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Identification of Virulence Factors in Edwardsiella Ictaluri

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Identification of virulence factors in *Edwardsiella ictaluri*

By

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Edwardsiella ictaluri is the causative agent of enteric septicemia of catfish (ESC), which is one of the most important diseases impacting the US catfish industry. Though this disease has been very common, progress has been slow to find an economical and practical treatment method. Our long-term goal is to determine the mechanisms of *E. ictaluri* virulence in ESC. The overall objective of this study was to identify *E. ictaluri* genes required for host encounter and serum resistance and to determine their roles in pathogenesis. The central hypothesis is that *E. ictaluri* must differentially regulate its genes to invade fish and evade host defenses, thus, mutation of these differentially expressed genes (DEG) should cause attenuation of *E. ictaluri* virulence. To test this hypothesis, we first determined the global gene expression patterns of the wild type (wt) *E. ictaluri* 93-146 and *Ei*AKMut02 mutant during catfish encounter and serum exposure using microarray analysis. Results indicated that in *E. ictaluri* wt, 377 and 16 DEGs were identified during host encounter and serum exposure, respectively. In *Ei*AKMut02, 82 and 296 DEGs were identified during host encounter and serum experiment. Through functional analysis using Blast2GO, PSORTb, Host Pathogen Interaction Database

(HPIDB), and Microbe Virulence Database (MVirDB), 38 DEGs in 9 KEGG pathways have been identified as potential virulence factors. The KEGG pathways represented were 1) bacterial secretion system including T3SS and T6SS, 2) ABC transporters including cystine transport system, iron complex transport system, d-methionine transport system, arginine transport system, thiamine transport system, and molybdate transport system, 3) protein export, 4) flagellar assembly, 5) two-component system, 6) bacterial chemotaxis, 7) ascorbate and aldarate metabolism, 8) phosphotransferase system, and 9) metabolic pathways. In order to understand their role in the *E. ictaluri* virulence, selected DEGs were in-frame deleted by allelic exchange, and their virulence and efficacy were characterized in channel catfish fingerlings. Our results showed that the virulence of *E. ictaluri ssaV* and *yscR* mutants was completely attenuated while their efficacies were moderate in catfish fingerlings. These results support that the T3SS and T6SS, ABC transporters, protein export, and flagella seem to be important in *E. ictaluri* virulence.

DEDICATION

I would like to dedicate this research to my parents Cuilan Liu and Yulin Lu and my family Guohua Yang, Junli Yang, and Lucia C. Yang.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

SPF: specific pathogen free

DEGs: differentially expressed genes

E. ictaluri: *Edwardsiella ictaluri*

E. coli: *Escherichia coli*

*Ei*AKMut02: *Edwardsiella ictaluri gcvP* transposon insertion mutant

HPIDB: host pathogen interaction database

MVirDB: microbe virulence database

FC: fold change

CHAPTER I

INTRODUCTION AND REVIEW OF RELEVANT LITERATURE

The catfish industry

Farm-raised channel catfish (*Ictalurus punctatus*) is number six in the "Top10" fish and seafood consumed, and catfish production is the largest aquaculture commodity in the United States. Channel catfish production is especially important in the Southeastern States. In 2010, Mississippi, Alabama, Arkansas, and Texas accounted for 94% of the total catfish sales (USDA, 2010). Mississippi has been the leading state in catfish production and catfish sales in Mississippi valued $218 million in 2010 (USDA, 2010). The catfish industry provides employment opportunities in Mississippi, especially in counties within the Delta (Dean et al., 2003; Tucker et al., 2004). The catfish industry, however, suffers great economic loss annually due to diseases caused by bacterial, viral, fungal, and parasitic pathogens. Enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri* is the most devastating disease in farm-raised catfish. ESC, existing in 60.6% of catfish operations, is the most prevalent disease of farm-raised channel catfish (USDA, 2003). In 2011, ESC accounted for 22.9% of the cases submitted to the Aquatic Research and Diagnostic Laboratory at Stoneville, MS (ARDL, 2011).

Edwardsiella ictaluri

E. ictaluri is a Gram negative rod-shaped anaerobic facultative bacterium. It belongs to the family *Enterobacteriaceae*. *E. ictaluri* is the causative pathogen of ESC (Hawke et al., 1981). In 1976, *E. ictaluri* was first identified from an epizootic of fish mortalities in Alabama and named as *E. ictaluri* (Hawke, 1979; Hawke et al., 1981). The size of *E. ictaluri* is 0.75 x 1.5 – 2.5 µm, and it is slightly motile at 25 -30 ^{o}C but not at higher temperatures. The bacterium grows slowly on agar plate, which takes 48 h at 30 ^{o}C. The genome of *E. ictaluri* 93-146 strain is slightly GC rich (57.5%) and consists of 3.81 million base pairs, containing 3,903 genes that encode 3,784 proteins (Williams et al., 2003). *E. ictaluri* is generally believed to be a facultative intracellular pathogen with the ability to survive inside professional phagocytic cells. Phagocytosed *E. ictaluri* has been observed in several pathological studies. In two of the studies, some of the phagocytosed bacteria appeared to be in binary division (Baldwin and Newton, 1993; Miyazaki and Plumb, 1985; Morrison and Plumb, 1994; Shotts et al., 1986a). These studies concluded that *E. ictaluri*'s ability to survive in phagocytes may be responsible for the development of a carrier state for ESC (Klesius, 1992) and could be a mechanism for dissemination (Miyazaki and Plumb, 1985; Shotts et al., 1986). Bertolini et al. investigated the serotype of *E. ictaluri* and concluded that *E. ictaluri* would be a good candidate for vaccine development as the tested strains are composed of a single serotype (Bertolini et al., 1990; Plumb and Vinitnantharat, 1989). Killed bacterins have only been efficacious under controlled laboratory conditions when given by injection (M.O. and J.A., 1986), which is not practical for commercial production. *E. ictaluri* can attach and penetrate host mucosal membranes rapidly and establish systemic infection, but the

precise mechanism by which this occurs is underexplored. Furthermore, little progress has been made to create effective vaccines to prevent ESC.

Enteric septicemia of catfish (ESC)

ESC has been a serious problem affecting the catfish industry since it was first described. ESC is the most prevalent disease in farm-raised channel catfish, causing the US a $60 million economic loss annually (Shoemaker et al., 2002). All sizes and ages of catfish can be affected by *E. ictaluri* during the late spring or early summer and during the fall when water temperatures are between 22-28 ^{o}C. *E. ictaluri* is host specific to some extent though it was also isolated from other fish species. The disease generally occurs in one of the two forms: acute form and chronic form (MacMillan, 1985; Newton et al., 1989; Shotts et al., 1986). In the acute form, farmers experience economic losses due to rapid mortalities (Shotts et al., 1986) because fish start dying 3-14 days post-infection by *E. ictaluri*. In the chronic form, farmers experience economic losses due to decreased production with fish manifesting signs 3-4 weeks after an acute outbreak (Newton et al., 1989). The dead fish in acute ESC show bacterial septicemia, while those that died of the chronic form show “hole in the head” lesions. Behaviorally, sick fish consume less food, swim listlessly, hang vertically at water surface, or spiral in water. The disease can be horizontally transferred from infected fish or fish that have died from ESC to naïve fish (Shotts et al., 1986). The only treatment currently available is to feed pellets containing oxytetracycline, sulfadimethoxine, or florfenicol antibiotics. However, one of the earliest clinical signs of ESC is anorexia. Therefore these antibiotics are only effective in limiting the spread of an outbreak and not in treating the disease because the

antibiotics are usually food-fed. Hence, vaccines became an alternative way to reduce or eliminate the impact of *E. ictaluri*.

Known virulence factors in *E. ictaluri*

Many virulence factors have been identified in *E. ictaluri* since the pathogen was isolated from farm-raised channel catfish in 1976. Early reports correlating *E. ictaluri* virulence with virulence factors included the *E. ictaluri* polysaccharide coat, which showed greater ability to degrade chondroitin (Stanley et al., 1994), fimbriae-like structures (Morrison and Plumb, 1994), chondroitinase (Cooper et al., 1996; Stanley et al., 1994), lipopolysaccharide O side chain (Lawrence et al., 2003; Lawrence et al., 2001), and outer membrane proteins, which were shown to be involved in the initial bacterial-host cell interactions (Skirpstunas and Baldwin, 2003). More recently, 37 *E. ictaluri* related genes were associated with infection or survival deficiency in catfish, which included genes involved in adhesion, fimbriae, lipopolysaccharide biosynthesis, type III secretion system (T3SS), and urease activity (Thune et al., 2007). Previous research in our laboratory has associated 10 *E. ictaluri* genes, involved in TCA cycle, one carbon metabolism, SoxS oxidative response system, and T3SS, with reduced virulence (Karsi et al., 2009). The mechanism of adherence is yet to be worked out, although genes encoding fimbriae and a T3SS have been identified (Thune et al., 2007).

E. ictaluri is resistant to normal catfish serum (Karsi and Lawrence, 2007; Ourth and Bachinski, 1987), which is at least partially mediated by the O polysaccharide portion of LPS (Lawrence et al., 2003). Evidence from in vitro studies suggests that *E. ictaluri* is capable of surviving in catfish neutrophils (Ainsworth and Dexiang, 1990; Karsi and Lawrence, 2007). The mechanism for intracellular survival of *E. ictaluri* is not

known, although it has been shown that it is not mediated by O polysaccharide (Lawrence et al., 2003). In one study, serial subcultivation resulted in failure to express 18.4 and 42.5 kDa proteins (Vinitnantharat et al., 1993). Williams et al. found that serial subcultivation results in failure of avirulent strains to express a 55 kDa outer membrane protein (OMP; Williams et al., 2003). Invasion of and survival within a host are all linked to coordinated gene expression of the pathogen. Although the number of virulence factors reported has been increasing and the complete *E. ictaluri* genome is available, more *E. ictaluri* virulence factors involved in recognition, attachment, and penetration of host mucosal membranes need to be identified.

Host defense mechanisms

The primary lymphoid organ in fish is bone marrow equivalent in primitive fishes and is the thymus in teleosts. Monocytes, macrophages, lymphocytes and surface Ig^+ cells were seen in the thymus of catfish through cytochemical staining (Petrie-Hanson and Ainsworth, 2000). The thymus prevents antigens from reaching the thymocytes by the tight junction inside the endothelium of the blood vessels within the organ and pharyngeal epithelium covering it. The secondary lymphoid organs are the kidney, spleen, gut-associated lymphoid tissues, and other organs contain lymphohemopoietic tissue. The kidney has haemopoietic tissue producing antibody. The system of sinusoids in haemopoietic tissue is phagocytic for carbon particles and immune complexes. Melanomacrophages centers inside the haemopoietic tissue possess a reticulin capsule, lymphocytes, and pyroninophilic cells which can take up antigens and immune complexes. Macrophages in spleen, enmeshed inside the reticulin fibers, can take up foreign materials such as carbon particles and bacteria. The reticulin fibers play important

roles in trapping immune complexes. GALT (gut-associated lymphoid tissue) and mucosal immune system in fish seem similar in all fish species histologically. Plasma cells have been seen in GALT, and the number of plasma cells increased upon antigenic challenge. Antigens in the gut caused an increase in number of intraepithelial leukocytes followed by inducing the production of specific antibodies in the mucosae and bile but not in serum. A protective immune response in skin mucus can be elicited thereafter (Zapata et al., 1996).

Fish has non-specific and specific immune systems. Non-specific immune system includes cellular defense and humoral defense. Non-specific cellular defense involves monocytes/machrophages, granulocytes, and nonspecific cytotoxic cells which lack specificity (Secombes, 1996). Non-specific humoral immune defense includes various substances inhibiting the growth of infectious microorganisms nonspecifically, such as lysozyme, interferon, C-reactive protein, transferrin, lectin and complement. These substances react with specific chemical groups or configurations but influence the growth of more than one microorganism (Yano, 1996). Specific cellular defense in fish is independent of antibody. The research on specific cell-mediated immunity in fish involves helper cell activity and requires the establishment of monocyte/macrophages as accessory cells in MLR, IL-1 signaling to helper cells, blast cell transformation and proliferation following MLR, T-cell mitogen or antigen stimulation, presence of IL-2 receptors and IL-2 signaling, secretion of IL-2 and other cytokines, MHC class I and II, T-cell receptor reaction with both MHC, and more studies in progress such as the establishment of a subpopulation of specific T-cells cytotoxic to MHC class I target cells (CD-8 T-cells), identification of CD-4, CD-8 and CD-3 molecules, confirmation of MHC

function in fish, and establishment of cell to cell contact and killing of specific MHC I restricted target cells (Manning and Nakanishi, 1996). Specific hormonal defense is the most thoroughly studied immune system and is antibody dependent. The functions of antibody effector include neutralization, precipitation and agglutination, opsonization, and complement - mediated functions. The complement system will be discussed in detail since *E. ictaluri* is resistant to the complement system in catfish serum (Kaattari and Piganelli, 1996).

The complement system existing in serum is an important part of the vertebrate immune system being composed of about 35 different kinds of proteins categorized into nine major components from C1 to C9. Functions of complement proteins in fish found so far are similar to their counterpart in mammals, and several complement proteins, such as C3 and factor B, even have multiple isoforms. The complement system can lyse foreign cells and opsonize foreign organisms and destroy by attracting phagocytes. The basic functions of bony fish complement system include virucidal activity, bactericidal activity, parasiticidal activity, opsonic activity, chemoattracting activity and inactivation of bacterial exotoxins (Yano, 1996).

In fish, three pathways of complement activation have been identified which are the classical complement pathway (CCP), alternative complement pathway (ACP), and the lectin complement pathway (LCP) (Holland and Lambris, 2002). First, CCP can be initiated by acute-phase protein such as ligand-bound C-reactive protein, by binding antibody to host cell surface, or by some viruses, bacteria and virus-infected cells (Merino et al., 1998; Petersen et al., 2000). C1 consists of C1q and C1s. C1q activated by binding IgM, an immunoglobulin exists in fish, will activate C1s. Activated C1s cleaves

C4 and C2 into C4a, C4b, C2a, and C2b. C3 convertase, C4bC2b enzymatic complex, cleaves C3 into C3a and C3b. C5 convertase, C4bC2bC3b, cleaves C5 into C5a and C5b. C5b, C3b, C6, C7, C8, and C9 form Membrane Attack Complex (MAC) which can attack the bacterial surface by forming a channel or pore in the membrane leading to cell lysis and death.

Second, ACP is directly activated by viruses, bacteria, fungi, or tumor cells without antibody binding. C3 is activated by H_2O followed by binding of the bacterial surface adjacent to protein B which is converted to Bb by protein D. C-H_2OBb produces C3b. The C3/C5 convertase produces C5b, C5a, and more C3b. Then MAC is formed and starts cytolysis. According to the mechanism mentioned, ACP is antibody independent.

Third, LCP can be activated by binding of mannose-binding lectin (MBL), a protein complex, and the serine proteases. The MBL connects proteases 1 and 2 (MASP-1 and MACP-2) to mannans on bacterial cell surface; therefore, the activation of LCP is independent of antibody also (Holland and Lambris, 2002). Some studies showed that at least 5 different bacterial species can avoid fish host complement systems. These bacterial species are *V. anguillarum* (Trust et al., 1981), *A. salmonicida* (Munn et al., 1982), *E. ictaluri* (Ourth and Bachinski, 1987), *Y. ruckeri* (Davies, 1991; Ourth and Bachinski, 1987) and *P. piscidida* (Magarinos et al., 1994). In *V. anguillarum*, the factors responsible for serum resistance appeared to be chromosome specific. However, the serum resistance in both *V. anguillarum* and *Y. ruckeri* is related to the presence of cell surface proteins. All virulent and some avirulent strains of *Y. ruckeri* tested are serum resistant, which suggest that there is an additional factor involved in the virulence of this pathogen. In *P. piscidida*, virulent strains can survive in normal fish serum, but avirulent

strains can only survive in the heat-treated serum from which the complement system was inactivated (Evelyn, 1996). A protein layer coating the cell and the long side chains of the *A. salmonicida* lipopolysaccharide (LPS) traverse the A layer. These two structures may prevent complement to reach susceptible target sites on *A. salmonicida* cells since the bacteria will be killed by normal serum if they lose the structures (Munn et al., 1982; Sakai and Kimura, 1985). The presence of large amount of sialic acid on *A. salmonicida* cells protects them by suppressing the activation of complement through the alternative pathway (Ourth and Bachinski, 1987). In *E. ictaluri*, sialic acid-induced suppression on the activation of the ACP was thought to be the mechanism to explain why *E. ictaluri* can survive in normal serum obtained from channel catfish (Ourth and Bachinski, 1987). Moreover, in virulent *E. ictaluri*, larger amount of surface proteins and polysaccharide capsular material were found more than that in avirulent strains (Stanley et al., 1994). These surface components most probably prevent bactericidal materials in normal serum, such as complement and lysozyme, from contacting with vulnerable sites on the bacteria surface (Evelyn, 1996).

Significance of research and objectives

E. ictaluri can attach and penetrate host mucosal membranes rapidly and establish systemic infection. This characteristic plays an important role in rapid spreading of disease and large number of mortalities. The ability of *E. ictaluri* to quickly regulate its pathogenic mechanisms in channel catfish could be the reason why *E. ictaluri* is well-adapted to the host environment. However, the *E. ictaluri* virulence factors mediating this rapid penetration and systemic invasion have not been explored completely. Lack of such knowledge is an important problem preventing development of new therapeutic strategies

that reduce or eliminate economic losses due to *E. ictaluri*. The overall objective of this study was to identify *E. ictaluri* genes required for host encounter and serum resistance and to determine their roles in pathogenesis. The central hypothesis of this proposal was that *E. ictaluri* must differentially regulate its genes to invade fish and evade host defenses. Thus, determination of DEGs would lead to new knowledge about virulence vaccines. The specific objectives of this study were:

1: Identify differentially expressed genes of E. ictaluri during host encounter and serum exposure. The working hypothesis for this aim was that global gene expression profiles in response to host and serum presence would show important early events in the pathogenesis of ESC.

2: Mutate selected E. ictaluri genes and determine virulence in channel catfish. The working hypothesis for this aim was that mutation of selected virulence genes in *E. ictaluri* would cause attenuation of the bacterial virulence.

CHAPTER II

GLOBAL GENE EXPRESSION ANALYSIS IN *EDWARDSIELLA ICTALURI* DURING CATFISH HOST ENCOUNTE

Abstract

Edwardsiella ictaluri is a Gram-negative facultative anaerobic rod, causing enteric septicemia of catfish (ESC). ESC is one of the most prevalent diseases in the catfish industry in the US. Studies on understanding the pathogenic mechanisms of *E. ictaluri* are necessary to identify virulence relevant genes in *E. ictaluri*. In this study, using microarray analysis, we determined differentially expressed genes (DEGs) in wt *E. ictaluri* during catfish fry encounter. A total of 377 DEGs were identified with a $p < 0.05$ and a fold change > 2 or < -2. All DEGs were analyzed further using Blast2GO, MVirDB, PSORTb, and HPIDB. We identified 15 DEGs to be potential virulence genes in *E. ictaluri*. Nine of these DEGs were involved in KEGG pathways known in *E. ictaluri*. Genes, *yscV*, *yscC*, *yscT*, *yscR*, and *yscN* are in the type three secretion system (T3SS); *icmF* is in type six secretion system (T6SS); *hmuV* is in iron complex transport system; D-methionine ABC transporter ATP-binding protein is in D-Methionine transport system; and *artP* is in arginine transport system.

Introduction

E. ictaluri is a Gram-negative facultative intracellular rod causing enteric septicemia of catfish (ESC) (Hawke, 1979; Hawke et al., 1981). The disease affects all ages of channel catfish (*Ictalurus punctatus*) during the late spring and fall when water temperatures are between 22 °C to 28 °C (Francis-Floyd et al., 1987). *E. ictaluri* can invade channel catfish through intestine (Baldwin and Newton, 1993; Newton et al., 1989; Shotts et al., 1986), olfactory sinus (Miyazaki and Plumb, 1985; Morrison and Plumb, 1994; Newton et al., 1989; Shotts et al., 1986), gills (Nusbaum and Morrison, 1996), and skin abrasions (Karsi et al., 2006; Menanteau-Ledouble et al., 2011).

The proposed mechanisms of *E. ictaluri* dissemination inside host include direct transport in blood or inside phagocytic cells (Thune et al., 1993). In catfish, disease manifests as acute enteritis and septicemia and chronic meningoencephalitis forms (MacMillan, 1985; Newton et al., 1989; Shotts et al., 1986). The acute form of ESC causes rapid and high mortalities (Shotts et al., 1986), while the chronic form of ESC reduces catfish production (Newton et al., 1989). Economic loss due to ESC in the catfish industry has been reported to be $60 million annually (Shoemaker et al., 2002). Currently, antibiotics and vaccines are being utilized in fish farms to treat or control ESC. Antibiotics are used as feed-additive, but they can only help control spread of the disease rather than treatment because sick fish are anorexic. A commercial vaccine (Aquavac-ESC) (Klesius and Shoemaker, 1999) is available to immunize catfish fry, but it provides a moderate level of protection (Shoemaker et al., 1999). ESC is still the most prevalent disease problem in catfish industry.

Although the number of virulence factors reported has been increasing and the complete *E. ictaluri* genome is available (Williams et al., 2012), little is known about the *E. ictaluri* virulence factors that are involved in recognition, attachment, and penetration of host mucosal membranes. Because *E. ictaluri* can establish systemic infection rapidly, identification of global gene expression in the early stages of infection could help identify novel virulence targets. Therefore, the purpose of this study was to analyze global gene expression patterns in *E. ictaluri* during the pathogen-fry encounter. We expect that our study will provide new insights on molecular adaptation of *E. ictaluri* during early stages of infection.

Materials and Methods

Bacterial strain and growth conditions

In this study, *E. ictaluri* 93-146 (wt) was used (Lawrence et al., 1997). *E. ictaluri* was streaked on brain heart infusion (BHI) (Difco) agar plate containing 12.5 µg/ml of colistin (Sigma) and incubated at 30 ^{o}C for 48 h. Small cultures were prepared by inoculating *E. ictaluri* colonies in 5 ml of BHI broth with 12.5 µg/ml of colistin and incubating for 16-18 h at 30 ^{o}C with shaking at 200 rpm. Large cultures were prepared by diluting the overnight cultures at a ratio of 1:1000 in 15 ml BHI broth.

Fry encounter model

All fish experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Mississippi State University. Specific pathogen free catfish fry were produced in the College of Veterinary Medicine at Mississippi State University. Two-week old fry (120) were divided into four containers

(30 fry / container). Eight *E. ictaluri* colonies were grown in BHI broth as described above and bacterial numbers for fish encounter were adjusted to be the same in each repeat according to cultures' optical densities (OD_{600}). Bacteria were collected by centrifugation at 10,000 rpm for 3 min, and then pellets were washed three times with ddH_2O. After the final wash, bacteria were resuspended in water used to hold fish fry. Four *E. ictaluri* cultures were added into four fry containers randomly to give a concentration of 1 x 10^7 CFU/ml water (Karsi et al., 2006). The remaining four *E. ictaluri* cultures (control) were added to the remaining containers without fry. Bacteria and fry were incubated for 3 h at room temperature under aeration. At the end of the incubation, the bacteria were collected by centrifugation and stabilized immediately using two volumes of RNAprotect Bacterial Reagent (Qiagen). After stabilization, the samples were centrifuged at 4,000 rpm for 20 min, and the bacteria were resuspended in 4 ml of RNAprotect Bacterial Reagent. The aliquots of the stabilized bacteria were prepared and stored at - 80 °C until RNA extraction.

Total RNA isolation and quality check

Total RNA was isolated from four biological replicates by using RNeasy Protect Bacteria Mini Kit (Qiagen). Contaminating bacterial DNA was eliminated using on-column DNase I treatment with RNase-Free DNase Set (Qiagen). The quality and concentration of the isolated total RNA were measured with NanoDrop 1000 (Thermo Scientific) and Agilent Bioanalyzer model 2100 Lab-on-a-Chip with RNA 6000 Nano Kit (Agilent Technologies). The ratios of 260/280 and 260/230 of all RNA samples were higher than 1.8. The concentration and integrity of all RNA samples met the requirements for microarray experiment set by NimbleGen.

cDNA preparation

Double-stranded cDNA was synthesized with Invitrogen SuperScript Double-stranded cDNA Synthesis Kit. 1 µl of 4 mg/ml RNase A solution was added to cleanup residual RNA template by incubating at 37 ^{o}C for 10 min. Treated with phenol:chloroform:isoamyl alcohol, the solution was centrifuged at 12,000 g for 5 min. The aqueous layer was transferred to a clean, labeled 1.5 ml tube. To precipitate cDNA, 16 µl of 7.5 M ammonium acetate, 7 µl of 5 mg/ml glycogen, and 326 µl of ice-cold absolute ethanol were added to the samples sequentially vortexing at each step. After centrifugation at 12,000 g for 20 min, 500 µl of ice-cold 80% ethanol (v/v) was used to wash the pellet twice with repeated inversion followed by centrifugation at 12,000 rpm for 5 min. The quality and integrity of cDNA was confirmed with NanoDrop 1000 and Agilent Bioanalyzer 2100 to meet the requirement for following step.

Microarray analysis

Microarray experiment was done using the custom microarray service of Roche NimbleGen (Madison, WI). After designing a 4-plex custom *E. ictaluri* array, microarray hybridization, array scan, data extraction, and preliminary data analysis were conducted at NimbleGen. All genes were interrogated with 9 probe pairs. Probe sets were selected to uniquely represent the gene transcripts and to achieve excellent hybridization characteristics.

NimbleGen One-color (Cy3) DNA Labeling Kit was used to label cDNA samples. Before hybridization for 4x72K array, Cy3 dye labeled cDNA samples were resuspended with 3.3 µl of Sample Tracking Control (STC) in the NimbleGen Hybridization Kit. Hybridization solution master mix was prepared for a 4x72K array: 29.5 µl of 2x

hybridization buffer, 11.8 µl of hybridization component A, and 1.2 µl of alignment oligo. 8.7 µl of hybridization solution master mix was used for 3.3 µl of each resuspended sample. The sample was then incubated at 95 ^{o}C for 5 min away from light and incubated again at 42 ^{o}C for 5 min or longer until ready for sample loading. X4 mixer was prepared and fixed in the slide bay of the hybridization system.

For hybridization, 8 µl of sample was loaded to A01 fill port on the slide with a Gilson microman pipette CP10. Samples were then repeat loaded into the A02 – A04 fill ports. All fill and vent ports on X4 mixers were covered with one mixer multi-port seal. After closing the bay clamp, the mixing panel on the hybridization system was turned on and the mix mode was set to B to start mixing. The samples were hybridized to the array at 42 ^{o}C for 16 – 20 h. NimbleGen wash buffer kit and NimbleGen array processing accessories were used for washing. The mixer-slide assembly was disassembled, and the slide was washed in 42 ^{o}C+2 ^{o}C Wash I, room-temperatured Wash II for 15 sec with vigorous constant agitation, and room-temperatured Wash III for 15 sec with vigorous constant agitation. After washing steps, the slides immediately were spin-dried in a microarray dryer for 30 sec. The edges were blot-dried with lint-free paper after removing the slides from the microarray dryer.

Microarray data extraction

MS 200 microarray scanner and MS 200 data collection software were used for scanning one-color NimbleGen arrays. The default parameters used in this study were shown in Table 2.1. 5 µm resolutionwas selected for scanning. The results were saved as .tif. DEVA software and was used for importing a scanner image and extracting the data. The .tif images and the design files for the study were imported into a new project in

DEVA software. A pair report (pair) was created, and RMA analysis function was performed using DEVA software. DEVA software normalized expression data using quantile normalization (Bolstad et al., 2003). Gene calls were generated with the Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003).

Table 2.1 Default parameters used for scanning NimbleGen microarray slide with MS 200 microarray scanner and MS 200 data collection software.

Parameter	**Default Setting**
Slide type	Roche NimbleGen
Speed/Sensitivity	High Speed
Channel 1	532
Channel 2	635
Laser Intensity 1	100%
Laser Intensity 2	100%
Autogain	Selected
Apply to slide 1/cycle 1	Selected

Microarray data analysis

Global transcription analysis of microarray data was conducted at Roy J. Carver Biotechnology Center, University of Illinois. NimbleScan software (v 2.6.0.0, Roche NimbleGen) was used to generate log2 normalized data values using the RMA algorithm (background correction, normalization and summarization) with the "Whole array" option, which aggregates the two on-chip technical replicates of each probe set. Statistical analysis for differential expression was done in R (http://www.R-project.org) using the limma package (Smyth, 2005). A cell-means model was fit to the two groups, taking into account the correlation due to arrays on the same chip (Smyth, 2004; Smyth et al., 2005). Pairwise comparisons were pulled as contrasts for the model, if they had raw p-values < 0.05 and +/- 2 FC in either pairwise comparison, which resulted in 451 probe sets.

NimbleGen provided only limited annotation for the probe sets on the array, thus further annotation was pulled from two databases: NCBI (NC_012779.gff downloaded on Jan 27, 2011) and Uniprot (http://www.uniprot.org/uniprot/?query= taxonomy:634503 downloaded on Jan 27, 2011). The GI IDs provided by NimbleGen were matched to GI ids from NCBI to extract Gene IDs, genomic location, protein products and protein ids. The Gene ids from NCBI were matched to Gene ids from Uniprot to gain protein Accesssion numbers, protein names, gene names, Gene Ontology terms, and Pathways. Forty Gene IDs matched to two different proteins in Uniprot; most of these IDs appeared to be similar or identical proteins and were resolved manually.

Bioinformatic analysis and pathway search

Among the DEGs, potential virulence genes were identified using Blast2GO, PSORTb, HPIDB, and MVirDB databases. A FASTA file containing the protein sequences of DEGs was used as the input. Blast2GO was used to improve the annotation of the DEGs that had been identified from the microarray experiment, and all DEGs were categorized into different functional groups (Conesa et al., 2005; Götz et al., 2008). Prediction of subcellular localization of the proteins encoded by the DEGs was conducted with PSORTb (Yu et al., 2010), which categorized all differentially expressed proteins into sub-cellular localizations in the bacterium. Host Pathogen Interaction Database (HPIDB) hosted at Mississippi State University (http://www.agbase.msstate.edu/hpi/main.html) (Kumar and Nanduri, 2010) was used to search genes involving in host-pathogen interactions. HPIDB includes experimental protein-protein interactions (PPIs) information from several public accessible databases and provides identification of PPIs between host and pathogen. Advanced BLAST search was used to perform BLASTP for

all DEGs against the full database. The Matrix was set as blosum 62. The cutoff valued was set to be 1E-10. All DEGs were blasted against microbial virulence database MvirDB (Zhou et al., 2007) to identify genes encoding putative virulence factors. MVirDB is a microbial database of protein toxins, virulence factors, and antibiotic resistance genes. The database combines DNA and protein sequence information from well-known virulence databases, including Tox-Prot, SCORPION, the PRINTS virulence factors, VFDB, TVFac, Islander, ARGO, and a subset of VIDA. In the current study, MVirDB database was downloaded from LLNL website and the protein sequences of DEGs were blasted against MVirDB database with Standalone blast using CLC Genomics Workbench v 4.0.2. An e-value of 10^{-10} and a 20% identity between subject sequence and query sequence were used as objective parameters to identify the virulence related genes. Subjective parameters including the function of the gene, pathway involved by the gene, etc. were also considered to determine virulence genes in *E. ictaluri*. All information from MVirDB/HPIDB/PSORTb/Blast2GO was integrated to determine target virulence or virulence related genes in *E. ictaluri*. Pathway analysis was performed for the identified virulence genes with KEGG pathway database (http://www.genome.jp/kegg-bin/show_organism?org=eic).

Results

Analysis of the *E. ictaluri* gene expression profiles after fry encounter

Through statistical analysis of microarray data, 10.26% of genes in *E. ictaluri* 93-146 genome showed significant changes in expression level ($p < 0.05$ and $FC > 2$ or < -2 in expression) upon bacterial encounter with the catfish host. Compared to *E. ictaluri* 93-146 without catfish fry, 377 genes were differentially expressed. 145 genes were up-

regulated (Appendix A), and 232 genes were down-regulated (Appendix B) in *E. ictaluri*. The greatest fold change of up-regulated genes was 6.83. The greatest fold change of down-regulated genes was 8.77. Of 145 up-regulated genes, 7 genes were up-regulated from 4 to 6 fold following host encounter; and 138 genes were up-regulated from 2 to 4 fold. Of 232 down-regulated genes, 7 genes were down-regulated from 6 to 8 fold following host encounter; 26 genes were down-regulated from 4 to 6 fold; and 189 genes were down-regulated from 2 to 4 fold.

Blast2GO

GeneOntology (GO) mapping for DEGs according to biological process, cellular components, and molecular function was performed with Blast2GO. The protein sequences of all 377 DEGs identified in this study were blasted against public and private sequence databases followed by InterProScan to complete the functional annotation depending on BLAST with protein domain information. 256 out of 377 DEGs were annotated by Blast2GO (Appendix C). Species distribution according to the top hits from BLASTP results showed that 364 DEGs match to *E. ictaluri*; 5 DEGs match to *Clostridium carboxidivorans*; 4 DEGs match to *E. tarda*; and 1 DEG matches to *Shigella dysenteriae*. The total number of GO terms was 1001 including 341 biological process terms (34.1%), 152 cellular component terms 15.2%), and 508 molecular function terms (50.7%). Annotated sequences were assigned to gene ontologies according to biological process (Figure 2.1), cellular component (Figure 2.2), and molecular function (Figure 2.3) at level 2. The results showed that metabolic process, cellular process, biological regulation, localization and response to stimulus are important annotation terms in biological process category; cell, macromolecular complex, organelle and extracellular

region in cellular component category; binding, catalytic activity, transporter activity, transcription regulator activity, electron carrier activity are the important annotation terms in molecular function category.

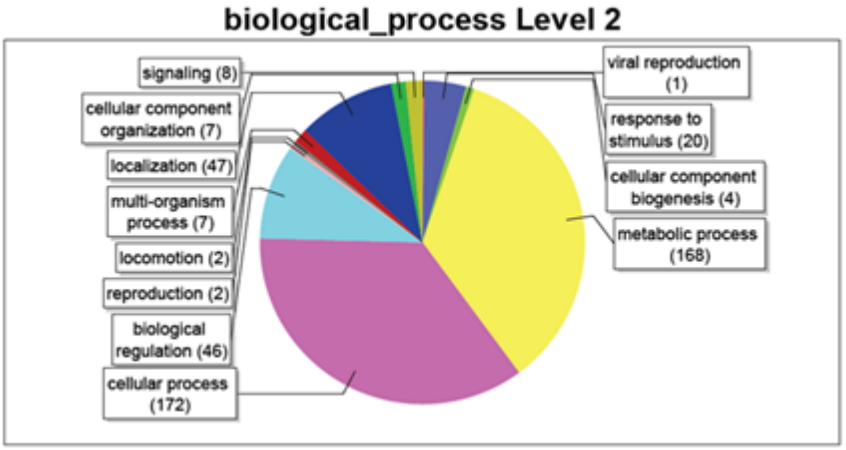

Figure 2.1 GO terms at level 2 according to biological process.

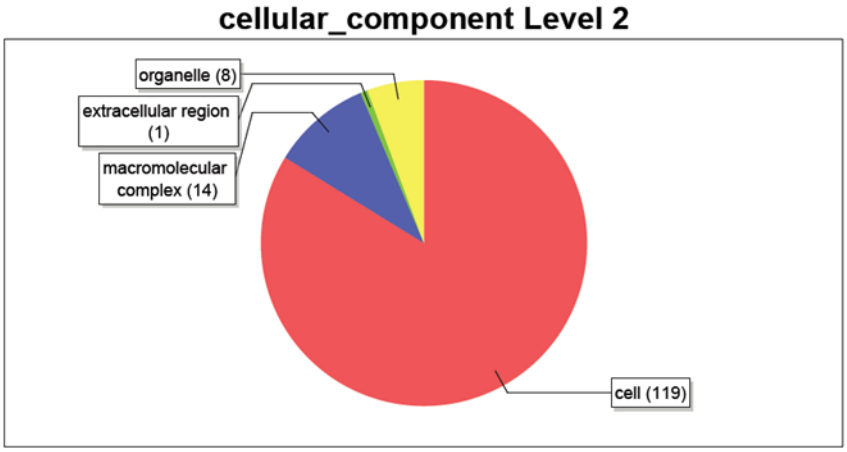

Figure 2.2 GO terms at level 2 according to cellular component.

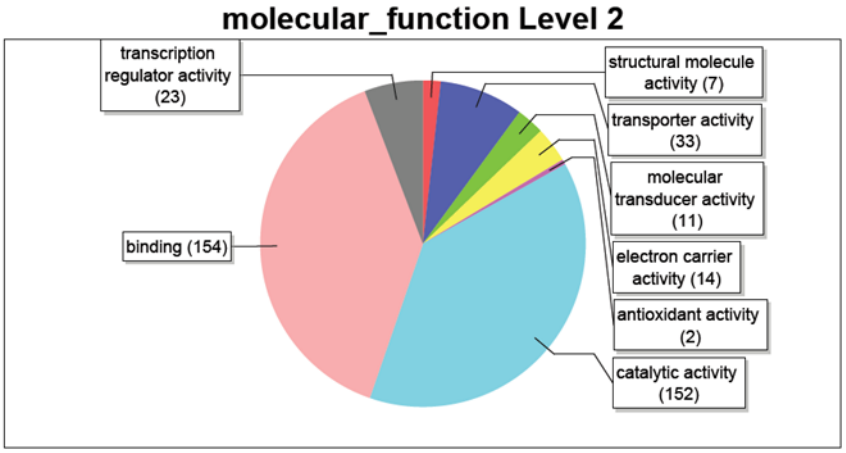

Figure 2.3 GO terms at level 2 according to molecular function.

PSORTb

PSORTb V3.0.2 predicts bacterial subcellular localization of target proteins. The protein sequences of all 377 DEGs identified in this study were submitted to PSORTb as a FASTA file. The subcellular locations of DEGs were predicted to be in cytoplasmic, periplasmic, cytoplasmic membrane, extracellular, outer membrane, and unknown categories. The result showed that there were 234 DEGs whose bacterial localizations were predicted while the remaining 143 DEGs were not predicted (Figure 2.4).

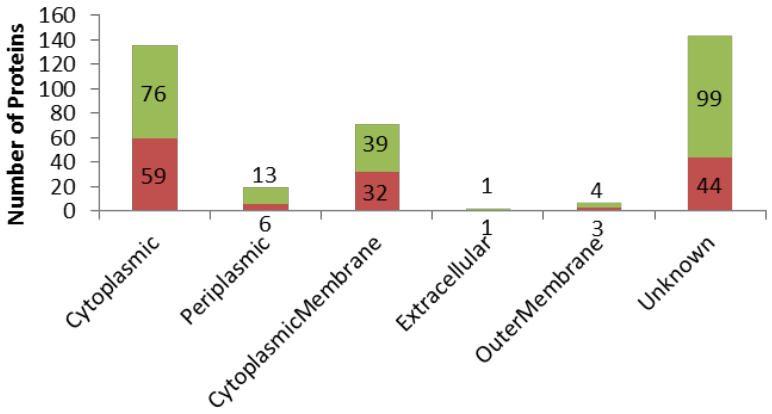

Figure 2.4 Bacterial localization prediction by PSORTb.

The digits on green area are the number of DEGs down-regulated, and the digits on the red area are the number of DEGs up-regulated.

From the PSORTb result, most of the DEGs were predicted to be in the cytoplasm, followed by cytoplasmic membrane, periplasmic, outer membrane, and extracellular. The 7 DEGs, which were predicted to locate on outer membrane in *E. ictaluri*, are listed in Table 2.2.

Table 2.2 7 DEGs located on outer membrane in *E. ictaluri* by PSORTb.

GI	Protein	Score
238918184	Maltoporin	10
238918845	Outer membrane lipoprotein blc	9.92
238918877	Type III secretion outer membrane pore, yscc/hrcc family, putative	10
238919522	Outer membrane protein slp	9.93
238919741	Hypothetical protein NT01EI_1845	9.92
238919771	Outer membrane protein N	10
238921206	Nucleoside-specific channel-forming protein tsx	10

HPIDB (Host Pathogen Interaction DataBase)

The top hit result showed that 138 of 377 DEGs were involved in host pathogen interactions. The DEGs mostly matched to *Yersinia pestis*, followed by *Bacillus*

anthracis, and *Francisella tularensis* (Figure 2.5). Upon searching against the full database, the remaining DEGs match to the following eukaryotes: *Homo sapiens, Rattus norvegicus*, and *Arabidopsis thaliana*.

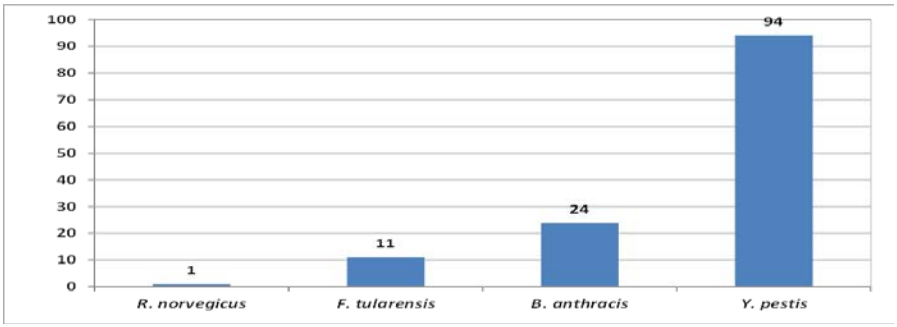

Figure 2.5 DEGs involved in host-pathogen interaction during host encounter experiment identified by HPIDB

MVirDB (Microbe Virulence DataBase)

56 DEGs match to known virulence factors (percent identity > 20% and E-value < 1E-20) (Appendix D). 7 DEGs belong to T3SS; 3 DEGs encode membrane proteins; 2 DEGs encode flagellin.

Potential virulence factors and pathway analysis

The results from Blast2GO, PSORTb, HPIDB, and MVirDB were pooled together and 15 DEGs were identified to be the genes most relevant to *E. ictaluri* pathogenesis (Table 2.3).

Table 2.3 Potential virulence factors identified from bioinformatic analysis in *E. ictaluri* during catfish host encounter

GI	Protein product	Symbol	Locus Taq
238918892	Secretion system apparatus protein ssav	Yscv	NT01EI_0958
238918794	Amino acid permease-associated region	Cadb	NT01EI_0858
238918253	Lysine/cadaverine transport protein	Cadb	NT01EI_0290
238918877	Type III secretion outer membrane pore, yscc/hrcc family	Yscc	NT01EI_0943
238920297	Flagellin	Fla	NT01EI_2407
238919771	Outer membrane protein N2	Ompn2	NT01EI_1875
238919801	Hypothetical protein	Hmuv	NT01EI_1905
238918894	Type III secretion apparatus protein, yscr/hrcr family, putative	Yscr	NT01EI_0960
238918896	Type III secretion apparatus protein, spar/ysct/hrct, putative	Ysct	NT01EI_0962
238918580	D-methionine ABC transporter, ATP-binding protein, putative	Dmabc	NT01EI_0632
238918937	Hypothetical protein	Hp1004	NT01EI_1004
238920767	Hypothetical protein	Hp2886	NT01EI_2886
238920408	Histidine transport ATP-binding protein hisp	Artp	NT01EI_2518
238920639	Hypothetical protein; type VI secretion system protein impl	Icmf	NT01EI_2751
238918891	Flagellum-specific ATP synthase	Yscn	NT01EI_0957

These 15 DEGs were searched against KEGG pathway database, and the bacterial secretion system (eic03070) and the ABC transporters (eic02010) pathways were represented. Six *E. ictaluri* genes were involved in the bacterial secretion system pathway, and three *E. ictaluri* genes were involved in the ABC transporters pathway (Table 2.4). Using tighter functional analysis with Module with the same six genes, 5 genes are involved in T3SS (eic_M00332) including yscV, yscC, yscR, yscT and yscN; icmF is involved in T6SS (K11891); hp1905 is involved in iron complex transport system (eic_M00240); NT01EI_0632 involved in D-Methionine transport system (eic_M00238); and artP is involved in Arginine transport system (eic_M00229) (Table 2.4).

Table 2.4 Pathways and Modules represented in *E. ictaluri* virulence genes during host encounter.

GI	Locus Taq	Pathway	Module
238918892	NT01EI_0958	Bacterial secretion system (eic03070)	Type III secretion system (eic_M00332)
238918877	NT01EI_0943	Bacterial secretion system (eic03070)	Type III secretion system (eic_M00332)
238919801	NT01EI_1905	ABC transporters (eic02010)	Iron complex transport system (eic_M00240)
238918894	NT01EI_0960	Bacterial secretion system (eic03070)	Type III secretion system (eic_M00332)
238918896	NT01EI_0962	Bacterial secretion system (eic03070)	Type III secretion system (eic_M00332)
238918580	NT01EI_0632	ABC transporters (eic02010)	D-Methionine transport system (eic_M00238)
238920408	NT01EI_2518	ABC transporters (eic02010)	Arginine transport system (eic_M00229)
238920639	NT01EI_2751	Bacterial secretion system (eic03070)	type VI secretion system protein ImpL (K11891*)
238918891	NT01EI_0957	Bacterial secretion system (eic03070)	Type III secretion system (eic_M00332)

*:A KEGG number is assigned to this gene product.

Discussion

Of the 3,903 genes in the *E. ictaluri* genome, 377 (10.26%) were differentially expressed during the presence of catfish fry compared to the control group (no catfish fry). Results showed that many genes were involved in recognition, attachment, and penetration of host mucosal membranes. Further analysis of these DEGs might provide more information on molecular adaptation of *E. ictaluri* during early stages of infection.

Bioinformatic analysis of 377 DEGs showed that 15 *E. ictaluri* genes are potential virulence genes (Table 2.3). Therefore, according to the findings in the current study, T3SS, T6SS, iron complex transport system, D-Methionine transport system, and arginine transport system may be important for *E. ictaluri*'s invasion of channel catfish. The 4 hypothetical proteins and those not found in any pathway may provide more information to understand the invasion mechanisms of *E. ictaluri* in the future. Previous studies have shown that outer membrane proteins are important for bacterial virulence.

Our finding of 7 outer membrane proteins differentially expressed in *E. ictaluri* infection provides more targets for further understanding this pathogen's virulence.

Our study also found that bacterial secretion systems T3SS and T6SS are important during infection. It has been reported that at least 6 types of protein secretion systems were found in Gram-negative bacteria. Type I and type III are *sec*-independent secretion pathways, while type II and type IV are *sec*-dependent secretion pathways (Hueck, 1998). Type V was named for the pathway used by autotransporter and similar proteins (Henderson et al., 2004). In proteobacteria, the type VI secretion system was reported to be important in the virulence of several human or fish pathogens (Filloux et al., 2008; Mougous et al., 2006; Pukatzki et al., 2006).

T3SS, first discovered in pathogenic strain of *Yesinia spp* (Michiels et al., 1990), seems to play very important roles in *E. ictaluri* infection during catfish host encounter experiment. The discussion below focused on T3SS and T6SS because 5 T3SS genes (*yscV*, *yscC*, *yscT*, *yscR*, and *yscN*) and 1 T6SS gene were identified as potential virulence factors.

YscR, YscV and YscT are inner membrane proteins in T3SS. YscR was reported to regulate expression of Low-Ca^{2+} Response Stimulon (LCRS) operons and secretion of LCRS proteins in *Y. pestis* (Fields et al., 1994). Oligorization of YscV needs the help of YscR and other two other export apparatus (Diepold et al., 2011). T3SS apparatus is also called injectisome due to its function in allowing bacteria to inject effector proteins into eukaryotic hosts by passing through two bacterial membranes and one eukaryotic membrane (Cornelis and Wolf-Watz, 1997; Galán and Collmer, 1999). YscV is an essential component of T3SS injectisome and is composed of eight transmembrane parts

and one C- terminal cytosolic domain (Diepold et al., 2011). YscT is a component of export apparatus in T3SS in *Y. pestis* and generally contains transmembrane helices (Diepold et al., 2011). In *Xanthomonas homologues*, YscT, however, has larger periplasmic domains (Berger et al., 2010). YscC is a secretin located on outer membrane and is involved in translocation of *Yersinia* outer proteins (Yops) into eukaryotic target cells (Plano and Straley, 1995). High-level expression of Low-Ca2+ Response Stimulon proteins in *Y. pestis* was also related to YscC (Goodin et al., 2005). YscN is an ATPase and is involved in the assembly and function of *Y. pestis* type III secretion apparatus. High levels of reporter gene activation can be obtained by the combination of YscN and YscL in *Y. pestis* (Jackson and Plano, 2000).

T6SSs were named as IcmF-associated homologous proteins previously and were found in at least 16 Gram-negative proteobacteria (Das and Chaudhuri, 2003). IcmF is an inner membrane protein in T6SS and was reported to be a protein contributing to stabilize the *Legionella pneumophila* T4SS (Sexton et al., 2004). The *icmF* gene in *E. ictaluri* may have the same function since it was somewhat conserved in many Gram-negative proteobacteria.

CHAPTER III

GLOBAL GENE EXPRESSION ANALYSIS IN *E. ICTALURI* UNDER SERUM EXPOSURE

Abstract

Edwardsiella ictaluri is a Gram-negative facultative anaerobic rod and the causative agent of enteric septicemia of catfish (ESC). *E. ictaluri* is one of the most prevalent pathogens of catfish, causing a significant economic loss in the catfish industry. *E. ictaluri* is resistant to complement system in catfish serum and can establish systemic septicemia rapidly. More studies are needed for understanding the mechanisms of *E. ictaluri* serum resistance. In the present study, we conducted microarray gene expression analysis in *E. ictaluri* treated with naive catfish serum. Our results showed that 16 differentially expressed genes (DEGs) were identified through microarray data analysis with a $p < 0.05$ and a fold change > 1.5 or < -1.5. Three genes have been up-regulated and 13 genes have been down-regulated. Two genes have been annotated and identified to be virulence genes through Blast2GO, MVirDB, PSORTb and HPIDB search. These two genes were found in two known *E. ictaluri* pathways. One was the type III secretion apparatus protein (GI 238918894), which is in T3SS (eic_M00332). The other was the type III transport ATP binding protein sfuC-like (GI 238920290), which is found in the cystine transport system (eic_M00234). This study showed that T3SS in *E. ictaluri* may play roles in resistance to fish complement system.

Introduction

ESC is one of the most prevalent diseases to cause economic losses in the catfish industry. All ages of farm-raised channel catfish are vulnerable to infection by *E. ictaluri* when water temperatures are between 22 ^{o}C to 28 ^{o}C (Francis-Floyd et al., 1987). *E. ictaluri* can invade channel catfish through the intestine (Baldwin and Newton, 1993; Newton et al., 1989; Shotts et al., 1986), olfactory sinus (Miyazaki and Plumb, 1985; Morrison and Plumb, 1994; Newton et al., 1989; Shotts et al., 1986), gills (Nusbaum and Morrison, 1996), and skin abrasions (Karsi et al., 2006; Menanteau-Ledouble et al., 2011). It has been reported that *E. ictaluri* has the capability to evade the host immune system and can replicate inside the host phagocytes (Baldwin and Newton, 1993). The dissemination of ESC in catfish is very rapid: *E. ictaluri* was found in the kidney tissue of catfish as soon as 15 min post-gastric lavage (Baldwin and Newton, 1993). The proposed mechanisms of *E. ictaluri* dissemination inside host include direct transport into blood or inside phagocytic cells (Thune et al., 1993). After immersion using bioluminescence, Karsi et al. (2006) also found that this pathogen spreads throughout the entire fish body within 60 h after infection.

E. ictaluri is resistant to the alternative complement system (Ourth and Bachinski 1987). The complement system in serum is an important part of the vertebrate immune system composed of about 35 different kinds of proteins categorized into nine major components from C1 to C9. The study of fish complement system is far from complete. Functions of complement proteins in fish found so far are similar to their counterpart in mammals, and several complement proteins even have multiple isoforms such as C3 and factor B. The complement system can lyse foreign cells and opsonize foreign organisms

for phagocytosis. The basic functions of bony fish complement system include virucidal, bactericidal, parasiticidal, opsonic, and chemoattracting activities. In fish, there are three pathways of complement activation, the classical complement pathway (CCP), alternative complement pathway (ACP), and the lectin complement pathway (LCP). Though the complement system in serum plays important roles to identify and aid in clearance of bacteria and foreign cells (Schmidt and Colten, 2000), mechanisms of *E. ictaluri* catfish serum resistance are still not understood completely. In this study, we identified differentially expressed genes (DEGs) in *E. ictaluri* after serum exposure. We expected that differentially expressed genes may provide new knowledge about *E. ictaluri* serum resistance.

Materials and Methods

Bacterial strain and growth conditions

In this study, *Edwardsiella ictaluri* 93-146 was used (Lawrence et al., 1997). *E. ictaluri* culture conditions were as described in chapter II.

Specific pathogen free serum preparation

Catfish serum was prepared from SPF channel catfish. Briefly, catfish weighing 1-2 kg were anesthetized in water containing 200 mg/liter of tricaine methane sulfonate (Argent Laboratories), and blood was collected at 1% of the body weight of catfish. After clot formation at room temperature, samples were transferred onto ice and incubated for an additional 30 min. The liquid phase was centrifuged at 3,000 rpm for 10 min at 4 ^{o}C using a with Sorvall® Legend™ RT centrifuge (Thermo Electron Corp., Asheville, NC). Catfish serum was aliquoted in 50 ml tubes and frozen at -80 ^{o}C until use. Complement

free catfish serum was prepared by heat treatment of normal serum at 65 ^{o}C for 45 min. Before serum exposure, heat treated and non-heat treated serum were placed in 30 ^{o}C water bath for 15 min to get a uniform temperature.

E. ictaluri serum exposure

E. ictaluri was exposed to normal and heat-treated catfish serum, and each treatment included four biological replicates. Briefly, eight *E. ictaluri* cultures were washed three times using 1.25 ml of cell wash buffer (10 mM TrisCl and 5 mM magnesium acetate). Normal and heat-inactivated serum (1.25 ml) were added onto *E. ictaluri* pellet, mixed, and incubated for 2 h at 30 ^{o}C. The serum in tubes was replaced with 1.25 ml of fresh serum and incubated a further 2 h. During incubation, the tubes were inverted 10 times every 30 min to mix bacteria and serum thoroughly. After the incubation, serum-bacteria mix was transferred to 15 ml centrifuge tubes containing 3 ml RNAprotect bacterial reagent and incubated at room temperature for 10 min. After this, serum-bacteria mix was aliquoted equally into four microcentrifuge tubes and frozen at -80 ^{o}C until total RNA isolation.

Microarray and bioinformatic analyses

Total RNA purification, cDNA synthesis, microarray experiments, and bioinformatic analysis have been conducted as described in chapter II.

Results and Discussion

E. ictaluri gene expression profiles following catfish serum exposure

16 DEGs have been identified in *E. ictaluri* exposed to the normal catfish serum compared to the *E. ictaluri* exposed to heat-inactivated serum ($p<0.05$ and FC > 1.5). The

proportion of DEGs was only 0.4344% of 3,903 genes in *E. ictaluri* genome. Three genes were up- regulated, while 13 genes were down-regulated (Table 3.1). Fold change was between 1.5 and 1.7 for up-regulated genes and between 1.5 and 2.5 for down-regulated genes.

Table 3.1 Up- and down-regulated genes identified in *E. ictaluri* during catfish serum exposure.

GI	Fold Change	P.Value	Protein_product	GeneName
238920912	1.69	0.003	Branched-chain amino acid transport system II carrier protein, putative	EiORF_3040
238921474	1.62	0.012	Hypothetical protein	EiORF_3626
238920156	1.55	0.023	Hypothetical protein	EiORF_2262
238919547	-1.50	0.010	Hypothetical protein	EiORF_1643
238918894	-1.55	0.022	Type III secretion apparatus protein, yscr/hrcr family, putative	EiORF_0960
238918895	-1.58	0.028	Esas	EiORF_0961
238918878	-1.59	0.048	Hypothetical protein	EiORF_0944
238919336	-1.59	0.015	Hypothetical protein	EiORF_1428
238920564	-1.60	0.017	Hypothetical protein	EiORF_2676
238920290	-1.65	0.006	ABC transporter, ATP-binding protein, putative	EiORF_2400
238921148	-1.71	0.014	Diaminopimelate decarboxylase, putative	EiORF_3289
238918881	-1.87	0.012	Hypothetical protein	EiORF_0947
238920289	-1.91	0.028	Hypothetical protein	EiORF_2399
238921731	-1.96	0.004	Aspartate--ammonia ligase, putative	EiORF_3900
238918103	-2.11	0.022	Cadmium-translocating P-type atpase, putative	EiORF_0127
238920803	-2.32	0.002	Glutamate/aspartate periplasmic-binding protein	EiORF_2929

The up-regulated genes included branched-chain amino acid transport system II carrier protein (BrnQ), and 2 hypothetical proteins. BrnQ is composed of 456 amino acids and is located on membrane. This protein functions as a multi-pass membrane protein and is a component of the LIV-II transport system for branched-chain amino acids. Functions of the other two hypothetical proteins are yet to be known.

Down-regulated proteins included T3SS apparatus protein YscR/HrcR, EsaS, ABC transporter, diaminopimelate decarboxylase, aspartate-ammonia ligase, cadmium-translocating P-type ATPase, glutamate/aspartate periplasmic-binding protein and 6 hypothetical proteins.

Blast2GO

Blast2GO analysis has annotated 10 DEGs out of 16 DEGs. The top hits for BLASTP results showed that all 16 DEG sequences match to *E. ictaluri*. The total number of GO terms was 41, including 13 biological process terms (31.7%), 8 cellular component terms (19.5%), and 20 molecular function terms (48.8%). Annotated sequences were assigned to GO terms according to biological process (Figure 3.1), cellular component (Figure 3.2), and molecular function (Figure 3.3) at level 2.

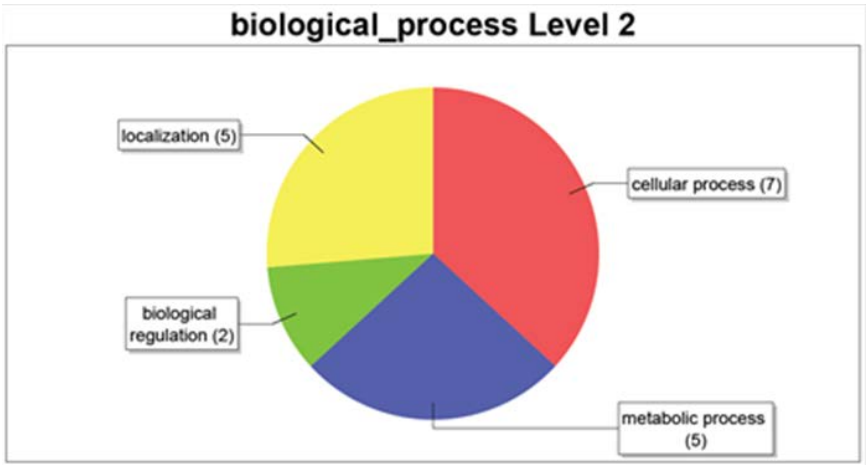

Figure 3.1 GO terms at level 2 according to biological process.

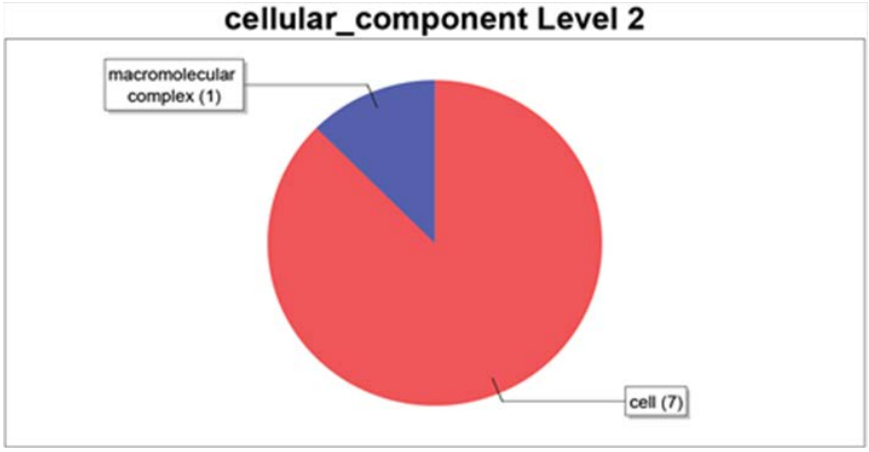

Figure 3.2 GO terms at level 2 according to cellular component.

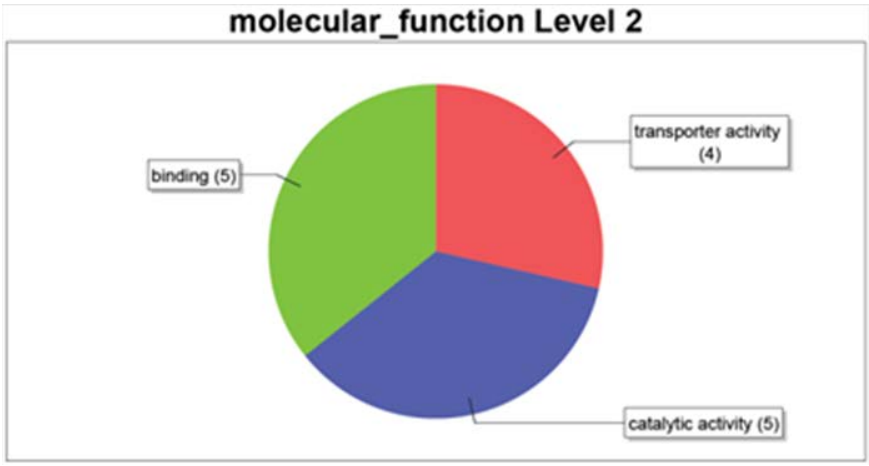

Figure 3.3 GO terms at level 2 according to molecular function.

PSORTb

PSORTb has predicted and categorized the location of 16 DEGs into six categorizations: cytoplasmic, periplasmic, cytoplasmic membrane, extracellular, outer membrane, and unknown (Figure 3.4). The PSORTb result showed that predicted DEGs were located at three subcellular locations: cytoplasmic membrane, cytoplasmic, and

periplasmic. The subcellular locations of the remaining DEGs were unknown. The DEGs on cytoplasmic membrane may function to transfer molecules into the cell. The unknown group may provide more accurate localization predictions when enough information is available for those proteins.

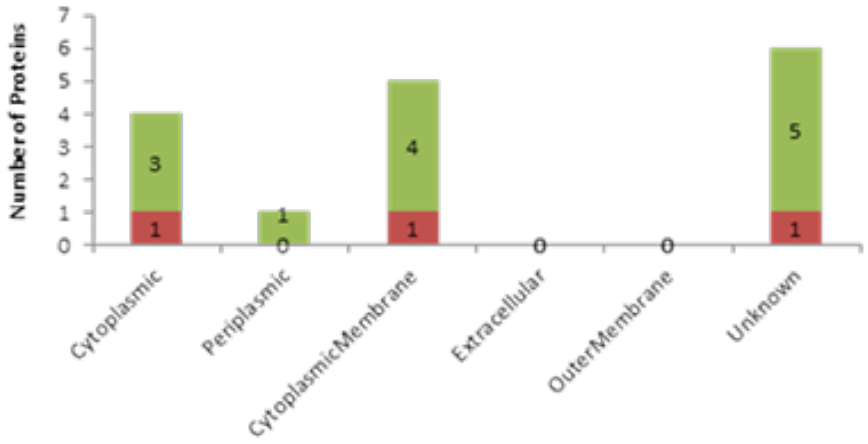

Figure 3.4 Prediction of bacterial localization for 16 DEGs.

The digits on top are the number of DEGs down-regulated and the digits on the bottom are the number of DEGs up-regulated.

HPIDB (Host Pathogen Interaction DataBase)

BLASTP has been conducted to search all 16 DEGs against full database. The top hit result suggests that 50% of differentially expressed proteins from serum exposure experiment were potentially involved in host pathogen interaction (Table 3.2).

Table 3.2 DEGs involved in host-pathogen interaction in *E. ictaluri* exposed to SPF catfish serum.

GI	Subject id	Organism
238920912	UNIPROT_AC:Q81T30	*Bacillus anthracis*
238921731	UNIPROT_AC:Q81S64	*Bacillus anthracis*
238918103	UNIPROT_AC:Q8D1J3	*Yersinia pestis*
238918894	UNIPROT_AC:P69980	*Yersinia pestis*
238919547	UNIPROT_AC:Q8D0G3	*Yersinia pestis*
238920290	UNIPROT_AC:Q7CI05	*Yersinia pestis*
238920803	UNIPROT_AC:Q7CJU9	*Yersinia pestis*
238921148	UNIPROT_AC:Q7CGZ1	*Yersinia pestis*

MVirDB (Microbe Virulence DataBase)

Only 2 DEGs encoding type III secretion apparatus protein (GI 238918894) and type III transport ATP binding protein sfuC like (GI 238920290) were identified to be virulence related in *E. ictaluri* (percent identity > 20% and E-value < 1E – 20). The result suggests that T3SS plays critical roles in *E. ictaluri* infection. Further study on these two genes is expected to shed light upon understanding of *E. ictaluri*'s virulence mechanisms.

Potential virulence factors and KEGG pathway

Among the 16 DEGs identified in *E. ictaluri* post catfish serum exposure, type III secretion apparatus protein YscR (GI 238918894; NT01EI_0960) and ATP binding protein sfuC like (GI 238920290; NT01EI_2400) have been identified to be potential virulence factors. Type III secretion apparatus protein YscR has been annotated and identified in MVirDB/HPIDB/PSORTb/Blast2GO. ATP binding protein sfuC like was found in MVirDB/HPIDB/PSORTb. These two proteins in *E. ictaluri* may play roles in resisting host serum during infection. T3SS apparatus protein YscR is located on the bacterial inner membrane and consists of 215 amino acids. This protein plays roles in

T3SS (eic_M00332) (http://www.genome.jp/kegg-bin/show_pathway?eic03070+NT01EI_0960) and may be involve in *E. ictaluri* virulence during catfish infection. According to Fields et al.'s study, in-frame deletion of *yscR* gene in *Yersinia pestis* caused defective secretion of certain virulence related proteins (Fields et al., 1994). sfuC might be a polar but membrane-bound protein and plays roles in iron transportation in iron-limited media (Angerer et al., 1990). *E. ictaluri sfuC* consists of 258 amino acids and belongs to the ABC transporter superfamily, playing role in cystine transport system (eic_M00234). SfuC has similar function as YecC, which is a component of FliY-YecS-YecC complex. SfuC is found in cystine transport system (eic_M00234), therefore it likely plays a role in the transportation of cystine in *E. ictaluri*. The result may suggest that cystine is a necessary amino acid during *E. ictaluri* infection or may also play roles in other aspects of *E. ictaluri*. This study indicates that type III secretion system and ABC transporter in *E. ictaluri* may play roles during host serum exposure.

CHAPTER IV

GLOBAL GENE EXPRESSION ANALYSIS OF *E. ICTALURI gcvP* MUTANT IN RESPONSE TO NAÏVE CHANNEL CATFISH

Abstract

Catfish aquaculture is a $423 million industry in the US (Hanson and Sites, 2012), which is particularly important in the Southern states of Mississippi, Alabama, Louisiana, and Arkansas. Enteric septicemia of catfish (ESC) is the most prevalent catfish disease, causing millions of dollars loss economically. *Edwardsiella ictaluri* is the causative agent of ESC, and its pathogenic mechanisms have not been understood completely. An *E. ictaluri gcvP* mutant (*Ei*AKMut02) has been produced in our lab and shown that it is completely attenuated and highly protective in catfish fingerlings. The *gcvP* is part of the glycine cleavage system, and we showed that it is important for both neutrophil and serum resistance in *E. ictaluri*. To better understand the importance of the *gcvP* in *E. ictaluri* virulence, we compared the global gene expression between the *E. ictaluri* wt and *gcvP* mutant after catfish fry encounter. There were 82 genes differentially expressed, including 13 up-regulated and 69 down-regulated genes. Bioinformatic analysis has been performed on all DEGs using Blast2GO, PSORTb, HPIDB, and MVirDB. Three of 82 DEGs showed homology to known virulence factors from other pathogenic bacteria. *ulaA* (GI238921506) functions in both ascorbate and aldarate metabolism (eic_00053) and phosphotransferase system (PTS; eic_02060) pathways. *ulaA* plays a role in ascorbate

degradation (ascorbate to D-xylulose-5P; eic_M00550) and is a component of the PTS system and/or ascorbate-specific II component (eic_M00283). *thiQ* (GI238918647) functions in ABC transporters pathway and plays a role in thiamine transport system (eic_M00189). Molybdate ABC transporter permease (GI238920722) also functions in ABC transporters pathway and plays a role in the molybdate transport system (eic_M00189).

Introduction

E. ictaluri is the causative pathogen of enteric septicemia of catfish (ESC) and is a Gram-negative facultative rod belonging to *Enterobacteriaceae* (Hawke, 1979; Hawke et al., 1981). All ages of channel catfish (*Ictalurus punctatus*) can be affected by this pathogen during the late spring and fall when water temperatures are between 22 to 28 °C (Francis-Floyd et al., 1987). Many virulence factors have been identified by different research groups (Cooper et al., 1996; Lawrence et al., 2003; Lawrence et al., 2001; Morrison and Plumb, 1994; Skirpstunas and Baldwin, 2003; Stanley et al., 1994; Thune et al., 2007). Our research has associated the glycine cleavage system with reduced virulence in *Ei*AKMut02, which contains a transposon insertion in its *gcvP* gene (Karsi et al., 2009). The GcvP protein is part of the glycine cleavage system, which catalyzes glycine oxidation reversibly to form 5, 10-methylenetetrahydrofolate, one of two sources of one carbon donor along with serine hydroxymethyltransferase. Glycine can induce the expression of the glycine cleavage enzyme system (Meedel and Pizer, 1974; Stauffer et al., 1994). A *gcvP* mutant cannot use glycine as a one carbon source and excrete glycine (Plamann et al., 1983). *E. ictaluri gcvP* is located downstream of *gcvH* and *gcvT*. *gcvP* is the third gene in the three gene operon encoding subunits of the glycine cleavage system.

The disturbance of the *gcvP* gene function in *Ei*AKMut02 mutant resulted in reduced resistance against catfish neutrophils, and catfish serum, and attenuated in catfish fingerlings compared to wild type (Karsi et al., 2009). Thus, the purpose of this work was to investigate differences between the *Ei*AKMut02 mutant and wt during fry encounter.

Materials and Methods

Bacterial strains and growth conditions

In this study, *Edwardsiella ictaluri* wild type 93-146 strain and *Ei*AKMut02 mutant were used (Karsi et al., 2009; Lawrence et al., 1997). *Ei*AKMut02 is a mutant of *E. ictaluri* 93-146, which was constructed by transposon insertion to the gene *gcvP* (Karsi et al., 2009). Both strains were grown in brain heart infusion (BHI) (Difco) agar and broth at 30 ^{o}C. Ampicillin (100 µg/ml), colistin (12.5 µg/ml) and gentamycin (12.5 µg/ml) antibiotics were added to the growth medium when needed.

Experimental procedures

Experimental fish, fry encounter, total RNA purification, cDNA synthesis, microarray experiments, and bioinformatic analysis have been conducted as described in chapter II. In *Ei*AKMut02 fry encounter experiment, *E. ictaluri* wt encountered with fry was used as control.

Fry encounter model

All fish experiments have been conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Mississippi State University. Specific pathogen free catfish fry were produced in the College of Veterinary Medicine at Mississippi State University. Two-week old fry (120) were divided into four containers

(30 fry / container). Eight *E. ictaluri* colonies were grown in BHI broth as described above and the bacterial numbers were adjusted according to cultures' optical densities (OD_{600}). The bacteria were collected by centrifugation at 10,000 rpm for 3 min, and then pellets were washed three times with ddH_2O. After the final wash, the bacteria were resuspended in the water in which the catfish fry were held. Four *E. ictaluri* cultures were added into four fry containers randomly to give a concentration of 1 x 10^7 CFU/ml water (Karsi et al., 2006). The remaining four *E. ictaluri* cultures (control) were added to the remaining containers without fry. The bacteria and fry were incubated for 3 h at room temperature under aeration. At the end of the incubation, the bacteria were collected by centrifugation and were stabilized immediately using two volumes of RNAprotect Bacterial Reagent (Qiagen). After stabilization, the samples were centrifuged at 4,000 rpm for 20 min and the bacteria were resuspended in 4 ml of RNAprotect Bacterial Reagent. Aliquots of the stabilized bacteria were prepared and stored at - 80 °C until RNA extractions.

Results and Discussion

Analysis of gene expression profiles in *Ei*AKMut02

Through microarray data analysis 2.2% of the *E. ictaluri Ei*AKMut02 genes (82 DEGs) significantly changed their expression level compared to *E. ictaluri* wt when they encountered with catfish fry ($p < 0.05$ and $FC > 2$). 13 genes were up-regulated (Table 4.1), and 69 genes were down-regulated (Table 4.2). The greatest fold change for up-regulated genes was 2.4 fold. The greatest fold change for down-regulated genes was 3 fold. The expression of all 13 up-regulated genes was increased 2-2.5 fold compared to *E. ictaluri* wt following host encounter. Of 69 down-regulated genes, 7 genes were down-

regulated from 2.5 to 3 fold following host encounter; 62 genes were down-regulated from 2.5 to 3 fold.

Table 4.1 Up-regulated genes in *Ei*AKMut02 compared to wild type during host encounter

GI	FC	P value	Protein_Product	GeneName
238921446	2.40	0.00	30S ribosomal protein S7, putative	EiORF_3598
238919616	2.30	0.01	Hypothetical protein	EiORF_1715
238921088	2.26	0.00	Ribosomal protein S16, putative	EiORF_3223
238921428	2.26	0.00	Hypothetical protein	EiORF_3580
238921429	2.17	0.01	Hypothetical protein	EiORF_3581
238920265	2.16	0.01	Hypothetical protein	EiORF_2374
238921447	2.11	0.01	30S ribosomal protein S12, putative	EiORF_3599
238921426	2.06	0.00	50S ribosomal protein L18, putative	EiORF_3578
238921430	2.06	0.01	50S ribosomal protein L5	EiORF_3582
238921445	2.03	0.00	Hypothetical protein	EiORF_3597
238919586	2.01	0.00	Outer membrane protein W	EiORF_1685
238919013	2.01	0.01	Hypothetical protein	EiORF_1079
238921420	2.00	0.00	30S ribosomal protein S4 (BS4)	EiORF_3571

Table 4.2 Down-regulated genes in *Ei*AKMut02 mutant strain compared to wild type during host encounter

GI	FC	P value	Protein_Product	GeneName
238919899	-2.00	0.02	Hypothetical protein	EiORF_2003
238918259	-2.00	0.04	Hypothetical protein	EiORF_0296
238918158	-2.00	0.01	Hypothetical protein	EiORF_0194
238918457	-2.01	0.02	Hypothetical protein	EiORF_0501
238920228	-2.01	0.04	Hypothetical protein	EiORF_2337
238918508	-2.01	0.01	Pyruvate formate lyase-activating enzyme	EiORF_0558
238918159	-2.01	0.02	Hypothetical protein	EiORF_0195
238919240	-2.01	0.01	Hypothetical protein	EiORF_1328
238920400	-2.01	0.02	Drug resistance transporter, Bcr/cfla family	EiORF_2510
238919429	-2.01	0.02	Hypothetical protein	EiORF_1523
238921507	-2.01	0.03	Hypothetical protein	EiORF_3659
238918024	-2.01	0.02	Type I restriction-modification system, R subunit	EiORF_0043
238919663	-2.02	0.01	Hypothetical protein	EiORF_1764
238920863	-2.03	0.01	Hypothetical protein	EiORF_2989
238921273	-2.04	0.04	Inner membrane protein yhjx	EiORF_3416
238920189	-2.06	0.01	Hypothetical protein	EiORF_2298

Table 4.2 (Continued)

238918654	-2.06	0.02	ABC-type multidrug efflux pump	EiORF_0712
238919474	-2.07	0.03	Hypothetical protein	EiORF_1568
238920043	-2.07	0.03	Hypothetical protein	EiORF_2147
238918278	-2.07	0.01	Hypothetical protein	EiORF_0315
238918813	-2.08	0.03	Hypothetical protein	EiORF_0876
238920554	-2.08	0.03	Hypothetical protein	EiORF_2666
238918281	-2.09	0.02	Hypothetical protein	EiORF_0318
238920301	-2.09	0.04	Hypothetical protein	EiORF_2411
238918081	-2.10	0.02	ECA biosynthesis protein wzye	EiORF_0100
238919439	-2.10	0.01	Hypothetical protein	EiORF_1533
238919940	-2.11	0.02	Hypothetical protein	EiORF_2044
238919941	-2.12	0.01	Hypothetical protein	EiORF_2045
238921167	-2.12	0.01	Hypothetical protein	EiORF_3308
238918181	-2.12	0.01	Hypothetical protein	EiORF_0217
238918293	-2.13	0.03	Hypothetical protein	EiORF_0330
238918296	-2.13	0.04	Hypothetical protein	EiORF_0333
238921151	-2.13	0.03	Hypothetical protein	EiORF_3292
238919925	-2.13	0.02	Hypothetical protein	EiORF_2029
238918460	-2.14	0.02	Altronate hydrolase	EiORF_0504
238921475	-2.14	0.01	Hypothetical protein	EiORF_3627
238920954	-2.14	0.04	Hypothetical protein	EiORF_3084
238921577	-2.16	0.04	Hypothetical protein	EiORF_3730
238920717	-2.16	0.01	Biotin synthase, putative	EiORF_2830
238918017	-2.16	0.03	Hypothetical protein	EiORF_0036
238918647	-2.17	0.01	Thiamine ABC transporter, ATP-binding protein, putative	EiORF_0704
238919342	-2.19	0.01	Hypothetical protein	EiORF_1434
238919927	-2.19	0.02	Replication of DNA	EiORF_2031
9230686	-2.20	0.02	Unknown	pEI1
238921338	-2.22	0.02	Hypothetical protein	EiORF_3482
238921170	-2.23	0.02	Hypothetical protein	EiORF_3311
238920986	-2.25	0.02	Hypothetical protein	EiORF_3116
238918225	-2.26	0.02	Hypothetical protein	EiORF_0261
238919463	-2.27	0.01	Hypothetical protein	EiORF_1557
238919960	-2.29	0.01	Hypothetical protein	EiORF_2064
238920091	-2.31	0.04	Hypothetical protein	EiORF_2197
238919759	-2.32	0.01	Hypothetical protein	EiORF_1863
238920949	-2.33	0.01	Hypothetical protein	EiORF_3079
238921272	-2.35	0.01	Hypothetical protein	EiORF_3415
238919749	-2.38	0.03	Hypothetical protein	EiORF_1853
238921506	-2.39	0.02	Ascorbate-specific permease iic component ulaa	EiORF_3658
238918276	-2.40	0.03	Arginine repressor, C- domain protein	EiORF_0313
238919951	-2.42	0.02	Ammonium transporter	EiORF_2055
238918260	-2.46	0.01	Hypothetical protein	EiORF_0297
238920722	-2.46	0.03	Molybdate ABC transporter, permease protein, putative	EiORF_2835
238920136	-2.46	0.01	Hypothetical protein	EiORF_2242
238919961	-2.49	0.02	Hypothetical protein	EiORF_2065
238920715	-2.50	0.01	Biotin biosynthesis protein bioc	EiORF_2828

Table 4.2 (Continued)

238918020	-2.51	0.01	Transposase A	EiORF_0039
238920944	-2.51	0.01	Hypothetical protein	EiORF_3074
238921700	-2.62	0.03	Hypothetical protein	EiORF_3861
238921180	-2.62	0.01	Hypothetical protein	EiORF_3321
238920836	-2.78	0.02	Hypothetical protein	EiORF_2962
238919102	-3.04	0.01	Hypothetical protein	EiORF_1173

Blast2GO

35 out of 82 differentially expressed genes have been annotated by Blast2GO (Appendix E). The top hits from BLASTP results showed that 79 DEGs match to *E. ictaluri*; 2 DEGs match to *E. tarda*; 1 DEG matches to *Clostridium carboxidivorans*. The total number of GO terms is 155 including 50 biological process terms (32.3%), 35 cellular component terms (22.6%), and 70 molecular function terms (45.2%). Annotated sequences were assigned to gene ontologies according to biological process (Figure 4.1), cellular component (Figure 4.2), and molecular function (Figure 4.3) at level 2.

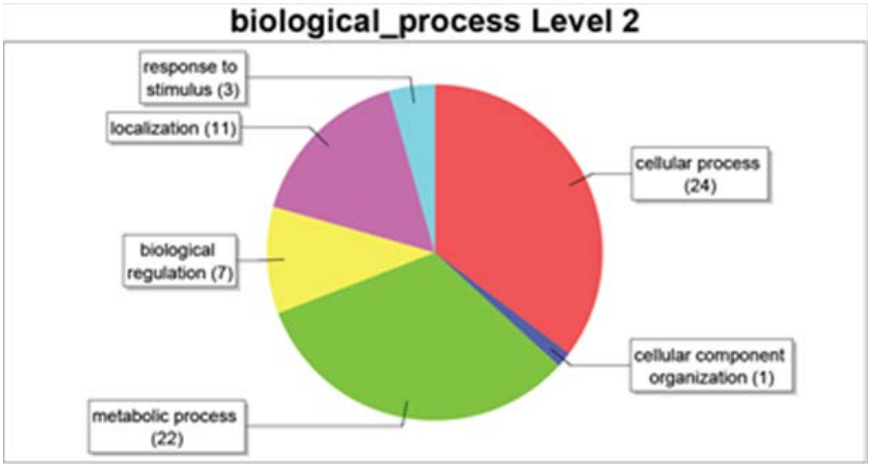

Figure 4.1 GO terms at level 2 according to biological process

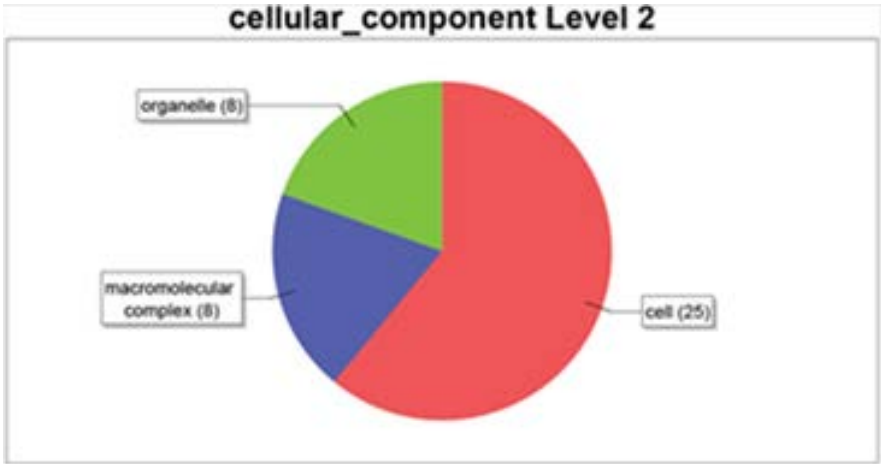

Figure 4.2 GO terms at level 2 according to cellular component

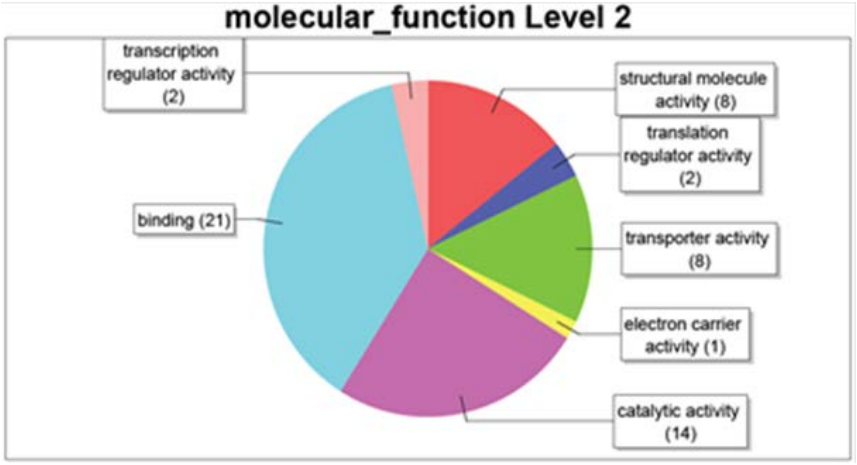

Figure 4.3 GO terms at level 2 according to molecular function

The results showed that the most common annotation terms in these three GO categories were cellular process, metabolic process, localization, cell, organelle, macromolecular complex, binding, catalytic activity, transporter activity, and structural molecule activity.

PSORTb

All 34 out of 82 DEGs were localized into six cellular locations: cytoplasmic, periplasmic, cytoplasmic membrane, extracellular, outer membrane, and unknown. (Figure 4.4).

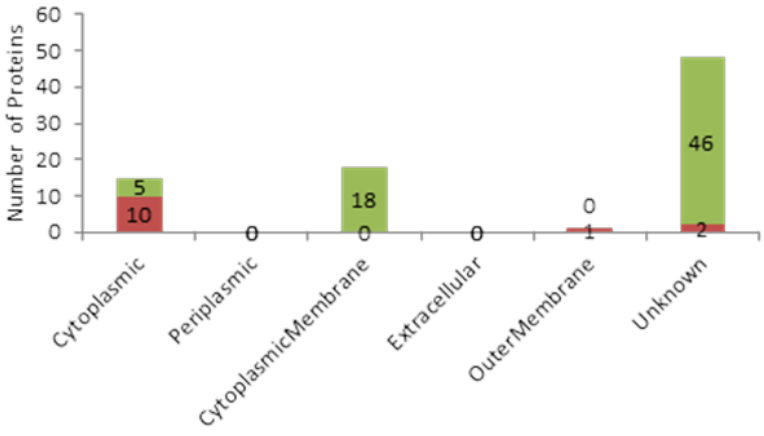

Figure 4.4 Bacterial localization prediction by PSORTb.

The digits on top are the number of DEGs down-regulated and the digits on the bottom are the number of DEGs up-regulated.

The result showed that only one gene, encoding outer membrane protein W (GI238919586), was located on outer membrane of *E. ictaluri*. The function of *ompW* in *E. ictaluri* is worth studying further because an *ompW* mutant of *V. cholera* 0139 strain SG25 by insertion caused a 10-fold reduction in colonization ability.

HPIDB (Host Pathogen Interaction DataBase)

The top hit result showed that 11 of 82 DEGs from this study were identified to be involved in host pathogen PPIs (Table 4.3). 9 of these 11 DEGs were involved in host pathogen PPIs in *Yersinia pestis*; 1 was found in *Bacillus anthracis*, and 1 in *Francisella*

tularensis subsp. tularensis (strain SCHU S4 / Schu 4); 71 DEGs were not found being involved in host pathogen PPIs.

Table 4.3 DEGs involved in host pathogen PPIs

Query id	e-value	Bit score	Organism	Protein product
238918647	7.00E-35	142	Bacillus *anthracis*	Thiamine ABC transporter, ATP-binding protein, putative
238920717	1.00E-88	322	*Francisella tularensis*	Biotin synthase, putative
238918020	3.00E-16	78.6	*Yersinia pestis*	Transposase A
238918081	0	682	*Yersinia pestis*	ECA biosynthesis protein wzye
238918460	0	776	*Yersinia pestis*	Altronate hydrolase
238920265	2.00E-67	250	*Yersinia pestis*	Hypothetical protein
238920400	2.00E-33	139	*Yersinia pestis*	Drug resistance transporter, Bcr/cfla family
238920722	1.00E-31	131	*Yersinia pestis*	Molybdate ABC transporter, permease protein, putative
238921167	1.00E-22	103	*Yersinia pestis*	Hypothetical protein
238921445	0	1299	*Yersinia pestis*	Hypothetical protein
238921506	3.00E-46	182	*Yersinia pestis*	Ascorbate-specific permease iic component ulaa

MVirDB (Microbe Virulence DataBase)

5 DEGs have been identified to be potential virulence factors in *E. ictaluri* *Ei*AKMut02 compared to wt during fish host encounter with a percent identity greater than 20% and an e-value less than 1E - 20. These five genes were protein STY4523, elongation factors GTPases, type IV pilus operon lipoprotein, PTS system enzyme IIC, and III ABC transporter ATP-binding protein (Table 4.4).

Table 4.4 Potential virulence factors identified in MVirDB in *Ei*AKMut02 compared to wt during host challenge.

Query	Number of hits	Lowest E-value	Description	Greatest identity %
238918281	10	2.15E-123	protein STY4523 [Salmonella enterica subsp. enterica serovar Typhi str. CT18]	44
238921445	45	4.25E-67	elongation factors GTPases	44
238918293	10	5.97E-35	type IV pilus operon lipoprotein [Yersinia enterocolitica subsp. enterocolitica 8081]	49
238921506	1	1.30E-30	PTS system enzyme IIC [Streptococcus agalactiae NEM316]	24
238918647	209	2.99E-26	III ABC transporter ATP-binding protein [Haemophilus influenzae Rd KW20]	55

Potential virulence factors and KEGG pathway

The results from Blast2GO, PSORTb, HPIDB, and MVirDB were compared with each other. The common DEGs appearing in multiple databases could contribute to the virulence of *E. ictaluri Ei*AKMut02 strain. Six DEGs were identified to be potential virulence factors (Table 4.5).

Table 4.5 Virulence factors found in multiple databases

GI	Protein Product	Database
238921506	ascorbate-specific permease iic component ulaa	MVirDB/PSORTb/HPIDB/B2G
238918647	thiamine ABC transporter, ATP-binding protein, putative	MVirDB/PSORTb/HPIDB/B2G
238921445	hypothetical protein	MVirDB/HPIDB/B2G
238918081	ECA biosynthesis protein WzyE	HPIDB/PSORTb/B2G
238920400	drug resistance transporter, Bcr/CflA family	HPIDB/PSORTb/B2G
238920722	molybdate ABC transporter, permease protein, putative	HPIDB/PSORTb/B2G

Three genes have been found in pathways through further analysis in KEGG pathway database. These three genes were ascorbate-specific permease iic component ulaA (GI238921506), thiamine ABC transporter/ATP-binding protein (GI238918647),

and molybdate ABC transporter/permease protein (GI238920722). *ulaA* has been found in two pathways: ascorbate and aldarate metabolism (eic_00053) and phosphotransferase system (PTS; eic_02060) pathways. UlaA protein can degrade ascorbate to D-xylulose-5P (eic_M00550) and is a component of PTS system and/or ascorbate-specific II (eic_M00283). *ulaA* belongs to the *ula* regulon, which consists of *ulaABCDEF* and is responsible for the utilization of L-ascorbate in *E. coli* (Yew and Gerlt, 2002). The function of the *ula* regulon was repressed by glucose through at least two mechanisms: cyclic AMP-cAMP receptor protein (cAMP-CRP) dependent and cAMP-CRP independent (Campos et al., 2004). Low level of utilizing L-ascorbate may be the reason why this *E. ictaluri* mutant's virulence was reduced because *ulaA* gene was down-regulated in *Ei*AKMut02 compared to *E. ictaluri* wt during host encounter.

Thiamine ABC transporter encoded by *thiQ* has been found in ABC transporters pathway and functions in thiamine transport system (eic_M00189). *thiQ* is an ATPase and a part of *thiBPQ* operon in *Salmonella typhimurium*, which was designed as *sfuABC* in *E coli*. Its protein product was required for the transport of thiamine and thiamine pyrophosphate (TPP) into the cells since the insertion mutation in *thiBPQ* operon caused defective in thiamine and TPP transport (Webb et al., 1998). The down-regulation of *thiQ* (down-regulated 2.17 FC) may suggest that *thiBPQ* operon was affected by *gcvP* mutation or that the infection in catfish may need low levels of thiamine or TPP inside the bacterium.

Molybdate ABC transporter permease has been found in ABC transporter pathway also, and functions in molybdate transport system (eic_M00189). Recently, the crystal structure of some ABC transporters, including type I ABC importer, and type II

ABC importer and ABC exporter, were made available (Locher, 2009). The molybdate/tungstate transporter ModBC from *A. fulgidus* is one of the type I ABC importers (Hollenstein et al., 2007). Vitamin B12 transporter BtuCD from *E. coli* (Locher et al., 2002), the metal-chelate-type transporter HI1470/1 from *H. influenza* (Pinkett et al., 2007), and BtuCD-F (Hvorup et al., 2007), are the three type II ABC importers with known crystal structure. The first high-resolution crystal structure of an ABC exporter is the multidrug transporter Sav1866 from *S. aureus* (Dawson and Locher, 2006). The signature sequence of the molybdate ABC transporter permease was identified to be located in the cytoplasmic loop between two transmembrane regions and might function in molybdate/tungstate without its binding protein in *E. coli* (Self et al., 2001).

CHAPTER V

GLOBAL GENE EXPRESSION ANALYSIS OF *Ei*AKMut02 MUTANT'S RESPONSE TO SPF CATFISH SERUM TREATMENT

Abstract

Edwardsiella ictaluri is the etiological agent of enteric septicemia of catfish, but its pathogenic mechanisms have not been understood completely. An *E. ictaluri gcvP* mutant (*Ei*AKMut02) has been produced in our lab and shown that it is completely attenuated and highly protective in catfish fingerlings. The *gcvP* is part of the glycine cleavage system, and we have shown that it is important for both neutrophil and serum resistance in *E. ictaluri*. To better understand importance of the *gcvP* in *E. ictaluri* virulence, we compared the global gene expression between the *E. ictaluri* wt and *gcvP* mutant after serum exposure. Our results showed that 296 genes in *Ei*AKMut02 have been differentially expressed (108 up-regulated and 188 down-regulated genes) post SPF serum exposure. Functional analysis has been performed on all differentially expressed genes with Blast2GO, PSORTb, HPIDB, and MVirDB. We have identified four virulence relevant factors, which are flagellar basal body L-ring protein, outer membrane protein N2, outer membrane protein N3, and ABC transporter/ATP binding protein.

Introduction

E. ictaluri is the causative pathogen of enteric septicemia of catfish (ESC) and is a Gram-negative facultative rod-shaped bacterium belonging to *Enterobacteriaceae* (Hawke, 1979; Hawke et al., 1981). Though many virulence factors have been identified by different research groups (Cooper et al., 1996; Lawrence et al., 2003; Lawrence et al., 2001; Morrison and Plumb, 1994; Skirpstunas and Baldwin, 2003; Stanley et al., 1994; Thune et al., 2007), ESC is still a devastating disease in catfish industry. Our previous research has shown that *Ei*AKMut02 mutant with *gcvP* insertion mutation is completely attenuated in catfish fingerlings. *gcvP* encodes a protein that is part of glycine cleavage system. The glycine cleavage system includes four subunits and this enzyme complex catalyzes glycine oxidation reversibly to form 5, 10-methylenetetrahydrofolate. 5, 10-methylenetetrahydrofolate is one of two sources of one carbon donor while the other one is serine hydroxymethyltransferase. Glycine can induce the expression of the glycine cleavage enzyme system (Meedel and Pizer, 1974; Stauffer et al., 1994). *gcvP* mutants cannot use glycine as a one carbon source and excrete glycine (Plamann et al., 1983). *E. ictaluri gcvP* is located downstream of *gcvH* and *gcvT*. *gcvP* is the third gene in the three gene operon which encodes subunits of the glycine cleavage system. The disturbance of *gcvP* function in *Ei*AKMut02 resulted in significant reduced resistance to both catfish neutrophils and catfish serum compared to wt (Karsi et al., 2009). Thus, in order to investigate the effect of *gcvP* mutation on *E. ictaluri* during the early stage of infection in catfish, we conducted microarray experiment in *Ei*AKMut02 and wt *E. ictaluri*.

Materials and methods

Bacterial strains and growth condition

In this study, the wild type strain of *Edwardsiella ictaluri* 93-146 and *Ei*AKMut02 mutant were used (Karsi et al., 2009; Lawrence et al., 1997). *Ei*AKMut02 is a mutant of *E. ictaluri* 93-146, which was constructed by transposon insertion to the gene *gcvP* (Karsi et al., 2009). Both strains were grown in brain heart infusion (BHI) (Difco) agar and broth at 30 ^{o}C. Ampicillin (100 µg/ml), colistin (12.5 µg/ml) and gentamycin (12.5 µg/ml) antibiotics were added to growth medium when needed.

Experimental procedures

Experimental fish, serum preparation, serum exposure assay, total RNA purification, cDNA synthesis, microarray experiments, and bioinformatic analysis have been conducted as described in chapter III.

Results and Discussion

Gene expression profiles of *Ei*AKMut02 following catfish serum exposure

296 genes were differentially expressed significantly in *Ei*AKMut02 compared to *E. ictaluri* wt strain when they were exposed to naïve catfish serum. The parameters to define DEGs were a $p < 0.05$ and a fold change > 1.5. The percentage of differentially expressed genes in *E. ictaluri* genome was 8.04%. 108 genes were up-regulated (Appendix F), while 188 genes were down-regulated (Appendix G) in *E. ictaluri* *Ei*AKMut02. The greatest fold change of up-regulated genes was 3. The greatest fold change of down-regulated genes was 4.2. Among 108 up-regulated genes, 32 genes were up-regulated from 2 to 3 fold; 76 genes were up-regulated from 1.5 to 2 fold compared to

E. ictaluri wt. Among 188 down-regulated genes, 8 genes were down-regulated from 3 to 4 fold; 44 genes were down-regulated from 2 to 3 fold; 136 genes were down-regulated from 1.5 to 2 fold compared to *E. ictaluri* wt.

Blast2GO

216 DEGs have been annotated by Blast2GO (Appendix H). A total of 908 annotations were found through Blast2GO including 306 biological terms (33.7%), 144 cellular component terms (15.9%), and 458 molecular function terms (50.4%). The top hits for BLASTP results showed that 289 DEGs matched to *E. ictaluri*, 3 DEGs matched to *E. tarda*, and 1 DEG matched to *Escherichia coli*, *Klebsiella sp*. and *Clostridium carboxidivorans*. Annotated sequences were assigned to gene ontologies according to biological process (Figure 5.1), cellular component (Figure 5.2), and molecular function (Figure 5.3). The results showed that metabolic process, cellular process, localization, biological regulation, response to stimulus, cell, macromolecular complex, organelle, catalytic activity, binding, transporter activity, structural molecule activity, and electron carrier activity were the most common annotation terms in these three GO categories.

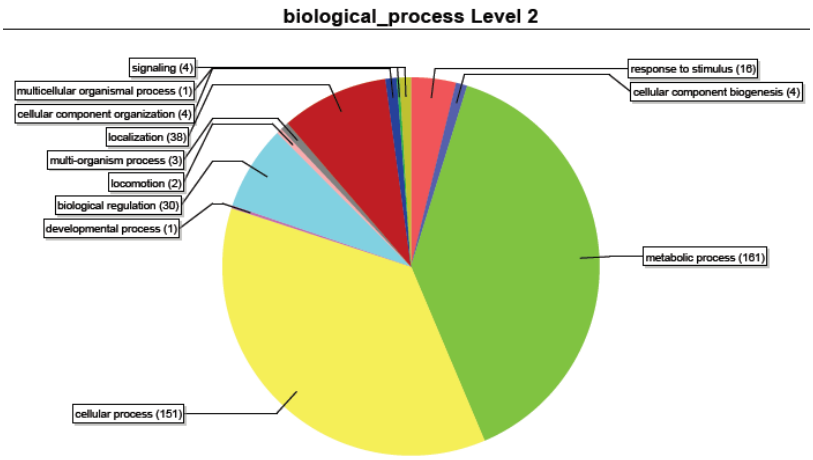

Figure 5.1 GO based on biological process for *Ei*AKMut02 and wt under serum exposure at level 2

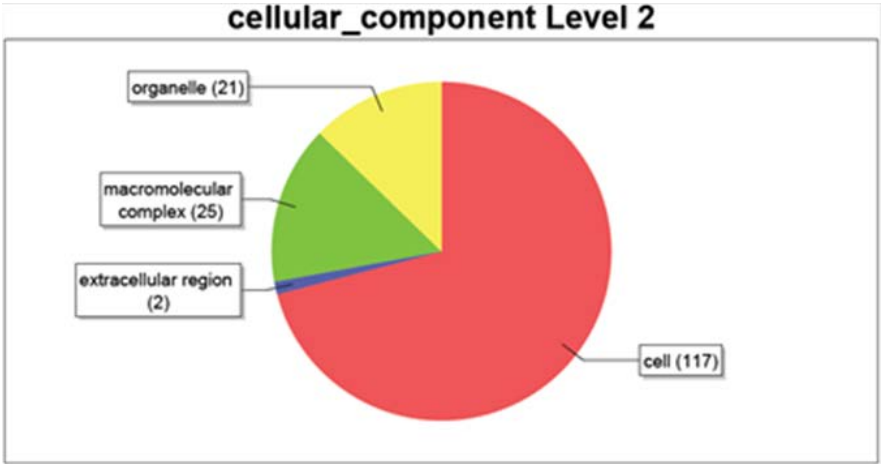

Figure 5.2 GO based on cellular component for *Ei*AKMut02 and wt under serum exposure at level 2

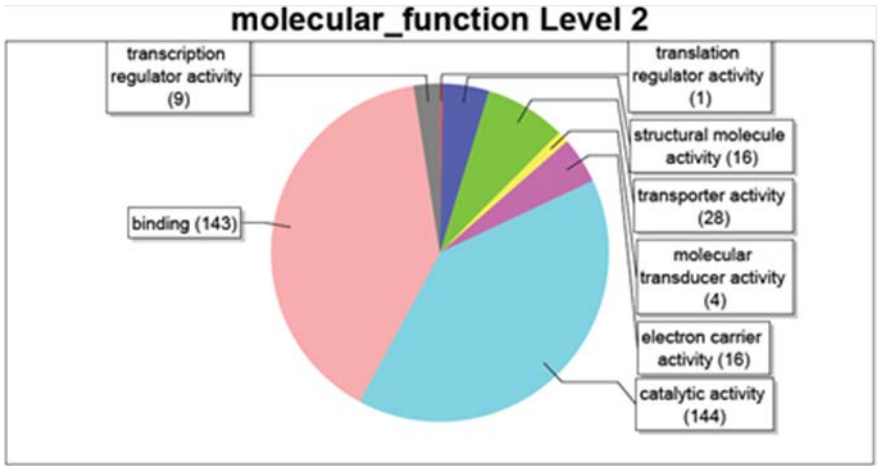

Figure 5.3 GO based on molecular function for *Ei*AKMut02 and wt under serum exposure at level 2

PSORTb

There were 209 (70.6%) DEGs in this study whose bacterial localization have been predicted (Figure 5.4).

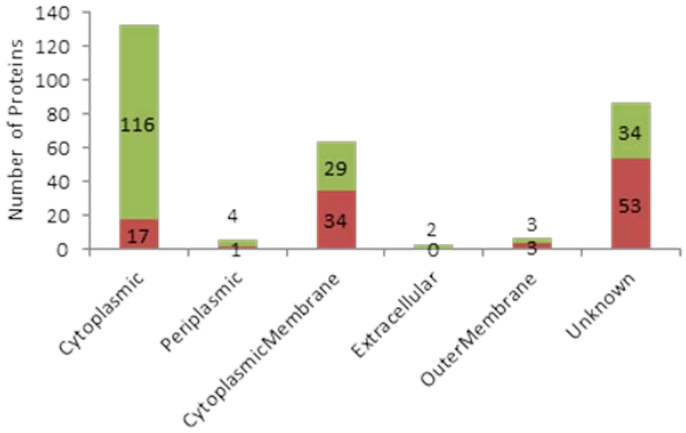

Figure 5.4 Bacterial localization prediction of 296 DEGs in *Ei*AKMut02 compared to wt under serum exposure by PSORTb.

The digits on top are the number of DEGs down-regulated, and the digits on the bottom are the number of DEGs up-regulated.

The results showed that 133 DEGs are located in cytoplasmic; 8 DEGs are located in periplasmic; 63 DEGs are located on cytoplasmic membrane; 3 DEGs are located in extracellular; 6 DEGs are located on outer membrane of *Ei*AKMut02; and 87 DEGs localizations have not been predicted. Outer membrane proteins are considered to be important for the virulence of bacterial pathogens. The 6 DEGs predicted to be located on *E. ictaluri* outer membrane are listed in Table 5.1.

Table 5.1 DEGs located on outer membrane of *Ei*AKMut02 compared to wt under SPF serum exposure

GI	**Protein produt**	**Localization**	**Score**
gi|238918213|ref|YP_002931727.1|	Hypothetical protein NT01EI_0249	OuterMembrane	9.52
gi|238918804|ref|YP_002932318.1|	Lipoprotein involved with copper homeostasis and adhesion	OuterMembrane	9.93
gi|238919262|ref|YP_002932777.1|	Flagellar basal body L-ring protein	OuterMembrane	9.92
gi|238919771|ref|YP_002933286.1|	Outer membrane protein N	OuterMembrane	10
gi|238919996|ref|YP_002933511.1|	Outer membrane lipoprotein pcp	OuterMembrane	9.93
gi|238921673|ref|YP_002935188.1|	Outer membrane protein N	OuterMembrane	10

HPIDB (Host Pathogen Interaction DataBase)

The top hit result showed that 43.6% (129) of DEGs in this study were involved in host pathogen interaction (Appendix I). 73 DEGs were matched to *Yersinia pestis*, 24 to *Bacillus anthracis*, 17 to *Francisella tularensis subsp. tularensis* (strain SCHU S4 / Schu 4), 1 to *Saccharomyces cerevisiae* (strain ATCC 204508 / S288c), and 1 to *Shigella flexneri*. The remaining 13 DEGs match to *Homo sapiens* and *Mus musculus*.

MVirDB (Microbe Virulence DataBase)

The protein sequences of 296 DEGs have been blasted against MVirDB database. We have considered the DEGs as virulence related genes in *E. ictaluri* if its percent

identity is greater than 20% with an e-value less than 1E - 20. In *Ei*AKMut02, 42 DEGs were matched to virulence factors from other pathogenic bacteria (Table 5.2).

Table 5.2 42 potential virulence factors identified from the 296 DEGs in *Ei*AKMut02 post SPF catfish serum exposure

Query	Greatest identity %	Lowest E-value	Description
gi|238918492	67	0.00E+00	Heptose 7-phosphate kinase/heptose 1-phosphate adenyltransferase [Haemophilus influenzae Rd KW20]
gi|238919172	100	0.00E+00	Full=Phosphoenolpyruvate-protein phosphotransferase; EC=2.7.3.9; altname: Full=Phosphotransferase system, enzyme I;
gi|238919742	88	0.00E+00	Kinase [Salmonella typhimurium LT2]
gi|238919957	100	0.00E+00	Full=Urease subunit alpha; EC=3.5.1.5; altname: Full=Urea amidohydrolase subunit alpha;
gi|238920314	83	0.00E+00	ATP synthase [Yersinia enterocolitica subsp. Enterocolitica 8081]
gi|238921110	73	0.00E+00	Mismatch repair protein [Salmonella typhimurium LT2]
gi|238921570	67	0.00E+00	Transporter [Salmonella typhimurium LT2]
gi|238920853	66	9.33E-163	Protein [Escherichia coli]
gi|238920246	51	1.39E-144	Repeat-containing protein [Salmonella typhimurium LT2]
gi|238918975	100	6.58E-144	Full=Export membrane protein secf; subname: Full=Protein-export membrane protein secf;
gi|238919771	100	2.58E-132	Full=Outer membrane protein S2; Flags: Precursor;
gi|238920284	41	5.21E-96	Synthetase D [Pseudomonas aeruginosa PAO1]
gi|238920346	57	5.90E-96	Pilk [Pseudomonas aeruginosa PAO1]
gi|238918051	58	2.07E-93	Heptosyltransferase I [Pseudomonas aeruginosa PAO1]
gi|238918052	49	4.03E-92	II [Pseudomonas aeruginosa PAO1]
gi|238918863	69	2.17E-90	Protein [Escherichia coli]
gi|238921673	76	1.04E-88	Full=Outer membrane protein F; altname: Full=Porin ompf; Flags: Precursor;
gi|238919262	94	4.40E-88	Basal body L-ring protein [Yersinia enterocolitica subsp. Enterocolitica 8081]
gi|238919298	88	1.40E-87	Component of hydroxymate-dependent iron ABC transporter [Yersinia pestis KIM]
gi|238918568	57	1.77E-62	Membrane receptor for ferric iron uptake [Escherichia coli O157:H7 EDL933]
gi|238920969	36	4.53E-60	S1C unassigned peptidase, has signal peptide
gi|238918653	48	1.47E-59	Protein [Escherichia coli]
gi|238920852	49	9.28E-59	Protein SA0116 [Staphylococcus aureus subsp. Aureus N315]
gi|238920016	50	5.48E-58	Synthase component I [Escherichia coli CFT073]

Table 5.2 (Continued)

gi|238921392	85	4.87E-55	Iron transport lipoprotein sirf [Staphylococcus aureus subsp. Aureus Mu50]
gi|238919490	30	3.23E-50	Full=SBT1 protein; subname: Full=Subtilisin-like protease;
gi|238918413	50	2.72E-48	Transcriptional regulator basr [Salmonella typhimurium LT2]
gi|238920924	47	4.48E-48	Resistance protein [Bordetella pertussis Tohama I]
gi|238920344	100	6.27E-47	Full=Integration host factor subunit beta; Short=IHF-beta;
gi|238918177	67	3.05E-46	Protein psie [Yersinia pestis CO92]
gi|238918421	100	4.72E-43	Full=Protein-export membrane protein secg; altname: Full=P12; altname: Full=Preprotein translocase band 1 subunit;
gi|238920481	45	6.01E-43	Full=Phosphotransferase system transporter enzyme I, frui;
gi|238920290	60	8.98E-39	III transport ATP binding protein sfuc like
gi|238918036	55	3.91E-37	Repair protein radc, putative [Vibrio cholerae O1 biovar El Tor str. N16961]
gi|238921255	38	4.82E-34	[Mycobacterium tuberculosis H37Rv]
gi|238920367	34	1.20E-30	Full=Epstein-Barr virus nuclear antigen EBNA-3C ;
gi|238921410	34	1.74E-29	Biosynthesis regulator flhf [Campylobacter jejuni subsp. Jejuni NCTC 11168]
gi|238918945	28	6.56E-29	Methylase hsdm, putative [Vibrio cholerae O1 biovar El Tor str. N16961]
gi|238918213	37	5.92E-25	Factor Hek [Escherichia coli]
gi|238920907	39	1.34E-24	Elongation factors gtpases
gi|238919371	44	9.59E-24	Receptor, binds flim, causes tumbling
gi|238921701	41	1.78E-22	Full=Putative general secretion pathway protein H;

Virulence factors and KEGG pathway

The differentially expressed genes in *Ei*AKMut02 compared to wt under serum exposure were analyzed with Blast2GO, PSORTb, HPIDB, and MVirDB. From the results, 15 virulence factors were identified (Table 5.3). Pathway analysis was conducted for these 15 virulence genes in the KEGG pathway database. 6 genes were identified in 6 different pathways (Table 5.4).

Table 5.3 Virulence factors identified through functional analysis in *Ei*AKMut02 after catfish serum exposrue

GI	Protein product	Locus Taq
238921570	Mg2+ transporter mgtb, putative	NT01EI_3723
238920246	Leucine-rich repeat protein	NT01EI_2355
238918975	Protein-export membrane protein secf, putative	NT01EI_1042
238919771	Outer membrane protein N	NT01EI_1875
238921673	Outer membrane protein N	NT01EI_3834
238919262	Flagellar L-ring protein flgh, putative	NT01EI_1352
238918653	Hypothetical protein	NT01EI_0711
238920314	Flil; flagellum-specific ATP synthase, putative	NT01EI_2424
238920284	Hypothetical protein	NT01EI_2394
238918177	Hypothetical protein	NT01EI_0213
238918421	Preprotein translocase, secg subunit, putative	NT01EI_0462
238920290	ABC transporter, ATP-binding protein, putative	NT01EI_2400
238920367	Anaerobic dimethyl sulfoxide reductase chain A, putative	NT01EI_2477
238921701	GTP-binding protein typa/bipa, putative	NT01EI_3862
238919371	Hypothetical protein(K03413 two-component system, chemotaxis family, response regulator chey)	NT01EI_1465

Table 5.4 Pathways represented in *Ei*AKMut02 after exposure to catfish serum

GI	Locus Taq	Pathway	Module
238918975	NT01EI_1042	Protein export (eic03060)	Sec (secretion) system (eic_M00335)
		Bacterial secretion system (eic03070)	N/A
238919262	NT01EI_1352	Flagellar assembly (eic02040)	N/A
238920314	NT01EI_2424	Flagellar assembly (eic02040)	N/A
238918421	NT01EI_0462	Protein export (eic03060)	Sec (secretion) system (eic_M00335)
		Bacterial secretion system (eic03070)	N/A
238920290	NT01EI_2400	ABC transporters (eic02010)	Cystine transport system (eic_M00234)
238919371	NT01EI_1465	Two-component system (eic02020)	N/A
		Bacterial chemotaxis (eic02030)	N/A

SecF (GI238918975) and SecG (GI238918421) play roles in protein export (eic_03060) and bacterial secretion system (eic_03070) pathways. SecF and SecG

contribute to sec (secretion) system (eic_M00335) based on module information. SecF together with SecD stabilize the inserted state of SecA in *E. coli* membrane (Economou et al., 1995). It was also reported that SecF and secD function together to stimulate protein export in *E. coli* (Pogliano and Beckwith, 1994). Deletion study of SecF and SecD in *E. coli* showed that SecF/D played roles in translocation of proteins, especially pro-proteins, into inverted inner membrane vesicles. SecF/D may also help maintain a proton electrochemical gradient in *E. coli* (Arkowitz and Wickner, 1994; Matsuyama et al., 1992). SecG is a component of *E. coli* preprotein translocase and makes the enzyme highly efficient through topology inversion (Nishiyama et al., 1996). SecG was identified to be a subunit of a trimeric complex of SecYEG, which was one membrane embedded part of preprotein translocase in *E. coli*. SecG was also found functioning in protein translocation directly in *E. coli* especially at low temperature (Nishiyama et al., 1994).

FlgH (GI238919262) and FliI (GI238920314) are involved in flagellar assembly pathway (eic_02040). FlgH was confirmed to be a lipoprotein and functions as the outer-membrane L-ring protein of the flagellar basal body in *Salmonella typhymurium* (Schoenhals and Macnab, 1996). FlgH is not necessary in all bacteria such as *Firmicutes* and *Spirochaetes* (Liu and Ochman, 2007). f*liI* is a component of the operon FliHIOPQR (Desvaux et al., 2006) and functions as the ATPase of the flagellar export apparatus (Fan and Macnab, 1996). To fully exert its ATPase activity, FliI ATPase forms a homo-hexameric ring structure (Claret et al., 2003; Minamino et al., 2006). FliL was found to be a component of the basal body and is located in the cytoplasmic membrane in Salmonella (Schoenhals and Macnab, 1999).

ABC transporter/ATP-binding protein (GI238920290) functions in ABC transporters pathway (eic_02010) and contributes to cystine transport system (eic_M00234) based on module search. A hypothetical protein (GI238919371) functions in both two-component system (eic_02020) and bacterial chemotaxis (eic_02030), which suggests that it may be important for the survivability of *E. ictaluri* inside and outside of the host.

CHAPTER VI

CONSTRUCTION AND EVALUATION OF ELEVEN *E. ICTALURI* IN-FRAME DELETION MUTANTS

Abstract

E. ictaluri is the etiological agent of enteric septicemia of catfish (ESC). Use of antibiotics as food additive is not effective since the first clinical sign of ESC is anorexia. In addition, antibiotic treatment may cause antibiotic resistant strains. Live attenuated vaccines have the potential as an alternative prevention method against ESC. However, only one commercial vaccine is available, and ESC is still the most prevalent disease of catfish industry. Here, we present the vaccine potential of novel *E. ictaluri* mutants. We have constructed 11 *E. ictaluri* mutants by in-frame deletion and have tested their virulence and efficacy in 8-week old channel catfish fingerlings by immersion challenge. Fish mortality data indicated that *yscR* and *ssaV* mutants were completely attenuated while the other 9 showed less or no attenuation compared to wild type strain. Efficacy testing indicated that *yscR* and *ssaV* mutants provided moderate protection compared to sham vaccinated group (94.05% mortality) since the relative percent survivals of fingerlings vaccinated with *yscR* and *ssaV* mutants were 42.90% and 40.09%, respectively. Our results support that the T3SS is important in *E. ictaluri* virulence.

Introduction

Enteric septicemia of catfish (ESC) is one of the most important diseases in the farm-raised channel catfish (*Ictalurus punctatus*) industry. ESC has been found in all states where catfish are raised. For the control of enteric septicemia, Romet, Terramycin and Aquaflor antibiotics may be applied as food additive. Though these antibiotics have the potential to prevent the spread of the ESC, they may not be effective to cure ESC as sick catfish become anorexic. Furthermore, new antibiotic resistant strains could develop. Thus, live attenuated vaccines are the alternative to control ESC. One commercial vaccine is available, but ESC is still the most prevalent disease in catfish aquaculture. More work is needed to develop safer and more immunogenic vaccines for ESC. As an extension of our work described in the previous chapters where we have identified potential virulence genes, here we report development and vaccine potential of 11 new mutants.

Materials and methods

Bacterial strains and plasmids

The wild type strain of *E. ictaluri* 93-146 was used as the parent strain to construct *E. ictaluri* mutants. Brain Heart Infusion (BHI) agar and broth (Difco, Sparks, MD) containing 12.5 µg/ml of colistin (Sigma) was used for culturing *E. ictaluri* parent strain and mutants colonies. *E. ictaluri* cultures were incubated at 30 ^{o}C at 200 rpm with aeration. *E. coli* CC118, and SM10 λ*pir* were used in cloning and conjugal transfer of suicide plasmid into *E. ictaluri*, respectively. *E. coli* strains were grown using LB broth and agar (Difco, Sparks, MD) at 37 ^{o}C for 18 h in a shaker incubator at 200 rpm.

Suicide plasmid pMEG-375 (*sacRB mobRP4* R6K *ori* Cm^r Amp^r) was used as the vector to generate and introduce in-frame deleted genes into *E. ictaluri* chromosome by allelic exchange. pMEG-375 includes ampicillin and chloramphenicol markers for the first step of mutant screening after the plasmid integrated into *E. ictaluri* genome. pMEG-375 also has *Bacillus subtilis sacB* gene encoding levane saccharase, which is lethal in most Gram-negative bacteria in the presence of sucrose (Gay et al., 1983). *sacB* gene is used for the secondary screening of mutant strains in which the plasmid was excised from *E. ictaluri* genomic DNA when they grew in a media containing sucrose. All strains and plasmids used and constructed in this study are listed in Table 6.1.

Table 6.1 Bacterial strains and plasmids

Strain	Relevant Characteristics	References
Edwardsiella ictaluri		
93146	Wild type; $pEI1^+$; $pEI2^+$; Col^r	(Lawrence et al., 1997)
*Ei*Δ*malE*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*malE*	This study
*Ei*Δ*typA*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*typA*	This study
*Ei*Δ*lamB*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*lamB*	This study
*Ei*Δ*blc*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*blc*	This study
*Ei*Δ*ssaV*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*ssaV*	This study
*Ei*Δ*yscR*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*yscR*	This study
*Ei*Δ*ompW*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*ompW*	This study
*Ei*Δ*ompN*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*ompN*	This study
*Ei*Δ*gpmA*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*gpmA*	This study
*Ei*Δ*hp2312*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*hp2312*	This study
*Ei*Δ*hp3311*	93146 derivative; $pEI1^+$; $pEI2^+$; Col^r; Δ*hp3311*	This study

Table 6.1 (Continued)

Escherichia coli		
CC118λ*pir*	Δ(*ara-leu*); *araD*; Δ*lacX74*; *galE*; *galK*; *phoA20*; *thi-1*; *rpsE*; *rpoB*; *argE*(Am); *recAl*; λ*pir*R6K	(Herrero et al., 1990)
SM10λ*pir*	*thi*; *thr*; *leu*; *tonA*; *lacY*; *supE*; *recA*; ::RP4-2-Tc::Mu; Km^r; λ*pir*R6K	(Miller and Mekalanos, 1988)
Plasmid		
pMEG-375	8142 bp, Amp^r, Cm^r, *lacZ*, R6K *ori*, *mob incP*, *sacR sacB*	(Dozois et al., 2003)
p*Ei*Δ*malE*	10527 bp, Δ*malE*, pMEG-375	This study
p*Ei*Δ*typA*	10318 bp, Δ*typA*, pMEG-375	This study
p*Ei*Δ*lamB*	10589 bp, Δ*lamB*, pMEG-375	This study
p*Ei*Δ*blc*	10255 bp, Δ*blc*, pMEG-375	This study
p*Ei*Δ*ssaV*	10260 bp, Δ*ssaV*, pMEG-375	This study
p*Ei*Δ*yscR*	10253 bp, Δ*yscR*, pMEG-375	This study
p*Ei*Δ*ompW*	10291 bp, Δ*ompW*, pMEG-375	This study
p*Ei*Δ*ompN*	10268 bp, Δ*ompN*, pMEG-375	This study
p*Ei*Δ*gpmA*	10337 bp, Δ*gpmA*, pMEG-375	This study
p*Ei*Δ*hp2312*	10272 bp, Δ*hp2312*, pMEG-375	This study
p*Ei*Δ*hp3311*	10283 bp, Δ*hp3311*, pMEG-375	This study

Genomic DNA and plasmid DNA isolation

E. ictaluri genomic DNA was purified using a Wizard Genomic DNA Purification Kit (Promega). The concentration and integrity of the genomic DNA was measured with NanoDrop 1000 (Thermo Scientific) and agarose gel electrophoresis. The purified genomic DNA was stored at 4 ^oC. Plasmid purification was done using a Qiaspin

Miniprep Kit (Qiagen). The concentration and quality of extracted plasmid were checked with NanoDrop 1000 and 1% agrose gel electrophoresis.

Preparation of competent cells

To prepare chemical competent cells, a single colony of *E. coli* CC118 λ*pir* was inoculated into 5 ml of LB broth and grown overnight. 4 ml of overnight culture was transferred to 50 ml of fresh LB broth and grown until OD600 reached 0.4 -0.5. The culture was chilled in ice-water for 10 min, after which the bacteria were collected by spinning at 3,000 rpm for 8 min at 4 ^{o}C. Pellet was washed twice with ice-cold 0.1 M $CaCl_2$. In the second wash, bacteria were incubated on ice for 30 min before centrifugation. After removal of the supernatant, the bacterial pellet was resuspended in 5 ml ice-cold 0.1 M $CaCl_2$ with 15% sterile glycerol. 200 µl bacterial suspension was aliquoted into 1.5 ml microcentrifuge tubes and quick frozen in liquid nitrogen. The aliquoted chemical competent *E. coli* CC118 λ*pir* was stored at -80 ^{o}C until use.

Electrocompetent *E. coli* SM10 λ*pir* cells were prepared as stabilized protocol (http://www.its.caltech.edu/~bjorker/Protocols/Prep_of_electocomp_cells.pdf) and then stored at -80 ^{o}C until use.

Selection of potential virulence genes in *E. ictaluri*

The virulence genes knocked out in this study were identified from our microarray and previous proteomic data analysis. 5 genes were selected from bioinformatics analysis; 5 genes were selected from the comparison between microarray and proteomic data (Dumpala, 2010); and1 gene was selected from the common DEGs

which have been identified in four microarray analysis discussed in Chapter II, III, IV and V.

In-frame gene deletion *in vitro*

Primers were designed with Primer3 V0.4.0 (http://frodo.wi.mit.edu/primer3/). All genes sequences plus 2,000 bp upstream and 2,000 bp downstream flanking regions and *E. ictaluri* whole genome were downloaded from http://www.ncbi.nlm.nih.gov/. The external forward and reverse primers were designed in the region of upstream 1,000 bp away from the very beginning of and down-stream 1,000 bp away from the very ending of the target gene. The internal reverse and forward primers were designed at the very beginning of the start codon and stop codon of the target gene, respectively. Then, the reverse complement of one of the internal primers was added to the 5' end of the other internal primer for overlap extension PCR to produce the spliced DNA fragment with in-frame deletion of the target gene. Unique restriction enzyme (RE) sites were added to the 5' end of each external primer for cloning purpose. Moreover, internal forward and reverse primers were designed in-frame to avoid disturbance of downstream genes in *E. ictaluri* chromosome. All primers used in this study are listed in Table 6.2.

Table 6.2 External and internal primers used in this study for construction of in-frame deletion mutants.

Primers	**Sequence** (5'→3')[b]	RE[a]
*EiT3SSssaV*_ext_F	AA**CCCGGG**ctatctgaaatgcctccacca	SmaI
*EiT3SSssaV*_ext_R	AA**TCTAGA**tatgcccgtcaagcagtgag	XbaI
*EiT3SSssaV*_int_F	TGAAAACGGTCAATAGCTGGCTGTTCACCACACCCGTACTC	
*EiT3SSssaV*_int_R	CCAGCTATTGACCGTTTTCA	
*EiT3SSp*_ext_F	AA**GAGCTC**tgtcaccgtcgatactcatct	SacI
*EiT3SSp*_ext_R	AA**CCCGGG**tgataatcagcatcagcagt	SmaI
*EiT3SSp*_int_F	CTGCTGATCGTAATGGGTACACTGCTGCTTTCGTACCGCTAA	
*EiT3SSp*_int_R	TGTACCCATTACGATCAGCAG	
*EiompW*_ext_F	AA**GAGCTC**agaatgatccccggcaagat	SacI
*EiompW*_ext_R	AA**TCTAGA**atatccccagttgcccattg	XbaI

Table 6.2 (Continued)

*EiompW*_int_F	GATGAAAAAGTGTAGCGTTGCGAGCCAGCACAGCATAAATACC	
*EiompW*_int_R	CGCAACGCTACACTTTTTCATC	
*EiompN*_ext_F	AA**GAGCTC**acacgggggatgtggtagaa	SacI
*EiompN*_ext_R	AA**TCTAGA**gctattttgccatgctggag	XbaI
*EiompN*_int_F	GTGAAACGTAATCTGCTGGCAATCGTTGCTCTGGGTCTGGT	
*EiompN*_int_R	TGCCAGCAGATTACGTTTCAC	
*EiompBLC*_ext_F	AA**GAGCTC**tcgctcggggtagcctatac	SacI
*EiompBLC*_ext_R	AA**TCTAGA**ctgaagaagaaggcgaggtc	XbaI
*EiompBLC*_int_F	CAAGGAGGAGAGAGCCGATGGCAGTAAGGCAGGCGTATATC	
*EiompBLC*_int_R	Catcggctctctcctccttg	
*Eihp*_3311_ext-F	AA**GAGCTC**actcgacctaacatgcggttc	SacI
*Eihp*_3311_ext-R	AA**TCTAGA**cgtggtatccgtggcatag	XbaI
*Eihp*_3311_int-F	TTGGGTATAGGTTGTTGGATTT	
*Eihp*_3311_int-R	AAATCCAACAACCTATACCCAATCGAAGACCAACAGTTTGAAT	
*Eihp*_2312_ext-F	AA**GAGCTC**ctctttttggtgccgttattgc	SacI
*Eihp*_2312_ext-R	AA**CCCGGG**ctatctcgcgggtggtcatc	SmaI
*Eihp*_2312_int-F	ACTGAGAGTGTTGCTGCATGA	
*Eihp*_2312_int-R	TCATGCAGCAACACTCTCAGTACCTTGACTGGGAATGAAGTG	
*Eihp*_0249_ext-F	AA**GAGCTC**atctgctccaacgcatacat	SacI
*Eihp*_0249_ext-R	AA**CCCGGG**ttaccgggtgatcctgatgg	SmaI
*Eihp*_0249_int-F	CATGACTTTACTCTGGGTGTGA	
*Eihp*_0249_int-R	TCACACCCAGAGTAAAGTCATGGGAGACCAGACCTACGAACAG	
*EinrsE*_ext-F	AA**GAGCTC**gctctatcgtattgccgtga	SacI
*EinrsE*_ext-R	AA**CCCGGG**ggcaggtcgatcttgttca	SmaI
*EinrsE*_int-F	TCTGTCCGTTTCAGCCGGTAA	
*EinrsE*_int-R	TTACCGGCTGAAACGGACAGATAGGCTGCCAGTCAACAGACTA	
*Eipgcm*_ext-F	AA**GAGCTC**atgccaaacttcatccaggtg	SacI
*Eipgcm*_ext-R	AA**CCCGGG**tttccagctaccgcctgtg	SmaI
*Eipgcm*_int-F	CCGCTGCATCATTATTATCTC	
*Eipgcm*_int-R	GAGATAATAATGATGCAGCGGGCGGAGCAGAACCAGTTTAGT	
*EitefG*_ext-F	AA**GAGCTC**ccaggagcttttaatggcaac	SacI
*EitefG*_ext-R	AA**TCTAGA**acctttgaagaacggagtatga	XbaI
*EitefG*_int-F	ATTGAAGCACGTGGCAAAT	
*EitefG*_int-R	ATTTGCCACGTGCTTCAATGTAACGCGAAATGGGTGTTGTA	
*EimalE*_ext_F	AA**CTGCAG**TGGGTGACGTAAATCATGGTG	PstI
*EimalE*_int_R	CAGACCGTTATACCCCTTGTC	
*EimalE*_int_F	GACAAGGGGTATAACGGTCTGGGTGAAATCATGCCGAACATC	
*EimalE*_ext_R	AA**GAGCTC**GGTAAAGATGACGGTCAGCAC	SacI
*EilamB*_ext_F	AA**CTGCAG**CTCTGGCTGTGGTAAATCGAC	PstI
*EilamB*_int_R	GGTATCACACTCGTTGCCAAG	
*EilamB*_int_F	CTTGGCAACGAGTGTGATACCACCTATGCGAACGGTAAGAAC	
*EilamB*_ext_R	AA**GAGCTC**CACCAGTCATCAAGCCACATCC	SacI
*EitypA*_ext_F	AATCTAGAACGGGTAGCAACTTCGTTCTG	XbaI
*EitypA*_int_R	AATGGCGATGTTACGCAGAT	
*EitypA*_int_F	ATCTGCGTAACATCGCCATTAAGCGTCATCTGACCGAGAAC	
*EitypA*_ext_R	AA**GAGCTC**GGAAAGCGGTAAATAGGGAGA	SacI

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

The PCR reaction volume was 25 µl, and the reaction was set as 100 ng *E. ictaluri* wt gDNA, 0.5 µl of 10 mM forward and reverse primers, 0.5 µl 10 mM dNTPs, 5 µl 5x buffer, 0.025 U goTaq DNA polymerase, and dd H2O. External forward and internal reverse primers were used to amplify product 1 (P1), while internal forward and external reverse primers were used to amplify product 2 (P2). The PCR cycles to amplify P1 and P2 were as follows: denaturation at 95^{o}C for 2 min, 30 cycles of 95 ^{o}C for 30 sec, 56 ^{o}C for 30 sec, and 72 ^{o}C for 2 min, and extension at 72 ^{o}C for 10 min. For overlap extension PCR, the reaction was set similarly except that 2 µl of equally mixed upstream and downstream PCR products (P1+P2) were used as template and annealing and extension times were 2 min and 3 min, respectively. All PCR products were checked with 1% agarose gel in 0.5 x TBE buffer. The external forward and reverse primers were used to amplify the recombinant fragment. The specific DNA band with the right size was recovered from the gel using Wizard® SV Gel and PCR Clean-Up System (Promega) or QIAquick Gel Extraction Kit (Qiagen) following manufacture's protocol. The recombinant DNA fragment was reamplified to obtain enough DNA.

The recombinant DNA (insert) and pMEG-375 plasmid (vector) were digested with the specific RE added to the external primers of each target gene. The digested insert and plasmid were checked with 1% agarose gel with the non-digested plasmid as control. 1 kb plus ladder from Invitrogen was used as a size marker. The insert and plasmid were gel-purified as above. 1% Agarose gel was used to check RE digestion efficiency with 1 µl of the RE digested product for both the insert and vector. The rest were gel purified as above to obtain the purified product following the protocol provided with the Kit. T4 ligase (Promega) was used to ligate digested in-frame deleted gene and pMEG-375

according to the molar ratio of vector:insert = 1 : 3. The ligation was performed at 4 ^{o}C overnight. 2 µl of the ligation was used for transformation with *E. coli* CC118 λ*pir* chemical competent cells and incubated for 1 h in SOC medium at 37 ^{o}C, 200 rpm. All pelleted bacteria were spread on LB agar plates containing 100 µg/ml ampicillin and incubated at 37 ^{o}C overnight. The following day, ampicillin resistant colonies were picked and cultured in LB broth with ampicillin at 37 ^{o}C overnight with aeration. Plasmids were isolated from the overnight culture and checked on agarose gel with empty pMEG-375 as control. The larger size plasmids were cut with REs for insert confirmation, followed by sequencing with BigDye Terminator v1.1 Cycle Sequencing Kit (Invitrogen). Frozen stocks were prepared for all colonies for *E. coli* CC118 λ*pir* including positive plasmids. The procedure for in vitro mutant construction was illustrated in Figure 6.1.

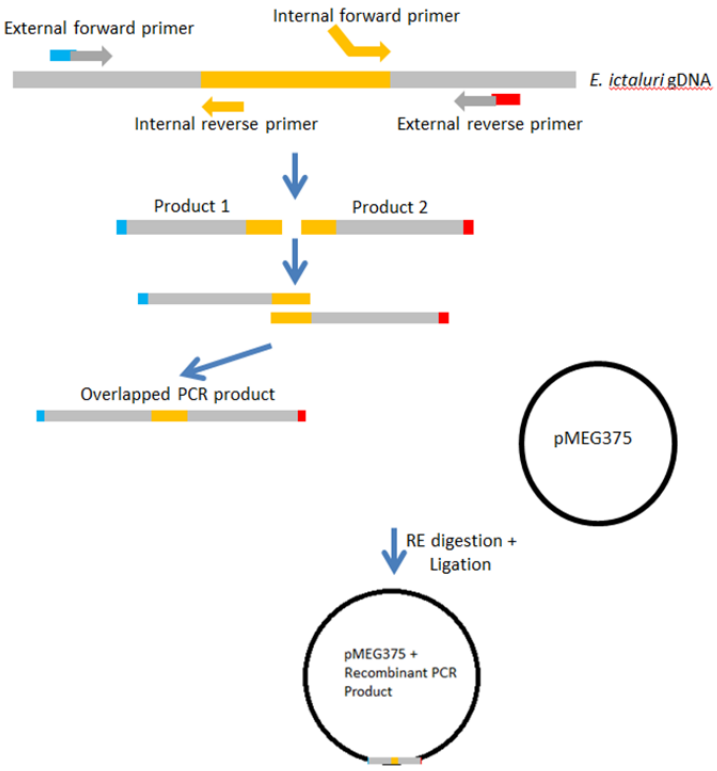

Figure 6.1 The process to construct *E. ictaluri* in-frame deletion mutants *in vitro*.

The arrows stand for primers; the blue and red tails at the end of external primers stand for the RE sites for cloning; the orange tail at the end of internal forward primer stands for the reverse complemented sequence of the internal reverse primer for producing the overlapped product.

The confirmed recombinant pMEG-375 containing the target insert was transferred into *E. coli* SM10 λ*pir* electro-competent cells with BioRad electroporator (BioRad), and isolated plasmid from *E. coli* SM10 λ*pir* was confirmed through gel electrophoresis. Frozen stocks were prepared for all colonies for *E. coli* SM10 λ*pir* strain including positive plasmid and kept at -80 ^{o}C. SOC medium was used for incubation after electroporation. The recombinant plasmid was then introduced into *E. ictaluri* wt genome by conjugation to allow the allelic exchange to occur through the homologous region between the cloned recombinant DNA fragment with in-frame deleted gene and *E.*

ictaluri chromosome. Two step screening method was conducted to screen the correct *E. ictaluri* mutant. In the first step, *E. ictaluri* colonies were selected using ampicillin because the entire plasmid incorporated into the *E. ictaluri* chromosome through a single crossover. In the second step, double cross-over mutants were selected on LB with 5% sucrose, 0.35% D-mannitol, and 12.5 µg/ml colistin because the suicide plasmid contains the sacB gene, which kills bacteria with integrated plasmid. At each step, colony PCR was used to check the genotype of colonies using external forward and reverse primers. For the mutants with right band observed from the colony PCR product, ampicillin sensitivity test was constructed to ensure the loss of plasmid. Final verification of the *E. ictaluri* mutants was done by sequencing using a gene specific primer designed next to the deleted region (Table 6.3).

Table 6.3 Primers used for sequencing to confirm the deletion region in *E. ictaluri* mutants.

Primer Name	Sequence
*EiyscR*_FS01	GGAGAACCCATCATGTCTCTG
*EiyscR*_S01	CAACACCACCGGTAAAGAGAG
*Eiblc*_FS01	GGAACGGGTGTAAAGGTAAGG
*Eiblc*_RS01	GCAGCAACGCTATCCTATCC
*EiompN*_FS01	AGTTCTCACTCGCAGCACCA
*EiompN*_RS01	TCACAATCCTCTATGCCTGTC
*Eihp2312*_FS01	CTCTCCTGCGATGTACCTTGA
*Eihp2312*_RS01	GAGATGCCCCACCATTTGAG
*EigpmA*_FS01	GGGTTATTGCATTCCACACACT
*EigpmA*_RS01	GAGTTAAGAAGGCGGGCTAGGT
*Eihp3311*_FS01	ACCTGCAAATCCACCACAAC
*Eihp3311*_RS01	AGTTCACCGCCAGACCATAA
*EiompW*_FS01	GATGTGATCTCGAATGAATCAA
*EiompW*_RS01	CTCGGTCGAAAACGATATGC
*EissaV*_FS01	CGATGCACTGTGGATTTCAG
*EissaV*_RS01	GCATGGGTGTCTCCTATCAGT
*EilamB*_Fs01	GTCGACTTTCACGGCTATGC
*EiLamB*_RS01	CTCAGCCCCTTACCACCAAG
*EimalE*_FS01	CGACAGGACTACAAGGACTGC
*EimalE*_RS01	GTGCCTGAGTCACGCTCTGA
*EitypA*_FS01	AGTCCCTCGCTTAATACGTG
*EitypA*_RS01	GTGGTATCACACCGACACGA

Virulence and efficacy of *E. ictaluri* mutants in catfish fingerlings

Specific pathogen free catfish were obtained from the MSU-CVM Specific Pathogen Free Catfish Hatchery. Virulence and efficacy trial were conducted as reported by our group (Karsi et al., 2009; Karsi and Lawrence, 2007; Karsi et al., 2006) by following institutional guidelines of Animal Care and Use Committee. 748 8-week old SPF channel catfish fingerlings (6.85 $\pm$ 0.45 cm, 2.86 $\pm$ 0.71 g) were stocked into 44 40-liter tanks at a rate of 17 fish/tank. Each treatment group (11 *E. ictaluri* in-frame deletion mutants) was randomly assigned to three tanks respectively, while positive (*E. ictaluri*

wt) and negative (BHI) control groups were randomly assigned to four tanks. After one week of acclimation, fish were challenged by immersion with 2.5 x 10^7 CFU/ml water for 1 h. Mortalities were recorded daily for 21 days, and the total percent mortalities were calculated for each treatment group.

To determine the efficacy of the *E. ictaluri* in-frame deletion mutants, the vaccinated and non-vaccinated groups were re-challenged with *E. ictaluri* wt (4.79 x 107 CFU/ml water) after 21 days post vaccination as described above. Fish mortalities were recorded daily for 14 days, and relative percent survival was determined for each group. Relative percent survival (RPS) was calculated according to the formula RPS = [1-(% mortality of vaccinated fish/% mortality of non-vaccinated fish)] x 100 (Amend, 1981) which expresses the proportion of fish saved due to vaccination.

Results

Selection of the *E. ictaluri* target genes for mutant construction

The 11 selected virulence genes were all identified from microarray experiment as described in material and method. The eleven genes include 3 genes encoding outer membrane proteins, 2 genes encoding T3SS proteins, 2 genes encoding hypothetical proteins, and *malE*, *lamB*, *typA*, and *gpmA* (Table 6.4).

Table 6.4 Eleven virulence genes being knocked out in this study.

Gene symble	GI	Protein product	Experiment	Deletion (%)
malE	238918180	Maltose ABC transporter periplasmic protein	Chapter II, IV	76.88
typA	238921701	GTP-binding protein typa/bipa	Chapter V	95.22
lamB	238918184	Maltoporin	Chapter II, IV	74.82
blc	238918845	Outer membrane lipoprotein blc	Chapter II, IV	79.14
ssaV	238918892	Type III secretion system apparatus protein ssav	Chapter II	93.88
yscR	238918894	Type III secretion system protein yscr	Chapter II, IV, III	83.80
ompW	238919586	Outer membrane protein W	Chapter IV	85.51
ompN	238919771	Outer membrane protein N	Chapter II, V	95.19
hp_2312	238920203	Hypothetical protein	Chapter II, IV	92.73
gpmA	238920733	Phosphoglyceromutase	Chapter II, IV	84.86
hp_3311	238921170	Hypothetical protein	Chapter II, IV, V	65.88

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

P1 and P2 of each gene have been amplified (Figure 6. 2) and in-frame deleted overlap extension products have been obtained (Figure 6. 3). All in-frame deletion recombinants for each target gene were successfully cloned into pMEG-375 suicide plasmid (Figure 6. 4).

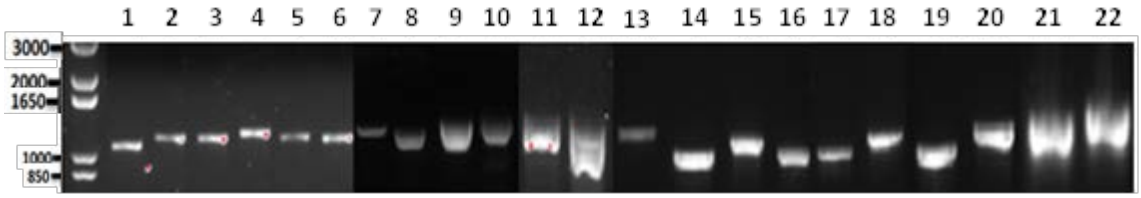

Figure 6.2 Agarose gel image of single PCR product 1, product 2 of 11 *E. ictaluri* genes.

1:*hp2312*-P2; 2:*hp2312*-P1; 3:*gpmA*-P1; 4:*gpmA*-P2; 5:*hp3311*-P2; 6:*hp3311*-P1; 7:*lamB*-P2; 8:*lamB*-P1; 9:*malE*-P2; 10:*malE*-P1; 11:*typA*-P2; 12:*typA*-P1; 13:*blc*-P1; 14:*blc*-P2; 15:*yscR*-P1; 16:*yscR*-P2; 17:*ompN*-P1; 18:*ompN*-P2; 19:*ssaV*-P1; 20:*ssaV*-P2; 21:*ompW*- P1; 22:*ompW*-P2

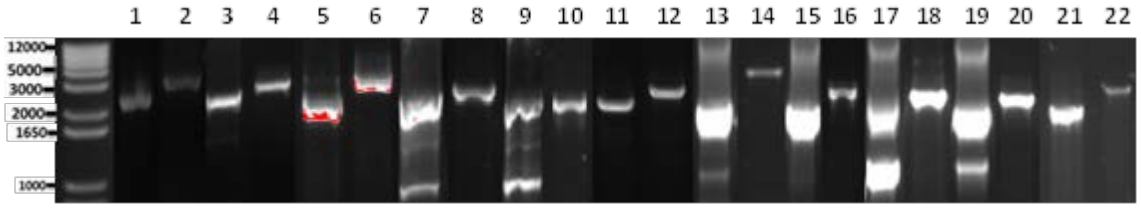

Figure 6.3 Agarose gel image of overlapped recombinant PCR product of 11 *E. ictaluri* genes.

1: Δ*malE*; 2: *malE*; 3: Δ*lamB*; 4: *lamB*; 5: Δ*typA*; 6: *typA*; 7: Δ*hp2312*; 8: *hp2312*; 9: Δ*hp3311*; 10: *hp3311*; 11: Δ*gpmA*; 12: *gpmA*; 13: Δ*ssaV*; 14: *ssaV*; 15: Δ*ompW*; 16: *ompW*; 17: Δ*yscR*; 18: *yscR*; 19: Δ*blc*; 20: *blc*; 21: Δ*ompN*; 22: *ompN*

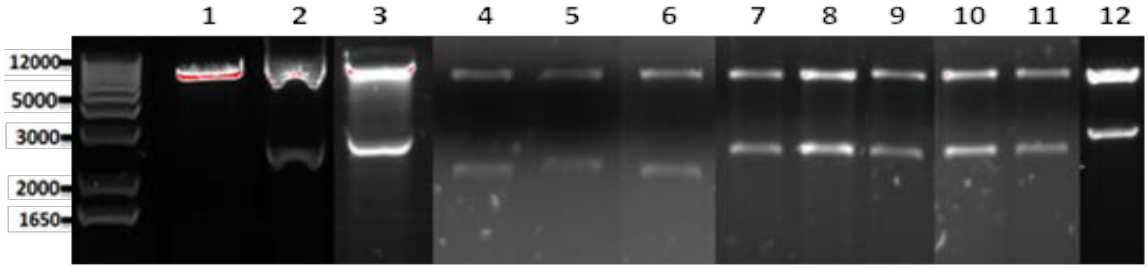

Figure 6.4 Confirmation of successfully cloning by RE digestion

1:linear pMEG-375; 2:p*Ei*Δ*malE*; 3:p*Ei*Δ*typA*; 4:p*Ei*Δ*hp2312*; 5:p*Ei*Δ*gpmA*; 6:p*Ei*Δ*hp3311*; 7:p*Ei*Δ*blc*; 8:p*Ei*Δ*ompW*; 9:p*Ei*Δ*ompN*; 10:p*Ei*Δ*ssaV*; 11:p*Ei*Δ*yscR*; 12:p*Ei*Δ*lamB*

E. ictaluri in-frame deletion mutants have been obtained for each gene listed in Table 6.1 above. The results of first and secondary selection of *E. ictaluri* mutant were confirmed with colony PCR (Figure 6.5; Figure 6.6).

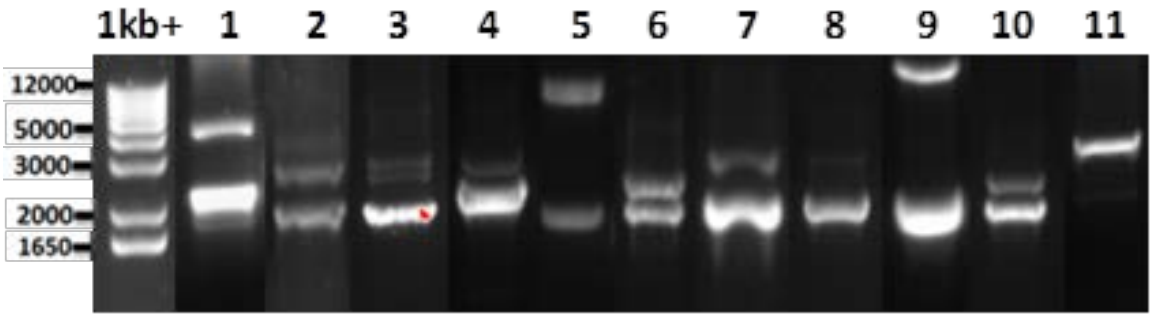

Figure 6.5 Colony PCR results after single crossover

1:*Ei*Δ*malE*; 2:*Ei*Δ*typA*; 3:*Ei*Δ*hp2312*; 4:*Ei*Δ*gpmA*; 5:*Ei*Δ*hp3311*; 6:*Ei*Δ*blc*; 7:*Ei*Δ*ompW*; 8:*Ei*Δ*ompN*; 9:*Ei*Δ*ssaV*; 10:*Ei*Δ*yscR*; 11:*Ei*Δ*lamB*

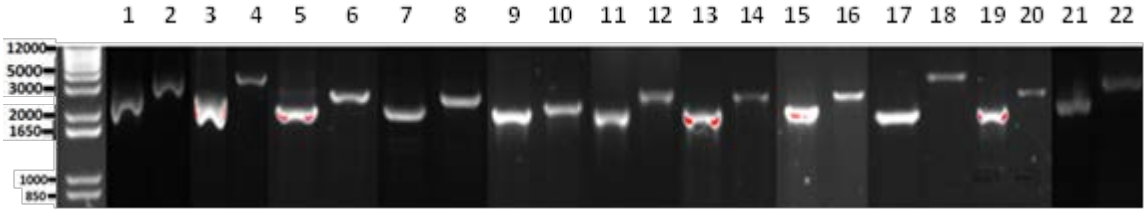

Figure 6.6 Colony PCR results after 2nd crossover under sucrose selection

1:*Ei*Δ*malE*; 2:*EimalE*; 3: *Ei*Δ*typA*; 4: *EitypA*; 5: *Ei*Δ*hp2312*; 6: *Eihp2312*; 7: *Ei*Δ*gpmA*; 8: *EigpmA*; 9: *Ei*Δ*hp3311*; 10: *Eihp3311*; 11: *Ei*Δ*blc*; 12: *Eiblc*; 13: *Ei*Δ*ompW*; 14: *EiompW*; 15: *Ei*Δ*ompN*; 16: *EiompN*; 17: *Ei*Δ*ssaV*; 18: *EissaV*; 19: *Ei*Δ*yscR*; 20: *EiyscR*; 21: *Ei*Δ*lamB*; 22: *EilamB*

Virulence and efficacy of *E. ictaluri* in-frame deletion mutants

The results revealed that *Ei*Δ*ssaV* and *Ei*Δ*yscR* have been attenuated completely (Figure 6.7). After 21 days post challenge, catfish mortality was 0% in the fish challenged with *Ei*Δ*ssaV* and *Ei*Δ*yscR*, respectively, while *E. ictaluri* wt caused 58.9%

mortality. For the other 9 *E. ictaluri* in-frame deletion mutants, their virulence was either less attenuated, not attenuated, or improved compared to the mortality of the wt strain.

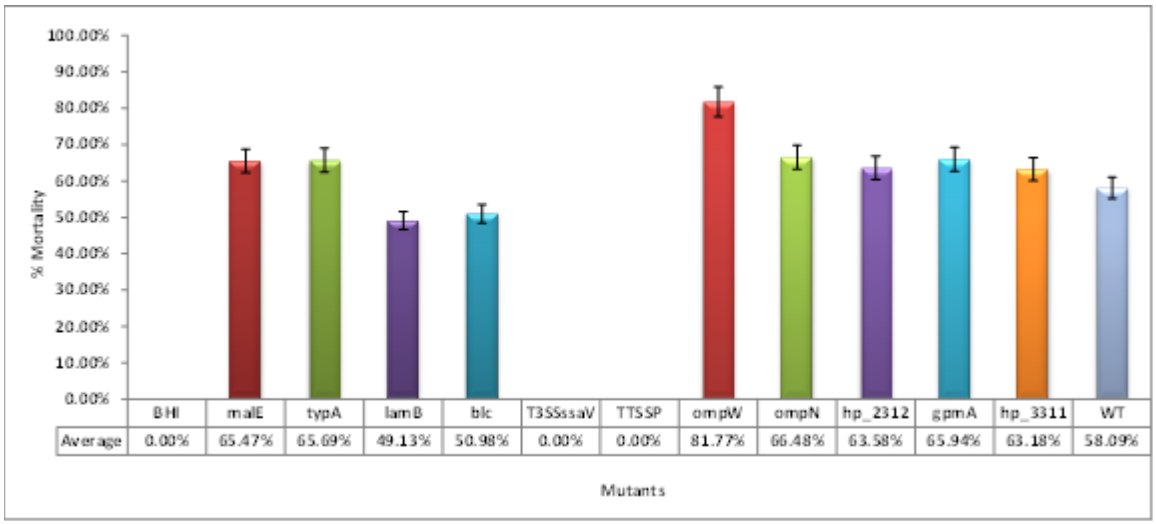

Figure 6.7 Percent mortalities of channel catfish fingerlings challenged with the 11 *E. ictaluri* in-frame deletion mutants and wt.

The evaluation of the mutants' efficacy using the channel catfish fingerlings from the virulence trial showed that mortality in the groups vaccinated with *E. ictaluri* mutants were lower than that of sham vaccinated group. The relative percent survival (RPS) rate of the groups vaccinated with *E. ictaluri* mutants was much higher than the RPS of sham vaccinated group (0%) (Figure 6.8).

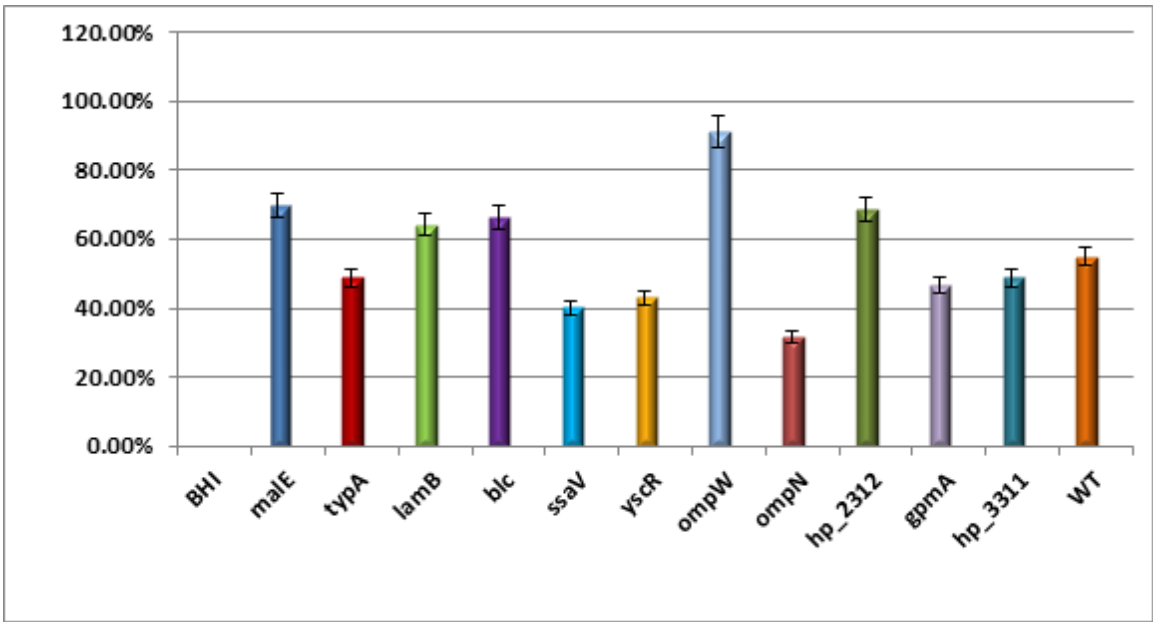

Figure 6.8 Percent survival of the channel catfish fingerlings vaccinated with the 11 *E. ictaluri* mutants post challenge with the *E. ictaluri* WT strain.

The low mortality and relative high RPS of *Ei*Δ*ssaV* and *Ei*Δ*yscR* indicated that the absence of these two genes reduced virulence of *E. ictaluri* and provided moderate protection in channel catfish fingerlings.

Discussion

The aim of this research was to construct and characterize 11 DEGs of *E. ictaluri*. The result of virulence and efficacy of *E. ictaluri* in-frame deletion mutants in fish trial showed that type III secretion system (T3SS) is important in the pathogenesis of *E. ictaluri*. In present study, two mutants from T3SS, *Ei*Δ*ssaV*, and *Ei*Δ*yscR*, gave 0% mortality in virulence trial with catfish fingerlings. YscR is a protein located on the bacterial inner membrane. In-frame deletion mutant of *yscR* in *Yersinia pestis* caused defective secretion of certain virulence related proteins (Fields et al., 1994). SsaV is

thought to be integral to the inner bacterial cell membrane and is part of the needle-like organelle that excretes pathogenic proteins across inner-membrane. The mutation of *ssaV* in *Salmonella* caused loss function of secreting SPI-2 (*Salmonella* pathogenicity island 2) T3SS effectors (Hansen-Wester et al., 2002; Nikolaus et al., 2001). Our result showed that *yscR* and *ssaV* were also important virulence genes in *E. ictaluri*'s pathogenesis because of the completely attenuated mortality of *Ei*Δ*yscR* and *Ei*Δ*ssaV* in the fingerling trial. The RPS of *Ei*Δ*yscR* and *Ei*Δ*ssaV* from the efficacy test were about 50% higher than that of sham-vaccinated group, but *Ei*Δ*yscR* and *Ei*Δ*ssaV* may not be effective live vaccine candidates. However, multiple genes in the *ssaV* and *yscR* mutants may be modified to optimize efficacy.

Outer membrane proteins give Gram-negative bacteria protection against tough environments and play important roles on translocation of solutes and proteins, as well as signal transduction across the membrane (Koebnik et al., 2000). Outer membrane protein W (ompW) was reported to provide significant protection in large yellow croakers when the fish being injected with purified ompW were challenged with *V. alginolyticus* ZJ04107 (Qian et al., 2007). It was also reported that a *V. cholera* 0139 strain SG25 *ompW* mutant by insertion caused 10-fold reduction in colonization ability (Nandi et al., 2005). The *Ei*Δ*ompW* mutant showed improved virulence in the fish virulence trial. This mutant caused high mortality (86.27%) in fish trial compared to 58.09% mortality of *E. ictaluri* wt. The deletion of *ompW* gene from *E. ictaluri* genome does not reduce this pathogen's virulence. Further studies are needed to resolve the function of *ompW* in bacterial virulence.

Blc is an outer membrane-bound lipoprotein, which binds fatty acids and phospholipids (Campanacci et al., 2006). When bacteria are at stationary growth phase and under high osmolarity, Blc was expressed to maintain the cell envelope integrity (Bishop et al., 1995). The deletion of *blc* from *E. ictaluri*'s genome did not cause any virulence attenuation perhaps because other genes may have compensated for the function of *blc*. Outer membrane protein N (ompN) was reported to be involved in adhesion and invasion that confers on pathogens' virulence. The *E. ictaluri* genome has three units of *ompN* (Neema et al., 2011). The deletion of one copy of this protein in *Ei*Δ*ompN* mutant caused 63.76% mortality similar to wt. The most possible reason may be the other two copies can still function normally.

In conclusion, our results indicate that the deletion of T3SS *ssaV* and T3SS *yscR* caused significant attenuation in *E. ictaluri* virulence. In the efficacy test, *Ei*Δ*ompW* provided good protection for catfish fingerlings when re-challenged with *E. ictaluri* wild type. The construction of double mutants of *ssaV* and *ompW* or *yscR* and *ompW* may prove to be effective vaccine candidates.

CHAPTER VII

CONCLUSIONS

This study aimed to identify new *E. ictaluri* virulence factors involved in recognition, attachment, and penetration of host mucosal membranes as well as in serum resistance. In *E. ictaluri* wild type host encounter experiment, 377 DEGs have been identified including 145 up-regulated genes and 232 down-regulated genes. In *E. ictaluri* wt exposed to SPF catfish serum, 16 DEGs have been identified including 3 up-regulated genes and 13 down-regulated genes. In *Ei*AKMut02 encountered with host and SPF catfish serum, 82 (13 up- and 69 down-regulated) and 296 DEGs (108 up- and 188 down-regulated) have been identified. In total, we have identified 38 potential *E. ictaluri* virulence factors. Second, 11 in-frame deletion mutants have been constructed and evaluated in catfish fingerlings. Among 11 *E. ictaluri* mutants, *Ei*Δ*ssaV*, *Ei*Δ*yscR* were completely attenuated, though they provided moderate protection against *E. ictaluri* wt.

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APPENDIX A

COMPLETE LIST OF UP-REGULATED GENES IN *E. ICTALURI* 93-146 DURING HOST ENCOUNTER

GI	Protein_product	FC
238921389	Putative carbamoyltransferase	6.83
238921388	Diaminopropionate ammonia-lyase	6.70
238921387	M20/dape family protein ygey	5.67
238921684	Argininosuccinate synthase, putative	5.43
238919743	Hypothetical protein	5.18
238921380	Hypothetical protein	4.64
238921232	Hypothetical protein	4.08
238920425	Hypothetical protein	3.98
238920953	Hypothetical protein	3.95
238920412	Arginine-binding periplasmic protein 2	3.75
238920875	Hypothetical protein	3.75
238919771	Outer membrane protein N	3.68
238918184	Maltoporin	3.62
238921683	Argininosuccinate lyase, putative	3.49
238921686	N-acetyl-gamma-glutamyl-phosphate reductase, putative	3.35
238921318	Ornithine carbamoyltransferase chain I, putative	3.23
238919760	Hypothetical protein	3.21
238921375	Guanine deaminase, putative	3.21
238920590	Phosphonopyruvate decarboxylase, putative	3.15
238921685	Acetylglutamate kinase, putative	3.12
238921385	Carbamate kinase, putative	3.07
238921379	Selenium metabolism protein ssna, putative	3.06
238921390	Hypothetical protein	3.02
238920591	Phosphoenolpyruvate phosphomutase, putative	3.00
238919889	Hypothetical protein	2.91
238920409	Arginine-binding periplasmic protein 1	2.88
238921170	Hypothetical protein	2.86
238920952	Hypothetical protein	2.84
238921349	Hypothetical protein	2.81
238919742	Pyruvate kinase, putative	2.79
238919970	Hypothetical protein	2.71
238920896	CTP synthase, putative	2.69
238920922	Hypothetical protein	2.69
238918794	Amino acid permease-associated region	2.68
238918110	Carbon starvation protein A	2.68
238920951	Hypothetical protein	2.67
238921206	Nucleoside-specific channel-forming protein tsx	2.65
238918136	Translation elongation factor Tu, putative	2.63
238920760	AHL-dependent transcriptional regulator	2.60

9230686	Unknown	2.60
238918070	Transcription termination factor Rho, putative	2.58
238919443	Putative transposase for insertion sequence element	2.55
238921243	Hypothetical protein	2.51
238921378	Oxidoreductases, FAD binding protein	2.51
238920247	Putative transposase for insertion sequence element	2.50
238921538	Putative ABC transporter, perplasmic amino acid binding protein	2.50
238918321	Hypothetical protein	2.49
238920111	Hypothetical protein	2.48
238920925	Hypothetical protein	2.47
238918241	Putative transposase for insertion sequence element	2.46
238921446	30S ribosomal protein S7, putative	2.45
238919232	Polysialic acid capsule biosynthesis protein neud	2.44
238921386	Dihydropyrimidinase, putative	2.42
238921759	Hypothetical protein	2.41
238921765	Ribonuclease P protein component, putative	2.40
238920083	Precorrin-6Y C5,15-methyltransferase	2.39
238919354	Hypothetical protein	2.37
238920408	Histidine transport ATP-binding protein hisp	2.37
238919417	Ispsy18, transposase	2.36
238920334	Xanthine permease	2.35
238921447	30S ribosomal protein S12, putative	2.32
238918057	Major facilitator superfamily MFS_1	2.32
238920352	Formate/nitrite transporter	2.31
238920926	Transposase, Mutator family	2.31
238919848	Hypothetical protein	2.31
238919362	Putative transposase for insertion sequence element	2.29
238921539	Polar amino acid ABC transporter, inner membrane subunit	2.29
238921376	Xanthine/uracil permease family protein	2.29
238919233	N-acetylneuraminate synthase	2.28
238918456	Hypothetical protein	2.28
238919950	Transposase, Mutator family	2.27
238918322	Hypothetical protein	2.26
238921138	Formate dehydrogenase, alpha subunit, putative	2.26
238920961	Ferritin and Dps	2.26
238920954	Hypothetical protein	2.25
238919655	Transposase, Mutator family	2.24
238918313	Transposase, Mutator family	2.24
238921139	4Fe-4S iron-sulfur binding domain protein	2.24
238920822	Lipoyl synthase, putative	2.24

238920941	Site-specific recombinase, phage integrase family	2.23
238920188	Hypothetical protein	2.23
238921554	Hypothetical protein	2.23
238918835	Putative L-2,4-diaminobutyrate decarboxylase	2.22
238919749	Hypothetical protein	2.21
238918699	Transcriptional regulator pdhr	2.20
238918155	Cation/acetate symporter actp, putative	2.20
238919415	Putative transposase for insertion sequence element	2.18
238918312	Transposase, Mutator family	2.18
238918458	Hypothetical protein	2.18
238918027	Transposase, Mutator family	2.18
238918807	Putative transposase for insertion sequence element	2.18
238919321	Phospholipase D3	2.17
238921654	Periplasmic repressor cpxp	2.17
238921303	Putative transposase for insertion sequence element	2.17
238918578	Lipoprotein, yaec family	2.17
238918907	Threonine dehydratase catabolic	2.17
238919066	Putative transposase for insertion sequence element	2.16
238921675	Hypothetical protein	2.15
238917984	Chromosomal replication initiator protein dnaa, putative	2.15
238918357	Hypothetical protein	2.15
238919545	Glyceraldehyde-3-phosphate dehydrogenase, type I, putative	2.15
238921148	Diaminopimelate decarboxylase, putative	2.14
238921195	Lysyl-trna synthetase, putative	2.14
238921474	Hypothetical protein	2.14
238919963	Transposase, Mutator family	2.13
238918580	D-methionine ABC transporter, ATP-binding protein, putative	2.13
238918451	Hypothetical protein	2.12
238918432	Inner membrane transport protein yhao	2.12
238920589	NAD-dependent aldehyde dehydrogenase, putative	2.10
238919388	Type II restriction enzyme	2.10
238921287	Glucuronide carrier protein	2.09
238920849	Hypothetical protein	2.09
238918222	Hypothetical protein	2.09
238921688	Acetylornithine deacetylase, putative	2.09
238921491	Phosphoenolpyruvate carboxykinase (ATP)	2.08
238920909	Periplasmic negative regulator of sigmae	2.08
9230685	Unknown	2.07
238918742	Prolipoprotein diacylglyceryl transferase, putative	2.07
238918514	Phosphopentomutase, putative	2.07

238919201	Hypothetical protein	2.07
238920821	Lipoyl(octanoyl) transferase, putative	2.07
238918435	Hypothetical protein	2.06
238920110	Is1 orf	2.06
238918065	Hypothetical protein	2.06
238921259	Cytochrome D ubiquinol oxidase subunit 1	2.05
238918325	Hypothetical protein	2.05
238921141	Hydrogenase nickel insertion protein hypa, putative	2.05
238918268	Hypothetical protein	2.04
238921381	Hypothetical protein	2.04
238918454	Hypothetical protein	2.03
238920035	Hypothetical protein	2.03
238921698	Hypothetical protein	2.03
238921295	Hypothetical protein	2.03
238920338	Hypothetical protein	2.03
238919866	DNA-binding transcriptional dual regulator	2.03
238920977	Is1 orf	2.02
238919399	Hypothetical protein	2.02
238920863	Hypothetical protein	2.02
238919550	Signal peptide peptidase sppa, 67K type, putative	2.02
238918180	Bacterial extracellular solute-binding protein, putative	2.02
9230687	Putative RNA one modulator protein	2.01
238918795	Orn/Lys/Arg decarboxylase family, putative	2.01
238921751	Phosphate ABC transporter, permease protein psta, putative	2.01
238921155	Hypothetical protein	2.01
238919416	Transposase, Mutator family	2.00

APPENDIX B

COMPLETE LIST OF DOWN-REGULATED GENES IN *E. ICTALURI* 93-146

DURING HOST ENCOUNTER

GI	Protein_product	FC
238919135	Peptidase M15B and M15C DD-carboxypeptidase vany/endolysin	-2.00
238918440	Hypothetical protein	-2.00
238918987	Hypothetical protein	-2.01
238919501	Crossover junction endodeoxyribonuclease ruvc, putative	-2.01
238921503	Hypothetical protein	-2.01
238919503	Holliday junction DNA helicase ruvb, putative	-2.02
238919309	Hypothetical protein	-2.02
238919748	Hypothetical protein	-2.03
238919254	Negative regulator of flagellin synthesis	-2.03
238918682	Cell division protein ftsa, putative	-2.03
238919207	Hypothetical protein	-2.03
238920639	Hypothetical protein	-2.03
238921241	Ribonuclease	-2.04
238920271	Ribonuclease, rnasee/rnaseg family , putative	-2.04
238919541	Hypothetical protein	-2.05
238919373	Hypothetical protein	-2.06
238920754	Hypothetical protein	-2.06
238920794	Asparagine synthase	-2.06
238918896	Type III secretion apparatus protein spar/ysct/hrct, putative	-2.06
238918887	Hypothetical protein	-2.06
238921434	Hypothetical protein	-2.07
238920432	D-alanyl-D-alanine carboxypeptidase dacc	-2.07
238918877	Type III secretion outer membrane pore, yscc/hrcc family, putative	-2.07
238921709	Hypothetical protein	-2.08
238918762	Hypothetical protein	-2.08
238919728	Phage shock protein A, putative	-2.08
238918705	Aconitate hydratase 2, putative	-2.08
238919084	Phosphomethylpyrimidine kinase, putative	-2.11
238919652	Hypothetical protein	-2.11
238918103	Cadmium-translocating P-type atpase, putative	-2.11
238918409	Ribosomal protein L27, putative	-2.11
238920646	Hypothetical protein	-2.11
238920524	Hypothetical protein	-2.12
238918798	Cytochrome c class I	-2.13
238919734	Hypothetical protein	-2.13
238919819	Hypothetical protein	-2.14
238920209	Trna (5-methylaminomethyl-2-thiouridylate)-methyltransferase, putative	-2.14
238919072	Hypothetical protein	-2.14
238920326	Peptidoglycan-binding domain 1 protein	-2.15

238921111	RNA polymerase sigma factor rpos, putative	-2.15
238919706	Hypothetical protein	-2.17
238920581	Hypothetical protein	-2.17
238920257	Aminodeoxychorismate lyase	-2.18
238920488	ABC transporter, periplasmic substrate-binding protein	-2.18
238919730	Phage shock protein C, putative	-2.19
238920773	KDP operon transcriptional regulatory protein kdpe	-2.19
238920297	Flagellin	-2.19
238920673	Transcriptional regulator, arsr family	-2.19
238919238	Hypothetical protein	-2.20
238918216	Copper/zinc superoxide dismutase, putative	-2.21
238920742	Tol-pal system-associated acyl-coa thioesterase, putative	-2.21
238920767	Hypothetical protein	-2.22
238919451	Hypothetical protein	-2.23
238919875	Hypothetical protein	-2.25
238921531	Hypothetical protein	-2.26
238920523	Hypothetical protein	-2.26
238919993	Pyridoxamine 5'-phosphate oxidase, putative	-2.26
238919345	Hypothetical protein	-2.27
238919562	Response regulator receiver protein	-2.27
238918867	Esal, putative	-2.27
238919040	DNA polymerase III subunit tau, putative	-2.28
238918898	Hypothetical protein	-2.28
238919215	Deoxycytidine triphosphate deaminase, putative	-2.29
238920014	Cation efflux system protein cusf	-2.29
238919375	Calcium/cation antiporter (caca)	-2.30
238919015	DNA-binding protein HU-beta	-2.31
238918392	3'(2'),5'-bisphosphate nucleotidase, putative	-2.33
238919836	Hypothetical protein	-2.33
238919783	Translation initiation factor IF-3, putative	-2.33
238919650	Universal stress protein family, putative	-2.34
238921702	Glutamine synthetase, type I, putative	-2.35
238919283	Hypothetical protein	-2.35
238918882	Hypothetical protein	-2.36
238919919	Hypothetical protein	-2.36
238920413	Methylated DNA protein cysteine S-methyltransferase family, putative	-2.36
238920876	Bifunctional protein fold, putative	-2.37
238921502	Hypothetical protein	-2.37
238919773	Hypothetical protein	-2.37
238918256	Hypothetical protein	-2.37

238919807	Hypothetical protein	-2.38
238918516	Soluble cytochrome b562	-2.38
238920204	Hypothetical protein	-2.38
238920104	Hypothetical protein	-2.40
238918937	Hypothetical protein	-2.40
238920231	Hypothetical protein	-2.41
238919020	HAD hydrolase, IIB family	-2.43
238921640	Hypothetical protein	-2.43
238919287	Hypothetical protein	-2.44
238919736	Hypothetical protein	-2.44
238919731	Hypothetical protein	-2.44
238918611	Hypothetical protein	-2.44
238921317	Hypothetical protein	-2.45
238918875	Hypothetical protein	-2.46
238918334	Hypothetical protein	-2.47
238918862	Hypothetical protein	-2.47
238919194	GTP cyclohydrolase I, putative	-2.47
238921051	Iron-sulfur cluster assembly protein isca, putative	-2.49
238920640	Hypothetical protein	-2.49
238919705	Hypothetical protein	-2.49
238920205	Prophage lambda integrase	-2.50
238920202	Hypothetical protein	-2.50
238920713	Hypothetical protein	-2.51
238919073	Hypothetical protein	-2.51
238918343	Hypothetical protein	-2.51
238918924	Hypothetical protein	-2.52
238920506	Hypothetical protein	-2.52
238918876	Type III secretion apparatus protein, yscd/hrpq family, putative	-2.53
238919209	Hypothetical protein	-2.56
238918485	Hypothetical protein	-2.56
238920215	Transcriptional regulatory protein phop	-2.56
238919704	Hypothetical protein	-2.58
238920382	Hypothetical protein	-2.58
238921634	Hypothetical protein	-2.58
238920998	Hypothetical protein	-2.58
238919762	Hypothetical protein	-2.61
238919288	Hypothetical protein	-2.61
238919801	Hypothetical protein	-2.62
238918254	Orn/Lys/Arg decarboxylase family, putative	-2.62
238919646	Hypothetical protein	-2.62

238920218	NAD-dependent deacetylase	-2.63
238921054	Iron-sulfur cluster assembly transcription factor iscr, putative	-2.63
238920974	Hypothetical protein	-2.67
238920381	ATP-dependent Clp protease ATP-binding subunit clpa, putative	-2.70
238919300	Hypothetical protein	-2.73
238918880	Escb	-2.76
238918253	Lysine/cadaverine transport protein	-2.76
238919535	Spovr family protein	-2.76
238919035	Hypothetical protein	-2.77
238921354	Hypothetical protein	-2.77
238919741	Hypothetical protein	-2.78
238919580	Hypothetical protein	-2.78
238919609	Hypothetical protein	-2.79
238919395	Hypothetical protein	-2.80
238921240	Barstar	-2.83
238921052	Fes cluster assembly scaffold iscu, putative	-2.84
238920054	Hypothetical protein	-2.89
238918707	Hypothetical protein	-2.89
238921530	Hypothetical protein	-2.89
238918574	Hypothetical protein	-2.89
238919905	Stability protein stbe	-2.90
238920200	Hypothetical protein	-2.93
238919540	Hypothetical protein	-2.94
238920332	Hypothetical protein	-2.94
238919502	Holliday junction DNA helicase ruva, putative	-2.95
238919918	Hypothetical protein	-2.95
238920679	Hypothetical protein	-2.96
238919452	Hypothetical protein	-2.98
238919912	Hypothetical protein	-3.01
238920272	Hypothetical protein	-3.01
238919997	Transcriptional regulator, marr family	-3.04
238919707	Hypothetical protein	-3.05
238918800	Hypothetical protein	-3.07
238918900	Hypothetical protein	-3.07
238920198	Hypothetical protein	-3.09
238918944	Hypothetical protein	-3.11
238920194	Hypothetical protein	-3.12
238920999	Hypothetical protein	-3.13
238919837	Hypothetical protein	-3.13
238919464	Hypothetical protein	-3.15

238918888	Hypothetical protein	-3.18
238920195	Hypothetical protein	-3.19
238919133	Hypothetical protein	-3.21
238919913	Hypothetical protein	-3.22
238918708	Hypothetical protein	-3.23
238918892	Regulatory protein	-3.29
238919009	Hypothetical protein	-3.33
238920199	Hypothetical protein	-3.41
238919626	Hypothetical protein	-3.42
238919177	Cell division protein zipa, putative	-3.45
238918186	Chorismate-pyruvate lyase, putative	-3.47
238920197	Hypothetical protein	-3.51
238919036	Hypothetical protein	-3.53
238920484	Hypothetical protein	-3.56
238918367	Trna delta(2) -isopentenylpyrophosphate transferase, putative	-3.59
238918732	RNA polymerase-binding protein dksa, putative	-3.60
238919832	Hypothetical protein	-3.61
238919700	Spheroplast protein Y	-3.73
238918845	Outer membrane lipoprotein blc	-3.73
238918681	Cell division protein ftsq	-3.73
238919446	Hypothetical protein	-3.79
238919184	Hypothetical protein	-3.81
238918861	Putative B-type cytochomre	-3.81
238919617	Hypothetical protein	-3.83
238920508	Transposon tn10 tetd protein (orfr)	-3.84
238918863	Hypothetical protein	-3.85
238918201	Repressor lexa, putative	-3.87
238919833	Hypothetical protein	-3.90
238920511	Hypothetical protein	-3.92
238919858	Uspa domain protein	-3.98
238918895	Esas	-4.03
238918890	Hypothetical protein	-4.07
238920510	Transcriptional regulator, tetr family	-4.08
238918868	Hypothetical protein	-4.10
238919142	Nitrate/nitrite response regulator protein narp	-4.10
238921353	Protein yedy	-4.11
238920001	Hypothetical protein	-4.14
238918921	Hypothetical protein	-4.22
238918894	Type III secretion apparatus protein, yscr/hrcr family, putative	-4.25
238919904	Hypothetical protein	-4.26

238920203	Hypothetical protein	-4.27
238919158	Dihydroxyacetone kinase, L subunit, putative	-4.27
238920003	Hypothetical protein	-4.33
238918889	Hypothetical protein	-4.36
238920474	Hypothetical protein	-4.38
238918870	Type III secretion apparatus lipoprotein, yscj/hrcj family, putative	-4.39
238918869	Hypothetical protein	-4.47
238919048	Acetate operon repressor	-4.49
238919587	Hypothetical protein	-4.59
238919157	PTS-dependent dihydroxyacetone kinase, dihydroxyacetone-bindingsubunit dhak	-4.74
238920733	Hypothetical protein	-4.96
238920002	Hypothetical protein	-4.98
238918878	Hypothetical protein	-5.04
238918893	Hypothetical protein	-5.22
238920936	DNA repair protein recn, putative	-5.27
238919187	Hypothetical protein	-5.34
238919616	Hypothetical protein	-5.35
238919303	Hypothetical protein	-5.41
238920599	Hypothetical protein	-5.49
238919311	Hypothetical protein	-5.49
238918871	Esai, putative	-5.60
238920238	Hypothetical protein	-5.69
238920230	Hypothetical protein	-5.77
238919522	Outer membrane protein slp	-5.85
238918891	Flagellum-specific ATP synthase	-5.94
238919826	Pyruvate:ferredoxin	-5.99
238920344	Integration host factor, beta subunit, putative	-6.05
238921352	Protein yedy	-6.11
238919788	Integration host factor, alpha subunit, putative	-6.12
238918872	Hypothetical protein	-6.68
238921575	Hypothetical protein	-6.81
238918873	Type III secretion apparatus needle protein, putative	-8.49
238918874	Esrc	-8.77

APPENDIX C

256 DEGs IN *E. ICTALURI* 93-146 ANNOTATED BY BLAST2GO DURING HOST ENCOUNTER

GI	GO ID	Protein product
238919734	GO:0016740	3-mercaptopyruvate sulfurtransferase
238918409	GO:0005840	50s ribosomal protein l27
238918937	GO:0009851	Abc transporter atp-binding protein
238921685	GO:0005737	Acetylglutamate kinase
238921688	GO:0005737	Acetylornithine deacetylase
238918705	GO:0003994	Aconitate hydratase 2
238920589	GO:0055114	Aldehyde dehydrogenase family protein
238921539	GO:0006810	Amino acid abc transporter
238920257	GO:0046656	Aminodeoxychorismate lyase
238920272	GO:0005737	Antibiotic biosynthesis monooxygenase
238920408	GO:0016887	Arginine abc atp-binding protein
238920409	GO:0006810	Arginine-binding periplasmic protein 1
238920412	GO:0006810	Arginine-binding periplasmic protein 2
238921683	GO:0005737	Argininosuccinate lyase
238921684	GO:0005737	Argininosuccinate synthase
238920794	GO:0004066	Asparagine synthase
238921389	GO:0016597	Aspartate ornithine carbamoyltransferase family protein
238920382	GO:0008233	Atp-dependent clp protease adaptor protein
238920381	GO:0009851	Atp-dependent clp protease atp-binding subunit
238919375	GO:0016021	Calcium proton antiporter
238918451	GO:0005886	Camphor resistance protein
238921385	GO:0008652	Carbamate kinase
238918110	GO:0016020	Carbon starvation protein
238919135	GO:0004180	Carboxypeptidase
238918155	GO:0005887	Cation acetate symporter
238919303	GO:0009432	Cell division inhibitor
238918681	GO:0051301	Cell division protein
238918682	GO:0005524	Cell division protein
238919177	GO:0007049	Cell division protein
238921640	GO:0007049	Cell division protein zapb
238921379	GO:0016810	Chlorohydrolase aminohydrolase
238918186	GO:0005737	Chorismate--pyruvate lyase
238917984	GO:0005737	Chromosomal replication initiator protein
238921052	GO:0005506	Cluster assembly scaffold
238920083	GO:0016491	Cobalt-precorrin-6y c -methyltransferase
238919020	GO:0008152	Cof-like hydrolase
238919617	GO:0060567	Cold shock protein
238918440	GO:0010181	Conserved protein
238918216	GO:0046872	Copper zinc superoxide dismutase
238920896	GO:0006541	Ctp synthetase
238919760	GO:0008610	Cyclopropane fatty acyl phospholipid synthase
238918798	GO:0020037	Cytochrome
238918862	GO:0022904	Cytochrome b561
238918516	GO:0000155	Cytochrome b562
238921259	GO:0055114	Cytochrome d ubiquinol oxidase subunit 1
238920432	GO:0006508	D-alanyl-d-alanine carboxypeptidase
238919215	GO:0009220	Deoxycytidine triphosphate deaminase

238921148	GO:0009089	Diaminopimelate decarboxylase
238921388	GO:0030170	Diaminopropionate ammonia-lyase
238921386	GO:0046872	Dihydropyrimidinase
238919157	GO:0006071	Dihydroxyacetone kinase
238919158	GO:0006071	Dihydroxyacetone l subunit
238918578	GO:0031225	Dl-methionine transporter substrate-binding subunit
238918580	GO:0009851	D-methionine abc atp-binding protein
238919040	GO:0009851	Dna polymerase iii subunits gamma and tau
238920936	GO:0005524	Dna repair protein
238921502	GO:0016226	Dna uptake protein
238920200	GO:0017111	Dna-binding protein
238919015	GO:0006350	Dna-binding protein hu-beta
238920506	GO:0003700	Dna-binding transcriptional regulator
238921390	GO:0017111	Dna-binding transcriptional regulator
238920230	GO:0006281	Dna-damage-inducible protein i
238921139	GO:0046872	Electron transport protein
238919207	GO:0004527	Endonuclease iv
238919819	GO:0016787	Esterase
238918762	GO:0003887	Exonuclease ix
238920326	GO:0008152	Family protein
238920297	GO:0005576	Flagellin
238919807	GO:0003824	Flavin reductase
238920524	GO:0010181	Flavodoxin nitric oxide synthase
238921138	GO:0055114	Formate dehydrogenase h
238920352	GO:0006810	Formate transporter 1
238918458	GO:0006064	Glucuronate isomerase
238921287	GO:0016020	Glucuronide transporter
238920484	GO:0006810	Glutamine abc transporter periplasmic protein
238921702	GO:0005737	Glutamine synthetase
238919545	GO:0006006	Glyceraldehyde-3-phosphate type i
238919833	GO:0005978	Glycogen synthesis protein
238919194	GO:0005737	Gtp cyclohydrolase i
238921375	GO:0008270	Guanine deaminase
238918611	GO:0016787	Had-superfamily hydrolase
238919311	GO:0009408	Heat shock protein
238919801	GO:0009851	Hemin transport system atp-binding protein
238919704	GO:0016787	Histidine triad protein
238919502	GO:0009432	Holliday junction dna helicase
238919503	GO:0009851	Holliday junction dna helicase
238919501	GO:0003676	Holliday junction resolvase
238921141	GO:0006464	Hydrogenase nickel incorporation protein
238918875	GO:0009405	Hypothetical protein NT01EI_0941 [Edwardsiella ictaluri 93-146]
238919646	GO:0016021	Inner membrane protein
238920523	GO:0016021	Inner membrane protein
238918392	GO:0016020	Inositol monophosphatase family protein
238918708	GO:0016020	Integral membrane protein
238919788	GO:0003677	Integration host alpha subunit
238920344	GO:0003677	Integration host factor subunit beta

238921051	GO:0005506	Iron-sulfur cluster assembly protein
238921054	GO:0046872	Iron-sulfur cluster assembly transcription factor
238920974	GO:0009061	Iron-sulfur cluster insertion protein
238920110	GO:0006313	Is1 orf
238920977	GO:0006313	Is1 orf
9230686	GO:0006313	Is4 family protein
238920849	GO:0003677	Is630 transposase
238918835	GO:0030170	L- -diaminobutyrate decarboxylase
238920821	GO:0005737	Lipoate-protein ligase b
238920822	GO:0005737	Lipoyl synthase
238919201	GO:0005886	Lrga family protein
238918253	GO:0016021	Lysine cadaverine antiporter
238918794	GO:0016021	Lysine cadaverine antiporter
238918254	GO:0030170	Lysine decarboxylase 1
238918795	GO:0030170	Lysine decarboxylase 1
238921195	GO:0005737	Lysyl-trna synthetase
238921387	GO:0046983	M20 family protein
238919970	GO:0055085	Major facilitator family transporter
238918057	GO:0055085	Major facilitator superfamily mfs_1
238919741	GO:0005624	Major outer membrane lipoprotein
238918184	GO:0015288	Maltoporin
238918180	GO:0055052	Maltose abc transporter periplasmic protein
238919184	GO:0016021	Manganese transport protein
238918357	GO:0055085	Mechanosensitive ion channel domain protein
238919036	GO:0016021	Membrane protein
238920876	GO:0009086	Methenyltetrahydrofolate cyclohydrolase
238920413	GO:0006281	Methylated-dna--protein-cysteine methyltransferase
238921381	GO:0005515	Molybdenum hydroxylase accessory family
238921352	GO:0046872	Molybdopterin binding
238920001	GO:0005737	Monothiol glutaredoxin
238918027	GO:0006313	Mutator family
238918312	GO:0006313	Mutator family
238918313	GO:0006313	Mutator family
238919416	GO:0006313	Mutator family
238919417	GO:0006313	Mutator family
238919655	GO:0006313	Mutator family
238919950	GO:0006313	Mutator family
238919963	GO:0006313	Mutator family
238920926	GO:0006313	Mutator family
238921686	GO:0005737	N-acetyl-gamma-glutamyl-phosphate reductase
238919232	GO:0055114	N-acetylneuraminate synthase
238919233	GO:0050462	N-acetylneuraminate synthase
238920218	GO:0070403	Nad-dependent deacetylase
238920599	GO:0044237	Nad-dependent epimerase dehydratase family protein
238919254	GO:0019861	Negative regulator of flagellin synthesis
238919142	GO:0000160	Nitrate nitrite response regulator protein
238921206	GO:0004872	Nucleoside-specific channel-forming protein tsx
238921318	GO:0006526	Ornithine carbamoyltransferase

238918845	GO:0005215	Outer membrane lipoprotein blc
238919771	GO:0009279	Outer membrane protein
238919522	GO:0019867	Outer membrane slp family
238918863	GO:0055114	Oxidoreductase molybdopterin binding protein
238919875	GO:0016805	Peptidase family c69
238919700	GO:0042597	Periplasmic stress adaptor protein
238919730	GO:0003677	Phage shock protein c
238920204	GO:0003677	Phage-related dna-binding protein
238918485	GO:0004810	Phosphatase
238921751	GO:0016021	Phosphate transporter permease subunit
238921491	GO:0005737	Phosphoenolpyruvate carboxykinase
238920591	GO:0050188	Phosphoenolpyruvate phosphomutase
238919133	GO:0016787	Phospholipase carboxylesterase
238919321	GO:0008152	Phospholipase d family protein
238919084	GO:0008972	Phosphomethylpyrimidine kinase
238920590	GO:0033980	Phosphonopyruvate decarboxylase
238918514	GO:0005737	Phosphopentomutase
238921538	GO:0006810	Polar amino acid abc periplasmic amino acid-binding protein
238918742	GO:0016021	Prolipoprotein diacylglyceryl transferase
238920205	GO:0008907	Prophage lambda integrase
238919550	GO:0006465	Protease 4
238918334	GO:0005515	Protein
238919009	GO:0003677	Protein
238919993	GO:0008615	Pyridoxamine 5 -phosphate oxidase
238918699	GO:0005622	Pyruvate dehydrogenase complex repressor
238919742	GO:0004743	Pyruvate kinase
238919826	GO:0030976	Pyruvate-flavodoxin oxidoreductase
238920760	GO:0000160	Quorum-sensing transcriptional regulator
238919866	GO:0043565	Regulatory protein
238918201	GO:0006281	Repressor
238919562	GO:0000160	Response regulator receiver protein
238921241	GO:0004521	Ribonuclease
238921698	GO:0004540	Ribonuclease bn
238920271	GO:0004540	Ribonuclease e
238921765	GO:0001682	Ribonuclease p
238921434	GO:0005840	Ribosomal protein l29
238921447	GO:0006412	Ribosomal protein s12
238921446	GO:0003735	Ribosomal protein s7
238921554	GO:0003676	Rna family
238921111	GO:0003700	Rna polymerase sigma factor
238918732	GO:0008270	Rna polymerase-binding transcription factor
238920511	GO:0016020	Secretion protein family protein
238918873	GO:0015031	Secretion system apparatus
238918867	GO:0046903	Secretion system apparatus protein
238918892	GO:0016021	Secretion system apparatus protein
238921378	GO:0055114	Selenate fad-binding subunit
238921380	GO:0055114	Selenate subunit
238919541	GO:0016787	Serine protein

238918432	GO:0006810	Serine transporter
238919848	GO:0055114	Short chain dehydrogenase
238920909	GO:0042597	Sigma-e factor regulatory protein
238920941	GO:0003677	Site-specific phage integrase family
238919072	GO:0008233	Spfh domain band 7 family protein
238920961	GO:0008199	Stress response dna-binding protein
238920488	GO:0005215	Substrate-binding transport protein
238921353	GO:0046872	Sulfite oxidase subunit
238921354	GO:0016491	Sulfite oxidase subunit
238918895	GO:0016021	T3ss structure protein
238920338	GO:0008690	Tetraacyldisaccharide 4 -kinase
238919707	GO:0016301	Thiamine kinase
238918268	GO:0030976	Thiamine pyrophosphate protein tpp binding domain protein
238919736	GO:0045454	Thiol peroxidase
238918861	GO:0022904	Thiosulfate reductase cytochrome b subunit (membrane anchoring protein)
238921530	GO:0006071	Thiosulfate sulfurtransferase
238918907	GO:0005737	Threonine dehydratase
238920742	GO:0005515	Tol-pal system-associated acyl- thioesterase
238920998	GO:0043565	Toxin-antitoxin antitoxin xre family
238918070	GO:0003723	Transcription termination factor rho
238918874	GO:0043565	Transcriptional family
238920508	GO:0043565	Transcriptional family
238920510	GO:0003700	Transcriptional family
238920673	GO:0005622	Transcriptional family
238919997	GO:0005622	Transcriptional regulator
238920215	GO:0000160	Transcriptional regulatory protein
238919048	GO:0003677	Transcriptional repressor
238919919	GO:0043565	Transcriptional xre family
238918898	GO:0016787	Transglycosylase signal peptide protein
238918136	GO:0005737	Translation elongation factor tu
238919783	GO:0005737	Translation initiation factor if-3
238918454	GO:0055085	Transport protein
238920035	GO:0006313	Transposase
238920111	GO:0003677	Transposase
238918241	GO:0006313	Transposase for insertion sequence element
238918807	GO:0006313	Transposase for insertion sequence element
238919066	GO:0006313	Transposase for insertion sequence element
238919362	GO:0006313	Transposase for insertion sequence element
238919415	GO:0006313	Transposase for insertion sequence element
238919443	GO:0006313	Transposase for insertion sequence element
238920247	GO:0006313	Transposase for insertion sequence element
238921303	GO:0006313	Transposase for insertion sequence element
238921243	GO:0016021	Trap subunit
238920209	GO:0005737	Trna (5-methylaminomethyl-2-thiouridylate)-methyltransferase
238918367	GO:0009851	Trna delta -isopentenylpyrophosphate transferase
238918900	GO:0000156	Two component transcriptional family
238920773	GO:0000160	Two component transcriptional winged helix family
238919388	GO:0009307	Type ii restriction enzyme

238918871	GO:0009306	Type iii secretion apparatus
238918894	GO:0016021	Type iii secretion apparatus family
238918880	GO:0006950	Type iii secretion low calcium response chaperone
238918877	GO:0009279	Type iii secretion outer membrane family
238918896	GO:0016021	Type iii secretion protein
238918870	GO:0009306	Type iii secretion system apparatus lipoprotein
238918891	GO:0015078	Type iii secretion system atpase
238918435	GO:0005515	U32 family
238921575	GO:0005886	Universal stress protein
238919858	GO:0005737	Universal stress protein e
238919650	GO:0006950	Universal stress protein f
238920334	GO:0055085	Xanthine permease
238921376	GO:0055085	Xanthine uracil permease family protein
238918103	GO:0008551	Zinc cadmium mercury lead-transporting atpase
238918065	GO:0003755	#Name?
238918921	GO:0004872	#Name?
238920733	GO:0005737	#Name?

APPENDIX D

56 DEGs REPRESENTED IN MVirDB IN *E. ICTALURI* 93-146 DURING HOST ENCOUNTER

Query	Description	Greatest identity %
gi|238918180|ref|YP_002931694.1|	Full=Maltose-binding periplasmic protein; altname: Full=MBP; altname: Full=MMBP; altname: Full=Maltodextrin-binding protein; Flags: Precursor;	100
gi|238918216|ref|YP_002931730.1|	Polysaccharide synthesis enzyme Cap8O [Staphylococcus aureus subsp. Aureus MW2]	66
gi|238918253|ref|YP_002931767.1|	Lysin/cadaverin transporter [Escherichia coli]	64
gi|238918254|ref|YP_002931768.1|	Cadaverin decarboxylase [Escherichia coli]	66
gi|238918440|ref|YP_002931954.1|	Protein STM3270 [Salmonella typhimurium LT2]	48
gi|238918580|ref|YP_002932094.1|	Of the ABC transporter complex bceab TC 3.A.1.123.5 involved in bacitracin export. Responsible for energy coupling to the transport system.	40
gi|238918794|ref|YP_002932308.1|	Lysin/cadaverin transporter [Escherichia coli]	66
gi|238918795|ref|YP_002932309.1|	Cadaverin decarboxylase [Escherichia coli]	67
gi|238918845|ref|YP_002932359.1|	Full=lipoprotein lpp20;	93
gi|238918863|ref|YP_002932377.1|	Protein [Escherichia coli]	69
gi|238918870|ref|YP_002932384.1|	III secretion system apparatus lipoprotein [Yersinia pestis CO92]	54
gi|238918874|ref|YP_002932388.1|	Family transcriptional regulator [Yersinia pestis CO92]	56
gi|238918876|ref|YP_002932390.1|	Type-III secretion protein [Yersinia pestis CO92]	50
gi|238918877|ref|YP_002932391.1|	Full=FLAGELLIN; subname: Full=Flagellin;	90
gi|238918891|ref|YP_002932405.1|	III secretion system atpase [Yersinia pestis CO92]	59
gi|238918892|ref|YP_002932406.1|	System apparatus protein ssav [Yersinia pestis CO92]	53
gi|238918894|ref|YP_002932408.1|	Type III secretion apparatus protein [Yersinia pestis CO92]	91
gi|238918896|ref|YP_002932410.1|	Type III secretion apparatus protein [Yersinia pestis CO92]	71
gi|238918900|ref|YP_002932414.1|	Two-component response regulator [Yersinia pestis CO92]	49
gi|238918937|ref|YP_002932451.1|	III ABC transporter ATP-binding protein [Haemophilus influenzae Rd KW20]	66
gi|238919015|ref|YP_002932529.1|	Binding	93
gi|238919142|ref|YP_002932656.1|	Component transcriptional regulatory protein devr [Mycobacterium tuberculosis H37Rv]	42
gi|238919232|ref|YP_002932747.1|	Protein [Streptococcus agalactiae 2603V/R]	36
gi|238919233|ref|YP_002932748.1|	Neuramic acid synthetase neub [Streptococcus agalactiae 2603V/R]	57
gi|238919254|ref|YP_002932769.1|	Factor flgm [Yersinia enterocolitica subsp. Enterocolitica 8081]	65
gi|238919311|ref|YP_002932826.1|	Nonribosomal peptide synthetase [Yersinia pestis KIM]	52
gi|238919321|ref|YP_002932836.1|	D	26
gi|238919416|ref|YP_002932931.1|	Full=IL-1 beta binding protein C9R; subname: Full=Interleukin 1 beta receptor;	36
gi|238919736|ref|YP_002933251.1|	Peroxidase [Vibrio cholerae O1 biovar El Tor str. N16961]	41

gi|238919742|ref|YP_002933257.1|	Kinase [Salmonella typhimurium LT2]	88
gi|238919760|ref|YP_002933275.1|	[Mycobacterium tuberculosis H37Rv]	39
gi|238919771|ref|YP_002933286.1|	Full=Outer membrane protein S2; Flags: Precursor;	100
gi|238919788|ref|YP_002933303.1|	Full=Integration host factor subunit alpha; Short=IHF-alpha;	100
gi|238919801|ref|YP_002933316.1|	Transport system ATP-binding protein [Yersinia pestis CO92]	55
gi|238919807|ref|YP_002933322.1|	Protein c4316 [Escherichia coli CFT073]	49
gi|238920035|ref|YP_002933550.1|	Transposase [Shigella flexneri 2a]	91
gi|238920215|ref|YP_002933730.1|	Regulator	82
gi|238920297|ref|YP_002933812.1|	Full=flagellin;	100
gi|238920326|ref|YP_002933841.1|	Protein STM0995 [Salmonella enterica subsp. Enterica serovar Typhimurium str. LT2]	51
gi|238920344|ref|YP_002933859.1|	Full=Integration host factor subunit beta; Short=IHF-beta;	100
gi|238920381|ref|YP_002933896.1|	Clp ATP-binding chain C [Listeria monocytogenes EGD-e]	53
gi|238920408|ref|YP_002933923.1|	III transport ATP binding protein sfuc like	69
gi|238920508|ref|YP_002934023.1|	Full=Carbamate kinase; EC=2.7.2.2;	46
gi|238920589|ref|YP_002934104.1|	Membrane protein [Yersinia pestis KIM]	35
gi|238920639|ref|YP_002934154.1|	[Pseudomonas aeruginosa PAO1]	29
gi|238920767|ref|YP_002934282.1|	Inner membrane protein [Salmonella typhimurium LT2]	55
gi|238920909|ref|YP_002934424.1|	Regulator for alginate biosynthesis mucb [Pseudomonas aeruginosa PAO1]	33
gi|238921111|ref|YP_002934626.1|	Polymerase sigma factor rpos [Salmonella typhimurium LT2]	84
gi|238921138|ref|YP_002934653.1|	Full=Epstein-Barr virus nuclear antigen EBNA-3C ;	44
gi|238921141|ref|YP_002934656.1|	Hydrogenase nickel incorporation protein hybf [Morganella morganii subsp. Morganii]	57
gi|238921195|ref|YP_002934710.1|	Lysil-trna synthetase lysu [Escherichia coli]	67
gi|238921206|ref|YP_002934721.1|	Precursor 1d [Burkholderia pseudomallei]	95
gi|238921318|ref|YP_002934833.1|	Full=nifr1 protein;	41
gi|238921385|ref|YP_002934900.1|	Full=Carbamate kinase-like protein yqea;	100
gi|238921390|ref|YP_002934905.1|	Full=Transcriptional regulator nifa family ;	78
gi|238921702|ref|YP_002935217.1|	Synthetase GLNA1 glutamine synthase GS-I [Mycobacterium tuberculosis H37Rv]	51

APPENDIX E

35 DEGs IN EiAKMUT02 ANNOTATED BY BLAST2GO DURING HOST ENCOUNTER

GI	GO ID	Protein product
gi|9230686|gb|AAF85957.1|AF244083_3	GO:0006313	Is4 family protein
gi|238918020|ref|YP_002931534.1|	GO:0003676	Transposase
gi|238918024|ref|YP_002931538.1|	GO:0055085	Type i restriction-modification enzyme r subunit
gi|238918081|ref|YP_002931595.1|	GO:0016021	Eca biosynthesis protein
gi|238918276|ref|YP_002931790.1|	GO:0005737	Arginine repressor
gi|238918281|ref|YP_002931795.1|	GO:0003677	Hypothetical protein NT01EI_0318 [Edwardsiella ictaluri 93-146]
gi|238918457|ref|YP_002931971.1|	GO:0006810	D-galactonate transporter
gi|238918460|ref|YP_002931974.1|	GO:0008789	Altronate hydrolase
gi|238918508|ref|YP_002932022.1|	GO:0006006	Pyruvate formate lyase activating enzyme
gi|238918647|ref|YP_002932161.1|	GO:0009851	Thiamine transporter atp-binding subunit
gi|238918654|ref|YP_002932168.1|	GO:0009851	Hemolysin secretion atp-binding protein
gi|238919586|ref|YP_002933101.1|	GO:0009279	Outer membrane protein w
gi|238919927|ref|YP_002933442.1|	GO:0016491	Dna replication protein gp18
gi|238919951|ref|YP_002933466.1|	GO:0016021	Ammonium transporter
gi|238920043|ref|YP_002933558.1|	GO:0016021	Cryptic c4-dicarboxylate transporter
gi|238920189|ref|YP_002933704.1|	GO:0003677	Phage antitermination protein q
gi|238920400|ref|YP_002933915.1|	GO:0015904	Multidrug resistance bcr family
gi|238920715|ref|YP_002934230.1|	GO:0008168	Biotin biosynthesis protein
gi|238920717|ref|YP_002934232.1|	GO:0005737	Biotin synthase
gi|238920722|ref|YP_002934237.1|	GO:0016021	Molybdate abc permease protein
gi|238921088|ref|YP_002934603.1|	GO:0005840	30s ribosomal protein s16
gi|238921151|ref|YP_002934666.1|	GO:0009279	Exported protein
gi|238921167|ref|YP_002934682.1|	GO:0005488	Adhesin hemagglutinin
gi|238921273|ref|YP_002934788.1|	GO:0006313	Oxalate formate antiporter
gi|238921338|ref|YP_002934853.1|	GO:0005886	Inner membrane protein
gi|238921420|ref|YP_002934935.1|	GO:0030371	Ribosomal protein s4
gi|238921426|ref|YP_002934941.1|	GO:0005840	Ribosomal protein l18
gi|238921428|ref|YP_002934943.1|	GO:0030371	30s ribosomal protein s8
gi|238921429|ref|YP_002934944.1|	GO:0003735	30s ribosomal protein s14
gi|238921430|ref|YP_002934945.1|	GO:0005840	50s ribosomal protein l5
gi|238921445|ref|YP_002934960.1|	GO:0005737	Translation elongation factor g
gi|238921446|ref|YP_002934961.1|	GO:0003735	Ribosomal protein s7
gi|238921447|ref|YP_002934962.1|	GO:0006412	Ribosomal protein s12
gi|238921506|ref|YP_002935021.1|	GO:0009401	Ascorbate-specific pts system enzyme iic
gi|238921507|ref|YP_002935022.1|	GO:0005737	L-ascorbate-specific enzyme iib component of pts

APPENDIX F

COMPLETE LIST OF UP-REGULATED GENES IN *EI*AKMUT02 COMPARED TO *E. ICTALURI* WT UNDER NORMAL SPF SERUM EXPOSURE

GI	FC	P.Value	Protein_product
238920048	3.02	0.04	Hypothetical protein
238921170	2.99	0.04	Hypothetical protein
238919357	2.95	0.04	Hypothetical protein
238920091	2.87	0.04	Hypothetical protein
238920836	2.67	0.04	Hypothetical protein
238918222	2.66	0.04	Hypothetical protein
238918020	2.62	0.04	Transposase A
238921490	2.55	0.02	Hypothetical protein
238921182	2.53	0.04	Hypothetical protein
238920400	2.51	0.05	Drug resistance transporter, Bcr/cfla family
238918276	2.5	0.05	Arginine repressor, C- domain protein
238918017	2.45	0.02	Hypothetical protein
238921541	2.44	0.05	Amino acid ABC transporter, permease protein, 3-TM region, His/Glu/Gln/Arg/opine
238921675	2.33	0.04	Hypothetical protein
238920443	2.3	0.03	Putative beta1,4-galactosyltransferase
238920135	2.24	0.04	Hypothetical protein
238921678	2.23	0.02	Hypothetical protein
238920442	2.22	0.04	Hypothetical protein
238921005	2.19	0.03	Hypothetical protein
238919417	2.19	0.04	Ispsy18, transposase
238921605	2.17	0.03	Hypothetical protein
238921577	2.14	0.04	Hypothetical protein
238918309	2.14	0.04	Hypothetical protein
238919415	2.13	0.05	Putative transposase for insertion sequence element
238918357	2.1	0.04	Hypothetical protein
238918919	2.06	0.03	Hypothetical protein
238918226	2.06	0.05	Hypothetical protein
238921728	2.05	0.04	Low affinity potassium transport system protein kup
238918111	2.02	0.02	Carbon starvation protein A
238921759	2.01	0.03	Hypothetical protein
238918173	2.01	0.04	Hypothetical protein
238921260	2	0.04	Rubredoxin
238919366	1.98	0.04	Hypothetical protein
238921607	1.98	0.01	Hypothetical protein
238921209	1.97	0.05	Glycine dehydrogenase, putative
238921606	1.96	0.02	Hypothetical protein
238921654	1.95	0.03	Periplasmic repressor cpxp
238921574	1.95	0.02	Hypothetical protein
238920924	1.94	0.04	Integrase core domain protein

238921569	1.93	0.03	Hypothetical protein
238920679	1.93	0.03	Hypothetical protein
238918273	1.93	0.03	Hypothetical protein
238921496	1.93	0.01	Hypothetical protein
238918006	1.92	0.03	Hypothetical protein
238918305	1.91	0.03	Hypothetical protein
238918024	1.91	0.02	Type I restriction-modification system, R subunit
238918112	1.91	0.05	Carbon starvation protein A-like protein, putative
238918975	1.9	0.02	Protein-export membrane protein secf, putative
238918019	1.89	0.04	Hypothetical protein
238920852	1.89	0.02	Hypothetical protein
238918321	1.88	0.01	Hypothetical protein
238921399	1.88	0	Homoserine O-succinyltransferase, putative
238918303	1.88	0.04	Hypothetical protein
238920612	1.87	0.03	Hypothetical protein
238921725	1.87	0.03	Ribose transport system permease protein rbsc
238921732	1.85	0.03	Asnc-family transcriptional regulator
238918036	1.84	0.02	DNA repair protein radc
238918289	1.84	0.03	Hypothetical protein
238919962	1.83	0.02	Hypothetical protein
238921626	1.82	0.05	Hypothetical protein
238921731	1.81	0.02	Aspartate--ammonia ligase, putative
238921632	1.81	0.04	Hypothetical protein
238918381	1.81	0.05	Hypothetical protein
238921730	1.8	0	Hypothetical protein
238920916	1.8	0.04	Hypothetical protein
238918130	1.79	0.03	Potassium uptake protein, trkh family
238921000	1.78	0.02	Baseplate assembly protein V (gpv)
238918260	1.77	0.03	Hypothetical protein
238919624	1.77	0.05	Hypothetical protein
238919957	1.77	0.04	Urease, alpha subunit, putative
238919371	1.75	0.04	Hypothetical protein
238921302	1.75	0.03	Hypothetical protein
238921507	1.74	0.04	Hypothetical protein
238921698	1.72	0.03	Hypothetical protein
238918177	1.72	0.01	Hypothetical protein
238921367	1.72	0	Hypothetical protein
238918455	1.71	0.03	Hypothetical protein
238920919	1.71	0.03	Hypothetical protein
238921414	1.7	0.02	Large-conductance mechanosensitive channel

238918947	1.69	0.04	Hsdr
238921002	1.69	0.03	Baseplate assembly protein J (gpj)
238921487	1.68	0.04	Ydhu
238921705	1.67	0.02	Hypothetical protein
238921345	1.67	0.05	Rod shape-determining protein mred, putative
238921673	1.67	0.03	Outer membrane protein N
238921638	1.67	0.03	Glycerol uptake facilitator protein
238921570	1.65	0.05	Mg2+ transporter mgtb, putative
238918413	1.65	0.03	Transcriptional regulatory protein basr/pmra
238921481	1.64	0.01	Hypothetical protein
238918806	1.64	0.03	Hypothetical protein
238918009	1.63	0.04	Hypothetical protein
238920853	1.63	0.03	Ibra
238919262	1.63	0.04	Flagellar L-ring protein flgh, putative
238921727	1.63	0.04	High affinity ribose transport protein rbsd
238919771	1.61	0.01	Outer membrane protein N
238921582	1.61	0.03	Hypothetical protein
238918590	1.61	0.03	Hypothetical protein
238921244	1.6	0.04	Tripartite ATP-independent periplasmic transporter dctq component
238920688	1.6	0.03	Methyl-accepting chemotaxis protein, putative
238918653	1.6	0.04	Hypothetical protein
238921603	1.58	0.02	Hypothetical protein
238920307	1.57	0.02	Hypothetical protein
238921299	1.56	0.03	Hypothetical protein
238920232	1.56	0.03	Hypothetical protein
238918942	1.55	0.05	N-acetylneuraminate transporter, sodium-glucose/galactose cotransporter
238918016	1.55	0.04	Hypothetical protein
238921687	1.53	0.05	Hypothetical protein
238919001	1.51	0.03	Hypothetical protein

APPENDIX G

COMPLETE LIST OF DOWN-REGULATED GENES IN *EI*AKMUT02 COMPARED TO *E. ICTALURI* WT UNDER NORMAL SPF SERUM EXPOSURE

GI	FC	P.Value	Protein_product
238918927	-1.50	0.02	Oxygen-insensitive NAD(P)
238918945	-1.50	0.01	Hypothetical protein
238921133	-1.51	0.03	Hydrogenase-4 component H
238918989	-1.51	0.02	Riboflavin biosynthesis protein ribd, putative
238918661	-1.51	0.03	3-isopropylmalate dehydrogenase, putative
238919183	-1.51	0.01	Pyrimidine nucleoside transport protein
238920822	-1.51	0.01	Lipoyl synthase, putative
238920907	-1.52	0.04	GTP-binding protein lepa, putative
238921410	-1.52	0.02	Methionyl-trna formyltransferase, putative
238920711	-1.52	0.00	Hypothetical protein
238921239	-1.53	0.02	Hypothetical protein
238918458	-1.53	0.02	Hypothetical protein
238918774	-1.53	0.02	Translation elongation factor Ts, putative
238921684	-1.54	0.02	Argininosuccinate synthase, putative
238919737	-1.54	0.05	Periplasmic murein peptide-binding protein
238919438	-1.55	0.01	Hypothetical protein
238920239	-1.55	0.00	Hypothetical protein
238921255	-1.55	0.01	Hypothetical protein
238919469	-1.55	0.02	Glutamyl-trna reductase, putative
238919606	-1.55	0.02	Cob(I)yrinic acid a,c-diamide adenosyltransferase, putative
238921379	-1.56	0.02	Selenium metabolism protein ssna, putative
238918030	-1.56	0.01	Ribonuclease PH, putative
238918948	-1.56	0.00	Atpase associated with various cellular activities, AAA_5
238921086	-1.56	0.05	Trna (guanine-N1)-methyltransferase, putative
238919603	-1.57	0.00	RNA pseudouridine synthase family protein
238920548	-1.57	0.02	[Ni/Fe] hydrogenase, small subunit
238918827	-1.57	0.00	Glutamine-dependent NAD(+)(+)
238919271	-1.57	0.01	Nicotinate phosphoribosyltransferase, putative
238921356	-1.57	0.02	Acetyl-coa carboxylase, biotin carboxyl carrier protein, putative
238920314	-1.58	0.02	Flagellum-specific ATP synthase, putative
238919382	-1.58	0.03	Hypothetical protein
238920483	-1.58	0.04	Hypothetical protein
238918213	-1.59	0.03	Hypothetical protein
238918421	-1.60	0.03	Preprotein translocase, secg subunit, putative
238920526	-1.60	0.01	Molybdopterin converting factor, subunit 1, putative
238920016	-1.60	0.01	Transcriptional activator protein copr
238918427	-1.60	0.02	Ribosomal protein S15, putative
238921083	-1.60	0.01	Hypothetical protein
238918409	-1.60	0.01	Ribosomal protein L27, putative
238920608	-1.60	0.00	Trna pseudouridine synthase A, putative
238919134	-1.60	0.03	Hypothetical protein
238919172	-1.60	0.05	Phosphoenolpyruvate-protein phosphotransferase, putative
238918031	-1.60	0.04	Orotate phosphoribosyltransferase, putative
238920333	-1.61	0.00	Amidohydrolase family, putative
238919374	-1.61	0.05	Hypothetical protein
238919071	-1.61	0.02	Hypothetical protein
238920217	-1.61	0.04	Hypothetical protein
238920090	-1.62	0.04	Hypothetical protein

238918492	-1.62	0.02	Bifunctional protein hlde, putative
238919489	-1.62	0.00	Hypothetical protein
238918349	-1.62	0.01	Translation elongation factor P, putative
238918501	-1.62	0.00	Ribosomal RNA small subunit methyltransferase C (2)
238918434	-1.63	0.00	Hypothetical protein
238919860	-1.63	0.00	Hypothetical protein
238920658	-1.64	0.01	Hypothetical protein
238918385	-1.64	0.01	Ribosomal protein S18, putative
238920001	-1.64	0.03	Hypothetical protein
238918034	-1.64	0.04	Bifunctional phosphopantothenoylcysteine decarboxylase/phosphopantothenate-cysteine ligase, putative
238919675	-1.65	0.03	Melibiose operon regulatory protein
238921089	-1.65	0.02	Signal recognition particle protein, putative
238921023	-1.65	0.00	GMP synthase
238918863	-1.65	0.01	Hypothetical protein
238921440	-1.66	0.03	Hypothetical protein
238921380	-1.67	0.04	Hypothetical protein
238920914	-1.68	0.04	ATP-dependent RNA helicase srmb
238918862	-1.68	0.00	Hypothetical protein
238919831	-1.68	0.00	Hypothetical protein
238919137	-1.69	0.00	Arsc family protein
238918708	-1.69	0.04	Hypothetical protein
238919180	-1.69	0.00	Glutamyl-trna synthetase, putative
238921143	-1.69	0.01	Hydrogenase assembly chaperone hypc/hupf, putative
238918399	-1.69	0.03	Inorganic diphosphatase, putative
238920879	-1.69	0.02	Cysteinyl-trna synthetase, putative
238920902	-1.69	0.05	Pyridoxine 5'-phosphate synthase, putative
238919005	-1.69	0.00	Hypothetical protein
238920240	-1.70	0.00	Orotidine 5'-phosphate decarboxylase, putative
238919991	-1.70	0.03	Pyridoxal kinase, putative
238919605	-1.70	0.03	Phosphatase, putative
238920349	-1.70	0.03	Phosphoserine aminotransferase, putative
238919298	-1.71	0.02	Beta-hydroxyacyl-(acyl-carrier-protein) dehydratase faba, putative
238920246	-1.71	0.00	Leucine-rich repeat protein
238921110	-1.71	0.04	DNA mismatch repair protein muts, putative
238918619	-1.72	0.01	Hypothetical protein
238919270	-1.72	0.02	Asparaginyl-trna synthetase, putative
238920290	-1.73	0.01	ABC transporter, ATP-binding protein, putative
238918361	-1.73	0.02	Oligoribonuclease
238918051	-1.73	0.02	Lipopolysaccharide heptosyltransferase I, putative
238921378	-1.73	0.01	Oxidoreductases, FAD binding protein
238920576	-1.73	0.01	5'-nucleotidase, putative
238921321	-1.74	0.01	Anaerobic glycerol-3-phosphate dehydrogenase subunit B
238918568	-1.74	0.01	Hypothetical protein
238917987	-1.75	0.02	DNA gyrase, B subunit, putative
238919557	-1.75	0.00	Exodeoxyribonuclease III, putative
238918692	-1.76	0.01	Guanosine monophosphate reductase, putative
238919490	-1.77	0.03	Aminotransferase, class II
238921389	-1.77	0.02	Putative carbamoyltransferase

238921441	-1.77	0.05	50S ribosomal protein L4
238920481	-1.78	0.00	Multiphosphoryl transfer protein (MTP)
238919824	-1.78	0.00	Glutamate synthase family, small subunit, putative
238918815	-1.78	0.01	Hypothetical protein
238920712	-1.78	0.01	Excinuclease ABC subunit B, putative
238918732	-1.79	0.01	RNA polymerase-binding protein dksa, putative
238918444	-1.79	0.01	Hypothetical protein
238921202	-1.79	0.01	Hypothetical protein
238918408	-1.79	0.01	Ribosomal protein L21, putative
238920609	-1.80	0.01	Aspartate-semialdehyde dehydrogenase, putative
238920524	-1.80	0.01	Hypothetical protein
238920578	-1.80	0.02	Hypothetical protein
238919121	-1.80	0.01	Uracil phosphoribosyltransferase, putative
238918851	-1.80	0.02	Gamma-glutamyl phosphate reductase
238920273	-1.81	0.03	Dihydroorotase, homodimeric type, putative
238919820	-1.81	0.02	Hypothetical protein
238921388	-1.82	0.01	Diaminopropionate ammonia-lyase
238920896	-1.82	0.03	CTP synthase, putative
238919041	-1.82	0.04	Hypothetical protein
238918255	-1.83	0.03	Glutathionylspermidine synthase
238920525	-1.84	0.01	Molybdopterin converting factor, subunit 2, putative
238921320	-1.84	0.00	Sn-glycerol-3-phosphate dehydrogenase subunit C, putative
238920212	-1.84	0.01	Adenylosuccinate lyase, putative
238919285	-1.84	0.00	Hypothetical protein
238921042	-1.85	0.01	Radical SAM enzyme, Cfr family
238920390	-1.85	0.04	Hydroxylamine reductase, putative
238917996	-1.87	0.02	Glycyl-trna synthetase, beta subunit, putative
238918804	-1.87	0.01	Lipoprotein nlpe
238919112	-1.88	0.02	Hypothetical protein
238919286	-1.89	0.01	Dihydroorotate oxidase, putative
238919980	-1.90	0.01	Hypothetical protein
238921637	-1.90	0.03	Glycerol kinase, putative
238920265	-1.91	0.03	Hypothetical protein
238919693	-1.91	0.03	Prop effector, putative
238920209	-1.94	0.03	Trna (5-methylaminomethyl-2-thiouridylate)-methyltransferase, putative
238920065	-1.94	0.04	Homoaconitate hydratase family protein, large subunit
238919466	-1.95	0.02	Hypothetical protein
238921392	-1.97	0.02	Single-stranded DNA-binding protein (SSB)
238919758	-1.97	0.00	Phosphoribosylglycinamide formyltransferase 2, putative
238920346	-1.98	0.00	Cytidylate kinase, putative
238920497	-2.00	0.00	Hypothetical protein
238919383	-2.01	0.01	Beta-aspartyl peptidase, putative
238918029	-2.02	0.00	Hypothetical protein
238919425	-2.02	0.01	Hypothetical protein
238919055	-2.03	0.00	Adenylate kinase
238920464	-2.04	0.01	Hypothetical protein
238919552	-2.04	0.02	Selenide, water dikinase, putative
238919488	-2.05	0.02	Arginyl-trna synthetase, putative

238920344	-2.05	0.01	Integration host factor, beta subunit, putative
238920953	-2.07	0.03	Hypothetical protein
238921387	-2.07	0.00	M20/dape family protein ygey
238921596	-2.08	0.01	Hypothetical protein
238920054	-2.08	0.04	Hypothetical protein
238919826	-2.08	0.00	Pyruvate:ferredoxin
238919974	-2.09	0.02	Fumarate hydratase, class II, putative
238921701	-2.09	0.04	GTP-binding protein typa/bipa, putative
238919120	-2.10	0.00	Phosphoribosylformylglycinamidine cyclo-ligase, putative
238921322	-2.11	0.00	Anaerobic glycerol-3-phosphate dehydrogenase subunit A
238921208	-2.11	0.00	Hypothetical protein
238918052	-2.12	0.03	Lipopolysaccharide heptosyltransferase II, putative
238918256	-2.12	0.03	Hypothetical protein
238919996	-2.14	0.00	Outer membrane lipoprotein pcp
238918139	-2.15	0.03	Ribosomal protein L11, putative
238919743	-2.15	0.01	Hypothetical protein
238919010	-2.16	0.01	Trigger factor, putative
238921352	-2.17	0.02	Protein yedy
238920729	-2.19	0.01	Galactose-1-phosphate uridylyltransferase, putative
238920367	-2.19	0.02	Anaerobic dimethyl sulfoxide reductase chain A, putative
238920520	-2.23	0.03	Ribonucleoside-diphosphate reductase, alpha subunit, putative
238919284	-2.25	0.01	Hypothetical protein
238919742	-2.25	0.01	Pyruvate kinase, putative
238921432	-2.26	0.01	50S ribosomal protein L14, putative
238918140	-2.28	0.01	Ribosomal protein L1, putative
238920615	-2.30	0.04	Hypothetical protein
238918353	-2.31	0.01	Fumarate reductase iron-sulfur subunit
238918629	-2.31	0.01	Xanthine dehydrogenase molybdenum-binding subunit
238920284	-2.41	0.00	Hypothetical protein
238918632	-2.48	0.02	Carbamoyl-phosphate synthase, small subunit, putative
238918354	-2.62	0.03	Fumarate reductase, flavoprotein subunit, putative
238918542	-2.64	0.01	Ribosomal protein L13, putative
238920969	-2.64	0.01	Protease do
238920480	-2.70	0.00	1-phosphofructokinase, putative
238921088	-2.86	0.01	Ribosomal protein S16, putative
238919782	-2.87	0.01	Threonyl-trna synthetase, putative
238918480	-3.27	0.00	Ribosomal protein S21, putative
238919729	-3.57	0.04	Phage shock protein B, putative
238920479	-3.60	0.00	Pts system fructose-specific eiibc component
238921446	-3.61	0.00	30S ribosomal protein S7, putative
238921024	-3.69	0.00	Inosine-5'-monophosphate dehydrogenase, putative
238919644	-3.77	0.00	Extracellular solute-binding protein family 5
238918384	-4.03	0.00	Ribosomal protein S6, putative
238921447	-4.21	0.00	30S ribosomal protein S12, putative

APPENDIX H

216 DEGS IN *EI*AKMUT02 ANNOTATED BY BLAST2GO COMPARED TO WT

UNDER NORMAL SPF SERUM EXPOSURE

GI	GO ID	Protein product
gi|238917987|ref|YP_002931501.1|	GO:0009851	Dna gyrase subunit b
gi|238917996|ref|YP_002931510.1|	GO:0005737	Glycyl-trna synthetase subunit beta
gi|238918009|ref|YP_002931523.1|	GO:0055085	Purine permease yice
gi|238918020|ref|YP_002931534.1|	GO:0003676	Transposase
gi|238918024|ref|YP_002931538.1|	GO:0055085	Type i restriction-modification enzyme r subunit
gi|238918030|ref|YP_002931544.1|	GO:0000175	Ribonuclease ph
gi|238918031|ref|YP_002931545.1|	GO:0005737	Orotate phosphoribosyltransferase
gi|238918034|ref|YP_002931548.1|	GO:0015937	Phosphopantothenate--cysteine ligase
gi|238918036|ref|YP_002931550.1|	GO:0006281	Dna repair protein
gi|238918051|ref|YP_002931565.1|	GO:0009103	Lipopolysaccharide heptosyltransferase i
gi|238918052|ref|YP_002931566.1|	GO:0009103	Adp-heptose:lps heptosyltransferase ii
gi|238918112|ref|YP_002931626.1|	GO:0016020	Carbon starvation protein
gi|238918130|ref|YP_002931644.1|	GO:0016021	Potassium transporter
gi|238918139|ref|YP_002931653.1|	GO:0006415	Ribosomal protein l11
gi|238918140|ref|YP_002931654.1|	GO:0003735	50s ribosomal protein l1
gi|238918177|ref|YP_002931691.1|	GO:0016021	Phosphate-starvation-inducible protein
gi|238918213|ref|YP_002931727.1|	GO:0016021	Adhesin virulence factor hek
gi|238918226|ref|YP_002931740.1|	GO:0006313	Transposase
gi|238918255|ref|YP_002931769.1|	GO:0003824	Glutathionylspermidine synthase
gi|238918276|ref|YP_002931790.1|	GO:0005737	Arginine repressor
gi|238918349|ref|YP_002931863.1|	GO:0043022	Translation elongation factor p
gi|238918353|ref|YP_002931867.1|	GO:0046872	Fumarate reductase iron-sulfur subunit
gi|238918354|ref|YP_002931868.1|	GO:0009061	Fumarate reductase flavoprotein subunit
gi|238918357|ref|YP_002931871.1|	GO:0055085	Mechanosensitive ion channel domain protein
gi|238918361|ref|YP_002931875.1|	GO:0003676	Oligoribonuclease
gi|238918384|ref|YP_002931898.1|	GO:0005840	Ribosomal protein s6
gi|238918385|ref|YP_002931899.1|	GO:0005840	30s ribosomal protein s18
gi|238918399|ref|YP_002931913.1|	GO:0004427	Inorganic pyrophosphatase
gi|238918408|ref|YP_002931922.1|	GO:0005840	50s ribosomal protein l21
gi|238918409|ref|YP_002931923.1|	GO:0005840	50s ribosomal protein l27
gi|238918413|ref|YP_002931927.1|	GO:0000160	Two-component system response regulator
gi|238918421|ref|YP_002931935.1|	GO:0015450	Preprotein translocase subunit
gi|238918427|ref|YP_002931941.1|	GO:0003735	Ribosomal protein s15
gi|238918434|ref|YP_002931948.1|	GO:0008233	U32 family
gi|238918458|ref|YP_002931972.1|	GO:0006064	Glucuronate isomerase
gi|238918480|ref|YP_002931994.1|	GO:0003735	30s ribosomal protein s21
gi|238918492|ref|YP_002932006.1|	GO:0019200	Bifunctional protein
gi|238918501|ref|YP_002932015.1|	GO:0005737	Ribosomal rna small subunit

		methyltransferase c
gi|238918542|ref|YP_002932056.1|	GO:0005840	Ribosomal protein l13
gi|238918568|ref|YP_002932082.1|	GO:0016887	Abc transporter atp-binding protein
gi|238918629|ref|YP_002932143.1|	GO:0055114	Xanthine dehydrogenase
gi|238918632|ref|YP_002932146.1|	GO:0005829	Carbamoyl-phosphate small subunit
gi|238918653|ref|YP_002932167.1|	GO:0009851	Hemolysin secretion atp-binding
gi|238918661|ref|YP_002932175.1|	GO:0005737	3-isopropylmalate dehydrogenase
gi|238918692|ref|YP_002932206.1|	GO:0046872	Gmp reductase
gi|238918708|ref|YP_002932222.1|	GO:0016020	Integral membrane protein
gi|238918732|ref|YP_002932246.1|	GO:0008270	Rna polymerase-binding transcription factor
gi|238918774|ref|YP_002932288.1|	GO:0005737	Translation elongation factor ts
gi|238918806|ref|YP_002932320.1|	GO:0003677	Site-specific phage integrase family
gi|238918815|ref|YP_002932329.1|	GO:0005529	Dna-binding transcriptional regulator
gi|238918827|ref|YP_002932341.1|	GO:0009851	Glutamine-dependent nad
gi|238918851|ref|YP_002932365.1|	GO:0055114	Gamma-glutamyl phosphate reductase
gi|238918862|ref|YP_002932376.1|	GO:0022904	Cytochrome b561
gi|238918863|ref|YP_002932377.1|	GO:0055114	Oxidoreductase molybdopterin binding protein
gi|238918927|ref|YP_002932441.1|	GO:0055114	Dihydropteridine reductase
gi|238918942|ref|YP_002932456.1|	GO:0004222	Sodium glucose cotransporter
gi|238918945|ref|YP_002932459.1|	GO:0006306	Type i restriction-modification m subunit
gi|238918947|ref|YP_002932461.1|	GO:0004386	Type i site specific deoxyribonuclease
gi|238918948|ref|YP_002932462.1|	GO:0016887	Atpase family protein
gi|238918975|ref|YP_002932489.1|	GO:0015450	Protein-export membrane protein
gi|238918989|ref|YP_002932503.1|	GO:0009231	Riboflavin biosynthesis protein
gi|238919010|ref|YP_002932524.1|	GO:0051083	Trigger factor
gi|238919041|ref|YP_002932555.1|	GO:0003677	Conserved protein
gi|238919055|ref|YP_002932569.1|	GO:0005737	Adenylate kinase
gi|238919071|ref|YP_002932585.1|	GO:0016020	Nodulation efficiency protein d
gi|238919120|ref|YP_002932634.1|	GO:0005737	Phosphoribosylformylglycinamidine cyclo-ligase
gi|238919121|ref|YP_002932635.1|	GO:0006223	Uracil phosphoribosyltransferase
gi|238919134|ref|YP_002932648.1|	GO:0005886	Membrane protein
gi|238919172|ref|YP_002932686.1|	GO:0005737	Phosphoenolpyruvate-protein phosphotransferase
gi|238919180|ref|YP_002932694.1|	GO:0005737	Glutamyl-trna synthetase
gi|238919183|ref|YP_002932698.1|	GO:0016020	Nucleoside permease
gi|238919262|ref|YP_002932777.1|	GO:0001539	Flagellar l-ring protein
gi|238919270|ref|YP_002932785.1|	GO:0004816	Asparaginyl-trna synthetase
gi|238919271|ref|YP_002932786.1|	GO:0005737	Nicotinate phosphoribosyltransferase
gi|238919284|ref|YP_002932799.1|	GO:0006629	Hypothetical protein NT01EI_1374 [Edwardsiella ictaluri 93-146]

gi|238919286|ref|YP_002932801.1|	GO:0019898	Dihydroorotate oxidase
gi|238919298|ref|YP_002932813.1|	GO:0006633	Beta-hydroxyacyl-(acyl-carrier-protein) dehydratase
gi|238919371|ref|YP_002932886.1|	GO:0019861	Response regulator receiver protein
gi|238919382|ref|YP_002932897.1|	GO:0016021	C4-dicarboxylate transporter
gi|238919383|ref|YP_002932898.1|	GO:0016810	Beta-aspartyl peptidase
gi|238919415|ref|YP_002932930.1|	GO:0006313	Transposase for insertion sequence element
gi|238919417|ref|YP_002932932.1|	GO:0006313	Mutator family
gi|238919466|ref|YP_002932981.1|	GO:0005737	Ribose-phosphate pyrophosphokinase
gi|238919466|ref|YP_002932981.1|	GO:0016301	
gi|238919469|ref|YP_002932984.1|	GO:0005737	Glutamyl-trna reductase
gi|238919488|ref|YP_002933003.1|	GO:0005737	Arginyl-trna synthetase
gi|238919490|ref|YP_002933005.1|	GO:0030170	Aminotransferase class i and ii
gi|238919552|ref|YP_002933067.1|	GO:0005524	Water dikinase
gi|238919557|ref|YP_002933072.1|	GO:0004519	Exodeoxyribonuclease iii
gi|238919603|ref|YP_002933118.1|	GO:0009982	23s rrna pseudouridylate synthase b
gi|238919605|ref|YP_002933120.1|	GO:0043755	Alpha-ribazole phosphatase
gi|238919606|ref|YP_002933121.1|	GO:0005737	Cob yrinic acid -diamide adenosyltransferase
gi|238919644|ref|YP_002933159.1|	GO:0005215	Nickel abc transporter periplasmic nickel-binding protein
gi|238919675|ref|YP_002933190.1|	GO:0043565	Melibiose operon regulatory protein
gi|238919693|ref|YP_002933208.1|	GO:0005737	Effector
gi|238919729|ref|YP_002933244.1|	GO:0006350	Phage shock protein b
gi|238919737|ref|YP_002933252.1|	GO:0005215	Periplasmic murein peptide-binding protein
gi|238919742|ref|YP_002933257.1|	GO:0004743	Pyruvate kinase
gi|238919758|ref|YP_002933273.1|	GO:0009152	Phosphoribosylglycinamide formyltransferase 2
gi|238919771|ref|YP_002933286.1|	GO:0009279	Outer membrane protein
gi|238919782|ref|YP_002933297.1|	GO:0005737	Threonyl-trna synthetase
gi|238919820|ref|YP_002933335.1|	GO:0030001	Zinc transport protein zntb
gi|238919824|ref|YP_002933339.1|	GO:0006537	Oxidoreductase fe-s binding subunit
gi|238919826|ref|YP_002933341.1|	GO:0030976	Pyruvate-flavodoxin oxidoreductase
gi|238919860|ref|YP_002933375.1|	GO:0008233	U32 family
gi|238919957|ref|YP_002933472.1|	GO:0005737	Urease subunit alpha
gi|238919962|ref|YP_002933477.1|	GO:0006313	Is1400 transposase a
gi|238919974|ref|YP_002933489.1|	GO:0045239	Fumarate hydratase
gi|238919980|ref|YP_002933495.1|	GO:0004329	Formate--tetrahydrofolate ligase
gi|238919991|ref|YP_002933506.1|	GO:0004340	Pyridoxamine kinase
gi|238919996|ref|YP_002933511.1|	GO:0019867	Outer membrane lipoprotein
gi|238920001|ref|YP_002933516.1|	GO:0005737	Monothiol glutaredoxin
gi|238920016|ref|YP_002933531.1|	GO:0005737	Dna-binding transcriptional activator

gi|238920065|ref|YP_002933580.1|	GO:0003861	Homoaconitate hydratase family protein
gi|238920209|ref|YP_002933724.1|	GO:0005737	Trna (5-methylaminomethyl-2-thiouridylate)-methyltransferase
gi|238920212|ref|YP_002933727.1|	GO:0006188	Adenylosuccinate lyase
gi|238920217|ref|YP_002933732.1|	GO:0005515	Cupin 4 family protein
gi|238920239|ref|YP_002933754.1|	GO:0006413	Translation initiation factor sui1
gi|238920240|ref|YP_002933755.1|	GO:0006207	Orotidine 5 -phosphate decarboxylase
gi|238920246|ref|YP_002933761.1|	GO:0020002	Leucine-rich repeat protein
gi|238920273|ref|YP_002933788.1|	GO:0004151	Dihydroorotase
gi|238920284|ref|YP_002933799.1|	GO:0016874	Lichenysin synthetase b
gi|238920290|ref|YP_002933805.1|	GO:0016887	Amino-acid abc transporter atp-binding protein
gi|238920314|ref|YP_002933829.1|	GO:0009851	Flagellum-specific atp synthase
gi|238920333|ref|YP_002933848.1|	GO:0018763	Hydroxydechloroatrazine ethylaminohydrolase
gi|238920344|ref|YP_002933859.1|	GO:0003677	Integration host factor subunit beta
gi|238920346|ref|YP_002933861.1|	GO:0005737	Cytidylate kinase
gi|238920349|ref|YP_002933864.1|	GO:0005737	Phosphoserine aminotransferase
gi|238920367|ref|YP_002933882.1|	GO:0055114	Anaerobic dimethyl sulfoxide reductase chain a
gi|238920390|ref|YP_002933905.1|	GO:0046872	Hydroxylamine reductase
gi|238920400|ref|YP_002933915.1|	GO:0015904	Multidrug resistance bcr family
gi|238920443|ref|YP_002933958.1|	GO:0009103	Glycosyl transferase family 25
gi|238920479|ref|YP_002933994.1|	GO:0016301	Phosphotransferase eiic
gi|238920480|ref|YP_002933995.1|	GO:0008662	1-phosphofructokinase
gi|238920481|ref|YP_002933996.1|	GO:0016301	Hpr family
gi|238920497|ref|YP_002934012.1|	GO:0005840	50s ribosomal protein l25
gi|238920520|ref|YP_002934035.1|	GO:0005971	Ribonucleoside-diphosphate alpha subunit
gi|238920524|ref|YP_002934039.1|	GO:0010181	Flavodoxin nitric oxide synthase
gi|238920525|ref|YP_002934040.1|	GO:0016740	Molybdopterin guanine dinucleotide biosynthesis protein
gi|238920526|ref|YP_002934041.1|	GO:0006790	Molybdopterin converting subunit 1
gi|238920548|ref|YP_002934063.1|	GO:0009375	Hydrogenase-2 small chain
gi|238920576|ref|YP_002934091.1|	GO:0008253	Metal dependent phosphohydrolase
gi|238920578|ref|YP_002934093.1|	GO:0008152	Had-superfamily subfamily variant 3
gi|238920608|ref|YP_002934123.1|	GO:0003723	Trna pseudouridine synthase a
gi|238920609|ref|YP_002934124.1|	GO:0005737	Semialdehyde dehydrogenase
gi|238920615|ref|YP_002934130.1|	GO:0006633	3-oxoacyl-
gi|238920688|ref|YP_002934203.1|	GO:0016021	Methyl-accepting chemotaxis sensory transducer
gi|238920711|ref|YP_002934226.1|	GO:0003676	Hypothetical protein NT01EI_2824 [Edwardsiella ictaluri 93-146]
gi|238920712|ref|YP_002934227.1|	GO:0005737	Excinuclease abc subunit b

gi|238920729|ref|YP_002934244.1|	GO:0008270	Galactose-1-phosphate uridylyltransferase
gi|238920822|ref|YP_002934337.1|	GO:0005737	Lipoyl synthase
gi|238920852|ref|YP_002934367.1|	GO:0003677	Immunoglobulin-binding regulator b
gi|238920853|ref|YP_002934368.1|	GO:0055114	Immunoglobulin-binding regulator a
gi|238920879|ref|YP_002934394.1|	GO:0005737	Cysteinyl-trna synthetase
gi|238920896|ref|YP_002934411.1|	GO:0006541	Ctp synthetase
gi|238920902|ref|YP_002934417.1|	GO:0008615	Pyridoxal phosphate biosynthetic protein
gi|238920907|ref|YP_002934422.1|	GO:0003924	Gtp-binding protein
gi|238920914|ref|YP_002934429.1|	GO:0009851	Atp-dependent rna helicase
gi|238920924|ref|YP_002934439.1|	GO:0003677	Transposase
gi|238920969|ref|YP_002934484.1|	GO:0055114	Protease do
gi|238921023|ref|YP_002934538.1|	GO:0006177	Gmp synthase
gi|238921024|ref|YP_002934539.1|	GO:0003938	Inosine 5 -monophosphate dehydrogenase
gi|238921042|ref|YP_002934557.1|	GO:0005737	Ribosomal rna large subunit methyltransferase n
gi|238921086|ref|YP_002934601.1|	GO:0005737	Trna (guanine-n -)-methyltransferase
gi|238921088|ref|YP_002934603.1|	GO:0005840	30s ribosomal protein s16
gi|238921089|ref|YP_002934604.1|	GO:0005525	Signal recognition particle protein
gi|238921110|ref|YP_002934625.1|	GO:0005524	Dna mismatch repair protein
gi|238921133|ref|YP_002934648.1|	GO:0051539	Hydrogenase-4 component h
gi|238921133|ref|YP_002934648.1|	GO:0046872	
gi|238921133|ref|YP_002934648.1|	GO:0009055	
gi|238921208|ref|YP_002934723.1|	GO:0016020	Sodium:alanine symporter family protein
gi|238921209|ref|YP_002934724.1|	GO:0004375	Glycine dehydrogenase
gi|238921255|ref|YP_002934770.1|	GO:0008610	Cyclopropane-fatty-acyl-phospholipid synthase
gi|238921260|ref|YP_002934775.1|	GO:0046872	Rubredoxin-type fe 4 protein
gi|238921320|ref|YP_002934835.1|	GO:0055114	Anaerobic glycerol-3-phosphate dehydrogenase subunit c
gi|238921321|ref|YP_002934836.1|	GO:0055114	Anaerobic glycerol-3-phosphate dehydrogenase subunit b
gi|238921322|ref|YP_002934837.1|	GO:0006072	Anaerobic glycerol-3-phosphate dehydrogenase subunit a
gi|238921345|ref|YP_002934860.1|	GO:0016021	Rod shape-determining protein
gi|238921352|ref|YP_002934867.1|	GO:0046872	Molybdopterin binding
gi|238921356|ref|YP_002934871.1|	GO:0006633	Acetyl- biotin carboxyl carrier protein
gi|238921378|ref|YP_002934893.1|	GO:0055114	Selenate fad-binding subunit
gi|238921379|ref|YP_002934894.1|	GO:0016810	Chlorohydrolase aminohydrolase
gi|238921380|ref|YP_002934895.1|	GO:0055114	Selenate subunit
gi|238921387|ref|YP_002934902.1|	GO:0046983	M20 family protein
gi|238921388|ref|YP_002934903.1|	GO:0030170	Diaminopropionate ammonia-lyase
gi|238921389|ref|YP_002934904.1|	GO:0016597	Aspartate ornithine carbamoyltransferase family protein

gi|238921392|ref|YP_002934907.1|	GO:0006260	Single-stranded dna-binding protein
gi|238921399|ref|YP_002934914.1|	GO:0005737	Homoserine o-succinyltransferase
gi|238921410|ref|YP_002934925.1|	GO:0006412	Methionyl-trna formyltransferase
gi|238921414|ref|YP_002934929.1|	GO:0016021	Large-conductance mechanosensitive channel
gi|238921432|ref|YP_002934947.1|	GO:0003735	50s ribosomal protein l14
gi|238921440|ref|YP_002934955.1|	GO:0005840	50s ribosomal protein l23
gi|238921441|ref|YP_002934956.1|	GO:0030371	50s ribosomal protein l4
gi|238921446|ref|YP_002934961.1|	GO:0003735	Ribosomal protein s7
gi|238921447|ref|YP_002934962.1|	GO:0006412	Ribosomal protein s12
gi|238921487|ref|YP_002935002.1|	GO:0022904	Thiosulfate reductase cytochrome b subunit
gi|238921507|ref|YP_002935022.1|	GO:0005737	L-ascorbate-specific enzyme iib component of pts
gi|238921541|ref|YP_002935056.1|	GO:0005886	Abc transporter permease component
gi|238921570|ref|YP_002935085.1|	GO:0015693	Magnesium-translocating p-type atpase
gi|238921582|ref|YP_002935097.1|	GO:0016301	Carbohydrate kinase
gi|238921596|ref|YP_002935111.1|	GO:0004222	Insulinase family protease
gi|238921605|ref|YP_002935120.1|	GO:0016020	Cellulose synthase
gi|238921626|ref|YP_002935141.1|	GO:0055085	Major facilitator superfamily mfs_1
gi|238921632|ref|YP_002935147.1|	GO:0016021	Conserved inner membrane protein
gi|238921637|ref|YP_002935152.1|	GO:0004370	Glycerol kinase
gi|238921638|ref|YP_002935153.1|	GO:0055085	Glycerol uptake facilitator protein
gi|238921673|ref|YP_002935188.1|	GO:0016020	Porin gram-negative type
gi|238921684|ref|YP_002935199.1|	GO:0005737	Argininosuccinate synthase
gi|238921698|ref|YP_002935213.1|	GO:0004540	Ribonuclease bn
gi|238921701|ref|YP_002935216.1|	GO:0003746	Gtp-binding protein
gi|238921725|ref|YP_002935240.1|	GO:0015407	Ribose transport system permease protein rbsc
gi|238921727|ref|YP_002935242.1|	GO:0005737	D-ribose pyranase
gi|238921728|ref|YP_002935243.1|	GO:0015079	Low affinity potassium transport system protein kup
gi|238921730|ref|YP_002935245.1|	GO:0005515	Protein viaa
gi|238921731|ref|YP_002935246.1|	GO:0005737	Aspartate--ammonia ligase
gi|238921732|ref|YP_002935247.1|	GO:0043565	Transcriptional family

APPENDIX I

129 DEGs INVOLVED IN HOST PATHOGEN INTERACTION IN EIAKMUT02

COMPARED TO WT UNDER NORMAL SPF SERUM EXPOSURE

Query id	Subject id	% identity	E-value	Bit score
gi|238917987|ref|YP_002931501.1|	UNIPROT_AC:Q5NHE7	65.55	0	1044
gi|238917996|ref|YP_002931510.1|	UNIPROT_AC:Q5NGR8	41.8	6.00E-153	536
gi|238918009|ref|YP_002931523.1|	UNIPROT_AC:Q8D1S0	85.06	0	763
gi|238918016|ref|YP_002931530.1|	UNIPROT_AC:Q7CLB3	76.92	3.00E-70	259
gi|238918020|ref|YP_002931534.1|	UNIPROT_AC:P69961	51.81	3.00E-16	78.6
gi|238918030|ref|YP_002931544.1|	UNIPROT_AC:Q8ZJP8	87.34	5.00E-114	405
gi|238918031|ref|YP_002931545.1|	UNIPROT_AC:Q8ZJP7	81.69	7.00E-102	365
gi|238918034|ref|YP_002931548.1|	UNIPROT_AC:Q96CD2	30.98	6.00E-15	77.4
gi|238918256|ref|YP_002931770.1|	UNIPROT_AC:Q7CGH1	71.62	1.00E-82	301
gi|238918349|ref|YP_002931863.1|	UNIPROT_AC:Q6KMS8	45.28	2.00E-36	146
gi|238918353|ref|YP_002931867.1|	UNIPROT_AC:Q5NIJ2	36.24	6.00E-37	149
gi|238918354|ref|YP_002931868.1|	UNIPROT_AC:Q7CKM6	85.84	0	1020
gi|238918357|ref|YP_002931871.1|	UNIPROT_AC:Q7CKM5	54.76	2.00E-23	102
gi|238918413|ref|YP_002931927.1|	UNIPROT_AC:Q7CJR6	42.78	1.00E-29	125
gi|238918458|ref|YP_002931972.1|	UNIPROT_AC:Q8ZIC6	83.8	0	832
gi|238918492|ref|YP_002932006.1|	UNIPROT_AC:Q8ZI60	82.32	0	787
gi|238918501|ref|YP_002932015.1|	UNIPROT_AC:Q0WJ78	29.94	4.00E-16	81.3
gi|238918568|ref|YP_002932082.1|	UNIPROT_AC:Q5NE72	63	0	730
gi|238918632|ref|YP_002932146.1|	UNIPROT_AC:Q8ZIL5	81.51	0	639
gi|238918653|ref|YP_002932167.1|	UNIPROT_AC:Q7CHU3	49.63	3.00E-68	253
gi|238918661|ref|YP_002932175.1|	UNIPROT_AC:Q8ZIG9	81.44	5.00E-179	622
gi|238918692|ref|YP_002932206.1|	UNIPROT_AC:Q9P2T1	66.47	5.00E-138	486
gi|238918774|ref|YP_002932288.1|	UNIPROT_AC:Q5NHX9	56.27	1.00E-77	285
gi|238918804|ref|YP_002932318.1|	UNIPROT_AC:Q7CH24	51.56	4.00E-71	263
gi|238918827|ref|YP_002932341.1|	UNIPROT_AC:Q7CJP7	82.28	0	804
gi|238918863|ref|YP_002932377.1|	UNIPROT_AC:Q8ZAW9	27.65	2.00E-13	71.6
gi|238918942|ref|YP_002932456.1|	UNIPROT_AC:A4QPH0	23.04	2.00E-20	96.7
gi|238918945|ref|YP_002932459.1|	UNIPROT_AC:Q5NFQ5	24.73	3.00E-17	85.9
gi|238918948|ref|YP_002932462.1|	UNIPROT_AC:Q8CKB2	52.1	2.00E-58	223
gi|238918975|ref|YP_002932489.1|	UNIPROT_AC:Q8D162	80.43	8.00E-146	511
gi|238918989|ref|YP_002932503.1|	UNIPROT_AC:Q7CK43	76.23	1.00E-164	574
gi|238919005|ref|YP_002932519.1|	UNIPROT_AC:Q81TU4	44.38	1.00E-30	127
gi|238919010|ref|YP_002932524.1|	UNIPROT_AC:Q5NH48	39.44	1.00E-85	312
gi|238919041|ref|YP_002932555.1|	UNIPROT_AC:Q5NGM5	49.07	1.00E-22	100
gi|238919055|ref|YP_002932569.1|	UNIPROT_AC:P54819	50.23	2.00E-57	217
gi|238919120|ref|YP_002932634.1|	UNIPROT_AC:P22102	47.54	9.00E-90	325
gi|238919121|ref|YP_002932635.1|	UNIPROT_AC:Q81JY5	47.34	1.00E-56	214
gi|238919172|ref|YP_002932686.1|	UNIPROT_AC:Q7CJF7	88.35	0	1014
gi|238919180|ref|YP_002932694.1|	UNIPROT_AC:Q8ZCK0	84.71	0	847
gi|238919183|ref|YP_002932698.1|	UNIPROT_AC:Q81XE1	56.27	2.00E-84	307
gi|238919262|ref|YP_002932777.1|	UNIPROT_AC:Q8ZFB2	79.55	5.00E-96	345
gi|238919270|ref|YP_002932785.1|	UNIPROT_AC:Q81L32	57.05	1.00E-155	545
gi|238919286|ref|YP_002932801.1|	UNIPROT_AC:Q81WF4	27.84	4.00E-12	67.8
gi|238919371|ref|YP_002932886.1|	UNIPROT_AC:Q8D0P1	90.7	1.00E-63	236
gi|238919417|ref|YP_002932932.1|	UNIPROT_AC:O68779	76.06	1.00E-28	119
gi|238919466|ref|YP_002932981.1|	UNIPROT_AC:Q5NH02	55.73	7.00E-104	372
gi|238919469|ref|YP_002932984.1|	UNIPROT_AC:Q8ZEX9	78.57	0	644

gi|238919488|ref|YP_002933003.1|	UNIPROT_AC:Q8ZEV7	79.03	0	966
gi|238919490|ref|YP_002933005.1|	UNIPROT_AC:Q81K67	33.25	7.00E-66	246
gi|238919552|ref|YP_002933067.1|	UNIPROT_AC:Q8ZEK1	81.5	2.00E-168	587
gi|238919557|ref|YP_002933072.1|	UNIPROT_AC:Q5NG93	28.15	5.00E-18	87
gi|238919644|ref|YP_002933159.1|	UNIPROT_AC:Q81L97	21.46	4.00E-18	88.6
gi|238919737|ref|YP_002933252.1|	UNIPROT_AC:Q7CIN4	46.95	6.00E-142	499
gi|238919742|ref|YP_002933257.1|	UNIPROT_AC:Q7CIS8	89.15	0	854
gi|238919758|ref|YP_002933273.1|	UNIPROT_AC:Q7CHW4	70.88	5.00E-161	562
gi|238919771|ref|YP_002933286.1|	UNIPROT_AC:Q7CHA0	64.06	2.00E-133	470
gi|238919782|ref|YP_002933297.1|	UNIPROT_AC:Q8ZDW5	89.41	0	1231
gi|238919824|ref|YP_002933339.1|	UNIPROT_AC:Q8D1E7	59.4	0	820
gi|238919826|ref|YP_002933341.1|	UNIPROT_AC:Q7CIP7	76.32	0	1872
gi|238919860|ref|YP_002933375.1|	UNIPROT_AC:Q81LK7	37.32	2.00E-46	183
gi|238919957|ref|YP_002933472.1|	UNIPROT_AC:Q9ZFR9	85.66	0.00E+00	1031
gi|238919974|ref|YP_002933489.1|	UNIPROT_AC:P07954	59.44	4.00E-159	556
gi|238919980|ref|YP_002933495.1|	UNIPROT_AC:P11586	48.34	1.00E-145	512
gi|238919996|ref|YP_002933511.1|	UNIPROT_AC:Q8D1N7	58.71	1.00E-25	111
gi|238920001|ref|YP_002933516.1|	UNIPROT_AC:Q86SX6	36.73	3.00E-20	92
gi|238920016|ref|YP_002933531.1|	UNIPROT_AC:Q81JJ0	37.23	6.00E-35	142
gi|238920065|ref|YP_002933580.1|	UNIPROT_AC:Q7CIL5	26.4	1.00E-23	106
gi|238920209|ref|YP_002933724.1|	UNIPROT_AC:Q81JE5	52.66	5.00E-113	403
gi|238920212|ref|YP_002933727.1|	UNIPROT_AC:Q5NIQ1	27.48	1.00E-17	86.7
gi|238920240|ref|YP_002933755.1|	UNIPROT_AC:P58644	76.21	2.00E-96	347
gi|238920246|ref|YP_002933761.1|	UNIPROT_AC:Q8VSA1	39.39	5.00E-89	324
gi|238920265|ref|YP_002933780.1|	UNIPROT_AC:Q7CJ25	76.97	2.00E-67	250
gi|238920273|ref|YP_002933788.1|	UNIPROT_AC:Q8ZFU4	69.45	6.00E-147	515
gi|238920284|ref|YP_002933799.1|	UNIPROT_AC:Q81QP7	33.55	3.00E-108	388
gi|238920290|ref|YP_002933805.1|	UNIPROT_AC:Q7CI05	69.76	1.00E-99	358
gi|238920314|ref|YP_002933829.1|	UNIPROT_AC:P00830	28	8.00E-33	137
gi|238920333|ref|YP_002933848.1|	UNIPROT_AC:Q81S14	31.26	2.00E-55	212
gi|238920346|ref|YP_002933861.1|	UNIPROT_AC:Q81SX6	46.67	5.00E-50	192
gi|238920349|ref|YP_002933864.1|	UNIPROT_AC:Q8ZGB4	73.28	4.00E-163	569
gi|238920367|ref|YP_002933882.1|	UNIPROT_AC:Q9X6B6	80.27	0	1382
gi|238920390|ref|YP_002933905.1|	UNIPROT_AC:Q8ZGE1	78.73	0	921
gi|238920400|ref|YP_002933915.1|	UNIPROT_AC:Q7CHC8	27.76	1.00E-33	139
gi|238920464|ref|YP_002933979.1|	UNIPROT_AC:Q7CHD9	65.48	1.00E-25	110
gi|238920479|ref|YP_002933994.1|	UNIPROT_AC:Q7CG44	42.04	2.00E-54	209
gi|238920481|ref|YP_002933996.1|	UNIPROT_AC:Q7CHE8	68.53	3.00E-139	490
gi|238920520|ref|YP_002934035.1|	UNIPROT_AC:Q7CH95	88.85	0	1436
gi|238920525|ref|YP_002934040.1|	UNIPROT_AC:O96007	37.04	2.00E-18	87
gi|238920578|ref|YP_002934093.1|	UNIPROT_AC:Q7CJ95	69.44	2.00E-84	306
gi|238920608|ref|YP_002934123.1|	UNIPROT_AC:Q8ZD27	76.45	6.00E-116	412
gi|238920609|ref|YP_002934124.1|	UNIPROT_AC:Q7CJA6	75.3	2.00E-150	527
gi|238920615|ref|YP_002934130.1|	UNIPROT_AC:Q7CJB1	83.74	0	684
gi|238920688|ref|YP_002934203.1|	UNIPROT_AC:Q8D0P4	46.02	2.00E-107	385
gi|238920712|ref|YP_002934227.1|	UNIPROT_AC:Q8ZGW7	84.73	0	1147
gi|238920729|ref|YP_002934244.1|	UNIPROT_AC:Q7CH58	82.51	3.00E-168	586
gi|238920822|ref|YP_002934337.1|	UNIPROT_AC:Q5NH21	46.79	4.00E-75	276
gi|238920879|ref|YP_002934394.1|	UNIPROT_AC:Q5NEL1	53.59	1.00E-147	518

gi|238920896|ref|YP_002934411.1|	UNIPROT_AC:Q5NHS1	61.9	0	700
gi|238920902|ref|YP_002934417.1|	UNIPROT_AC:Q8ZCP4	79.01	1.00E-109	391
gi|238920907|ref|YP_002934422.1|	UNIPROT_AC:Q81LR7	58.28	0	750
gi|238920914|ref|YP_002934429.1|	UNIPROT_AC:Q5NGA0	37.91	4.00E-70	261
gi|238920969|ref|YP_002934484.1|	UNIPROT_AC:Q7CKD3	78.17	0	734
gi|238921023|ref|YP_002934538.1|	UNIPROT_AC:Q81VE0	51.35	5.00E-161	563
gi|238921024|ref|YP_002934539.1|	UNIPROT_AC:P12268	40.74	2.00E-88	321
gi|238921042|ref|YP_002934557.1|	UNIPROT_AC:Q7CJM9	83.72	0	687
gi|238921086|ref|YP_002934601.1|	UNIPROT_AC:Q8ZBU9	90.98	6.00E-123	435
gi|238921089|ref|YP_002934604.1|	UNIPROT_AC:P14576	31.77	8.00E-55	210
gi|238921110|ref|YP_002934625.1|	UNIPROT_AC:Q8ZBQ3	81.81	0	1415
gi|238921208|ref|YP_002934723.1|	UNIPROT_AC:Q81QN8	41.04	7.00E-88	320
gi|238921209|ref|YP_002934724.1|	UNIPROT_AC:Q8ZHI8	77.48	0	1529
gi|238921320|ref|YP_002934835.1|	UNIPROT_AC:Q8CWI1	79.95	0	693
gi|238921322|ref|YP_002934837.1|	UNIPROT_AC:Q8CWG4	26.02	2.00E-19	93.2
gi|238921352|ref|YP_002934867.1|	UNIPROT_AC:Q8ZAW9	67.44	1.00E-28	120
gi|238921379|ref|YP_002934894.1|	UNIPROT_AC:Q81S14	25.56	1.00E-22	103
gi|238921380|ref|YP_002934895.1|	UNIPROT_AC:Q8D1R3	32.29	2.00E-46	183
gi|238921387|ref|YP_002934902.1|	UNIPROT_AC:Q8ZA85	29.6	7.00E-14	74.3
gi|238921392|ref|YP_002934907.1|	UNIPROT_AC:Q5NE96	42.39	3.00E-27	116
gi|238921399|ref|YP_002934914.1|	UNIPROT_AC:Q81JP7	48.84	9.00E-84	305
gi|238921410|ref|YP_002934925.1|	UNIPROT_AC:Q8ZDX8	30.32	4.00E-25	110
gi|238921432|ref|YP_002934947.1|	UNIPROT_AC:P62829	40	4.00E-11	62
gi|238921541|ref|YP_002935056.1|	UNIPROT_AC:Q7CI04	29.13	3.00E-12	65.9
gi|238921570|ref|YP_002935085.1|	UNIPROT_AC:Q7CG67	28.3	2.00E-87	319
gi|238921637|ref|YP_002935152.1|	UNIPROT_AC:Q81U58	60	0	658
gi|238921638|ref|YP_002935153.1|	UNIPROT_AC:Q92482	39.16	1.00E-42	169
gi|238921673|ref|YP_002935188.1|	UNIPROT_AC:Q8D051	45.48	3.00E-81	297
gi|238921684|ref|YP_002935199.1|	UNIPROT_AC:Q81KV7	43.36	5.00E-92	333
gi|238921701|ref|YP_002935216.1|	UNIPROT_AC:Q5NFP9	69.49	0	887
gi|238921725|ref|YP_002935240.1|	UNIPROT_AC:Q81V35	52.79	3.00E-83	304
gi|238921727|ref|YP_002935242.1|	UNIPROT_AC:Q81V37	43.17	8.00E-33	134
gi|238921731|ref|YP_002935246.1|	UNIPROT_AC:Q81S64	47.2	5.00E-77	283