

Infection and immunization of zebrafish (*D. rerio*). The zebrafish challenges were performed by the methodology described earlier (47), with modifications (55, 57). The water temperature was 26 ± 1°C and the fish were acclimated for 2 weeks prior to the experiment. Groups of twelve adult zebrafish (average weight, 0.5 g) were sedated in 100 mg/L tricaine methanesulfonate (MS-222, Sigma) and injected intramuscularly (i.m.) with 10 μl of the bacterial suspension per fish. A 3/10-cc U-100 ultrafine insulin syringe with a 0.5-in.-long (ca. 1-cm-long) 29-gauge needle (catalog no. BD-309301; VWR) was used to inject the fish. Two sets of controls were used: fish that were not injected and fish that were injected with 10 μl of sterile phosphate-buffered saline containing 0.01% gelatin (BSG) (12). Moribund fish demonstrating clinical signs were euthanized, necropsied, and plated for enumeration of bacterial loads in various organs (47). Survivors of each dose at 4 weeks post i.m. inoculation were challenged with 10^5 CFU of *E. ictaluri* (100 LD_{50}). The fish were fed twice daily with TetraMin Tropical Fish Flake Feed. During the experiments, the fish were observed daily, and every other day water quality was monitored for pH, NO_2, and NO_3 with standard kits (Lifegard Aquatics® Water Testing Strips). The LD_{50} was calculated by the method of Reed-Muench (1). Fish care and use was performed in accordance with the

requirements of the Arizona State University Institutional Animal Care and Use Committee.

Intracoelomic infection and immunization of catfish (*I. punctatus*). Specific-pathogen-free channel catfish fingerlings were used with a mean weight of 18.5 ± 1.3 g. The animals were randomly assigned to treatment groups of 10-25 fish each in 100-liter tanks. Each tank was equipped with a re-circulating, biofiltered, mechanical filtered, and U.V. water treated system with 12 h light cycle per day. The water temperature was set at 28 ± 1°C during the first two weeks of acclimatization and during the course of the experiments. The fish were fed daily with commercial Aquamax grower 400 (Purina Mills Inc., St. Louis, MO). During the experiments, the fish were observed daily, and every other day water quality was monitored for pH, NO_2, and NO_3 with standard kits (Lifegard Aquatics® Water Testing Strips). Catfish were infected by the intracelomic (i.c.) route with 10^5 to 10^8 CFU of *E. ictaluri* strains (fish were not fed until 1 h after infection). The fish were anesthetized with tricaine methanesulfonate buffered (pH 7.5) (MS-222, Sigma; 100 mg/L of water) prior to handling. The LD_{50} was calculated by the method of Reed-Muench (1). Moribund animals were euthanatized and then necropsied to evaluate presence of *E. ictaluri* in kidney, spleen and liver.

Immersion immunization of catfish. Catfish fingerlings were immersed in a solution of *E. ictaluri* Δ*fur-35* containing 10^7 CFU/ml for 30 min. Six weeks post-immunization, fish were challenged by bath with 10^7 CFU/ml of *E. ictaluri* wild type (10 LD_{50}) for 30 min. The animals were fasted 24 h prior to oral inoculation and 1 h post inoculation. Non-immunized animals were used as a control. During the experiments, the fish were observed daily. The LD_{50} was calculated by the method of Reed-Muench (1).

Oral immunization of catfish. Catfish were fasted for 24 h before oral inoculation with the respective *E. ictaluri* strains. Fish were anesthetized with tricaine methanesulfonate buffered (pH 7.5) (100 mg/L of water) prior to handling. The animals were orally inoculated with 100 μl of the corresponding bacterial suspension (see below). BSG (12) was used as a control. The fish were not fed until 1 h after inoculation. The LD_{50} was calculated by the method of Reed-Muench (1).

Determination of IgM titers in serum and skin mucus. Cutaneous mucus and blood samples were collected both at two weeks prior to immunization and four weeks post immunization as described previously (55). Ten i.c. immunized fish and ten immersion-immunized fish were sampled individually. The experiment was repeated twice. *E. ictaluri* outer membrane proteins diluted in 20 mM Tris-OH (pH 8.0) and purified *E. ictaluri* LPS were independently applied to polyvinyl chloride 96-well

plates both at 100 ng per well. The plates were incubated overnight at 4°C, washed once with 200 μl of PBS–0.05% Tween (PBS-T) per well, and blocked with 1% sea buffer (Thermo, Rockford, IL) diluted in PBS (1 h at room temp). Catfish serum samples diluted in PBS (1:2) and undiluted mucus samples were plated in triplicate wells at 100 μl/well. Triplicate control wells on each plate contained diluted sera from immunized and non-immunized catfish. Mucus assays also included wells of pooled mucus from non-exposed fish. The plates were incubated overnight at 4°C and washed five times with PBS-T. The biotinylated rabbit anti-catfish Ig antibody was applied at 0.25 to 0.50 μg/well. The plates were incubated for 1 h at room temp and were washed five times with PBS-T. Application of the primary antibody was followed by application of streptavidin-alkaline phosphatase conjugate (Southern Biotech, Birmingham, AL) diluted in PBS (1:50,000). Enzyme substrate *p*-nitrophenyl phosphate diethanolamine (100 μl; Sigma) was added and incubated for 30 min at room temperature. The reaction was stopped with 50 μl of 3M NaOH. The absorbance (A_{405}) values were determined on a kinetic microplate reader (model V-max; Molecular Devices Corp., Sunnyvale, Calif.) at 30 min and 1 h. The immunized fish were challenged 6 weeks post immunization as described previously.

4. *Results*

Sequence analysis of *fur.* An analysis of DNA and protein sequences, structural alignment, predicted 3D structure, and predicted binding residues revealed that *E. ictaluri* and *E. tarda* Fur proteins have several key differences compared to other bacterial iron regulator protein families (Figs 1-4). This provides evidence that these Fur proteins are members of a distinctive bacterial iron regulator protein family, shaped by the gene reduction theory. The *E. ictaluri fur* gene sequence from J100 *E. ictaluri* wild type is identical to the sequence from the *E. ictaluri* 93-146 strain published sequence at the NCBI. In comparison to *E. coli* and *Salmonella fur* genes, the *E. ictaluri fur* gene has a smaller open reading frame (ORF), and has a guanine plus cytosine (G+C) content of 57%, 10% higher than the *Escherichia coli* (G+C 48.5%) and *Salmonella* (G+C 47.7%) *fur* genes (Fig. 1). The *fur* gene sequences from the fish isolated *E. tarda* PPD130/91 and *E. tarda* EIB202 (70) contain 333 identical base pair in contrast with the human isolated *E. tarda* ATCC 23685 that contains a longer *fur* gene (435 bp) similar to the rest of the enterics (Fig. 1). The *E. ictaluri fur* promoter region contains a Crp binding site indicating that Crp might participate in Fur regulation (Fig. 2).

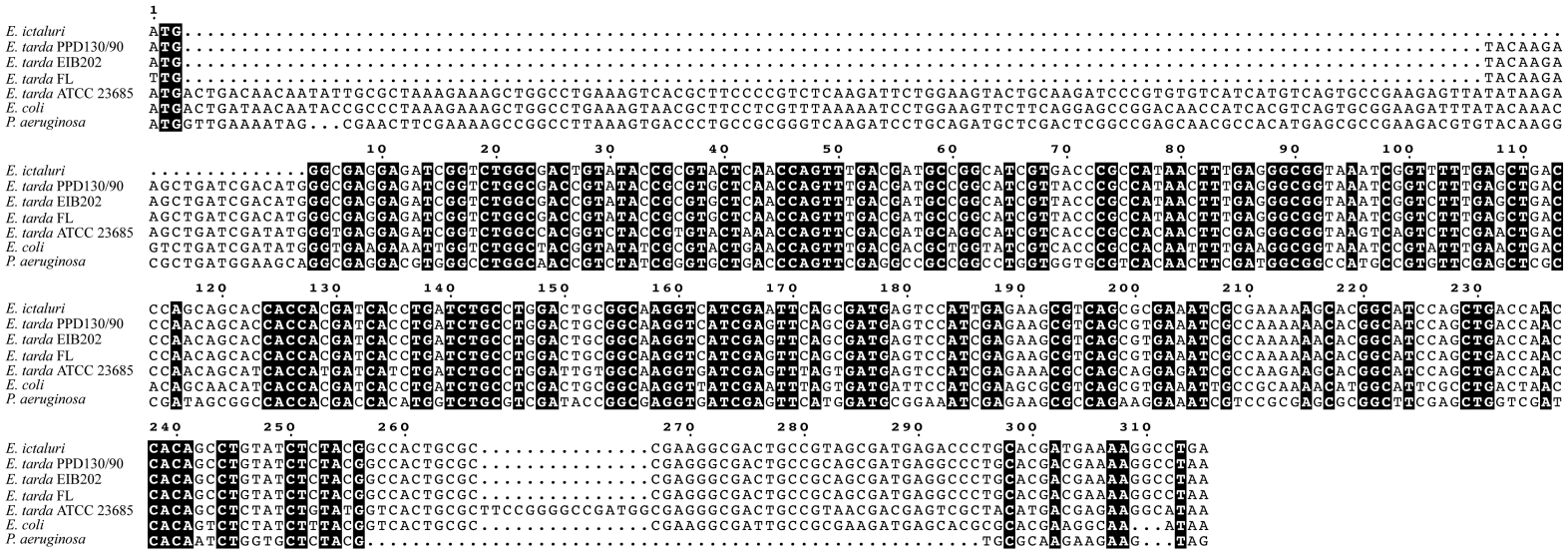


Figure 1. Alignment of representative *fur* gene.

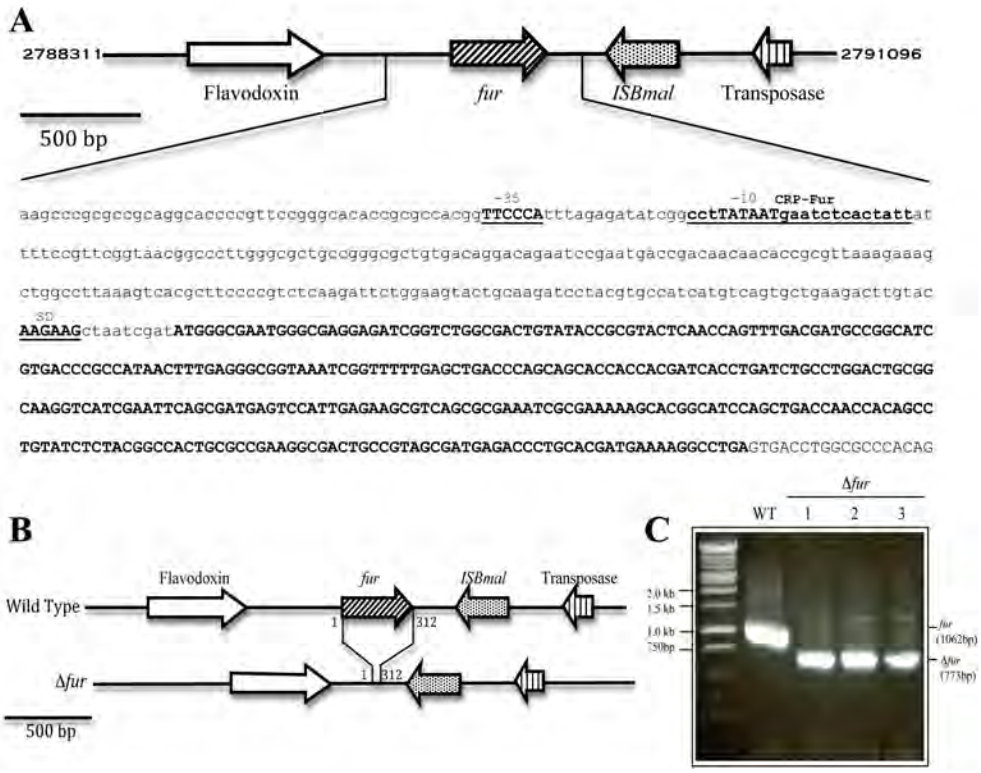


Figure 2. *E. ictaluri fur* gene. A. Genetic map and promoter analysis of *E. ictaluri fur* gene. In bold is the gene sequence of the *E. ictaluri fur* gene. The predicted –35, –10 and the Shine Delgarno (SD) region are indicated. The predicted Crp and Fur binding box in the positive strand are indicated in bold. The arrows in the map indicate the size of the gene and the transcription direction; B**.** Deletion map of *fur* gene indicates the 311 bp in frame deleted; C. Genotype verification of *E. ictaluri* Δ*fur-35* by PCR.

The Fur protein from fish isolated *E. tarda* PPD130/91 and *E. tarda* EIB202 (70) contains 111 identical residues in contrast with the longer Fur proteins in *E. coli* (145 residues), *Salmonella* Typhimurium (150 residues), *Yersinia pestis* (148 residues) and other enterics (Fig. 3). Interestingly the human isolated strain *E. tarda* ATCC 23685 contains a longer Fur protein (145 residues) similar to the rest of the enterics, indicating it may be more adapted to humans than fish. *E. ictaluri* 93-146 and *E. ictaluri* J100 contain a shorter Fur with 104 identical residues (Fig. 3). Alignment of these sequences showed that the N-terminal helices, α1, α2, and α3, at the DNA binding domain are missing in *E. ictaluri*, as well as in the *E. tarda* fish isolated Fur proteins (Fig. 3). The α4 helix at the Fur DNA binding domain is still intact in *E. ictaluri* and *E. tarda* fish isolates, sharing about 90% identity with other Fur bacterial species (Fig. 3). Structural protein alignment between functional representative bacterial Fur proteins revealed that 37 amino acid residues (~36%) are strictly conserved out of 104 residues in *E. ictaluri* Fur (Fig. 3). *E. ictaluri* Fur has 29%, 53%, 54%, 57%, and 93% amino acid similarity to the Fur of *Pseudomonas*, *Escherichia coli, S. enterica*, *Y. pestis*, and *E. tarda,* respectively.

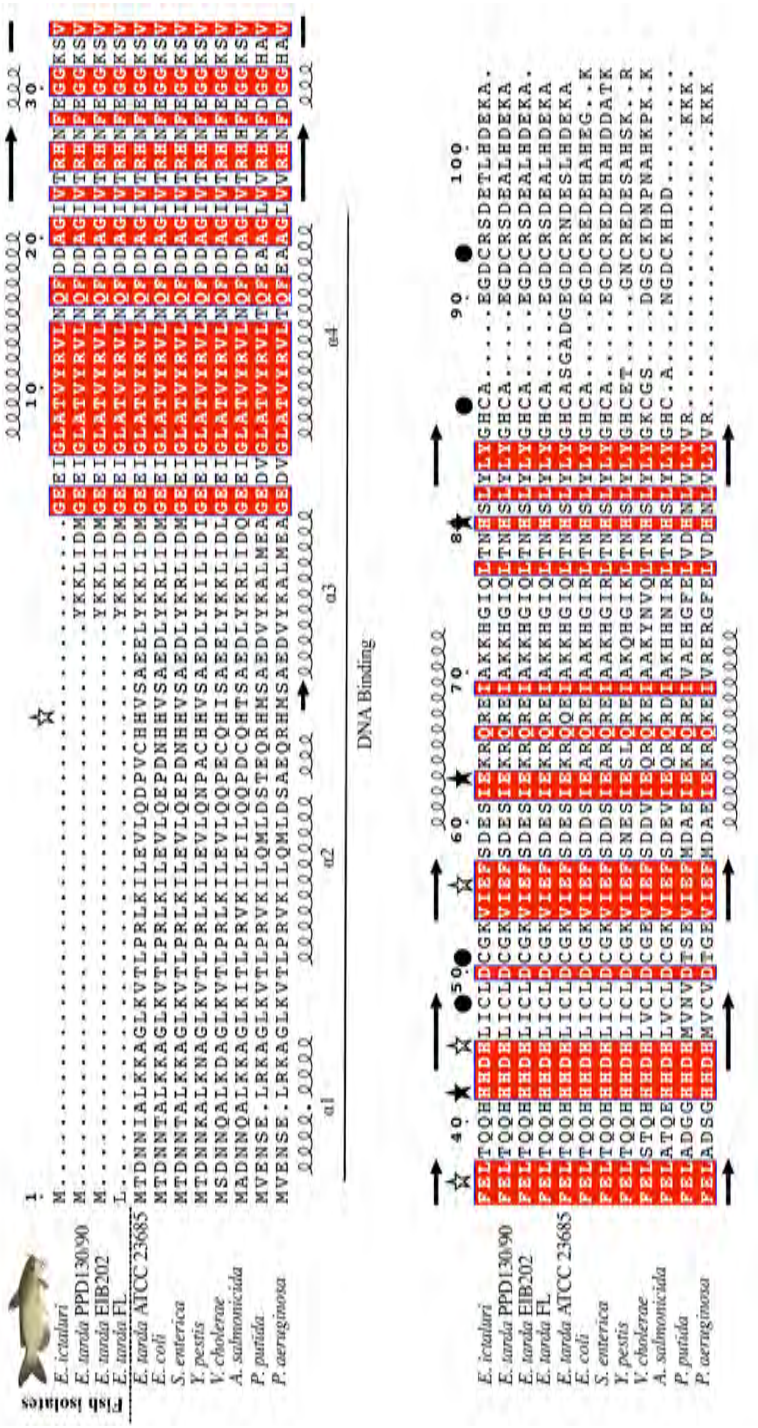


Figure 3. *E. ictaluri* Fur alignment and secondary structure. The white stars indicate the residues related to the Zn^{+2} binding pocket. The

black stars indicate the residues related to the Fe^{+2} binding pocket. The black circles indicate the cysteine residues related to *E. coli* Fe^{+2} and Zn^{+2} binding pockets. The secondary structure at the top of the alignment corresponds to the *E. ictaluri* Fur. The secondary structure at the bottom of the alignment corresponds to the *Escherichia coli* Fur protein. Spirals represent α- helices and arrows represent β-sheets.

In terms of phylogeny, the fish isolated *Edwardsiella* Fur proteins belong to a distinctive group compared to the rest of the Enterobacteriaceae family (Fig. 2). Although, the Enterobacteriaceae share a common Fur ancestor, the only phylogenic group with a short Fur protein among enterics is *Edwardsiella* (Fig. 4), indicting that Fur might have undergone host bacterial pathogen specialization.

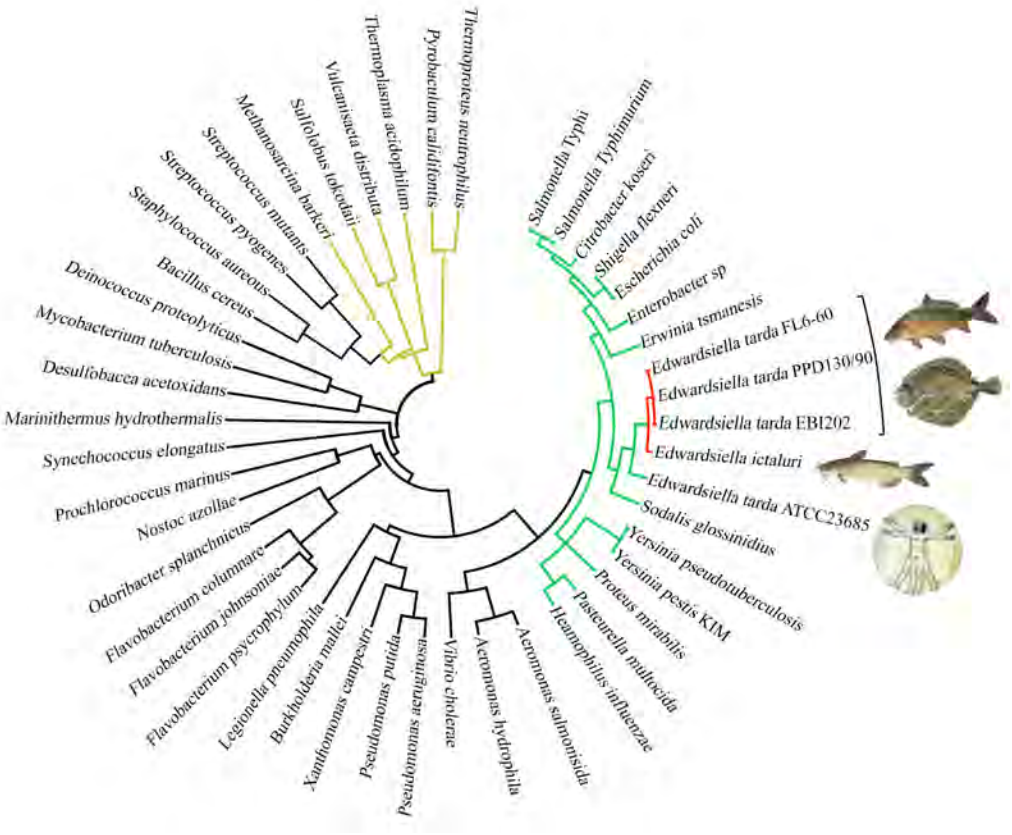


Figure 4. Molecular phylogenetic analysis of *fur*. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (65). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed (15). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was less than 100 or less than one fourth of the total number of sites, the maximum parsimony method was used;

otherwise the BIONJ method with MCL distance matrix was used. The analysis involved 48 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 546 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (64). The gold line indicates a representative Archeae *fur* evolutionary path, green line indicates the Enterobacteriaceae *fur* family evolution path, and the red line indicates the fish isolated *Edwardsiella fur* evolution path.

Structural protein analysis showed that *Edwardsiella* Fur contains two domains, like other Fur proteins (32, 48). The DNA binding domain harbors the Zn^{2+} bond residues, and the dimerization domain harbors the Fe^{2+} bond residues (Figs. 3 and 5A-B). We observed that *E. ictaluri* Fur, as well as the *E. tarda* Fur (only fish isolated strains), do not contain the α-helices α1, α2, and α3 (Fig. 3). Analysis of the residues related to the Zn^{2+} and Fe^{2+} bounds showed they are located in similar positions as their corresponding residues in both *E. coli* Fur and *Pseudomonas* Fur proteins. Specifically, the *E. coli* Fur related residues Cys92 and Cys95 required for the Zn^{2+} bond (17, 32) (in *E. ictaluri* Fur Cys48 and Cys51; Figs. 5A and 5C) and the residues Cys132 and Cys88 required for the Fe^{2+} bond (17, 32) (in *E. ictaluri* Fur Cys93 and Cys88; Figs. 5A and 5C) are located at the loop region in *E. ictaluri* Fur (Figs. 5A and 5C). The *Pseudomonas* Fur related residues His24, His86, Asp88, and Glu107 required for the Zn^{2+} bond (48) (in *E. ictaluri* Fur Glu36, His45, and Glu56; Figs. 5B and 5D)

and the residues His32, Glu80, His89, and Glu100 required for the Fe^{2+} bond (48) (in *E. ictaluri* Fur His42, Glu63, His80, and Glu90; Figs. 5D and 5E) are located in their respective domains within *E. ictaluri* Fur (Figs. 5B, 5D and 5E). In *P. aeruginosa* the Fur residue His32 is required for the Fe^{+2} bond and essential for its activity (4). But the N-terminal region, where His32 would normally be located, is absent in the *E. ictaluri* Fur protein (Fig. 3). Spectroscopic and biochemical data have shown that the structural zinc site in *P. aeruginosa* and *E. coli* Fur proteins are different, which might be related to their specific function in the different organisms. However, in both *E. coli* and *P. aeruginosa* Fur proteins, the N-terminal region is essential for Fur activity (4, 9). On the other hand, the importance of the cysteines is different in *E. coli* Fur and in *P. aeruginosa* Fur, because the Cys92 and Cys95 bound to Zn^{2+} in *E. coli* Fur are essential for its activity (8). The single cysteine residue in the *P. aeruginosa* Fur is dispensable for its in vivo activity (33) and is absent in other *Pseudomans* Fur, like *P. putida* Fur (Fig. 3). Based on the above analysis we believe that *E. ictaluri* Fur is a distinct Fur protein among the Fur family. Further structural information on the *E. ictaluri* Fur protein is necessary for a better understanding of the specific structure-function relationship within the Fur family.

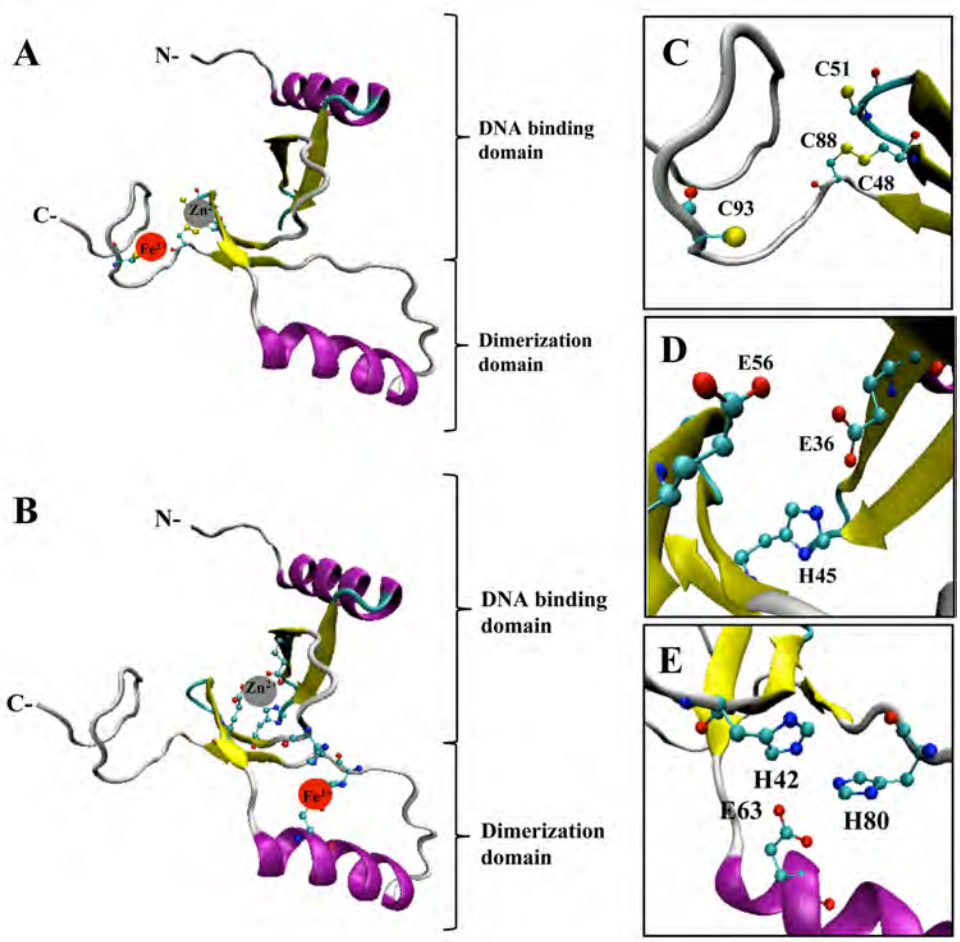


Figure 5. *E. ictaluri* Fur protein functional analysis. A. Predicted *E. ictaluri* Fur protein displaying the (C51; C48) Zn^{+2} and (C88; C93) Fe^{+2} binding pockets; B. Predicted *E. ictaluri* Fur protein displaying the (E36; H45; E56) Zn^{+2} and (H42; E63; H80) Fe^{+2} binding pockets; C. Residues C51 and C48 related to Zn^{+2} and residues C88 and C93 related to Fe^{+2} binding pockets; D. Residues E36, H45, and E56 related to Zn^{+2} binding pocket; E.

Residues H42, E63 and H80 related to Fe^{+2} binding pocket. The residues E36, H45, and E56 related to Zn^{+2} and the residues H42, E63, and H80 related to Fe^{+2} binding pockets are related to the *Pseudomonas* Fur protein. The residues C51 and C48 related to Zn^{+2} and residues C88 and C93 related to Fe^{+2} binding pockets related to *Escherichia coli* Fur protein are misplaced in the overall *E. ictaluri* Fur protein structure.

Complementation of the *fur* gene. The structural analysis of *E. ictaluri* Fur indicated that the overall domain organization is different than other Fur-family members, but it has the same set of key residue groups and likely a similar mechanism as other Fur proteins (Figs. 3-5). To further evaluate the functionality of *E. ictaluri* Fur we complemented Δ*fur* mutants of *Salmonella enterica* with the *E. ictaluri* P_{fur}-*fur* gene cloned into the low copy number plasmid pEZ136 (Table 1). *S*. *enterica* serovar Typhimurium Δ*fur-44* was utilized for complementation assays (Table 1). *S. enterica* Δ*fur* mutants present a constitutive synthesis of IROMPs and secretion of siderophores (Fig. 6A-6C). *E. ictaluri* P_{fur}-*fur* complements *S. enterica* Δ*fur-44* mutants, repressing IROMPs and siderophores synthesis in an iron dependent fashion (Fig. 6). This indicates that the missing N-terminal region, (containing the α1, α2, and α3 helices) is not required for DNA binding, and that the residues missing in this region are not essential for the Zn^{2+} bond required for DNA recognition (59).

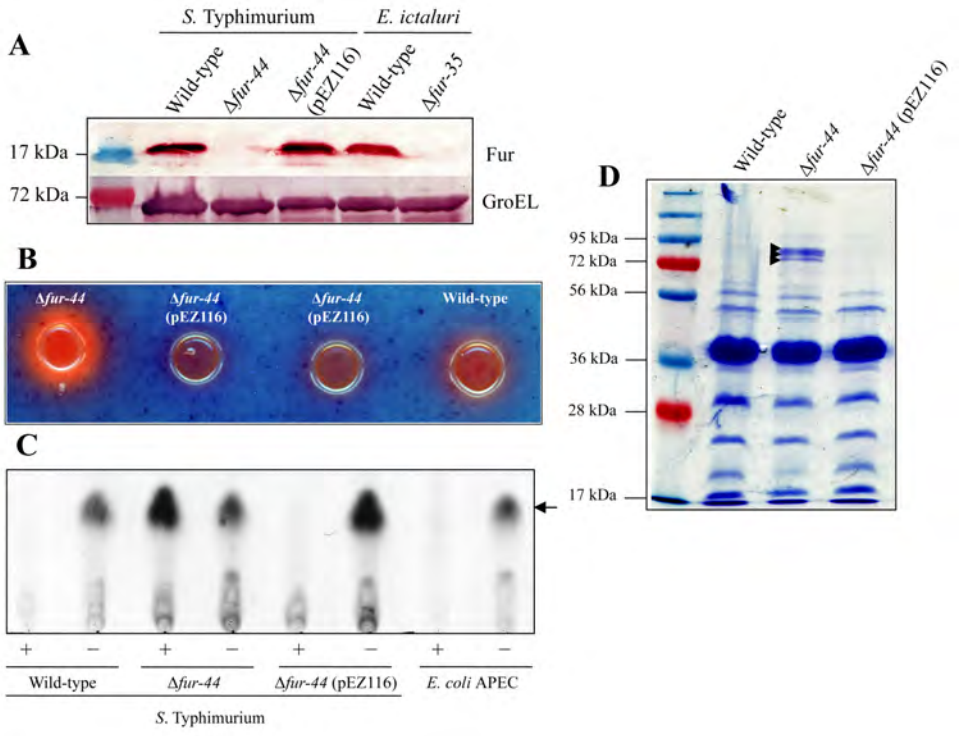


Figure 6. Complementation of *Salmonella* Typhimurium Δ*fur* mutants with *E. ictaluri fur* gene cloned in pEZ116. A. Synthesis of Fur verified by western blot analysis. GroEL was used as control; B. Non-detection of siderophores in CAS indicator agar plates; C. Detection of secreted siderophores in *Salmonella* Typhimurium strains grown under iron-rich (+) and iron-limited (–) conditions by TLC; D. Outer membrane protein profile of *S*. Typhimurium Δ*fur* complemented with *E. ictaluri fur* gene cloned in pEZ136. The arrows indicated the Fur regulated *S.* Typhimurium IROMPs proteins.

Siderophore synthesis in *E. ictaluri*. Results from CAS liquid and plate assays showed that *E. ictaluri* does not synthesize detectable siderophores (Fig. 7A). Although the *E. ictaluri* chromosome contains a ferric enterobactin transport protein (FepE; siderophores receptor; NC_012779.1) and a TonB-dependent ferrichrome receptor protein (FcuA; NC_012779.1), TLC analysis showed that *E. ictaluri* does not secrete either catechol or hydroxylamine related siderophores, or heme binding molecules (data not shown), regardless of the presence of iron in the growth media or Fur protein (Fig. 7B).

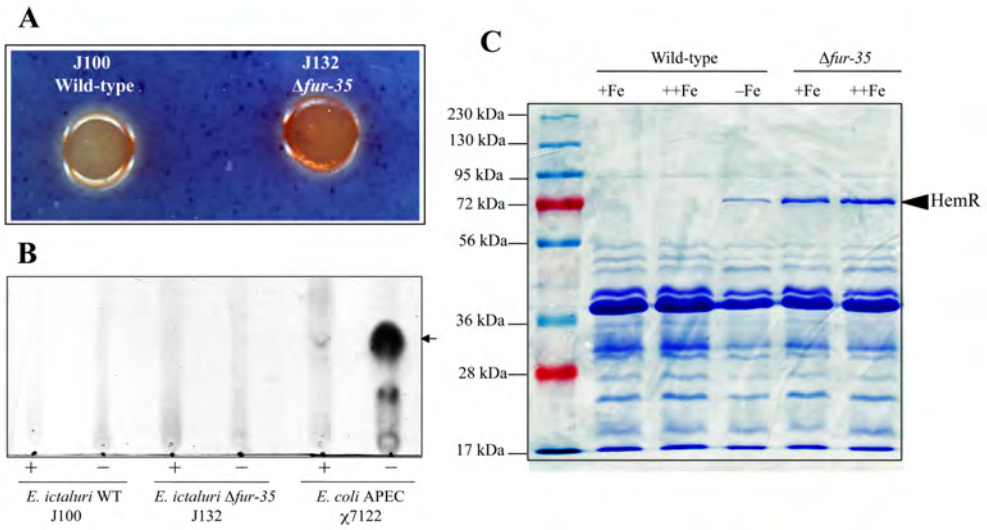
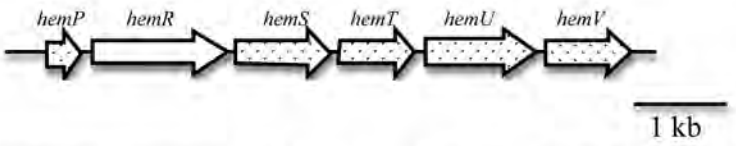


Figure 7. Siderophore and Fur regulated IROMPs in *E. ictaluri*. A. Detection of siderophores in CAS indicator agar plates; B. Detection of secreted siderophores in *E. ictaluri* strains grown under iron-rich (+) and iron-limited (–) conditions by TLC; C. Outer membrane profiles of *E. ictaluri* strains grown under iron-rich (+Fe (BHI broth), ++Fe (BHI broth +150 μM $FeSO_4$)) and iron-limited (–Fe) conditions.

Fur iron regulated outer membrane proteins and heme uptake machinery. *E. ictaluri* wild type grown in iron limiting conditions and *E. ictaluri* Δ*fur-35* mutants both up regulate an IROMP of ~72 kDa, indicating that this protein is iron-Fur dependent and related to iron acquisition (Fig 7C). Protein identification indicated that this IROMP corresponds to a TonB-dependent heme receptor protein (HemR) (Fig. 8). The gene encoding HemR is part of the *hemPRSTUV* operon, which contains the genes required for the synthesis of the heme uptake machinery (Fig. 8).



Protein	Hypothetical function	BLAST (ID%:Positives%)	pI	Mass (Da)
HemP	Hemin uptake	*E. tarda* (86:94) *P. stuartii* (68:76)	10.29	8345.85
HemR	TonB dependent Hemin receptor	*E. tarda* (94:97) *Y. mollaretii* (61:75)	6.13	72904.86
HemS	Hemin degradation/storage	*E. tarda* (92:93) *Y. pestis* (62:76)	6.19	38018.77
HemT	Periplasmatic hemin-binding protein	*E. tarda* (91:94) *Y. pestis* (62:77)	9.56	28631.10
HemU	Hemin ABC transporter, permease	*E. tarda* (91:94) *Y. pestis* (62:77)	9.40	35576.78
HemV	Hemin ABC transporter, ATPase	*E. tarda* (96:96) *Y. intermedia* (67:77)	9.26	28914.41

Figure 8. *E. ictaluri* heme uptake operon gene map and protein description.

Structural analysis of the iron uptake proteins reveled that these are similar to the putative *E. tarda* heme uptake system and to the *Yersinia* heme uptake system (Fig. 8). The outer membrane receptor HemR contains a β-barrel structure with a cork required to transfer the heme into the cell (Fig 9). Structural analysis of HemR predicts a signal sequence peptide in the N-terminal region (Fig. 10), which was observed in 8% SDS-PAGE gel analysis (data not shown). The predicted model of heme transport is described in Figure 9A and consists of the following steps. First heme is transferred into the cell mediated by HemR where it is captured by HemT, a periplasmic hemin binding protein. It is then transported through the cytoplasmic membrane by the ATP dependent HemU-HemV cytoplasmic membrane ABC transport complex. Once heme is transferred to the cytoplasm, it is captured by HemS, a predicted heme degradation-storage protein (Fig. 9A).

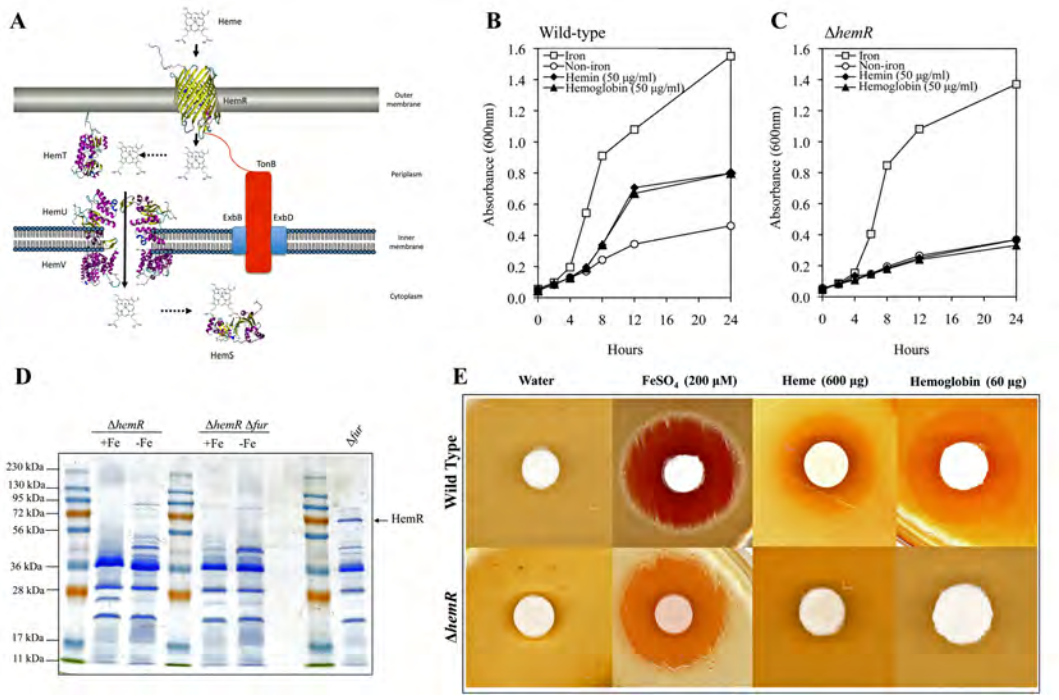


Figure 9. Heme and hemoglobin uptake is mediated by HemR. A. Proposed model for *E. ictaluri* heme transport system; B. Growth of *E. ictaluri* wild type under iron-limited conditions supplemented with heme or hemoglobin; C. Growth of *E. ictaluri* Δ*hemR* mutant under iron-limited conditions supplemented with heme or hemoglobin; D. Outer membrane protein profile of *E. ictaluri* Δ*hemR* and *E. ictaluri* Δ*hemR* Δ*fur* mutants grown under iron-rich (+Fe) and iron-limited conditions (–Fe); E. Growth zones of *E. ictaluri* wild type and *E. ictaluri* Δ*hemR* mutant around discs soaked in 10 µl of $FeSO_4$, heme, and hemoglobin in BHI agar supplemented with 150 µM of 2'2' -dipyridyl.

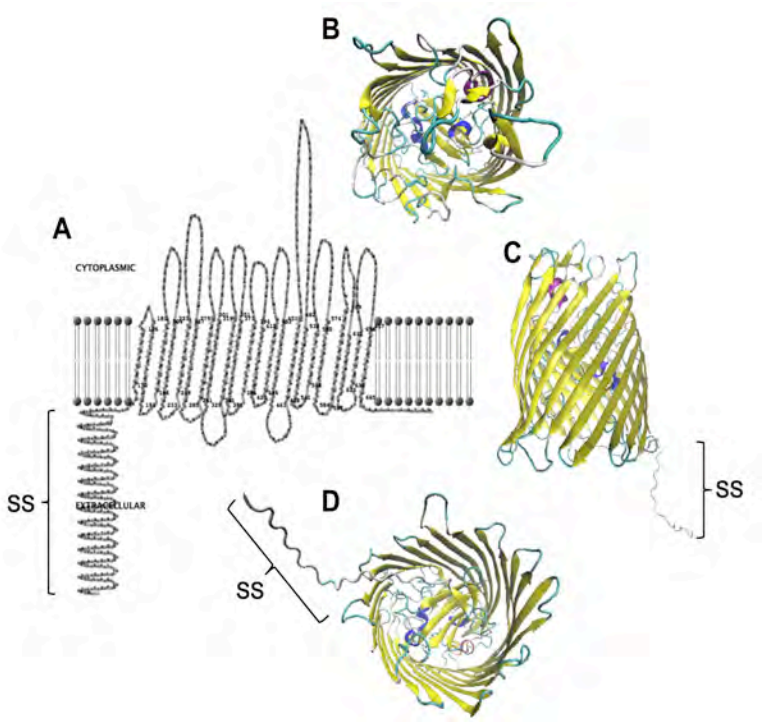


Figure 10. *E. ictaluri* HemR topology and 3D structure. A. HemR topology; B-D. 3D structure of HemR. SS. Secretion signal sequence.

***E. ictaluri* uses heme and hemoglobin as an iron source mediated by HemR.** To determine whether *E. ictaluri* has a functional heme-hemoglobin uptake system, we constructed an *E. ictaluri* Δ*hemR* mutant (thereby disrupting heme-hemoglobin receptor synthesis) (Fig. 9B). *E. ictaluri* iron depleted-cells grow poorly in iron-depleted media. *E. ictaluri* wild-type iron depleted-cells were able to grow in iron-depleted media when it is supplemented with heme or hemoglobin as iron source (Figs.

9C and 9E). In contrast, *E. ictaluri* Δ*hemR* iron depleted-cells are not able to grow in iron-depleted media supplemented with heme or hemoglobin (Figs. 9D and 9E). Although *E. ictaluri* utilizes heme, hemoglobin is the preferred iron source, requiring a lower concentration of hemoglobin to support growth (Fig. 9C and 9E). These results confirm that *E. ictaluri* has a heme-hemoblobin iron uptake system (Fig. 9).

Fur regulated genes. Fur binds to promoter DNA containing a 19 bp Fur box with the consensus sequence 5'-GATAATGATAATCATTATC-3'; this sequence can be described as three adjacent hexamers of the sequence 5'-GATAAT-3', with the third being in a reverse orientation, or symbolically "F-F-X-R", where 'F' stands for the forward sequence "GATAAT", 'R' stands for the reverse sequence "ATTATC" and 'X' stands for any nucleotide (30). We identified a Fur box with an F-F-X-R orientation at the *hem* operon, between the -4 and +14 region in the *hemP* gene (Fig. 10A). The Fur binding box in the F-F-X-R conformation found in *E. ictaluri* is similar to the *Pseudomonas aeruginosa* Fur binding box (40), (Fig. 11A).

Using the consensus hexamers for the *E. ictaluri* Fur box, we identified six different promoters not related to the *hemPRSTUV* operon, which could be regulated by the *E. ictaluri* Fur protein (Fig. 11B). These are promoters for Mg^{+2} transport (*mgtB*), manganese transport (*mntH*), fatty acid degradation (*fadR*), heme synthesis (*hemF*), and glycolysis (*gmpA*). The expression of *hemR*, *mgtB*, *mntH*, *hemF*, and *gmpA*

genes in the absence of iron in wild-type *E. ictaluri*, as well as in our Δ*fur* mutant, were confirmed by RT-PCR (Fig. 11C). Although we predicted a Fur binding box at the *fadR* promoter, its expression does not depend on either iron or the Fur protein (Fig. 11C).

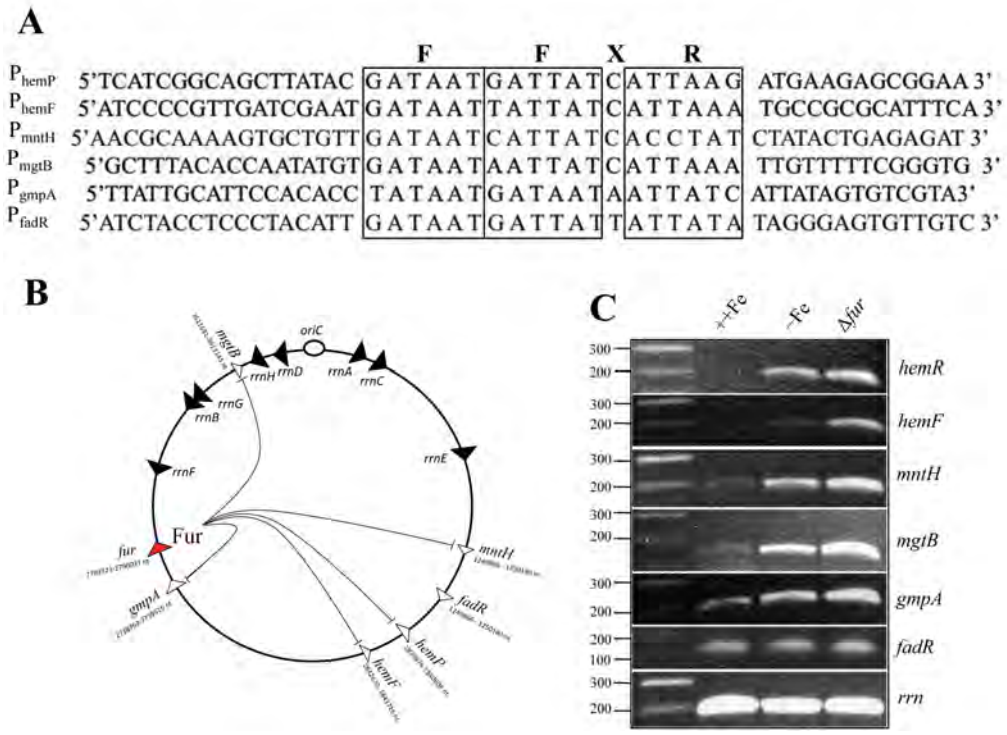


Figure 11. Genes regulated by Fur in *E. ictaluri*. A. Operon maps regulated by Fur; B. Predicted chromosomal genes/operons regulated by Fur. The arrows indicate the direction of the gene. Bold arrows indicate the ribosomal RNA operons. The red arrow indicates the *fur* gene and the white arrows indicate the genes in operons regulated by the Fur protein; C. RT-PCR of genes regulated by Fur.

Virulence of *E. ictaluri* Δ*fur-35* mutants in the fish host. Zebrafish (*Danio rerio*) is not the natural host of *E. ictaluri*, but has been established as a reliable model system to evaluate *E. ictaluri* virulence (47, 55, 57). We found that *E. ictaluri* Δ*fur* was not fully virulent with an LD_{50} of 10^4 CFU, a 10-fold increase over wild type in zebrafish (Figs. 12A-12B). The *E. ictaluri* Δ*fur* mutant was also evaluated in catfish (*I. punctatus*), the natural host of *E. ictaluri*. We found that *E. ictaluri* Δ*fur* i.c. administered to catfish were attenuated with a 1000-fold LD_{50} increase over the wild type (Figs. 12C-12D). *E. ictaluri* Δ*fur-35* delivery by immersion immunization was fully attenuated in frys and fingerlings (data not shown).

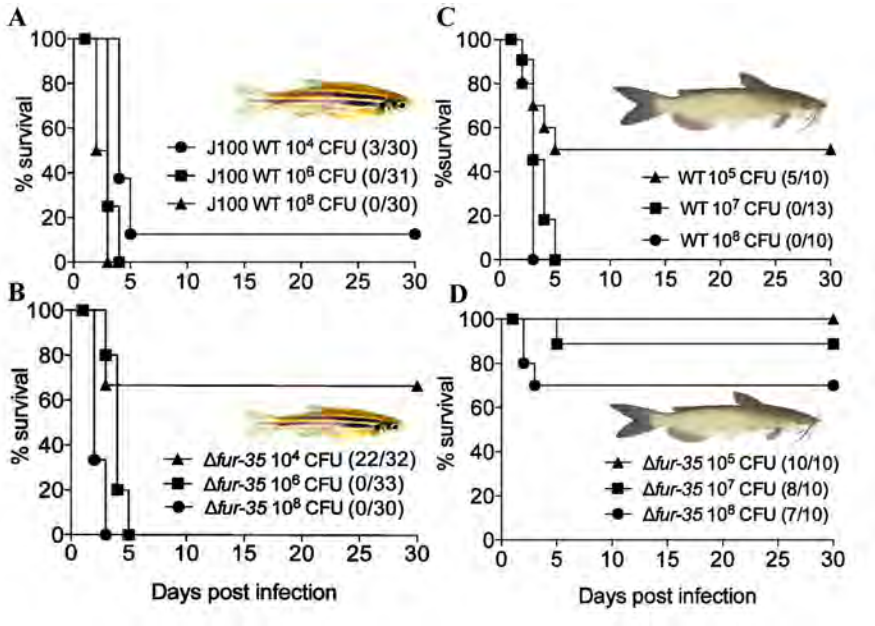


Figure 12. Virulence of *E. ictaluri* Δ*fur* in fish hosts. A. Zebrafish i.m. infected with *E. ictaluri* wild type; B. Zebrafish i.m. infected with *E. ictaluri* Δ*fur-35*; C. Catfish i.c. infected with *E. ictaluri* wild type; D. Catfish i.c. infected with *E. ictaluri* Δ*fur-35*.

Immune protection of *E. ictaluri* Δ*fur* mutants in the fish host. The ideal live attenuated bacterial vaccine should be totally attenuated and immunogenic. Synthesis of IROMPs is up-regulated inside of the host after invasion and these are not constantly exposed to the immune system. We hypothesized that constant synthesis of IROMPs by *E. ictaluri* could trigger a protective immune response and so evaluated whether fish i.c. immunized with *E. ictaluri* Δ*fur* were protected against a wild-type *E. ictaluri* i.c challenge. We determined that *E. ictaluri* Δ*fur* does

not confer immune protection against i.c. challenge (Fig. 13A), but does confer full protection against immersion challenge. Catfish immersion immunized or oral immunized with *E. ictaluri* Δ*fur* survived the bath challenge (Fig. 13B). Catfish i.c. immunized presented significant levels of IgM titers, either in the blood or in the mucus (Fig. 13C). However, these IgM levels were not protective for i.c. challenged fish (Fig. 13A). Immersion immunized catfish presented significant levels of systemic IgM and low levels of skin IgM titers (Fig. 13D). These levels were sufficient to protect the fish against the immersion challenge (Fig. 13B).

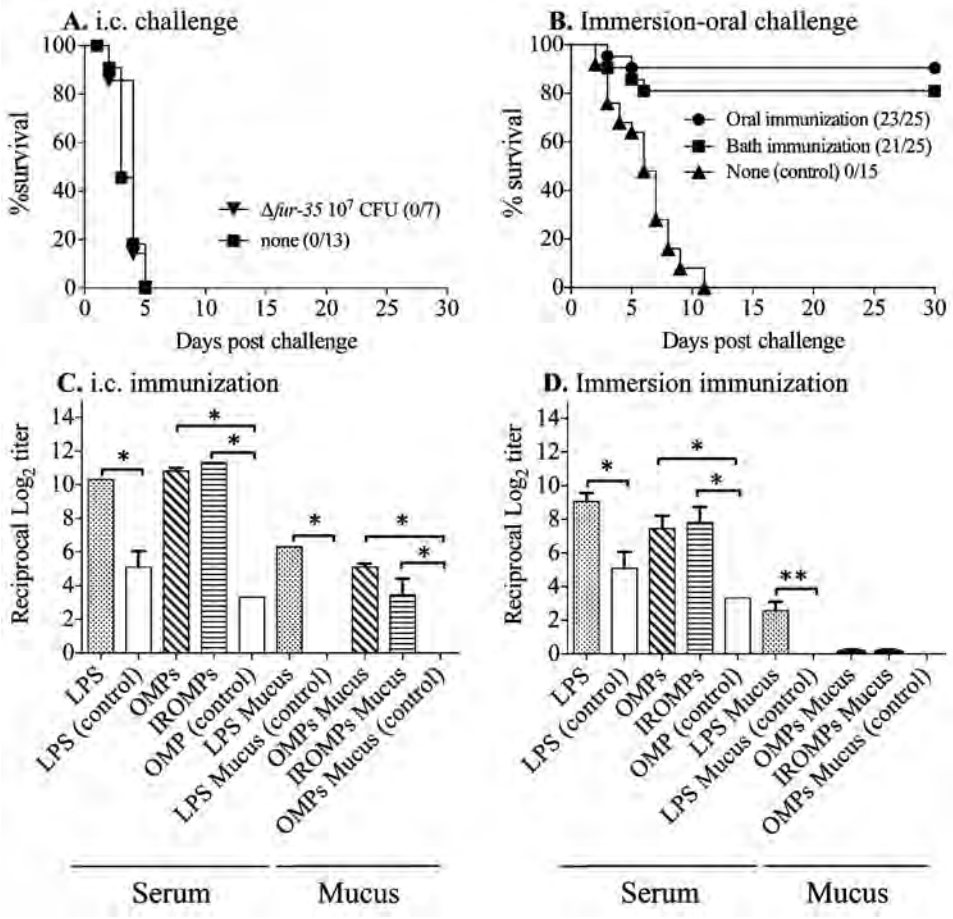


Figure 13. Immune protection of *E. ictaluri* Δ*fur-35* in catfish host. A. *E. ictaluri* wild type i.c. challenged 6 weeks post i.c. vaccination with *E. ictaluri* Δ*fur-35* (10^7 CFU/dose); B. *E. ictaluri* wild type immersion challenged 6 weeks post immersion or oral vaccination with *E. ictaluri* Δ*fur-35* (10^7 CFU/dose); C. Catfish i.c. immunized with *E. ictaluri* Δ*fur-35* (10^7 CFU/dose) (n=22); D. Catfish bath immunized with *E. ictaluri* Δ*fur-35* (10^7 CFU/ml, 30 min) (n=24). The samples were taken 30 days post immunization. The samples correspond to two independent experiments with 10 to 14 animals each. **P*<0.001; ***P*<0.05

5. *Discussion*

The ferric uptake regulator (Fur) is a global regulatory protein that is involved in diverse aspects of bacterial life. It is a metalloregulatory protein that requires Fe^{+2} or other divalent transition metal ions like Zn^{+2}, as a cofactor (3, 39, 70). Fur possesses three functional domains, the helix-turn-helix DNA-binding domain, the protein-protein dimerization domain, and the metal ion-responsive domain. This last domain is essential for Fur dimerization to form a functional protein (9, 47, 51). It has already been suggested by Pohl et al. (47) that the N-terminal helix is required for efficient DNA binding at the Fur box. This is supported by mutagenesis studies. For example *P. aeruginosa* Fur, having Ala10 mutated to glycine (a much poorer helix former) was found to be unable to bind to the Fur box at the *pvdS* gene promoter (4). In *E. coli* it was shown that proteolytic cleavage of the 8 or 9 N-terminal residue resulted in a protein with reduced DNA-binding affinity and specificity (9). However as we have shown, the N-terminal region of *E. ictaluri* Fur is missing, as a long with the His32 residue, which interacts with Fe^{2+} in *P. aeruginosa* Fur. This indicates that the binding residues for Fe^{+2} and other divalent ions are different than those used in *E. coli* and *Pseudomonas* Fur proteins.

In terms of phylogeny, the sequenced *E. tarda* isolated from humans contains a longer *fur* gene, in contrast with the reported *E. tarda* *fur* genes isolated from fish, which contain a shorted version and

appear to have a common ancestor with the *E. ictaluri fur* gene (Fig. 2). Numerous examples of genome reduction have been documented in the transition from free-living bacteria to parasitic life style (35). Pathogenic bacteria seem to have embarked on some of the same processes of gene degradation and deletion that have led to extensive genome shrinkage in host restricted pathogenic groups. For example, large numbers of pseudogenes have been identified in both *Y. pestis* (42) and *S. enterica* serovar Typhi (41). Perhaps the emergence of a shorter *fur* gene in the *Edwardsiella* genera is an adaptation to the fish host. *E. ictaluri* was first described by Hawke in 1979 (20), and then characterized in 1982 (21), during the industrial expansion of the catfish aquaculture industry (67). So, an alternate hypothesis is that the reduction of the *fur* gene could be part of a process of *E. ictaluri* host specialization due to intensive fish aquaculture. This observation raises the question of how this shorter *fur* gene increases the fitness of *Edwardsiella* in the fish host and how it appears during the evolution to host adaptation.

Although *E. ictaluri* Fur is missing the N-terminal region, it is fully functional in *S. enterica*, complementing the regulation of siderophore and IROMP synthesis in an iron dependent fashion (Fig. 4). This indicates that a single β-strand in the DNA binding domain is enough for DNA binding and gene repression. Detailed studies are required to evaluate this hypothesis. Nevertheless, the fact that *E. ictaluri fur* complements *S. enterica* indicates a recent divergence from the Fur

phylogenetic trunk and supports the idea that *E. ictaluri fur* might be part of an ongoing process of host specialization.

As mentioned previously iron acquisition mechanisms, like siderophores, are essential for bacterial pathogens to overcome host defenses. However, we did not detect siderophore synthesis in *E. ictaluri*, regardless of the presence or absence of iron in the growth media or the presence or absence of the *fur* gene (Fig. 5). This observation reinforces the hypothesis of genome degradation during the process of host specialization of *E. ictaluri* to catfish, where siderophore genes might be lost. Nevertheless, in absence of iron *E. ictaluri* up regulates a heme-hemoglobin system in a Fur dependent fashion (Figs. 5-7). During pathogenesis clearly there is a battle between *E. ictaluri* and the catfish host for iron (32, 33, 44, 45, 62), were the *E. ictaluri* heme-hemoglobin system becomes a lethal weapon contributing to the systemic infection of the fish. However, this heme-hemoglobin acquisition system needs to be tightly regulated and synthesized in the precise time and location within the host. We observed that *E. ictaluri* presents some preference for hemoglobin over heme (Fig. 7), yet how and why *E. ictaluri* has this preference remains unclear. Further studies are required to evaluate heme-hemoglobin transport and utilization.

Fur protein is a global regulator that controls genes related to iron and other metal divalent ion acquisition and genes related to bacterial catabolism, affecting virulence. We determined that *E. ictaluri* Fur

regulates genes related with iron acquisition and heme synthesis as well as Mg^{+2} and Mn^{+2} uptake. However, Fur not only regulates iron metabolism and virulence. Fur regulation of *gmpA*, encoding the phosphoglycerate mutase, has previously been described in *E. coli* (18) and *Salmonella* (66) and here we described its regulation in *E. ictaluri.* This indicates that Fur participates in the general regulation of metabolic pathways, linking bacterial growth to the supply of key nutrients like iron. We found a cyclic AMP receptor protein (Crp) binding box in the *fur* promoter region (Fig. 1), which is conserved in *E. coli* and *Salmonella fur* promoters (18, 66). Crp may link expression of the Fur regulator to the availability of carbon sources.

The main virulence factors described in *Edwardsiella* are the type III secretion system, required for cell invasion and survival in *Edwardsiella* containing vesicles in macrophages, and the type VI secretion system, required for full virulence (64, 65, 71). The *Edwardsiella* type III and VI secretion systems, as well as the transcriptional regulators EsrB and EsrC, are very similar between *E. tarda* and *E. ictaluri* (64, 65, 71). Recently the linkage of Fur with virulence in *E. tarda* has been reported (6). Fur regulates the type VI secretion system mediated repression of *evpP* gene expression (type VI secretion effector), binding to its promoter region and blocking the binding of the EsrC activator (6). We did not find either an *E. ictaluri evpP* gene ortholog or a Fur binding box at the promoter region of the *evpA* gene or *E. ictaluri* type VI secretion

system (data not shown). Protein-protein interaction between Fur and EsrC, or between Fur and EsrB response regulators was described in *E. tarda* (6). Although we did not find a Fur binding box in the type III secretion system or in the type VI secretion system of *E. ictaluri*, it is possible that Fur influences the virulence of *E. ictaluri* mediated protein-protein interaction as was described in *E. tarda*.

Salmonella Δ*fur* mutants are attenuated in mammals when administered orally (49) or intraperitoneally (15), but are not very immunogenic (11). However, Δ*fur* mutants constitutively synthesize their IROMPs, exposing them to the immune system of the host. Although *E. ictaluri* Δ*fur* is not an efficient vaccine compared to the recently described *E. ictaluri* Δ*crp* mutant (54) the *fur* gene is an evolutionary conserved regulon that controls iron homeostasis and bacterial virulence. Therefore, deletion of *fur* is an effective modification for live attenuated vaccines, and can be combined with other deletions, like *crp* to improve the efficiency of vaccines.

In summary, we conclude that *E. ictaluri* does not secret detectable siderophores in the growth conditions tested, regardless of the presence or absence of Fur and iron. Fur regulates a heme-hemoglobin uptake system in *E. ictaluri* and deletion of *fur* can be successfully used as a means to attenuate *E. ictaluri* in order to develop effective immersion live attenuated vaccines for the aquaculture industry.

6. *Acknowledgments*

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CHAPTER IV

THE ASPARTATE-SEMIALDEHYDE DEHYDROGENASE OF *EDWARDSIELLA ICTALURI* AND ITS USE AS BALANCED-LETHAL SYSTEM IN FISH VACCINOLOGY

1. *Abstract*

asdA mutants of Gram-negative bacteria have an obligate requirement for diaminopimelic acid (DAP), which is an essential constituent of the peptidoglycan layer of the cell wall of these organisms. In environments deprived of DAP, i.e., animal tissues, they will undergo lysis. Deletion of the *asdA* gene has previously been exploited to develop antibiotic-sensitive strains of live attenuated recombinant bacterial vaccines. Introduction of an Asd^+ plasmid into a Δ*asdA* mutant makes the bacterial strain plasmid-dependent. This dependence on the Asd^+ plasmid vector creates a balanced-lethal complementation between the bacterial strain and the recombinant plasmid.

E. ictaluri is an enteric gram-negative fish pathogen that causes enteric septicemia in catfish. Because *E. ictaluri* is a nasal/oral invasive intracellular pathogen, this bacterium is a candidate to develop a bath/oral live recombinant attenuated *Edwardsiella* vaccine (RAEV) for the catfish aquaculture industry. As a first step to develop an antibiotic-

sensitive RAEV strain, we characterized and deleted the *E. ictaluri asdA* gene. *E. ictaluri* Δ*asdA01* mutants exhibit an absolute requirement for DAP to grow. The *asdA* gene of *E. ictaluri* was complemented by the *asdA* gene from *Salmonella*. Several Asd^+ expression vectors with different origins of replication were transformed into *E. ictaluri* Δ*asdA01*. Asd^+ vectors were compatible with the pEI1 and pEI2 *E. ictaluri* native plasmids. The balanced-lethal system was satisfactorily evaluated in vivo. Recombinant GFP, PspA, and LcrV proteins were synthesized by *E. ictaluri* Δ*asdA01* harboring Asd^+ plasmids. Here we constructed a balanced-lethal system, which is the first step to develop an antibiotic-sensitive RAEV for the aquaculture industry.

2. *Introduction*

Aspartate β-semialdehyde dehydrogenase (Asd; EC 1.2.1.11), a highly conserved homodimeric enzyme encoded by the *asd* gene, is involved in the conversion of β-aspartyl phosphate to aspartate β-semialdehyde. Asd is an enzyme common to the biosynthesis of the essential amino acids lysine, threonine, methionine, and isoleucine. It also performs a key step in the production of diaminopimelic acid (DAP), a required component for the peptidoglycan synthesis of Gram-negative and some Gram-positive bacterial cell walls (38, 39, 45, 51) and an immediate precursor to lysine. *asd* mutants have an obligate requirement for

DAP, and in the absence of DAP they undergo lysis. This has been demonstrated by gene-knockout studies with *Legionella pneumophila* (21), *Salmonella* Typhimurium (17) and *Streptococcus mutans* (8).

The Asd enzyme is also found in plants, where lysine is synthesized via the DAP pathway (23, 53). In contrast, mammalian cells neither synthesize nor use DAP as a substrate in any metabolic pathway, and lysine is not synthesized since it is an essential amino acid that is obtained from dietary sources (7, 9, 21). Also lysine, threonine, methionine, and isoleucine are essential amino acids in the diet of teleostei fish (14, 19, 20, 32, 34, 37), suggesting the absence of both the DAP/lysine synthesis pathway and Asd enzyme in fish cells.

Since DAP is absent from mammalian tissues, deletion of the *asd* gene has been exploited to develop a balanced-lethal system for vaccine delivery vehicles using a cloned *asd* gene as a selective marker in place of antibiotic-resistance markers, which are totally impractical in vivo (17). Introduction of an Asd^+ plasmid into *asd* mutants makes the bacterial strain plasmid-dependent. This dependence on the Asd^+ plasmid vector creates a balanced-lethal complementation between the bacterial strain and the recombinant plasmid (36). Asd^+ vectors introduced into live recombinant attenuated *Salmonella* vaccines have been used to deliver heterologous antigens (13). The construction of live attenuated recombinant bacterial vaccines not only require the absence of antibiotic-resistance markers in their recombinant plasmid, but also in

their chromosomal deletions.

Edwardsiella ictaluri, a Gram-negative bacterial pathogen, is the cause of enteric septicemia in catfish, which causes losses estimated at $50-80 million annually (48). The current USDA licensed vaccine, live *E. ictaluri* AQUAVAC-ESC® (Intervet Inc.), has been selected by multiple passages in increased concentrations of the antibiotic rifampicin (2, 28, 47). The selected spontaneous mutant strain presented an attenuated phenotype missing part of the lipopolysaccharide (LPS) (30, 42). Although there are FDA and USDA regulations against the use of antibiotic resistance in live attenuated bacterial vaccines for birds, mammals, and humans, the catfish industry currently allows antibiotic-resistant vaccine strains. Despite the fact that the current vaccine against enteric septicemia in catfish is antibiotic resistant, by using this vaccine we have learned that *E. ictaluri* live attenuated vaccines can be easily delivered to young fish and stimulate both humoral and cellular immunity of long duration (46, 48). These results provide guidance to design live attenuated antibiotic–sensitive vaccines for the catfish aquaculture.

As a first step in developing an antibiotic-sensitive live recombinant *E. ictaluri* vaccine strain (RAEV), we adapted suicide vector technology (15) to *E. ictaluri* to construct defined unmarked chromosomal deletion mutations, for instance the *asd* deletion. Two *E. ictaluri asd* genes were identified, a functional a*sdA* and a non-functional *asdB* pseudogene. The *asdA* gene was deleted by using the described suicide vector

technology. Using Asd^+ expression vectors (13), we developed a balance-lethal system compatible with *E. ictaluri* native plasmids, to express and secrete heterologous proteins through the type II secretion system. The virulence of the *E. ictaluri* Δ*asdA* mutant, harboring an $AsdA^+$ expression vector, was evaluated in vivo in the catfish (*Ictalurus punctatus*) and in the zebrafish (*Danio rerio*) host models. Here we report the first balanced-lethal vector-host system in *E. ictaluri*, a key in constructing antibiotic-sensitive live RAEV for the catfish industry.

3. *Material and methods*

Bacterial strains, plasmids, media, and regents. The bacterial strains and plasmids are listed in Table 1 and 2, respectively. Bacteriological media and components are from Difco (Franklin Lakes, NJ). Antibiotics and reagents are from Sigma (St. Louis, MO). LB broth (tryptone, 10 g; yeast extract 5 g; NaCl, 10 g; 1 g dextrose, 1L ddH_2O,) (4), Bacto-Brain Heart Infusion broth (BHI), and Trypticase Soy Broth (TSB), were used routinely. When required, the media were supplemented with 1.5% agar, 5% sucrose, colistin sulphate (Col; 12.5 μg/ml), ampicillin (Amp; 100 μg/ml), chloramphenicol (Cm; 25 μg/ml), or kanamycin (Km; 50 μg/ ml). Fish broths were prepared with fresh homogenized catfish tissues (liver, spleen, kidney, and meat) to 1% in BHI and filter sterilized (0.22 μm). Bacterial growth was monitored spectrophotometrically and/or by

plating. Oligonucleotides were from IDT (Coralville, IA). Restriction endonucleases were from New England Biolabs. Taq DNA polymerase (New England Biolabs) was used in all PCR tests. Qiagen products (Hilden, Germany) were used to isolate plasmid DNA, gel-purify fragments or purify PCR products. T4 ligase, T4 DNA polymerase and shrimp alkaline phosphatase (SAP) were from Promega.

Table 1

Bacterial strains used in this study

Strain	Relevant characteristics	Source or reference
Escherichia coli		
χ6212	F⁻ Δ(*argF-lacZYA*)-*U169 glnV44* l⁻ *deoR* f80d*lacZΔM15 gyrA96 recA1 relA1 endA1* Δ*asdA4* Δ(*zhf-2*::Tn*10*) *thi-1 hsdR17*; Tet^r	(11)
χ7213	*thr-1 leuB6 fhuA21 lacY1 glnV44 recA1* Δ*asdA4* D(*zhf-2*::Tn*10*) *thi-1* RP4-2-Tc::Mu [λ*pir*]; Km^r	(40)
χ7232	*endA1 hsdR17 (rK-, mk+) supE44 thi-1 recA1 gyrA relA1* Δ*(lacZYA-argF) U169* λ*pir deoR (f80dlac*Δ*(lacZ)M15)*	Lab collection
Edwardsiella ictaluri		
J100	Wild-type; pEI1$^+$; pEI2$^+$ API20E 40040057; smooth LPS; Col^r DAP$^+$	(39)
J102	Wild-type; pEI1$^+$; pEI2$^+$ API20E 40040057; smooth LPS; Col^r DAP$^+$	ATCC 33202
J111	J102 derivative; Δ*asdA01*; pEI1$^+$; pEI2$^+$ API20E 40040057; smooth LPS; Col^r DAP$^-$	This study
J112	J100 derivative; Δ*asdA01*; pEI1$^+$; pEI2$^+$ API20E 40040057; smooth LPS; Col^r DAP$^-$	This study
Salmonella enterica		
χ3761	*S*. Typhimurium UK-1; wild-type	(12)
χ8958	*S*. Typhimurium UK-1 Δ*asdA33*	Lab collection
χ9112	*S*. Typhi ISP1820 Δ*asdA33*	Lab collection
χ9124	*S*. Typhi Ty2 Δ*asdA33*	Lab collection
Yersinia pestis		
χ10006	Δ*asdA12*	Lab collection

Table 2

Plasmid used in this study

Plasmids	Relevant characteristics	Source or reference
pYA248	3,000 bp, contains 1,071 bp of *S. mutans asdA gene;* p15A *ori*	(36)
pYA575	5,730 bp, contains ~1,330 bp of *S. mutans* DNA inserted between the EcoRI and HindIII sites of pBR322 plasmid, Amp, Tet, pBR *ori*	(25)
pYA3341	2595 bp, plasmid Asd^+; pUC *ori*	(12)
pYA3493	3113 bp, plasmid Asd^+; pBR *ori* β-lactamase signal sequence-based periplasmic N- terminal secretion plasmid	(13)
pYA3620	3169 bp, plasmid Asd^+; pBR *ori* β-lactamase signal sequence-based periplasmic N- and C-terminal secretion plasmid	(12)
pYA3994	pBR *ori*, Asd^+, GFP^+ 3113 bp,	Lab collection
pYA3840	323 bp DNA encoding the LcrV in pYA3493	(5)
pYA4088	852 bp DNA encoding the α-helical region of PspA aa 3-285 in pYA3493	(60)
pRE112	5,173 bp, Cm, *sacB, oriV, oriT*	(15)
pMEG-375	8,142 bp, Cm, Amp, *lacZ*, R6K *ori, mob incP, sacR sacB*	(44)
pACYC184	4,245 bp, Tet, Cm, p15A *ori*	(10)
pEZ101	Δ*asdA01*, pR112	This study
pEZ102	Δ*asdA01*, pMEG-375	This study
pEZ140	SD-*asdA*, Cm, pACYC184	This study
pEZ142	P_{asdA}-*asdA*, Cm, pACYC184	This study

Sequence analysis. Nucleotide Basic Local Alignment Search Tool (BLAST) was performed based on the sequences of the putative *asd* genes present in the genome sequence of *E. ictaluri* 93-146 accessed from NCBI's Entrez Genome database (NC_012779).

Asd sequences used were obtained from NCBI's Entrez Protein database. Amino acid sequence alignments were performed using the CLC Free Workbench software tool (v. 6.1 CLC bio A/S, Aarhus, Denmark). Protein structural-based alignments were performed by using the web-based interface for ESPript v.2.2 located at http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi (18). Phylogenetic position of *E. ictaluri* AsdA protein was performed with CLC Free Workbench version using the unweighted pair group method with arithmetic mean (UPGMA). Bootstrap analysis was performed with 100 resamplings.

The 3D structure of *E. ictaluri* AsdA protein was predicted by using position specific iterative - BLAST (PSI-BLAST) alignment and HHpred (49).

Construction and characterization of *asdA* mutants. The recombinant suicide vector pEZ102 (Table 1) carrying the linked flanking regions (5' 361 bp and 3' 422 bp) to generate an in-frame deletion of the *asdA* gene was constructed as described in (44). The Δ*asdA01* defined deletion mutation encompasses a 1,104 base pair deletion including the ATG start codon but not including the TAG stop codon. Primers (primer 1) 5'-

ACATGCATGCAATGCCGTCAACGCCGCAGAAT-‘3 and (primer 2) 5’-CCGCTCGAGATGCACTCCTGCCTTGGATGGTGA -‘3 were designed to amplify the upstream *asdA* flanking region (361 bp). A SphI site was included in the primer 1 (underlined) and a XhoI site was included in primer 2 (underlined). The downstream *asdA* flanking region (422 bp) was amplified by primers (primer 3) 5’-CCGCTCGAGTGAGGCTACTGCTCTAGCCCGTGC -‘3 and (primer 4) 5’-TCGTCTAGAGCCAGATAGATTTGATGTTGTCTCTGCTGC -‘3. A XhoI site was included in primer 3 (underlined) and XbaI site was included in primer 4. The flanking regions were amplified from *E. ictaluri* J100, ligated, cloned into pRE112 and pMEG-375, and then digested with SphI and XbaI. The resulting plasmids were designated pEZ101 and pEZ102, respectively. To construct the *E. ictaluri* Δ*asdA01* mutant, the suicide plasmid was conjugationally transferred from *Escherichia coli* χ7213 (41) to *E. ictaluri* wild-type strains J100 and J102. Strains containing single-crossover plasmid insertions (*E. ictaluri asdA*::pEZ102) were isolated on BHI agar plates containing Col, Amp, and DAP. Loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected by using the *sacB*-based sucrose sensitivity counter-selection system (15). The colonies were screened for Amp^s, Col^r and for growth only in presence of DAP. DAP^- colonies were screened by PCR using primer 1 and 4. Biochemical profiles of *E. ictaluri* strains were

determined using the API 20E system (bioMériux, Marcy l'Etoile, France).

Complementation of *asdA* gene. The *asdA* gene of *E. ictaluri*, with and without its promoter, was cloned into a pAYCY184 vector (10) by inactivating the Tet cassette at the BamHI and XbaI restriction sites. The primers used to amplify *asdA* with its promoter (P_{asdA}-*asdA*) were 5' – TCGTCTAGAATCTTGTAAGTTTGAGGATTA – 3' (upstream) and 5' – CGGGATCCTCAGCATGCGGCGCAACGGCTC – 3' (downstream). An XbaI and BamHI site were included in these primers, respectively (underlined). To amply the *E. ictaluri* Shine-Dalgarno (SD)-*asdA* promoter-less the upstream primer 5' – TCGTCTAGAAGGCAGGAGTGCATATGAAAAA – 3' was used with the downstream primer previously described. An XbaI site was included in this primer (underlined). The *E. ictaluri* promoter-less *asdA* includes the SD AGGA region, 6 bp upstream from the ATG start codon (SD-*asdA*). The resulting plasmids, pEZ140 (SD-*asdA*) and pEZ146 (P_{asd}-*asdA*) were used to complement different Δ*asdA* mutant strains. Also *asd* from S*treptococcus mutans*, cloned into pYA575 (25) and pYA248 (36), was used to evaluate complementation of *E. ictaluri* Δ*asdA01* mutants.

To create a balanced-lethal system in *E. ictaluri*, several Asd^+ expression vectors harboring the SD-*asdA* gene sequence from *Salmonella* Typhimurium UK-1 with different origins of replication, (Table 1) (13) were transformed into *E. ictaluri* Δ*asdA01* to evaluate their

complementation and stability. The growth rate of the complementing strains was evaluated in the absence of DAP. Plasmid stability was evaluated for fifty generations as described by Konjufca et al. (29).

Expression of heterologous antigens by *E. ictaluri* Δ*asdA01.* Asd^+ expression vectors encoding different heterologous proteins (Table 1) were transformed into *E. ictaluri* Δ*asdA01* to evaluate the expression and secretion of foreign proteins. First, the green fluorescent protein (GFP) was used to evaluate protein synthesis in the *E. ictaluri* Δ*asdA01* strain. The vector pYA3994 $AsdA^+$ GFP^+ without a peptide secretion signal sequence was transformed into *E. ictaluri* Δ*asdA01* (Table 1). The synthesis of GFP was evaluated by fluorescent microscopy. The synthesis of LcrV and PspA was evaluated by western blot and the secretion was evaluated by subcellular fractionation (27).

Western blot analysis. To evaluate the synthesis of heterologus proteins by *E. ictaluri*, the strains were grown in 3 ml of BHI at 28°C with aeration (180 r.p.m.). The samples were collected when the culture reached the absorbance of 1.0 ($O.D_{600}$ 1.0~$1x10^8$ cfu/ml). One ml of culture was collected and prepared for western blot analysis (43). The total proteins were normalize by using a nanodrop spectrophotometer (ND-1000, NanoDrop) at 25 mg/μl and separated by 10% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto

nitrocellulose membranes (43). Fat-free milk powder solution (5%, wt/vol) in PBS supplemented with 0.05% of Tween 20 (PBS-T) was used for blocking. The membrane was incubated individually with a primary mouse anti-RpoD monoclonal antibody (1:1,000) (Neoclone), rabbit anti-LcrV polyclonal antibody (1:1,000) (AbCAM), or rabbit anti-PspA polyclonal antibody (1:10,000), for 1 h at room temperature, washed three times with PBS-T, and then incubated with a 1:10,000 dilution of alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG) (Sigma) or anti-rabbit immunoglobulin G (IgG) (Sigma). Color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Amaresco).

***Edwardsiella* subcellular fractionation.** Cultures were grown in BHI at 28°C static to an OD_{600} of 0.6 and centrifuged at 7,000 rpm for 10 min. Periplasmic fractions were prepared by a modification of the lysozyme-osmotic shock method (59) as previously described (27). The supernatant fluid was saved for analysis of secreted proteins. Equal volumes of periplasmic, cytoplasmic, and supernatant fractions and total lysate samples were separated by SDS-PAGE for western blot analysis.

Determination of LD_{50} in zebrafish animal host. Zebrafish infections were performed by the methodology described by Petri-Hanson et al. (40) with modifications. The temperature of the water was 26 ± 1

°C and the fish were acclimated during 2 weeks prior to the start of the experimentation. Adult zebrafish (average weight, 0.5 g) were sedated in 100 mg/L tricaine methanesulfonate (MS-222, Sigma) and then injected intramuscularly (i.m.). Groups of zebrafish (typically 15 fish per group) were injected i.m. with 10 μl of the bacterial suspension (10^3-10^9 CFU) into each fish. A 3/10-cc U-100 ultrafine insulin syringe with a 0.5-in.-long (ca. 1-cm-long) 29-gauge needle (catalog no. BD-309301; VWR) was used to inject the fish. Two sets of controls were used: fish that were injected with 10 μl of sterile phosphate-buffered saline containing 0.01% gelatin (BSG) (12) and fish that were not injected. Moribund fish demonstrating clinical signs were euthanized, necropsied, and bacteria isolated as previously described (40). The fish were fed twice daily with TetraMin Tropical Fish Flake Feed. During the experiments, the fish were observed daily, and every other day water quality was monitored for pH and NO_2 with standard kits. The LD_{50} was calculated by the method of Reed-Muench (1). Fish care and use was performed in accordance with the requirements of the Arizona State University, Institutional Animal Care and Use Committee.

Determination of LD_{50} in catfish animal host. Specific-pathogen-free channel catfish (*Ictalurus punctatus*) fingerlings were used with a mean weight of 18.5 ± 1.3 g. The animals were randomly assigned to treatment groups of 6 to 8 fish each in 100 liter tanks. Each tank was equipped with a self-contained, recirculating, biofiltered, mechanical filtered, and

U.V. water treated system with 12 h of illumination daily. The water temperature was 28 ± 1 °C during the 2 weeks of acclimatization and during the experiments. The fish were fed daily with commercial Aquamax grower 400 (Purina Mills Inc., St. Louis, MO). During the experiments, the fish were observed daily, and every other day water quality was monitored for pH and NO_2 with standards kits. Catfish were infected with 10^3 to 10^9 CFU of *E. ictaluri* strains (fish were not fed until 1 h after infection) orally and intra coelomic (i.c.). The fish were anesthetized with tricaine methanesulfonate (MS-222, Sigma; 100 mg/L of water) prior to handling. The LD_{50} was calculated by the method of Reed-Muench (1). Moribund animals were necropsied to evaluate presence of *E. ictaluri* in kidney, spleen and liver. Fish care and use was performed in accordance with the requirements of the Arizona State University, Institutional Animal Care and Use Committee.

Bacteria preparation. Bacterial strains were grown overnight in standing cultures that were diluted 1:20 in prewarmed BHI broth and grown with mild aeration (180 r.p.m.) at 28°C to an OD_{600} of 0.8 to 0.9 (~10^8 CFU/ml). Bacteria were sedimented 10 min by centrifugation (7,000 r.p.m.) at room temperature and resuspended in BSG (12) to densities appropriate for the inoculation.

4. *Results*

Sequence analysis. To develop a balanced-lethal system we first characterized the *asd* genes present in *E. ictaluri*. The genome of *E. ictaluri* has two *asd* gene sequences, *asdA* (gene ID 7960734) and *asdB* (gene ID 7959931). Sequence and structural alignment between functional representative bacterial Asd proteins reveals that 22 amino acid residues (~6%) are strictly conserved out of 367 residues in *E. ictaluri* AsdA (Fig. 1). *E. ictaluri* AsdA has 28%, 81%, 82%, 84%, and 97% amino acid similarity to the Asd of *Streptococcus mutans*, *Salmonella enterica*, *Escherichia coli, Yersinia* (*Y. pestis* and *Y. ruckeri*), and *E. tarda,* respectively. The overall domain organization of *E. ictaluri* AsdA is similar to other Gram-negative Asd-family members, presenting an N-terminal domain comprising the NAD binding site and a C-terminal catalytic domain (Fig. 1). The same set of key functional groups in the active sites (Cys-135, Gln-162, Glu-241, Arg-267, and His-274) are conserved in *E. ictaluri* AsdA and likely have the same catalytic mechanism as other Asd enzymes (Fig. 1 and Fig. 2).

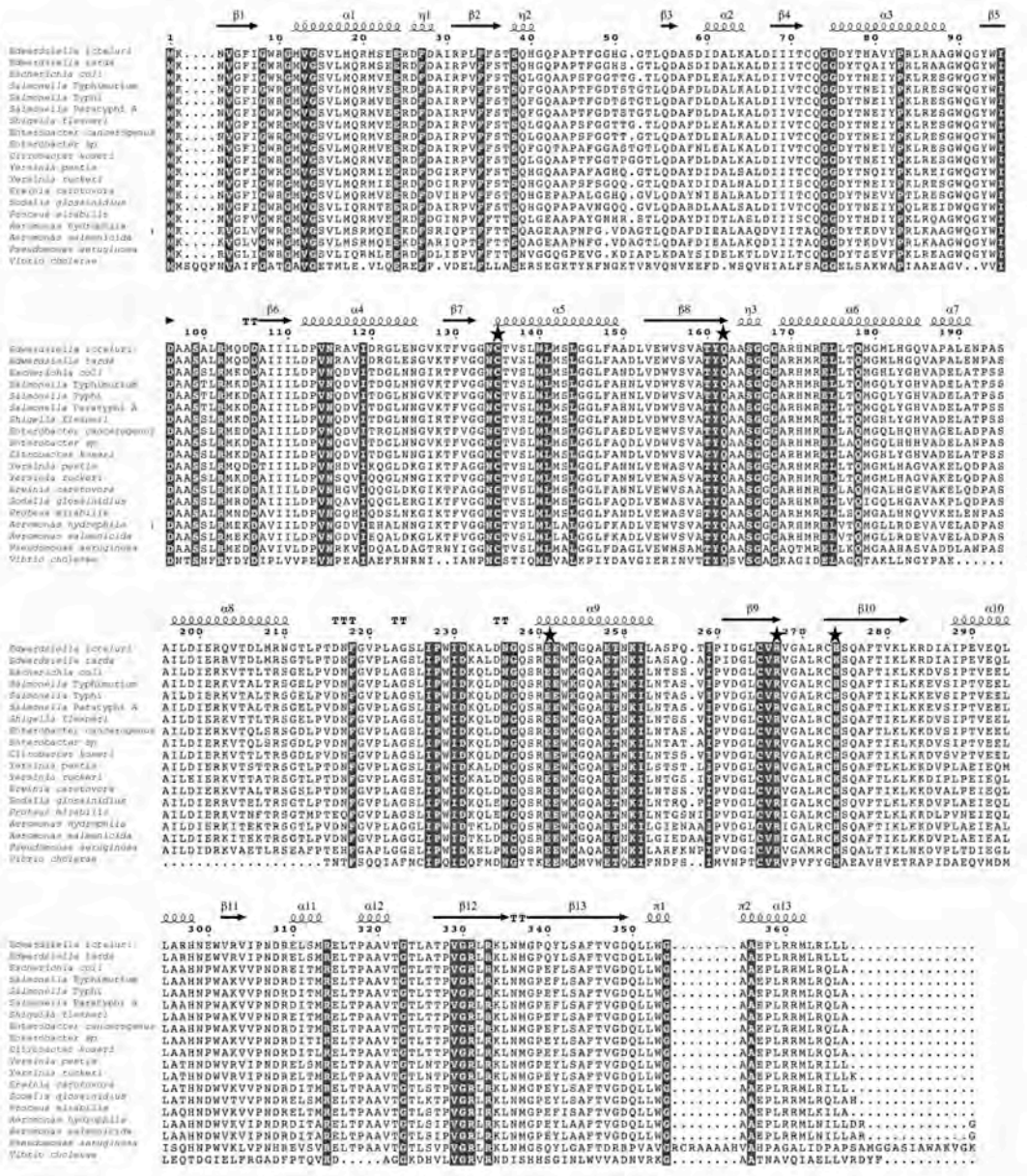

Figure 1. Sequence alignment among representative members of the AsdA family. The secondary structure at the top of the alignment corresponds to the *E. ictaluri* AsdA enzyme (spirals represent α-helix; arrows represent β-sheet). Conserved amino acids residues are indicated in grey. The stars indicated the key catalytic active site residues

(Cys-135, Gln-162, Glu-241, Arg-267, and His-274). The AsdA sequences were obtained from NCBI's Entrez Protein database for *Edwardsiella ictaluri* YP_002935083.1; *Edwardsiella tarda* YP_003297386.1; *Escherichia coli* AP_004358.1; *Salmonella* Typhi NP_807591.1; *Salmonella* Paratyphi A YP_152515.1; *Salmonella* Typhimurium AAB69392.1; *Shigella flexnieri* YP_690789.1; *Shigella sonnei* YP_312455.1; *Citrobacter koseri* YP_001456333.1; *Enterobacter cancerogenus* ZP_05969786.1; *Enterobacter* sp. YP_001178547.1; *Yersinia pestis* NP_671174.1; *Yersinia ruckeri* ZP_04615435.1; *Proteus mirabilis* YP_002152826.1; *Aeromonas hydrophila* ABK39477.1; *Aeromonas salmonicida* YP_001142146.1; *Sodalis glossinidius* YP_456010.1; *Vibrio cholerae* YP_001217562.1; *Pseudomonas aeruginosa* NP_251807.1; *Erwinia carovora atrosepticum* YP_052242.1.

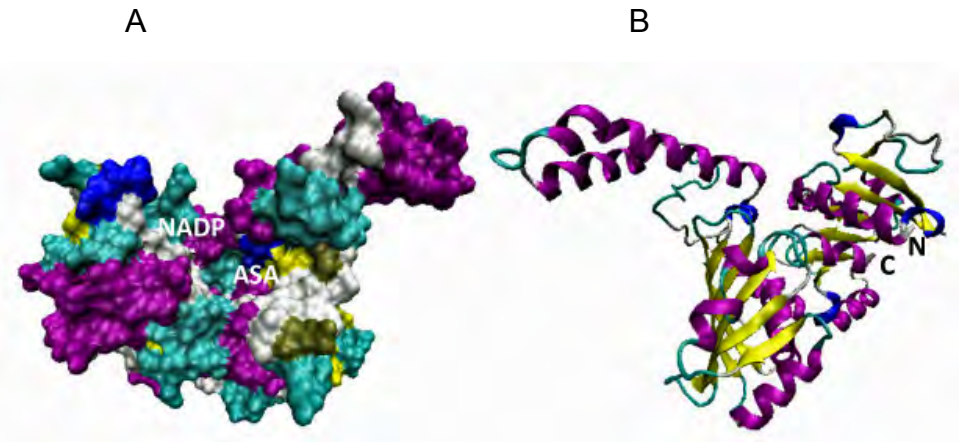

Figure 2. 3D structure model of the *E. ictaluri* AsdA protein. A. 3D surface map of AsdA; color-coded to show the secondary structure. Purple, α-helix; yellow, β-sheet; white and blue, loops. NADP and ASA (β-aspartyl phosphate) binding site are shown; B. 3D E. ictaluri AsdA structure. N- and C- terminal regions are indicated.

The sequence and structural alignment between representative bacterial AsdB proteins reveals that 52 amino acid residues (~15%) are strictly conserved out of 336 in *E. ictaluri* AsdB (Fig. 3). The *E. ictaluri* AsdB has 30%, 32%, 40%, 75%, and 99% amino acid similarity to the AsdB of *Streptococcus mutans*, *Mycobacterium marinum, Vibrio cholerae, Y. pestis*, and *E. tarda,* respectively. In contrast to AsdA, the overall domain organization of *E. ictaluri* AsdB is similar to other Gram-positive Asd-family members. However, *E. ictaluri* AsdB lacks key functional groups in the active sites (Cys-135, Gln-162, and Arg-267) and likely has

no catalytic activity.

The guanine plus cytosine (G+C) content found in the *E. ictaluri asdA* gene was 62%, significantly higher than the 54% of G+C found in the *Escherichia coli asdA* gene. Overall DNA comparison of the *asdA* gene showed that the *E. ictaluri asdA* gene shared 72% identity with the *Escherichia coli asdA* gene.

In terms of phylogeny, the bacterial Asd family is subdivided into two structural branches consisting of the enzymes from Gram-negative and Gram-positive bacteria (52) (Fig. 4). The *E. ictaluri* AsdA enzyme belongs to the Gram-negative branch, in contrast to AsdB that belongs to the Gram-positive branch (Fig. 4). *Edwardsiella* species comprise a linage that diverged from the ancestral trunk before the divergence of some other enteric bacteria, such as *Salmonella* and *Escherichia* (6, 55). The phylogenetic position of the *E. ictaluri* AsdA enzyme corresponds with the *E. ictaluri* genome phylogenetic position (Fig. 4). Inside of the AsdB branch, a non-functional AsdB branch composed of *Edwardsiella* and *Yersinia* AsdB sequences was identified (Fig. 4), indicating that these non-functional AsdB proteins may have a common origin.

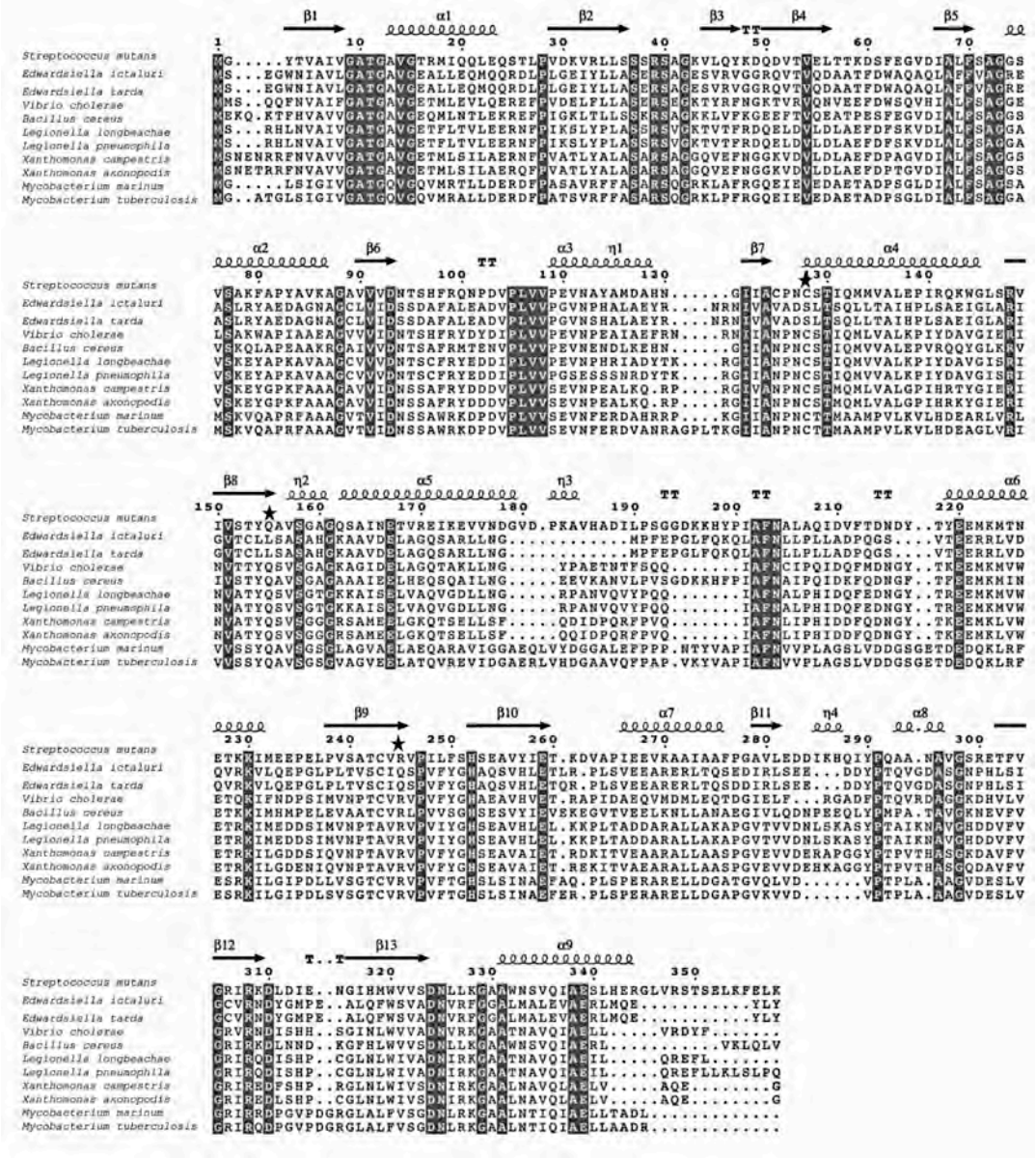

Figure 3. Sequence alignment among representative members of the AsdB family. The secondary structure at the top of the alignment corresponds to the *S. mutans* AsdB enzyme (spirals represent α-helix; arrows represent β-sheet). Conserved amino acids residues are indicated

in grey. The stars indicated the key catalytic active site residues not present in AsdB from Edwardsiella. The AsdB sequences were obtained from NCBI's Entrez Protein database for *Streptococcus mutans* NP_721384.1; *Edwardsiella ictaluri* YP_002934124; *Edwardsiella tarda* YP_003296462; *Vibrio cholerae* YP_001217630.1; *Bacillus cereus* YP_085142.1; *Legionella longbeachae* CBJ10915; *Legionella pneumophila* YP_096311.1; *Xanthomonas axonopodis* NP_643032.1; *Xanthomonas campestris* NP_637897.1; *Mycobacterium tuberculosis* NP_218225.1; *Mycobacterium marinum* YP_001853481.1.

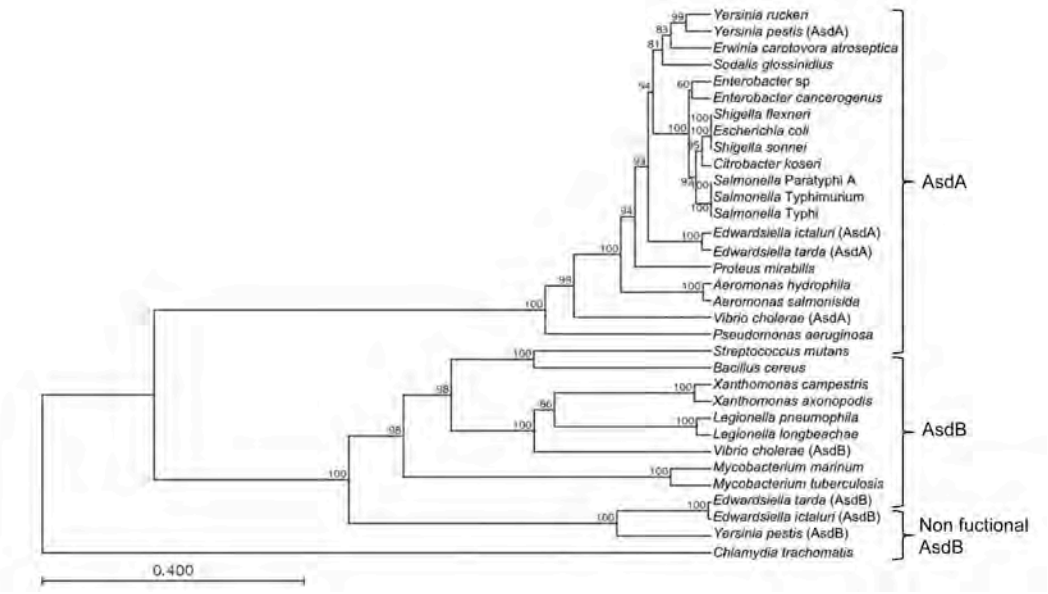

Figure 4. Phylogenetic tree constructed by the unweighted pair group method with arithmetic mean. Bootstrap values indicate the number of times that a given node was detected out of 100. The Asd sequences were obtained from NCBI's Entrez Protein database for *Edwardsiella*

ictaluri YP_002935083.1; *Edwardsiella tarda* YP_003297386.1; *Escherichia coli* AP_004358.1; *Salmonella* Typhi NP_807591.1; *Salmonella* Paratyphi A YP_152515.1; *Salmonella* Typhimurium AAB69392.1; *Shigella flexnieri* YP_690789.1; *Shigella sonnei* YP_312455.1; *Citrobacter koseri* YP_001456333.1; *Enterobacter cancerogenus* ZP_05969786.1; *Enterobacter* sp. YP_001178547.1; *Yersinia pestis* NP_671174.1; *Yersinia ruckeri* ZP_04615435.1; *Proteus mirabilis* YP_002152826.1; *Aeromonas hydrophila* ABK39477.1; *Aeromonas salmonicida* YP_001142146.1; *Sodalis glossinidius* YP_456010.1; *Vibrio cholerae* YP_002810714.1; *Pseudomonas aeruginosa* NP_251807.1; *Erwinia carovora atrosepticum* YP_052242.1; *Streptococcus mutans* NP_721384.1; *Edwardsiella ictaluri* YP_002934124; *Edwardsiella tarda* YP_003296462; *Vibrio cholerae* YP_001217630.1; *Bacillus cereus* YP_085142.1; *Legionella longbeachae* CBJ10915; *Legionella pneumophila* YP_096311.1; *Xanthomonas axonopodis* NP_643032.1; *Xanthomonas campestris* NP_637897.1; *Mycobacterium tuberculosis* NP_218225.1; *Mycobacterium marinum* YP_001853481.1; *Chlamydia trachomatis* YP_002887982.1.

Construction and characterization of *asdA* mutants. The construction of *E. ictaluri* Δ*asdA* mutants was performed first by using pEZ101, a pR112 (Cm) base suicide vector (Table 1). pEZ101 was conjugated from *Escherichia coli* χ7213 to *E. ictaluri* J100 and *E. ictaluri* J102

using the methods described for *E. ictaluri* (33) and *Escherichia coli* (35). The selection of transconjugants was carried out in BHI agar supplemented with Col, DAP, and Cm. We did not recover transconjugants by using pEZ101. Therefore, we constructed and used pEZ102, a pMEG-375 (Cm, Amp) base suicide vector (Table 1). The selection of transconjugants was carried out in BHI agar supplemented with Col, DAP, Amp or Cm. Transconjugants were recovered in the presence of Amp, but not in the presence of Cm. Transconjugants Amp^r, harboring pEZ102 (Amp, Cm), were sensitive to Cm. We determined that *E. ictaluri* is highly sensitive to Cm. Small colonies (>0.5mm) harboring pEZ102 were recovered in a Cm concentration below 1 μg/ml. Using BHI agar supplemented with Col, DAP, and Cm (1 μg/ml), transconjugants were not recovered using pEZ101 (Cm) or pEZ102 (Amp, Cm). Certainly, these results indicate that Cm selection and Cm-base suicide vectors are not useful to genetically manipulate *E. ictaluri*.

Single colonies of *E. ictaluri* transconjugants harboring pEZ102 (Col^r, Amp^r), were grown in BHI, TSB or LB supplemented with DAP and Col at 28°C for 6 h with aeration (180 r.p.m.). The selection was performed in BHI, TSA and LB agar plates supplemented with DAP, Col, and 5% sucrose at 28°C for 4-5 days. BHI sucrose selection agar did not provide selection, due to *E. ictaluri* overgrowth. TSA and LB sucrose selection agar presented a satisfactory selection. Positive mutants were screened for Col^r, Amp^s, and DAP^-. Several *E. ictaluri* Δ*asdA*

mutants were recovered from TSA and LB sucrose-selection agar plates. The genotype was verified by PCR, and the phenotype by growth in presence of DAP and no growth in absence of DAP (Fig. 5). The biochemical profile, evaluated by API20E, did not present any difference between the wild type and Δ*asdA01* mutant strains. *E. ictaluri* strains were identified as *Edwardsiella* sp (code 4004000). These results confirmed that the AsdB present in *E. ictaluri* is non-functional, since deletion of *asdA* is enough to preclude cell growth in the absence of DAP. Thus, *asdB* can be considered a pseudo gene in *E. ictaluri*.

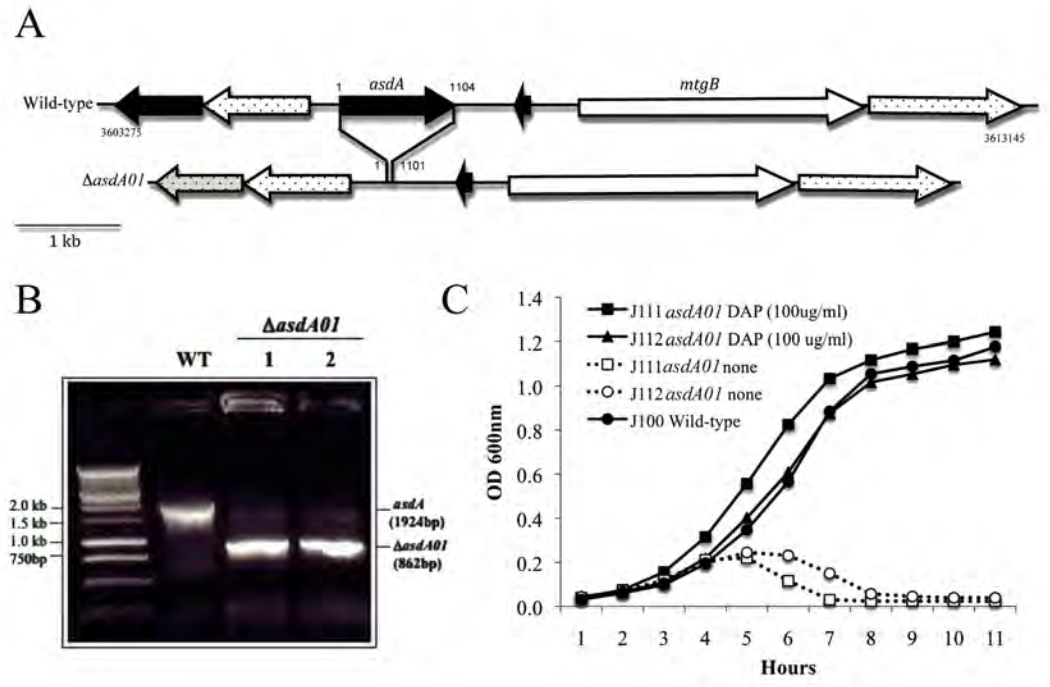

Figure 5. Deletion of *asdA* gene in *E. ictaluri*. A. Deletion map of Δ*asdA01*; B. Genotype verification of J112 Δ*asdA01* by PCR; C. Phenotype of *E. ictaluri* J111 Δ*asdA01* and J112 Δ*asdA01* mutants. The strains were grown in BHI at 28°C with agitation (180 r.p.m.).

We evaluated reutilization of DAP by the *E. ictaluri* Δ*asdA01* mutants released from lysed Δ*asdA* cells grown in absence of DAP. Washed cells of *E. ictaluri* J112 Δ*asdA01* were diluted from 10^1 to 10^{10} CFU/ml in BHI Col. The estimated minimum number of *E. ictaluri* Δ*asdA01* cells needed to support growth in absence of DAP was $1.3x10^8$ – $2.7x10^8$ CFU/ml. This is because of DAP-less death and reuse of DAP to permit growth on media without DAP.

The amount of DAP in the cell wall of *Escherichia coli* has been estimated at ~$3.5x10^6$ molecules (56). Based on the results obtained for the minimum number of *E. ictaluri* Δ*asdA01* cells needed to support growth in the absence of DAP, and the calculated amount of DAP molecules per cell of *Escherichia coli*, we estimated that the minimum number of DAP molecules to support growth is ~$4.5x10^{14}$-$9.5x10^{14}$ molecules of DAP/ml in the growth media. We evaluated the growth of *E. ictaluri* Δ*asdA01* in 10^{10} to 10^{20} molecules of DAP/ml in BHI Col. *E. ictaluri* Δ*asdA01* did not grow in concentrations below 10^{14} molecules of DAP/ml. Our previous estimation about the minimum number of DAP molecules required to support growth was confirmed, indicating that the amount of DAP in the cell wall of *E. ictaluri* is similar to *Escherichia coli*.

It has been reported that lysine, threonine, methionine, and isoleucine are essential amino acids in the diet of teleostei fish (14, 19, 20, 32, 34, 37), suggesting the absence of the DAP/lysine synthesis pathway in fish cells. We tested the growth of *E. ictaluri* J112 Δ*asdA01* in different catfish broths (1% of catfish liver, spleen, kidney and meat in BHI) in presence and absence of DAP. *E. ictaluri* J112 Δ*asdA01* was not able to grow in fish broth not supplemented with DAP. *E. ictaluri* J100 wild-type, used as control, grew in all fish broth conditions. This result supports the idea that as mammalian cells, fish cells neither synthesize nor use DAP as substrate in any metabolic pathway.

Complementation of *E. ictaluri asdA* gene and *E. ictaluri* Δ*asdA01* mutant. The structural analysis of *E. ictaluri* AsdA indicated that the overall domain organization is similar to other AsdA-family members and has the same set of key active-site functional groups and therefore the same catalytic mechanism as other Asd enzymes (Fig. 1 and Fig. 2). To evaluate the likely broad functionality of *E. ictaluri* AsdA enzyme, *asdA* mutants of *Escherichia coli*, *Salmonella enterica* (serovars Typhimurium, and Typhi), *Y. pestis,* and *E. ictaluri,* were complemented with the *E. ictaluri asdA* gene. Because overproduction of AsdA enzyme increases generation times (13, 26) and synthesis of Asd enzyme is proportional to the copy number of the complementing plasmid, *asdA* mutants were complemented with *E. ictaluri asdA* gene with (P_{asdA}-*asdA*) and without its promoter (SD-*asdA*), this last to decrease Asd synthesis, cloned into p15A *ori* plasmid (pACYC184; Table 2).

Escherichia coli, *S. enterica* and *Y. pestis* Δ*asdA* mutants complemented with *E. ictaluri* SD-*asdA* presented similar growth rates compared to wild type (Fig. 6), indicating full complementation. *E. ictaluri* Δ*asdA01* mutants complemented with SD-*asdA* presented a significantly lower growth rate than the wild type (Fig. 6). This could be due to overproduction or underproduction of Asd. It has been reported that SD-*asd* constructions do not enable Δ*asdA* strains to survive in absence of DAP if the origin of plasmid replication (*ori*) is from pSC101 or p15A. In other words, with these lower- copy-number replicons, the amount

of Asd enzyme synthesized is insufficient to enable growth in absence of lysis (13). To evaluate if the decrease in the generation time of the SD-*asdA* complemented *E. ictaluri* Δ*asdA01* strain was due to overproduction or underproduction of AsdA, complementation with P_{asdA}-*asdA* (pEZ142) was performed. Complementation of *E. ictaluri* Δ*asdA01* mutants with pEZ142 decreased the growth rate even more than complementation with SD-*asdA*. These results suggest that the decreased growth rate in the *E. ictaluri* Δ*asdA01* complemented with its own *asdA* gene is due to overproduction of AsdA. There are differences in the SD regions that could justify part of the difference in the growth rate of *E. ictaluri* Δ*asdA01* complemented with its own SD-*asd* gene. The SD region of *E. ictaluri asdA* gene has an optimal spacing (6 nt) between the SD region and the ATG initiation codon of the mRNA (11) in contrast to the other bacterial species complemented with *E. ictaluri* SD-*asdA* (Fig. 6)

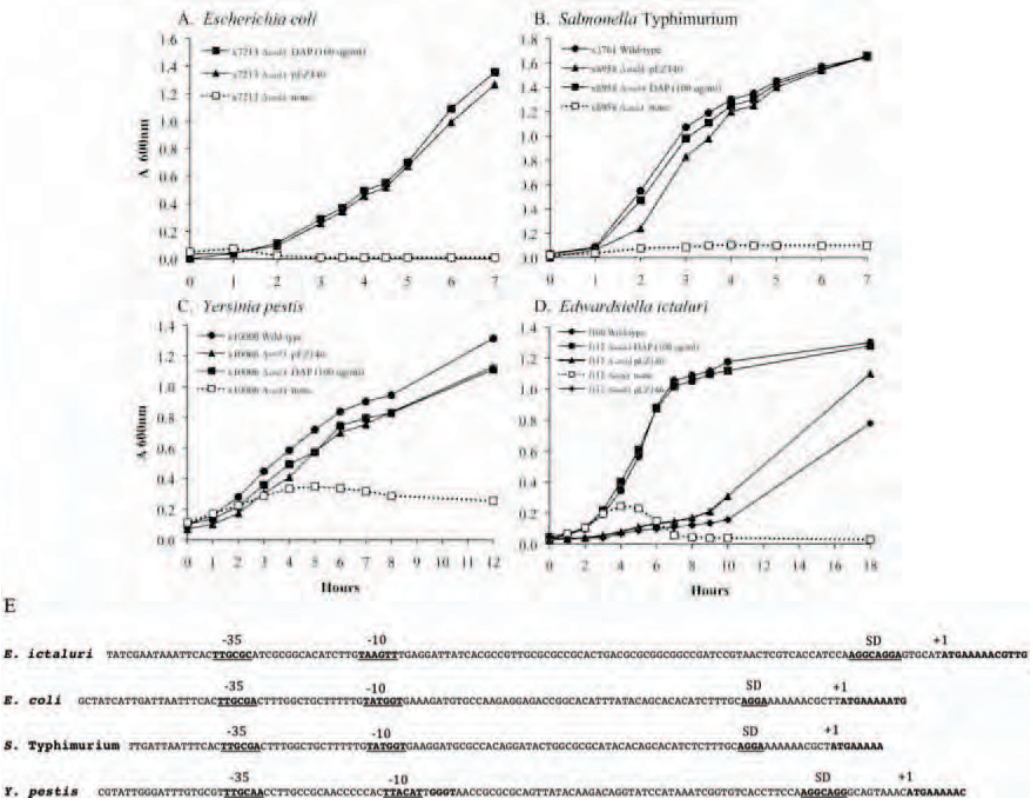

Figure 6. Complementation of representative Δ*asdA* mutant strains with *E. ictaluri asdA* gene. (A-D) Growth of representative Δ*asdA* mutant strains complemented with *asdA* from *E. ictaluri*. pEZ140 (SD-*asdA*); pEZ142 (P_{asdA}-*asdA*); The strains were grown in BHI at 28°C with agitation (180 r.p.m.); (E) Promoter region of *asdA* gene from *E. ictaluri* and representative strains.

Complementation of *E. ictaluri* Δ*asdA01* mutants by Gram-positive AsdB enzyme was also evaluated. *Streptococcus mutans asdB* region (including the full promoter), cloned into pYA575 (25) and *S.*

mutans SD-*asdB*, cloned into pYA248 (35) complemented *E. ictaluri* Δ*asdA01* mutants. However these strains presented lower growth rates than the wild type (Fig. 7). *E. ictaluri* Δ*asdA01* mutants complemented with SD-*asdB* (pYA248), presented the lowest growth rate, suggesting that *S. mutans* AsdB is probably required in higher levels to fully complement *E. ictaluri* or *S. mutans* AsdB do not interact efficiently with *E. ictaluri* aspartokinase enzymes to transfer the β-aspartyl phosphate to Asd.

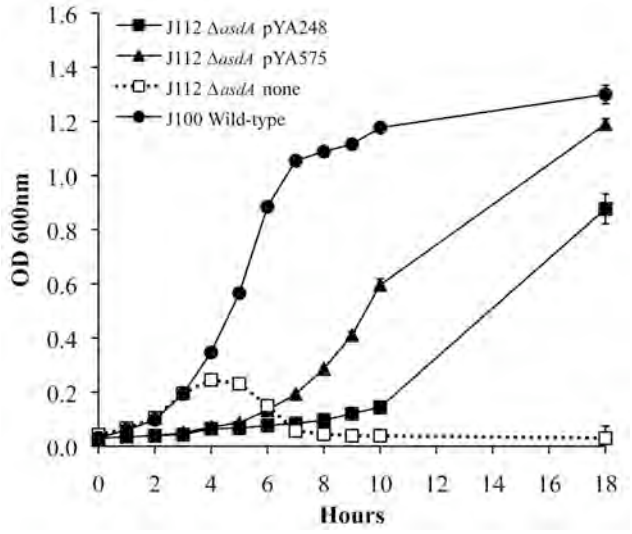

Figure 7. Growth of *E. ictaluri* Δ*asdA01* complemented with *asdB* from *Streptococcus mutans*. The strains were gown in BHI at 28°C with agitation (180 r.p.m.).

Complementation by Asd$^+$ vectors to develop a balanced-lethal system in *E. ictaluri.* The *asdA* gene from *E. ictaluri* complemented *S. enterica* Δ*asdA* mutants, in addition the Asd enzymes from *E. ictaluri* and *S. enterica* share 81% similarity. Therefore, we used the Asd$^+$ vectors utilized in live recombinant attenuated *Salmonella* vaccines (13) to develop a balanced-lethal system in *E. ictaluri*. The Asd$^+$ vectors utilized in this study possess only the SD-*asdA* gene from *S*. Typhimurium with a modified start codon from ATG to GTG. *E. ictaluri* Δ*asdA01* mutants were complemented with the *asdA* gene from *S.* Typhimurium (Fig. 8). The growth rate of *E. ictaluri* Δ*asdA01* complemented with different copy number of Asd$^+$ vectors was similar to the wild type in all cases (Fig. 8). The Asd$^+$ vectors were compatible with the native plasmids of *E. ictaluri* (Fig. 8) and stable for at least 80 generations. These results show the first balanced-lethal system in *E. ictaluri*.

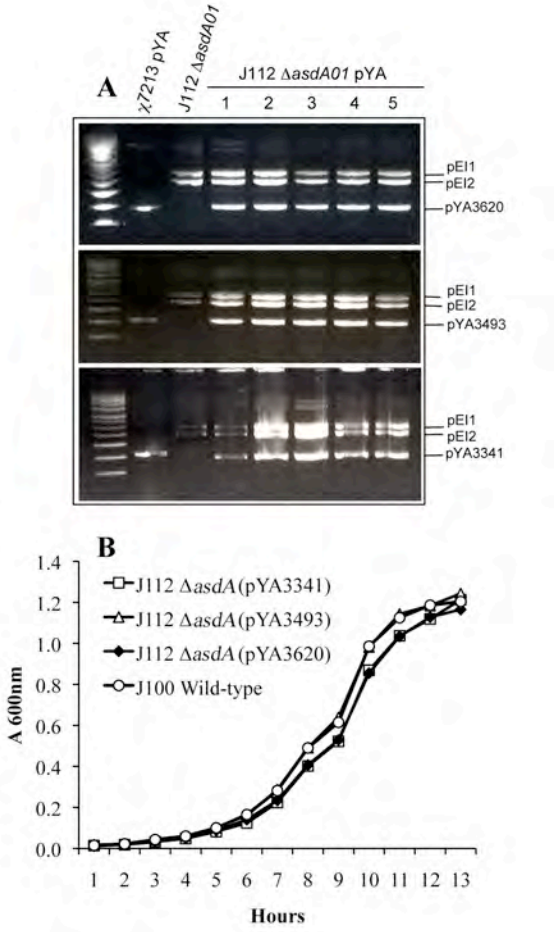

Figure 8. Complementation of *asdA* gene with Asd$^+$ vectors. A. Plasmid profile of *E. ictaluri* Δ*asdA01* complemented with AsdA$^+$ vectors of different copy number. pEI1 (5.7 kb), pEI2 (4.9 kb), pYA3620 (3169 bp), pYA3493 (3113 bp), pYA3341 (2595 bp); Supercoiling ladder, from the top to the bottom: 16,210 bp, 14,174 bp, 12,138 bp, 10,102 bp, 8,066 bp, 7,045 bp, 6,030 bp, 5,012 bp, 3,990 bp, 2,972, 2,067 bp; B. Growth of *E. ictaluri* Δ*asdA01* complemented with different AsdA$^+$ vectors; The strains were grown in BHI at 28°C with agitation (180 r.p.m.).

Expression of genes encoding GFP protein in the AsdA$^+$ vector. The synthesis of heterologous proteins, for instance GFP, cloned into Asd$^+$ vectors was evaluated in *E. ictaluri* Δ*asdA01* to potentially develop live *E. ictaluri* recombinant vaccines. First, the synthesis of heterologous proteins was evaluated by using the GFP$^+$ Asd$^+$ vector pYA3994 (Table 2). *E. ictaluri* Δ*asdA01* mutant strains harboring the GFP$^+$ Asd$^+$ vector grew in absence of DAP and synthesized GFP$^+$ as expected (Fig. 9). The GFP$^+$ Asd$^+$ vector was compatible with the native plasmids of *E. ictaluri* in the relaxed conformation (Fig. 9). The GFP$^+$ Asd$^+$ vector was stable in *E. ictaluri* Δ*asdA01* strains for at least 80 generations. The expression of LcrV and PspA heterologous proteins using AsdA$^+$ vectors was also evaluated (see below).

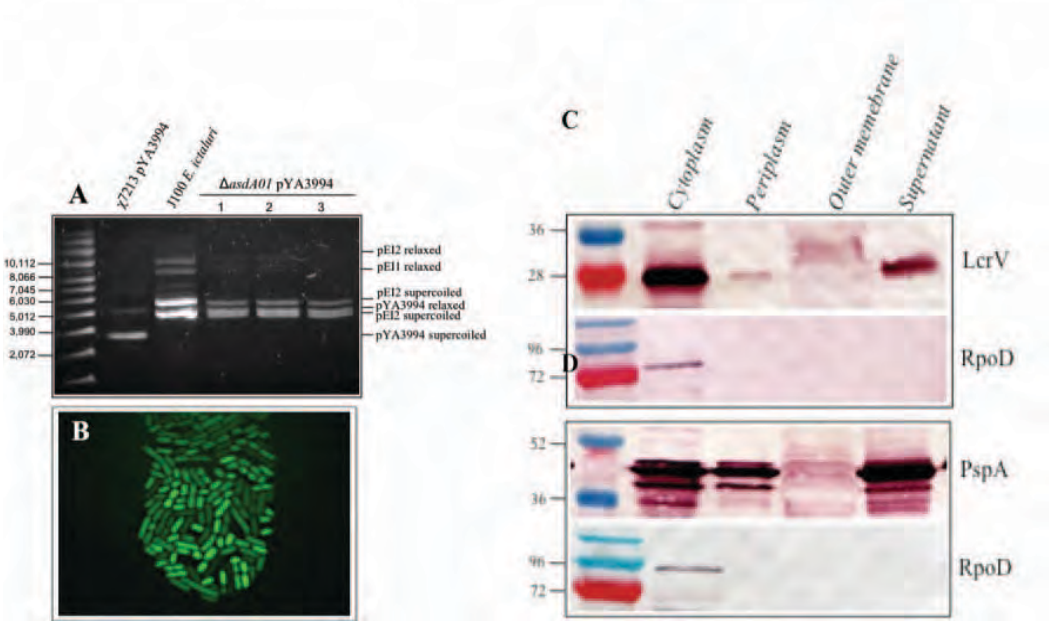

Figure 9. Synthesis of heterologous antigens in *E. ictaluri* J112 Δ*asdA01* by using AsdA^{+} expression vectors. A. Plasmid profile of J112 (pYA3994); B. Expression of GFP J112 (pYA3994); C. Expression and secretion of *Y. pestis* LcrV antigen by J112 (pYA3840); D. Expression and secretion of *S. pneumoniae* PspA-Rx1 antigen by J112 (pYA4088).

Secretion of heterologous proteins. Secretion of the heterologous antigens by live attenuated recombinant bacterial vaccines has been shown to enhance immunogenicity against the heterologous antigen (27). The synthesis and secretion of heterologous proteins was evaluated by using the proteins derived from Gram-positive and Gram-negative bacterial strains. PspA-Rx1 from *Streptococcus pneumoniae* was utilized as a Gram-positive representative and LcrV from *Yersinia pestis* was

utilized as a Gram-negative representative. The heterologous antigens, PspA-Rx1 and LcrV fused to β-lactamase signal sequence, were expressed from the Asd^+ vectors pYA4088 and pYA3841, respectively (Table 2). Both heterologous proteins were secreted through the type II secretion system. No difference in the growth rate was observed between the recombinant *E. ictaluri* and the wild-type strain J100.

Virulence of *E. ictaluri* Δ*asdA01* strain complemented with the $AsdA^+$ plasmid vector in catfish host and zebrafish host models. The idea to develop a balanced-lethal system in a pathogenic bacterial strain is to synthesize heterologous proteins protective antigens, without the use of antibiotic-resistant genes, in either the plasmid or in the bacterial chromosome. This is the first step towards developing live recombinant bacterial vaccines. The ideal balanced-lethal system should present nearly the same level of virulence as the wild-type strain with regard to invasion and colonization of lymphoid tissues. We evaluated the virulence of *E. ictaluri* Δ*asdA01* mutants with and without the balanced-lethal system in the catfish and zebrafish host models. We used pYA3493 $AsdA^+$ since this vector has been used successfully in live recombinant *Salmonella* vaccines (5, 12, 59). *E. ictaluri* Δ*asdA01* was attenuated at the high dose of 10^8 CFU, but still produced some mortality in catfish (Table 3). *E. ictaluri* Δ*asdA01* at a high dose (10^8 CFU) was not attenuated in zebrafish and all the fish died (Table 4). However, at lower doses (10^7-10^4) *E. ictaluri*

Δ*asdA01* was totally attenuated in zebrafish (Table 4). *E. ictaluri* Δ*asdA01* harboring the Asd^+ vector pYA3493 increased the LD_{50} one log-fold, from 10^4 CFU to 10^5 CFU in orally infected catfish, and two log-fold, from 10^3 CFU to 10^5 CFU, in zebrafish (Tables 3 and 4). Catfish i.p. infected with *E. ictaluri* Δ*asdA01* harboring the Asd^+ vector pYA3493 presented the same level of virulence as *E. ictaluri* wild type (Table 3). From moribund orally infected catfish, *E. ictaluri* Δ*asdA* (pYA3493) $AsdA^+$ was recovered from the head-kidney, spleen and liver, indicating that *E. ictaluri* Δ*asdA* (pYA3493) $AsdA^+$ colonized these lymphoid tissues.

Table 3

Survival of catfish (*I. punctatus*) infected with *E. ictaluri* wild type and *E. ictaluri* Δ*asdA01* with and without Asd^+ vectors. The catfish were infected i.p. with 100 μl and orally with 20 μl of the respective *E. ictaluri* strain

	Experiment #1 i.p.		Experiment #2 Oral	
E. ictaluri strains	Dose (CFU/ml)	Survivors/ Total	Dose (CFU/ml)	Survivors /Total
J100 wild-type	$1.5x10^8$	0/6	$1.2x10^8$	1/7
	$1.5x10^6$	0/6	$1.2x10^6$	2/7
	$1.5x10^4$	1/6	$1.2x10^4$	4/7
J112 Δ*asdA01*	$3.0x10^8$	3/7*	$1.7x10^8$	7/8*
J112 Δ*asdA01* (pYA3493)	$2.1x10^8$	0/6	$1.8x10^8$	3/7
	$2.1x10^6$	2/6	$1.8x0^6$	4/7
	$2.1x10^4$	2/6	$1.8x10^4$	5/7
BSG (Control)	None	6/6	None	6/6

* death within 48 h

Table 4

Survival of zebrafish (*D. rerio*) infected with wild-type and *E. ictaluri* Δ*asdA01* with and without Asd^+ vectors. The zebrafish were infected i.m. with 10 μl of the respective *E. ictaluri* strain.

	Experiment #1		**Experiment #2**	
	i.m.		**i.m.**	
***E. ictaluri* strains**	**Dose (CFU/ml)**	**Survivors /Total**	**Dose (CFU/ml)**	**Survivors /Total**
J100 wild-type	$1.5x10^8$	0/25	$1.2x10^7$	0/25
	$1.5x10^6$	0/25	$1.2x10^3$	13/25
	$1.5x10^4$	2/25	$1.2x10^2$	22/25
J112 Δ*asdA01*	$3.0x10^8$	0/10	$1.7x10^7$	1/5
			$1.7x10^6$	10/10
			$1.7x10^4$	10/10
J112 Δ*asdA01* (pYA3493)	$2.1x10^8$	0/10	$1.8x10^8$	0/5
	$2.1x10^6$	0/10	$1.8x0^6$	0/5
	$2.1x10^4$	10/10	$1.8x10^4$	5/5
BSG (Control)	None	10/10	None	5/5

5. *Discussion*

To develop a balanced-lethal system in *E. ictaluri*, we first characterized the *asdA* and *asdB* genes present in the genome of *E. ictaluri* (Fig. 1). Deletion of the *asdA* gene precluded the growth of *E. ictaluri* in absence of DAP (Fig. 2), indicating that *asdB* does not encode for a functional protein related to DAP synthesis. This is consistent with the bioinformatic analysis (Figs. 3 and 4), which showed that the AsdB enzyme lacked several key amino acid residues at the catalytic active site.

The phylogeny of Asd has two branches, AsdA related with Gram-negatives and AsdB related with Gram-positives (52). We found a particular group of non-functional AsdB genes in *Edwardsiella* and *Yersinia*. The common origin of AsdB in these bacteria suggests that the genes might have lost their activity through evolution, and that *asdB* could be considered a pseudogene in *Edwardsiella* and *Y. pestis*.

Suicide vector technology has been successfully used in several enteric bacteria to develop antibiotic-sensitive mutants (15). Using this technology it was possible to construct defined deletion mutations in the absence of antibiotic-resistance markers for the first time in *E. ictaluri* (Fig. 4). During this process, we determined that *E. ictaluri* is extremely sensitive to Cm, even in the presence of the *cat* gene. The *cat* gene confers high-level resistance to Cm in most bacterial species. It codes for an enzyme called chloramphenicol acetyltransferase

which inactivates Cm by covalently linking one or two acetyl groups, derived from acetyl-S-coenzyme A, to the hydroxyl groups on the chloramphenicol molecule (31). This might indicate that chloramphenicol acetyltransferase is not functional or inefficient in *E. ictaluri*. Further studies are required to answer this.

The current live attenuated *E. ictaluri* vaccine is a rifampicin-resistant strain (28). Antibiotic resistance in live attenuated bacterial vaccines present a threat to both the animal and to human health, due to the horizontal transmission of genes, in this case by transduction. Recently lytic bacteriophages have been isolated from catfish ponds against *E. ictaluri* (54). This suggests that temperate phages for *E. ictaluri* that can establish lysogeny might be present in these environments and could spread rifampicin resistance to native environmental bacterial flora. Here we have described a methodology to genetically engineer *E. ictaluri* without the use of antibiotic-resistance genes in the final strain. This advancement opens up the field of *E. ictaluri* live attenuated vaccine development and will provide opportunities for further research into the pathogenesis of this important organism.

Although, *E. ictaluri* Δ*asdA01* is complemented with its own *asdA* gene, the complemented strain did not grow at the same rate as the parental wild-type strain, presenting a higher growth rate. To achieve the right amount of native AsdA in *E. ictaluri* using Asd^+ vectors requires further studies. However, *E. ictaluri* Δ*asdA01* was fully

complemented by the *Salmonella* SD-*asdA* gene, allowing the development of a balanced-lethal system.

One of the major difficulties in the construction of a balanced-lethal system in *E. ictaluri* is the incompatibility of the Asd^+ vectors with cryptic plasmids present in the bacterial strain. *E. ictaluri* possesses two native autonomous small plasmids, pEI1 and pEI2 (16), that have been implicated in virulence (50). The Asd^+ expression vectors were compatible with pEI1 and pEI2 native plasmids of *E. ictaluri*, indicating that the origin of replication of these plasmids, ColE1 *ori* and ColE2 *ori*-like*,* respectively (16), are compatible with p15A ori, pBR *ori* and pUC *ori*.

E. ictaluri was described by Hawke in 1979 (22), and recently sequenced (NCBI's Entrez Genome database NC_012779). Most of its genes encode for putative functions. *E. ictaluri* possesses the machinery for the type II secretion system in its genome. Therefore we evaluated the secretion of proteins by using a β-lactamase signal sequence at the N-terminal end of a recombinant protein (13), a signal required for a protein to be secreted through the system mentioned above. Recombinant proteins, cloned in the $AsdA^+$ vector and using the β-lactamase signal sequence, were secreted in a similar fashion (Fig. 9) as for a *Salmonella* recombinant vaccine (27), suggesting that the type II secretion system in *E. ictaluri* is fully functional.

Salmonella Δ*asdA* mutants are totally attenuated in mice orally infected with 10^8 CFU per dose (13). *E. ictaluri* Δ*asdA01* mutants

were not fully attenuated in catfish i.c. or orally infected (Table 3). Zebrafish i.m. infected with doses of 10^8 CFU succumbed to *E. ictaluri* Δ*asdA01* mutant infection (Table 4). Lower doses of *E. ictaluri* Δ*asdA01* mutants (10^6-10^4 CFU) were totally attenuated (Table 4). It has been reported that *E. ictaluri* contain toxins, like hemolysin (57, 58). We believe that the mortality caused by *E. ictaluri* Δ*asdA01* mutants is due to a toxic shock-like effect caused by the toxins realized after this DAP dependent mutant lyse in vivo. These toxins probably are not LPS related, since fish and amphibians are resistant to the toxic effects of LPS (3, 24). *E. ictaluri* Δ*asdA01* ($pAsd^+$) was attenuated by one log-fold in catfish animal host model (orally infected), and two log-fold in zebrafish. The next step in the construction and design of a live recombinant *E. ictaluri* vaccine is the attenuation of the bacterial strain without altering colonization of lymphoid tissues and immunogenicity. From moribund orally infected catfish, *E. ictaluri* Δ*asdA* ($pAsdA^+$) were recovered from the head kidney, spleen and liver, indicating that *E. ictaluri asdA* ($pAsdA^+$) colonize lymphoid tissues. The increase in attenuation in catfish orally infected with *E. ictaluri* Δ*asdA* ($pAsdA^+$) could be used together with other genetic modifications to attenuate *E. ictaluri* in regard to constructing a live RAEV.

In summary, we have described methods to genetically engineer *E. ictaluri* without the use of antibiotic-resistant genes in the final strain. This opens up the field of RAEV development and will provide opportunities for further research into *E. ictaluri* pathogenesis. We have developed

an antibiotic-sensitive recombinant *E. ictaluri* strain, using suicide vector technology (15) and Asd^+ expression vectors (13). This first balanced-lethal vector-host system in *E. ictaluri* is key in constructing antibiotic-sensitive live RAEV for the catfish industry.

6. *Acknowledgments*

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CHAPTER VI

DISCUSSION AND CONCLUSIONS

The process of evolution produces a pattern of relationships between species. As lineages evolve and split and modifications are inherited, their evolutionary paths diverge. This produces a branching pattern of evolutionary relationships. The tree of life is divided into tree domains, Eucarya, Archaea, and, Bacteria (previously grouped with Archaea and called prokaryotic kingdom). The Enterobacteriaceae family, belong to the Bacteria domain. Enteric bacterial pathogens of mammals and birds present evolutionary conserved regulatory mechanisms that control virulence factors, among them are the cyclic adenosine 3',5'-monophosphate receptor protein (Crp) and the ferric uptake regulatory protein (Fur). Also, several essential genes required for cell growth, like the aspartate-semialdehyde dehydrogenase (*asd*), are evolutionary conserved between the bacteria domain. These evolutionary conserved genes described in chapters II to IV can be considered universal measures regarding the development of live attenuated bacteria recombinant vaccines for different hosts, but with restrictions within the bacteria domain to different families. Although, the DNA sequence of these genes varies between species, the functional activity of the encoded proteins remains the same. It has been suggested that the evolutionary fitness of a protein depends on the stability of its structure allowing the

protein to perform a function, such a catalyzing a chemical reaction or binding to a ligand (6). Although, Crp, Fur, and, AsdA are present in the entire bacteria domain, they do not execute the exact same function in all families.

Chapter II describes Crp, the most conserved protein studied here. cAMP and cAMP-binding domains are conserved from bacteria to humans as an ancient ubiquitous signaling mechanism to translate extracellular stress signals into appropriate biological responses (1). Proteins harboring a cAMP-binding domain that covalently links to the DNA binding domain are conserved in prokaryotes, like Crp, which is broadly distributed among bacteria. As mentioned in chapter II, Crp has almost no variation through the bacterial domain evolutionary tree and it regulates a similar family of genes in both non-pathogenic and pathogenic bacteria, but in bacterial pathogens, Crp regulates genes related to virulence.

As mentioned in chapter III, the Fur protein controls iron uptake and in bacterial pathogens, Fur has participation in virulence control. Although, Fur conserves its function through evolution as a repressor, its protein structure has adapted to different intra-cellular bacterial environments. For instance, in *Edwardsiella* from fish isolated, Fur has lost part of the N-terminal (13); the *Flavobacterium columnare* Fur amino acid sequence has significant differences (11). Nevertheless, all Fur proteins conserve the α-helix domain required for DNA binding. The Fur protein has significant variations in the residues related to Fe^{+2} and Zn^{+2}

binding and this is likely related to a balance between stability and activity of the protein.

Usually, the *asdA* gene is in the chromosome of Gram-negative bacteria and *asdB* is in the chromosome of Gram-positive bacteria. In some Gram-negative species like *Edwardsiella*, *Yersinia*, and *Vibrio*, *asdB* is also present, but in *Edwardsiella* and *Yersinia asdB* is a pseudogene, and the synthesis of diaminopimelic acid (DAP) depends on AsdA (12). In contrast, in *Vibrio anguillarum*, a Gram-negative non-enteric fish pathogen, both *asd* genes are functional, but not required for cell growth. Thus, a *Vibrio anguillarum* Δ*asdA* Δ*asdB* mutant is DAP independent, indicating differences in the cell wall structure (Santander, unpublished data). This report correlates with the cell wall structure modifications described in *V. cholerae* in comparison to enteric bacteria (3, 9).

In terms of vaccinology, I conclude that (**i**) a Δ*crp* deletion can be considered as a universal tool for the development of live attenuated bacterial vaccines, since this deletion lead to attenuation and immunogenicity mediated an inactivation-activation cascade of genes related to virulence and probably to immune suppression; (**ii**) deletion of the *fur* gene can be considered as a supplementary measure to develop live attenuated bacterial vaccines for the aquaculture industry. As reported before in mammals and birds (4, 7, 10), Δ*fur* mutants are not highly immunogenic and *E. ictaluri* Δ*fur* shows no protection against the intra coelomic (i.c.) *E. ictaluri* wild-type challenge in catfish. However, *E.*

ictaluri Δ*fur-35* protects immunized fish against bath challenged, indicating that *E. ictaluri* Δ*fur-35* triggers immune protection at the mucosal immune level of the fish. Therefore, in terms of safety and efficacy, deletion of the *fur* gene needs to be combined with other deletion to be used as bath vaccine for the aquaculture industry. On the other hand, it has been suggested that Fur is inactive in the intestinal tract of zebrafish larvae (8). This observation does not correlates with the current knowledge about availability of Fe^{+2} in this organ, in which the small intestine conditions are anaerobic and therefore replete with free Fe^{2+}, leading to an active Fur protein that represses genes involved in iron uptake. However, larvae-tissues are very thin and oxygen transport is not required in the early development stages of fish (2, 5, 14). Thus, presumably the intestinal tract of the fish larvae is iron limited due to aerobic conditions and iron uptake by the fish larva and by the bacterial flora, leading to inactivation of Fur protein and up-regulation of Fur repressed genes, as have been suggested for the *E. coli* and zebrafish larvae interaction (8). Therefore, attenuation and immune protection at the early stage of the fish by the *E. ictaluri* Δ*fur-35* mutant must be evaluated to consider it as a measure for vaccine development for the global aquaculture industry; (**iii**) deletion of the *asdA* gene can be used to create a balanced-lethal system and recombinant vaccines with restriction to the Enterobacteriaceae family. Although, presumably the structure of the cell wall of Gram-negative bacteria is conserved, it clearly presents unknown differences

between families of the bacteria domain; (**iv**) combination of *crp*, *fur* and *asdA* deletions can be considered as general tools regarding development of attenuated recombinant bacterial vaccines restricted to the Enterobacteriaceae family. In the case of *E. ictaluri*, combinations of these deletions can be use to develop a RAEV are: J129 Δ*crp-10* Δ*asdA01*, J155 Δ*fur-35* Δ*asdA01*, and J157 Δ*crp-10* Δ*fur-35* Δ*asdA01*. However, the efficacy of these vaccines needs to be evaluated.

In summary, here I have described three evolutionary conserved genes in *E. ictaluri*, *crp, fur,* and *asd*, that can be used to develop live recombinant attenuated bacterial vaccines for the aquaculture industry. These vaccines can be applied by immersion-immunization to the larvae or fingerlings, and later booster the juveniles or adults by oral-food immunization. On the other hand, these attenuate vaccines could serve as vectors to deliver antigens against different pathogens, like bacteria, viruses, and parasites, or deliver DNA plasmid-vector vaccines not only to protect the fish against diseases, but also control the fish life cycle.

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APPENDIX
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 315
Tempe, Arizona 85287-6111
Phone: (480) 965-4387 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**12-1242R**
Protocol Title:	**Live Attenuated Vaccine for the Fish Aquaculture Industry**
Principal Investigator:	**Roy Curtiss III**
Date of Action:	**1/21/2012**

The animal protocol review was considered by the Committee and the following decisions were made:

- ☐ The original protocol was APPROVED as presented.
- ☒ The revised protocol was APPROVED as presented.
- ☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- ☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be considered when these issues are clarified and the revised protocol is submitted.
- ☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- ☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- ☐ The Committee requests you to contact _____ to discuss this proposal.
- ☐ A copy of this correspondence has been sent to the Vice President for Research.
- ☐ Amendment was approved as presented.

Documentation of Level III Training will need to be provided to the IACUC office before the participant can perform procedures independently. For more information on Level III requirements see
https://researchintegrity.asu.edu/training/animals/levelthree

Total # of Animals:	**13,170**	**Pain Level:**	**C-3,645; E-2,745** **C-3,840; E-2,940**	**Species:**	**Zebrafish** **Catfish**
Sponsor:	**USDA**				
Award #:	**022772**				
Approval Period:	**01/21/2012 – 01/20/2015**				

Signature: C. Miller for D. Murphy Date: 1/24/12
IACUC Chair or Designee

Original: Principal Investigator
Cc: IACUC Office
IACUC Chair