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Identification of the *Edwardsiella ictaluri* genes causing impaired growth in complex medium

By

Safak Kalindamar

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

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Safak Kalindamar

2013

Identification of the *Edwardsiella ictaluri* genes causing impaired growth in complex medium

By

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Edwardsiella ictaluri is the causative agent of enteric septicemia of catfish (ESC). Although some virulence mechanisms in *E. ictaluri* have been identified, further research is needed to discover new virulence genes, which could be used to develop safe and efficacious live vaccines. Here, we report production of growth deficient *E. ictaluri* mutants on complex agar media and identification of genes causing this growth deficiency.

The overall goal of this project is to generate growth deficient *E. ictaluri* mutants and identify genes causing this growth deficiency on complex media. Mutants exhibiting slow growth in complex media may be potential candidates for vaccine development. In this study, 56 unique *E. ictaluri* genes have been identified. 32 of them showed host protein binding properties while 30 of them were found to be involved in bacterial virulence in other pathogenic bacteria.

DEDICATION

I would like to dedicate this research to my parents Pakize Kalindamar and Selahattin Kalindamar, and my brother Donem Kalindamar.

ACKNOWLEDGEMENTS

I want to thank my major professor Dr. Attila Karsi for mentorship, encouragement, and support during my research. I am also thankful to my committee members Dr. Mark L. Lawrence and Dr. Andy D. Perkins for their guidance and critical review of my research. I am grateful to the Ministry of National Education of the Republic of Turkey for financial support during my Master of Science studies.

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LIST OF SYMBOLS

E. ictaluri: *Edwardsiella ictaluri*

E. coli: *Escherichia coli*

MvirDB: Microbial virulence database

HPIDB: Host pathogen interaction database

CHAPTER I

INTRODUCTION

The catfish industry

Aquaculture is a rapidly developing agricultural industry in the US with 1.4 billion dollars of sale value (USDA, 2009). Channel catfish is the major aquaculture commodity in the Southeastern United States, especially in Mississippi, Alabama, Arkansas and Louisiana, which accounted for $341 million in 2011 (USDA, 2012). Farm raised catfish production is one of the most economically important agriculture activities in Mississippi with production acreage of 51,200 and sales of $165 million from 180 catfish operations in 2012 (Mississippi State University, 2012). However, bacterial diseases, enteric septicemia of catfish (ESC) in particular, cause significant economic losses in the catfish industry. *Edwardsiella ictaluri* is the causative agent of ESC (Hawke, 1998) and it is well-adapted to channel catfish.

Edwardsiella ictaluri

E. ictaluri belongs to the *Edwardsiella* genus and is a member of the *Enterobacteriaceae* family (Hawke, 1979). It is a Gram-negative, facultative anaerobe, catalase positive, lactose negative, and glucose fermentative intracellular rod first isolated from an outbreak in Alabama and Georgia (Hawke et al., 1981b). *E. ictaluri* is motile by peritrichous flagella at 25 ^{o}C but non-motile at higher temperatures. *E. ictaluri* is a slow-

growing bacterium on agar plates due to its requirement of a rich and complex medium for growth (Collins et al., 1996). *E. ictaluri* typically requires incubation for 48 h at the optimal growth temperature of 25 ºC (Hawke et al., 1981a). Although members of *Edwardsiella* genus has capability of infecting variety of animal species, *E. ictaluri* is well adopted to channel catfish (Janda et al., 1991).

Enteric septicemia of catfish (ESC)

Enteric septicemia is a serious problem accounting for $20-$30 million annual loss in the United States. Approximately, one third of all disease cases diagnosed in the Southern-Eastern United States is ESC (Hawke, 1998).

Clinical signs and treatment

Two forms of ESC have been described in farm-raised catfish production; an acute form and a chronic form. The acute (septicemic) form is related to the gut route of infection and causes necrosis and ulceration in various organs after the bacteria enter the bloodstream through the intestine. The chronic (encephalitic) form is related to the nerve route of infection and causes meningoencephalitis in which an infection spreads from meninges to the skull and the skin and leads to “hole in the head” lesions (MacMillan, 1985; Newton et al., 1989; Shotts et al., 1986). In the acute form, rapid mortalities are typically observed with few external signs. Clinically affected fish may occasionally hang head up in the water and exhibit corkscrew spiral swimming. In the chronic form, disease progresses more slowly than the acute form, death occurring three to four weeks after an acute outbreak (Newton et al., 1989; Shotts et al., 1986).

Ormetoprim-sulfadimethoxine, florfenicol and oxytetracycline antibiotics have been approved for treatment of ESC, but because one of the earliest clinical signs of ESC is anorexia, treatment with these oral antibiotics is not effective. Also, *E. ictaluri* isolates could develop resistance to these antibiotics (McGinnis et al., 2003; Taylor et al., 1991). Therefore, vaccination could be an alternative approach for preventing ESC and reducing for antibiotic use.

Vaccination against ESC

Studies have shown that live attenuated vaccines are effective, because they stimulate the immune response by themselves or by the heterologous antigens they carry. In particular, attenuation is generally based on deletion of virulence factors or mutations of genes encoding metabolic enzymes vital for microorganism (Detmer et al., 2006; Judson et al., 2000). Live attenuated vaccines have the potential to be used as an alternative method for providing a high level of protection against ESC. Live attenuated vaccines simulate a natural infection so that protective antigens are expressed without causing infection in the host. Chondroitinase (Cooper et al., 1996), auxotrophic (*aroA*) (Thunea et al., 1999) and *purA* (Lawrence et al., 1997) mutants have been determined to be live attenuated vaccine candidates for *E. ictaluri*. Also, there is a commercial live attenuated ESC vaccine (Aquavac-ESC), but ESC still continues to be a major problem in catfish aquaculture. Thus, there is a need to develop novel live-attenuated vaccines. For this aim, it is important to identify which virulence genes require alteration to attenuate the organism.

CHAPTER II

IDENTIFICATION OF THE *EDWARDSIELLA ICTALURI* GENES CAUSING IMPAIRED GROWTH IN COMPLEX MEDIUM

Abstract

Edwardsiella ictaluri is the causative agent of enteric septicemia of catfish (ESC). Although some virulence mechanisms in *E. ictaluri* have been identified, further research is needed to discover new virulence genes, which could be used to develop safe and efficacious live vaccines. Here, we report production of growth deficient *E. ictaluri* mutants on complex agar media and identification of genes causing this growth deficiency.

Introduction

Enteric septicemia of catfish (ESC) is a devastating disease that leads to millions of dollars lost due to production losses and treatment costs. Antibiotics were used commonly as a control agent of ESC. However, antibiotic use is not effective to treat the sick fish by medicated feed due to onset of anorexia. Also, antibiotic use causes emergence of antibiotic resistant strains, which is not desirable (Hawke, 1998). Live attenuated vaccines have the potential as an alternative method for providing a high level of protection against ESC. Live attenuated vaccines simulate of a natural infection so that protective antigens are expressed without causing infection in the host. There is a

commercial live attenuated ESC vaccine, Aquavac-ESC (RE33). However, ESC is still the major disease in the catfish industry. Thus, there is a need to develop novel live-attenuated mutants. For this, it is important to identify which virulence genes require alternation.

High-throughput methods can be used to identify possible virulence factors and genes essential for an organism's survival. Random transposon mutagenesis is one of the high-throughput methods that provide genome-wide insertion mutant libraries. The insertion disrupts genes and its function can be evaluated. Several studies have reported that transposons can be used to describe essential genes for virulent bacteria (Judson et al., 2000; Liberati et al., 2006). In this research, we report identification of potential virulence genes of *E. ictaluri* by transposon mutagenesis. Mutants exhibiting slow growth in complex media may be potential candidates for vaccine development.

Materials and Methods

Bacterial strains, plasmids and media

Bacterial strains and plasmids are listed in Table 1. Transposon insertion mutants were generated in wild type *Edwardsiella ictaluri* 93-146 with pAKgfplux1 (Karsi et al., 2007) by using *Escherichia coli* SM10-λ*pir* with pMAR2xT7 transposon (Figure 1) (Liberati et al., 2006). *E. coli* was grown at 37 ^{o}C with Luria-Bertani (LB) broth and agar plates. *E. ictaluri* was grown at 28 ^{o}C with brain-heart infusion (BHI) broth and agar plates.

Table 1 Bacterial strains and plasmids.

Strain or plasmid	Description	Source
Escherichia coli		
SM10 λ*pir*	Km^r; *thi; thr; leu; tonA; lacY; supE; recA;*::RP4-2-Tc::Mu; λ*pir* R6K	(de Lorenzo et al., 1994)
Edwardsiella ictaluri		
93-146	Wild-type; pEI1; pEI2; Col^r	(Lawrence et al., 1997)
Plasmids		
pAKgfplux1	Amp^r; *lacZ;* P_{lac}; P_{T7}; *gfpmut3a; luxCDABE*	(Karsi et al., 2007)
pMAR2xT7	R6K replicon; *Himar I*; T7 promoters; Amp^r; Gen^r	(Liberati et al., 2006)

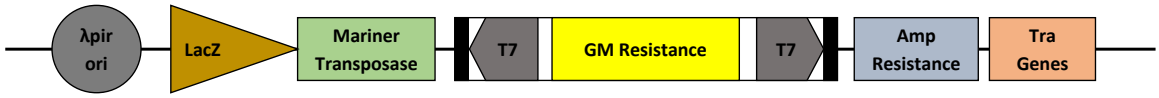

Figure 1 Structure of pMAR2xT7.

Construction of transposon insertion library

Conjugation

To prepare a transposon insertion library, conjugation was performed between *E. coli* SM10-λ*pir* with pMAR2xT7 (donor) and *E. ictaluri* 93-146 wild type containing pAKgfpLux1 (recipient). *E. coli* SM10-λ*pir* was grown in 2 ml Luria-Bertani (LB) broth containing 100 µg/ml of ampicillin at 37 ^oC overnight (16-18 h) with continuous shaking at 200rpm. *E. ictaluri* was grown in 2 ml brain-heart infusion (BHI) broth containing 12.5 µg/ml of colistin at 28 ^oC overnight (16-18 h) with continuous shaking at 200 rpm. The next day, 100 µl of overnight culture of *E. coli* SM10-λ*pir* was inoculated to 5 ml fresh

LB (no antibiotics). 400 µl of overnight culture of *E. ictaluri* 93-146 was inoculated to 5 ml fresh BHI (no antibiotics). The donor and recipient cultures were grown for 2 h and the OD_{600} measured. When OD_{600} reached 0.6., 1000 µl of donor and 1000 µl of recipient culture were collected separately by centrifugation at 12,000 rpm for 2 min and pellets were washed 3 times with BHI broth (no antibiotics). Pellets of donor and recipient were resuspended in 1,000 µl of BHI and 125 µl of donor and 500 µl of recipient bacteria were mixed in a 1.5 ml micro-centrifuge tube and spun at 12,000 rpm for 2 min (donor to recipient ration = 1:4). Small punched pieces of sterile 0.45 µM filter papers were placed on BHI agar plate (no antibiotics). Supernatant was removed from the donor and recipient mixture and spun briefly one more time to collect liquid, in which bacteria were dissolved by pipetting up and down several times to obtain viscous slurry of bacteria. A drop of viscous bacteria mixture was added onto the center of the filter and the plate incubated at 28 ºC for 1 h, then inverted and incubated at 28 ºC for 24 h. The next day, filters were transferred into 1.5 ml sterile centrifuge tube and washed with 825 µl BHI containing 100 µg/ml of ampicillin, 12.5 µg/ml of gentamicin and 25 µg/ml colistin. 25 µl of bacteria was diluted with 225 µl of water (10 times dilution). Then, 50 µl of diluted culture was spread onto selective BHI plates containing 100 µg/ml of ampicillin, 12.5 µg/ml of gentamicin and 25 µg/ml colistin. Plates were incubated at 28 ºC for 36-48 h. Various size colonies (very small to very large) were observed on the plate and 250 smallest colonies (growth deficient) were picked, cultured in BHI broth with appropriate antibiotics at 28 ºC for two days, and frozen at -80 ºC.

Transposon end mapping

Genomic DNA isolation

100 µl frozen culture was diluted in 1 ml distilled water (ddH_2O) and mixed well. Bacteria were collected by centrifugation at 20,817 g for 3 min. Entire supernatant except 50 µl was removed and bacterial pellet was dissolved by vortexing. Each sample was transferred to 200 µl PCR tubes and cells were incubated at 100 °C for 10 min by using the Applied Biosystems 2720 Thermal Cycler. Samples containing genomic DNA and bacterial cell debris were transferred into 1.5 ml micro-centrifuge tubes and gDNA was isolated by removing bacterial debris at 20,817 g for 10 min. Supernatant was used as a gDNA template in subsequent PCR reactions.

PCR amplification of transposon ends

Single primer PCR was performed to amplify the transposon end and flanking bacterial DNA (Karlyshev et al., 2000). Total PCR volume per reaction was 25 µl, containing 100 ng of genomic DNA, 13.87 µl of ddH_2O, 0.5 µl of 10 mM pMAR2xT7 specific reverse R1 primer (5`-CCGTATGCCCAACTTTGTATAGA-3`), 0.5 µl of 10 mM dNTPs (Promega, Madison, WI), 5 µl of 7.5 mM 5X Green GoTaq Reaction Buffer (Promega, Madison, WI), and 0.025 U of Taq DNA Polymerase (Promega, Madison, WI). PCR amplification was performed using the Applied Biosystems 2720 Thermal Cycler. The following PCR parameters was used: denaturation at 94 °C for 1 min, followed by 20 cycles of 94 °C 30s, 50 °C 30s, and 72 °C for 3 min; 30 cycles of 94 °C for 30s, 30 °C for 30 and 72 °C for 2 min; and 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min, followed by a 7 min extension at 72 °C.

Agarose gel electrophoresis

The amplified single primer PCR products were analyzed using the agarose gel electrophoresis. 5 X TBE buffer (54 g of Tris base, 27.5 g of boric acid and 20 ml 0.5M EDTA dissolved in ddH_2O up to 1 L). To prepare a 1 % agarose gel, 1 g of agarose was dissolved in 100 ml 0.5 X TBE buffer and boiled in microwave until a clear solution was observed. The molten agar was cooled, and 5 µl SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) was added, which was then poured into a gel apparatus with a comb in place. After agar solidify, it is covered with 0.5 X TBE buffer and 2 µl of single primer PCR product mixed with a loading dye were loaded in each well. Gel electrophoresis was run at constant voltage of 110 V for 90 min and bands are visualized under UV in a BioRAD ChemiDOC XRS^+ (BioRAD, Hercules, CA). Sizes of fragments were estimated by using 6 µl of 1 kb^+ DNA ladder (Promega, Madison, WI).

ExoSAP-IT treatment and sequencing

Single primer PCR products were cleaned by using 4 µl ExoSAP-IT PCR (Affymetrix, Santa Clara, CA) for 10 µl of PCR product. Treatment was done at 37 °C for 15 minutes and reaction was stopped by increasing the temperature to 80 °C for 15 min. Sequencing was conducted at Eurofins MWG Operon (Huntsville, AL) using the transposon specific nested R3 primer (5`- TCTCGGCTTGAACGAATTGTT-3`). Total 161 transposon inserted mutants were sequenced.

Bioinformatics analysis

Transposon sequence removal and sequence trimming based on sequence quality scores were done by using the Sequencher v4.10.1 (Gene Codes Corp., Ann Arbor, MI).

Trimmed sequences were searched against the available *E. ictaluri* genome using BlastX (Williams et al., 2012b). Using the GI numbers, a FASTA file containing all protein sequences were downloaded from the Genbank and used for Blast2GO, PSORTb, HPIDB, and MVirDB queries. Gene Ontology (GO) annotation, visualization and metabolic and cellular processes were determined by using Blast2GO (Conesa et al., 2005). PSORTb v3.0 was used to predict bacterial protein subcellular localization (Yu et al., 2010). Host Pathogen Interaction Database (HPIDB) including experimental protein-protein interaction (PPIs) information from several public accessible databases (Kumar et al., 2010) was used to search genes involving in host-pathogen interactions. MVirDB was used to identify potential virulence genes (Zhou et al., 2007).

Results

Blastx

Total 250 single primer PCR reactions were run and 151 showed amplification. Sequencing of the 151 PCR products produced 94 good sequences. Blastx search of these 94 sequences resulted in 56 unique genes with transposon insertion (Table 2).

Table 2 56 unique *E. ictaluri* genes with transposon insertion.

Gene ID	NT	Gene	E-value	Freq.
Eis001	NT01EI_1281	NAD-dependent malic enzyme (NAD-ME)	2.00E-72	
Eis002	NT01EI_1721	PTS system, mannose/fructose/sorbose family, IIB component	2.00E-106	
Eis004	NT01EI_0182	Hypothetical protein NT01EI_0182	7.00E-57	
Eis006	NT01EI_0085	ATP-dependent DNA helicase Rep, putative	2.00E-114	
Eis009	NT01EI_1236	Coproporphyrinogen III oxidase, aerobic	6.00E-32	
Eis011	NT01EI_3690	ABC transporter, perplasmic amino acid binding protein, putative	1.00E-74	
Eis013	NT01EI_2795	Translocator protein, LysE family	2.00E-115	
Eis018	NT01EI_0377	Aspartate ammonia-lyase, putative	7.00E-145	
Eis024	NT01EI_3505	Dihydrouridine synthase Dus	1.00E-129	
Eis027	NT01EI_0408	tRNA delta(2)-isopentenylpyrophosphate transferase, putative	5.00E-94	
Eis028	NT01EI_0277	Transposase, IS4 family protein	1.00E-73	4
Eis029	NT01EI_2683	Hypothetical protein NT01EI_2683	2.00E-88	
Eis033	pEI2_p2	Putative Rep protein	2.00E-123	5
Eis035	pEI1_p4	Putative RNA one modulator protein	9.00E-19	
Eis037	NT01EI_2355	Hypothetical protein NT01EI_2355 (E3 ubiquitin-protein ligase SspH2)	3.00E-129	2
Eis038	NT01EI_1334	Hypothetical protein NT01EI_1334 (Type III secretion system leucine rich repeat)	5.00E-67	2
Eis039	NT01EI_3177	FeS assembly protein IscX, putative	3.00E-38	
Eis041	NT01EI_0943	Type III secretion outer membrane pore, YscC/HrcC family, putative	8.00E-116	
Eis048	NT01EI_3148	hypothetical protein NT01EI_3148	4.00E-80	
Eis055	NT01EI_2314	Prophage lambda integrase, putative	8.00E-128	
Eis059	NT01EI_1941	Hypothetical protein NT01EI_1941	3.00E-70	
Eis065	NT01EI_2281	Excinuclease ABC subunit C, putative	3.00E-48	
Eis068	NT01EI_0448	Polyprenyl synthetase	1.00E-115	
Eis080	NT01EI_0981	Hypothetical protein NT01EI_0981	8.00E-129	9
Eis086	NT01EI_1237	N-acetylmuramoyl-L-alanine amidase AmiA, putative	3.00E-67	
Eis107	NT01EI_0475	DEAD box containing helicase	1.00E-112	
Eis110	NT01EI_1332	Hypothetical protein NT01EI_1332	4.00E-26	2
Eis131	NT01EI_2157	Hypothetical protein NT01EI_2157	4.00E-66	
Eis152	NT01EI_0224	Transporter, major facilitator family	1.00E-16	4
Eis154	NT01EI_3522	Selenate reductase, FAD-binding subunit, putative	2.00E-73	
Eis155	NT01EI_0725	Transcriptional regulator FruR, putative	3.00E-134	
Eis156	NT01EI_2381	Ribonuclease, RNaseE/RNaseG family , putative	2.00E-04	
Eis157	NT01EI_0144	Twin-arginine translocation protein subunit TatB, putative	8.00E-07	
Eis158	NT01EI_0022	Hypothetical protein NT01EI_0022	8.00E-46	
Eis171	NT01EI_3723	Magnesium-translocating P-type ATPase, putative	0	
Eis172	NT01EI_3786	Hypothetical protein NT01EI_3786	1.00E-26	
Eis173	NT01EI_3265	Acyltransferase/AMP-dependent synthetase and ligase protein family	0	2

Table 2 (continued)

Eis174	NT01EI_3721	Hypothetical protein NT01EI_3721	3.00E-25	
Eis175	NT01EI_3774	IS1 transposase	7.00E-93	
Eis176	NT01EI_0962	Type III secretion apparatus protein SpaR/YscT/HrcT, putative	5.00E-129	
Eis180	NT01EI_3103	UPF0126 domain protein	7.00E-12	
Eis183	NT01EI_3105	Chloride transporter, chloride channel (ClC) family	6.00E-166	
Eis184	NT01EI_0419	RNA methyltransferase, TrmH family, group 3	9.00E-27	
Eis185	NT01EI_3386	TRAP transporter, DctM subunit	7.00E-85	
Eis192	NT01EI_3147	Hypothetical protein NT01EI_3147	0	2
Eis194	NT01EI_1981	Hypothetical protein NT01EI_1981	9.00E-86	3
Eis195	NT01EI_0376	Anaerobic C4-dicarboxylate transporter DcuA, putative	2.00E-159	11
Eis207	NT01EI_1817	Spermidine/putrescine transport system permease protein PotB, putative	4.00E-132	
Eis210	NT01EI_0800	Prolipoprotein diacylglyceryl transferase, putative	5.00E-79	
Eis220	NT01EI_2076	Hypothetical protein NT01EI_2076	6.00E-20	
Eis222	NT01EI_0768	Hypoxanthine phosphoribosyltransferase, putative	5.00E-127	
Eis223	NT01EI_1086	Extracellular solute-binding protein, family 5	2.00E-142	
Eis230	NT01EI_3769	Phosphoglycerate transporter family protein	0	
Eis232	NT01EI_2010	Hypothetical protein NT01EI_2010	2.00E-136	3
Eis233	NT01EI_2530	Putative permease, membrane region	4.00E-81	
Eis235	NT01EI_3289	Diaminopimelate decarboxylase, putative	9.00E-36	

Blast2GO

The protein sequences of all 56 genes were blasted against public and private sequence databases followed by InterProScan to complete the functional annotation depending on BLAST with protein domain information. Annotated sequences were assigned to gene ontologies according to biological process (Figure 2), cellular component (Figure 3), and molecular function (Figure 4) at level 2. The results showed that single-organism process, metabolic process, cellular process, and localization categories contained higher number of proteins. Similarly, cell and membrane in cellular component category; and binding, catalytic activity, and transporter activity in molecular function category contained higher numbers of proteins.

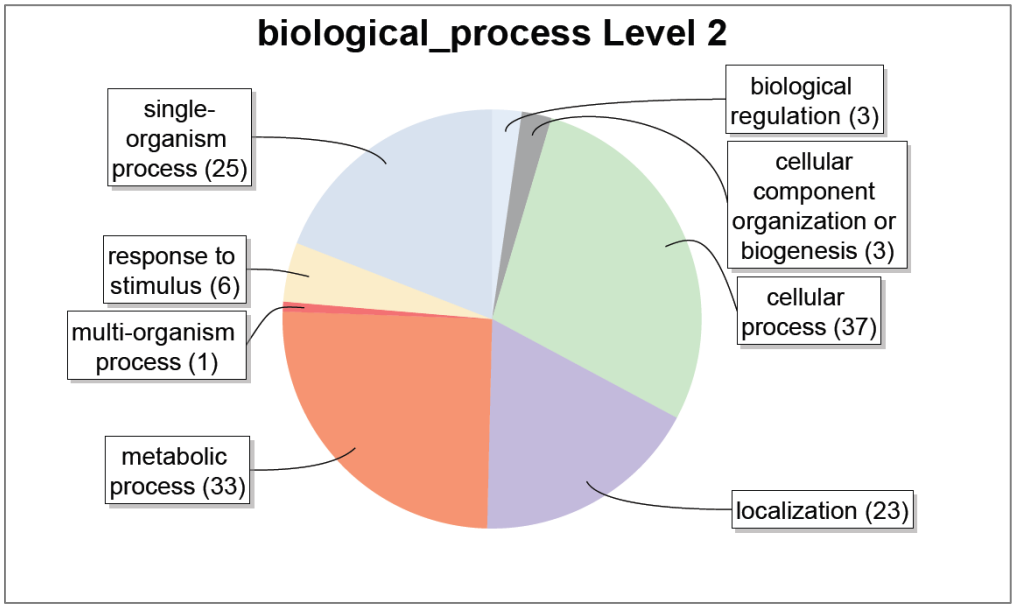

Figure 2 GO terms at level 2 according to biological process.

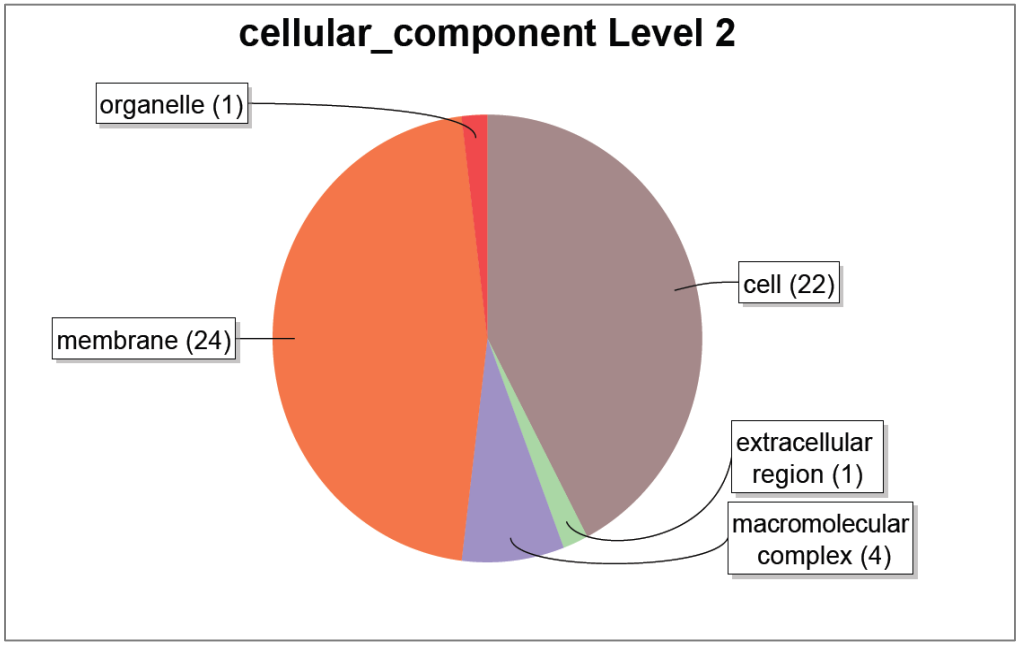

Figure 3 GO terms at level 2 according to cellular component.

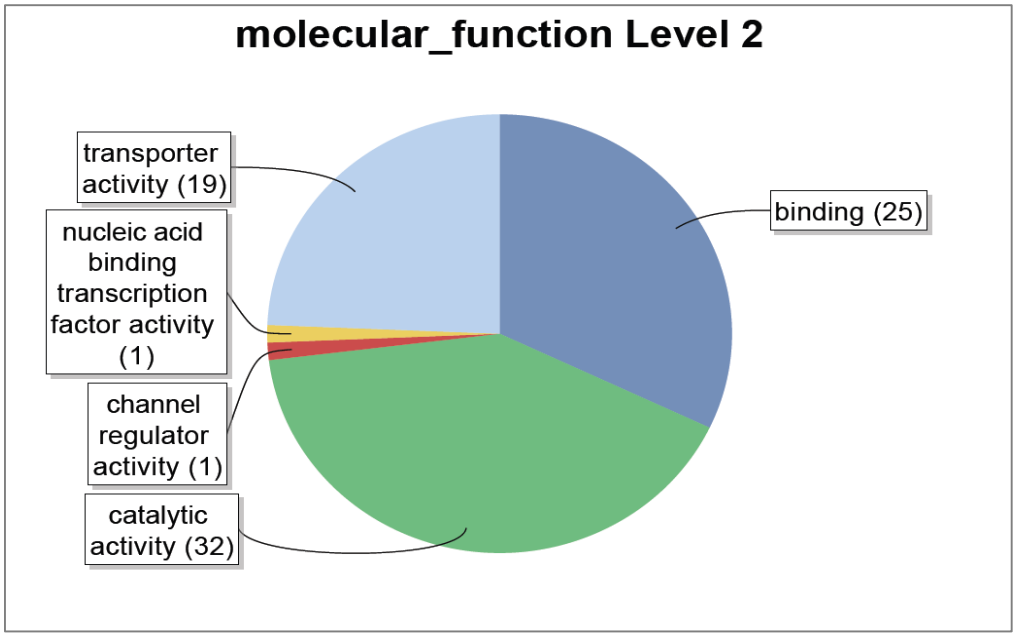

Figure 4 GO terms at level 2 according to molecular function.

PSORTb

The subcellular locations of 56 proteins are shown in Figure 5. Location of 15 proteins could not be predicted (unknown category). Of the 41 proteins, most of them were localized to cytoplasm and cytoplasmic membrane. Outer membrane and periplasm contained very few proteins.

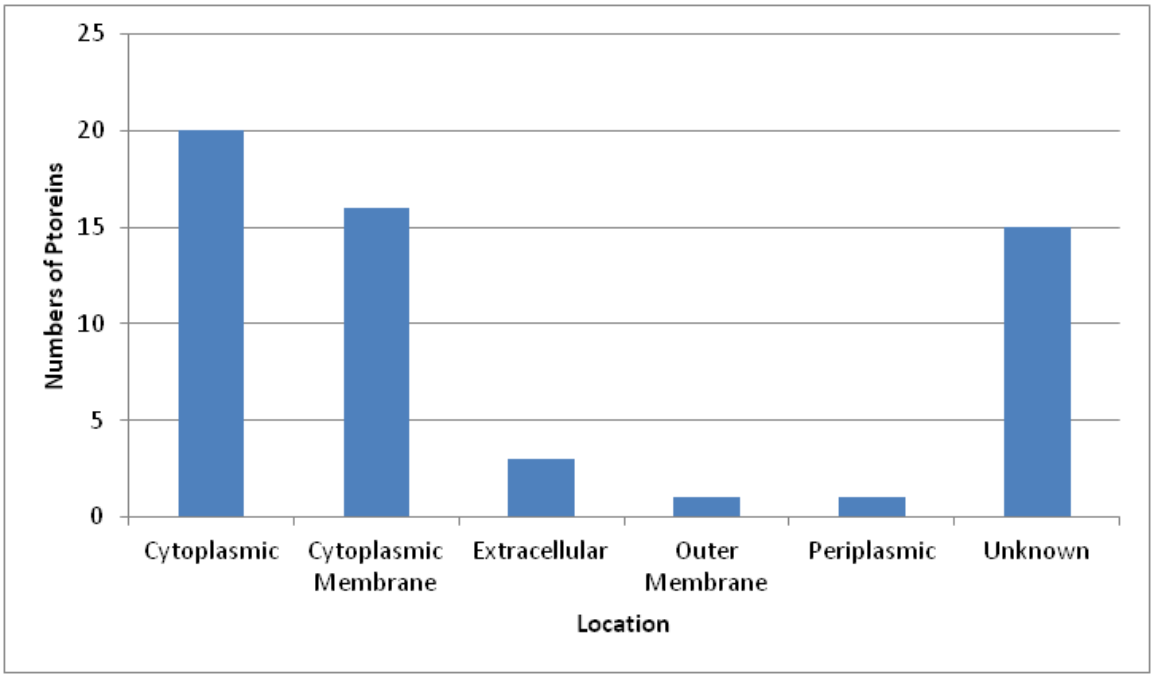

Figure 5 Bacterial localization prediction by PSORTb.

HPIDB (Host Pathogen Interaction Database)

32 out of 56 transposon insertions were involved in host pathogen interactions (Table 3). Proteins mostly matched to *Yersinia pestis*, followed by *Bacillus anthracis*, *Francisella tularensis SCHU S4, Escherichia coli K12* and *Shigella flexneri* (Figure 6).

Table 3 30 genes involved in host pathogen interactions.

Gene ID	Accession No	Protein	Organism	GI	E-value
Eis152	YP_019321.1	Oxalate:formate antiporter, putative	*Bacillus anthracis*	47527972	2.00E-20
Eis155	YP_017710.1	Sugar-binding transcriptional regulator, LacI family	*Bacillus anthracis*	47526361	2.00E-21
Eis207	YP_002347936.1	Inner membrane permease T of sulfate/thiosulfate ABC transporter	*Yersinia pestis*	218930061	1.00E-12
Eis011	YP_017492.1	Amino acid ABC transporter, amino acid-binding protein	*Bacillus anthracis*	47526143	1.00E-15
Eis013	NP_670988.1	Putative threonine efflux protein	*Yersinia pestis*	22127565	5.00E-11
Eis171	YP_002345523.1	Putative cation transport protein	*Yersinia pestis*	218927648	4.00E-88
Eis223	YP_002345598.1	HTH-type transcriptional regulator SgrR	*Yersinia pestis*	218927723	2.00E-46
Eis176	NP_857736.1	Yop proteins translocation protein T	*Yersinia pestis*	31795275	9.00E-31
Eis107	YP_022388.1	ATP-dependent RNA helicase, DEAD/DEAH box family	*Bacillus anthracis*	47531039	1.00E-84
Eis024	NP_842644.2	tRNA-dihydrouridine synthase	*Bacillus anthracis*	27000059	0 6.00E-62
Eis110	NP_858359.2	E3 ubiquitin-protein ligase ipaH9.8	*Shigella flexneri*	56404031	6.00E-90
Eis086	NP_667964.1	N-acetylmuramoyl-l-alanine amidase II	*Yersinia pestis*	22124541	1.00E-39
Eis184	YP_016695.1	RNA methyltransferase, TrmH family, group 3	*Bacillus anthracis*	47525346	6.00E-43
Eis180	YP_002345138.1	Putative membrane protein	*Yersinia pestis*	218927263	4.00E-20
Eis006	YP_170066.1	ATP-dependent DNA helicase	*Francisella tularensis*	56708170	1.00E-171
Eis009	YP_170044.1	Coproporphyrinogen-III oxidase, aerobic	*Francisella tularensis*	56708148	9.00E-52
Eis027	YP_169650.1	tRNA dimethylallyltransferase	*Francisella tularensis*	56707754	8.00E-76
Eis041	YP_002345337.1	Possible type III secretion protein	*Yersinia pestis*	218927462	4.00E-154
Eis173	NP_994169.1	Bifunctional protein aas	*Yersinia pestis*	45442630	0
Eis235	YP_002345851.1	Diaminopimelate decarboxylase	*Yersinia pestis*	218927976	7.00E-177
Eis156	NP_669066.1	RNase E	*Yersinia pestis*	22125643	0
Eis183	NP_668136.1	H(+)/Cl(-) exchange transporter ClcA	*Yersinia pestis*	22124713	0
Eis065	NP_669748.1	UvrABC system protein C	*Yersinia pestis*	22126325	0
Eis002	YP_002346757.1	PTS enzyme IIAB, mannose-specific	*Yersinia pestis*	218928882	3.00E-149
Eis222	YP_646612.1	Hypoxanthine phosphoribosyltransferase	*Yersinia pestis*	108810845	6.00E-84
Eis068	YP_491372.1	Octaprenyl-diphosphate synthase	*Escherichia coli*	388479180	2.00E-157
Eis001	YP_002346527.1	NAD-dependent malic enzyme	*Yersinia pestis*	218928652	0
Eis230	YP_001608410.1	Putative regulatory protein	*Yersinia pestis*	162418146	0
Eis018	NP_667943.1	Aspartate ammonia-lyase (Aspartase)	*Yersinia pestis*	22124520	0
Eis233	YP_002346351.1	Putative transport protein YPO1326/y2857/YP_1266	*Yersinia pestis*	218928476	0

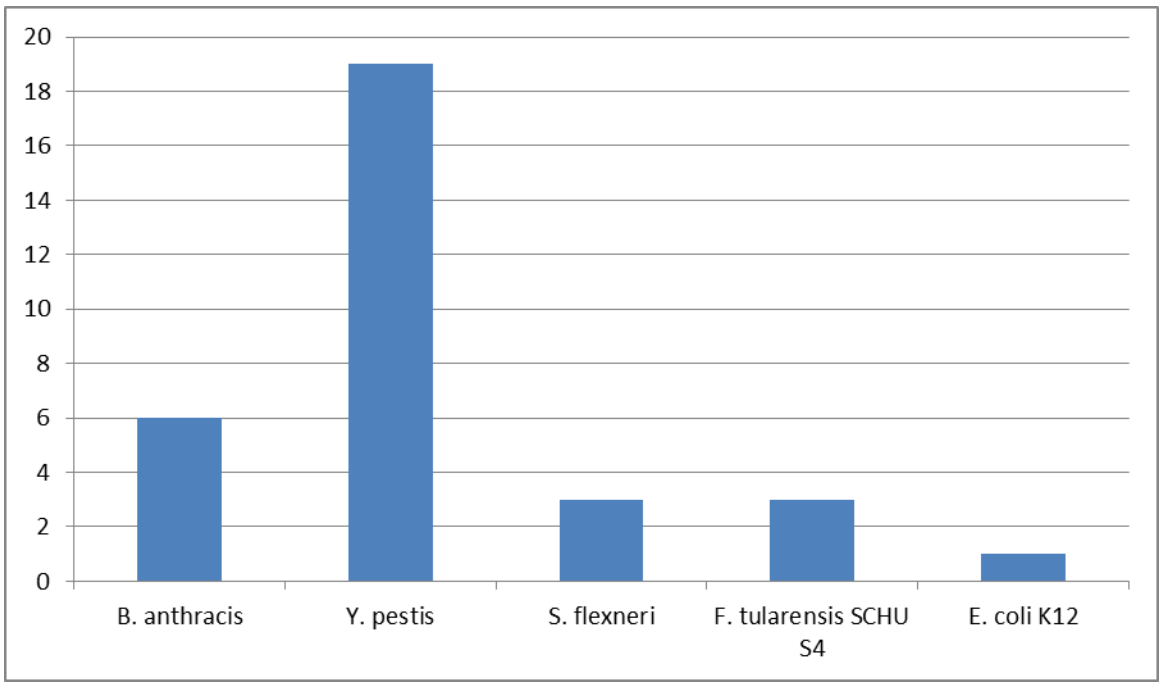

Figure 6 Species *E. ictaluri* proteins showed similarity in HPIDB.

MVirDB (Microbial Virulence Database)

30 proteins were determined to be similar to known virulence factors from other bacteria in the microbial virulence database (Table 4).

Table 4 Virulence factors.

Gene ID	NT	GI	Number of hits	Lowest E-value	Accession (E-value)	Protein
Eis041	NT01EI_0943	238918877	250	0	131220	Type III secretion outer membrane pore, YscC/HrcC family
Eis110	NT01EI_1332	238919243	91	0	115433	Hypothetical protein NT01EI_1332
Eis171	NT01EI_3723	417359287	22	0	110875	Magnesium-translocating P-type ATPase
Eis192	NT01EI_3147	238921017	10	0	120131	Hypothetical protein NT01EI_3147
Eis037	NT01EI_2355	417359148	192	9.13E-144	115433	Hypothetical protein NT01EI_2355 (E3 ubiquitin-protein ligase SspH2)
Eis038	NT01EI_1334	238919245	267	3.95E-121	115433	Hypothetical protein NT01EI_1334 (Type III secretion system leucine rich repeat)
Eis001	NT01EI_1281	238919198	6	1.97E-95	128674	NAD-dependent malic enzyme (NAD-ME)
Eis195	NT01EI_0376	238918338	5	8.13E-87	115771	Anaerobic C4-dicarboxylate transporter DcuA
Eis173	NT01EI_3265	238921126	221	8.89E-61	131711	Acyltransferase/AMP-dependent synthetase and ligase protein family
Eis185	NT01EI_3386	238921243	5	1.74E-56	115847	TRAP transporter, DctM subunit
Eis107	NT01EI_0475	238918430	11	2.34E-56	128147	DEAD box containing helicase
Eis230	NT01EI_3769	238921614	250	1.16E-46	118100	Phosphoglycerate transporter family protein
Eis028	NT01EI_0277	417358948	8	4.26E-43	120092	Transposase, IS4 family protein
Eis175	NT01EI_3774	238921619	34	6.93E-41	118297	IS1 transposase
Eis184	NT01EI_0419	238918378	8	2.71E-40	120591	RNA methyltransferase, TrmH family, group 3
Eis176	NT01EI_0962	238918896	144	9.04E-38	116182	Type III secretion apparatus protein SpaR/YscT/HrcT
Eis157	NT01EI_0144	238918120	250	4.66E-29	130356	Twin-arginine translocation protein subunit TatB
Eis068	NT01EI_0448	238918407	2	4.13E-26	119169	Polyprenyl synthetase
Eis155	NT01EI_0725	238918667	9	4.05E-12	120505	Transcriptional regulator FruR, putative
Eis086	NT01EI_1237	238919166	4	9.61E-12	117141	N-acetylmuramoyl-L-alanine amidase AmiA
Eis006	NT01EI_0085	417358934	40	9.81E-12	117150	ATP-dependent DNA helicase Rep, putative
Eis235	NT01EI_3289	238921148	20	2.44E-10	110259	Diaminopimelate decarboxylase, putative
Eis055	NT01EI_2314	238920205	22	7.18E-08	122349	Prophage lambda integrase, putative
Eis207	NT01EI_1817	238919714	59	1.13E-07	110338	Spermidine/putrescine transport system permease protein PotB, putative
Eis080	NT01EI_0981	417358998	10	1.52E-06	115431	Hypothetical protein NT01EI_0981
Eis222	NT01EI_0768	238918710	44	1.67E-06	126608	Hypoxanthine phosphoribosyltransferase
Eis018	NT01EI_0377	238918339	4	1.96E-06	120511	Aspartate ammonia-lyase
Eis011	NT01EI_3690	238921538	9	2.98E-04	110969	ABC transporter, perplasmic amino acid binding protein
Eis131	NT01EI_2157	238920051	21	9.55E-03	115941	Hypothetical protein NT01EI_2157
Eis223	NT01EI_1086	238919019	16	9.91E-03	120571	Extracellular solute-binding protein, family 5

Potential virulence factors

The results from Blast2GO, PSORTb, HPIDB, and MVirDB were pooled together in Table 5.

Table 5 Potential virulence factors identified with bioinformatics analysis.

Gene ID	GI	NT	Blastx E value	MVirDB E value	Gene	Freq.	Localization	HPIDB Protein	Organism
Eis171	21892764	YP_002345523.1	0	0	Magnesium-translocating P-type ATPase		Cytoplasmic Membrane	Putative cation transport protein	*Yersinia pestis*
Eis110	56404031	NP_858359.2	4.00E-26	0	Hypothetical protein NT01EI_1332	2	Extracellular	E3 ubiquitin-protein ligase ipaH9.8	*Shigella flexneri*
Eis041	21892746	YP_002345337.1	8.00E-116	0	Type III secretion outer membrane pore, YscC/HrcC famil		OuterMembrane	Possible type III secretion protein	*Yersinia pestis*
Eis192	23892101	NT01EI_3147	0	0	Hypothetical protein NT01EI_3147	2	Unknown		
Eis037	56404031	NP_858359.2	3.00E-129	9.13E-144	Hypothetical protein NT01EI_2355 (E3 ubiquitin-protein ligase SspH2)	2	Extracellular	E3 ubiquitin-protein ligase ipaH9.8	*Shigella flexneri*
Eis038	56404031	NP_858359.2	5.00E-67	3.95E-121	Hypothetical protein NT01EI_1334 (Type III secretion system leucine rich repeat)	2	Extracellular	E3 ubiquitin-protein ligase ipaH9.8	*Shigella flexneri*
Eis001	21892865	YP_002346527.1	2.00E-72	1.97E-95	NAD-dependent malic enzyme (NAD-ME)		Cytoplasmic	NAD-dependent malic enzyme	*Yersinia pestis*
Eis195	23891833	NT01EI_0376	2.00E-159	8.13E-87	Anaerobic C4-dicarboxylate transporter DcuA	11	Cytoplasmic Membrane		
Eis173	45442630	NP_994169.1	0	8.89E-61	Acyltransferase/AMP-dependent synthetase and ligase protein family	2	Cytoplasmic Membrane	Bifunctional protein aas	*Yersinia pestis*
Eis185	23892124	NT01EI_3386	7.00E-85	1.74E-56	TRAP transporter, DctM subunit		Cytoplasmic Membrane		
Eis107	47531039	YP_022388.1	1.00E-112	2.34E-56	DEAD box containing helicase		Cytoplasmic	ATP-dependent RNA helicase, DEAD/DEAH box family	*Bacillus anthracis*
Eis230	16241814	YP_001608410.1	0	1.16E-46	Phosphoglycerate transporter family protein		Cytoplasmic Membrane	Putative regulatory protein	*Yersinia pestis*
Eis028	41735894	NT01EI_0277	1.00E-73	4.26E-43	Transposase, IS4 family protein	**4**	Unknown		

Table 5 (continued)

Eis175	238921619	NT01EI_3774	7.00E-93	6.93E-41	IS1 transposase	Unknown		
Eis184	47525346	YP_016695.1	9.00E-27	2.71E-40	RNA methyltransferase, TrmH family, group 3	Cytoplasmic	RNA methyltransferase, TrmH family, group 3	*Bacillus anthracis*
Eis176	31795275	NP_857736.1	5.00E-129	9.04E-38	Type III secretion apparatus protein SpaR/YscT/HrcT	Cytoplasmic Membrane	Yop proteins translocation protein T	*Yersinia pestis*
Eis157	238918120	NT01EI_0144	8.00E-07	4.66E-29	Twin-arginine translocation protein subunit TatB	Cytoplasmic Membrane		
Eis068	388479180	YP_491372.1	1.00E-115	4.13E-26	Polyprenyl synthetase	Cytoplasmic	Octaprenyl-diphosphate synthase	*Escherichia coli*
Eis155	47526361	YP_017710.1	3.00E-134	4.05E-12	Transcriptional regulator FruR	Cytoplasmic	Sugar-binding transcriptional regulator, LacI family	*Bacillus anthracis*
Eis086	22124541	NP_667964.1	3.00E-67	9.61E-12	N-acetylmuramoyl-L-alanine amidase AmiA	Unknown	N-acetylmuramoyl-l-alanine amidase II	*Yersinia pestis*
Eis006	567081700	YP_170066.1	2.00E-114	9.81E-12	ATP-dependent DNA helicase Rep	Cytoplasmic	ATP-dependent DNA helicase	*Francisella tularensis*
Eis235	218927976	YP_00234585 1.1	9.00E-36	2.44E-10	Diaminopimelate decarboxylase	Cytoplasmic	Diaminopimelate decarboxylase	*Yersinia pestis*
Eis055	238920205	NT01EI_2314	8.00E-128	7.18E-08	Prophage lambda integrase	Cytoplasmic		
Eis207	218930061	YP_00234793 6.1	4.00E-132	1.13E-07	Spermidine/putrescine transport system permease protein PotB	Cytoplasmic Membrane	Inner membrane permease T of sulfate/thiosulfate ABC transporter	*Yersinia pestis*
Eis080	417358998	NT01EI_0981	8.00E-129	1.52E-06	Hypothetical protein NT01EI_0981	9 Cytoplasmic		
Eis222	108810845	YP_646612.1	5.00E-127	1.67E-06	Hypoxanthine phosphoribosyltransferase	Cytoplasmic	Hypoxanthine phosphoribosyltransferase	*Yersinia pestis*
Eis018	22124520	NP_667943.1	7.00E-145	1.96E-06	Aspartate ammonia-lyase	Cytoplasmic	Aspartate ammonia-lyase (Aspartase)	*Yersinia pestis*
Eis011	47526143	YP_017492.1	1.00E-74	2.98E-04	ABC transporter, perplasmic amino acid binding protein	Periplasmic	Amino acid ABC transporter, amino acid-binding protein	*Bacillus anthracis*
Eis131	238920051	NT01EI_2157	4.00E-66	9.55E-03	Hypothetical protein NT01EI_2157	Unknown		
Eis223	218927723	YP_00234559 8.1	2.00E-142	9.91E-03	Extracellular solute-binding protein, family 5	Unknown	HTH-type transcriptional regulator SgrR	*Yersinia pestis*

Discussion

Bioinformatics analysis of 56 unique genes with transposon insertion showed majority of proteins were already identified as virulence factors in other pathogenic bacteria, which may also be involved in virulence in *E. ictaluri.* Type 3 secretion systems

(T3SS), twin-arginine translocation (Tat), and ATP-binding cassette (ABC) transporter seems to be important for *E. ictaluri* virulence and invasion of channel catfish.

Although virulence proteins are located in outer membrane or periplasm in Gram-negative bacteria, we observed most of the proteins located in the cytoplasm and cytoplasmic membrane where metabolic processes take place. This could be due to selection of growth impaired mutants.

Previous studies have shown that protein secretion systems provide a well adaptation to environment in different processes. In Gram-negative bacteria, these secretion pathways involve in protein export to host cell. It has been reported that *E. ictaluri* genome has virulence mechanisms including T3SS and Tat (Williams et al., 2012a). Our study found that *yscC/hrcC* proteins in outer membrane, Type 3 secretion system leucine rich repeat protein in extracellular and *spaR/yscT/hcrT* protein in cytoplasm are components of T3SS. *yscT* is a type 3 secretion system protein in *Yersinia spp.* and located in cytoplasm which has extended and helical regions that may form membrane-bound subunits (Hensel et al., 1997). Ubiquitinylation of proteins is an important process that regulates inflammatory response in eukaryotic cells. E3 ubiquitin-protein ligase has a role in T3SS that provides a strategy to exploit host cell ubiquitin pathway (Hicks et al., 2010).

Tat pathway located in periplasmic space and involved in translocation of proteins including components of respiratory complexes using a proton gradient as an energy source (Muller, 2005). A recent study showed that *tatB* mutants exhibited slow growth under low-iron conditions and observed a 10-fold decrease in growth. Tat pathway is also related to type 2 protein secretion (T2SS) that allows the virulence factors export from

periplasm (Rossier et al., 2005). TatABCE protein subunit tatB was identified as a potential virulence factor.

ABC transporter pathway has a significant role for exporting substrates such as toxins, proteases and lipases directly from inner to outer membrane. ABC transporter periplasmic amino acid binding protein in *E. ictaluri* may have a role in virulence of bacterium. Spermidine/putrescine transport system is another ABC transporter pathway. Spermidine and putrescine are polyamines that have a role of stabilization of DNA and adjust the translation of some genes by affecting their mRNA. These polyamines are important for swarming (Kurihara et al., 2009). PotABCD is a spermidine and putrescine importer system in Gram-negative bacteria. The membrane spanning protein potB may have a role for virulence in *E. ictaluri*.

CHAPTER III

CONCLUSIONS

The overall goal of this project is to generate growth deficient *E. ictaluri* mutants and identify genes causing this growth deficiency on complex media. Mutants exhibiting slow growth in complex media may be potential candidates for vaccine development. In this study, 56 unique *E. ictaluri* genes have been identified. 32 of them showed host protein binding properties while 30 of them were found to be involved in bacterial virulence in other pathogenic bacteria. Among these 30 proteins, Type 3 secretion systems (T3SS), twin-arginine translocation (Tat), and ATP-binding cassette (ABC) transporter have been noted.

In conclusion, results indicate presence of large amount of virulence related genes in growth deficient *E. ictaluri* library. It is necessary to verify if these genes are also virulence factors in *E. ictaluri* by using fish infection studies.

REFERENCES

Collins, L.A., & Thune, R. L. (1996). Development of a Defined Minimal Medium for the Growth of Edwardsiella ictaluri. *Applied and Environmental Microbiology, 62*(3), 848-852.

Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., & Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics, 21*(18), 3674-3676. doi: 10.1093/bioinformatics/bti610

Cooper, R. K., Shotts, E. B., & Nolan, L. K. (1996). Use of a Mini-Transposon to Study Chondroitinase Activity Associated with Edwardsiella ictaluri. *Journal of Aquatic Animal Health, 8*(4), 319-324.

de Lorenzo, V., & Timmis, K. N. (1994). Analysis and construction of stable phenotypes in gram negative bacteria with Tn5 and Tn10-derived minitransposons. *Methods Enzymology, 235*, 386-405.

Detmer, A., & Glenting, J. (2006). Live bacterial vaccines--a review and identification of potential hazards. *Microb Cell Fact, 5*, 23. doi: 10.1186/1475-2859-5-23

Hawke, J. P. (1979). A bacterium associated with disease of pond cultured channel catfish, Ictalurus punctatus. *Journal of the Fisheries Research Board of Canada, 36*, 1508-1512.

Hawke, J. P. (1998). ESC - Enteric Septicemia of Catfish. SRAC Publication: Southern Regional Aquaculture Center.

Hawke, J. P., McWhorter, A. C., Steigerwalt, A. G., & Brenner, D. J. (1981a). Edwardsiella ictaluri sp. nov., the Causative Agent of Enteric Septicemia of Catfish. *International Journal of Systematic Bacteriology, 31*(4).

Hawke, J. P., McWhorter, A. C., Steigerwalt, A. G., & Brenner, D. J. . (1981b). Edwardsiella ictaluri sp. nov., the Causative Agent of Enteric Septicemia of Catfish *International Journal of Systematic Bacteriology 31*(4), 396-400.

Hensel, M., Shea, J. E., Raupach, B., Monack, D., Falkow, S., Gleeson, C., . . . Holden, D. W. (1997). Functional analysis of ssaJ and the ssaK/U operon, 13 genes encoding components of the type III secretion apparatus of Salmonella Pathogenicity Island 2. *Mol Microbiol, 24*(1), 155-167.

Hicks, S. W., & Galan, J. E. (2010). Hijacking the host ubiquitin pathway: structural strategies of bacterial E3 ubiquitin ligases. *Curr Opin Microbiol, 13*(1), 41-46. doi: 10.1016/j.mib.2009.11.008

Janda, J. M., Abbott, S. L., Kroske-Bystrom, S., Cheung, W. K., Powers, C., Kokka, R. P., & Tamura, K. (1991). Pathogenic properties of Edwardsiella species. *J Clin Microbiol, 29*(9), 1997-2001.

Judson, N., & Mekalanos, J. J. (2000). Transposon-based approaches to identify essential bacterial genes. *Trends Microbiol, 8*(11), 521-526.

Karlyshev, A. V., Pallen, M. J., & Wren, B. W. (2000). Single-primer PCR procedure for rapid identification of transposon insertion sites. *Biotechniques, 28*(6), 1078, 1080, 1082.

Karsi, A., & Lawrence, M. L. (2007). Broad host range fluorescence and bioluminescence expression vectors for Gram-negative bacteria. *Plasmid, 57*(3), 286-295. doi: 10.1016/j.plasmid.2006.11.002

Kumar, R., & Nanduri, B. (2010). HPIDB--a unified resource for host-pathogen interactions. *BMC Bioinformatics, 11 Suppl 6*, S16. doi: 10.1186/1471-2105-11-S6-S16

Kurihara, S., Suzuki, H., Tsuboi, Y., & Benno, Y. (2009). Dependence of swarming in Escherichia coli K-12 on spermidine and the spermidine importer. *FEMS Microbiol Lett, 294*(1), 97-101. doi: 10.1111/j.1574-6968.2009.01552.x

Lawrence, M. L., Cooper, R. K., & Thune, R. L. (1997). Attenuation, persistence, and vaccine potential of an Edwardsiella ictaluri purA mutant. *Infect Immun, 65*(11), 4642-4651.

Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., . . . Ausubel, F. M. (2006). An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. *Proc Natl Acad Sci U S A, 103*(8), 2833-2838. doi: 10.1073/pnas.0511100103

MacMillan, J.R. (1985). Infectious diseases in channel catfish culture. In C. S. Tucker (Ed.), (pp. 405-496.): New York, Elsevier Science Publishers.

McGinnis, A., Gaunt, P., Santucci, T., Simmons, R., & Endris, R. (2003). In vitro evaluation of the susceptibility of Edwardsiella ictaluri, etiological agent of enteric septicemia in channel catfish, Ictalurus punctatus (Rafinesque), to florfenicol. *J Vet Diagn Invest, 15*(6), 576-579.

Mississippi State University, Division of Agriculture, Forestry, Natural Resources. (2012). Catfish Water Acres in Mississppi.

Muller, M. (2005). Twin-arginine-specific protein export in Escherichia coli. *Res Microbiol, 156*(2), 131-136. doi: 10.1016/j.resmic.2004.09.016

Newton, J. C, Wolfe, L. G, Grizzle, J. M, & Plumb, J. A. (1989). Pathology of experimental enteric septicemia in channel catfish, Ictalurus punctatus (Rafinesque), following immersion-exposure to Edwardsiella ictaluri. *Journal of Fish Disease, 12*, 335-347.

Rossier, O., & Cianciotto, N. P. (2005). The Legionella pneumophila tatB gene facilitates secretion of phospholipase C, growth under iron-limiting conditions, and intracellular infection. *Infect Immun, 73*(4), 2020-2032. doi: 10.1128/IAI.73.4.2020-2032.2005

Shotts, E. B., Blazer, V. S., & Waltman, W. D. (1986). Pathogenesis of experimental Edwardsiella ictaluri infections in channel catfish (Ictalurus punctatus). *Canadian Journal of Fish Aquatic Science, 43*, 36-42.

Taylor, P.W., & Johnson, M.R. (1991). Antibiotic resistance in Edwardsiella ictaluri *American Fisheries Society Fish Health Section Newsletter,* pp. 3-4.

Thunea, R. L., Fernandez, D. H. , & Battista, J. R. (1999). An aroA Mutant of Edwardsiella ictaluri Is Safe and Efficacious as a Live, Attenuated Vaccine. *Journal of Aquatic Animal Health, 11*(4), 358-372.

USDA, Census of Agriculture, 2007. (2009). United States Summary and State Data. *Geographic Area Series, 1*(51), AC-07-A-51.

USDA, National Agricultural Satatistics Service. (2012). Catfish Processing.

Williams, M. L. , Gillaspy, A. F., Dyer, D. W. , Thune, R. L., Waldbieser, G. C., Schuster, S. C., . . . Lawrence, M. L. . (2012a). Genome Sequence of Edwardsiella ictaluri 93-146, a Strain Associated with a Natural Channel Catfish Outbreak of Enteric Septicemia of Catfish. *Journal of Bacteriology, 194*(3), 740-741.

Williams, M. L., Gillaspy, A. F., Dyer, D. W., Thune, R. L., Waldbieser, G. C., Schuster, S. C., . . . Lawrence, M. L. (2012b). Genome sequence of Edwardsiella ictaluri 93-146, a strain associated with a natural channel catfish outbreak of enteric septicemia of catfish. *J Bacteriol, 194*(3), 740-741. doi: 10.1128/JB.06522-11

Yu, N. Y., Wagner, J. R., Laird, M. R., Melli, G., Rey, S., Lo, R., . . . Brinkman, F. S. (2010). PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics, 26*(13), 1608-1615. doi: 10.1093/bioinformatics/btq249

Zhou, C. E., Smith, J., Lam, M., Zemla, A., Dyer, M. D., & Slezak, T. (2007). MvirDB--a microbial database of protein toxins, virulence factors and antibiotic resistance genes for bio-defence applications. *Nucleic Acids Res, 35*(Database issue), D391-394. doi: 10.1093/nar/gkl791