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Understanding molecular aspects of catfish-pathogen interactions

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UNDERSTANDING MOLECULAR ASPECTS OF CATFISH-PATHOGEN INTERACTIONS

By

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The catfish industry suffers losses primarily due to enteric septicemia of catfish and columnaris disease caused by *Edwardsiella ictaluri* and *Flavobacterium columnare*, respectively. Understanding the host-pathogen interactions is vital for prevention and eradication of these diseases. Hence, the *overall objective* of this study was to analyze whole cell proteomes of these two bacteria, and to determine the changes in *E. ictaluri* protein expression against *in vitro* iron-restriction and host serum treatment. High-throughput proteomic analysis of these bacteria was conducted using two-dimensional liquid chromatography followed by electrospray ionization tandem mass spectrometry (2-D LC ESI MS/MS) and two-dimentional gel electrophoresis coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (2-DE MALDI TOF/TOF). Identified proteins were clustered into functional groups using clusters of orthologous groups, and subcellular locations as well as possible functional relationships were determined. A total of 788 unique *E. ictaluri* and 621 unique *F. columnare* proteins were identified, which represented 12 and 28 pathways, respectively.

Vertebrate hosts tend to chelate free iron of their body and make the environment hostile for bacteria. Hence, reduced availability of iron may cause significant stress for pathogens and is considered a signal that leads to alteration in virulent gene expression. Similarly, *E. ictaluri* might use the catfish blood stream effectively for quick systemic invasion. Hence, exposure to catfish serum components might reveal the ability of *E. ictaluri* to protect against host defense mechanisms. Using two-dimensional difference gel electrophoresis, responses of *E. ictaluri* due to *in vitro* iron-restriction and host serum treatment were determined. A total of 50 and 19 proteins were identified to be differentially expressed due to *in vitro* iron-restriction and catfish serum treatment, respectively. Among the differentially expressed proteins, several putative virulent determinants, immunogenic proteins, chaperones, and housekeeping genes were noted. To initiate functional studies, four differentially expressed *E. ictaluri* genes (*lamB*, *glyS*, *malE,* and *sdhA*) were mutated by in-frame deletion. Results from this study provided experimental evidence for many predicted proteins. In addition, identification of differentially expressed proteins provided targets for further functional analysis, which could help elucidate pathogenic mechanisms of *E. ictaluri*.

Keywords: *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Ictalurus punctatus*, Proteomic analysis, 2-D LC ESI MS/MS, 2-DE MALDI TOF/TOF, 2-D DIGE, Iron-restriction, Serum, Complement, In-frame mutation

DEDICATION

I would like to dedicate this research to my parents Ramana Reddy Dumpala and Rajya Lakshmi Dumpala, my wife Divya Swetha and my son Rohan Reddy Dumpala.

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TABLE OF CONTENTS

DEDICATION ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER

1. GENERAL INTRODUCTION AND REVIEW OF PERTINENT LITERATURE 1

Catfish industry 1
Edwardsiella ictaluri 2
Enteric septicemia of catfish (ESC) 3
Virulence factors 5
Flavobacterium columnare 8
Role of iron in determining potential virulence factors of bacteria 12
Effect of serum components on bacterial protein expression 14
Significance of research and objectives 19
REFERENCES 21

2. PROTEOME ANALYSIS OF *EDWARDSIELLA ICTALURI* 33

Abstract 33
Introduction 34
Materials and Methods 35
Total protein extraction 35
Trypsin digestion 36
MS/MS analysis 36
Protein identification 37
2-DE analysis 37
In-gel trypsin digestion and MALDI PMF 38
Identification of COGs and protein locations 39
Pathway analysis 39
Results 39
Protein identification using 2-D LC and 2-DE 39

Functional classification of identified proteins 42
Cellular localization of identified proteins 44
Pathway analysis 45
Discussion 49
REFERENCES 55

3. PROTEOMIC ANALYSIS OF THE FISH PATHOGEN *FLAVOBACTERIUM COLUMNARE* 61

Abstract 61
Introduction 62
Materials and Methods 64
Protein extraction 64
2-D LC ESI MS/MS analysis 65
2-DE and MALDI MS analysis 65
Identification of COGs, protein locations, and pathways 66
Results 66
Protein identification using 2-D LC and 2-DE 66
Functional classification of identified proteins 67
Subcellular localization of identified proteins 71
Pathway analysis 71
Discussion 74
REFERENCES 81

4. IDENTIFICATION OF DIFFERENTIALLY REGULATED PROTEINS OF *EDWARDSIELLA ICTALURI* DURING IRON-RESTRICTION 88

Abstract 88
Introduction 89
Materials and Methods 91
Iron-restricted growth and total protein extraction 91
Labeling of proteins 92
Protein separation using 2-DE 92
Analysis of 2-D DIGE gel images 93
Preparative gel electrophoresis and protein identification 94
Functional modeling of differentially expressed proteins 94
Results 95
Identification of differentially expressed proteins 95
Functional modeling of differentially expressed proteins 96
Discussion 105
REFERENCES 112

5. IDENTIFICATION OF DIFFERENTIALLY REGULATED PROTEINS OF *EDWARDSIELLA ICTALURI* DURING SERUM TREATMENT 119

Abstract 119
Introduction 120
Materials and Methods 122
Serum preparation 122
Serum treatment and total protein extraction 123
Labeling of proteins 124
Protein separation using 2-DE 124
Analysis of 2-D DIGE gel images 125
Preparative gel electrophoresis and protein identification 125
Results 126
Discussion 129
REFERENCES 136

6. IN-FRAME DELETION OF SELECTED *EDWARDSIELLA ICTALURI* GENES 141

Abstract 141
Introduction 141
Materials and Methods 142
Bacterial strains and media 142
Plasmid and *E. ictaluri* genes 143
Primer design and gene amplification for mutation analysis 144
Gene Mutation using splicing by overlap extension (SOEn) PCR 147
Cloning 147
Ligation and Transformation of *E. coli* CC118 λpir 148
Verification of cloning 148
Results and Discussion 150
Future research 153
REFERENCES 154

7. CONCLUSION 155

APPENDIX

A. *EDWARDSIELLA ICTALURI* PROTEINS IDENTIFIED USING 2-D LC 159

B. *EDWARDSIELLA ICTALURI* PROTEINS IDENTIFIED USING 2-DE 191

C. *FLAVOBACTERIUM COLUMNARE* PROTEINS IDENTIFIED USING 2-D LC 203

D. *FLAVOBACTERIUM COLUMNARE* PROTEINS IDENTIFIED USING 2-DE 234

E. PATHWAYS SIGNIFICANTLY REPRESENTED IN
FLAVOBACTERIUM COLUMNARE PROTEIN DATASET 245

LIST OF TABLES

2.1 Putative virulence related proteins of *Edwardsiella ictaluri* 42

2.2 Outer membrane proteins of *Edwardsiella ictaluri* identified in this study 47

2.3 List of pathways significantly represented in *Edwardsiella ictaluri* proteome 48

2.4 Genes represented in *Edwardsiella ictaluri* lipopolysaccharide biosynthesis pathway 48

3.1 *Flavobacterium columnare* proteins that may have role in pathogenesis 69

3.2 Outer membrane proteins of *Flavobacterium columnare* predicted by PSORTb 73

4.1 Differentially expressed proteins of *Edwardsiella ictaluri* in response to *in vitro* iron-restriction 98

4.2 List of pathways significantly represented by differentially expressed *Edwardsiella ictaluri* proteins in response to *in vitro* iron-restriction 104

5.1 Differentially expressed proteins of *Edwardsiella ictaluri* in response to catfish serum 129

6.1 *Edwardsiella ictaluri* genes selected for in-frame deletion mutation 144

6.2 Primers used in this study 146

6.3 PCR reaction set up 147

LIST OF FIGURES

2.1 Master image of *Edwardsiella ictaluri* proteome obtained by 2-DE 41

2.2 Comparison of COG categories between proteins identified in this study (788 unique proteins by 2-D LC MS/MS and 2-DE analysis) and predicted proteins from the *Edwardsiella ictaluri* genome (3,945 ORFs) 44

2.3 Subcellular locations of *Edwardsiella ictaluri* proteins determined by PSORTb prediction 46

2.4 Glycolysis/gluconeogenesis pathway in *Edwardsiella ictaluri* 49

3.1 2-D map of proteins extracted from *Flavobacterium columnare* 68

3.2 Comparison of COG categories between proteins identified in this study and predicted proteins from the *Flavobacterium columnare* genome 70

3.3 Subcellular locations of *Flavobacterium columnare* proteins determined by PSORTb prediction 72

3.4 Amino acid metabolism related pathways in *Flavobacterium columnare* 74

4.1 Fluorescent difference gel electrophoresis (2-D DIGE) of *Edwardsiella ictaluri* grown in *in vitro* iron-restriction condition 97

4.2 Biological process gene ontology (GO) Slim of differentially expressed proteins of *Edwardsiella ictaluri* grown in *in vitro* iron restriction condition 100

4.3 Molecular function gene ontology (GO) Slim of differentially expressed proteins of *Edwardsiella ictaluri* grown in *in vitro* iron-restriction condition 101

4.4 Subcellular locations of *Edwardsiella ictaluri* proteins differentially expressed due to *in vitro* iron-restriction were predicted using PSORTb 103

4.5 Protein interaction network of differentially expressed *Edwardsiella ictaluri* proteins due to *in vitro* iron-restriction by Pathway Studio 6.0 105

5.1 Fluorescent difference gel electrophoresis (2-D DIGE) of *Edwardsiella ictaluri* upon treatment with catfish serum 128

6.1 Plasmid map of pDS132 143

6.2 Primer design strategy for in-frame deletion of maltoporin (*lamB*) of *Edwardsiella ictaluri* 145

6.3 Construction of in-frame deletion mutant of *Edwardsiella ictaluri* 149

6.4 Ethidium bromide stained agarose gel showing bands of *Xba*I digested pDS132 with insert 151

6.5 General structure and physical map of the pDS132 with insert 152

CHAPTER 1

GENERAL INTRODUCTION AND REVIEW OF PERTINENT LITERATURE

Catfish industry

Aquaculture is one of the fastest growing agricultural commodities in the United States (US) with sales value of $1.4 billion from 6,409 operations (Agcensus, 2007). Catfish farming is one of the most economically important forms of aquaculture in the US with sales of 214 million pounds of fresh and frozen whole fish, fillets, and other products in 2009 (NASS, 2010). The catfish industry is one of the most important agricultural activities in the Southeastern United States, especially in Mississippi, Alabama, Arkansas, and Louisiana with production acreage for all four states constituting around 94 percent of the entire catfish production acreage in the US. In 2008, Mississippi was the national leader in sales of catfish with $206 million from 427 catfish operations (NASS, 2009). The catfish industry is the primary source of employment in the state of Mississippi, especially in counties within the Delta (Dean et al., 2003; Tucker et al., 2004). The catfish industry suffers losses primarily due to enteric septicemia of catfish (ESC) and columnaris diseases caused by *Edwardsiella ictaluri* and *Flavobacterium columnare*, respectively (USDA, 2003). More than 78% of all catfish operations have reported ESC and columnaris infections (USDA, 1997). The Thad Cochran National Warm water Aquaculture Center-Aquatic Diagnostic Laboratory at Stoneville, MS

reported that over the past six years, ESC and columnaris diseases have accounted for 37.6% and 44.0% of all diagnostic submissions, respectively.

Edwardsiella ictaluri

Edwardsiella ictaluri is the gram-negative facultative intracellular etiological agent of ESC (Hawke, 1979; Hawke et al., 1981). It is a short, pleomorphic rod, measuring about 0.75×1.5-2.5 μM and is weakly motile at 25-30°C and non-motile at higher temperatures. *E. ictaluri* was first isolated through the examination of ailing channel catfish (*Ictalurus punctatus*) from Alabama and Georgia that were submitted to the Southeastern Cooperative Fish Disease Laboratory at Auburn University. Based on biochemical characterization and DNA-DNA homology, *E. ictaluri* was located in the genus *Edwardsiella*, which belongs to the *Enterobacteriaceae* family. *E. ictaluri* can be readily distinguished from *E. tarda*, a common member of the genus *Edwardsiella*, through biochemical analyses (Waltman et al., 1986). *E. ictaluri* is catalase-positive, glucose-fermenter, indole, lactose, and cytochrome oxidase-negative, and reduces nitrate to nitrite (Shotts and and Teska, 1989). Biochemically, biophysically, and serologically *E. ictaluri* is a homogenous species (Fernandez et al., 2001). *E. ictaluri* is also known to harbor two plasmids designated as pEI1 and pEI2 (Lobb and Rhoades, 1987; Newton et al., 1988). *E. ictaluri* is observed to be susceptible to most of the antibiotics active against gram-negative bacteria like aminoglycosides, newer penicillin's, cephalosporins, quinolones, tetracyclines, nitrofurantoin, chloramphenicol, and potentiated sulfonamides. Resistance has been observed against colistin and sulfonamides, which were considered as effective against gram-positive bacteria (Waltman and Shotts, 1986). *E. ictaluri* is known to survive for over 90 days at 25°C in sterilized pond-bottom mud (Plumb and

Quinlan, 1986a). Previous research identified and characterized two unique bacteriophages that showed host-specificity for *E. ictaluri* (Walakira et al., 2008). Recently, Williams and Lawrence (2010) developed a PCR-based assay that could be used as a diagnostic tool for identification of *E. ictaluri*.

Enteric septicemia of catfish (ESC)

Enteric septicemia of catfish is considered one of the most important bacterial diseases of farm-raised channel catfish in the US, especially in the southeast. ESC has been frequently diagnosed in Mississippi, Alabama, Louisiana, Arkansas, Georgia, and Florida and less frequently diagnosed in Texas, California, Arizona, Virginia, Kentucky, Idaho, Indiana, and Maryland. Usually ESC is seen, but not confined to, water temperatures that range from 18-28°C. Previous research has shown that the highest mortalities were recorded at water temperature 25°C, with lower mortalities at 23 and 28°C whereas very few/no mortalities at 17, 21, and 32°C (Francis-Floyd et al., 1987). It is likely these temperature limitations made ESC a seasonal disease, with outbreaks typically seen in the "ESC Window", late spring to early summer, also precluding *E. ictaluri* from being a pathogen to humans or other warm-blooded animals (Janda et al., 1991). Channel catfish are severely affected by ESC, whereas other *Ictalurids* like white catfish (*Ameiurus catus*), blue catfish (*Ictalurus furcatus*), and brown bullhead (*Ameiurus nebulosus*) are less susceptible (Plumb, 1999a). ESC affects channel catfish grown in all types of cultural conditions of all sizes, but more commonly seen in fingerlings (up to 15 centimeters). Recently, research conducted by Wise et al. (2008) suggested that the mortality rate of catfish increases upon feeding, when *E. ictaluri* is exposed via an oral route.

Potential portals of entry for *E. ictaluri* in catfish include the olfactory sac, intestine (Baldwin and Newton, 1993; Newton et al., 1989; Shotts et al., 1986), gills (Lawrence et al., 1997; Nusbaum, 1996), and skin injuries (Karsi et al., 2006). Upon 24 h post-infection, electron microscopic immunocytochemistry of the mucosal surface and epithelium of the olfactory organs of catfish confirmed the presence of *E. ictaluri,* indicating the olfactory organ as one of the portals of entry of *E. ictaluri* in catfish (Morrison and Plumb, 1994). The earliest lesions of catfish fingerlings, challenged with 5×10^8 colony-forming units (CFU)/ml of *E. ictaluri* by immersion for 1 h, are enteritis and olfactory sacculitis (Newton et al., 1989). Nusbaum and Morrison (1996) indicated that the gills of catfish should be considered as a potential route of entry for *E. ictaluri*. Also, using bioluminescence imaging, novel attachment sites like eroded fin arches, skin epithelia, and injured skin were identified (Karsi et al., 2006).

ESC in catfish occurs as mild, chronic or acute. Affected fish show reduced feeding activity, listless swimming at the surface with 'head-up, tail-down' posture, spiraling, and occasional rapid swimming at the surface (Jarboe et al., 1984). During the later stages of infection, due to the effect of *E. ictaluri* on brain, fish may swim in corkscrew circles, chasing their tails "head-chasing-tail". External signs of catfish affected with ESC include petechial hemorrhages under the jaw, on the operculum, and in the ventral or belly region. Hemorrhages can also be seen at the base of the fins. Abdominal distention, pale gills, exophthalmia, and small white depigmented areas (ranging from pinhead to about half the size of a dime) appear on skin, progressing into an inflamed cutaneous ulcer (Jarboe et al., 1984; Plumb, 1999b). During the chronic form of the disease, infection spreads from the meninges to the skull and finally to the skin

between the frontal bones of the skull/posterior to, or between the eyes leading to a classic “hole-in-the-head” lesion. Internal signs include numerous petechial hemorrhages over the visceral organs such as the liver, intestines, supporting mesenteries, adipose tissue, and abdominal serosa. The abdominal cavity is filled with a cloudy, straw color and/or red bloody fluid. The liver may have a characteristic pale area of necrosis, hypertrophied kidney, or a dark red spleen.

Virulence factors

Research conducted on *E. ictaluri* has identified various virulence factors, including flagella, extracellular capsular polysaccharide, lipopolysaccharide (LPS), outer membrane proteins (OMPs), hemolysins, and chondroitinases. Flagella of *E. ictaluri* isolates derived from channel catfish was first isolated and purified using acid dissociation and cell solubilization. Flagellar filaments of uniform lengths with basal bodies and hooks were noticed upon cell solubilization (Newton and Triche, 1993). Flagella has been identified as a potential virulence determinant in bacteria (Jonson et al., 2005). Stanley et al. (1994) showed that virulent isolates of *E. ictaluri* expressed higher amounts of capsular material and surface proteins, and they established greater ability to degrade chondroitin when compared to avirulent isolates. Chemical characterization of LPS, a known antigenic polysaccharide of gram-negative bacteria, from *E. ictaluri* was analyzed by SDS-PAGE, gas chromatography, and spectrophotometry. Comparison of *E. ictaluri* LPS with other bacteria revealed that LPS from *E. ictaluri* was the rough type, compared with that of *Salmonella typhimurium* and *Escherichia coli* which were both of the smooth type (Weete et al., 1988). Using transposon mutagenesis, a O-polysaccharide (OPS)-negative *E. ictaluri* 93-146 mutant was shown to be avirulent in channel catfish

(Lawrence et al., 2001). It was also shown that, OPS resist killing of *E. ictaluri* by regular catfish serum, but not by catfish neutrophils (Lawrence et al., 2003). Though previous research has shown that hemolytic activity of *E. ictaluri* is not related to virulence, it was noted that hemolysin activity of a virulent strain was significantly higher compared to an attenuated strain (Williams et al., 2003). However, further studies have shown that there is no significant difference in virulence between the wild type and the hemolysin deficient *E. ictaluri* mutant (Williams and Lawrence, 2005). Using signature-tagged mutagenesis of *E. ictaluri*, virulence-related genes of *E. ictaluri* including three genes involved in LPS biosynthesis, three genes involved in type III secretion systems, and two genes involved in urease activity were determined (Thune et al., 2007).

Outer membrane proteins (OMPs) often act as important virulence determinants in gram-negative bacteria. Sodium N-lauroyl sarcocinate soluble OMP profiles of *E. ictaluri* isolates from various species of fish including channel catfish were examined by electrophoresis (Newton et al., 1990). Two major OMPs of *E. ictaluri*, 36 and 18·5 kDa molecular weight, were detected in cell extract preparations. The immune response of channel catfish to the purified 36 kDa outer membrane protein was significantly less than that of the crude membrane protein (Vinitnantharat et al., 1993). Rabbit antisera produced against *E. ictaluri* OMPs with apparent molecular weights of 22, 31, and 59 kDa has blocked virulent *E. ictaluri* invasion against cells from fathead minnow (*Pimephales promelas*) (Skirpstunas and Baldwin, 2003). The OMP fraction of virulent *E. ictaluri* strain AL-93-75 was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bader et al., 2004). They also confirmed immunogenicity of 97, 80, and 19 kDa proteins by western blot analysis using vaccinated catfish sera.

Their vaccine studies revealed that OMP of *E. ictaluri* provided only marginal protection against ESC with no significant improvement in protection even with a booster dose. Similarly, production of chondroitin sulfatase in *E. ictaluri* is a potential virulence determinant. Injection of transposon mutant *E. ictaluri* MI15, deficient in chondroitinase activity, to catfish developed no signs of ESC even upon injection of a known virulent parent strain of *E. ictaluri* 2 weeks after initial mutant injection (Cooper et al., 1996). *E. ictaluri* encode a putative urease pathogenicity island and is able to produce urea and ammonium transporters. Urease activity may not be required for uptake or survival of *E. ictaluri* inside the head kidney-derived macrophages, but may be required for intracellular replication (Booth et al., 2009). Urease activity may aid in neutralization of the acidic environment of the phagosome of macrophages. The virulence and vaccine efficacy of several mutants obtained by a high-throughput bioluminescence screening procedure were recently reported (Karsi et al., 2009). Ten unique genes, including genes encoding a negative regulator of sigmaE activity, a glycine cleavage system protein, tricarboxylic acid cycle enzymes, an O polysaccharide biosynthesis enzyme, proteins encoded on the native plasmid pEI1, and a fimbrial chaperon protein were among the mutated *E. ictaluri* genes, some of which have shown vaccine potential.

In quest of finding an effective solution for severe losses incurred by the catfish industry due to ESC, scientists started searching for prophylactic methods since the late 1980's to prevent ESC in catfish. Due to biochemical and serological homogeneity of *E. ictaluri* strains, several studies emphasized usage of killed whole cell preparations as a vaccine and have obtained equivocal results. The effect of various water temperatures on the level of antibody response in channel catfish immunized with formalin killed *E.*

ictaluri was determined (Plumb et al., 1986b). They showed that catfish vaccinated by immersion were more protected than other methods like intraperitoneal injections. A purified component of *E. ictaluri* LPS was tested as a potential fish vaccination, but results indicated that very poor protection was induced in catfish unless Freund's complete adjuvant was added (Thune et al., 1997a). An attempt was made to vaccinate channel catfish 12 days post hatch with a formalin killed vaccine by immersion and/or immersion followed by an oral booster. However, this vaccine delivered no significant protection (Thune et al., 1997b). A modified live vaccine, using a rifampicin-resistant strategy, that can be administered by immersion to 3-9 month old catfish is available (Klesius and Shoemaker, 1999). Shoemaker et al., (1999) demonstrated the efficacy of *Edwardsiella ictaluri* RE-33 vaccine on 7 to 31 days post hatch channel catfish and showed a relative percent survival of 45 to 79.5.

Flavobacterium columnare

Flavobacterium columnare is a long, slender gram-negative rod in the family *Flavobacteriaceae*, one of the main phyletic lines within the *Bacteroidetes* group from the domain Bacteria (Bernardet et al., 1996). Previously this bacterium has been referred to by different names including *Bacillus columnaris*, *Flexibacter columnaris*, *Cytophaga columnaris* (Bernardet and Grimont, 1989). The current name *Flavobacterium columnare* was adopted following molecular characterization of archived strains. Several species in *Flavobacteriaceae* cause diseases in fish. *F. columnare* is the causative agent of columnaris disease (Bernardet, 1997), which exists both in fresh and brackish water throughout the world (Plumb, 1999b). *F. columnare* has been distributed ubiquitously in fresh water environments and has a tendency to cause disease in fish in conjunction with

mechanical and/or environmental insults in cultured, ornamental, and wild fish populations (Shotts and Starliper, 1999). Columnaris disease was first described by Herbert Spencer Davis (1922), from the Mississippi River, and named the causative organism as *Bacillus columnaris*. It is considered as one of the oldest known diseases of warm water fish in North America. Columnaris disease is also considered as one of the most important bacterial diseases of commercially raised channel catfish in the United States (USDA, 2003). Seventy percent of farmers from the Southeastern United States listed columnaris disease or mixed infection along with ESC as the cause of greatest economic losses to the catfish industry (USDA, 2003).

F. columnare is able to infect catfish under a variety of circumstances like any age group, water conditions, and season of the year (Griffin, 1992). The disease commonly occurs in catfish when water temperatures that ranges from 25-32°C during the spring, summer, and fall. However, higher mortalities were noticed in outbreaks especially during spring and autumn and are most likely associated with poor environmental conditions (Moore et al., 1990; Wakabayashi, 1991). Columnaris disease is most commonly associated with stress such as high water temperatures, elevated organic loads, crowded conditions, and excessive handling (Wakabayashi, 1991). Columnaris disease is also known as Cotton-Wool, Cotton-Mouth, Flexibacter, and Mouth Fungus based on the site of infection and appearance of infected tissues. Lesion along the dorsal fin which extends laterally on either sides of the abdomen is referred to a saddleback lesion. The initial external symptoms seen in fish affected with columnaris disease are appearance of white or grayish white spots over the head, fins, oral cavity, and/or necrosis of the gills. More often, yellowish brown mucus-like growth is seen

inside the mouth of affected fish and is referred to as Cotton-Mouth. Incubation period of acute columnaris disease in fish is less than 24 h, yielding mortalities ranging from 10 to 100% starting from two to three days post exposure, based on temperature and other environmental conditions (Holt et al., 1975). *F. columnare* infections can be chronic, causing a gradual increase in mortalities in channel catfish, but more often the disease appears suddenly and causes mortalities within a few days (Austin and Austin, 1999).

Extensive research has been conducted on *F. columnare* classification and phylogeny, isolation, identification, detection, and characterization of *F. columnare* strains. *F. columnare* was first cultured and characterized by Ordal and Rucker (1944). They have successfully developed methods for the isolation and culture of the *F. columnare* (Ordal and Rucker, 1944). Sequence signature analysis of the 16S ribosomal RNA gene has shown that *F. columnare, F. psychrophilum*, and *Flexibacter maritimus* are closely related. These 3 bacteria share a common descent and represent a distinct group within the division *Bacteroides*, named *Flavobacterium* (Bader and Shotts, 1998). Darwish et al. (2004) designed a pair of PCR primers based on species-specific regions in the 16S rRNA gene of *F. columnare*. For research puposes, addition of polymyxin B and neomycin was used to obtain more discrete colonies of *C. columnaris* (Fijan, 1969). Research has also shown that adding tobramycin at 1 mg/ml to Shieh medium significantly improved selective isolation of *F. columnare* (Decostere et al., 1997). Bader et al., (2003) developed a primer set for detecting *F. columnare* in various samples, tested under many testing conditions. Shoemaker et al. (2005) developed a rapid (seven minutes) protocol for identification of *F. columnare* using whole-cell fatty acid profiles. Recently, a multiplex PCR method was developed to simultaneously identify 3 important

bacterial fish pathogens, *F. columnare*, *E. ictaluri*, and *Aeromonas hydrophila* in infected fish (Panangala et al., 2007). Recently, pulse field gel electrophoresis was used to construct dendrograms of more than 30 *F. columnare* strains, identifying two major groups, a more virulent group with > 60% mortality and a less virulent group with < 9% mortality (Soto et al., 2008).

Efforts have also been made to understand the mechanisms of virulence employed by *F. columnare*. *F. columnare* is known to produce a capsule and the thickness of the capsule seems to be correlated with virulence. Capsule thicknesses of the high and low virulent strains of *F. columnare* were shown to be 120–130 nm and 80-90 nm, respectively (Decostere et al., 1999). *F. columnare* produces several extracellular proteases that are believed to be important virulence factors contributing to the bronchial and cutaneous necrosis. However, the role of these proteases have not been definitively elucidated (Newton et al., 1997). The major component of the extracellular proteases of *F. columnaris* are zinc metalloproteases (Bertolini and Rohovec, 1992). Also fibrillar structures associated with peripheral layers of *F. columnare* may play a role in the gliding motility (Pate and Ordal, 1967). Recently, it was observed that rifampicin-resistant *F. columnare* mutants lost their high molecular weight lipopolysaccharide (LPS) bands displayed by virulent isolates. Thus suggesting the LPS in *F. columnare* may play an important role in columnaris disease pathogenesis (Zhang et al., 2006). Chondroitin AC lyase activity was found to be significantly related to the virulence of the strains of *F. columnare* in a temperature dependent manner (Stringer-Roth et al., 2002; Suomalainen et al., 2006). Virulence of *F. columnare* was not plasmid associated (Suomalainen et al., 2006). Recently, Staroscik et al., (2008) developed a protocol to introduce the *E. coli* -

Flavobacterium shuttle vector pCP29 into *F. columnare* by conjugal mating which may facilitate deeper understanding of the virulence mechanisms of *F. columnare*. Using suppression subtractive hybridization, genetic differences between the high virulence strain and the low virulence strain of *F. columnare* were identified (Li et al., 2010). The sarcosine-insoluble outer membrane protein fraction of *F. columnare* was analyzed by Liu et al. (2008) and were identified 14 outer membrane proteins.

Role of iron in determining potential virulence factors of bacteria

Iron is an essential micro element for almost all living organisms and is involved in various metabolic processes like sugar, protein, energy, and DNA metabolism, growth, and response to oxidative stress (Mey et al., 2005). The ability of an organism to acquire iron from the host is the major determinant as to whether a bacterium is able to survive, invade, and establish an infection. Hence considerable research has been undertaken to understand the interrelationships between pathogen, host, and iron (Ratledge and Dover, 2000). Pathogenic bacteria are capable of obtaining all of its nutrients from the host easily except iron. Iron is unique, as it is not freely available at pH 7, due to its existence as the ferric form (FeIII) with low solubility ($1.4x10^{-9}$M) (Chipperfield and Ratledge, 2000). In all vertebrate hosts, iron is bound to glycoproteins like serum transferrin, lactoferrin or lactotransferrin, ensuring no free iron is available in circulation (Crichton and Ward, 1998). Hence, withholding iron from pathogenic bacteria is one of the innate immune mechanisms of the host (Payne, 1993; Weinberg, 1993). However, pathogenic bacteria are able to overcome this innate immune mechanism of host and multiply in the host due to the development of various mechanisms to acquire iron from the host. Bacteria developed various strategies to aquire iron from the host. Certain bacteria are

able to obtain host iron by direct contact, reduce, and uptake from host iron sources such as transferrin, lactoferrin, and heme proteins. Another stategy is by synthesis of ultra-high-affinity compounds known as siderophores to the surrounding environment to aquire iron. Siderophores are specific Fe (III)-chelating agents that are produced by many bacteria but not all, in response to an iron deficiency. Siderophores are able to chelate iron attached to host molecules such as ferritin, transferrin, and lactoferritin but not from heme. Gram-negative bacteria are able to synthesize a variety of high-affinity receptors, specific for iron-siderophore complexes. Further, translocation of these complexes into the periplasm is an active process requiring the 'Ton' system which is powered by a proton motive force (Larsen et al., 1996; Occhino et al., 1998; Nicholson and Beall, 1999). Production of hemolysins releasing iron complexed to intracellular heme and hemoglobin is another mechanism for iron acquisition adopted by bacteria. These mechanisms of iron acquisition are closely linked to virulence of pathogenic bacteria.

For successful invasion, colonization, and establishment of a disease by pathogenic bacteria, it has to sense the surrounding environment and respond appropriately with alterations in the expression of membrane proteins and virulence determinants. Along with other environmental factors, changes in iron concentrations serve as an important environmental signal to bacteria for an alteration in the expression of virulence determinants. Numerous studies have shown a relationship between the expressions of virulence determinants to iron availability. Several bacterial species regulate virulent gene expression based on iron availability by a single regulatory gene, fur (ferric uptake regulator). Fur functions, in an iron-dependent manner, by binding to promoter regulatory elements known as Fur boxes. In many bacteria, Fur has been known

to regulate more than 40 genes involved in various pathways (Grifantini et al., 2003; McHugh et al., 2003) including virulence along with iron acquisition and storage. Fur-operated promoters were usually repressed under iron-rich conditions and were activated under iron-restricted conditions. Based on the above phenomenon, a significant number of attempts were made to determine the role of iron in expression of virulent determinants in various bacteria such as α-hemolysin and Shiga-like toxin-1 (Calderwood and Mekalanos, 1987) in *E. coli*, Shiga toxin in *Shigella dysenteriae* (Dubos and Geiger, 1946; Van Heyningen and Gladstone, 1953), hemolysin and outer membrane proteins in *Vibrio cholera* (Sciortino and Finkelstein, 1983; Stoebner and Payne, 1988), outer membrane proteins of *Neisseria gonorrhoeae* (West and Sparling, 1985), diphtheria toxin by *Corynebactenium diphtheniae* (Russell et al., 1984; Tai et al., 1990) and exotoxin A, elastase, and alkaline protease by *Pseudomonas aeruginosa* (Bjorn et al., 1978; Bjorn et al., 1979).

Effect of serum components on bacterial protein expression

The immune system of vertebrates is operated by innate and acquired systems in regard to infection. Initial microbial invasions in all vertebrates were countered by innate defense mechanisms that preexist in all organisms. The innate immune system is capable of acting within minutes of infection. Acquired/adaptive immune defenses come into play only when the innate immune mechanisms are bypassed, evaded, or overwhelmed. Though the innate immune system is considered primitive, it is crucial for the first line of defense against microbial invasion before the acquired immune systems become functional (Hoffmann et al., 1999). Microorganisms which overcome the host's physical barriers are faced immediately by tissue phagocytic macrophages, which are equipped

with membrane bound pattern recognition receptors (PRRs), such as Toll like receptors, macrophage mannose receptors, NOD-like receptors, CD14-receptor for bacterial LPS, and glucan receptors. This in turn leads to an inflammatory response, which causes the accumulation of plasma/serum proteins that provide circulating or humoral innate immunity.

Serum is a blood component without blood cells and clotting factors. Serum consists of several factors like proteins (i.e., complement, lysozyme, antibodies, beta-lysin, fibronectin, transferrin, and lactoferrin), electrolytes, antigens, hormones, and other exogenous substances. In late 19th century, Hans Ernst August Buchner, a German bacteriologist was the first to demonstrate that a substance in blood serum was capable of destroying bacteria. He further noted that the bacteriolytic power of serum was lost when heated to 56°C and named the heat-labile substance *alexin*. Later alexins were renamed as 'complement' by Paul Ehrlich.

The complement system is composed of a large number of distinct plasma proteins that react with one another to opsonize pathogens and induce a series of inflammatory responses that help to fight against infection. Complement system aid in opsonization and killing of bacteria by antibodies. The primary role of the complement system in serum is to identify and aid in the clearance of bacteria and foreign cells (Schmidt and Colten, 2000). Complement proteins exist as precursor zymogens in the serum, distributed throughout body fluids, which are activated through a triggered-enzyme cascade. Complement can be activated by three distinct pathways. First, the classical pathway is triggered either by antibody or by binding of C1q to the surface of a pathogen. Second, the MB-lectin pathway is triggered by mannan-binding lectin, a

normal serum constituent, which binds to an encapsulated bacterium. Third, the alternative pathway is triggered directly on the surface of bacteria. Though three pathways trigger upon encounter with different molecules, they converge to generate a similar set of effector molecules. The complement system protects the body against infection by generating numerous activated complement proteins that bind to bacterial surfaces then opsonizing bacteria for engulfment by phagocytes bearing complement receptors. Similarly, several activated complement proteins may act as chemoattractants to recruit more phagocytes. Finally, the terminal components of complement assemble to form a membrane-attack complex and create pores in the bacterial membrane.

Lysozyme, a 14.4-kDa protein also known as muramidase or N-acetylmuramide glycanhydrolase, is the enzyme that damages the bacterial cell wall by catalyzing the hydrolysis of β 1, 4 linkages between N-acetyloglucosamine and N-acetylmuramic acid. In serum, lysozyme along with complement are involved in lysis of gram-positive and a few gram-negative bacteria (Ogundele, 1998). Research has shown that lysozyme enhanced killing of bacteria is by the antibody-complement system (Davis et al., 1966). Membrane disruption is the most important anti microbial mechanism shown by lysozyme (During et al., 1999). In contrast, research conducted by Davis et al. (1968) and others showed that lysozyme had little or no role in lysis of gram-negative bacteria (Michael and Braun, 1964; Gemsa et al., 1966; Davis et al., 1968). Lysozyme and the antibody-complement system in cooperation with beta-lysin in isotonic serum is known to kill *E. coli* efficiently (Donaldson et al., 1974).

Beta-lysins, trace serum proteins like high-density lipoproteins (HDL), lipopolysaccharide binding protein (LBP), and septin may play a role in reducing the

biological activity of bacterial lipopolysaccharide (LPS). Interaction of serum HDL with bacterial LPS may lead to formation of LPS-HDL complexes, which may contribute to a reduction in endotoxic activity of LPS by preventing LPS (lipid A) from generating crucial transmembrane signals within the cell (Baumberger et al., 1991). It is known that non-lipid components of serum disintegrates bacterial LPS, facilitating its interaction with HDL (Ulevitch et al., 1979). The serum protein LBP is shown to bind with the lipid A moiety of bacterial LPS, forming LPS-LBP complexes which are recognized by CD14 receptor of monocytes and macrophages (Wright et al., 1991), and may reduce the production of tumor necrosis factor upon binding with monocytes and macrophages (Schumann et al., 1990). Wright et al. (1992) proposed a system named 'septin' for recognition of LPS-LBP complexes by CD14 receptors of monocytes and macrophages. They suggested that a septin protease cascade is required for opsonization of LPS.

The primary function of the complement system along with lysozyme, beta-lysin, and other endogenous serum proteins is to kill bacteria. Hence, the ability of bacteria to evade from complement mediated lysis is considered an important virulent determinant. Previous research has shown that bacteria primarily use three strategies to evade complement mediated lysis. The first strategy is binding with complement inhibitors. For example, several bacteria have acquired the ability to bind to C4b-binding protein (C4BP), a known inhibitor of classical and lectin pathways in *E. coli* (Johnsson et al., 1996; Prasadarao et al., 2002). Similarly, bacteria like *H. influenzae* has been shown to bind with Factor H and Factor H-like protein 1 (FHL-1), known regulators of the alternative pathway by binding with C3b (Hallstrom et al., 2008). Vitronectin, another serum glycoprotein, plays an important role in the regulation of the terminal pathways of

complement and MAC (Schvartz et al., 1999). *H. influenza* (Hallstrom et al., 2006) and *Moraxella catarrhalis* (Attia et al., 2006) are known to use vitronectin to prevent the formation of MAC. As complement protein C3 is a crucial component, which links all three complementary pathways, the second strategy used by bacteria to evade complementary lysis is to target and incapacitate the C3 component. *M. catarrhalis* neutralizes C3 and interfers with the alternate pathway (Nordstrom et al., 2004). Lee et al. (2004) proposed that *Staphylococcus aureus* produces a secretary protein called extracellular fibrinogen binding protein that binds to the C3d domain of the C3 complementary molecule, inhibiting deposition over the bacterial surface. The third strategy is the production of proteases such as cysteine protease SpeB (Terao et al., 2008), and streptococcal C5a peptidase (Ji et al., 1996) which specifically cleave important complement factors.

Another fundamental component of the innate immune system of all classes of life is cationic antimicrobial peptides (CAMPs). Most of these CAMPs are less than 10 kDa in size, possess an overall net positive charge, are hydrophobic, and are membrane active (Hultmark, 2003). Modes of action adopted by CAMPs to kill bacteria or foreign cells may include disrupting membranes, interfering with metabolism, and targeting cytoplasmic components. In turn, bacteria use various evasion strategies to avoid antimicrobial peptide mediated lysis (Brogden, 2005). Microorganisms like *Salmonella* modify its cell wall Lipid A to promote resistance to CAMPs (Guo et al., 1997). *S. aureus* reduce the net negative charge by introducing basic amino groups so that CAMPs may not effectively adhere to bacterial surfaces (Peschel et al., 1999). Some other microorganisms like *Klebsiella pneumoniae* increase production of capsular

polysaccharides which may limit the interaction of CAMPs with the bacterial surface (Campos et al., 2004).

Significance of research and objectives

Enteric septicemia of catfish (ESC) and columnaris diseases, caused by *Edwardsiella ictaluri* and *Flavobacterium columnare*, respectively, are the major bacterial diseases incurring significant economical losses to the commercial catfish industry. Hence, understanding the virulence mechanisms of these pathogens is vital for the prevention and eradication of these diseases. Previously, a significant number of studies have been conducted on identification of various virulence factors of *E. ictaluri* and *F. columnare*. Most of these studies have focused mainly on identification of one or two specific virulence related proteins, yet this approach is not efficient and incapable of disclosing en masse virulence related proteins and their differential expression upon interaction with host factors. Thus, the *critical gap* in the knowledge base is a lack of understanding of how these bacteria react and produce virulence determinants when treated with host factors. Filling this gap may aid our understanding of the precise mechanism of pathogenesis of these bacteria at the molecular level. Hence, a comprehensive proteomic analyses along with differential protein expression studies are imperative. The *overall objective* of this study was to analyze whole cell proteomes of *E. ictaluri* and *F. columnare*, and understand the ability of *E. ictaluri* to alter its protein expression against *in vitro* iron-restriction and host serum treatment. Identification of differentially expressed proteins of *E. ictaluri* should advance our knowledge on its pathogenesis, which may lead to the development of novel treatment strategies. Therefore the primary objectives of the present study were:

1. Analyze whole cell proteomes of *E. ictaluri* and *F. columnare.*

   The *working hypothesis* was that thorough analysis of *E. ictaluri* and *F. columnare* proteomes using high-throughput proteomic analysis would aid to accelerate functional and comparative studies to delineate pathogenic mechanisms of these pathogens. This objective was accomplished using the complementary technologies of two dimensional liquid chromatography electrospray ionization tandem mass spectrometry (2-D LC ESI MS/MS) and two dimensional gel electrophoresis coupled with matrix assisted laser desorption ionization time of flight mass spectrometry (2-DE MALDI TOF/TOF MS).

2. Identify responses of *E. ictaluri* when interacted with catfish serum and *in vitro* iron-restriction.

   The *working hypothesis* was that differentially expressed proteins of *E. ictaluri* upon interaction with catfish serum and *in vitro* iron-restriction would play an important role in pathogenesis. This objective was accomplished using two dimensional difference gel electrophoresis (2-D DIGE) coupled with MALDI TOF/TOF MS.

3. Mutate selected *E. ictaluri* genes to determine their role in *E. ictaluri* virulence.

   The *working hypothesis* was that mutation of selected *E. ictaluri* genes was expected to alter the virulence of *E. ictaluri*, revealing their possible involvement in *E. ictaluri* pathogenesis. In-frame deletion mutation of selected *E. ictaluri* genes were accomplished by overlap extension PCR.

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CHAPTER 2

PROTEOME ANALYSIS OF *EDWARDSIELLA ICTALURI*

Abstract

Edwardsiella ictaluri is a facultative intracellular gram-negative bacterium causing enteric septicemia of catfish (ESC), one of the most prevalent diseases affecting farm-raised channel catfish in the United States. Despite its economic importance, studies addressing high-throughput proteomics were not possible because of a lack of a comprehensive protein database. Here, we report the first high-throughput proteomics analysis of *E. ictaluri* using 2-D LC ESI MS/MS and 2-DE MALDI TOF/TOF MS. Proteins identified in this study and predicted from the whole *E. ictaluri* genome were clustered into functional groups using clusters of orthologous groups (COGs) and their subcellular locations were predicted. Possible functional relationships among proteins were determined using pathway analysis. The total number of unique *E. ictaluri* proteins identified using both 2-D LC and 2-DE approaches was 788, of which 15.48% (122) were identified by both methods while 78.43% (618) and 6.09% (48) were unique in 2-D LC and 2-DE, respectively. COG groupings and subcellular localizations were quite similar between our data set and proteins predicted from the whole genome. Twelve pathways were significantly represented in our dataset ($p < 0.05$). Results from this study provided experimental evidence for many proteins that were predicted from the *E. ictaluri* genome

annotation, and they should accelerate future functional and comparative studies aimed at understanding virulence mechanisms of this important pathogen.

Introduction

Enteric septicemia of catfish (ESC) is one of the most prevalent bacterial diseases observed in the commercial catfish industry in the USA. *Edwardsiella ictaluri* is the gram-negative facultative intracellular etiological agent of ESC (Hawke, 1979; Hawke et al., 1981), which occurs as either an acute or chronic disease in channel catfish (MacMillan, 1985; Miyazaki and plumb, 1985; Shotts et al., 1986; Newton et al., 1989). Potential portals of entry for *E. ictaluri* in catfish include the olfactory sac (Miyazaki and plumb, 1985; Shotts et al., 1986; Morrison and plumb, 1994), intestine {Shotts et al., 1986; Newton et al., 1989; Baldwin and Newton, 1993), gills (Nusbaum and Morrison, 1996; Lawrence et al., 1997), and skin injuries (Karsi et al., 2006). Proposed mechanisms of pathogen dissemination include direct transport in blood or inside phagocytic cells (Thune et al., 1993; Booth et al., 2006).

Previous research conducted on *E. ictaluri* virulence factors include flagella {Newton and Triche, 1993), extracellular capsular polysaccharide (Stanley et al., 1994), lipopolysaccharide (LPS) (Weete et al., 1988; Lawrence et al., 2003; Lawrence et al., 2001; Arias et al., 2003; Newton and Triche, 1993b; Williams et al., 2003), outer membrane proteins (OMP) (Newton et al., 1990; Skirpstunas and Baldwin, 2003; Vinitnantharat et al., 1993; Williams et al., 2003; Bader et al., 2004), hemolysins (Williams and Lawrence, 2005), and chondroitinase (Cooper et al., 1996). Antigens of *E. ictaluri* were previously studied using 1-D SDS PAGE or Western blot analysis {Plumb and Klesius, 1988; Klesius and Horst, 1991; Baldwin et al., 1997). *E. ictaluri* antigenic

and nonantigenic proteins using 2-D PAGE were also studied (Moore and thune, 1999; Moore et al., 2002).

E. ictaluri pathogenesis relies on its ability to regulate its proteome to avoid host defenses, and thus, study of the *E. ictaluri* proteome is important for understanding its pathogenic mechanisms. However, global analysis of the *E. ictaluri* proteome using high-throughput proteomics was not possible due to a lack of a comprehensive protein database from *E. ictaluri*. Sequencing of the *E. ictaluri* genome by our group resulted in a comprehensive translational database and paved the way for global proteome analysis. In the present study, we report the first comprehensive protein expression analysis of total cellular proteins of *E. ictaluri* using the complementary technologies of 2-D LC ESI MS/MS and 2-DE MALDI TOF/TOF MS. Results of this study should accelerate functional and comparative studies to delineate pathogenic mechanisms of *E. ictaluri*.

Materials and Methods

Total protein extraction

Clinical *E. ictaluri* isolate 93-146 was cultured on brain heart infusion (BHI) agar plates for 48 h. Six colonies were grown in 12 ml BHI separately at 30°C under continuous shaking at 200 rpm. Growth of bacteria was monitored by measuring optical density at 600 nm (OD_{600}), and bacteria were harvested at mid-exponential phase (OD_{600} 0.6) by centrifugation at 3,750 rpm for 15 min at room temperature.

Two buffers, Triton X-100 and urea-CHAPS, were used for protein extraction. Three pellets were resuspended in Triton X-100 buffer (2% triton X-100, 2.6 g/mL sodium azide, 0.1 M tris-HCl, 8mM PMSF pH 8.0) in separate tubes. The other three

pellets were separately resuspended in urea-CHAPS buffer (7 M urea, 50 mM tris-HCl, 2% CHAPSO, 8 mM PMSF pH 8.0). Cells were lysed on ice by applying ten intermittent pulses of 10 s with a sonicator, and bacterial homogenates were centrifuged at 14,000 rpm for 5 min at 4°C to remove cell debris and unbroken cells.

Proteins from supernatant were precipitated by trichloroacetic acid/acetone, and the resultant protein pellets were resuspended in rehydration buffer (7 M urea, 20 mM tris-HCl, 5 mM EDTA, 5 mM $MgCl_2$, 4% CHAPS, and 5 mM PMSF pH 8.0). Protein concentrations were estimated using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ) following manufacturer's instructions.

Trypsin digestion

100 µg of protein was precipitated by trichloroacetic acid/acetone, and protein pellets were resuspended in 100 mM ammonium bicarbonate with 5% HPLC grade acetonitrile (ACN), reduced with 5 mM DTT at 65°C for 5 min, alkylated with 10 mM iodoacetamide at 30°C for 30 min, and digested with trypsin (1:50 w/w) at 37°C for 16 h. The resultant peptides were desalted using a peptide macrotrap (Michrom BioResources, Auburn, CA) and eluted using a 0.1% trifluoroacetic acid (TFA) and a 95% ACN solution. Desalted peptides were dried in a vacuum centrifuge, and stored at -80^{o}C. Prior to 2-D LC ESI MS/MS analysis, pellets were resuspended in 20 µL of 5% ACN and 0.1% formic acid.

MS/MS analysis

Mass spectrometric analysis was accomplished using a ProteomeX Workstation (Thermo Scientific, Waltham, MA) as described previously (McCarthy et al., 2005).

Protein identification

To identify proteins, searches were conducted using TurboSEQUEST (BioWorks Browser 3.2, Thermo Scientific). Mass spectra and tandem mass spectra were searched against an *in silico* trypsin digested *E. ictaluri* protein database containing 3,945 open reading frames (ORFs) from the draft annotation of our *E. ictaluri* genome project (http://micro-gen.ouhsc.edu/e_ictal/e_ictal_home.htm). Mass changes due to cysteine carbamidomethylation (C, 57.02 Da) and methionine mono- and di-oxidation (15.99 Da and 32 Da) were included in the search criteria. The peptide (MS precursor ion) mass tolerance was set to 1.5 Da, and the fragment ion (MS/MS) mass tolerance was set to 1.0 Da. Peptide matches were included only if they were ≥6 amino acids long and had $\Delta Cn >$ 0.1 and Sequest cross correlation (Xcorr) scores of 1.5, 2.0, and 2.5 for charge states +1, +2, and +3, respectively (Gygi et al, 1999). We further used the reverse database (decoy search strategy) functionality of BioWorks 3.2 and searched MS/MS data against a reversed subset of the original protein database using the same search criteria as described above (Elias and Gygi, 2007), which allowed us to estimate the probability of false identification of peptides based on the real data from the experiment (Park et al., 2006; Peng et al., 2003). Protein probabilities were calculated according to the published literature (Nesvizhskii et al., 2003; MacCoss et al., 2002; Lopez-Ferrer et al., 2004).

2-DE analysis

For isoelectric focusing (IEF), precast 17 cm pH 3-10 NL immobilized pH gradient (IPG) strips (Bio-Rad, Hercules, CA) were used. Protein extraction was done from four mid-exponential phase bacterial cultures as described above using urea-CHAPS buffer (7 M urea, 50 mM tris-HCl, 2% CHAPSO, 8 mM PMSF pH 8.0). Protein

pellets were resuspended in 350 µL of freshly prepared rehydration buffer (7 M urea, 2 M thio urea, 2% CHAPS, 1:50 carrier ampholytes, 0.3% DTT) and quantified using a 2-D Quant Kit (GE Healthcare). 800-1000 µg of solubilized proteins were loaded onto each IPG strip for in-gel rehydration. IEF was performed in a Protean IEF cell (Bio-Rad, Hercules, CA) at 23°C, 500 V for 15 min; linear ramp to 10,000 V for 3 h; and 10,000 V until a total of 70,000 Vh was reached. After IEF, IPG strips were equilibrated in 6 M urea, 30% glycerol, 50 mM Tris-HCl, 2% SDS, 2% DTT, at pH 8.8, and with a trace of bromophenol blue for 15-20 min followed by equilibration containing 2.5% iodoacetamide (IAA) instead of 2% DTT for 15-20 min. Once equilibrated, strips were transferred onto 12% SDS-PAGE and sealed with 0.5% agarose in electrophoresis buffer. Electrophoresis was performed using a PROTEAN II XL system (Bio-Rad) at a constant current of 10mA/gel for the first 15 min followed by 25mA/gel at 20°C until the dye front reached the lower end of the gel. Resolved proteins were visualized by Coomassie brilliant blue R-250 and scanned using the ProteomeWorks Spot Cutter System (Bio-Rad). Images were analyzed using PDQuest 7. 4. 0 (Bio Rad) including gel cropping, anchor spots selection, alignment, and background subtraction. A master gel generated by the software and unique sample spot protein (SSP) numbers were assigned to spots, which were excised and transferred into a 96-well plate using the ProteomeWorks Spot Cutter System.

In-gel trypsin digestion and MALDI PMF

In-gel trypsin digestion and MALDI PMF analysis were performed as previously described (Chaudhary et al., 2007)

Identification of COGs and protein locations

All identified proteins from our dataset and predicted whole *E. ictaluri* proteome were organized into functional groups using annotation based on COG using the COGnitor tool available at NCBI (Tatusov et al., 2000; Tatusov et al., 1997; Tatusov et al., 2001). The subcellular locations of proteins from our dataset as well as from whole E. ictaluri proteome were predicted using PSORTb v2.0.4 (Gardy et al., 2005; Gardy et al., 2003).

Pathway analysis

Pathways with significant protein representation were identified in experimental and predicted *E. ictaluri* proteome using Pathway Studio 4.0 software (Ariadne Genomics, Rockville, MD). Due to a lack of molecular interaction database for *E. ictaluri* in Pathway Studio, all *E. ictaluri* proteins were mapped to their corresponding *E. coli* orthologs by identifying reciprocal-best-BLAST hits with greater than 50% similarity. The resulting ortholog map file was used to predict interactions between *E. ictaluri* proteins. As an initial screening method, we used the "Find pathways" tool to identify pathways that had a significant number of identified proteins. We used $P < 0.05$ to select pathways with significant protein coverage.

Results

Protein identification using 2-D LC and 2-DE

Analysis of proteins identified using Triton X-100 and urea-CHAPS buffers indicated that the proteome coverage in both buffers was quite similar. Approximately 74% of proteins were common to both buffers. 2-D LC analysis yielded 740 unique *E.*

ictaluri proteins (Appendix A) representing 18.76% of the predicted *E. ictaluri* proteome, which has 3,945 ORFs.

2-DE analysis of four CBB stained gels showed approximately 675 spots. 236 common spots were excised and analyzed (Figure 2.1). We were able to identify 224 (95%) of the excised spots (Appendix B). 2-DE analysis yielded 170 unique *E. ictaluri* proteins while 54 (24.12%) of the identified spots appeared to be modifications of identified proteins.

Comparison of 2-D LC and 2-DE analysis indicated that 618 (78.43%) proteins were only detected by 2-D LC, and 48 (6.09%) proteins were only detected by 2-DE, while 122 proteins (15.48%) were detected by both methods. The total number of unique proteins identified by both 2-D LC and 2-DE was 788 (618 unique to 2-D LC; 48 unique to 2-DE; 122 common to both). Analysis of identified proteins revealed several potential virulence proteins from *E. ictaluri* (Table 2.1).

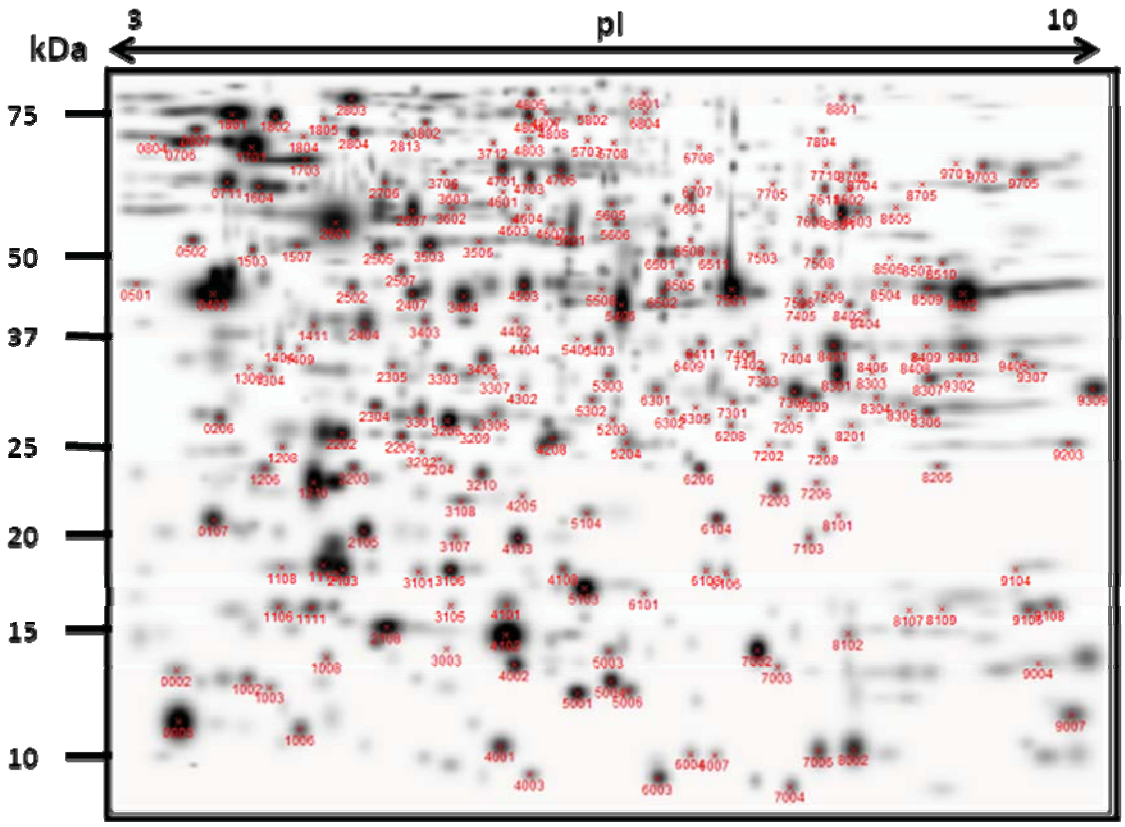

Figure 2.1 Master image of *Edwardsiella ictaluri* proteome obtained by 2-DE.

Spots marked with "x" were given sample spot protein (SSP) numbers, excised, and analyzed by mass spectrometry.

Table 2.1

Putative virulence related proteins of *Edwardsiella ictaluri*

Protein name	**ORF number**	**COG[a]**	**Location**	**Approach**
Antigenic protein Et 32	ORF00307	-	Unknown	2-DE
Catalase/peroxidase HPI (KatG)	ORF00626	P	Unknown	2-D LC
Copper/zinc superoxide dismutase (SodC)	ORF02977	P	Periplasmic	Both
E. tarda virulence protein A (EvpA)	ORF01194	S	Cytoplasm	Both
E. tarda virulence protein B (EvpB)	ORF01195	S	Cytoplasm	2-D LC
E. tarda virulence protein C (EvpC)	ORF01196	-	Unknown	Both
E. ictaluri hemolysin (EihA)	ORF01576	M	Outer mem	2-D LC
E. ictaluri hemolysin (EihB)	ORF01577	M	Outer mem	2-D LC
Iron-cofactored superoxide dismutase (SodB)	ORF00515	P	Unknown	2-D LC
Lipopolysaccharide heptosyltransferase I	ORF00870	M	Cytoplasm	2-D LC
Probable hemagglutinin-related protein	ORF01448	-	Cytoplasm	2-D LC
Putative TTSS chaperone (EseE)	ORF00649	-	Unknown	Both
Putative TTSS effector protein C (EseC)	ORF00647	-	Unknown	2-D LC
Putative TTSS translocon filament protein (EseB)	ORF00645	-	Unknown	2-D LC
Putative TTSS translocon protein (EseD)	ORF00648	-	Unknown	2-D LC
Tia invasion determinant	ORF02980	M	Outer mem	Both
Tol-Pal system beta propeller repeat protein TolB	ORF02087	N	Periplasmic	2-D LC
Type III secretion apparatus lipoprotein (YscJ/HrcJ)	ORF00661	N	Unknown	2-D LC
Type III secretion system chaperone (LcrH/SycD)	ORF00646	R	Unknown	Both
Type V secretory pathway, adhesin AidA	ORF02032	M	Unknown	2-D LC
Urease accessory protein UreG	ORF02233	OK	Cytoplasm	2-D LC

a)COG categories are as follows: P, inorganic ion transport and metabolism; S, function unknown; M, cell wall/membrane/envelop biogenesis; N, cell motility; R, general function prediction only; O, post-translational modification and protein turnover, chaperones; K, transcription.

Functional classification of identified proteins

Comparison of functional distribution of our protein dataset including 788 unique proteins and predicted whole *E. ictaluri* proteome with 3,945 ORFs are summarized in Figure 2.2. A total of 178 (22.59%, 178/788) proteins from our dataset and 609 (15.44%, 609/3,945) proteins from predicted whole proteome were classified as information storage and processing related categories (J, K, and L). Cellular processes and signaling

related categories (D, M, N, O, T, U, and V) included 146 (18.53%) and 563 (14.27%) proteins from our protein dataset and whole proteome database, respectively. A total of 282 (35.79%) and 1,149 (29.13%) proteins from our protein dataset and predicted whole proteome, respectively, were represented in metabolism related categories (C, E, F, G, H, I, P, and Q). Poorly characterized COG groups (R and S) contained 90 (11.42%) and 461 (11.69%) proteins in our protein dataset and predicted whole proteome, respectively. Percent representation of major COG groups was higher in our protein dataset than the predicted proteome due to higher number of proteins assigned to "No hit" (non-significant short or low-complexity sequences) and "No COGs" (proteins do not belong to any of the currently-defined COGs) groups in the predicted proteome (11.68% vs 29.48%).

The COG categories with the highest percent coverage from our protein dataset were translation, ribosomal structure and biogenesis (J, 10.91%) and "No COGs" (10.91%), which were followed by amino acid transport and metabolism (E, 9.26%). By contrast, the highly represented group in the predicted whole proteome was "No COGs" (26.01%), which was followed by the category of proteins involved in amino acid transport and metabolism (E, 7.35%). Six (0.76%) proteins from our protein dataset and 137 (3.47%) proteins from whole proteome were assigned to "No hit" category.

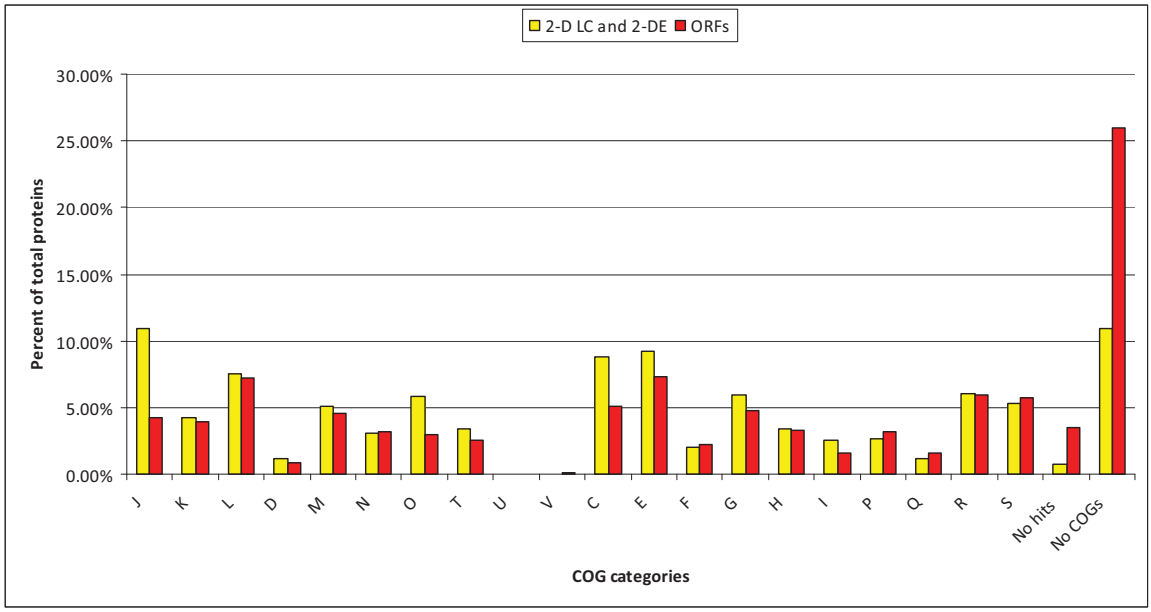

Figure 2.2 Comparison of COG categories between proteins identified in this study (788 unique proteins by 2-D LC MS/MS and 2-DE analysis) and predicted proteins from the *Edwardsiella ictaluri* genome (3,945 ORFs).

Percentages were calculated by dividing the number of proteins in the particular COG category by the number of unique proteins in each analysis (788 proteins identified in this study and 3,945 ORFs predicted from the draft genome). COG categories are as follows: J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination, and repair; D, cell cycle control, cell division, chromosome partitioning; M, cell wall/membrane/envelop biogenesis; N, cell motility; O, post-translational modification and protein turnover, chaperones; T, signal transduction mechanisms; U, intracellular trafficking, secretion, and vesicular transport; V, defense mechanisms; C, energy production and conversion; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown. "No hit" group indicates non-significant short or low-complexity sequences and "No COGs" indicates query proteins not belong to any of the currently-defined COGs.

Cellular localization of identified proteins

Cellular location of *E. ictaluri* proteins identified in this study and predicted from the whole genome were determined (Figure 2.3). Distribution of proteins in predicted

cellular locations were generally similar in all groups. For example, the number of proteins predicted to be "extracellular" was the lowest in all groups. Most proteins were predicted to be "cytoplasmic" origin in all groups except predicted proteome (ORFs), in which the number of proteins with "unknown" cellular location was higher. In 2-DE, proteins predicted to be in "cytoplasmic membrane" were less than that of "outer membrane", while this was the opposite in 2-D LC and ORFs groups. Outer membrane proteins of *E. ictaluri* identified in this study are summarized in Table 2. 2.

Pathway analysis

Pathways significantly represented in the *E. ictaluri* proteome based on proteins identified by 2-D LC and 2-DE were determined. Using predicted functions based on ortholog matching with *E. coli*, all 788 proteins were imported into Pathway Studio, and 94 pathways were identified. Eleven pathways belonging to metabolism and one pathway in genetic information processing were significant ($P< 0.05$) (Table 2.3). Significant pathways contained 190 proteins from our dataset. Of the significantly represented pathways, purine metabolism contained the highest number (30) of proteins, and the reductive carboxylate cycle had the fewest (10). Genes involved in lipopolysaccharide biosynthesis pathway are summarized in Table 2.4.

Of all the pathways, the glycolysis/gluconeogenesis pathway was the most significant pathway in our dataset (P = 1.28E-14, Figure. 2.4), which is followed by the aminoacyl-tRNA synthetases pathway (P = 5.16E-07).

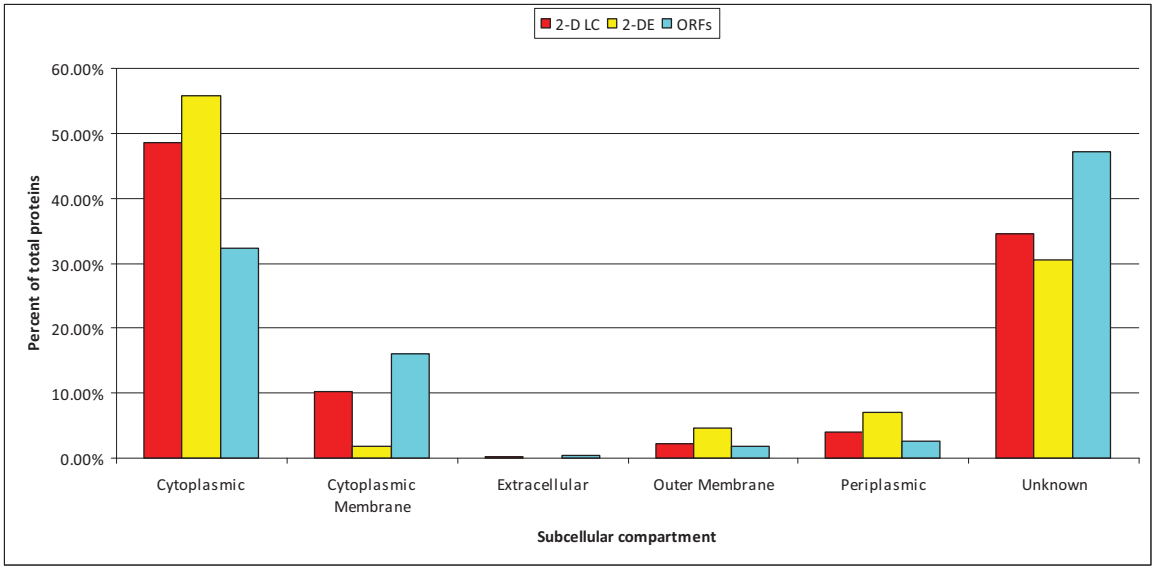

Figure 2.3 Subcellular locations of *Edwardsiella ictaluri* proteins determined by PSORTb prediction.

Percentages were calculated by dividing the number of proteins in the particular subcellular location by the number of unique proteins in each analysis. 2-D LC and 2-DE indicates proteins identified in this study while ORFs indicates proteins predicted from the whole genome.

Table 2.2

Outer membrane proteins of *Edwardsiella ictaluri* identified in this study

Protein Name	**ORF number**	**Approach**	**Spot number**
Chaperone protein Skp	ORF03844	2-D LC	
Conserved hypothetical protein	ORF00069	2-D LC	
E. ictaluri hemolysin (EihA)	ORF01576	2-D LC	
E. ictaluri hemolysin (EihB)	ORF01577	2-D LC	
Fkbp-type 22 kDa peptidyl-prolyl cis-trans isomerase	ORF03257	Both	0206
Hypothetical protein	ORF00273	2-D LC	
Large exoproteins involved in heme utilization or adhesion	ORF01613	2-D LC	
Lipoprotein Nlpi, contains TPR repeats	ORF03217	2-D LC	
Maltoporin	ORF03010	2-D LC	
Outer membrane protein A (OmpA)	ORF02587	Both	3202
Outer membrane protein assembly factor (YaeT)	ORF03843	2-D LC	
Outer membrane protein F (OmpF)	ORF02553	Both	0501
Outer membrane protein N (OmpN)	ORF02706	Both	1503
Outer membrane protein precursor PalA	ORF01447	2-D LC	
Outer membrane protein TolC	ORF03032	2-DE	5605
Peptidoglycan-associated lipoprotein	ORF02086	Both	6103
Serine protease secreted autotransporter toxin (Sat)	ORF00364	2-D LC	
Tia invasion determinant	ORF02980	Both	1210
TonB-dependent vitamin B12 receptor	ORF01185	2-DE	7804

Table 2.3

List of pathways significantly represented in *Edwardsiella ictaluri* proteome

Pathway name	Protein no.[a)]	*P* value[b)]	Classification
Aminoacyl-tRNA synthetases	19/28 (67.86%)	5.16E-07	Genetic Information Processing-Translation
Carbon fixation	13/17 (76.47%)	2.74E-03	Metabolism-Energy
Citrate cycle	12/21 (57.14%)	8.57E-03	Metabolism-Carbohydrate
Glycine, serine and threonine metabolism	23/46 (50.00%)	3.99E-03	Metabolism-Aminoacid
Glycolysis/gluconeogenesis	12/41 (29.27%)	1.28E-14	Metabolism-Carbohydrate
Lipopolysaccharide biosynthesis	14/53 (26.42%)	1.14E-02	Metabolism-Glycan Biosynthesis
Oxidative phosphorylation	13/36 (36.11%)	2.33E-03	Metabolism-Energy
Pentose phosphate pathway	13/23 (56.52%)	2.76E-02	Metabolism-Carbohydrate
Phenylalanine, tyrosine and tryptophan biosynthesis	12/25 (48.00%)	2.52E-02	Metabolism-Aminoacid
Purine metabolism	30/76 (39.47%)	3.30E-02	Metabolism-Nucleotide
Pyruvate metabolism	19/29 (65.52%)	1.87E-02	Metabolism-Carbohydrate
Reductive carboxylate cycle	10/19 (52.63%)	6.67E-03	Metabolism-Energy

a) Gene numbers represented in our dataset and predicted whole proteome, respectively. Numbers in brackets indicate percentage of proteins represented in our dataset as compared to the whole proteome in that particular pathway.

b) Probability of pathway calculated for our dataset.

Table 2.4

Genes represented in *Edwardsiella ictaluri* lipopolysaccharide biosynthesis pathway

Name	Description	Connectivity	COG[a)]
kdsA	Bifunctional 3-deoxy-7-phosphoheptulonate synthase/chorismate mutase	6	M
htrB	Lipid A biosynthesis lauroyl acyltransferase	16	N
queA	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	6	J
lpxD	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	27	M
kdsB	3-deoxy-manno-octulosonate cytidylyltransferase	2	M
ibpB	Lipid A biosynthesis lauroyl acyltransferase	26	O
rfaD	ADP-L-glycero-D-mannoheptose-6-epimerase, NAD(P)-binding	6	MG

a) COG categories are as follows:.M, cell wall/membrane/envelop biogenesis; N, cell motility; J, translation, ribosomal structure and biogenesis; O, post-translational modification and protein turnover, chaperones; G, carbohydrate transport and metabolism.

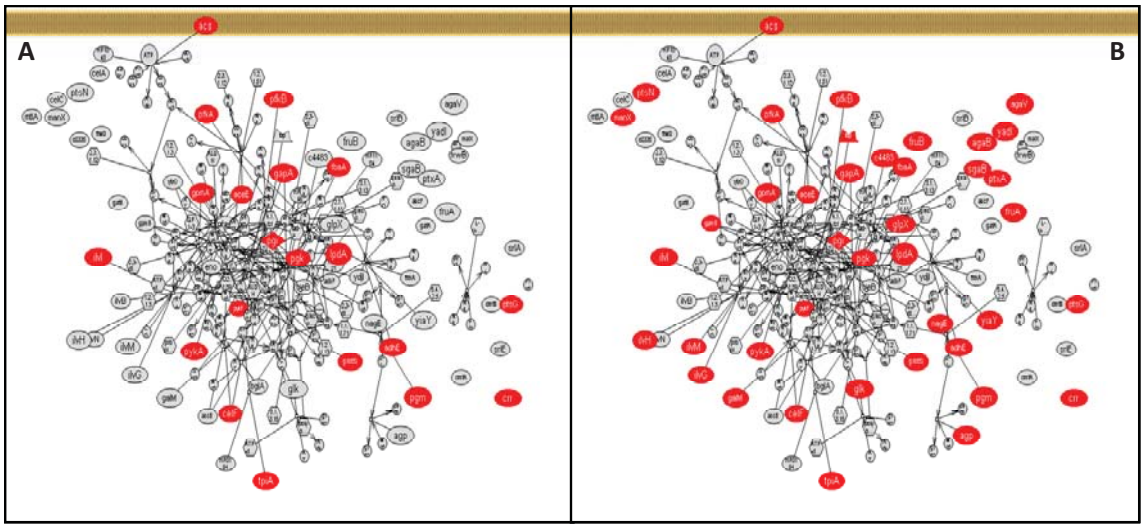

Figure 2.4 Glycolysis/gluconeogenesis pathway in *Edwardsiella ictaluri*.

Entities shown in oval represent genes involving in this pathway. Ovals filled with red represent genes determined in this study (A) and in predicted proteome (B).

Discussion

This research analyzed the whole cell proteome of *Edwardsiella ictaluri* using complementary technologies of 2-D LC and 2-DE. An important goal of proteomics is to understand cellular function at the protein level. A comprehensive proteome along with a good 2-D reference map is essential for future comparative and functional proteomic investigations. Currently, the most popular methods employed to explore the proteome of a cell is by protein separation using 2-D LC (gel-free) or 2-DE (gel-based) followed by MS analysis (Gorg et al., 2004). Use of more than one methodology in large-scale proteomic investigations can increase the percentage of protein identification due to unique proteins detected in each approach (Chong and Wright, 2005; Vanrobaeys et al., 2005; Salzano et al., 2007; Chong and Wright, 2005; Wolff et al., 2006). Therefore, in the present study, we employed the complementary techniques of 2-DE and 2-D LC and

identified 788 unique proteins from *E. ictaluri* (618 unique to 2-D LC, 48 unique to 2-DE, and 122 common to both), which provided experimental evidence for about one fifth (19.98%) of the predicted 3,945 *E. ictaluri* ORFs.

In the 2-D LC method, we compared two widely used buffers in terms of their proteome coverage. The first buffer contained a non-ionic detergent (Triton X-100) commonly used for solubilization of membrane proteins, while the second buffer contained a zwitterionic, non-denaturing detergent (CHAPS) commonly used for solubilization and stabilization of proteins. The number of proteins determined using triton X-100 buffer (612) was quite similar to the number identified using urea-CHAPS (615). Also, 74% of all proteins were common to both buffer sets. Therefore, we did not observe any advantage of using either buffer in terms of protein coverage.

Ribosomal proteins, one of the most abundantly expressed protein types in cells, were detected in high numbers. In the *E. ictaluri* proteome, 73 proteins were predicted as ribosomal proteins. In the present study, we identified 39 and 9 unique ribosomal proteins using 2-D LC and 2-DE, respectively. Along with ribosomal proteins, we also identified several proteins involved in translational machinery, including several aminoacyl-tRNA synthases, translation elongation factors (EF) such as EF-G (ORF02296), EF-Ts (ORF03835), EF-Tu (ORF00244), EF-G (ORF02296), EF-2, EF-GTPases (ORF02295, ORF03061), and EF-P (ORF03297), and translation initiation factors such as initiation factor-3 (Inf C) (ORF02693).

Although grown without the presence of any host/host factors, several potential virulence proteins were detected in our analysis. Most virulence related proteins were detected with the 2-D LC method, although a few were detected using 2-DE. It is

possible that virulence related proteins are expressed at low levels under the culture conditions we used, and the sensitivity of 2-DE was not sufficient for detection. On the other hand, only 35% of the spots were cut and analyzed from the SDS-PAGE gel, and it is possible that the virulence related proteins were not picked. Regardless, 2-D LC demonstrated the ability to detect virulence related genes under our growth conditions. Use of 2-D DIGE may increase the sensitivity of 2-DE allowing detection of less abundant proteins (Gade et al., 2003).

EseB, EseC, and EseD are type III secretion system effector proteins that have been identified from *Edwardsiella tarda* (Srinivasa Rao et al., 2004). We identified an *E. ictaluri* protein orthologous to EseE (ORF00649) using both approaches, and we detected EseB (ORF00645), EseC (ORF00647), and EseD (ORF00648) orthologs using 2-D LC. *E. tarda* virulence proteins (Evp), especially EvpB and EvpC, are important for replication in phagocytes (Srinivasa Rao et al., 2004; Tan et al., 2005). We identified EvpA (ORF01194), EvpB (ORF01195), and EvpC (ORF01196) orthologs in *E. ictaluri* using both techniques. Recently, a type III secretion system, a urease accessory system, and LPS biosynthesis proteins were identified as important virulence factors in *E. ictaluri* by signature-tagged mutagenesis (Thune et al., 2007). In the present study, we identified an *E. ictaluri* protein similar to type III secretion low calcium response chaperone LcrH/SycD (ORF00646), a protein similar to type III secretion apparatus lipoprotein, YscJ/HrcJ family (ORF00661), and urease accessory protein UreG (ORF02233).

Catalase and superoxide dismutase (SOD) are important bacterial defense mechanisms allowing survival of *E. tarda* in phagocytes to protect them from the 'respiratory burst' of phagocytes (Srinivasa Rao et al., 2003). In the present study, we

identified both catalase/peroxidase HPI (KatG) (ORF00626) as well as iron-containing SOD [Fe] (ORF00515) and copper/zinc superoxide dismutase (Sod C) (ORF02977). These genes may also contribute to survival of *E. ictaluri* in phagocytes.

Based on ortholog matching with *E. coli*, identified *E. ictaluri* proteins and the predicted *E. ictaluri* proteome were classified into COGs to assign functions. The majority of identified proteins (35.79%) were housekeeping proteins in "metabolism" categories. It was not surprising that the percentage of proteins assigned to the 'metabolism', 'cellular processes and signaling', and 'information storage and processing' categories was higher from our protein dataset when compared to the predicted proteome because we harvested proteins from bacteria when they were metabolically active at mid-log phase. It was also not surprising that a higher percentage of proteins from the predicted proteome were not assigned to COG groups compared to our identified proteins. Proteins with unknown function may be expressed only under specialized conditions like host environment or stressed conditions and may not be expressed under laboratory culture conditions.

We used the PSORTb algorithm to predict the subcellular location of the proteins identified using both 2-D LC and 2-DE approaches and the predicted whole proteome. Higher percentages of cytoplasmic proteins were detected, which is expected because they are not hydrophobic and thus do not hinder either of the protein separation techniques. However, cytoplasmic membrane proteins were identified in higher percentages using 2-D LC (10.27%) when compared to 2-DE (1.76%). This is not surprising because hydrophobic membrane proteins are typically difficult to identify using 2-DE. Hydrophobic proteins were found to be under-represented in 2-DE based

proteome analyses of membrane protein fractions from *Escherichia coli* (Fountoulakis and Gassser, 2003; Lai et al., 2004; Molloy et al., 2000) and *Bacillus subtilis* (Bunai and Yamane, 2005; Eymann et al., 2004; Wolff et al., 2006). However, even though 2-D LC was better at detecting cytoplasmic membrane proteins than 2-DE, neither approach achieved 16.07%, which is the percentage of cytoplasmic proteins in the predicted proteome. This might be due to the hydrophobic nature of membrane proteins or because cytoplasmic membrane proteins are not as abundant. For future functional studies involving proteins from this compartment, specialized isolation techniques may be needed. Both methods were more effective at identifying outer membrane proteins (OMPs). *E. ictaluri* OMPs are important for adherence and internalization (Newton et al., 1990; Vinitnantharat et al., 1993; Skirpstunas and Baldwin, 2003; Williams et al., 2003; Bader et al., 2004).

From Pathway Studio analysis, we identified pathways that were significantly represented in our protein dataset. The glycolysis/gluconeogenesis pathway was the most represented metabolic pathway identified in our dataset, followed by the aminoacyl-tRNA synthetases pathway. This higher representation of metabolic and translational process related pathways is expected because we isolated proteins from *E. ictaluri* at their metabolically active state. Interestingly, lipopolysaccharide biosynthesis pathway, which may be one of the most important virulence-related systems in *E. ictaluri* pathogenesis (Newton and Triche, 1993; Lawrence et al., 2001; Arias et al., 2003; Lawrence et al., 2003; Williams et al., 2003), was significantly represented in our dataset. Lipopolysaccharide (LPS) is an important component of the gram-negative bacterial envelope; it is an immunodominant antigen, especially in members of the

Enterobacteriaceae. Carbon fixation and reductive carboxylate cycle (CO_2 fixation) pathways classified as energy metabolism related pathways were significantly represented in our dataset. *E. ictaluri* is not autotrophic, but Pathway Studio places 23 proteins in these pathways. We found these proteins to have functions in multiple pathways and thus, their primary function may not be carbon fixation in *E. ictaluri*.

As was reported for other bacterial species, overall results from the present study demonstrated that use of both 2-D LC and 2-DE approaches improves proteome coverage, although 2-D LC provided good proteome coverage by itself. The methods used provided good coverage of gram-negative cell compartments, with the possible exception of the cytoplasmic membrane. Ortholog matching with *E. coli* was an effective method for allowing assignment of putative protein functions and pathway analysis. This comprehensive proteome analysis of *E. ictaluri* will serve as a reference for further functional studies. Results from this study also provided experimental evidence for many *E. ictaluri* proteins that had only been predicted, which will improve the accuracy of the *E. ictaluri* genome annotation.

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CHAPTER 3

PROTEOMIC ANALYSIS OF THE FISH PATHOGEN *FLAVOBACTERIUM COLUMNARE*

Abstract

Flavobacterium columnare causes columnaris disease in cultured and wild fish populations worldwide. Columnaris is the second most prevalent disease of the commercial channel catfish industry in the United States. Despite its economic importance, little is known about the expressed proteins and virulence mechanisms of *F. columnare*. Here, we report the first high throughput proteomic analysis of *F. columnare* using 2-D LC ESI MS/MS and 2-DE MALDI TOF/TOF MS. Proteins identified in this study and predicted from the draft *F. columnare* genome were clustered into functional groups using clusters of orthologous groups (COGs), and their subcellular locations were predicted. Possible relationships among proteins were determined using pathway analysis. The total number of unique *F. columnare* proteins identified using both 2-D LC and 2-DE approaches was 621, of which 10.95% (68) were identified by both methods, while 77.29% (480) and 11.75% (73) were unique in 2-D LC and 2-DE, respectively. COG groupings and subcellular localizations were quite similar between our data set and proteins predicted from the whole genome. Twenty eight pathways were significantly represented in our dataset ($P < 0.05$). Results from this study provide experimental evidence for many proteins that were predicted from the *F. columnare* genome

annotation, and they should accelerate functional and comparative studies aimed at understanding virulence mechanisms of this important pathogen.

Introduction

Flavobacterium columnare is a long, slender gram-negative rod in the family *Flavobacteriaceae*, one of the main phyletic lines within the *Bacteroidetes* group from the domain Bacteria (Bernardet et al., 1996). Several species in *Flavobacteriaceae* cause disease in fish. *F. columnare* is the causative agent of columnaris disease (Bernardet, 1997), which exists both in fresh and brackish water throughout the world (Plumb, 1999). Outbreaks may result in high mortality, especially during spring and autumn and are most likely associated with poor environmental conditions causing stress (Moore, 1990; Wakabayashi, 1991). Stressful conditions are common in commercial aquaculture where production is kept at maximum levels.

Columnaris disease generally begins as an external infection on the skin, fins, gills, or oral cavity (Austin and Austin, 1999). On the skin and fins, lesions are characterized by dull, grayish-white or yellow erosive lesions that can progress to deep ulcers in the underlying muscle. External infection often is concurrent with systemic infection and sub acute mortalities (Plumb, 1999). In some cases, systemic infection with little or no visible external or internal pathological signs may occur (Hawke and Thune, 1992). *F. columnare* infections can be chronic, but more often, the disease appears suddenly and causes mortalities within a few days (Austin and Austin, 1999).

A substantial amount of work has been done on *F. columnare* phylogeny (Bernardet and Grimont, 1989; Bernardet et al., 1996), isolation, identification, and detection of *F. columnare* (Ordal and Rucker, 1944; Fijan, 1969; Griffin, 1992; Decostere

et al., 1997; Bader et al., 2003; Darwish et al., 2004; Shoemaker et al., 2005; Panangala et al., 2006; Yeh et al., 2006; Panangala et al., 2007), and characterization of *F. columnare* strains (Shamsudin and Plumb, 1996; Decostere et al., 1998; Thomas-Jinu and Goodwin, 2004; Darwish and Ismaiel, 2005; Schneck and Caslake, 2006; Olivares-Fuster et al., 2007; Soto et al., 2008). In addition, genetic tools for the manipulation of *F. columnare* have recently been reported (Staroscik et al., 2008). Efforts have been made to understand the mechanisms of virulence employed by the organism (Newton et al., 1997; Stringer-Roth et al., 2002; Bader et al., 2005; Suomalainen et al., 2006), but much remains poorly understood. *F. columnare* produces several extracellular proteases that are believed to be important virulence factors contributing to the bronchial and cutaneous necrosis (Christison and Martin, 1971; Griffin, 1991; Bertolini and Rohovec, 1992; Newton et al., 1997), but the role of the proteases has not been definitively elucidated. A surface capsular material that may be involved in adhesion has been described (Pate and Ordal, 1967). Lipopolysaccharide (LPS) may also play an important role in columnaris pathogenesis (Zhang et al., 2006). Chondroitin lyase activity was found to be significantly related to strain virulence in a temperature dependent manner (Suomalainen et al., 2006).

Although, 14 *F. columnare* outer membrane related proteins were reported recently (Liu et al., 2008), little is known about the expressed *F. columnare* proteins. In the present study, we report the first protein expression analysis from *F. columnare* using the complementary technologies of 2-D LC ESI MS/MS and 2-DE MALDI TOF/TOF MS. Results of this study provide experimental evidence for the predicted *F. columnare*

proteins and should accelerate functional and comparative studies to delineate pathogenic mechanisms of *F. columnare*.

Materials and Methods

Protein extraction

F. columnare growth medium (FCGM) [tryptone (8.00 g), yeast extract (0.80 g), $MgSO_4$ 7 H_2O (1.00 g), $CaCl_2$ 2 H_2O (0.74 g), NaCl (5.00 g), and sodium citrate (1.50 g) per liter] was used to grow *F. columnare*. Isolate ATCC 49512 was streaked on FCGM agar plates (FCGM medium plus 8.00 g agar per liter) and incubated at 30°C for 48 h. Four colonies were grown in 12 ml FCGM broth separately at 30°C under continuous shaking at 200 rpm. Growth of bacteria was monitored by measuring optical density at 600 nm (OD_{600}), and bacteria were harvested at mid-exponential phase (OD_{600} 0.6) by centrifugation at 3,750 rpm for 15 min at 30°C.

The four bacterial pellets were separately resuspended in cold urea-CHAPS buffer (7 M urea, 50 mM tris-HCl, 2% CHAPS, 8 mM PMSF pH 8.0), and cells were lysed immediately on ice by applying ten intermittent pulses of 10 s with a sonicator. Bacterial homogenates were centrifuged at 14,000 rpm for 5 min at 4°C to remove cell debris and unbroken cells. Proteins from supernatant were precipitated by trichloroacetic acid/acetone, and the resultant protein pellets were resuspended in rehydration buffer (7 M urea, 20 mM tris-HCl, 5 mM EDTA, 5 mM $MgCl_2$, 4% CHAPS, and 5 mM PMSF pH 8.0). Protein concentrations were estimated using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ).

2-D LC ESI MS/MS analysis

100 µg of protein was digested with trypsin (1:50 w/w) at 37°C for 16 h. The resultant peptides were desalted, dried in a vacuum centrifuge, and resuspended in 20 µL of 5% acetonitrile and 0.1% formic acid. Mass spectrometric analysis was accomplished using a ProteomeX Workstation (Thermo Scientific, Waltham, MA) as described previously (McCarthy et al., 2005). The auto-annotated *F. columnare* draft genome was obtained from the J. Craig Venter Institute. The *F. columnare* protein database with 2,882 open reading frames (ORFs) is available on our website (http://www.miangel.msstate.edu), while the *F. columnare* genome project is hosted at The Oklahoma University Health Sciences Center (http://microgen.ouhsc.edu). To identify proteins, mass spectra and tandem mass spectra were searched against the *in silico* trypsin digested *F. columnare* protein database as previously reported (Dumpala et al., 2009). Protein identifications and associated MS data were submitted to the PRoteomics IDEntifications database (PRIDE) database (Accession number: 9749).

2-DE and MALDI MS analysis

Proteins were extracted from four mid-exponential phase bacterial cultures as described in section 2.1. Protein pellets were resuspended in 350 µL of freshly prepared rehydration buffer (7 M urea, 2 M thio urea, 2% CHAPS, 1:50 carrier ampholytes, 0.3% DTT) and quantified using 2-D Quant Kit (GE Healthcare). For in-gel rehydration, 800-1000 µg of solubilized proteins were loaded onto each IPG strip (17 cm pH 3-10 NL). Isoelectric focusing (IEF), in-gel trypsin digestion, and MALDI MS analysis were performed as previously described (Chaudhary et al., 2007; Dumpala et al., 2009).

Identification of COGs, protein locations, and pathways

All identified proteins from our dataset and the predicted whole *F. columnare* proteome were organized into COG functional groups using COGnitor tool (Tatusov et al., 1997; Tatusov et al., 2000; Tatusov et al., 2001). The subcellular locations of proteins from our dataset as well as from the whole F. columnare proteome were predicted using PSORTb v2.0.4 (Gardy et al., 2003; Gardy et al., 2005). Pathways with significant protein representation in our experimentally derived protein dataset were identified using Pathway Studio (Ariadne Genomics, Rockville, MD). Due to a lack of a molecular interaction database for *F. columnare* in Pathway Studio, we custom built a database with all *F. columnare* proteins being mapped to their corresponding *E. coli*, *F. psychrophilum*, and *F. johnsoniae* orthologs by identifying reciprocal-best-BLAST hits with greater than 50% similarity. The resulting ortholog map file was used to predict pathways in *F. columnare* proteins. We used $P \leq 0.05$ to select pathways with significant representation.

Results

Protein identification using 2-D LC and 2-DE

2-D LC analysis yielded a total 548 *F. columnare* proteins (Appendix C) representing 19.01% of the predicted *F. columnare* proteome (2,882 ORFs). 2-DE analysis of four CBB stained gels showed approximately 600 spots. Among the common spots, 192 were excised and analyzed from one of these gels. We were able to identify 182 (94.79%) of the excised spots (Appendix D), which represented 141 unique *F. columnare* proteins. In 2-DE analysis, 41 (22.53%) proteins were identified in multiple spots, probably due to post-translational modifications and processing. Together, 2-D LC

and 2-DE analyses resulted in the identification of 621 *F. columnare* proteins, 480 (77.29%) of which were identified only by 2-D LC, while 73 (11.75%) were identified only by 2-DE (Figure 3.1), and 68 (10.95%) were detected by both approaches. Several of the identified proteins may have the potential to play important roles in *F. columnare* pathogenesis (Table 3.1).

Functional classification of identified proteins

Functional classifications of the 621 identified unique proteins and the 2,882 predicted *F. columnare* proteins are summarized in Figure 3.2. A total of 88 (14.17%, 88/621) proteins from our dataset and 372 (12.91%, 372/2,882) proteins from the predicted whole proteome were classified as information storage and processing related categories (J, K, and L). Cellular processes and signaling related categories (D, M, N, O, and T) included 112 (18.04%) and 370 (12.84%) proteins from our protein dataset and the whole proteome database, respectively. A total of 197 (31.72%) and 658 (22.83%) proteins from our protein dataset and the predicted whole proteome, respectively, were represented in metabolism related categories (C, E, F, G, H, I, P, and Q). Poorly characterized COG groups (R and S) contained 70 (11.27%) and 318 (11.03%) proteins in our protein dataset and the predicted whole proteome, respectively. "No COGs" (proteins not belong to any of the currently-defined COGs) and general function prediction only (R) were the top two represented categories in our protein dataset (23.51% and 8.70%, respectively) and in the whole proteome (36.33% and 7.15%, respectively). Eight (1.29%) proteins from our protein dataset and 117 (4.06%) proteins from the whole proteome were assigned to "No hit" (non-significant short or low-complexity sequences) category.

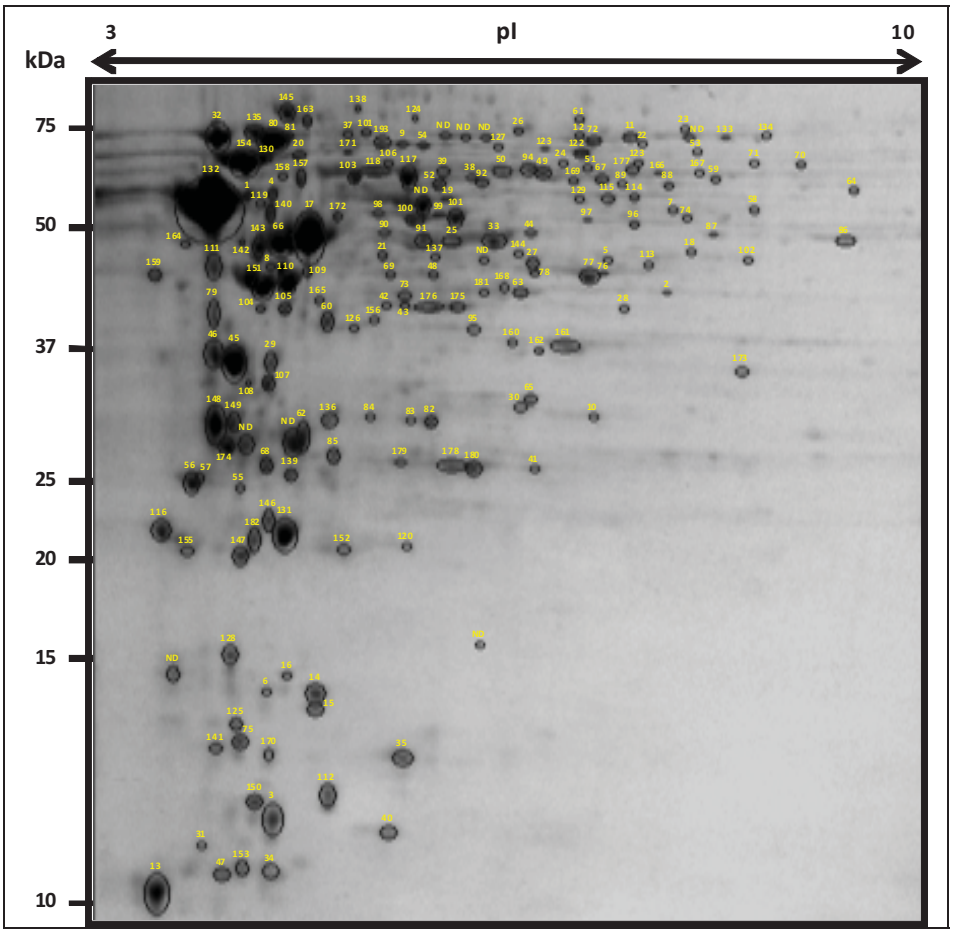

Figure 3.1 2-D map of proteins extracted from *Flavobacterium columnare*.

Circled spots were excised and analyzed by MS. Numbers indicate proteins with known IDs while NDs indicate unidentified proteins.

Table 3.1

Flavobacterium columnare proteins that may have role in pathogenesis.

ORF #	Protein name	Approach/ SN[a)]	Role category[b)]
ORF00560	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, YrbI family	2-D LC	Cell envelope (A)
ORF01244	3-deoxy-D-manno-octulosonate cytidylyltransferase	2-D LC	Cell envelope (A)
ORF02664	Amidinotransferase family protein	2-D LC	Cellular processes (E)
ORF01484	CHU large protein; gliding motility-related protein; putative adhesin AidA-related	2-D LC	Cellular processes (C)
ORF01824	DTDP-4-dehydrorhamnose 3,5-epimerase	2-DE/120	Cell envelope (A)
ORF00198	Extracellular elastinolytic metalloproteinase	2-D LC	Cellular processes (D)
ORF01481	Fibronectin type III domain protein	2-D LC	Cellular processes (D)
ORF02336	GldL	Both/152	Cellular processes (C)
ORF02335	GldM	2-D LC	Cellular processes (C)
ORF02334	GldN	2-DE/151	Cellular processes (C)
ORF02678	Glucose-1-phosphate thymidylyltransferase	2-D LC	Cell envelope (A)
ORF00396	Glycosyl transferase, group 1	2-D LC	Cell envelope (A)
ORF02332	Glycosyl transferase, putative	2-D LC	Cell envelope (A)
ORF00532	GTP-binding protein TypA/BipA	2-DE/36, 37	Cellular processes (F)
ORF01035	Hemagglutinin	2-D LC	Cellular processes (E)
ORF02801	Lipid-A-disaccharide synthase	2-D LC	Cell envelope (A)
ORF01136	LPS biosynthesis protein , RfbU family	2-D LC	Cell envelope (A)
ORF02060	Mannosyltransferase	2-D LC	Cell envelope (A)
ORF00475	Membrane protein, putative	2-D LC	Cellular processes (E)
ORF01989	Monofunctional biosynthetic peptidoglycan transglycosylase	2-D LC	Cell envelope (B)
ORF02568	PepSY-associated TM helix family	2-D LC	Cellular processes (C)
ORF02672	Polysaccharide export outer membrane protein	2-D LC	Cell envelope (A)
ORF01163	Tetraacyldisaccharide 4'-kinase	2-D LC	Cell envelope (A)
ORF01370	UDP-3-0-acyl N-acetylglucosamine deacetylase	2-D LC	Cell envelope (A)
ORF01367	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	2-D LC	Cell envelope (A)
ORF02676	UDP-glucose 6-dehydrogenase	Both/169	Cell envelope (A)
ORF02536	UDP-N-acetylglucosamine 2-epimerase	2-D LC	Cell envelope (A)
ORF00211	Universal stress protein family protein	2-DE/10	Cellular processes (F)

a) SN indicates spot number marked on the 2-DE image (Figure 1).

b) A, Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides; B, Biosynthesis and degradation of murein sacculus and peptidoglycan; C, Chemotaxis and motility; D, Pathogenesis; E, Toxin production and resistance; F, Adaptations to atypical conditions.

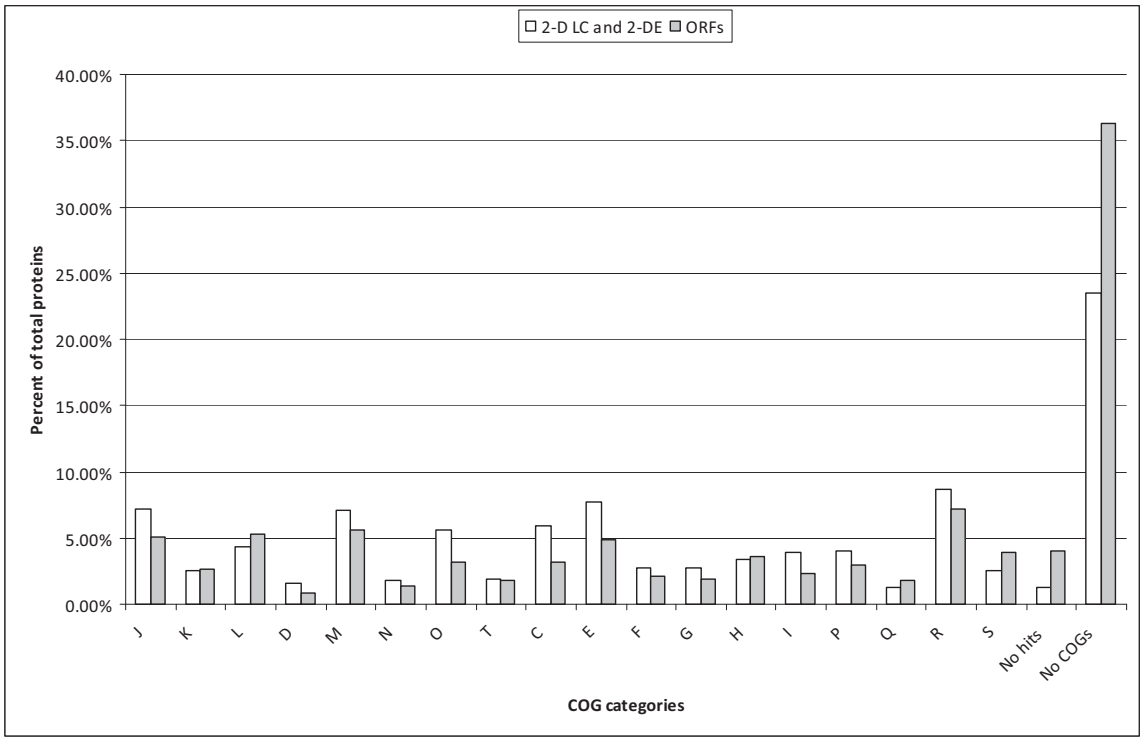

Figure 3.2 Comparison of COG categories between proteins identified in this study and predicted proteins from the *Flavobacterium columnare* genome.

Percentages were calculated by dividing the number of proteins in the particular COG category by the number of unique proteins in each analysis. COG categories are as follows: J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination, and repair; D, cell cycle control, cell division, chromosome partitioning; M, cell wall/membrane/envelop biogenesis; N, cell motility; O, post-translational modification and protein turnover, chaperones; T, signal transduction mechanisms; U, intracellular trafficking, secretion, and vesicular transport; V, defense mechanisms; C, energy production and conversion; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown. "No hit" group indicates non-significant short or low-complexity sequences and "No COGs" indicates query proteins not belong to any of the currently-defined COGs.

Subcellular localization of identified proteins

Subcellular locations of *F. columnare* proteins identified in this study and predicted from the draft genome were determined (Figure 3.3). 2-DE and 2-D LC generally resulted in similar coverage of subcellular compartments compared to the predicted proteome, except 2-DE gave better coverage of cytoplasmic proteins and poorer coverage of cytoplasmic membrane proteins. 2-D LC had better representation of proteins from both membrane compartments and extracellular proteins. Both 2-D LC and 2-DE had lower representation of the "unknown" subcellular location proteins than the percentage from the predicted proteome. In this study, 41 proteins were predicted to locate in the *F. columnare* outer membrane by PSORTb (Table 3.2).

Pathway analysis

We used Pathway Studio analysis to gain insight into various pathways represented in *F. columnare* proteins identified by 2-D LC and 2-DE analysis. Twenty seven pathways belonging to metabolism and one pathway in genetic information processing were significantly represented ($P< 0.05$, Appendix E). Out of 28 pathways, eight related to amino acid metabolism (Figure 3.4), five carbohydrate metabolism, four cofactor and vitamin metabolism, four xenobiotics biodegradation and metabolism, two glycan biosynthesis and metabolism, two nucleotide metabolism, one energy metabolism, one biosynthesis of secondary metabolites, and one related to translational process. 277 proteins from our dataset were integral to these pathways. Of the significantly represented pathways, purine metabolism contained the highest number of proteins (24 proteins), while the biotin metabolism pathway had the fewest (4 proteins). Of all the

pathways, folate biosynthesis pathway was the most significant pathway in our dataset (P = 2.25E-09), which was followed by the purine metabolism pathway (P = 1.44E-07).

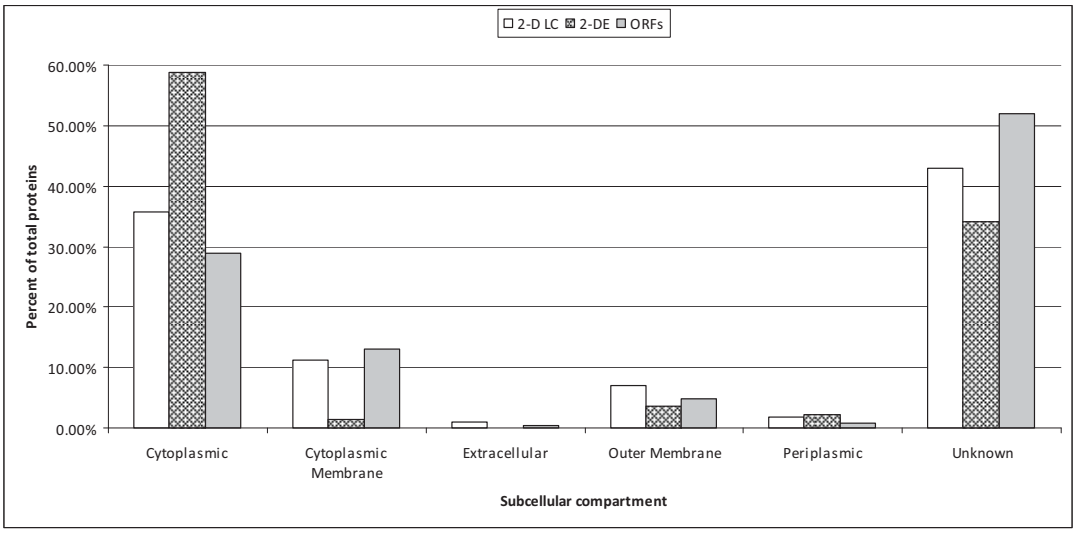

Figure 3.3 Subcellular locations of *Flavobacterium columnare* proteins determined by PSORTb prediction.

Percentages were calculated by dividing the number of proteins in the particular subcellular location by the number of unique proteins in each analysis (548 proteins in 2D-LC, 141 proteins in 2-DE, and 2,882 ORFs predicted from the draft genome). 2-D LC and 2-DE indicates proteins identified in this study, while ORFs indicates proteins predicted from the whole genome. Unknown category includes proteins with multiple subcellular locations or unknown location.

Table 3.2

Outer membrane proteins of *Flavobacterium columnare* predicted by PSORTb.

Protein name	**ORF #**	**COG[a)]**	**Approach/SN[b)]**
Conserved hypothetical protein	ORF02881	-	2-D LC
Conserved hypothetical protein	ORF00226	-	2-D LC
Conserved hypothetical protein	ORF01938	D	2-D LC
Conserved hypothetical protein	ORF00224	-	2-D LC
Conserved hypothetical protein	ORF02143	-	2-D LC
Conserved hypothetical protein	ORF02328	R	2-D LC
Extracellular elastinolytic metalloproteinase	ORF00198	-	2-D LC
Fjo24	ORF02628	-	2-D LC
Hypothetical protein	ORF02366	-	2-D LC
Hypothetical protein	ORF01273	-	2-D LC
Hypothetical protein	ORF01272	-	2-D LC
Hypothetical protein	ORF01485	-	2-D LC
Hypothetical protein	ORF02042	P	2-D LC
Lipoprotein, putative	ORF01143	-	2-D LC
Lipoprotein, putative	ORF02619	-	Both/166, 167
Omp assembly complex, YaeT protein	ORF02858	M	2-D LC
OmpA	ORF02005	M	Both/132
OmpA	ORF00341	M	2-D LC
OmpA family protein	ORF00085	M	2-D LC
OmpA/MotB family	ORF00937	M	2-D LC
OmpA/MotB family	ORF01639	N	2-D LC
OmpA/MotB family	ORF00225	M	2-DE/11, 12
Outer membrane efflux protein	ORF00073	MN	2-D LC
Outer membrane insertion C- signal domain protein	ORF00187	-	2-D LC
Peptidyl-prolyl cis-trans isomerase C	ORF02829	O	2-D LC
Putative TonB-dependent receptor	ORF00266	P	2-D LC
RagA protein, putative	ORF00258	P	2-D LC
Secreted protein	ORF02176	-	2-DE/141
Snf2 family helicase	ORF00742	KL	2-D LC
Tetratricopeptide repeat domain protein	ORF01855	R	2-D LC
TonB-dependent outer membrane receptor	ORF00779	P	2-D LC
TonB-dependent outer membrane receptor	ORF02179	P	2-D LC
TonB-dependent outer membrane receptor	ORF00147	P	2-D LC
TonB-dependent outer membrane receptor	ORF02172	P	2-D LC
TonB-dependent outer membrane receptor	ORF02232	P	2-D LC
TonB-dependent outer membrane receptor	ORF01690	P	2-D LC
TonB-dependent outer membrane receptor, putative	ORF00704	P	2-D LC
TonB-dependent outer membrane receptor, putative	ORF02424	P	2-D LC
TonB-dependent receptor plug domain protein	ORF01433	P	2-D LC
TonB-dependent receptor, putative	ORF01511	P	2-D LC
Transcriptional regulator, AraC family protein	ORF01879	R	2-D LC

a)COG categories are as follows: M, cell wall/membrane/envelop biogenesis; D, cell cycle control, cell division, chromosome partitioning; P, inorganic ion transport and metabolism; S, function unknown; N, cell motility; R, general function prediction only; O, post-translational modification and protein turnover, chaperones; K, transcription; L, replication, recombination and repair; -, "No COGs".

b)SN indicates spot number marked on the 2-DE image (Figure 1).

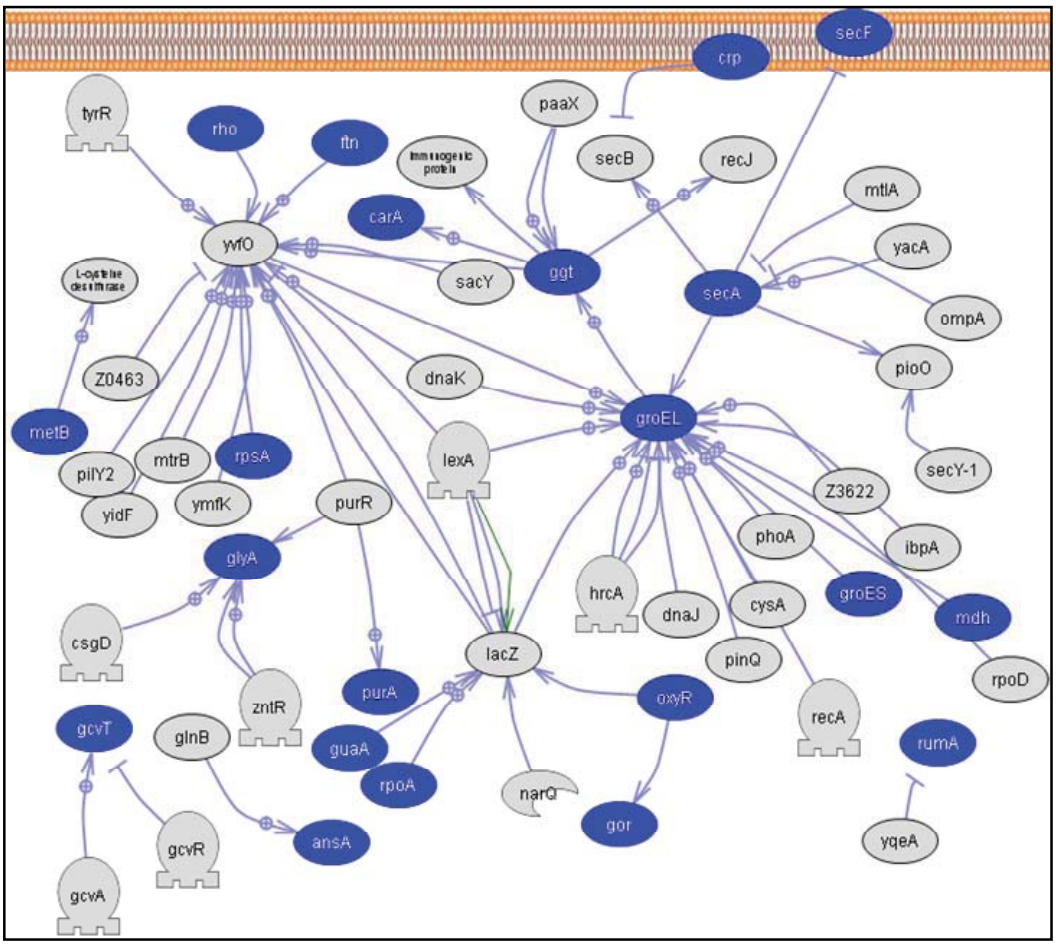

Figure 3.4 Amino acid metabolism related pathways in *Flavobacterium columnare*.

Amino acid metabolism related pathways include glutamate; histidine; selenoamino acid; alanine and aspartate; glycine, serine, and threonine metabolism; lysine biosynthesis; lysine degradation; and valine, leucine, and isoleucine degradation pathways. Entities shown in oval represent genes involved in this pathway. Filled ovals represent proteins detected in this study. Unconnected genes were removed from the figure to reduce complexity.

Discussion

This research analyzed the soluble cell proteome of *Flavobacterium columnare* using complementary technologies of 2-D LC and 2-DE. Proteomic data from this study has provided experimental evidence for the expression of many *F. columnare* proteins that were previously only predicted from the draft genome sequence. Data from this study

provides a picture of the expressed *F. columnare* proteome during log-phase growth in FCGM along with a 2-D reference map, which will provide an important basis for comparison under other environmental conditions.

Currently, the most popular methods employed to explore protein expression utilize protein separation by 2-D LC (gel-free) or 2-DE (gel-based) followed by MS analysis (Gorg et al., 2004). Use of more than one methodology in proteomic investigations improves proteome coverage because each method identifies a set of unique proteins (Wolff et al., 2006; Salzano et al., 2007). Therefore, in the present study, we employed the complementary techniques of 2-DE and 2-D LC to identify 621 unique proteins from *F. columnare* (480 unique to 2-D LC, 73 unique to 2-DE, and 68 common to both), which provided experimental evidence for more than one fifth (21.55%) of the predicted 2,882 *F. columnare* ORFs.

Ribosomal proteins, one of the most abundantly expressed protein types in cells, were detected in high numbers. In the draft *F. columnare* genome sequence, 60 proteins were predicted as ribosomal proteins. In the present study, we identified 14 and 3 unique ribosomal proteins using 2-D LC and 2-DE, respectively. Along with ribosomal proteins, we also identified several proteins involved in translational machinery, including several aminoacyl-tRNA synthases, translation initiation factors such as translation initiation factor IF-2 (InfB) (ORF00214), and elongation factors (EF) such as transcription elongation factor GreA (ORF00178), translation elongation factor Tu (ORF00244), translation elongation factor Ts (ORF00695), translation elongation factor P (ORF01368), and translation elongation factor G (ORF02228).

Outer membrane proteins (OMPs) often act as important virulence factors in gram-negative bacteria. Therefore, identification of such molecules provides targets for future studies to increase our understanding of *F. columnare* pathogenesis. Moreover, OMPs may also play a crucial role in interacting with other molecules. In the present study, we identified 41 unique OMPs using 2-D LC and 2-DE analysis, which includes many of the 14 OMPs previously identified by Liu et al. (2008). TonB-dependent outer membrane receptors function in the import of iron-siderophore complexes and vitamin B12 across the outer membrane in gram-negative bacteria (Letain and Postle, 1997; Braun, 1998). We identified six OmpA related proteins, which are the most abundant protein components of outer membranes of gram-negative bacteria (Molloy et al., 2001). OmpA is predicted to be a nonspecific diffusion channel, allowing small solutes to cross the outer membrane (Sugawara and Nikaido, 1992).

In *F. columnare,* proteases, adherence factors, and chondroitin AC lyase have been reported as virulence factors (Newton et al., 1997; Decostere, 1999). The *F. columnare* genome encodes eight putative secreted metalloproteases/peptidases probably involved in virulence and/or in the destruction of host tissues. In this study, we identified only one extracellular elastinolytic metalloproteinase (ORF00198) using 2-D LC, which is probably due to the absence of host/host factors during *F. columnare* growth and/or isolation of the bacterial cell proteins, but not the secreted proteins. Chondroitin AC lyase was not detected, but we did detect fibronectin type III domain protein (ORF01481), a potential adherence factor that may be involved in cell surface binding of *F. columnare*. LemA (ORF00319) is also shown to be a putative virulence factor in several animal and plant bacteria, especially in *Pseudomonas* (Hrabak and Willis, 1992; Tan et al., 1999).

F. columnare moves over surfaces by gliding motility. Thirteen gliding motility related proteins are encoded in the *F. columnare* draft genome, out of which we detected four (GldN, GldM, GldL and gliding motility-related protein). Detection of only four gliding motility proteins may be due to planktonic growth of *F. columnare* in broth culture.

Production of reactive oxygen species (ROS) by macrophages is one of the most effective defense mechanisms of host against bacterial pathogens (Secombes, 1996). Superoxide dismutases, bifunctional catalase-peroxidases, thiol peroxidases, and proteins of the peroxy-redoxin family are important bacterial defense mechanisms allowing survival of *F. psychrophilum* against phagocytes (Sanchez-Moreno et al., 1989; Kawai et al., 2000; Nematollahi et al., 2003; Nematollahi et al., 2005; Duchaud et al., 2007). In the present study, we identified copper/zinc superoxide dismutase (SodC) (ORF02131), superoxide dismutase [Mn] (ORF02002), catalase/peroxidase HPI (KatG) (ORF02145), cytochrome c551 peroxidase (ORF01733), thiol peroxidase (ORF00815), and glutathione peroxidase (ORF00555). SodC is a periplasmic enzyme that converts superoxide radicals to hydrogen peroxide and water. Sod [Mn] is expressed only under aerobic conditions (Touati, 1988) and is believed to be involved in effective prevention of DNA damage in *Escherichia coli* (Hopkin et al., 1992). Expression of these proteins in *F. columnare* suggests its ability to resist ROS.

The predicted *F. columnare* proteins were classified into COGs to assign functions. The majority of proteins identified in the current study (31.72%) were housekeeping proteins in “metabolism” related categories (‘metabolism’, ‘cellular processes and signaling’, and ‘information storage and processing’). This was not

surprising because we harvested proteins from bacteria when they were metabolically active at mid log phase. As compared to the predicted whole proteome, our experimental dataset had relatively lower coverage of the *F. columnare* proteins that were not assigned to COG groups. Proteins with unknown function may be expressed only under specialized conditions like host environment or stressed conditions and may not be expressed under laboratory culture conditions. Analysis of metabolism-related proteins in our list implies that amino acids, instead of carbohydrates, could be the major sources of carbon or energy. The FCGM medium we used for bacterial growth is a good source of amino acids, nitrogen, and certain salts like $MgSO_4$ and $CaCl_2$ but it lacks readily available carbohydrate sources. Most of the metabolic proteins indentified in our study were amino acid kinases, including five histidine kinases and five aminotransferases. Only three carbohydrate kinases involved in core carbohydrate metabolisms were identified. We found 18 peptidases, which may be useful in utilizing available amino acids as a major source of carbon. Previous studies on *F. psychrophilum* also suggest that this bacterium was unable to use readily available carbohydrates as a major energy source (Bernardet and Kerouault, 1989).

We used the PSORTb algorithm to predict the subcellular locations of *F. columnare* proteins. Many proteins were mapped to the cytoplasm, which is expected because these proteins are not hydrophobic, and thus solubility does not hinder protein isolation and separation. 2-DE was particularly effective at detection of cytoplasmic proteins. By contrast, cytoplasmic and outer membrane proteins were identified in higher percentages using 2-D LC when compared to 2-DE. The same buffer solution containing 7M urea and 2% CHAPSO was used to solubilize proteins during isolations for both 2D-

LC and 2-DE, so it appears that the difference is not due to the isolation procedure, but rather the separation techniques. Other studies have reported that hydrophobic membrane proteins are typically difficult to identify using 2-DE. Hydrophobic proteins were found to be under-represented in 2-DE based proteome analyses of membrane protein fractions from *E. coli* (Molloy et al., 2000; Fountoulakis and Gasser, 2003; Lai et al., 2004) and *Bacillus subtilis* (Eymann et al., 2004; Bunai and Yamane, 2005; Wolff et al., 2006). It is important to keep in mind that we chose to analyze only 192 common spots (30.81%) out of approximately 600 spots identified on our gels, so the bias toward cytoplasmic proteins in our 2-DE results may be because these proteins were more abundant or consistently present in gels. However, for future functional studies of membrane proteins, specialized isolation techniques may be needed. The ease and higher sensitivity of 2-D LC for detection of hydrophobic membrane proteins may make this the preferred method for studying membrane proteins.

From the Pathway Studio analysis, we identified pathways that were significantly represented in our protein dataset. Folate biosynthesis was the most significantly represented metabolic pathway, which was followed by the purine metabolism pathway. Higher representation of metabolic and translational process related pathways is expected because proteins were isolated from *F. columnare* during a metabolically active state. Amino acid metabolism related pathways (glutamate; histidine; selenoamino acid; alanine and aspartate; glycine, serine, and threonine metabolism; lysine biosynthesis; lysine degradation; and valine, leucine, and isoleucine degradation) are highly represented in our dataset compared to carbohydrate metabolism. Moreover, we have identified 18 peptidases in our protein list suggesting breakdown of host proteins and

usage of resultant amino acids as a major source of energy, carbon, and nitrogen by *F. columnare*. Similarly, Glycosylphosphatidylinositol (GPI)-anchor biosynthesis and LPS biosynthesis pathways were significantly represented in our dataset, which may play an important role in cell wall synthesis. Moreover, LPS biosynthesis may be one of the important virulence-related systems in *F. columnare* pathogenesis. LPS is an important component of the cell envelope in gram-negative bacteria and is an immunodominant antigen.

Our main objective was to identify the expressed proteins of *F. columnare* during normal growth. We showed for the first time the expression of 621 proteins from *F. columnare*, which provides experimental evidence for many proteins that were predicted from the *F. columnare* genome annotation. This information could accelerate functional and comparative studies aimed at understanding virulence mechanisms of this important pathogen.

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CHAPTER 4

IDENTIFICATION OF DIFFERENTIALLY REGULATED PROTEINS OF *EDWARDSIELLA ICTALURI* DURING IRON-RESTRICTION

Abstract

Edwardsiella ictaluri is a facultative intracellular gram-negative bacterium that causes enteric septicemia of catfish (ESC). ESC is one of the most important bacterial diseases affecting farm-raised channel catfish. Iron is an essential inorganic nutrient of bacteria and is crucial for bacterial invasion. Reduced availability of iron by the host may cause a significant stress for bacterial pathogens and is considered a signal that leads to significant alteration in virulence gene expression. However, the precise effect of iron-restriction on *E. ictaluri* protein expression is unknown. The purpose of this study was to determine differentially expressed proteins of *E. ictaluri* during iron-restricted conditions. We applied two dimensional difference gel electrophoresis for determining differentially expressed proteins and MALDI TOF/TOF MS for protein identification. Gene ontology and pathway-based functional modeling of differentially expressed proteins was also presented. A total of 50 unique differentially expressed proteins at a minimum of 2-fold ($p \leq 0.05$) difference in expression due to iron-restriction were determined. The numbers of up- and down-regulated proteins were 37 and 13, respectively. We noted several genes like *esrB*, *lamB*, *malM, malE, fdaA*, and tonB-dependent heme/hemoglobin receptor family proteins that may act as virulent determinants of *E. ictaluri*. The present study

revealed altered proteins of *E. ictaluri* in response to iron-restriction. These proteins may play crucial roles in the pathogenesis of *E. ictaluri*.

Introduction

Edwardsiella ictaluri is a facultative intracellular gram-negative bacterium that causes enteric septicemia of catfish (ESC). ESC is one of the most prevalent bacterial diseases affecting farm-raised channel catfish in the United States (Hawke et al., 1981). ESC can occur either as an acute or a chronic disease in channel catfish (*Ictalurus punctatus*), and is capable of causing high mortalities (Miyazaki and Plumb, 1985; Shotts et al., 1986; Newton et al., 1989). Previously, attempts have been made to identify potential virulence factors of *E. ictaluri,* suggesting various virulent determinants, including extracellular capsular polysaccharide (Stanley et al., 1994), lipopolysaccharide (LPS) (Weete et al., 1988; Newton and Triche, 1993; Lawrence et al., 2001; Arias et al., 2003; Lawrence et al., 2003; Williams et al., 2003), outer membrane proteins (OMP) (Newton et al., 1990; Vinitnantharat et al., 1993; Skirpstunas and Baldwin, 2003; Williams et al., 2003; Bader et al., 2004), hemolysins (Williams and Lawrence, 2005), and chondroitinase (Waltman et al., 1986; Stanley et al., 1994; Cooper et al., 1996). Previous research has also shown that *E. ictaluri* is able to survive and replicate inside catfish neutrophils and macrophages (Miyazaki and Plumb, 1985; Shotts et al., 1986; Ainsworth and Chen, 1990; Baldwin and Newton, 1993; Stanley et al., 1994).

Iron is an essential micro element for almost all living organisms and is involved in various metabolic processes like sugar, protein, energy, and DNA metabolism, growth, and response to oxidative stress (Mey et al., 2005). Reduced availability of iron may cause a significant stress for bacterial pathogens and is considered a signal that leads to

significant changes in gene expression. In 1982, Neilands reviewed the changes in the expression of outer membrane proteins of *Escherichia coli* along with other enteric bacteria in response to iron-restricted environments (Neilands, 1982). Vertebrate hosts tend to chelate free iron using high affinity proteins like ferritin, transferrin, heme proteins and make the environment hostile for bacteria to grow (Ratledge and Dover, 2000; Lenco et al., 2007). This innate mechanism of iron-restriction by the host is one of the crucial means of host defense against virulent bacterial invasion (Payne, 1993; Weinberg, 1993). In turn, low levels of iron in the environment triggers a signal for the pathogen, leading to expression of more virulence determinants (Litwin and Calderwood, 1993). Moreover, previous research has also shown that in many gram-negative bacteria, iron, in association with its ferric uptake regulator (fur), acts as a regulatory molecule in virulent determinant expression (Griffiths and Chart, 1999). Based on the above phenomenon, a significant number of attempts were made and succeeded in identification of potential virulent determinants in bacteria such as *E. coli* (Bindereif and Neilands, 1985; Calderwood and Mekalanos, 1987), *Shigella dysenteriae* (Dubos and Geiger, 1946), *Vibrio cholera* (Sigel and Payne, 1982; Sciortino and Finkelstein, 1983; Stoebner and Payne, 1988; Goldberg et al., 1990), *Neisseria meningitidis* (Dyer et al., 1988), *Pseudomonas aeruginosa* (Bjorn et al., 1978; Bjorn et al., 1979; Poole et al., 1993).

Although a considerable number of attempts were made previously to identify various virulence determinants of *E. ictaluri*, further research disclosing en masse potential virulence factors on this economically important bacterium is required. Recently, we analyzed and annotated the sub-proteome of *E. ictaluri* strain 93-146 (Dumpala et al., 2009). Similar to other bacteria, *E. ictaluri* pathogenesis may rely on its

ability to regulate its proteome for successful invasion and establishment of disease. Thus, there is a need to understand how *E. ictaluri* react and express virulence determinants when grown under iron-restricted conditions. Lack of such knowledge is an important problem for elucidation of the mechanisms of pathogenesis of ESC at molecular level.

The objective of the present study was to identify differentially expressed proteins of *E. ictaluri* grown under *in vitro* iron-restricted conditions to enlighten the possible mechanisms of pathogenesis of *E. ictaluri*. We anticipate our study to be a starting point for further functional studies. For example, identified genes could be mutated and mutant phenotypes could be determined.

Materials and Methods

Iron-restricted growth and total protein extraction

E. ictaluri strain 93-146 was grown on brain heart infusion (BHI) broth or agar medium. Chelating agent 2,2′-dipyridyl (Sigma, St. Louis, MO.) at a final concentration of 100 mM was used to sequest iron from the medium (Davies et al., 1992; Sabri et al., 2006). A single colony was used to establish control (grown on regular BHI) and treatment (grown on iron-restricted BHI) groups of each replica and cultures were harvested at mid-exponential phase (OD_{600} 0.6) by centrifugation at 3,750 rpm for 15 min at 30°C.

Six bacterial pellets (3 control and 3 treatment) were washed thrice using standard cell wash buffer (10mM TrisCl and 5mM Magnesium acetate) at 30°C and were resuspended in 750 uL of urea-CHAPS buffer (8 M urea, 30 mM tris-HCl, 4% CHAPS, 8

mM PMSF pH 8.0). Cells were lysed on ice by applying ten intermittent pulses of 10 s with a sonicator and bacterial homogenates were centrifuged in a refrigerated tabletop centrifuge (4°C) at 14,000 rpm for 5 min to remove cell debris and unbroken cells.

Proteins from supernatant were precipitated by trichloroacetic acid/acetone, and the resultant protein pellets were resuspended again in urea-CHAPS buffer. The pH of the lysates was adjusted to 8.5 using 50 mM sodium hydroxide. Protein concentrations were estimated using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ) following the manufacturer's instructions.

Labeling of proteins

Protein samples were labeled using a CyDye DIGE Fluor minimal labeling kit (GE Healthcare) according to the kit's manual. Briefly, 50 μg of protein from an internal standard (equal mixture (8.33 μg) of all 6 samples), control, and treatment were mixed with 400 pmol of Cy2, Cy3, or Cy5 dyes, respectively, and protein-dye mixtures were incubated on ice in the dark for 30 min. Labeling reaction was terminated by adding 1 μl of 10 mM lysine, mixing well, and incubating samples in the dark for 10 min.

Protein separation using 2-DE

For isoelectric focusing (IEF), precast 17 cm pH 3-10 NL immobilized pH gradient (IPG) strips (Bio-Rad, Hercules, CA) were used. Each of the labeled protein samples from control, treatment, and internal standard were combined with rehydration buffer containing 7M urea, 2M thio urea, 4% CHAPS, 1:50 carrier ampholyte, 2% DTT. Mixed samples were loaded onto each IPG strip for in-gel rehydration. IEF was performed in a Protean IEF cell (Bio-Rad, Hercules, CA) in the dark at 23°C, 500 V for

15 min; linear ramp to 10,000 V for 3 h; and 10,000 V until a total of 70,000 Vh was reached. After IEF, IPG strips were equilibrated in 6 M urea, 30% glycerol, 50 mM Tris-HCl, 2% SDS, 2% DTT, at pH 8.8, and with a trace of bromophenol blue for 15-20 min followed by equilibration containing 2.5% iodoacetamide (IAA) instead of 2% DTT for 15-20 min. Once equilibrated, strips were transferred onto 12% SDS-PAGE gels (Jule Inc., Milford, CT) and sealed with 0.5% agarose in electrophoresis buffer. Electrophoresis was performed using a PROTEAN II XL system (Bio-Rad) at a constant current of 10mA/gel for the first 15 min followed by 24mA/gel at 20 °C until the dye front reach the lower end of the gel.

Analysis of 2-D DIGE gel images

After electrophoresis, DIGE gels were scanned using a Typhoon 9410 imager (GE Healthcare). Excitation and emission filters used for each dye were as follows: Cy2 (488 nm/520 nm), Cy3 (532 nm/580 nm), and Cy5 (633 nm/670 nm). Acquired images were analyzed using DeCyder 5.0 software (GE Healthcare). Briefly, spots were detected using the differential in-gel analysis (DIA) module. Spot matching between gels and statistical analysis of protein-abundance changes were conducted using the biological variation analysis (BVA) module. Among the 3 replicates, the gel with the highest number of spots was assigned as the master gel. All the spots which were matched automatically were also manually compared among all 3 replicate gels to minimize false spot matching. Statistical significance was calculated using the Student's t-test with applied false discovery rate and a significance threshold of $p < 0.05$. Only spots showing at least a 2-fold change in spot intensity and were consistent in all three replicate gels were considered as differentially expressed and chosen for protein identification.

Preparative gel electrophoresis and protein identification

Preparative 2-DE gels were prepared exactly as described above, except that the IPG strips were loaded with 500 µg of protein. Resultant gels were stained using Deep Purple Total Protein Stain (GE Healthcare) according to the manufacturers' protocols. Briefly, gels were fixed overnight in 15% v/v ethanol and 1% w/v citric acid followed by staining for 1 h in 1:200 parts of Deep Purple and 100 mM sodium borate solution at pH 10.5. Gels were then washed for 30 min with 15% v/v ethanol in water, acidified for 30 min using a solution containing 15% v/v ethanol and 1% w/v citric acid. Stained gels were scanned using a Typhoon 9410 imager using a 532 nm laser and a 610 nm BP30 emission filter. In-gel trypsin digestion and MALDI PMF was performed as previously described (Chaudhary et al., 2007).

Functional modeling of differentially expressed proteins

We used various gene ontology (GO) resources like GORetriever and GOanna available at AgBase (McCarthy et al., 2006) for obtaining biological processes and molecular functional annotations of differentially expressed proteins. Using GORetriever tool, we obtained all GO annotations for proteins with existing GO annotation. Proteins with no existing GO annotations but with a sequence similarity of >80% with presumptive orthologs were annotated using Goanna tool. Obtained GO biological processes and molecular function annotations were manually summarized to more generalized categories based on the ancestor chart for GO terms at QuickGO (Binns et al., 2009). The subcellular locations of differentially expressed proteins were predicted using PSORTb v3.0.0 (Gardy et al., 2005). To gain insight into various biological pathways that were significantly represented by our differentially expressed proteins, we

used Pathway Studio 6.0 (Ariadne, Rockville, MD) as previously reported (Dumpala et al., 2009). In addition, "build pathway" function was used to build a biological interactions network of up- and down-regulated proteins.

Results

Identification of differentially expressed proteins

The DIGE analysis detected around 2,200 spots in each replicate and after automatic matching and manual verification of each spot, only those spots which were matched in all 3 replicate gels were subjected to statistical analysis. Analysis of these spots revealed that, 131 spots (92 up- and, 39 down-regulated spots) were shown to be differentially expressed with a minimum of 2-fold, $p < 0.05$ in the iron-restricted conditions compared to bacteria grown in regular BHI media. Among the 131 spots, 71 spots (54 up- and 17 down-regulated) matched to a preparative gel were cut for MS analysis, 65 (91.54%) positive ID's with C. I. % > 99 were identified (Figure. 4.1). Fifteen proteins were represented in more than one spot, probably due to post-translational modifications and processing. In conclusion, we were able to determine 50 (37 up- and 13 down-regulated) unique differentially expressed *E. ictaluri* proteins under *in vitro* iron-restricted conditions (Table 4.1).

We noted several genes like *esrB, lamB, malM, malE, fda, aspA, dsbA, ompA, oppA*, and tonB-dependent heme/hemoglobin receptor family protein that may potentially act as virulent determinants of *E. ictaluri*.

Functional modeling of differentially expressed proteins

GO annotation of 50 unique differentially expressed proteins and manual slimming based on GO terms resulted in 14 biological process (Figure 4.2) and 14 molecular function (Figure 4.3) categories. Up-regulated proteins were represented in 12 biological processes, whereas down-regulated proteins were represented only in 7 biological processes. The top three biological process categories represented by highere numbers of up-regulated proteins were carbohydrate metabolic process, oxidation reduction, and cellular metabolic process. Two of these categories (cellular metabolic process and oxidation reduction) were also among the top three biological processes that down-regulated proteins were assigned. Interestingly, carbohydrate metabolic processes, including the highest number of up-regulated proteins, did not include any down-regulated proteins.

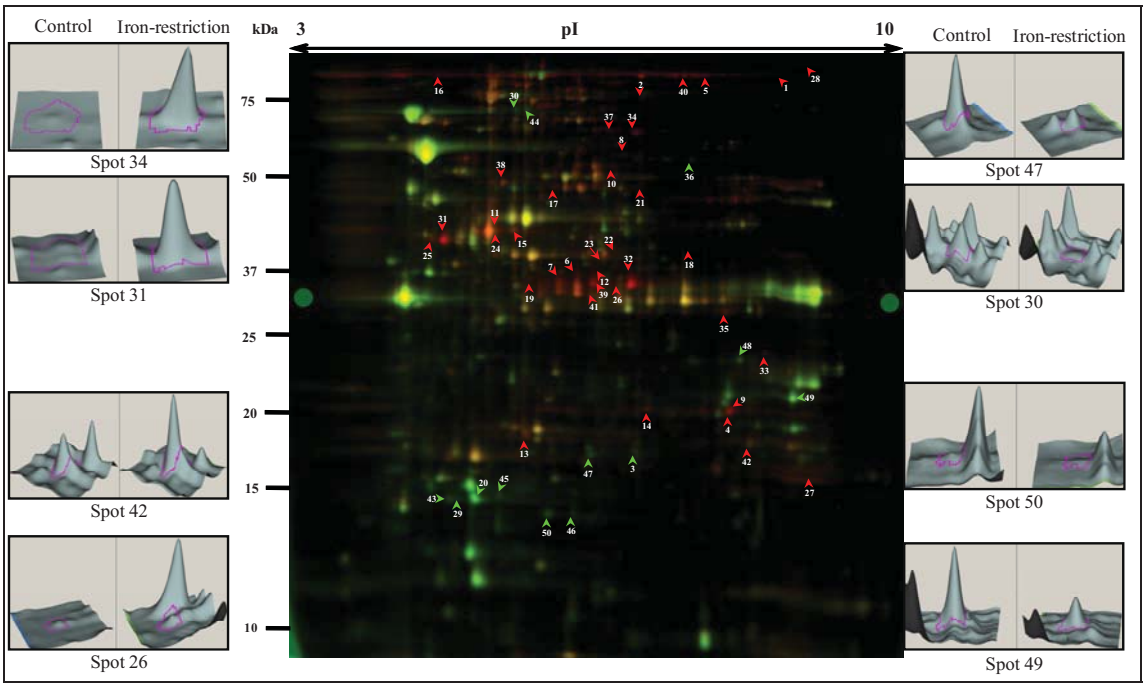

Figure 4.1 Fluorescent difference gel electrophoresis (2-D DIGE) of *Edwardsiella ictaluri* grown in *in vitro* iron-restriction condition.

Fifty µg of soluble *E. ictaluri* protein grown in regular brain heart infusion (BHI) media was labeled with Cy3, grown in BHI with chelator 2,2'-dipyridyl was labeled with Cy5, and the pooled internal standard with Cy2. Spots shown in red and green arrow head are up- and down-regulated (≥2 fold), respectively. 3D images of 2 spots with maximum and minimum fold up-regulated proteins were shown on left top and bottom corners of gel image, respectively. 3D images of 2 spots with maximum and minimum fold down-regulated proteins were shown on Right top and bottom corners of gel image, respectively.

Table 4.1

Differentially expressed proteins of *Edwardsiella ictaluri* in response to *in vitro* iron-restriction

Process/GI number	Protein #	Spot ID	Fold difference	CI %	Protein name	Protein MW/PI	Pep. Count	Protein score	GENE name
Alcohol metabolic process									
238919566	1	194/195	3.82/2.68	100	Aldehyde-alcohol dehydrogenase 2	95992.7/6.41	28	413	NT01EI_1665
Biosynthetic process									
238919324	2	350/346	2.89/2.49	100	Bifunctional polymyxin resistance protein ArnA, putative	73954/5.67	26	264	arnA/NT01EI_1415
238920260	3	1972	-4.14	100	3-oxoacyl-[acyl-carrier-protein] reductase, putative	25567.1/5.95	9	244	NT01EI_2369
Carbohydrate metabolic process									
238921292	4	1811	5.48	100	N-acetylmuramoyl-L-alanine amidase AmiD	28647.3/6.6	11	220	NT01EI_3435
238920353	5	182	3.63	100	Formate acetyltransferase, putative	85051.6/5.65	22	154	NT01EI_2463
238921224	6	1347/1352/1316	3.03	100	Fructose-bisphosphate aldolase, putative	39129.7/5.65	14	382	fba or fda/NT01EI_3367
238918053	7	1301	3.55	99.99	ADP-glyceromanno-heptose 6-epimerase, putative	34791/5.29	9	77	hldD/NT01EI_0072
238918174	8	715	2.45	100	Glucose-6-phosphate isomerase	61392.9/6.06	19	243	pgi/NT01EI_0210
238920733	9	1809	3.05	100	Hypothetical protein NT01EI_2846	28196.7/6.56	18	454	gpmA/NT01EI_2846
238921491	10	699/690	3.16/3.24	100	Phosphoenolpyruvate carboxykinase (ATP)	59171.9/5.77	28	571	pckA/NT01EI_3643
Nucleoside/nucleotide metabolic process									
238918513	11	1034	2.84	100	Thymidine phosphorylase, putative	46793.9/5.33	14	139	NT01EI_0563
238918595	12	1236	2.33	100	Hypothetical protein NT01EI_0651	38512.4/8.34	12	209	NT01EI_0651
238918515	13	1888	2.17	100	Purine nucleoside phosphorylase, putative	25636.8/5.4	11	292	deoD/NT01EI_0565
238918109	14	1801	3.33	100	Uridine phosphorylase, putative	27335.9/6.07	13	645	NT01EI_0133
238918514	15	1051	2.17	100	Phosphopentomutase, putative	44429.2/5.33	20	341	deoB/NT01EI_0564
Oxidation reduction									
238918700	16	147	2.46	100	Pyruvate dehydrogenase; acetyl-transferring, homodimeric type, putative	99427.8/5.55	20	169	NT01EI_0758
238918702	17	819/833	2.51	100	Dihydrolipoyl dehydrogenase, putative	50803.5/5.64	19	397	NT01EI_0760
238919229	18	1144	4.49	100	Udp-glucose 6-dehydrogenase	43359.6/6.09	11	113	NT01EI_1312
238918818	19	1288	3.08	100	1,3-propanediol dehydrogenase	40188.1/5.45	15	467	NT01EI_0882
238920005	20	2096	-3.67	100	Superoxide dismutase	21120.4/5.26	5	364	Sod_Fe/NT01EI_2109
Phosphorylation									
238921741	21	713	3.68	100	ATP synthase subunit alpha/ AltName: F-ATPase subunit alpha	55190.7/5.59	24	491	atpA/NT01EI_3910
238920582	22	1171	2.1	100	Acetate kinase, putative	43096/5.9	17	517	NT01EI_2694
238920730	23	1243	2.45	100	Galactokinase, putative	41138.9/5.83	18	411	NT01EI_2843
Translation									
238921444	24	1082	2.28	100	Elongation factor Tu	43262.2/5.15	19	672	NT01EI_3596
238918136	25	1095	3.71	100	Translation elongation factor Tu, putative	43262.2/5.15	23	866	NT01EI_0167
238919786	26	1289	2.02	100	Phenylalanyl-tRNA synthetase, alpha subunit, putative	36890.7/5.9	25	581	pheS/NT01EI_1890
238921441	27	2020	2.47	100	50S ribosomal protein L4	22068.8/9.72	7	198	rplD/NT01EI_3593
238918424	28	102	6.95	100	Translation initiation factor IF-2, putative	98155.7/5.72	21	158	infB/NT01EI_0467
238921430	29	2095	-2.95	100	RecName: Full=50S ribosomal protein L5	20333.7/9.59	12	248	rplE/NT01EI_3582
238917996	30	428/406	-2.61/-4.78	100	Glycyl-tRNA synthetase, beta subunit, putative	75997.9/5.35	37	633	glyS/NT01EI_0014

Table 4.1 (continued)

Transport									
238918184	31	1099	8.77	100	Maltoporin	46962.3/5.18	18	576	lamB/NT01EI_0220
238918180	32	1314/1303	8.73	100	Bacterial extracellular solute-binding protein, putative	43474.5/6.48	24	494	malE/NT01EI_0216
238918185	33	1570	4.86	100	Maltose operon periplasmic protein	31714.5/8.77	8	121	malM/NT01EI_0221
238919805	34	551/541	17.95	100	TonB-dependent heme/hemoglobin receptor family protein	72860.4/6.13	33	457	chuA/NT01EI_1909
238920966	35	1442	2.74	100	ABC transporter, substrate binding protein	37823.6/7.79	14	352	NT01EI_3096
238919569	36	675	-2.82	100	Periplasmic oligopeptide-binding protein	61472.2/6.82	12	136	oppA/NT01EI_1668
Tricarboxylic acid cycle									
238920751	37	540	6.3	100	Succinate dehydrogenase, flavoprotein subunit, putative	64419.1/5.94	29	500	sdhA/NT01EI_2870
238918339	38	795	3.25	100	Aspartate ammonia-lyase, putative	52454.8/5.33	17	436	aspA/NT01EI_0377
Others									
238921325	39	1353	2.1	100	Glycerophosphoryl diester phosphodiesterase	40878.5/6.01	17	303	NT01EI_3469
238920583	40	188	4.22	100	Phosphate acetyltransferase	76925/5.46	21	221	NT01EI_2695
238918772	41	1364	2.48	100	Methionine aminopeptidase, type I, putative	29710.1/5.63	12	298	NT01EI_0835
238918900	42	1935	2	100	Hypothetical protein NT01EI_0965	23510.7/6.92	14	359	esrB/NT01EI_0965
238919128	43	2094	-3.03	100	Glycine cleavage system transcriptional repressor	20825.4/5.03	7	82	gcvR/NT01EI_1199
238921227	44	407	-2.51	100	Transketolase 1 (TK 1)	72356/5.66	19	197	tktA/NT01EI_3370
238921714	45	2099	-3.35	99.99	Thiol:disulfide interchange protein DsbA	22948.7/5.79	5	79	dsbA/NT01EI_3876
238921092	46	2148	-2.74	100	Hypothetical protein NT01EI_3227	18959.4/5.6	9	330	luxS/NT01EI_3227
238919302	47	1968	-4.79	100	Outer membrane protein A	38075.3/8.79	12	200	ompA/NT01EI_1392
238919598	48	1868	-2.68	100	Hypothetical protein NT01EI_1697	27900.5/8.98	15	307	NT01EI_1697
238920203	49	1596/1598	-2.78/-2.34	100	Hypothetical protein NT01EI_2312	31984.4/6.92	21	656	NT01EI_2312
238920625	50	2159	-2.33	100	Hypothetical protein NT01EI_2737	19363.8/5.29	9	123	eip20/NT01EI_2737

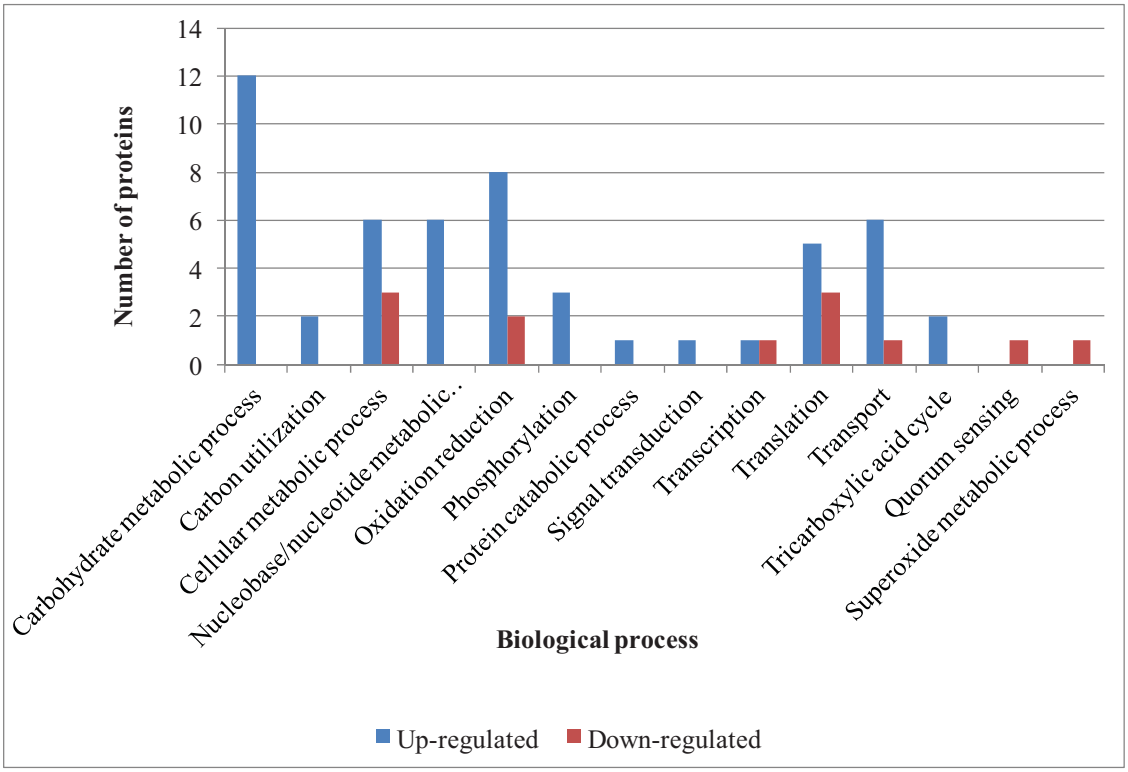

Figure 4.2 Biological process gene ontology (GO) Slim of differentially expressed proteins of *Edwardsiella ictaluri* grown in *in vitro* iron restriction condition.

All biological process GO annotations of up- and down-regulated proteins were summarized to more generalized GO categories based on ancestor chart for GO terms at QuickGO. Number of proteins involved in various generalized GO biological process categories was represented.

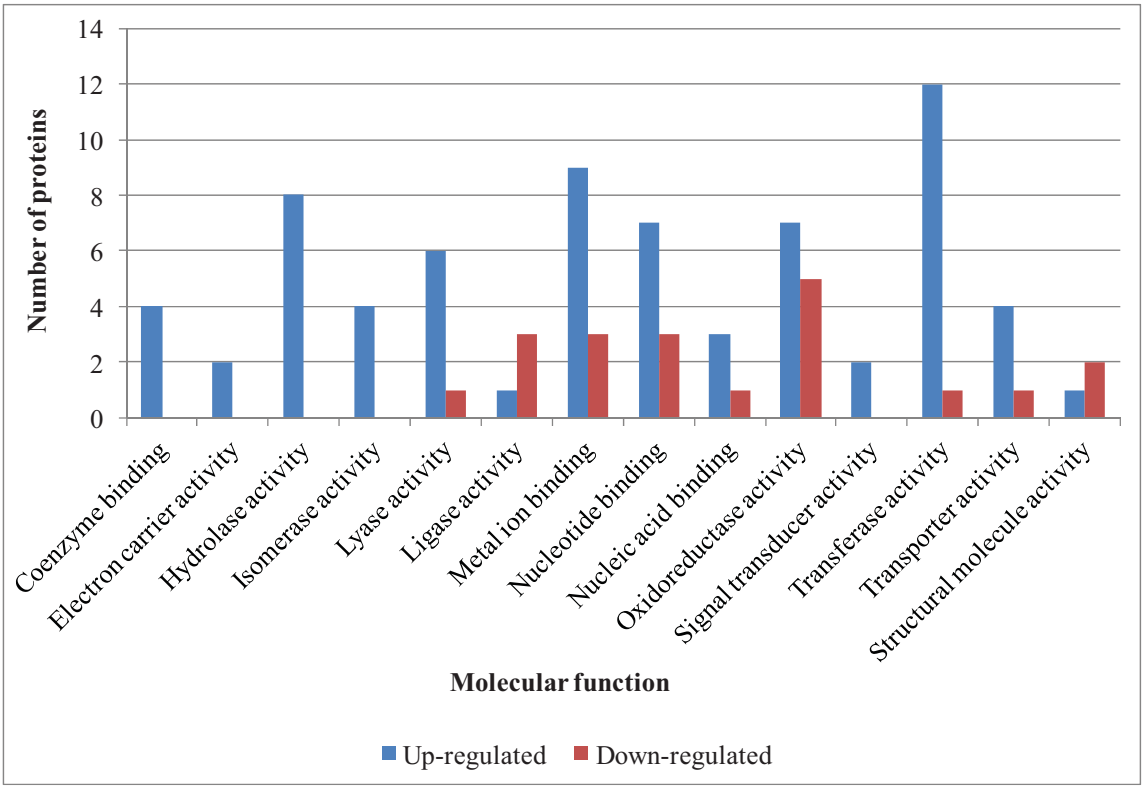

Figure 4.3 Molecular function gene ontology (GO) Slim of differentially expressed proteins of *Edwardsiella ictaluri* grown in *in vitro* iron-restriction condition.

All molecular functional GO annotations of up- and down-regulated proteins were summarized to more generalized GO categories based on ancestor chart for GO terms at QuickGO. Number of proteins involved in various generalized GO molecular functional categories was shown.

Up-regulated proteins were represented in all 14 molecular functional categories, whereas, down-regulated proteins were represented only in 9 molecular functional categories. In the molecular function grouping, transferase activity, metal ion binding, and hydrolase activity were the top three categories represented by up-regulated proteins. Only four down-regulated proteins were in these groups while most (11/13) down-regulated proteins were categorized under oxidoreductase activity, metal ion binding, and nucleotide binding.

Subcellular locations of differentially expressed proteins were predicted using PSORTb (Figure 4.4). Higher numbers of up- and down-regulated proteins were predicted to be located at the cytoplasm and periplasm, excluding those proteins of unknown location.

Pathways with significant representation of differentially expressed proteins were determined ($p \leq 0.05$). Ten pathways related to carbohydrate, amino acid, lipid, and nucleotide metabolism were significantly represented (Table 4.2). We built a pathway using a pathway reconstruction algorithm- "build pathway" available at Pathway Studio analyzing the shortest paths of up- and down-regulated proteins with biological interactions such as binding interactions, post-translational regulation, and expression regulation. Cellular processes such as pathogenesis, virulence, secretion, biofilm, motility, regulation of signal transduction, protein folding, glycolysis, gluconeogenesis, growth rate, catabolism, transcription termination, respiration, proteolysis, apoptosis, and cell survival were predominantly represented in the differential protein expression interaction network (Figure 4.5).

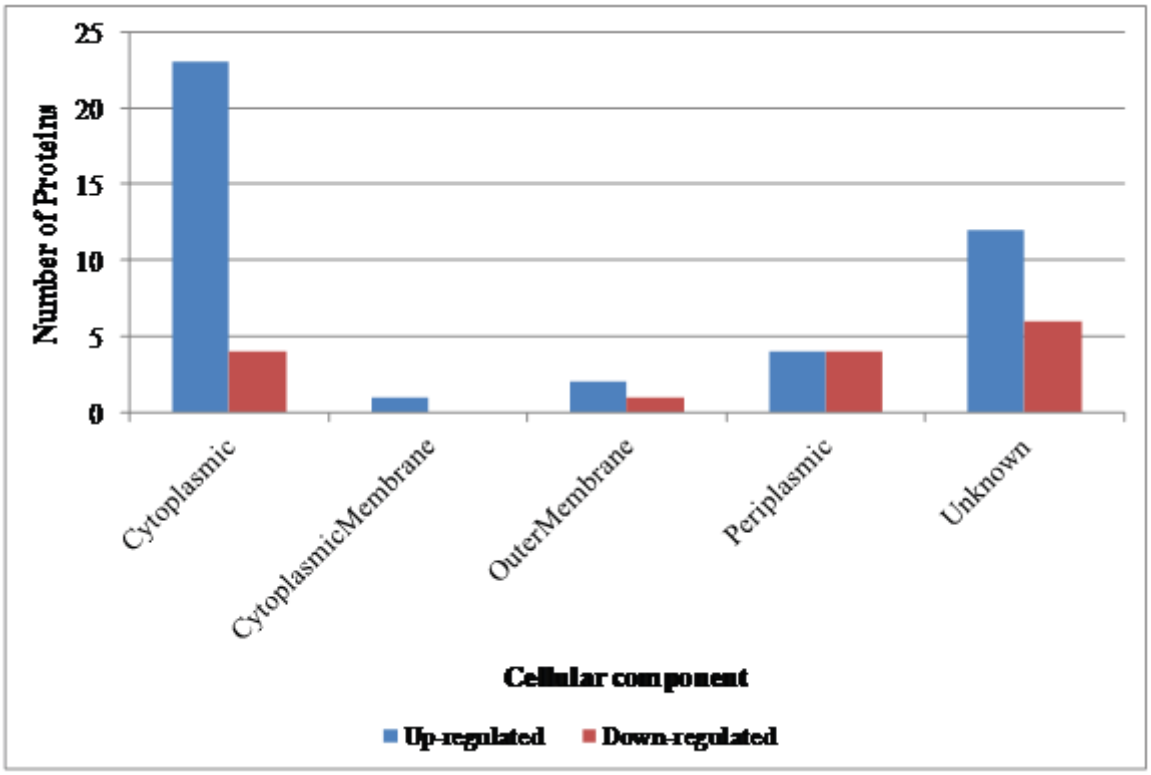

Figure 4.4 Subcellular locations of *Edwardsiella ictaluri* proteins differentially expressed due to *in vitro* iron-restriction were predicted using PSORTb.

Number of differentially expressed proteins, identified in this study, predicted to be located in various subcellular locations was shown. Unknown category includes proteins with multiple subcellular localizations or unknown location.

Table 4.2

List of pathways significantly represented by differentially expressed *Edwardsiella ictaluri* proteins in response to *in vitro* iron-restriction.

Name	**# of proteins**	**p-value**	**Classification**
Glycolysis / Gluconeogenesis	8	1.29E-06	Carbohydrate Metabolism
Pyruvate metabolism	8	2.31E-06	Carbohydrate Metabolism
Pentose phosphate pathway	5	2.66E-04	Carbohydrate Metabolism
Citrate cycle (TCA cycle)	3	1.06E-02	Carbohydrate Metabolism
Butanoate metabolism	4	1.51E-02	Carbohydrate Metabolism
Propanoate metabolism	3	4.01E-02	Carbohydrate Metabolism
Glycerolipid metabolism	3	1.96E-02	Lipid Metabolism
Selenoamino acid metabolism	6	5.49E-05	Metabolism of Other Amino Acids
Taurine and hypotaurine metabolism	2	4.57E-02	Metabolism of Other Amino Acids
Purine metabolism	5	2.14E-02	Nucleotide Metabolism

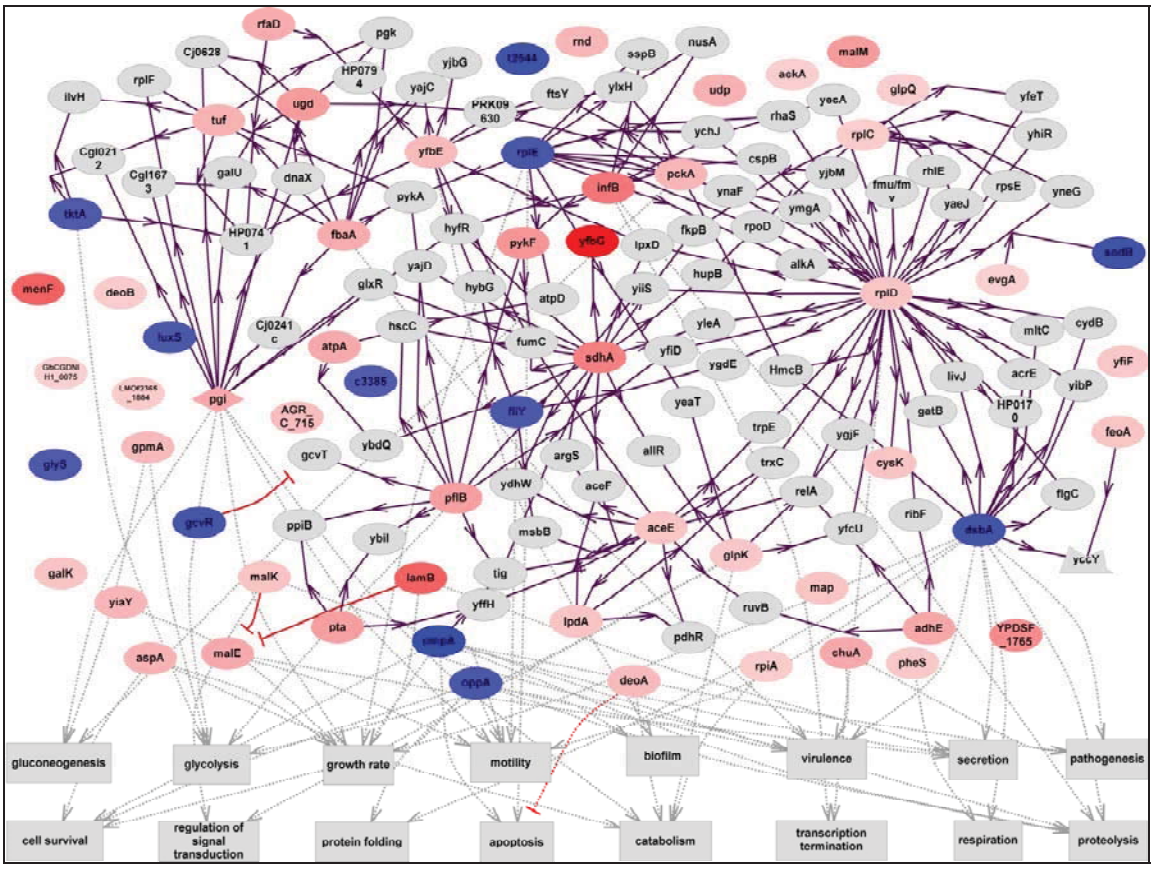

Figure 4.5 Protein interaction network of differentially expressed *Edwardsiella ictaluri* proteins due to *in vitro* iron-restriction by Pathway Studio 6.0.

Entities shown in red and blue were up- and down-regulated, respectively, due to *in vitro* iron-restriction. Intensity of entity color indicates the fold-difference in protein expression.

Discussion

The purpose of the present study was to determine differential protein expression of *E. ictaluri* grown under iron-restricted and normal growth conditions and investigate their possible role in pathogenesis. We determined 50 unique proteins with altered expression (37 up- and 13 down-regulated) in *E. ictaluri* in response to iron-restriction. It is known that iron is an essential micronutrient which acts as a cofactor for various

enzymes involved in oxidative and electron transport process. Hence, iron is essential for pathogenic bacteria in order to establish an infection.

Similar to human and other vertebrates, fluctuation of iron availability in a gastric environment is possible in catfish due to rapid changes in pH, iron absorption, and chelation (Ernst et al., 2005). Similar to other bacteria, *E. ictaluri* has a ferric uptake regulator (fur) gene in its genome (GI number 238920780), which may regulate iron transport and storage (Griffiths and Chart, 1999; Braun, 2001). Transport proteins, especially cation, are highly expressed in iron-restricted conditions compared to control. TonB-dependent heme/hemoglobin receptor family protein, with its 18-fold higher expression in iron-restricted growth conditions, may act as a crucial factor in an iron acquisition mechanism of *E. ictaluri*. Similar to other bacteria, *E. ictaluri* may also rely more on siderophore dependent mechanisms of iron acquisition. During iron-stress these bacteria secrete siderophores, low molecular weight iron-chelating compounds, to its surrounding environment which chelate iron and form siderophore-iron complexes (Neilands, 1982). These siderophore-iron complexes are then recognized by specific outer membrane receptors like TonB and transported across the membranes (Larsen et al., 1997; Moeck and Coulton, 1998). Bruan (2001) suggested that these iron-complexes from the periplasm were transported across the inner membrane by an ABC transporter. Most of the bacterial extracellular solute-binding proteins fall into either iron uptake or carbohydrate transport (Hansmeier et al., 2006). Hence, putative bacterial extracellular solute-binding protein may be an integral component of siderophore-type iron ABC transporters in *E. ictaluri*. TonB-dependent heme/hemoglobin receptor family protein in *E. ictaluri* might have both receptor and transporter activity along with its involvement in

transduction of environmental signals, and a possible role in pathogenicity similar to several bacterial pathogens (Koebnik, 2005; Ferguson et al., 2007). Research conducted by Wang et al. (2008) in *Vibrio alginolyticus* demonstrated that mutants of TonB complex exhibited attenuation in virulence compared to wild-type in zebrafish (*Danio rerio*). Similarly, maltoporin (LamB), a member of the sugar porin family, aid in transport of maltose and other maltodextrins across the outer membrane in *E. coli* (Wang et al., 1997). Mutation studies of the *lamB* gene of enteropathogenic *E. coli* showed that mutants were deficient in adherence to HEp-2 cells (Subramanian et al., 2008). It was also shown that LamB is an important outer membrane protein in *E. coli* for obtaining tetracycline resistance (Zhang et al., 2008). Based on our understanding, proteins involved in the acquisition of iron are closely associated with virulence of several bacteria; it is likely that transporter proteins identified in the present study might be important in *E. ictaluri* pathogenesis.

Translational proteins like elongation factor Tu (EF-Tu), a three-domain GTPase, is crucial during the elongation phase of mRNA translation. The EF-Tu complexed with GTP and aminoacyl-tRNA delivers tRNA to the ribosome. It is known that EF-Tu might play a role in protein-folding during stress (Caldas et al., 1998). It is also proposed that EF-Tu might sense and respond to stress (Yu et al., 1986). Hence, EF-Tu may assume the role of translational regulation allowing it to trigger the synthesis of stress-induced proteins and to thwart the translation of unnecessary proteins. During starvation/stress in *E. coli*, EF-Tu was shown to be methylated and become membrane associated (Young and Bernlohr, 1991). Previous research has also shown that EF-Tu might act as a virulence factor in *P. aeruginosa* (Kunert et al., 2007). During those conditions EF-Tu

may acquire a possible role in the organism's response to stress and growth regulation, in addition to its primary role in regulation of translation.

N-acetylmuramoyl-L-alanine amidase is an outer membrane lipoprotein which catalyzes cleavage of the bond between muramic acid and L-alanine of murein. Similarly, ADP-L-glycero-D-mannoheptose-6-epimerase is involved in the lipopolysaccharide (LPS) biosynthesis pathway and is responsible for synthesis of the ADP-heptose precursor of core LPS (Kneidinger et al., 2002). Glucose-6-phosphate isomerase, phosphoenolpyruvate carboxykinase, hypothetical protein NT01EI_2846, which is also named as Phosphoglyceromutase (GpmA), and Fructose 1, 6-bisphosphate aldolase (FbA) are known to be involved in the glycolysis/gluconeogenesis pathway. Vassinova and Kozyrev, (2000) suggested that transcription of GpmA is regulated by Fur in *E. coli*. It has also been shown that Fba of *E. ictaluri* has antigenic properties and is expressed during the infectious process of ESC in catfish (Moore et al., 2002). Similarly, research conducted by Ling et al. (2004) also revealed that on respiratory challenge with virulent *Streptococcus pneumonia* in mice, Fba is able to elicit significant levels of immune response. Succinate dehydrogenase catalyzes the oxidation of succinate to fumerate in the tricarboxylic acid cycle. Aspartate ammonia-lyase is an anerobic enzyme which catalyzes the amination of fumarate to generate L-aspartate. Mutational studies conducted by Jacobsen et al. (2005) showed that aspartate ammonia-lyase plays an important role in the pathogenesis of *Actinobacillus pleuropneumoniae* in pigs (Jacobsen et al., 2005).

Research conducted by Wang et al. (2009) confirmed that the conserved hypothetical protein (*esrB*) and iron concentrations regulate transcription of the *E. tarda* virulence protein in *Edwardsiella tarda*. Thiol: disulfide interchange protein DsbA,

which was -3.35 fold down-regulated, was a protein-folding catalyst which aids in correct folding of surface-presented virulence factors like adherence factors, toxins, and components of the type III secretory system (Yu and Kroll, 1999). The observed down-regulation of periplasmic oligopeptide-binding protein (oppA), an ATP-dependent ABC Superfamily of transporters involved in oligopeptide uptake, is in agreement with reduced metabolism of bacteria due to stress caused by the limitation of available iron (Madsen et al., 2006). Research conducted by Lee et al. (2009) revealed that the *dsbA* mutant of *Pseudomonas putida* exhibited enhanced extracellular matrix production and biofilm formation. Down-regulation of OmpA (4.79 fold), is consistent with findings of *Clamydia pneumonia* an iron-limitation model (Timms et al., 2009). Similarly, superoxide dismutase (Sod_Fe) was shown to be positively regulated by the Fur–Fe^{+} complex in many bacterial species (Vasil, 2007; Jung and Kwon, 2008). Hence in the present study, down regulation of superoxide dismutase was expected.

GO annotation and manual slimming of up-regulated proteins resulted in a higher number of biological processes (12) and molecular functional categories (14) compared to down-regulated proteins (7 and 9, respectively). This was expected as the number of unique proteins that were up-regulated (42) was high compared to those that were down-regulated (15). Up-regulated proteins were highly represented in the carbohydrate metabolic process. This might be due to a higher expression of proteins involved in glycolysis/gluconeogenesis, pyruvate metabolism, and in synthesis of cell wall structures like peptidoglycan and LPS. A down-regulation of proteins involved in cellular metabolic processes, oxidation reduction, and translational processes indicate possible reduction in the metabolism of *E. ictaluri* due to iron starvation stress. A higher number of up-

regulated proteins were predicted to be located in cytoplasm as they are hydrophilic and thus do not interfere in 2-DE separation techniques. DsbA, Sod_Fe, periplasmic oligopeptide-binding protein, and conserved hypothetical protein were the four down-regulated proteins predicted to be located in periplasm.

Protein interaction networks of differentially expressed proteins were built using Pathway Studio. Differentially expressed proteins were involved in cellular processes like virulence, pathogenesis, secretion, biofilm, and regulation of signal transduction. Up-regulated genes like *fbaA* and *chuA* and down-regulated genes like *oppA*, *ompA*, and *dsbA* were involved in virulence and pathogenesis processes suggesting that differentially expressed proteins during iron-restriction may play an important role in *E. ictaluri* pathogenesis. Down-regulation of several proteins involved in cellular processes like cell survival, motility, and growth rate may be expected with reduced metabolism due to iron-limitation stress.

It is always a challenge to elucidate how bacteria adopt various adaptive mechanisms to invade, colonize, and successfully establish a disease in the host. It is certain that *E. ictaluri* will encounter several environmental stresses in the gastric environment of catfish like iron starvation and fluctuation in pH during the initial course of its pathogenesis. With an objective to thoroughly elucidate the response of *E. ictaluri* to iron-restriction, we used 2D-DIGE technology to investigate post-transcriptional changes. We therefore hypothesized that analysis of *E. ictaluri* response towards iron-restriction conditions may aid in enlightening the possible mechanisms of pathogenesis of *E. ictaluri.* In the present study, we noted several differentially expressed proteins that were previously shown to be involved in the pathogenesis of other gram-negative bacteria

including *E. ictaluri.* Future experiments determining the role of these differentially expressed proteins should provide important information regarding the mechanisms used by *E. ictaluri* during colonization and establishment of ESC in catfish.

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CHAPTER 5

IDENTIFICATION OF DIFFERENTIALLY REGULATED PROTEINS OF *EDWARDSIELLA ICTALURI* DURING SERUM TREATMENT

Abstract

Edwardsiella ictaluri is a facultative, intracellular, gram-negative bacterium that causes enteric septicemia of catfish (ESC). *E. ictaluri* is resistant to the innate defense mechanisms in catfish serum. Hence, *E. ictaluri* might effectively use blood stream of catfish for establishment of infection. However, the precise mechanisms of *E. ictaluri* adaptation to host serum factors are not known. Exposure to catfish serum and determination of changes at the protein level might reveal important information about *E. ictaluri* adaptation mechanisms against host defense systems present in serum. The primary objective and purpose of the present study was to identify differentially expressed proteins of *E. ictaluri* on exposure to naive catfish serum obtained from specific pathogen free catfish. We used two dimensional difference gel electrophoresis technology followed by in-gel trypsin digestion and MALDI TOF/TOF analysis for identification of the differentially expressed proteins. We identified total of 19 differentially expressed proteins (7 up- and 12 down-regulated, respectively) at a minimum of 1.5-fold ($p < 0.05$) change in protein expression. Among those were four putative immunogenic proteins, two chaperones, eight proteins involved in the translational process, two nucleic acid degradation and integration proteins, two

intermediary metabolism proteins, and one iron ion binding protein. Present proteomic analysis identified proteins that maybe involved in *E. ictaluri* adaptation against host factors present in serum. Further research focusing on functions of these differentially expressed proteins might reveal their role in *E. ictaluri*-host adaptation.

Introduction

Edwardsiella ictaluri is a facultative, intracellular, gram-negative bacterium belonging to the *Enterobacteriaceae. E. ictaluri* is the causative agent of enteric septicemia of catfish (ESC) and the bacterium was first characterized and named by Hawke et al. (1981). ESC is considered as one of the most important bacterial diseases of the farm-raised channel catfish (*Ictalurus punctatus*) in the United States. Catfish farming is one of the most economically important forms of aquaculture, with a production of 446 million pounds of live weight food size fish in 2009 (NASS, 2010). Losses incurred to worldwide catfish industry due to ESC accounts for millions of dollars every year (Wagner et al., 2002). Because of the disease's economic importance, studies addressing the molecular mechanism of pathogenesis of *E. ictaluri* are underway. *E. ictaluri* is able to evade the host immune system and are able to replicate inside the phagocytes of catfish (Ainsworth and Chen, 1990; Baldwin and Newton, 1993). Using bioluminescence, Karsi et al. (2006) found that *E. ictaluri* is able to spread entire fish body within 60h post infection through immersion. The dissemination of ESC in catfish is rapid as *E. ictaluri* is able to accumulate in the kidney tissue of catfish as early as 15 min after gastric lavage (Baldwin and Newton, 1993). *E. ictaluri* probably uses the blood stream of catfish effectively to reach various organs. For survival and successful establishment of infection *E. ictaluri* must first adopt and counter the challenges from host immune mechanisms.

Hence, exposure of *E. ictaluri* to catfish serum might reveal its ability to survive host defense mechanisms.

Serum of normal animals contains several factors that are reactive against a vast array of pathogenic microorganisms. These naturally occurring humoral immune substances include acute-phase proteins, complement, lysozyme, antibodies, interferon, beta-lysin, cationic antimicrobial peptides (CAMPs), transferrin, lactoferrin, and ion-binding proteins. The primary function of serum host factors is to kill the bacteria through cell lysis, disruption of plasma membrane, inhibition of growth by iron binding and clearance by opsonization. The primary role of the complement system in serum is to identify and aid in clearance of bacteria and foreign cells (Schmidt and Colten, 2000). The complement system can be activated by three distinct pathways; the classical, the MB-lectin, and the alternative pathways. Activated complement proteins may act as chemoattractants to recruit more phagocytes, and the terminal components of activated complement proteins assemble to form a membrane-attack complex and create pores in the bacterial membrane. Previous research has shown that bacteria use primarily three strategies to evade complement mediated lysis of bacteria. The first strategy is binding with complement inhibitors such as C4b-binding protein (C4BP) (Johnsson et al., 1996; Prasadarao et al., 2002), Factor H and Factor H-like protein 1 (FHL-1) (Hallstrom et al., 2008), and vitronectin (Schvartz et al., 1999). The second strategy is inactivation of C3, a crucial component of all complement pathways (Lee et al., 2004; Nordstrom et al., 2004). The third strategy is production of proteases that specifically cleave important complement factors (Ji et al., 1996; Terao et al., 2008).

Another fundamental component of the innate immune system of mammals and non-mammalian vertebrates is CAMPs. CAMPs bind to the surface of gram-negative bacteria cells through electrostatic interactions with the negatively charged groups of lipopolysaccharides (LPS)(Gunn, 2001) and then traverse to the inner membrane and form a pore, which leads to destabilization of the outer membrane (Shai, 1999).

Ability of bacteria to evade serum host factors is considered to be an important virulence mechanism. However, mechanisms of *E. ictaluri* adaptation to evade from serum host factors are unknown. The purpose of the present study was to identify differentially expressed proteins of *E. ictaluri* on encounter with host serum components. Differential protein expression analysis of *E. ictaluri* towards naive catfish serum may aid in understanding the possible mechanisms of pathogenesis. We anticipate our study to be a starting point for further functional studies on understanding the ability of *E. ictaluri* to counter lethal host serum factors.

Materials and Methods

Serum preparation

Catfish serum was collected from specific-pathogen-free (SPF) channel catfish obtained from the SPF fish laboratory at the College of Veterinary Medicine, Mississippi State University. Around 30 SPF catfish weighing 2-3 kg were anesthetized in water containing 200 mg/L of tricaine methane sulfonate (Argent Laboratories). Blood from each fish at approximately 1% of body weight was drawn from a tail vein. Serum was prepared as described previously (Karsi and Lawrence, 2007), aliquoted as single-use vials, and stored at -80°C until use.

Serum treatment and total protein extraction

E. ictaluri strain 93-146 was grown on brain heart infusion (BHI) broth or agar medium. Three replicates of controls and treatments, obtained from three colonies (each replicate of control and treatment was obtained from single colony), were harvested at mid-exponential phase (OD_{600} 0.6) by centrifugation at 3,750 rpm for 15 min at 30°C. Six bacterial pellets (three controls and three treatments) were washed thrice using standard cell wash buffer (10mM TrisCl and 5mM Magnesium acetate) at 30°C in a 1.5 ml microcentrifuge tubes. Total time of incubation of *E. ictaluri* with catfish serum (heat treated and non-heat treated) was 6 h. All 3 control pellets were dissolved in 1.25 ml of heat treated (65°C for 45 min) catfish serum, whereas 3 treatment pellets were dissolved in regular catfish serum (non-heat treated) each. Heat treated and non-heat treated catfish serum, at room temperature and on ice, respectively, were brought to 30°C before adding the bacterial pellet. While in incubation, bacteria was harvested every 2 h and resuspended in fresh, heat treated and non-heat treated serum in respective tubes. All controls and treatments were mixed by inverting 10 times every 30 min and returned with lids open to the 30°C incubator. After 6 h incubation, bacteria were harvested and washed thrice using standard cell wash buffer. After final wash, pellets were resuspended in urea-CHAPS buffer (8 M urea, 30 mM tris-HCl, 4% CHAPS, 8 mM PMSF pH 8.0). Cells were lysed on ice by applying ten intermittent pulses of 10 s with a sonicator, and bacterial homogenates were centrifuged in a refrigerated tabletop centrifuge (4°C) at 14,000 rpm for 5 min to remove cell debris and unbroken cells.

Proteins from supernatant were precipitated by trichloroacetic acid/acetone, and the resultant protein pellets resuspended again in urea-CHAPS buffer. The pH of the

lysates was adjusted to 8.5 using 50 mM sodium hydroxide. Protein concentrations were estimated using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol for DIGE.

Labeling of proteins

Protein samples were labeled according to the GE Healthcare manual for DIGE (GE Healthcare, Piscataway, NJ). Briefly, 50 µg sample of protein from control (*E. ictaluri* treated with non-heat treated serum), treatment (*E. ictaluri* treated with heat treated serum), and an internal standard (equal mixture (8.33 µg) of all 6) were mixed with 400 pmol of Cy3, Cy5, or Cy2 dyes, respectively, and then the protein-dye mixtures were incubated on ice in the dark for 30 min. The labeling reaction was terminated by adding 1 µl of 10 mM lysine, mixing well, and incubating samples in the dark for 10 min.

Protein separation using 2-DE

For isoelectric focusing (IEF), precast 17 cm pH 3-10 NL immobilized pH gradient (IPG) strips (Bio-Rad, Hercules, CA) were used. Each of the labeled protein samples from control, treatment, and internal standard were combined with rehydration buffer containing 7M urea, 2M thio urea, 4% CHAPS, 1:50 carrier ampholyte, and 2% DTT. Mixed samples were loaded onto each IPG strip for in-gel rehydration. IEF was performed in a Protean IEF cell (Bio-Rad, Hercules, CA) in the dark at 23°C, 500 V for 15 min; linear ramp to 10,000 V for 3 h; and 10,000 V until a total of 70,000 Vh was reached. After IEF, IPG strips were equilibrated in 6 M urea, 30% glycerol, 50 mM Tris-HCl, 2% SDS, 2% DTT, at pH 8.8, and with a trace of bromophenol blue for 15-20 min followed by equilibration containing 2.5% iodoacetamide (IAA) instead of 2% DTT for

15-20 min. Once equilibrated, strips were transferred onto 12% SDS-PAGE with low fluorescent glass (JULE, INC. Milford, CT) and sealed with 0.5% agarose. Electrophoresis was performed using a PROTEAN II XL system (Bio-Rad) at a constant current of 10mA/gel for the first 15 min followed by 24mA/gel at 20°C until the dye front reached the lower end of the gel.

Analysis of 2-D DIGE gel images

After electrophoresis, DIGE gels were scanned using a Typhoon 9410 imager (GE Healthcare). Excitation and emission filters used for each dye were as follows: Cy2 (488 nm/520 nm), Cy3 (532 nm/580 nm), and Cy5 (633 nm/670 nm). Acquired images were analyzed using DeCyder 5.0 software (GE Healthcare). Briefly, spots were detected using the differential in-gel analysis (DIA) module. Spot matching among gels and statistical analysis of protein-abundance changes were conducted using the biological variation analysis (BVA) module. Among the three replicates, the gel with the highest number of spots was assigned as the master gel. All the spots that were matched automatically were also compared manually among all three replicate gels to minimize false spot matching. Statistical significance was calculated using the Student's t-test with applied false discovery rate and a significance threshold of $p < 0.05$. Only spots showing at least a 1.5-fold change in spot intensity and consistent in all three replicate gels were considered as differentially expressed and chosen for protein identification.

Preparative gel electrophoresis and protein identification

Preparative 2-DE gels were prepared exactly as described above, except that the IPG strips were loaded with 500 μg of protein. Resultant gels were stained using Deep

Purple Total Protein Stain (GE Healthcare) according to the manufacturers' protocols. Briefly, gels were fixed overnight in 15% v/v ethanol and 1% w/v citric acid followed by staining for 1 h in 1:200 parts of Deep Purple and 100 mM sodium borate solution at pH 10.5. Gels were then washed for 30 min with 15% v/v ethanol in water, and acidified for 30 min using a solution containing 15% v/v ethanol and 1% w/v citric acid. Stained gels were scanned using a Typhoon 9410 imager using a 532 nm laser and a 610 nm BP30 emission filter. In-gel trypsin digestion and MALDI PMF was performed as described previously (Chaudhary et al., 2007).

Results

Proteins expressed by *E. ictaluri* on treatment with heat treated and non-heat treated catfish serum were analyzed on a same gel, and quantitative comparison made with an internal standard. The DIGE analysis detected around 2,100 spots in each replicate, and after automatic matching and manual verification of each spot, only those spots which were matched in all three replica gels were subjected to statistical analysis. Analysis of these gels revealed that 83 spots (34 up- and 49 down-regulated) were shown to be differentially expressed with a minimum of 1.5-fold change in expression, $p \leq 0.05$. Forty two spots (21 up- and 21 down-regulated), which were matched to a preparative gels, were excised, trypsin digested, and subjected to MALDI PMF for identification. Out of 42 spots, we were able to identify 21 proteins with a cross confidence interval (C.I. %) ≥ 90. Among those 21 proteins, two proteins were identified in more than one spot, probably due to post-translational modifications and processing. Altogether, 19 proteins (7 up- and 12 down-regulated) differentially expressed by *E. ictaluri* on treatment with catfish serum were identified.

The expression level of differentially expressed proteins of *E. ictaluri* varied from -3.35 fold to 2.82 fold on treatment with catfish serum. Among the differentially expressed proteins, we noted eight proteins belonging to translation process [Ribosomal protein S1 (Rps1 and RpsA), Ribosomal protein S2 (Rps2), FusA/EF-G, RplC, RplO, RplD, and EF-Tu], four immunogenic proteins with a potential role in lipopolysaccharide and peptidoglycan metabolism (ArnA, BipA, AmiD, and IDH), two chaperones (DnaK and Thioredoxin domain protein), two proteins involved in nucleic acid degradation and integration (PNPase and Integrase), two proteins involved in intermediary metabolism (Tkt/TK 1 and AsnB), and a protein involved in iron ion binding (IspG) (Table 1). Of all the up-regulated proteins, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (IspG) was the highly up-regulated (2.82 fold) protein followed by Bifunctional polymyxin resistance protein ArnA (2.22 fold). Among the down-regulated proteins, Ribosomal protein S2 was highly differentially expressed (-3.35 fold) followed by GTP-binding protein TypA/BipA (-3.23 fold).

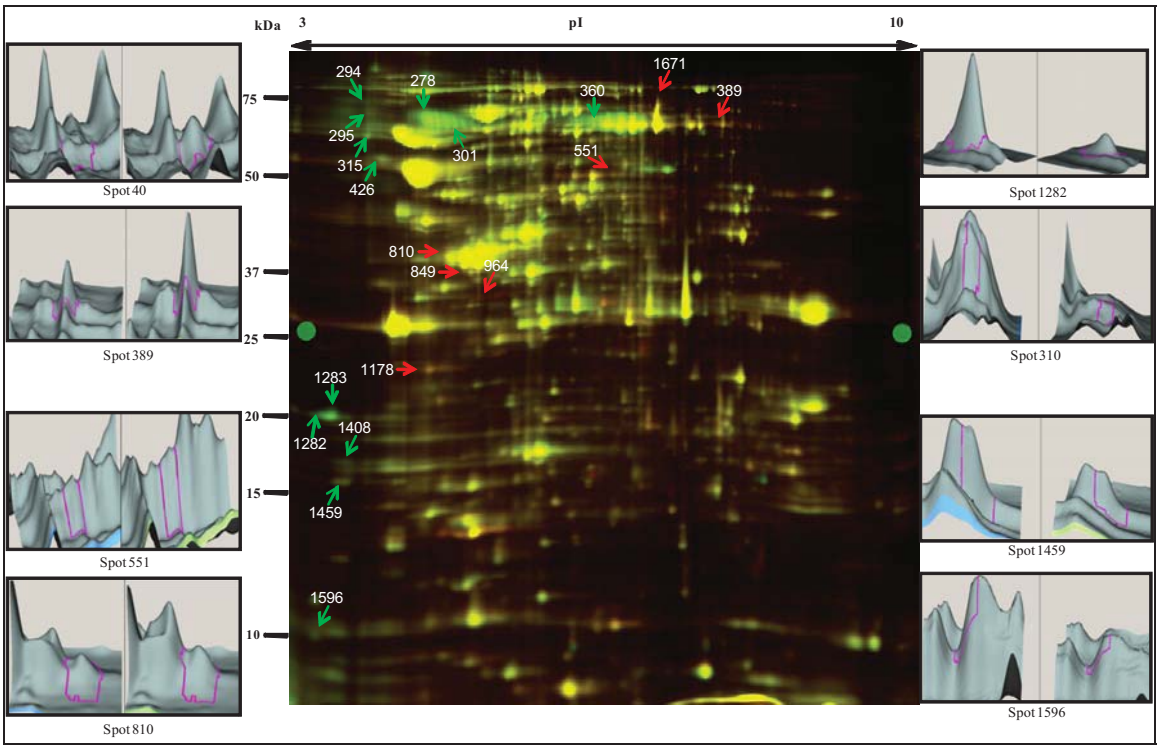

Figure 5.1 Fluorescent difference gel electrophoresis (2-D DIGE) of *Edwardsiella ictaluri* upon treatment with catfish serum.

Fifty µg of soluble *E. ictaluri* protein treated with heat treated catfish serum was labeled with Cy3, non-heat treated catfish serum was labeled with Cy5, and the pooled internal standard with Cy2. Spots shown in red and green arrows are up- and down-regulated (≥1.5 fold), respectively. 3D images of two spots with maximum and minimum fold up-regulated proteins are shown on left top and bottom corners of gel image, respectively. 3D images of 2 spots with maximum and minimum fold down-regulated proteins are shown on right top and bottom corners of gel image, respectively.

Table 5.1

Differentially expressed proteins of *Edwardsiella ictaluri* in response to catfish serum

GI number	Spot ID	Fold difference	C.I. %	Protein name	Mol wt	pI	Gene symbol/Functional category
238918773	1282	-3.35	100.0	Ribosomal protein S2, putative	26295.6	6.61	rps2:Ribosome
238921445	278	-2.03	100.0	Elongation factor G (EF-G)	77423.2	5.2	fusA:Translation elongation
238920345	315	-1.91	100.0	Ribosomal protein S1, putative	61119.6	4.89	rspA:Ribosome
238921442	1408	-1.89	100.0	Hypothetical protein NT01EI_3594	21354.4	9.8	rplC:Ribosome
238920345	426/325	-1.78	100.0	Ribosomal protein S1, putative	61119.6	4.89	rps1:Ribosome
238921423	1596	-1.76	99.0	50S ribosomal protein L15	15199.3	11.14	rplO:Ribosome
238921441	1459	-1.57	100.0	50S ribosomal protein L4	22068.8	9.72	rplD:Ribosome
238918136	810	1.59	100.0	Translation elongation factor Tu, putative (EF-Tu)	43262.2	5.15	tufA:Translation
238921701	301	-3.23	99.0	GTP-binding protein TypA/BipA, putative	67038.2	5.18	typA/bipA:Immunogen
238920114	1283	-2.06	100.0	N-acetylmuramoyl-L-alanine amidase	32859	7.82	amiD/ampD:Peptidoglycan catabolism/ Immunogen
238920206	849	1.75	100.0	Isocitrate dehydrogenase, NADP-dependent, putative	45494.3	5.16	icd/idh:TCA cycle/Immunogen
238919324	389	2.22	100.0	Bifunctional polymyxin resistance protein ArnA, putative	73954	5.67	arnA:LPS biosynthesis/Immunogen
238918615	295	-2.25	100.0	Chaperone protein dnaK/Heat shock protein 70/HSP70	68467.3	4.79	dnaK:Chaperone
238920890	1178	1.69	93.9	Thioredoxin domain protein	31183.1	4.8	Chaperone
238921227	360/390	-1.96	100.0	Transketolase 1 (TK 1)	72356	5.66	tkt:Intermediary metabolism
238920794	551	1.52	100.0	Asparagine synthase	62102	5.97	asnB:Intermediary metabolism
238918428	294	-1.87	100.0	Polyribonucleotide nucleotidyltransferase	76443.6	5.2	pnp:Nucleic acid degradation
238919949	1671	1.87	90.5	Integrase	41910.1	9.95	int:DNA integration
238921039	964	2.82	100.0	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	40678.4	6.35	ispG:Iron ion binding

Discussion

The purpose of the present study was to determine differential protein expression of *E. ictaluri* on treatment with naïve, heat treated and regular catfish serum. We performed a thorough analysis of these differentially expressed proteins with an emphasis on their role in combating *E. ictaluri* against serum complement mediated lysis. We determined 19 differentially expressed proteins (7 up- and 12 down-regulated, respectively) in *E. ictaluri* in response to catfish serum treatment.

We noted the down-regulation of a considerable number of ribosomal proteins and protein involved in translational machinery. Though we harvested and started treating *E. ictaluri* during mid-exponential phase, during serum treatment we anticipate that bacteria might transform to a stationary phase in which the rate of protein synthesis drops to about 20% in the first hour and remains roughly constant for several hours (Reeve et al., 1984). Moreover, treatment of *E. ictaluri* with catfish serum is considered a significant stress, in which down-regulation of ribosomal proteins is expected (Yus et al., 2009). Ribosomal proteins that were the components of the 30S ribosomal subunit (Rps1, Rps2, and RpsA) and 50S ribosomal subunit (RplC, RplO, and rplD) were down regulated in a range varying from -1.54 to -3.35 fold. Rps1 protein is the largest of the ribosomal proteins, binds to the leader sequence of mRNAs upstream of the Shine-Dalgarno region, and plays crucial role in stabilizing the mRNA on the ribosome (Sengupta et al., 2001). It is shown that, in *E. coli*, Rps2 is essential for the binding of Rps1 to the 30s ribosomal subunit (Moll et al., 2002). Along with ribosomal proteins, translational elongation factor G (EF-G/FusA) is down regulated. EF-G is an essential protein that facilitates the GTP-dependent translocation of the ribosome by one codon along the mRNA molecule. It is shown that FusA is identified as a *Helicobacter pylori* antigen showing a higher seropositivity in duodenal ulcer patients (Lin et al., 2007). Using serological proteomic analysis, Vytvytska et al. (2002) identified also that FusA and EF-Tu in *Staphylococcus aureus* are highly immunogenic antigens.

Though translational proteins (ribosomal and EF-G) were down-regulated in *E. ictaluri,* which may be due to a stationary growth phase, EF-Tu was shown to be up-regulated. Previous research demonstrated clearly that EF-Tu, a GTP binding protein

known to be involved in protein translation, may be involved in several functions depending on its cytoplasmic or surface location. Jacobson and Rosenbusch (1976) suggested association of EF-Tu with the membrane of *E. coli*. Previous research on *Pseudomonas aeruginosa* has shown that EF-Tu binds to the complement regulator factor H and plasminogen and acts as a virulence factor (Kunert et al., 2007). Further functional studies on *E. ictaluri* are required to confirm the role of EF-Tu (92% sequence similarity with EF-Tu of *P. aeruginosa*) that may bind to a catfish serum protein (similar to CFH protein of *Denio rerio* with 98% sequence similarity with human Factor H protein) to evade from complement mediated lysis in the host. ET-Tu is also observed as a common antigen in many *lactobacilli* (Nakamura et al., 1997) and in *Mycoplasma mycoides* (Alonso et al., 2002). Research conducted on *E. coli* has also shown that EF-Tu may bind to unfolded and denatured proteins, as do other molecular chaperones involving in protein folding and renaturation during stress (Caldas et al., 1998).

Among the four differentially expressed immunogenic proteins, Bifunctional polymyxin resistance protein (ArnA) and NADP-dependent isocitrate dehydrogenase (IDH) were up-regulated. Bifunctional enzyme, with both formyl-transferase and UDP-GlcA decarboxylase activities, catalyzes the oxidative decarboxylation of UDP-glucuronic acid (UDP-GlcUA) to UDP-4-keto-arabinose (UDP-Ara4O) and the addition of a formyl group to UDP-4-amino-4-deoxy-L-arabinose (UDP-L-Ara4N) to form UDP-L-4-formamido-arabinose (UDP-L-Ara4FN) (Raetz et al., 2007). Attachment of the modified arabinose to the lipid A moiety of gram-negative bacteria may acquire resistance to the CAMPs of the host innate immune system and polymixin, a cationic peptide antibiotic (Yan et al., 2007). Up-regulation of ArnA was noticed also in *E.*

ictaluri grown under *in vitro* iron-restricted conditions (unpublished data). Higher expression of ArnA, which is essential for both lipid A modification with Ara4N and polymyxin resistance, by *E. ictaluri* strain 93-146 may be the reason for acquiring natural resistance to polymyxin E (colistin). Isocitrate dehydrogenase (ICD/IDH), with kinase and phosphatase activity, controls a branch point between TCA cycle and glyoxylate cycle pathways of central metabolism. Previous research has reported immunodominant, immunosensitive, and the immunospecific nature of ICD in *Mycobacterium tuberculosis* (Banerjee et al., 2004). Recently, Hussain et al. (2008) highlighted the ability of ICD to interact and elicit a significant humoral immune response in individuals infected with *H. pylori*. Further studies emphasizing the functional role of ArnA and ICD of *E. ictaluri* may aid in elucidating the virulence mechanisms of *E. ictaluri*.

N-acetylmuramoyl-L-alanine amidase (AmiD) and GTP-binding protein (TypA/BipA) are the immunogenic proteins down-regulated in *E. ictaluri* on exposure to catfish serum. Down-regulation of AmiD in the present study is not surprising as it plays role in the catabolic process of peptidoglycan. Peptidoglycan is a semi rigid macromolecule of cytoplasmic membrane that confers shape to the bacterial cell and protects against high internal osmotic pressure. AmiD is an outermembrane lipoprotein suggested to play a role in murein recycling by cleaving the bond between muramic acid and L-alanine within murein, muropeptides, and anhydro-muropeptides (Uehara and Park, 2007). BipA is homologous to the C terminal domains of the elongation factor EF-G and is also down-regulated along with EF-G. Research has shown that BipA is involved in pathogenesis of bacteria along with its role in modulating the structure or function of the ribosome (Krishnan and Flower, 2008). Farris et al. (1998) reported that

BipA in enteropathogenic *E. coli* is a virulence regulator that controls flagellar mediated cell motility and its resistance to antibacterial effects of host defense proteins. Further detailed studies of AmiD and BipA of *E. ictaluri* are required to establish the role in the pathogenesis of *E. ictaluri* infection.

Between two differentially expressed chaperone proteins, DnaK (equivalent of the 70-kDa heat shock protein, HSP70) protein involved in RNA degradation and the DNA replication process was down-regulated (-2.25 fold). Whereas, Thioredoxin domain protein involved in posttranslational modification, protein turnover was up-regulated (1.69 fold) in *E. ictaluri*. Bacterial HSPs represent one of the major targets of the host's immune response. Research has shown that immunization of mice with proteins containing DnaK-specific sequences protected against *Borrelia burgdorferi* infection (Bey et al., 1995). However, down regulation of DnaK in *E. ictaluri* on extended treatment in this study may be a protective mechanism as the effect of DnaK is growth phase dependent. Blum et al. (1992) reported a progressive decrease in cell viability with increasing induced over expression of DnaK in *E. coli* during the stationary phase. Thioredoxin domain protein, periplasmic in gram-negative bacteria, is known to play a role in defense against oxidative killing by hydrogen peroxide in *Neisseria gonorrhoeae* (Achard et al., 2009). The authors suggested a reduction in intracellular survival of bacteria in epithelial cells upon mutation of a corresponding gene. Hence, up-regulation of thioredoxin domain protein in *E. ictaluri* upon treatment with serum is expected as thioredoxin domain protein may play role in protecting *E. ictaluri* against oxidative killing inside the phagocytes.

Down-regulation of polyribonucleotide nucleotidyltransferase (PNPase, -1.87 fold) is striking as reduced activity of PNPase leads to an increase in steady-state levels and half-lives of mRNA. It is known that extensive degradation of macromolecules, especially RNA, is of primary biological importance for the survival of cells during stress (Maruyama and Okamura, 1972). Integrase gene, which is up-regulated in this study, is shown to be located in the virulence-associated region in the genome of *Dichelobacter nodosus* (Bloomfield et al., 1997). Similarly, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (IspG) is highly up-regulated (2.82-fold), which has a role in iron ion binding and the non-mevalonate pathway of isoprenoid biosynthesis Zepeck et al., (2005). It is known that acquisition of iron, an essential scarce microelement in host, is required to survive and establish an infection in host. Hence, further research on these genes of *E. ictaluri* may aid in increasing our understanding of molecular mechanisms involved in *E. ictaluri* pathogenesis.

Aspargine synthase (AsnB), which catalyzes conversion of aspartate to asparagines, is up-regulated in this study. It is known to be involved in Alanine, aspartate and glutamate metabolism, and in Nitrogen metabolism pathways. Research conducted by Ren and Liu, (2006) has suggested that AsnB is involved in natural resistance to multiple drugs by *Mycobacterium smegmatis*. Similarly, up-regulation of AsnB in the study may suggest an increased level of antibiotic resistance to *E. ictaluri* in catfish. Up-regulation of AsnB was shown also in mammary pathogenic *E. coli* grown in fresh milk (Lippolis et al., 2009). Down regulation of another protein, transketolase 1 (TK 1), which is integral to pentose phosphate pathway, is expected in this study as it is shown to be negatively regulated in bacteria exposed to host factors (Song et al., 2008). Previous

research has shown also that transketolase has been down regulated during stationary growth phage in *E. coli* (Jung et al., 2005). TK 1 in *S. aureus* is also reported to be immunogenic (Brady et al., 2006).

Differential protein expression analysis presented in the present study showed changes in *E. ictaluri* proteins on treatment with catfish serum. Several differentially expressed proteins, like EF-Tu, and ArnA, may be targets for further functional studies.

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CHAPTER 6

IN-FRAME DELETION OF SELECTED *EDWARDSIELLA ICTALURI* GENES

Abstract

Genes differentially expressed by *E. ictaluri* 93-146 in previous chapters were selected for functional analysis to elucidate their role in the pathogenesis of *E. ictaluri*. Genes selected for functional analysis were *malE, glyS, lamb*, and *sdhA*. For predicting the function of the above genes in *E. ictaluri*, in-frame deletions were performed using overlap extension PCR. Deleted mutant product was cloned into a suicidal vector, pDS132. I anticipate that trails to test the virulence of these mutants versus wild type on challenging the catfish might be a promising study, revealing possible involvement of these genes in *E. ictaluri* pathogenesis.

Introduction

Functional analysis of genes is important for revealing their roles in bacteria. Eliminating functions of genes by mutating open reading frames (ORFs) and observing the resultant phenotype is a common method for predicting gene function in bacteria. In-frame deletions of ORFs prevent any adverse effect to downstream genes and are thus preferred. In previous chapters, I have identified differentially expressed *E. ictaluri* proteins due to *in vitro* iron-restriction and on serum treatment. To initiate functional studies and understand possible roles of these proteins through phenotypical observation

(virulence), selected genes encoding identified protein were subjected to in-frame mutational analysis. The *working hypothesis* was that mutation of these genes encoding differentially expressed proteins of *E. ictaluri* may help us to observe changes in the virulence of *E .ictaluri*. Observing reduced virulence in mutant *E .ictaluri* may also be a strong indication for a possible link between genes and bacterial virulence. Mutants with reduced virulence may have potential for use as live attenuated vaccine candidates against enteric septicemia of catfish while aiding in elucidating the mechanism of pathogenesis.

Materials and Methods

Bacterial strains and media

Bacterial strains used in this research were *Edwardsiella ictaluri* 93-146, *Escherichia coli* CC118 λ*pir*, and *E. coli* SM10 λ*pir*. *E. ictaluri* colonies were grown on Brain Heart Infusion (BHI) agar plates at 30°C, whereas *Escherichia coli* colonies were grown on Luria-Bertani (LB) agar plates at 37°C. *E. ictaluri* and *E. coli* cultures were grown in BHI and LB broth, respectively, at the same temperatures indicated above and under continuous shaking at 200 rpm. Frozen stocks were maintained at -80°C in appropriate media with 20% (v/v) glycerol. When required for selection, appropriate antibiotics added to agar plates, and cultures included chloramphenicol (cat), ampicillin (amp), and colistin (col) at 30 ug/ml, 100 µg/ml, and 12.5 µg/ml, respectively. Bacterial phenotypes for antibiotic selection were as following: *E. ictaluri* 93-146 (col^r, amp^s, cat^s), *E. coli* CC118 λ*pir* (cat^s, col^s, amp^s), and *E. coli* SM10 λ*pir*. (cat^s, col^s, amp^s)

Plasmid and *E. ictaluri* genes

The suicide plasmid pDS132 used in cloning (Figure 6.1) (Philippe et al., 2004) is a derivative of suicide plasmid pCVD442 (Donnenberg and Kaper, 1991). *E. ictaluri* genes selected for in-frame deletion mutation are shown in Table 6.1.

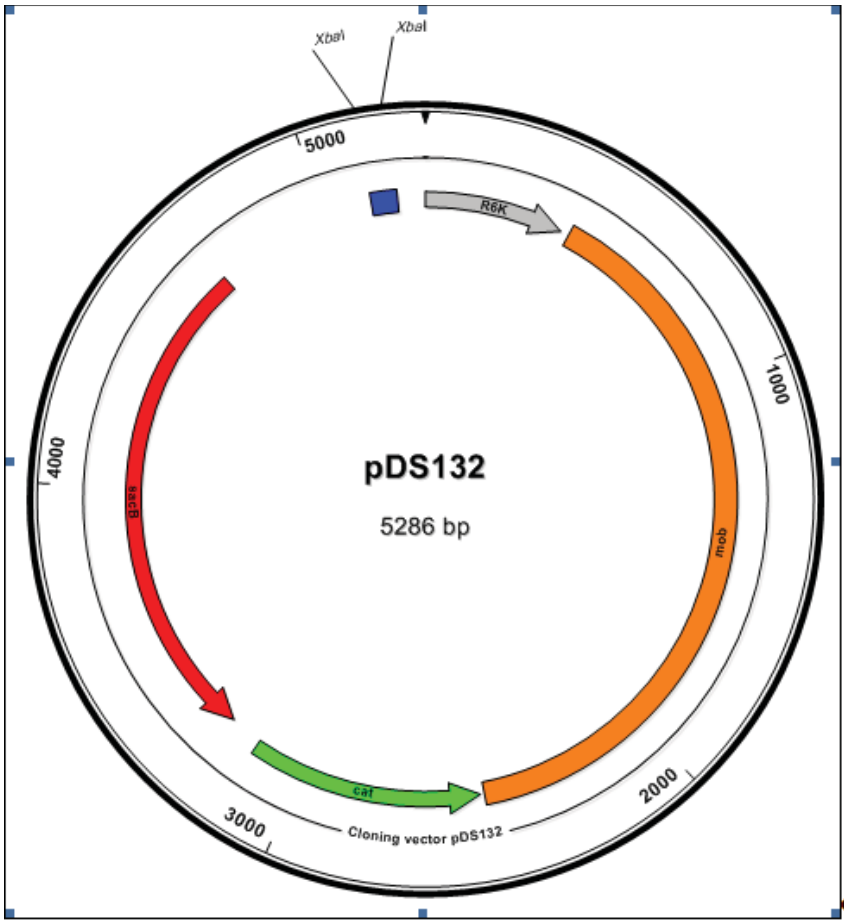

Figure 6.1 Plasmid map of pDS132.

pDS132 carries origin of replication (*R6K*), the *mob* region (mobilisation), the *cat* gene conferring chloramphenicol resistance, the *sacB* gene encoding levane saccharase, and a multiple cloning site.

Table 6.1

Edwardsiella ictaluri genes selected for in-frame deletion mutation

Accession Number	**Gene Symbol**	**Gene Name**
238867748	*malE*/e*t38*	Bacterial extracellular solute-binding protein
259511924	*glyS*	Glycyl-tRNA synthetase, beta subunit
238867752	*lamB*	Maltoporin
238870320	*sdhA*	Succinate dehydrogenase, flavoprotein subunit

Primer design and gene amplification for mutation analysis

For each gene, two pairs of primers were designed using Primer3 software available online (http://frodo.wi.mit.edu/primer3/). External forward (Ext-For) primer for each gene was designed in the region between 1000-1100 bp upstream to the start codon of the target gene, whereas external reverse (Ext-Rev) primer was designed in the region around 1000-1100 bp downstream to the stop codon of the same gene. Internal reverse (Int-Rev) primer of each gene was designed in the region of 100-200 bp downstream to the start codon of the target gene, whereas internal forward (Int-For) primer was designed in the region of 100-200 bp upstream of the stop codon and were shown in Figure 6.2. *Xba*I restriction enzyme site (tctaga) with two additional adenine nucleotides added to the 5' end of both external primers. An overlap sequence, a reverse complement of Int-Rev primer, was added to the 5' end of the Int-For primer for permitting overlapping extension. While designing internal primers, measures were taken to determine the region of deletion is in-frame. Primers for all the genes are listed in Table 6.2.

Figure 6.2 Primer design strategy for in-frame deletion of maltoporin (*lamB*) of *Edwardsiella ictaluri*.

Maltoporin sequence is represented in upper case whereas up-stream and down-stream sequences are represented in lower case. Primer locations indicating the direction are marked using red arrow.

Table 6.2

Primers used in this study

Gene Name	Primer Symbol	Primer Sequence
malE	Ext-For	5'-aatctagaTGGGTGACGTAAATCATGGTG-3'
	Int-Rev	5'-CAGACCGTTATACCCCTTGTC-3'
	Int-For	5'-gacaaggggtataacggtctgGGTGAAATCATGCCGAACATC-3'
	Ext-Rev	5'-aatctagaGGTAAAGATGACGGTCAGCAC-3'
glyS	Ext-For	5'-aatctagaGCAGTCTTCAGCGCATCATAC-3'
	Int-Rev	5'-CAGTTCTGTGCGGAAGTTGTC-3'
	Int-For	5'-gacaacttccgcacagaactgGTCGATGACTTCTTCGAGCA-3'
	Ext-Rev	5'-aatctagaGCATGAACTATGTGCCGGATC-3'
lamB	Ext-For	5'-aatctagaCTCTGGCTGTGGTAAATCGAC-3'
	Int-Rev	5'-GGTATCACACTCGTTGCCAAG-3'
	Int-For	5'-cttggcaacgagtgtgataccACCTATGCGAACGGTAAGAAC-3'
	Ext-Rev	5'-aatctagaACCAGTCATCAAGCCACATCC-3'
sdhA	Ext-For	5'-aatctagaGCCTGGGCAACATCTAATCA-3'
	Int-Rev	5'-GGTCGGAAACACTTTGGACAG-3'
	Int-For	5'-ctgtccaaagtgtttccgaccCTGTGCCATACCCTGTACCTG-3'
	Ext-Rev	5'-aatctagaGGGATCGGTCAAGTAGTCCTC-3'

Genomic DNA of *E. ictaluri* 93-146 was isolated using the Wizard DNA purification kit (Promega, Madison, WI) according to the manufacturer's protocol. Upstream and downstream PCR products were obtained using the Ext-For primer plus Int-Rev primer and Int-For plus Ext-Rev primers, respectively, by using the GoTaq polymerase (Promega). The concentrations and components of PCR are shown in Table 6.3. A PCR program used to obtain initial PCR products included initial denaturation of 95°C for 2 min, and 30 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 2 min. Final extension is done at 72°C for 10 min and the reaction was kept at 10°C after final extension. PCR products were gel purified using a Wizard DNA purification kit (Promega) according to manufacturer's recommendation.

Table 6.3

PCR reaction set up

PCR Components	**Final**
Template(50ng/ul)	100 ng
dd H_2O	Varies
For-Primer(10mM)	0.4 mM
Rev-Primer(10mM)	0.4 mM
dNTPs(10mM)	0.2 mM
Buffer(5x)	1 x
goTaq(5U/ul)	0.025 U
Total volume	25 ul

Gene Mutation using splicing by overlap extension (SOEn) PCR

From the up- and down-stream PCR products obtained from the initial set of reactions, gene SOEn was performed using Ext-For plus Ext-Rev primers. The program used in SOEing PCR is 1 cycle of 94^{o}C for 10 min; 30 cycles of 94^{o}C for 30 sec, 55^{o}C for 2 min, 72^{o}C 3 min; then 72^{o}C for 30 min, 4^{o}C for infinity. If necessary, another run of PCR was performed with product obtained from initial SOEing PCR reaction again using Ext-For plus Ext-Rev primers with PCR program:1 cycle of 95^{o}C for 2 min; 30 cycles of 95^{o}C for 30 sec, 56^{o}C 30 sec, 72^{o}C 3 min; then 72^{o}C for 10 min, 4^{o}C infinity. Resultant SOEing PCR product was gel purified before use in cloning process.

Cloning

Cloning procedures were depicted in Figure 6.3. SOEn product and pDS132 vector were digested with *Xba*I (Promega) following manufacturer's protocol. For removal of residual enzyme and buffer, 1% Agarose gel purification was performed using Wizard gel purification and PCR clean-up Kit (Promega).To avoid self ligation, linear

pDS132 was treated with thermo sensitive alkaline phosphatase (TSAP) (Promega), in accordance with manufacturer's protocol.

Ligation and Transformation of *E. coli* CC118 λpir

T4 DNA ligase (Promega) was used to clone a mutated SOEn product into *Xba*I cut and TSAP treated pDS132. The ligation mixture was electroporated into electrocompetent *E. coli* CC118 *λpir*. During electroporation, 400 Ω and 2.5kv settings were used for a 0.2 cm cuvette. After recovery *E. coli* CC118 *λpir* was spun down, and 25 µl were spread on LB agar with 30µl/ml of cat and incubated at 37°C overnight. Only bacteria with pDS132, which incur cat resistance to *E. coli* CC118, can grow on the above plates. Resultant colonies were picked and cultured in LB broth with cat at 37°C overnight. Plasmid DNA was isolated from the overnight bacterial cultures with Qiaprep Spin Mini Kit (Qiagen, Valencia, CA), according to manufacturer's protocol.

Verification of cloning

One µL of isolated plasmid DNA was run on agarose gel with circular empty plasmid to obtain initial confirmation about the presence of insert in the vector. Plasmids run higher than the empty vector were digested with *Xba*I and ran 1% agarose gel to verify the size of insert. An empty *Xba*I digested linear pDS132 and 1kb+ ladder were included on the agarose gel as controls. After verification of inserts and their sizes, two sequencing reactions with Ext-For and Ext-Rev were set up for each gene, and obtained sequences were compared with the pDS132 and original *E. ictaluri* gene sequences for final verification. Purified pBS132 with correct insert was electroporated to

electrocompetent *E. coli* SM10 *λpir* donor for conjugal transformation of suicide vector carrying mutated genes to *E. ictaluri* 93-146.

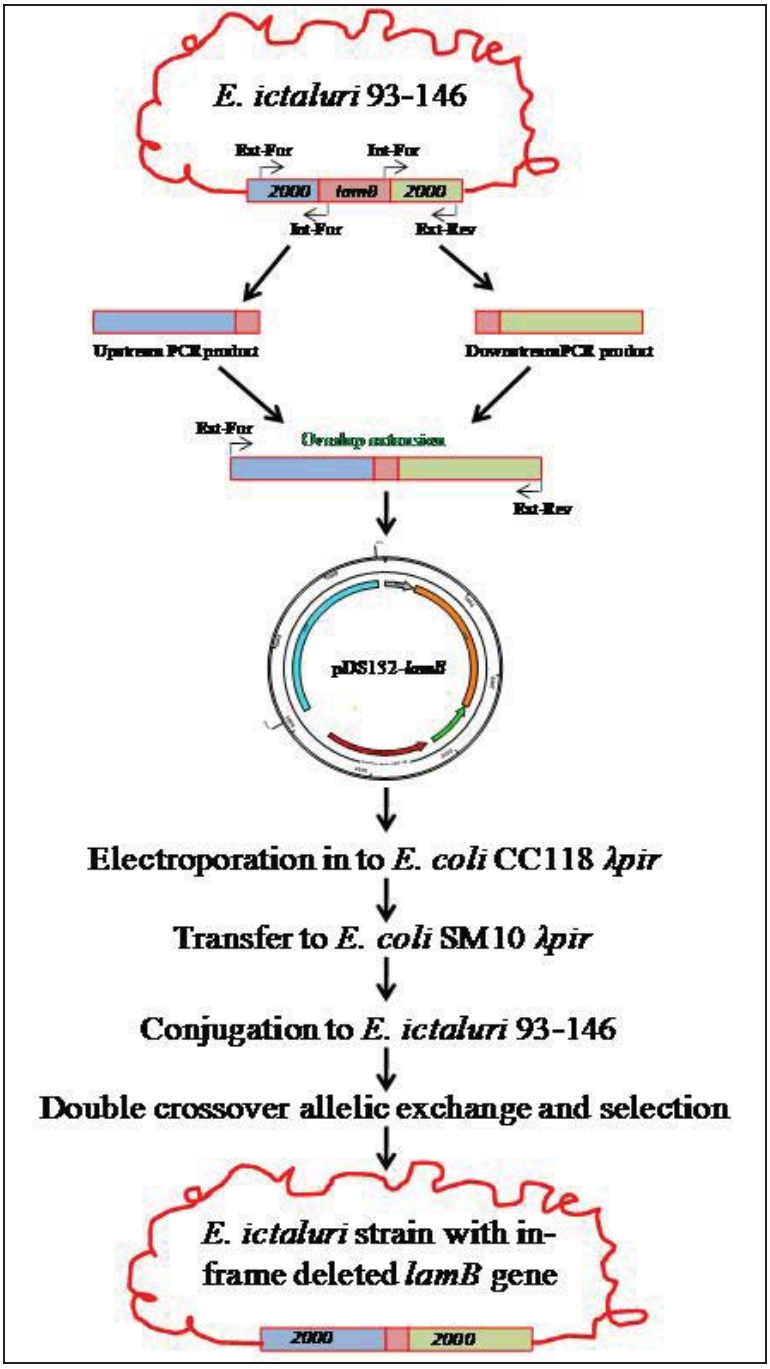

Figure 6.3 Construction of in-frame deletion mutant of *Edwardsiella ictaluri*.

Results and Discussion

In this study, using in-frame deletion of maltoporin (*lamB*), bacterial extracellular solute-binding protein (*malE*), glycyl-tRNA synthetase, beta subunit (*glyS*), and succinate dehydrogenase, flavoprotein subunit (*sdhA*) genes using overlap-extension PCR was established. The fundamental reason for choosing in-frame deletion was to avoid polar effects on the downstream genes of the operon.

For cloning process, in this study a suicidal vector pDS132 was used. Previously this vector has been has been successfully used in allelic exchange studies in bacteria (Naughton et al., 2009; Steyert and Pineiro, 2007; Zheng et al., 2008). Allelic exchange cross-over is a two-step procedure with first step being homologous recombination integrating the plasmid within the target based on sequence, followed by a second step being excision via a second cross-over of allelic exchange. pDS132 carries the replication of origin (R6K), which was able to replicate only in *pir* strains such as SM10 *λpir* or CC118 *λpir* producing the π protein, the product of the *pir* gene. pDS132 carries sucrose-sensitivity system, *Bacillus subtilis sacB* gene which encodes levane saccharase, lethal in most gram-negative bacteria in the presence of sucrose (Gay et al., 1983). This is useful for selecting plasmid excision in the cells growing in a media containing sucrose. pDS132 also carries the plasmid mobilization region (mob), chloramphenicol resistance conferring gene (cat), and several unique restriction endonuclease sites at multiple cloning site region useful for cloning.

In overlap extension PCR incorporation of complementary sequences to Int-For primer in the initial PCR reaction generation will aid in obtaining a “fused” mutational DNA fragment. During the first denature and annealing of the SOEing PCR, one strand

of each PCR product acts as primer on the other due to addition of complementary sequence, and extension of this overlap yields the mutation product. Due to short overlap between the two PCR products, annealing may occur at low frequency, hence addition of Ext-For plus Ext-Rev primers may aid in yielding more fusion products.

After successful cloning of in-frame deleted mutant product to the suicidal vector pDS132, 1% agarose gel was ran to verify the approximate size of the insert, which are shown in Figure 6.4. For further confirmation of correct construct, DNA sequencing of four genes was performed, and results were analyzed using Lasergene and are shown in Figures 6.5.

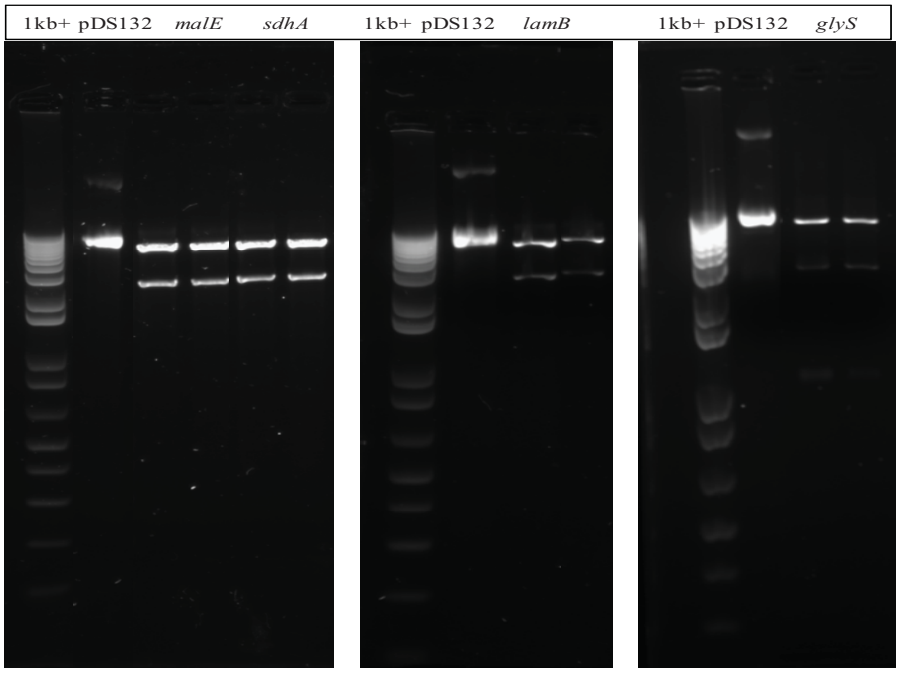

Figure 6.4 Ethidium bromide stained agarose gel showing bands of *Xba*I digested pDS132 with insert.

For each gel loaded with 1kb+ ladder and pDS132 plasmid (control), pDS132 with mutant insert of *malE, sdhA, lamb* and *glyS* was labeled accordingly over the well.

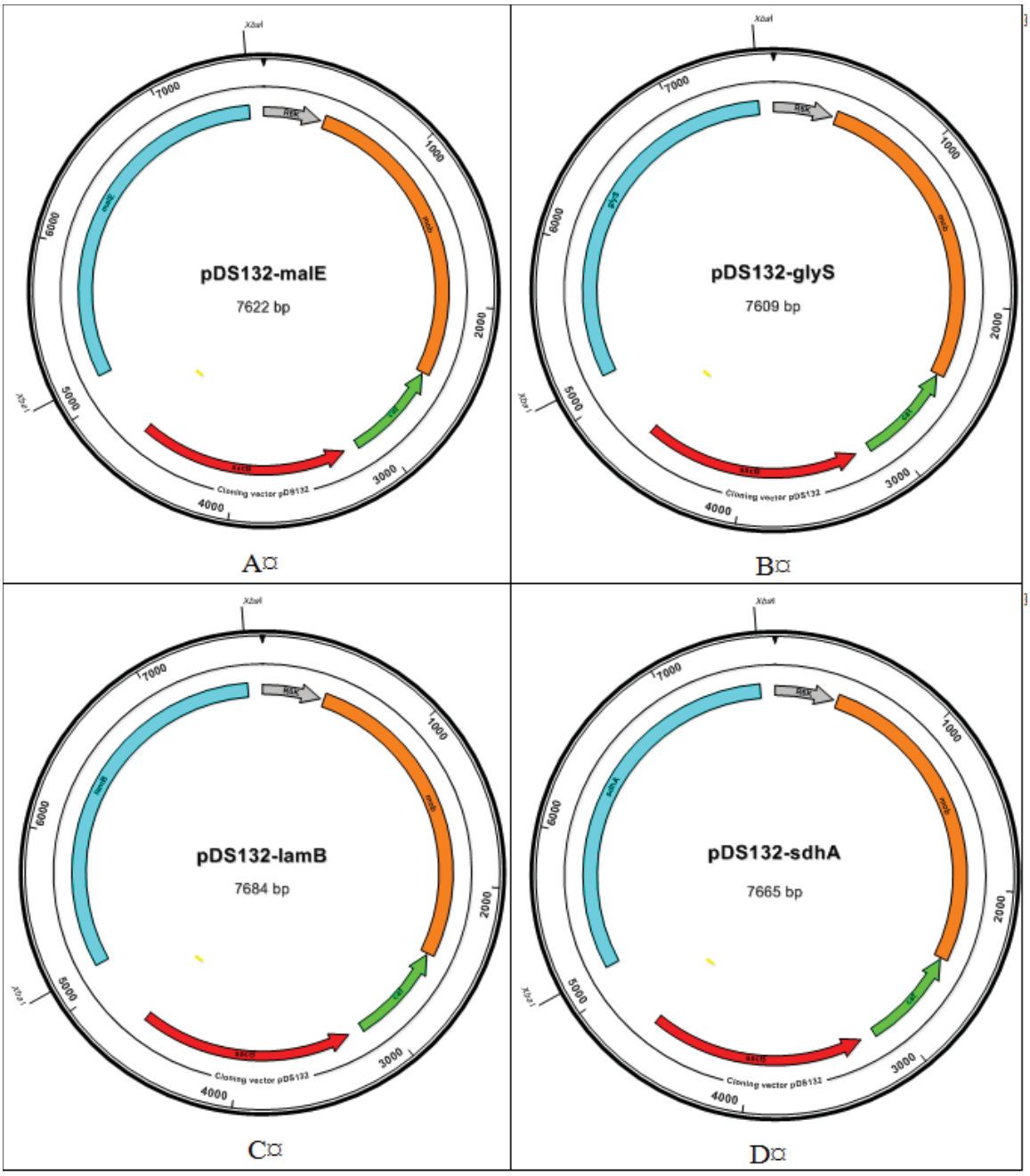

Figure 6.5 General structure and physical map of the pDS132 with insert.

A, pDS132 with *malE* mutant; B, pDS132 with *glyS* mutant; C, pDS132 with *lamB* mutant; D, pDS132 with *sdhA* mutant.

Future research

For obtaining mutant *E. ictaluri* strains, suicidal plasmids carrying mutated genes will be transferred to *E. ictaluri* 93-146 by conjugation using the procedure described previously (Karsi et al., 2006).

Selection of *E. ictaluri* mutants is a two step procedure (Reyrat et al., 1998). Briefly, in the first step, pDS132 with mutant insert integrates to genomic DNA of *E. ictaluri* by a single crossover. At this stage, *E. ictaluri* will be chloramphenicol resistant due to cat gene on the pDS132. In the second step, propagation of *E. ictaluri* carrying the *sacB* gene on its chromosome in BHI with 10% (w/v) sucrose will induce loss of the pDS132 vector by a second crossover event. Colonies that grow on this medium will be tested for chloramphenicol sensitivity to ensure the loss of plasmid. Chromosome gene deletion will be further confirmed by genomic PCR and DNA sequencing using Ext-For and Ext-Rev primers.

After obtaining *E. ictaluri* mutants, virulence trials will be conducted in channel catfish to observe evidence of changes in the virulence due to deletion of selected gene. Briefly, catfish fingerlings will be stocked at a rate of 20 per tank. Immersion challenges will be conducted for each mutant, and fish mortalities and the amount of mutant *E. ictaluri* in catfish will be quantified as described previously (Karsi et al., 2006; Karsi et al., 2009).

To demonstrate attenuation is exclusively due to the mutated genes, the wild-type genes will be cloned into pBBRMCS4 and transferred to mutants to restore the function of the mutated gene. Mutants containing the complementation plasmid will be used in fish challenge studies to determine restoration of virulence.

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CHAPTER 7

CONCLUSION

Considering the economic importance of diseases caused by *Edwardsiella ictaluri* and *Flavobacterium columnare* in catfish and with the recent availability of comprehensive proteomic databases, this is a most opportune time to study the proteomic aspects of these bacteria. The *overall objective* of this study was to analyze whole cell proteomes of *E. ictaluri* and *F. columnare*, and understand how *E. ictaluri* is able to alter its proteome upon interaction with catfish serum and *in vitro* iron-restriction. The present study reports the first comprehensive protein expression analysis of *E. ictaluri* and *F. columnare* using gel-based and gel-free approaches. Proteomic analysis reported expression of 788 unique *E. ictaluri* and 621 unique *F. columnare* proteins, which provides experimental evidence for many proteins that had only been predicted, which may improve the accuracy of their genome annotations.

Proteomic analysis of *E. ictaluri* also identified the virulence related proteins and outer membrane proteins that may have a potential role in pathogenesis. It is known that *E. ictaluri* is able to replicate inside host phagocytes. The present study identified the expression of EvpB and EvpC, which were shown to be important in *Edwardsiella tarda* for replication in phagocytes. The type III secretion system (TTSS) was also identified as one of the important virulence mechanisms in other bacteria. Expression of EseB, EseC, and EseD proteins that may act as effector proteins of TTSS were identified in our *E.*

ictaluri protein dataset. Expression of catalase and SOD, which may aid in protection of *E. ictaluri* from the "respiratory burst" of phagocytes, was identified. The present study also demonstrated that use of both gel-based and gel-free approaches improve proteome coverage, although 2-D LC provided good coverage by itself. It was also shown that cytoplasmic membrane proteins, due to their hydrophobic nature, were identified in lesser percentages using 2-DE compared with 2-D LC. Further, it was noted that ortholog matching of proteins expressed by these bacteria with closely related well annotated bacteria such as *E. coli* was an effective method for pathway analysis. Twelve pathways were significantly represented from our *E. ictaluri* proteomic dataset. The lipopolysaccharide biosynthesis pathway, which may be one of the important virulence-related systems in *E. ictaluri* pathogenesis, was significantly represented.

Similarly, proteomic analysis of *F. columnare* also demonstrated few putative virulence related proteins. Proteases, adherence factors, motility related proteins, and defense proteins were considered as putative virulence determinants. In this study, the expression of putative virulence proteins such as extracellular elastinolytic metalloproteinase (protease), fibronectin type III domain protein (potential adherence factor), GldN, GldM, GldL and gliding motility-related protein (motility), SodC, Sod[Mn], KatG, and peroxidases (defense against reactive oxygen species produced by macrophages) was confirmed. Expression of a greater number of aminoacid kinases, aminotransferase, and peptidases than carbohydrate kinases indicates that amino acids, instead of carbohydrates, could be the major sources of carbon or energy in *F. columnare*. Pathway analysis also supports this supposition due to the greater number of amino acid related metabolic pathways compared with carbohydrate metabolic pathways.

Comprehensive proteome analysis and good 2-D reference maps of *E. ictaluri* and *F. columnare* might serve as a reference and accelerate further functional studies aimed at understanding virulence mechanisms of these important pathogens.

It is always a challenge to elucidate how bacteria employ various adaptive mechanisms to invade, colonize, and successfully establish infection in a host. Vertebrate hosts tend to chelate free iron, which is an essential microelement for almost all living organisms, and there by makes the environment hostile for bacteria to grow. Reduced availability of iron may cause a significant stress for bacterial pathogens and is considered a signal that leads to significant changes in virulent gene expression. The present study revealed differential expression of 50 *E. ictaluri* proteins in response to iron-restriction. Among those several proteins that are known to be involved in various pathogenesic process in other bacteria, the following were found in the present study: TonB-dependent heme/hemoglobin receptor family protein (siderophore dependent iron acquisition mechanism and possible role in pathogenicity), bacterial extracellular solute-binding protein/MalE (transport iron-complexes from periplasm across inner membrane), LamB (OMP aid in adherence), EF-Tu (sense and respond to stress, virulence factor), proteins involved in LPS biosynthesis process, Fba (antigenic), aspartate ammonia-lyase (pathogenesis), and DsbA (Biofilm formation).

Similarly, it is known that *E. ictaluri* might effectively use the catfish's blood stream for quick systemic infection. Hence for survival and successful establishment of the septicemic condition in catfish, it is certain that *E. ictaluri* might have to counter the challenges from catfish serum proteins. Hence, a study was conducted to identify differentially expressed proteins of *E. ictaluri* upon encounter with catfish serum.

Nineteen differentially expressed proteins with putative immunogenic proteins (ArnA, BipA, AmiD, and IDH), chaperones (DnaK and Thioredoxin domain protein), translational process proteins, nucleic acid degradation and integration proteins, intermediary metabolism proteins, and iron ion binding protein were identified. Studies on other bacteria have shown that EF-Tu may bind to the complement regulator factor H and plasminogen and acts as a virulence factor. Studies on other bacteria have also shown that ArnA might aid in acquiring resistance to CAMPs and polymixins, indicating possible roles in *E. ictaluri*. The present study may be a starting point for further functional studies. For example, identified genes could be mutated, and mutant phenotypes could be analyzed for usage as potential vaccine candidates.

Hence, to initiate functional studies and understand possible roles of differentially expressed proteins, respective genes (*malE, lamb, sdhA, glyS*) were selected for mutational analysis to observe changes in the virulence of *E .ictaluri*.

Conclusively, the present study is crucial in identifying targets for further research on elucidation of pathogenic mechanisms of *E. ictaluri* and for extending preventive and therapeutic options.

APPENDIX A

EDWARDSIELLA ICTALURI PROTEINS IDENTIFIED USING 2-D LC

Serial No	ORF No	Protein name	Σxcorr	Coverage	MW	No of peptide ions	COG category	Subcellular location	Identified by
1	ORF03304	Chaperonin GroL	342.30	72.58	57393.6	480	O	Cytoplasmic	Both
2	ORF01996	Cysteine synthase A	220.31	84.33	34140.0	400	E	Unknown	Both
3	ORF02587	Outer membrane protein A	200.37	67.14	38098.9	130	M	OuterMembrane	Both
4	ORF02296	Translation elongation factor G	170.28	38.32	77471.6	83	J	Cytoplasmic	Both
5	ORF03674	Chaperone protein Dnak	158.40	41.42	68508.8	114	O	Cytoplasmic	Both
6	ORF00647	EseC	150.32	37.29	50537.5	52	NO related COG	Unknown	2-D LC
7	ORF02276	Phosphopyruvate hydratase	140.26	55.2	45745.8	80	G	Cytoplasmic	Both
8	ORF01652	Phosphoglycerate kinase	128.28	55.56	40925.8	25	G	Cytoplasmic	Both
9	ORF02553	Outer membrane protein F	120.21	35.92	40848.2	57	NO related COG	OuterMembrane	Both
10	ORF03055	Ribosomal protein L1	110.29	52.14	24720.5	39	J	Unknown	Both
11	ORF03543	Amino-acid ABC transporter periplasmic component	100.30	51.36	27917.4	87	E	Periplasmic	Both
12	ORF01529	Peroxiredoxin	98.31	66.5	22213.2	84	O	Cytoplasmic	Both
13	ORF00684	Glyceraldehyde-3-phosphate dehydrogenase, type I	90.32	38.07	35449.2	73	G	Cytoplasmic	Both
14	ORF01195	EvpB	90.27	29.7	54457.1	42	S	Cytoplasmic	2-D LC
15	ORF01724	Negative regulator of beta-lactamase expression	90.25	44.53	28664.9	38	M	Cytoplasmic	Both
16	ORF00402	Isocitrate dehydrogenase, NADP-dependent	88.33	26.86	45523.1	32	C	Cytoplasmic	2-D LC
17	ORF03060	Elongation factor Tu	88.21	69.4	14862.1	49	JE	Cytoplasmic	2-D LC
18	ORF02295	GTPases - translation elongation factors	80.31	55.61	23635.5	197	JE	Cytoplasmic	Both
19	ORF01194	EvpA	80.22	52.94	19375.8	38	S	Cytoplasmic	Both
20	ORF00214	Ribosomal protein S3	78.25	40.34	26026.0	22	J	Cytoplasmic	2-D LC
21	ORF01848	Ribosomal protein S1	74.22	21.9	61156.6	19	J	Cytoplasmic	2-D LC
22	ORF00486	Adenosine deaminase	70.39	41.74	36573.3	50	F	Cytoplasmic	2-D LC
23	ORF02486	Triosephosphate isomerase	70.30	51.18	26706.2	64	G	Unknown	Both
24	ORF00225	Ribosomal protein S5	70.27	34.94	17442.1	39	J	Unknown	2-D LC
25	ORF02980	Tia invasion determinant	70.24	33.81	22480.6	41	M	OuterMembrane	Both
26	ORF01651	Fructose-bisphosphate aldolase, class II	68.33	29.89	39154.3	41	G	Unknown	Both
27	ORF01051	Probable N-acetylmuramoyl-L-alanine amidase YbjR	68.30	27.89	32879.3	30	M	Cytoplasmic	Both

28	ORF03218	Polyribonucleotide nucleotidyltransferase	68.25	14.29	76490.8	26	J	Cytoplasmic	2-D LC
29	ORF01488	Trigger factor	66.18	23.33	48073.3	15	O	Cytoplasmic	Both
30	ORF03517	Chaperone protein htpg	60.39	19.1	70622.9	14	JE	Cytoplasmic	Both
31	ORF03241	Malate dehydrogenase, NAD-dependent	60.29	39.42	32348.2	27	C	Unknown	Both
32	ORF02303	Fkbp-type peptidyl-prolyl cis-trans isomerase fkpa	60.27	38.24	28698.3	38	O	Periplasmic	Both
33	ORF02105	Citrate synthase I	60.24	17.56	48218.9	32	ER	Cytoplasmic	Both
34	ORF00231	Ribosomal protein S4	60.23	34.95	23420.8	18	J	Cytoplasmic	2-D LC
35	ORF02297	Ribosomal protein S7	60.23	44.23	17620.3	17	J	Unknown	2-D LC
36	ORF00220	Ribosomal protein L5	60.21	37.43	20346.5	22	J	Unknown	2-D LC
37	ORF00223	50S ribosomal protein L6	60.19	32.77	18884.7	22	J	Unknown	2-D LC
38	ORF03133	Phosphopentomutase	56.17	27.27	44456.9	7	G	Cytoplasmic	2-D LC
39	ORF03105	Ribosomal protein L13	50.34	38.03	16025.5	90	J	Cytoplasmic	2-D LC
40	ORF03020	Glucose-6-phosphate isomerase	50.32	8.93	61431.3	10	G	Cytoplasmic	2-D LC
41	ORF00406	Hypothetical protein	50.32	23.61	32003.9	23	No related COGs	Unknown	Both
42	ORF03455	Glutamine synthetase, type I	50.29	15.57	51679.2	21	E	Cytoplasmic	Both
43	ORF00022	S-Ribosylhomocysteinase (Luxs)	50.28	42.11	18971.5	51	T	Cytoplasmic	2-D LC
44	ORF01196	EvpC	50.27	44.17	17781.9	37	No related COGs	Unknown	Both
45	ORF02097	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase	50.27	24.81	43199.3	15	C	Cytoplasmic	Both
46	ORF00515	Superoxide dismutase [Fe]	50.26	29.69	21133.5	70	P	Unknown	2-D LC
47	ORF03834	Ribosomal protein S2	50.25	20.34	26312.0	20	J	Cytoplasmic	Both
48	ORF02346	Phosphoenolpyruvate carboxykinase (ATP)	50.24	18.37	59208.5	10	C	Cytoplasmic	2-D LC
49	ORF03305	Chaperonin GroS	50.24	74.23	10339.8	119	O	Cytoplasmic	Both
50	ORF01187	Had superfamily (subfamily iiib) phosphatase	50.24	22.82	26760.3	18	R	Unknown	2-D LC
51	ORF01654	Transketolase	50.22	11.9	72401.6	6	G	Cytoplasmic	2-D LC
52	ORF00208	Ribosomal protein S10	50.18	48.54	11766.5	21	J	Unknown	2-D LC
53	ORF03760	Pyruvate dehydrogenase E1 component	50.18	7.67	99489.6	5	C	Cytoplasmic	2-D LC

54	ORF02977	Copper/zinc superoxide dismutase	48.39	49.75	21147.1	50	P	Periplasmic	Both
55	ORF00232	DNA-directed RNA polymerase, alpha subunit	48.24	21.58	36519.5	12	TK	Cytoplasmic	Both
56	ORF03762	Dihydrolipoamide dehydrogenase	48.23	13.37	53533.4	12	C	Cytoplasmic	Both
57	ORF02291	Peptidyl-prolyl cis-trans isomerase B	48.20	51.22	18191.3	15	O	Cytoplasmic	Both
58	ORF02610	Bifunctional polymyxin resistance Arna protein (Polymyxin resistanceprotein pmri)	46.34	14.42	73999.8	13	MG	Cytoplasmic	Both
59	ORF00213	Ribosomal protein L22	40.31	30.91	12173.2	18	J	Cytoplasmic	2-D LC
60	ORF00196	Conserved domain protein	40.30	69.57	7425.3	90	K	Cytoplasmic	Both
61	ORF01508	6,7-dimethyl-8-ribityllumazine synthase	40.29	30.13	16146.5	23	H	Unknown	Both
62	ORF00219	Ribosomal protein L24	40.28	37.5	11302.1	79	J	Unknown	2-D LC
63	ORF00209	Ribosomal protein L3	40.24	31.1	22350.5	39	J	Unknown	2-D LC
64	ORF03765	Aconitate hydratase 2	40.23	9.94	92900.9	7	C	Unknown	2-D LC
65	ORF02884	Putative Universal stress protein A homolog	40.21	20.86	15909.2	5	T	Cytoplasmic	2-D LC
66	ORF03416	ATP synthase F1, alpha subunit	40.21	15.01	55224.7	7	C	Unknown	Both
67	ORF03247	Inorganic pyrophosphatase	40.20	26.7	19676.4	12	C	Cytoplasmic	Both
68	ORF02000	Glucose-specific phosphotransferase enzyme IIA component	40.19	51.48	18382.2	11	G	Cytoplasmic	Both
69	ORF01998	Phosphocarrier protein Hpr	40.19	62.35	9061.2	7	G	Cytoplasmic	Both
70	ORF00233	Ribosomal protein L17	40.15	22.66	14394.5	7	J	Unknown	2-D LC
71	ORF00212	Ribosomal protein L2	40.15	19.34	29987.4	12	J	Unknown	2-D LC
72	ORF03414	ATP synthase F1, beta subunit	40.14	15.43	50042.9	4	C	Cytoplasmic	Both
73	ORF03384	Periplasmic component of the Tol biopolymer transport system	38.30	13.89	47594.0	10	E	Unknown	2-D LC
74	ORF01305	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	38.22	27.2	28213.8	19	G	Unknown	Both
75	ORF02906	Multimeric flavodoxin Wrba	38.21	24.08	20279.7	4	R	Unknown	2-D LC
76	ORF00222	Ribosomal protein S8	38.20	45.38	14118.6	20	J	Unknown	2-D LC

77	ORF00416	3-oxoacyl-[acyl-carrier-protein] synthase 1	34.18	11.41	42471.1	5	IQ	Cytoplasmic	2-D LC
78	ORF00973	Aldehyde dehydrogenase B	30.32	12.38	55626.1	4	C	Cytoplasmic	2-D LC
79	ORF01315	Serine hydroxymethyltransferase	30.29	13.67	45526.5	8	E	Cytoplasmic	Both
80	ORF01638	Glycine cleavage system T protein	30.28	13.64	40937.6	5	E	Cytoplasmic	Both
81	ORF01487	ATP-dependent Clp protease, proteolytic subunit Clpp	30.28	25.73	22951.4	9	NO	Cytoplasmic	Both
82	ORF00039	Ribosomal subunit interface protein	30.27	30.1	11615.1	4	J	Cytoplasmic	2-D LC
83	ORF03515	Adenylate kinase	30.27	24.77	23492.6	9	F	Cytoplasmic	Both
84	ORF00931	Uridine phosphorylase	30.26	20.39	27353.1	6	F	Cytoplasmic	Both
85	ORF00026	Ribosomal protein S16	30.25	52.44	9000.2	63	J	Unknown	2-D LC
86	ORF01332	Nucleoside diphosphate kinase	30.24	36.36	15686.6	14	F	Cytoplasmic	Both
87	ORF02735	Pyruvate kinase	30.23	7.23	50809.3	17	G	Cytoplasmic	Both
88	ORF00644	Conserved hypothetical protein	30.22	34.78	13186.9	6	D	Cytoplasmic	2-D LC
89	ORF01768	Ribosomal protein L32	30.22	44.64	6294.2	40	J	Unknown	2-D LC
90	ORF03576	Aldehyde-alcohol dehydrogenase	30.22	16.26	34270.3	4	C	Unknown	2-D LC
91	ORF03260	Ribosomal protein L9	30.22	26.85	15753.0	12	J	Cytoplasmic	Both
92	ORF00646	Type III secretion low calcium response chaperone Lcrh/Sycd	30.21	10.97	17514.7	7	R	Unknown	Both
93	ORF00855	Ribosomal protein L28	30.21	23.08	8946.4	26	J	Cytoplasmic	2-D LC
94	ORF00210	Ribosomal protein L4/L1 family	30.19	20.4	22082.4	5	J	Unknown	Both
95	ORF03835	Translation elongation factor Ts	30.19	14.74	30601.0	6	J	Cytoplasmic	Both
96	ORF01982	Glucans biosynthesis protein G	30.18	10.58	56570.0	7	P	Periplasmic	Both
97	ORF03577	Aldehyde-alcohol dehydrogenase	30.18	9.85	60065.4	6	C	Cytoplasmic	2-D LC
98	ORF02758	Putative reductase	30.17	12.89	31754.7	4	S	Cytoplasmic	2-D LC
99	ORF03846	Beta-hydroxyacyl-(acyl-carrier-protein) dehydratase FabZ	30.17	16.56	18567.6	4	I	Cytoplasmic	Both
100	ORF00889	Thioredoxin	30.17	41.67	11587.4	5	OC	Cytoplasmic	Both
101	ORF00967	Glycerophosphoryl diester phosphodiesterase	30.16	9.47	40904.0	3	C	Periplasmic	2-D LC
102	ORF02219	Serine phosphatase RsbU,	30.16	7.78	30246.6	5	TK	Unknown	2-D LC

		regulator of sigma subunit							
103	ORF03837	Ribosome recycling factor	30.16	27.03	20764.7	4	J	Cytoplasmic	Both
104	ORF02877	Ribosomal L25p family	30.16	54.26	10435.8	4	J	Unknown	Both
105	ORF01853	L-asparaginase, putative	30.15	7.16	36791.6	7	EJ	Periplasmic	2-D LC
106	ORF02508	6-phosphofructokinase	30.15	8.44	34952.8	3	G	Cytoplasmic	2-D LC
107	ORF01894	Dead/deah box helicase, N-terminal	30.13	6.14	114646.7	3	L	Unknown	2-D LC
108	ORF00649	EseE	28.38	42.52	14641.0	14	NO related COG	Cytoplasmic	Both
109	ORF03262	Ribosomal protein S6	28.27	16.79	15270.0	5	J	Cytoplasmic	Both
110	ORF00626	Catalase/peroxidase HPI	28.26	11.74	79695.3	9	P	Unknown	2-D LC
111	ORF02281	Protein YBBN	28.16	15.85	31202.6	5	O	Cytoplasmic	2-D LC
112	ORF03415	ATP synthase F1, gamma subunit	28.16	9.76	31755.5	4	C	Unknown	Both
113	ORF02024	Periplasmic nitrate reductase, large subunit	28.13	6.63	92240.8	3	C	Periplasmic	2-D LC
114	ORF01641	Xaa-Pro aminopeptidase	26.21	11.14	49262.7	3	E	Cytoplasmic	2-D LC
115	ORF00014	Alanyl-tRNA synthetase	26.19	6.74	95543.4	11	J	Cytoplasmic	2-D LC
116	ORF01422	Hypothetical protein	26.15	64.15	6188.5	3	No Hits	Unknown	2-D LC
117	ORF00501	Tyrosyl-tRNA synthetase	26.15	14.49	47677.7	4	J	Cytoplasmic	Both
118	ORF01484	ATP-dependent protease La	26.14	7.53	87411.7	5	O	Cytoplasmic	2-D LC
119	ORF02898	Sensor kinase protein RcsC	26.13	6.4	109546.7	3	T	CytoplasmicMembrane	2-D LC
120	ORF03053	50S ribosomal subunit protein L7/L12	24.20	55.56	5543.4	3	J	Unknown	2-D LC
121	ORF01208	Lipoprotein, putative	24.13	4.96	141366.6	4	S	CytoplasmicMembrane	2-D LC
122	ORF01684	Xanthosine permease	22.13	6.92	56326.7	12	GEPR	CytoplasmicMembrane	2-D LC
123	ORF01793	ABC-type polar amino acid transport system, ATPase component	20.33	19.38	28548.8	6	Q	CytoplasmicMembrane	2-D LC
124	ORF00645	EseB	20.32	15.15	21590.1	16	NO related COG	Unknown	2-D LC
125	ORF03227	Dihydropteroate synthase	20.30	7.58	29480.7	8	H	Cytoplasmic	2-D LC
126	ORF03061	GTPases - translation elongation factors	20.28	11.03	29964.8	22	JE	Cytoplasmic	Both
127	ORF03564	Dps family protein	20.28	18.07	18818.2	9	L	Unknown	2-D LC
128	ORF00230	Ribosomal protein S11	20.27	33.33	13858.9	8	J	Unknown	2-D LC
129	ORF00648	EseD	20.26	15.03	20950.1	7	NO related COG	Unknown	2-D LC

130	ORF02305	Fkbp-type peptidyl-prolyl cis-trans isomerase slyd	20.25	25.62	21570.5	9	O	Cytoplasmic	2-D LC
131	ORF01662	S-adenosylmethionine synthetase	20.24	8.5	44913.8	7	H	Cytoplasmic	2-D LC
132	ORF02087	Tol-Pal system beta propeller repeat protein TolB	20.24	7.21	46047.7	6	N	Periplasmic	2-D LC
133	ORF03831	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	20.24	17.15	29926.0	4	E	Cytoplasmic	Both
134	ORF01776	Antibiotic biosynthesis monooxygenase	20.23	26.04	10568.9	4	S	Cytoplasmic	Both
135	ORF02432	Universal stress protein A	20.22	24.83	16097.3	6	T	Cytoplasmic	Both
136	ORF00029	Ribosomal protein L19	20.22	23.48	13128.2	11	J	Cytoplasmic	2-D LC
137	ORF01657	Agmatinase, putative	20.22	12.5	33258.4	2	E	Cytoplasmic	2-D LC
138	ORF01622	Lysyl-tRNA synthetase	20.21	6.73	57739.2	10	J	Cytoplasmic	Both
139	ORF03918	Prpf, Acnd-accessory	20.20	7.16	41216.8	2	S	Cytoplasmic	2-D LC
140	ORF03162	Multifunctional CCA protein	20.20	8.45	46363.6	6	J	Cytoplasmic	2-D LC
141	ORF01843	Conserved domain protein	20.20	31.88	7502.3	10	K	Cytoplasmic	Both
142	ORF02500	ATP-dependent protease HslV	20.20	16.56	17559.9	15	O	Cytoplasmic	Both
143	ORF01416	Ferritin and Dps	20.19	6.9	19826.3	22	L	Cytoplasmic	Both
144	ORF03700	Chaperone SurA	20.19	9.45	47857.0	7	O	Periplasmic	Both
145	ORF02111	Conserved hypothetical protein	20.18	7.29	27065.5	4	S	Cytoplasmic	2-D LC
146	ORF02356	Protein GntY	20.18	15.71	21204.7	2	O	Cytoplasmic	2-D LC
147	ORF00044	Chaperone ClpB	20.18	4.78	95200.2	2	O	Cytoplasmic	2-D LC
148	ORF03132	Purine nucleoside phosphorylase	20.18	14.35	25653.4	11	F	Cytoplasmic	Both
149	ORF01401	Glutamate-1-semialdehyde-2,1-aminomutase	20.17	6.79	45864.4	2	H	Cytoplasmic	Both
150	ORF00102	Conserved hypothetical protein	20.17	26.71	16492.7	4	No related COGs	Unknown	Both
151	ORF03167	Ribosomal protein S21	20.17	15.49	8513.9	3	J	Cytoplasmic	2-D LC
152	ORF02099	Succinate dehydrogenase iron-sulfur protein	20.16	5.88	26596.6	2	C	Cytoplasmic	2-D LC
153	ORF02298	Ribosomal protein S12	20.16	10.48	13797.0	3	J	Unknown	2-D LC
154	ORF01648	Conserved hypothetical protein	20.16	10.88	26006.3	3	S	Periplasmic	Both
155	ORF00758	3-deoxy-8-phosphooctulonate synthase	20.16	12.61	25968.7	2	M	Cytoplasmic	Both
156	ORF01326	Ferredoxin, 2Fe-2S type, ISC system	20.15	36.04	12409.9	3	C	Cytoplasmic	2-D LC

157	ORF02520	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	20.15	5.84	56470.5	6	G	Cytoplasmic	2-D LC
158	ORF01034	Single-stranded DNA-binding protein	20.14	14.29	19681.6	2	L	Unknown	2-D LC
159	ORF02270	Signal transduction histidine-protein kinase BarA	20.13	2.83	102657.5	3	T	CytoplasmicMembrane	2-D LC
160	ORF02928	Hydrogenase-2 large chain	20.13	8.64	62689.3	2	C	Unknown	2-D LC
161	ORF01444	Autonomous glycyl radical cofactor	20.13	25.98	14193.0	2	R	Cytoplasmic	2-D LC
162	ORF03832	Protein-P-II uridylyltransferase	20.13	5.09	101643.0	2	O	Unknown	2-D LC
163	ORF03824	Lysine decarboxylase family protein	20.13	9.25	50711.0	2	R	Cytoplasmic	2-D LC
164	ORF01999	Phosphoenolpyruvate-protein phosphotransferase	20.12	6.09	63340.4	2	G	Cytoplasmic	2-D LC
165	ORF03131	Soluble cytochrome b562	20.12	20.31	13683.4	3	C	Periplasmic	2-D LC
166	ORF03052	Ribosomal protein L7/L12 C-terminal domain	20.12	35.62	7537.7	2	J	Unknown	Both
167	ORF02038	Conserved hypothetical protein	20.12	3.98	70039.1	2	R	Unknown	2-D LC
168	ORF03054	50S ribosomal protein L10	20.12	13.33	17827.7	2	J	Unknown	Both
169	ORF00330	ATP phosphoribosyltransferase	20.11	8.03	33248.7	2	E	Cytoplasmic	2-D LC
170	ORF00992	Cyclic diguanylate phosphodiesterase (EAL) domain protein	20.11	2.38	71541.3	2	T	CytoplasmicMembrane	2-D LC
171	ORF00217	Ribosomal protein S17	18.37	45.31	7401.7	13	J	Cytoplasmic	2-D LC
172	ORF01201	Chaperone ClpB	18.33	4.35	95836.9	3	O	Cytoplasmic	2-D LC
173	ORF00890	Transcription termination factor Rho	18.23	11.69	46934.8	6	K	Cytoplasmic	Both
174	ORF03176	Conserved hypothetical protein	18.23	27.07	14521.3	4	S	Unknown	Both
175	ORF01408	Protease do	18.23	5.52	48674.4	4	O	Periplasmic	2-D LC
176	ORF03050	DNA-directed RNA polymerase, beta' subunit	18.21	2.28	154785.8	2	K	Cytoplasmic	2-D LC
177	ORF02137	Glutaminyl-tRNA synthetase	18.19	4.5	63481.6	2	J	Cytoplasmic	Both
178	ORF01940	UDP-N-acetylglucosamine 4-epimerase	18.16	15.79	36159.0	2	MG	Unknown	2-D LC
179	ORF02454	Protein yhjk	18.15	4.52	74318.3	3	T	Cytoplasmic	2-D LC
180	ORF02778	Spheroplast protein Y	18.15	9.3	19226.8	2	S	Periplasmic	2-D LC
181	ORF02533	Sulfatase	18.15	5.36	61000.0	2	R	CytoplasmicMembrane	2-D LC
182	ORF00631	EsrA	18.14	4.17	100154.4	3	T	CytoplasmicMembrane	2-D LC

183	ORF03228	Cell division protease FtsH	18.14	4.47	71569.6	2	O	CytoplasmicMembrane	2-D LC
184	ORF01030	Diaminopropionate ammonia-lyase	18.14	9.77	43740.0	5	E	Unknown	2-D LC
185	ORF00025	Signal recognition particle protein	18.14	9.93	49834.8	2	N	Cytoplasmic	2-D LC
186	ORF03467	Phosphoenolpyruvate carboxylase	18.14	1.37	98768.3	2	C	Cytoplasmic	2-D LC
187	ORF02272	GTP pyrophosphokinase (ATP:GTP 3'-pyrophosphotransferase)(ppgpp synthetase I) ((P)ppgpp synthetase)	18.14	3.34	84362.7	3	TK	Cytoplasmic	2-D LC
188	ORF01993	DNA ligase, NAD-dependent	18.13	6.7	73406.3	2	L	Cytoplasmic	2-D LC
189	ORF01439	DNA repair protein RecN	18.13	8.32	61652.4	2	L	Cytoplasmic	2-D LC
190	ORF00364	Serine protease sat	18.13	1.54	173208.6	2	M	OuterMembrane	2-D LC
191	ORF03812	Exodeoxyribonuclease V, alpha subunit	18.13	6	67315.3	2	L	Unknown	2-D LC
192	ORF02292	Cysteinyl-tRNA synthetase	18.13	9	57359.7	2	J	Cytoplasmic	2-D LC
193	ORF00923	HTH-type transcriptional regulator GntR	18.13	14.07	37025.6	2	K	Cytoplasmic	2-D LC
194	ORF02009	NADP-dependent malic enzyme	18.13	4.48	82002.3	2	C	Unknown	2-D LC
195	ORF03036	Acetate-CoA ligase	18.12	4.45	72181.4	2	I	Cytoplasmic	2-D LC
196	ORF02909	Molybdenum cofactor biosynthesis protein C	18.12	23.46	17358.2	2	H	Unknown	2-D LC
197	ORF00842	Type IV secretory pathway, Virb4 components	18.12	1.15	107159.0	2	N	Cytoplasmic	2-D LC
198	ORF02787	Tail-specific protease	18.11	3.82	76182.0	2	M	Unknown	2-D LC
199	ORF00614	Conserved hypothetical protein	18.11	18.85	13507.4	2	K	Cytoplasmic	2-D LC
200	ORF03651	Acyl-CoA synthetase	18.11	1.91	97608.6	2	C	Cytoplasmic	2-D LC
201	ORF02769	Spermidine/putrescine-binding periplasmic protein	18.11	13.07	39233.2	2	E	Periplasmic	2-D LC
202	ORF02925	Peptidase family C69	16.19	9.43	54874.1	2	NO related COG	Unknown	2-D LC
203	ORF03223	Transcription elongation factor	16.17	6.67	54731.3	3	K	Cytoplasmic	Both
204	ORF02642	D-lactate dehydrogenase	16.16	10	36944.2	3	CHR	Cytoplasmic	2-D LC
205	ORF01023	Pyridine nucleotide-disulphide oxidoreductase domain protein	16.16	4.25	115480.8	4	ER	Cytoplasmic	2-D LC
206	ORF00454	Acetate kinase	16.15	8.77	43123.1	7	C	Cytoplasmic	2-D LC

207	ORF01482	Peptidyl-prolyl cis-trans isomerase D	16.15	4.31	67980.4	3	O	Unknown	2-D LC
208	ORF03926	Cobyric acid synthase CobQ	16.15	5.89	55207.3	2	H	Cytoplasmic	2-D LC
209	ORF01249	Cobw/P47K family protein	16.15	9.56	39626.0	2	R	Cytoplasmic	2-D LC
210	ORF02448	Transcriptional regulator, LysR family protein	16.14	12.46	33662.5	3	K	Cytoplasmic	2-D LC
211	ORF00793	Trimethylamine-N-oxide reductase TorA	16.14	6.37	94034.8	2	C	Periplasmic	2-D LC
212	ORF03475	Hybrid peroxiredoxin hyPrx5	16.14	15.98	27325.9	3	O	Unknown	2-D LC
213	ORF00733	Protein of unknown function	16.14	11.11	33208.9	4	S	Unknown	2-D LC
214	ORF01455	Conserved hypothetical protein	16.14	34.86	12017.8	2	S	Unknown	2-D LC
215	ORF02751	Peptide transport periplasmic protein SapA	16.14	5.58	62013.3	2	EHR	Periplasmic	2-D LC
216	ORF03609	Hydrogenase-4 component B	16.13	3.63	66086.7	4	CP	CytoplasmicMembrane	2-D LC
217	ORF02592	Helicase IV	16.13	6.29	79229.2	2	L	CytoplasmicMembrane	2-D LC
218	ORF01510	Protein YbaD	16.12	19.46	17025.6	12	K	Cytoplasmic	2-D LC
219	ORF02447	D-alanine--D-alanine ligase A	16.12	10.21	42010.9	4	M	Cytoplasmic	2-D LC
220	ORF01955	Methionyl-tRNA synthetase	16.11	4.14	72867.5	2	J	Cytoplasmic	2-D LC
221	ORF02184	Crotonobetainyl-CoA dehydrogenase	16.11	8.16	42464.1	2	I	Cytoplasmic	2-D LC
222	ORF03785	Polysaccharide deacetylase domain protein	16.11	6.15	49624.8	2	G	Unknown	2-D LC
223	ORF00661	Type III secretion apparatus lipoprotein, YscJ/HrcJ family	14.14	15.23	26983.3	2	N	Unknown	2-D LC
224	ORF01882	ABC transporter, CydDC cysteine exporter (CydDC-E) family, permease/ATP-binding protein C	14.14	5.57	62878.1	3	Q	CytoplasmicMembrane	2-D LC
225	ORF00671	Conserved hypothetical protein	14.14	10	20832.6	2	R	Cytoplasmic	2-D LC
226	ORF03721	2-isopropylmalate synthase	14.13	4.18	57806.1	2	E	Cytoplasmic	2-D LC
227	ORF02413	ProteinYiiM	14.13	8.48	25373.9	2	S	Unknown	2-D LC
228	ORF00710	ATP-dependent helicase, DinG family protein	14.13	2.17	70725.2	6	L	Unknown	2-D LC
229	ORF00820	DNA topoisomerase I	14.13	5.03	74298.1	3	L	Cytoplasmic	2-D LC
230	ORF01850	3-phosphoshikimate 1-carboxyvinyltransferase	14.13	5.84	46053.5	2	E	CytoplasmicMembrane	2-D LC
231	ORF02658	FAD linked oxidases, C-terminal domain protein	14.13	2.24	112921.3	2	C	Cytoplasmic	2-D LC
232	ORF03611	Hydrogenase-4 component G	14.13	7.07	64279.0	2	C	Unknown	2-D LC

233	ORF00179	ABC-type sugar transport systems, permease components	14.11	11.05	20691.7	2	No related COGs	CytoplasmicMembrane	2-D LC
234	ORF03022	Asparate kinase, monofunctional class	14.10	6.46	47902.9	2	E	Cytoplasmic	2-D LC
235	ORF03016	ABC-type maltose transport systems, permease component	14.09	4.05	32318.4	4	G	CytoplasmicMembrane	2-D LC
236	ORF03379	Conserved hypothetical protein	12.11	10.46	26725.5	2	No related COGs	Unknown	2-D LC
237	ORF01646	Ribose 5-phosphate isomerase A	10.37	12.33	22837.4	5	G	Unknown	2-D LC
238	ORF03029	Camp phosphodiesterase	10.29	8.73	30678.5	4	R	Cytoplasmic	2-D LC
239	ORF01352	GMP synthase [glutamine-hydrolyzing]	10.28	4.19	58165.3	2	R	Cytoplasmic	2-D LC
240	ORF00052	Xanthine phosphoribosyltransferase	10.27	10.53	16932.3	5	F	Cytoplasmic	2-D LC
241	ORF03413	ATP synthase F1, epsilon subunit	10.25	20.86	15179.4	1	C	Cytoplasmic	Both
242	ORF03658	Aerobic respiration control protein ArcA	10.25	8.82	27314.9	3	E	Cytoplasmic	Both
243	ORF03251	Methionine-S-sulfoxide reductase	10.24	9.91	23395.1	5	O	Unknown	Both
244	ORF03482	Inhibitor of vertebrate lysozyme	10.23	9.93	16627.0	7	No related COGs	Unknown	2-D LC
245	ORF02595	Protein YccU	10.23	11.51	14975.5	1	R	Unknown	2-D LC
246	ORF00947	Peptidase E	10.23	9.54	26723.4	1	E	Cytoplasmic	2-D LC
247	ORF00780	Glutathione synthase	10.23	6.01	35267.3	3	HJ	Unknown	Both
248	ORF00187	Lipoprotein, putative	10.23	10.4	13344.2	1	S	Unknown	2-D LC
249	ORF03199	Protein ElaB	10.23	17.82	10936.1	2	No Related COGs	Cytoplasmic	Both
250	ORF03297	Translation elongation factor P	10.22	6.94	18845.3	1	J	Cytoplasmic	2-D LC
251	ORF02443	Protein involved in catabolism of external DNA	10.22	4.64	32246.7	12	R	Cytoplasmic	2-D LC
252	ORF03307	Aspartate ammonia-lyase	10.22	3.14	52488.1	1	E	Cytoplasmic	2-D LC
253	ORF02123	Phosphoglucomutase, alpha-D-glucose phosphate-specific	10.21	2.38	59000.1	2	G	Cytoplasmic	2-D LC
254	ORF02776	Diadenosine tetraphosphate	10.21	21.55	12710.5	5	FGR	Unknown	2-D LC
255	ORF02128	Flavodoxin	10.21	10.86	19423.6	3	C	Cytoplasmic	2-D LC
256	ORF02129	Ferric uptake regulation protein	10.21	20.19	11864.1	1	P	Cytoplasmic	2-D LC
257	ORF02695	Threonyl-tRNA synthetase	10.21	2.78	49988.4	4	J	Cytoplasmic	2-D LC

258	ORF01840	3-deoxy-D-manno-octulosonate cytidylyltransferase	10.20	9.62	28170.9	1	M	Cytoplasmic	Both
259	ORF03595	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	10.20	22.29	16603.0	1	I	Cytoplasmic	2-D LC
260	ORF00880	Branched-chain amino acid aminotransferase	10.20	6.15	33931.4	2	EH	Cytoplasmic	Both
261	ORF00229	Ribosomal protein S13p/S18e	10.20	15.25	13177.3	5	J	Unknown	2-D LC
262	ORF01665	Protein PmbA	10.20	3.59	48002.6	1	R	Cytoplasmic	2-D LC
263	ORF03743	Cell division protein FtsZ	10.20	2.59	40536.7	3	D	CytoplasmicMembrane	Both
264	ORF02994	Transcriptional regulator CriR	10.20	12.72	25612.0	1	TK	Cytoplasmic	2-D LC
265	ORF01636	Glycine dehydrogenase	10.20	2.08	104901.4	2	E	Cytoplasmic	2-D LC
266	ORF00677	Selenide, water dikinase	10.19	4.31	36605.6	1	E	Cytoplasmic	2-D LC
267	ORF00288	Transposase and inactivated derivatives	10.19	16.96	12941.0	3	NO related COG	Cytoplasmic	2-D LC
268	ORF02716	Conserved domain protein	10.19	17.39	7434.3	37	K	Cytoplasmic	2-D LC
269	ORF01485	ATP-dependent Clp protease, ATP-binding subunit Clpx	10.19	3.22	47454.1	2	O	Cytoplasmic	2-D LC
270	ORF00565	Conserved hypothetical protein	10.19	3.18	38908.6	2	No related COGs	Unknown	2-D LC
271	ORF02693	Translation initiation factor IF-3	10.19	8.55	17303.0	7	J	Cytoplasmic	2-D LC
272	ORF00215	Ribosomal protein L16	10.19	11.76	15263.1	2	J	Unknown	2-D LC
273	ORF01565	Hypothetical protein	10.19	20.99	9257.1	1	No related COGs	Cytoplasmic	2-D LC
274	ORF01044	Cytochrome o ubiquinol oxidase, subunit I	10.18	1.81	74406.8	6	C	CytoplasmicMembrane	2-D LC
275	ORF00327	Histidine biosynthesis bifunctional protein HisB	10.18	5.57	40198.8	1	E	Unknown	2-D LC
276	ORF00777	Conserved hypothetical protein	10.18	6.78	26133.6	1	R	Cytoplasmic	2-D LC
277	ORF01876	Outer membrane lipoprotein carrier protein LolA	10.18	10.78	22568.3	1	M	Periplasmic	2-D LC
278	ORF03274	HflC protein	10.18	5.69	37322.4	1	O	Unknown	2-D LC
279	ORF00046	Gamma-glutamyl phosphate reductase	10.18	3.65	38471.0	4	E	Cytoplasmic	2-D LC
280	ORF00741	Arginyl-tRNA synthetase	10.18	2.08	63482.3	1	J	Cytoplasmic	2-D LC
281	ORF01826	Zn-dependent hydrolases, including glyoxylases	10.18	8.84	24110.1	1	R	Cytoplasmic	2-D LC
282	ORF03381	Small heat shock protein IbpB	10.17	12.9	17485.7	1	O	Unknown	2-D LC

283	ORF03761	Pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase	10.17	2.11	55105.1	1	C	Cytoplasmic	Both
284	ORF02086	Peptidoglycan-associated lipoprotein	10.17	8.82	18739.1	5	M	OuterMembrane	Both
285	ORF00042	Ribosomal large subunit pseudouridine synthase D	10.17	5.83	37124.3	1	J	Cytoplasmic	2-D LC
286	ORF02740	Periplasmic murein peptide-binding protein	10.17	3.71	60252.0	1	NO related COG	Periplasmic	Both
287	ORF03829	Flavodoxins	10.17	13.42	16097.9	1	C	Cytoplasmic	Both
288	ORF01686	Transcriptional regulator, LysR family	10.17	4.06	36285.7	3	K	Unknown	2-D LC
289	ORF03069	D-methionine-binding lipoprotein MetQ	10.17	7.01	29428.4	1	R	Unknown	2-D LC
290	ORF01856	Formate acetyltransferase	10.17	3.29	85105.3	1	C	Cytoplasmic	Both
291	ORF00685	Aldose 1-epimerase	10.17	4.03	32954.9	1	G	Unknown	Both
292	ORF03102	Glutathione S-transferase	10.16	6.57	24452.1	4	O	Cytoplasmic	Both
293	ORF00224	Ribosomal protein L18	10.16	19.66	12799.6	1	J	Cytoplasmic	2-D LC
294	ORF02765	Peptidase T	10.16	2.18	44583.6	2	E	Cytoplasmic	2-D LC
295	ORF02321	Phosphoglycolate phosphatase, bacterial	10.16	6.14	23864.3	3	R	Unknown	2-D LC
296	ORF01738	Protein Ybis	10.16	18.75	10674.1	1	S	Unknown	2-D LC
297	ORF02749	Phage shock protein A	10.16	6.79	25532.9	1	I	Cytoplasmic	2-D LC
298	ORF03275	Membrane protease subunits, stomatin/prohibitin homologs	10.16	6.68	45810.9	1	O	Unknown	2-D LC
299	ORF02555	Asparaginyl-tRNA synthetase	10.16	2.06	43309.5	2	J	Cytoplasmic	2-D LC
300	ORF01880	Thioredoxin-disulfide reductase	10.16	3.72	34557.9	2	O	Unknown	Both
301	ORF02504	Ribosomal protein L31	10.16	22.86	7713.8	1	J	Unknown	2-D LC
302	ORF02095	Succinyl-CoA synthetase alpha chain	10.16	4.48	29571.9	6	C	CytoplasmicMembrane	Both
303	ORF01775	Ribonuclease E	10.16	1.31	117128.6	2	J	Cytoplasmic	2-D LC
304	ORF03057	Transcription antiterminator	10.16	28.57	7926.9	2	K	Unknown	Both
305	ORF00460	Di- and tricarboxylate transporters	10.16	4.89	66545.1	1	P	CytoplasmicMembrane	2-D LC
306	ORF03418	ATP synthase F0, B subunit	10.15	9.62	17316.9	1	C	Cytoplasmic	2-D LC
307	ORF03644	Conserved hypothetical protein	10.15	2.28	47395.6	1	S	Cytoplasmic	2-D LC
308	ORF02728	Thiosulfate reductase	10.15	4.49	82749.2	1	R	Periplasmic	2-D LC
309	ORF03043	DNA-binding protein HU-	10.15	16.67	9461.8	1	L	Unknown	2-D LC

		alpha							
310	ORF01383	Phage Tail Protein X	10.15	25.37	7848.0	1	No related COGs	Cytoplasmic	2-D LC
311	ORF03072	Prolyl-tRNA synthetase	10.15	5.07	63413.8	1	J	Cytoplasmic	2-D LC
312	ORF03844	Chaperone protein skp	10.15	13.98	20883.7	1	M	OuterMembrane	2-D LC
313	ORF01493	Protein /Y1016/	10.15	16.56	18401.9	1	S	Cytoplasmic	Both
314	ORF02313	Peptidyl-prolyl cis-trans isomerase	10.15	7.98	20499.5	1	O	Periplasmic	2-D LC
315	ORF02381	Aerobic glycerol-3-phosphate dehydrogenase	10.15	2.79	56229.5	1	C	Cytoplasmic	2-D LC
316	ORF03562	Protein YciI	10.15	26.8	10424.6	1	S	Unknown	2-D LC
317	ORF03560	Intracellular septation protein A	10.15	14.44	20656.8	1	D	CytoplasmicMembrane	2-D LC
318	ORF00698	Universal stress protein F	10.15	17.48	15889.3	1	T	Cytoplasmic	2-D LC
319	ORF02666	Phospho-2-dehydro-3-deoxyheptonate aldolase	10.15	8.62	38310.5	1	E	Cytoplasmic	2-D LC
320	ORF00321	Trimethylamine-N-oxide reductase	10.15	4.58	90584.1	1	C	Periplasmic	2-D LC
321	ORF00393	Transcriptional regulatory protein PhoP	10.15		25496.2	3	TK	Cytoplasmic	Both
322	ORF01978	Molybdopterin synthase sulfurylase MoeB	10.15	11.97	27722.0	1	H	Cytoplasmic	2-D LC
323	ORF00305	NAD(P) transhydrogenase subunit beta	10.14	1.94	48920.9	3	C	CytoplasmicMembrane	2-D LC
324	ORF01255	Ecotin	10.14	9.47	18998.2	1	No Related COGs	Periplasmic	2-D LC
325	ORF01448	Probable hemagglutinin-related protein	10.14	6.69	28923.3	1	No related COGs	Cytoplasmic	2-D LC
326	ORF02518	Rhodanese domain protein	10.14	11.03	15926.3	1	P	Unknown	2-D LC
327	ORF01413	Multidrug resistance protein A	10.14	4.59	42856.6	1	Q	Unknown	2-D LC
328	ORF01604	MafB5	10.14	10.05	22197.6	1	NO related COG	Unknown	2-D LC
329	ORF01756	Pts system, glucose-specific iibc component	10.14	2.94	50669.2	1	G	CytoplasmicMembrane	2-D LC
330	ORF00641	Conserved hypothetical protein	10.14	17.21	14230.0	1	NO related COG	Cytoplasmic	2-D LC
331	ORF02756	Peptide transport system ATP-binding protein SapF	10.14	11.48	30405.0	1	EP	Cytoplasmic	2-D LC
332	ORF03612	Hydrogenase-4 component H	10.14	13.51	20336.5	1	C	Unknown	2-D LC

333	ORF03817	Dinucleotide-utilizing enzymes involved in molybdopterin and thiamine biosynthesis family 1	10.14	11.81	28985.1	1	H	Unknown	2-D LC
334	ORF02171	Protein Plu1293	10.14	17.24	9888.3	3	S	Cytoplasmic	2-D LC
335	ORF03286	Phosphatidylserine decarboxylase	10.14	10.16	33519.1	1	I	Unknown	2-D LC
336	ORF01029	Peptidase, M20/M25/M40 family	10.14		45245.1	3	E	Cytoplasmic	2-D LC
337	ORF02390	DNA invertase	10.14	27.78	5959.9	1	L	Cytoplasmic	2-D LC
338	ORF03273	Putative acyltransferase, putative	10.14	7.84	42106.8	1	I	CytoplasmicMembrane	2-D LC
339	ORF03257	Fkbp-type 22 kda peptidyl-prolyl cis-trans isomerase	10.14	8.25	22277.9	1	O	OuterMembrane	Both
340	ORF00950	Xaa-Pro dipeptidase	10.14	2.71	49906.1	1	E	Cytoplasmic	2-D LC
341	ORF02328	Hypothetical protein	10.14	19.81	11449.3	1	No related COGs	Unknown	2-D LC
342	ORF02668	HugX	10.14	16.76	19499.3	1	NO related COG	Cytoplasmic	2-D LC
343	ORF02018	Zn-dependent alcohol dehydrogenase	10.14	7.01	39930.8	1	CR	Cytoplasmic	2-D LC
344	ORF00672	Exodeoxyribonuclease III	10.14	4.46	30715.6	1	L	Cytoplasmic	2-D LC
345	ORF03357	Inner membrane protein YicG	10.14	12.62	22292.6	1	S	CytoplasmicMembrane	2-D LC
346	ORF00567	Conserved hypothetical protein	10.14	11.76	18155.4	1	No related COGs	Unknown	2-D LC
347	ORF01938	UDP-glucose 4-epimerase	10.14	5.99	36460.8	1	M	Unknown	2-D LC
348	ORF02636	Hypothetical protein	10.14	50	4438.0	2	No hits	Unknown	2-D LC
349	ORF01552	HsdR	10.14	0.92	124104.7	1	L	Cytoplasmic	2-D LC
350	ORF03084	LppC superfamily	10.14	1.9	67441.8	1	R	Unknown	2-D LC
351	ORF02326	Shikimate kinase	10.14	6.4	19557.9	2	E	Cytoplasmic	2-D LC
352	ORF02202	IbrB	10.14	13.81	24168.3	1	K	Cytoplasmic	2-D LC
353	ORF00197	Hypothetical protein	10.14	41.46	4654.2	1	No related COGs	Unknown	2-D LC
354	ORF03302	YvbY	10.13	12.55	24920.5	1	S	Cytoplasmic	2-D LC
355	ORF01621	Glyoxalase family protein	10.13	13.76	21588.2	1	E	Unknown	2-D LC
356	ORF01895	Restriction modification system DNA specificity domain:Filamentation induced by cAMP protein Fic	10.13	5.69	37731.1	1	S	Unknown	2-D LC
357	ORF01369	DNA adenine methylase	10.13	6.35	29379.8	2	L	Cytoplasmic	2-D LC

358	ORF01605	Large exoproteins involved in heme utilization or adhesion	10.13	15.09	10753.8	1	No hits	Unknown	2-D LC
359	ORF00693	D-amino acid dehydrogenase small subunit	10.13	13.19	45366.7	1	E	Unknown	2-D LC
360	ORF00461	5'-nucleotidase YfbR	10.13	12.56	25592.2	1	R	Cytoplasmic	2-D LC
361	ORF00995	Protein YhdH	10.13	6.07	32934.0	1	CR	Unknown	2-D LC
362	ORF02045	Zinc metalloprotease	10.13	6.16	55270.2	1	O	CytoplasmicMembrane	2-D LC
363	ORF02947	NADH-quinone oxidoreductase, f subunit	10.13	5.36	49154.1	1	C	Cytoplasmic	2-D LC
364	ORF00607	Conserved hypothetical protein	10.13	17.01	16895.1	1	NO related COG	Unknown	2-D LC
365	ORF00073	NTP pyrophosphohydrolases including oxidative damage repair enzymes	10.13	21.65	11484.9	1	LR	Unknown	2-D LC
366	ORF00717	6-phosphogluconolactonase	10.13	9.52	24600.0	1	G	Unknown	2-D LC
367	ORF00874	L-threonine 3-dehydrogenase	10.13	9.09	37137.8	1	ER	Cytoplasmic	2-D LC
368	ORF03490	D-lactate dehydrogenase	10.13	3.14	64215.9	1	C	Unknown	2-D LC
369	ORF00996	Protein YedY	10.13	2.1	37473.6	3	R	Unknown	Both
370	ORF01779	Membrane protein, putative	10.13	10.32	27176.3	1	R	CytoplasmicMembrane	2-D LC
371	ORF02020	Nitrate/tmao reductases, membrane-bound tetraheme cytochrome c subunit	10.13	7.77	23755.1	1	C	CytoplasmicMembrane	2-D LC
372	ORF01542	Amino acid transporters	10.13	3.52	49570.4	1	E	CytoplasmicMembrane	2-D LC
373	ORF03845	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	10.13	5.59	35566.6	1	M	Cytoplasmic	2-D LC
374	ORF01062	Acetyl-CoA acetyltransferase	10.13	8.38	40303.2	1	I	Unknown	2-D LC
375	ORF01526	Queuine tRNA-ribosyltransferase	10.13	3.96	42845.8	1	J	Cytoplasmic	2-D LC
376	ORF02030	Acriflavine resistance protein B	10.13	2.18	100366.4	1	Q	CytoplasmicMembrane	2-D LC
377	ORF03217	Lipoprotein Nlpi, contains TPR repeats	10.13	10.2	33612.4	1	R	OuterMembrane	2-D LC
378	ORF03714	Iron	10.13	5.81	34240.4	1	P	Periplasmic	2-D LC
379	ORF03238	Ribosomal protein L21	10.13	29.27	9236.7	1	J	Cytoplasmic	2-D LC
380	ORF03617	Formate dehydrogenase, alpha subunit	10.13	3.76	62431.4	2	R	Unknown	2-D LC
381	ORF03548	Anthranilate synthase component I	10.13	2.86	57114.9	1	EH	Cytoplasmic	2-D LC

382	ORF02384	Thiosulfate sulfurtransferase GlpE	10.13	23.85	11875.2	1	P	Unknown	2-D LC
383	ORF03378	Glycyl-tRNA synthetase, beta subunit	10.13	3.34	76044.8	1	J	Cytoplasmic	2-D LC
384	ORF01696	Hydrogenase-1 small chain	10.13	5.9	40725.3	1	C	Unknown	2-D LC
385	ORF00586	Conserved hypothetical protein	10.13	18.32	13968.1	1	No related COGs	Unknown	2-D LC
386	ORF02197	Probable transposase for transposon	10.13	9.94	19986.0	1	No related COGs	Unknown	2-D LC
387	ORF00306	NAD(P) transhydrogenase, alpha subunit	10.13	5.71	54267.9	1	C	CytoplasmicMembrane	2-D LC
388	ORF01506	Thiamine-monophosphate kinase	10.13	9.6	35029.6	1	H	Cytoplasmic	2-D LC
389	ORF02979	Conserved hypothetical protein	10.13	8.45	31784.2	1	N	Cytoplasmic	2-D LC
390	ORF01576	Hemolysin	10.13	1.18	167081.3	1	M	OuterMembrane	2-D LC
391	ORF02488	Conserved hypothetical protein	10.13	21.68	16030.1	1	S	CytoplasmicMembrane	2-D LC
392	ORF03284	Oligoribonuclease	10.13	9.58	19262.7	1	F	Cytoplasmic	2-D LC
393	ORF03359	DNA-directed RNA polymerase, omega subunit	10.13	23.08	10252.5	1	K	Cytoplasmic	2-D LC
394	ORF02464	Dipeptide transport ATP-binding protein DppD	10.13	4.91	35733.1	1	EP	Unknown	2-D LC
395	ORF02753	ABC-type antimicrobial peptide transport system, permease component	10.13	17.5	17814.2	1	EP	CytoplasmicMembrane	2-D LC
396	ORF01541	Hypothetical chaperone protein YegD	10.13	6.89	48969.4	1	O	Cytoplasmic	2-D LC
397	ORF03166	Gcp	10.13	7.62	36242.3	1	O	Extracellular	2-D LC
398	ORF03581	Protein hnr	10.13	5.11	39124.4	1	T	Cytoplasmic	2-D LC
399	ORF00462	Aspartate/tyrosine/aromatic aminotransferase	10.13	3.45	45704.3	1	E	Cytoplasmic	2-D LC
400	ORF03759	Pyruvate dehydrogenase complex repressor	10.13	7.48	29061.0	1	K	Cytoplasmic	2-D LC
401	ORF01385	Terminase, endonuclease subunit	10.13	13.36	24554.9	1	No related COGs	Unknown	2-D LC
402	ORF03235	GTP-binding protein Obg/Cgta	10.13	6.38	43599.5	1	R	Cytoplasmic	2-D LC
403	ORF00514	Nlpc/P60 family protein	10.13	5.28	32290.4	1	M	Unknown	2-D LC
404	ORF00056	Peptidase T	10.13	5.76	45354.2	1	E	Cytoplasmic	2-D LC
405	ORF02988	TRNA-dihydrouridine synthase A	10.12		38702.6	1	R	Cytoplasmic	2-D LC

406	ORF03271	Adenylosuccinate synthetase	10.12	2.78	47032.4	3	F	Cytoplasmic	2-D LC
407	ORF03172	Dead/deah box helicase, N-terminal	10.12	0.92	123506.2	1	L	Unknown	2-D LC
408	ORF03051	DNA-directed RNA polymerase, beta subunit	10.12	1.49	150609.2	1	K	Cytoplasmic	2-D LC
409	ORF00600	Conserved hypothetical protein	10.12	0.84	286863.5	1	NO related COG	Unknown	2-D LC
410	ORF02609	Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase	10.12	7.29	37016.6	1	M	Unknown	2-D LC
411	ORF01764	3-oxoacyl-(acyl-carrier-protein) reductase	10.12	3.69	25583.3	1	QR	Cytoplasmic	Both
412	ORF03056	Ribosomal protein L11	10.12	9.86	14848.2	1	J	Unknown	2-D LC
413	ORF01932	N-acylneuraminate cytidylyltransferase	10.12	2.4	47515.2	1	M	Cytoplasmic	2-D LC
414	ORF00870	Lipopolysaccharide heptosyltransferase I	10.12	3.43	36260.2	1	M	Cytoplasmic	2-D LC
415	ORF01358	Toprim	10.12	2.15	102394.5	1	NO related COG	Unknown	2-D LC
416	ORF03810	Protease 3	10.12	2.08	106928.8	1	O	Periplasmic	2-D LC
417	ORF02199	Transposase and inactivated derivatives	10.12	7.91	29087.6	2	L	Unknown	2-D LC
418	ORF01530	Maltodextrin glucosidase	10.12	3.52	67590.6	1	G	Cytoplasmic	2-D LC
419	ORF01712	Lipoprotein, putative	10.12	7.55	34864.9	1	No related COGs	Unknown	2-D LC
420	ORF01524	Protein-export membrane protein SecD	10.12	3.56	66887.0	2	N	CytoplasmicMembrane	2-D LC
421	ORF02067	Protein YaiE	10.12	22.11	10492.8	1	S	Unknown	2-D LC
422	ORF02991	Zinc uptake regulation protein	10.12	15.29	17784.3	1	P	Cytoplasmic	2-D LC
423	ORF03342	Ribonuclease PH	10.12		25236.8	1	J	Cytoplasmic	2-D LC
424	ORF03705	DNA polymerase II	10.12	2.44	88119.3	1	L	Cytoplasmic	2-D LC
425	ORF03735	Phospho-N-acetylmuramoyl-pentapeptide-transferase	10.12	3.06	40067.7	1	M	CytoplasmicMembrane	2-D LC
426	ORF00218	Ribosomal protein L14	10.12	16.26	13540.9	1	J	Unknown	2-D LC
427	ORF01338	TPR repeat	10.12	3.88	22446.3	1	S	Unknown	Both
428	ORF01354	Integrase	10.11	2.75	45655.2	1	L	Unknown	2-D LC
429	ORF00053	Aminoacyl-histidine dipeptidase	10.11	4.32	51924.0	1	E	Unknown	2-D LC
430	ORF03771	Carbonic anhydrase	10.11	8.64	24996.5	1	P	Cytoplasmic	2-D LC

431	ORF02359	Zn-dependent hydrolases of the beta-lactamase fold	10.11	5.65	40155.4	2	R	Unknown	2-D LC
432	ORF02941	NADH-quinone oxidoreductase chain l	10.11	2.93	66375.8	2	CP	CytoplasmicMembrane	2-D LC
433	ORF02986	Replicative DNA helicase	10.11	2.55	52771.3	1	L	Unknown	2-D LC
434	ORF03351	Conserved hypothetical protein	10.11	10.82	21591.6	2	No related COGs	Unknown	2-D LC
435	ORF03010	Maltoporin	10.11	3.77	46991.5	1	NO related COG	OuterMembrane	2-D LC
436	ORF03566	Conserved hypothetical protein	10.11	4.98	26093.7	1	NO related COG	Cytoplasmic	2-D LC
437	ORF03214	Inner membrane transport protein YhaO	10.11	2.84	46370.3	1	NO related COG	CytoplasmicMembrane	2-D LC
438	ORF02741	Thiol peroxidase	10.11	14.37	17753.2	1	O	Unknown	Both
439	ORF02499	Heat shock protein HslVU, ATPase subunit HslU	10.11	3.16	49578.3	1	O	Unknown	2-D LC
440	ORF01596	Transposase and inactivated derivatives	10.11	7.31	34750.5	1	L	Unknown	2-D LC
441	ORF01829	Hypothetical protein	10.11	22.08	8750.5	1	No related COGs	Unknown	2-D LC
442	ORF00949	NAD(P)H-flavin reductase (FMN reductase)(Aquacobalamin reductase) (NAD(P)H:flavin oxidoreductase)	10.11	8.15	26416.1	1	HC	Unknown	2-D LC
443	ORF03558	Eps9H	10.11	11.22	23286.1	1	NO related COG	Cytoplasmic	2-D LC
444	ORF03048	Thiamine biosynthesis protein ThiC	10.11	2.95	72057.2	1	H	Cytoplasmic	2-D LC
445	ORF02764	NAD	10.11	5.08	22392.2	1	R	Cytoplasmic	Both
446	ORF03667	Transaldolase	10.11	1.89	34842.5	2	G	Unknown	Both
447	ORF02474	Pyrroline-5-carboxylate reductase	10.11	5.58	27991.4	1	E	Cytoplasmic	2-D LC
448	ORF03507	Fimbrial protein	10.11	6.2	37695.1	1	NO related COG	Unknown	2-D LC
449	ORF02060	Exonuclease SbcC	10.11	1.7	140921.5	2	L	Unknown	2-D LC
450	ORF03724	Putative long-chain-fatty-acid-CoA ligase homolog	10.11	3.15	67907.1	1	I	CytoplasmicMembrane	2-D LC
451	ORF02019	Ribose 5-phosphate isomerase	10.11	6.13	17826.3	1	G	Cytoplasmic	2-D LC

		B							
452	ORF02233	Urease accessory protein UreG	10.11	9.09	22786.2	1	OK	Cytoplasmic	2-D LC
453	ORF02407	Cell division protein FtsY	10.11	2.34	50948.5	1	N	Cytoplasmic	2-D LC
454	ORF03851	DNA polymerase III alpha subunit	10.11	1.55	130165.0	1	L	Cytoplasmic	2-D LC
455	ORF00963	Anaerobic glycerol-3-phosphate dehydrogenase subunit B	10.11	2.38	45099.1	1	E	Cytoplasmic	2-D LC
456	ORF00441	Phosphoesterase, putative subfamily	10.11	4.28	20578.6	1	R	Unknown	2-D LC
457	ORF01403	IscA protein	10.11	10.53	12117.6	1	S	Unknown	2-D LC
458	ORF01420	Alpha/beta hydrolase fold	10.11	5.59	31415.5	2	R	Unknown	Both
459	ORF02680	Hypothetical protein	10.11	16.28	4899.7	2	No hits	Unknown	2-D LC
460	ORF02880	Inner membrane protein YejM	10.11	1.55	65488.9	1	R	CytoplasmicMembrane	2-D LC
461	ORF01494	2-dehydropantoate 2-reductase	10.11	6.89	33639.5	1	H	Cytoplasmic	2-D LC
462	ORF03346	Type I restriction-modification system, S subunit, putative	10.10	3.25	64574.9	1	L	Unknown	2-D LC
463	ORF02955	Mismatch repair ATPase	10.10	4.76	27661.1	1	M	Unknown	Both
464	ORF03477	Hydrogen peroxide-inducible genes activator	10.10	4.56	34613.0	1	K	Cytoplasmic	2-D LC
465	ORF03365	AsmA family	10.10	1.6	62005.4	1	No related COGs	Unknown	2-D LC
466	ORF03712	ABC-type multidrug efflux pump	10.10	6.83	36787.1	1	Q	CytoplasmicMembrane	2-D LC
467	ORF03152	Conserved hypothetical protein	10.10	17.05	10209.6	1	S	Cytoplasmic	2-D LC
468	ORF01404	Inner membrane protein Yads	10.10	11.33	21450.9	1	S	CytoplasmicMembrane	2-D LC
469	ORF02436	Periplasmic sensor signal transduction histidine kinase	10.10	2.87	54438.5	2	T	CytoplasmicMembrane	2-D LC
470	ORF03226	Phosphoglucosamine mutase	10.10	2.02	47326.0	1	G	Cytoplasmic	2-D LC
471	ORF01725	Monofunctional chorismate mutase	10.10	6.11	20707.5	1	E	Periplasmic	Both
472	ORF00019	Phosphatase YqaB	10.10	12.77	20821.7	1	R	Cytoplasmic	2-D LC
473	ORF03675	Chaperone protein Dnaj	10.10	4.51	41261.4	1	O	Cytoplasmic	2-D LC
474	ORF03376	Hypothetical protein	10.10	17.07	4751.7	1	No related COGs	Unknown	2-D LC
475	ORF00999	Acetyl-coa carboxylase, biotin carboxyl carrier protein	10.10	14.94	16051.5	1	I	Unknown	2-D LC
476	ORF02181	Carnitinyl-CoA dehydratase	10.10	6.13	27664.6	1	I	Unknown	2-D LC
477	ORF01890	ATP-dependent endonuclease	10.10	1.98	63342.6	1	L	Cytoplasmic	2-D LC

		of the OLD family							
478	ORF02337	Intracellular growth attenuator protein	8.19	3.57	74287.2	31	No related COGs	Unknown	2-D LC
479	ORF00526	Transcriptional activator protein CopR	8.18	8.81	25392.4	4	TK	Cytoplasmic	2-D LC
480	ORF02496	Transcriptional regulator	8.17	15	9452.6	2	S	Cytoplasmic	2-D LC
481	ORF01577	EihB	8.16	3.94	61682.4	1	N	OuterMembrane	2-D LC
482	ORF01038	Aromatic-amino-acid aminotransferase	8.16		44097.7	4	E	Cytoplasmic	2-D LC
483	ORF03100	Beta-galactosidase, beta subunit	8.15	8.23	17800.4	3	S	Cytoplasmic	2-D LC
484	ORF03023	Methionine synthase	8.15	1.3	134308.9	1	E	Cytoplasmic	2-D LC
485	ORF03729	MraZ protein	8.15	15.79	17535.0	1	S	Cytoplasmic	2-D LC
486	ORF03410	Glucosamine--fructose-6-phosphate aminotransferase, isomerizing	8.15	3.76	46648.1	4	M	Cytoplasmic	2-D LC
487	ORF00731	Aspartyl-tRNA synthetase	8.15	2.37	65505.4	1	J	Cytoplasmic	2-D LC
488	ORF03843	Outer membrane protein assembly factor YaeT	8.15	2.26	88396.1	1	M	OuterMembrane	2-D LC
489	ORF01068	Hypothetical protein	8.15	4.74	28669.6	2	No related COGs	Unknown	2-D LC
490	ORF01935	Lst, putative	8.15	4.79	38749.8	1	NO related COG	Unknown	2-D LC
491	ORF01817	Flagellar export protein FliJ	8.15	14.38	17099.0	1	N	Unknown	2-D LC
492	ORF02544	Flagellar basal-body rod protein FlgF	8.15	10.36	26693.9	1	N	Unknown	2-D LC
493	ORF02902	Ribonucleoside-diphosphate reductase, alpha subunit	8.15	4.34	85739.7	1	F	Cytoplasmic	2-D LC
494	ORF00135	Putative cryptic C4-dicarboxylate transporter DcuD	8.14	8.36	29904.6	2	C	CytoplasmicMembrane	2-D LC
495	ORF02044	PerM	8.14	8.36	39890.2	1	R	CytoplasmicMembrane	2-D LC
496	ORF03222	Translation initiation factor 2	8.14	2.89	98215.4	2	J	Cytoplasmic	2-D LC
497	ORF02598	Acylphosphatase	8.14	19.54	9719.0	1	C	Unknown	2-D LC
498	ORF03941	Propanediol utilization	8.14	9.43	38397.9	1	NO related COG	Cytoplasmic	2-D LC
499	ORF00568	Helix-turn-helix motif	8.14	21.57	11207.8	1	K	Unknown	2-D LC
500	ORF01067	Signal transduction histidine-protein kinase AtoS	8.14	4.92	64315.8	1	T	CytoplasmicMembrane	2-D LC
501	ORF01591	Nucleoprotein/polynucleotide-	8.14	9.76	18389.8	1	No Related	Unknown	2-D LC

		associated enzyme					COGs		
502	ORF03680	Isoleucyl-tRNA synthetase	8.14	2.22	105790.8	1	J	Cytoplasmic	2-D LC
503	ORF03405	Phosphate ABC transporter, permease protein PstA	8.14	10.27	32052.1	1	P	CytoplasmicMembrane	2-D LC
504	ORF03389	Chromosomal replication initiator protein DnaA	8.14	9.94	52054.0	1	L	Cytoplasmic	2-D LC
505	ORF02282	Short chain dehydrogenase	8.14	9.2	28134.4	1	QR	Unknown	2-D LC
506	ORF03747	Preprotein translocase, SecA subunit	8.14	2	101771.0	1	N	CytoplasmicMembrane	2-D LC
507	ORF01828	Putative peptidoglycan binding domain protein	8.14	5.53	57664.8	3	S	Unknown	2-D LC
508	ORF00312	Hypothetical protein	8.14	43.59	4728.8	1	No hits	Unknown	2-D LC
509	ORF02163	DNA polymerase III, delta subunit	8.14	9.01	39004.9	1	L	Unknown	2-D LC
510	ORF02182	Probable crotonobetaine/carnitine-CoA ligase	8.14	5	57753.6	1	IQ	Unknown	2-D LC
511	ORF02690	Phenylalanyl-tRNA synthetase, alpha subunit	8.14	2.75	36913.7	1	J	Cytoplasmic	2-D LC
512	ORF03423	Glucose-inhibited division protein A	8.14	4.77	69961.5	1	D	Cytoplasmic	2-D LC
513	ORF01534	Proline-specific permease ProY	8.14	4.73	50677.8	1	E	CytoplasmicMembrane	2-D LC
514	ORF00069	Conserved hypothetical protein	8.14	3.84	36809.1	6	No related COGs	OuterMembrane	2-D LC
515	ORF03533	Peptidase S49	8.14	6.03	38336.1	2	NO	Unknown	2-D LC
516	ORF03386	DNA gyrase, B subunit	8.14	1.24	89927.4	2	L	Unknown	2-D LC
517	ORF03348	Type I restriction-modification system, R subunit	8.13	3.99	70241.8	2	L	Unknown	2-D LC
518	ORF02985	Hypothetical protein	8.13	86.49	3802.4	2	No hits	Unknown	2-D LC
519	ORF03665	Na+/alanine symporter	8.13	32.1	9203.0	1	E	Unknown	2-D LC
520	ORF03531	DNA topoisomerase I	8.13	1.27	96608.1	1	L	Unknown	2-D LC
521	ORF03679	Riboflavin biosynthesis protein RibF	8.13	6.37	34615.4	1	H	Cytoplasmic	2-D LC
522	ORF02100	Succinate dehydrogenase, flavoprotein subunit	8.13	2.89	64459.6	1	C	Periplasmic	2-D LC
523	ORF00273	Hypothetical protein	8.13	4.86	56371.7	1	NO related COG	OuterMembrane	2-D LC
524	ORF02028	Nitrate/nitrite response regulator protein NarP	8.13	10.7	23598.8	1	TK	Cytoplasmic	2-D LC

525	ORF03282	Carbohydrate kinase family	8.13	4.63	51391.9	1	G	Unknown	2-D LC
526	ORF02445	Methyl-accepting chemotaxis protein I	8.13	5.33	55705.4	1	N	CytoplasmicMembrane	2-D LC
527	ORF01031	Aspartate/ornithine carbamoyltransferase, Asp/Orn binding domain family	8.13	2.53	44003.2	2	E	Cytoplasmic	2-D LC
528	ORF00365	GTP cyclohydrolase II	8.13	4.59	21801.9	1	H	Cytoplasmic	2-D LC
529	ORF02556	Nicotinate phosphoribosyltransferase	8.13	7.23	45537.8	1	H	Unknown	2-D LC
530	ORF01361	Hypothetical protein	8.13	16.52	25792.9	1	NO related COG	Unknown	2-D LC
531	ORF00302	Peptidase U32	8.13	3.67	73205.3	2	O	Unknown	2-D LC
532	ORF03156	Lipid A biosynthesis lauroyl acyltransferase	8.13	3.56	36082.1	1	N	CytoplasmicMembrane	2-D LC
533	ORF00720	Pyruvate kinase	8.13	4.58	51798.1	1	G	Cytoplasmic	2-D LC
534	ORF01528	Acyl carrier protein phosphodiesterase	8.13	7.73	24132.7	1	S	Unknown	2-D LC
535	ORF01365	Phage tail tape measure protein, core region	8.13	2.45	101427.0	1	N	CytoplasmicMembrane	2-D LC
536	ORF01567	Transcriptional regulator	8.13	3.9	34492.0	2	K	Cytoplasmic	2-D LC
537	ORF02157	Glutamate-aspartate ABC transporter ATP-binding component GltL	8.13	12.86	26504.6	1	E	Cytoplasmic	2-D LC
538	ORF02275	CTP synthase	8.13	5.87	60038.5	1	F	Cytoplasmic	Both
539	ORF02329	Hypothetical protein	8.13	15.29	17736.4	1	No related COGs	Unknown	2-D LC
540	ORF03869	Integrase	8.13	18.33	6949.0	1	No related COGs	Unknown	2-D LC
541	ORF00888	ATP-dependent RNA helicase RhlB	8.13	5.19	47177.6	1	LKJ	Unknown	Both
542	ORF02071	Multidrug resistance protein MdtC	8.13	1.66	110509.1	1	Q	CytoplasmicMembrane	2-D LC
543	ORF03661	Bifunctional aspartokinase/homoserine dehydrogenase I (AKI-HDI)	8.13	3.29	88244.9	1	E	Cytoplasmic	2-D LC
544	ORF00060	Glutaminase 1	8.13	8.04	32905.2	1	E	Unknown	2-D LC
545	ORF01166	Hypothetical protein	8.13	32.47	8633.9	1	No related COGs	Unknown	2-D LC
546	ORF00010	Glycoside hydrolase, family 3,	8.13	0.97	179206.9	1	G	Unknown	2-D LC

		N-terminal:Glycoside hydrolase, family 3, C-terminal							
547	ORF00061	DNA polymerase IV	8.13	9.74	17571.2	1	L	Cytoplasmic	2-D LC
548	ORF03098	Putative N-acetylmannosamine-6-phosphate 2-epimerase	8.13	7.23	25238.5	1	G	Unknown	2-D LC
549	ORF02734	Hypothetical protein	8.13	69.23	4387.2	1	No related COGs	Unknown	2-D LC
550	ORF03559	Sulfate permease family protein	8.13	2.23	63116.4	1	P	CytoplasmicMembrane	2-D LC
551	ORF01502	Exodeoxyribonuclease VII, small subunit	8.13	25	8888.9	8	L	Unknown	2-D LC
552	ORF03850	Ribonuclease HII	8.13	12.18	21297.6	11	L	Cytoplasmic	2-D LC
553	ORF01570	Putative glucokinase regulator homolog	8.13	6	31417.0	1	R	Unknown	2-D LC
554	ORF01711	ATPase	8.13	5.62	27339.3	1	G	CytoplasmicMembrane	2-D LC
555	ORF00915	DNA helicase II	8.13	3.2	81613.0	1	L	Cytoplasmic	2-D LC
556	ORF01004	TRNA-dihydrouridine synthase B	8.13	6.29	37051.4	5	R	Cytoplasmic	2-D LC
557	ORF03668	Succinate-semialdehyde dehydrogenase	8.13	2.69	51696.8	1	C	Cytoplasmic	2-D LC
558	ORF03545	Conserved hypothetical protein	8.13	27.71	9292.7	1	NO related COG	Cytoplasmic	2-D LC
559	ORF00667	Pentapeptide mxkdx repeat protein	8.12	20.62	10650.4	1	NO related COG	Unknown	2-D LC
560	ORF03215	Cold-shock dead box protein a	8.12	4.42	71334.4	2	LKJ	Unknown	2-D LC
561	ORF01026	Xanthine dehydrogenase accessory factor, putative subfamily, putative	8.12	2.15	54736.7	1	O	Unknown	2-D LC
562	ORF03814	N-acetylmuramoyl-L-alanine amidase AmiC	8.12	3.93	42492.3	2	M	Unknown	2-D LC
563	ORF00552	Hypothetical protein	8.12		91914.9	3	No related COGs	Cytoplasmic	2-D LC
564	ORF00723	High-affinity zinc uptake system protein ZnuA	8.12	5.69	36451.3	1	P	Periplasmic	2-D LC
565	ORF02706	Outer membrane protein N	8.12	2.95	40953.6	4	M	OuterMembrane	Both
566	ORF01447	PalA	8.12	1.1	197099.0	1	M	OuterMembrane	2-D LC
567	ORF01351	Inosine-5'-monophosphate dehydrogenase	8.12	2.05	51764.0	4	F	Cytoplasmic	Both

568	ORF01845	Lipid A export permease/ATP-binding protein MsbA	8.12	2.23	64521.4	1	Q	CytoplasmicMembrane	2-D LC
569	ORF01766	3-oxoacyl-[acyl-carrier-protein] synthase 3	8.12	6.14	31200.0	5	I	CytoplasmicMembrane	2-D LC
570	ORF01454	Recombination protein RecR	8.12	7.96	21747.7	1	L	Cytoplasmic	2-D LC
571	ORF02973	Nitrate reductase, alpha subunit	8.12	0.74	136444.9	1	C	Unknown	2-D LC
572	ORF02689	Phenylalanyl-tRNA synthetase, beta subunit	8.12	1.51	86940.1	3	J	Cytoplasmic	2-D LC
573	ORF03076	Trp operon repressor	8.12	10.91	12522.2	4	K	Unknown	2-D LC
574	ORF01598	Putative transposase for insertion sequence element	8.12		11182.1	1	L	Cytoplasmic	2-D LC
575	ORF03189	Glucuronate isomerase	8.12	1.92	53291.3	1	G	Cytoplasmic	2-D LC
576	ORF00023	Inner membrane protein YfjD	8.12	3.27	47716.3	1	L	CytoplasmicMembrane	2-D LC
577	ORF02015	Dihydroxyacetone kinase	8.12	12	10508.8	4	G	Unknown	2-D LC
578	ORF01847	Integration host factor beta-subunit	8.12	21.21	7526.6	1	L	Cytoplasmic	2-D LC
579	ORF03461	Multidrug resistance protein MdtM	8.12	2.93	44525.8	1	GEPR	CytoplasmicMembrane	2-D LC
580	ORF02371	HTH-type transcriptional regulator MalT	8.12		103772.1	1	K	Cytoplasmic	2-D LC
581	ORF02289	Hypothetical protein	8.12	9.3	9449.1	1	NO related COG	Unknown	2-D LC
582	ORF00639	Regulatory protein	8.12	2.77	74791.7	1	N	CytoplasmicMembrane	2-D LC
583	ORF01976	GTP cyclohydrolase I	8.12	7.27	24617.2	1	H	Cytoplasmic	2-D LC
584	ORF03187	Altronate hydrolase	8.12	4.44	53985.1	1	G	Cytoplasmic	2-D LC
585	ORF00632	EsrB	8.12	5.14	23525.1	1	TK	Cytoplasmic	2-D LC
586	ORF00272	Transposase and inactivated derivatives	8.12	8.28	19655.5	1	L	Unknown	2-D LC
587	ORF01966	Exodeoxyribonuclease I	8.12	5.07	54060.0	1	L	Cytoplasmic	2-D LC
588	ORF00411	Ospd3	8.12	3.44	45860.2	1	No related COGs	Unknown	2-D LC
589	ORF01729	Type I restriction enzyme R protein (Hsdr)	8.12	0.97	118140.9	1	L	Cytoplasmic	2-D LC
590	ORF00746	Molecular chaperone	8.12	10.33	23942.6	1	No related COGs	Unknown	2-D LC
591	ORF01321	Cysteine desulfurase Iscs	8.12	3.47	44896.1	1	E	Cytoplasmic	Both
592	ORF00806	Biodegradative arginine decarboxylase	8.12		84224.0	1	E	Unknown	2-D LC
593	ORF03813	Amino-acid N-	8.12	2.27	48712.8	1	E	Cytoplasmic	2-D LC

		acetyltransferase							
594	ORF02032	Type V secretory pathway, adhesin AidA	8.12	1.06	113585.7	1	M	Unknown	2-D LC
595	ORF00602	Kila-N domain family	8.12	12.37	21080.4	1	NO related COG	Cytoplasmic	2-D LC
596	ORF02442	Histidine triad	8.12		17824.2	1	FGR	Cytoplasmic	2-D LC
597	ORF02512	Sensor protein CpxA	8.12	4.33	52103.3	1	T	CytoplasmicMembrane	2-D LC
598	ORF00447	NAD-dependent aldehyde dehydrogenase	8.12	3.48	50734.2	1	C	Cytoplasmic	2-D LC
599	ORF02946	NADH-quinone oxidoreductase, chain g	8.12	1.43	100390.1	1	C	Unknown	2-D LC
600	ORF02550	Flagellar hook-associated protein 3	8.11	3.54	33885.8	8	N	Extracellular	2-D LC
601	ORF03121	Conserved hypothetical protein	8.11		21264.7	1	S	Unknown	2-D LC
602	ORF02930	Fe-S-cluster-containing hydrogenase components 1	8.11	2.45	35588.4	2	C	Periplasmic	2-D LC
603	ORF01613	Large exoproteins involved in heme utilization or adhesion	8.11	2.43	82231.8	4	M	OuterMembrane	2-D LC
604	ORF00422	Erythronate-4-phosphate dehydrogenase	8.11	5.6	40520.4	4	E	Cytoplasmic	2-D LC
605	ORF02572	Conserved hypothetical protein	8.11	7.03	20521.7	1	NO related COG	Unknown	2-D LC
606	ORF00718	Glucose-6-phosphate 1-dehydrogenase	8.11	3.67	55848.1	2	G	Cytoplasmic	2-D LC
607	ORF01193	Conserved hypothetical protein	8.11	20	8663.9	7	No related COGs	Unknown	2-D LC
608	ORF03318	Conserved hypothetical protein	8.11	0.71	143437.8	1	No related COGs	Unknown	2-D LC
609	ORF00358	Transposase and inactivated derivatives	8.11	7.6	37651.6	1	L	Cytoplasmic	2-D LC
610	ORF02441	Oligopeptidase A	8.11	1.32	76328.9	1	E	Cytoplasmic	2-D LC
611	ORF03692	Carbamoyl-phosphate synthase, large subunit	8.11	1.68	117439.5	1	EF	Unknown	2-D LC
612	ORF00385	Transcription-repair coupling factor	8.11	1.73	129623.5	1	LK	CytoplasmicMembrane	2-D LC
613	ORF00754	Extracellular solute-binding protein, family 1	8.11	5.99	42173.7	5	N	Unknown	2-D LC
614	ORF02150	PhoH family protein	8.11	3.42	39422.8	1	T	Cytoplasmic	2-D LC
615	ORF01340	GTP-binding protein EngA	8.11	2.23	54982.4	1	R	Cytoplasmic	2-D LC

616	ORF03476	Soluble pyridine nucleotide transhydrogenase (STH)(NAD(P)(+) transhydrogenase [B-specific])	8.11	3.43	51648.0	1	C	Cytoplasmic	2-D LC
617	ORF03158	Adenylate cyclase, putative	8.11		48260.8	1	S	Unknown	2-D LC
618	ORF00762	Glutamyl-tRNA reductase	8.11	3.57	46407.5	1	H	Cytoplasmic	2-D LC
619	ORF03026	DNA topoisomerase IV, A subunit	8.11	2.5	84463.2	2	L	Cytoplasmic	2-D LC
620	ORF02319	Siroheme synthase	8.11	3.9	49998.2	1	H	Unknown	2-D LC
621	ORF03278	TRNA delta(2)-isopentenylpyrophosphate transferase	8.11	4.1	35479.4	3	J	Cytoplasmic	2-D LC
622	ORF02696	Choline transporter	8.11	19.23	5613.4	1	NO related COG	Unknown	2-D LC
623	ORF02694	Threonyl-tRNA synthetase	8.11	6.67	25523.1	2	J	Cytoplasmic	2-D LC
624	ORF01320	Iron-sulfur cluster assembly transcription factor IscR	8.11	10.3	17823.0	2	K	Cytoplasmic	2-D LC
625	ORF00245	Zn-finger domain associated with topoisomerase type I	8.11	11.29	20421.7	1	L	Cytoplasmic	2-D LC
626	ORF00570	Phage regulatory protein, Rha family, putative	8.11	3.46	29757.9	1	S	Unknown	2-D LC
627	ORF01816	Flagellar protein export atpase FliI	8.11	4.65	48649.2	1	N	Cytoplasmic	2-D LC
628	ORF00445	Phosphoenolpyruvate phosphomutase	8.11	5.92	33945.8	1	G	Cytoplasmic	Both
629	ORF01830	Chromosome partition protein MukB	8.11	0.94	168774.3	1	D	Cytoplasmic	2-D LC
630	ORF02576	Ion transport family protein, putative	8.11	4.74	28363.2	1	P	CytoplasmicMembrane	2-D LC
631	ORF03290	Putative lysyl-tRNA synthetase homolog GenX	8.11	5.23	36802.3	1	J	Cytoplasmic	2-D LC
632	ORF01114	Transcriptional activator of tdc operon	8.11	4.55	34751.0	3	K	Unknown	2-D LC
633	ORF02074	Transcriptional regulatory protein BaeR	8.11	4.94	27856.2	1	TK	Cytoplasmic	2-D LC
634	ORF03822	Putative RNA 2'-O-ribose methyltransferase MtfA	8.11	3.74	42673.9	1	R	Unknown	2-D LC
635	ORF03823	Exodeoxyribonuclease-9	8.11	9.49	27897.7	1	L	Cytoplasmic	2-D LC
636	ORF03174	N-6 DNA methylase	8.11	1.65	87656.0	1	L	Unknown	2-D LC

637	ORF02373	4-alpha-glucanotransferase	8.11	3.16	78404.6	1	G	Cytoplasmic	2-D LC
638	ORF03180	Putative ribosomal RNA small subunit methyltransferase D (rrna (guanine-N(2)-)-methyltransferase)	8.11	5.25	41599.4	1	J	Unknown	2-D LC
639	ORF02374	Glucose-1-phosphate adenylyltransferase	8.11	4.34	48783.3	1	G	Cytoplasmic	2-D LC
640	ORF02096	Succinyl-coa synthetase beta chain	8.11	2.58	41349.1	1	C	Cytoplasmic	2-D LC
641	ORF01105	Chemotaxis protein CheZ	8.11	7.51	24098.2	1	N	Cytoplasmic	2-D LC
642	ORF03210	STM-proteaseA	8.11	5.2	29581.4	1	O	Cytoplasmic	2-D LC
643	ORF02703	6-phosphofructokinase isozyme 2	8.11	7.26	33033.6	1	G	Unknown	2-D LC
644	ORF03522	Isocitrate dehydrogenase kinase/phosphatase	8.11	4.09	20307.0	2	NO related COG	Cytoplasmic	2-D LC
645	ORF00522	Cation efflux system protein CusA	8.11	1.42	114891.7	1	P	CytoplasmicMembrane	2-D LC
646	ORF01697	Hypothetical protein	8.11	12.5	5900.9	1	NO related COG	Unknown	2-D LC
647	ORF03194	Uxu operon transcriptional regulator	8.11	9.02	29383.2	1	K	Cytoplasmic	2-D LC
648	ORF03622	Hydrogenase accessory protein HypB	8.11	5.11	39208.9	2	OK	Cytoplasmic	2-D LC
649	ORF03081	Phosphoserine phosphatase	8.11	5.85	35180.5	1	E	Cytoplasmic	2-D LC
650	ORF00382	NADH dehydrogenase	8.11	4.38	46795.6	1	C	Unknown	2-D LC
651	ORF03736	UDP-N-acetylmuramoylalanine--D-glutamate ligase	8.11	3.2	47462.2	1	M	Unknown	Both
652	ORF00351	Penicillin-binding protein 6	8.11	4.44	49015.2	1	M	CytoplasmicMembrane	2-D LC
653	ORF00702	Septum site-determining protein MinC	8.10		21657.7	1	D	Unknown	2-D LC
654	ORF02085	YbgF protein	8.10	5.16	27337.2	1	S	Unknown	2-D LC
655	ORF01282	TRNA-dihydrouridine synthase C	8.10	5.03	35395.0	1	R	Cytoplasmic	2-D LC
656	ORF01527	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	8.10	3.36	39577.6	1	J	Unknown	2-D LC
657	ORF02652	Protein AegA	8.10	3.24	73491.6	1	ER	Cytoplasmic	2-D LC
658	ORF01119	Transcriptional regulator	8.10	6.35	33610.9	1	K	Cytoplasmic	2-D LC
659	ORF02498	1,4-dihydroxy-2-naphthoate	8.10	7.54	32745.7	1	H	CytoplasmicMembrane	2-D LC

		octaprenyltransferase							
660	ORF00251	Hypothetical protein	8.10	2.95	45029.4	1	NO related COG	Unknown	2-D LC
661	ORF03375	DNA-3-methyladenine glycosylase 1	8.10		22123.2	2	L	Unknown	2-D LC
662	ORF02585	Protein YcbG	8.10	11.84	18313.9	1	S	Cytoplasmic	2-D LC
663	ORF02377	Glycogen debranching enzyme GlgX	8.10	3.68	64613.1	1	G	Cytoplasmic	2-D LC
664	ORF01888	Homolog of VirK	8.10	3.46	35869.2	1	S	Unknown	2-D LC
665	ORF01551	GTPase subunit of restriction endonuclease	8.10	1.07	95221.3	1	L	Unknown	2-D LC
666	ORF03095	Arylsulfate sulfotransferase	8.10	3.88	74831.2	1	No related COGs	Unknown	2-D LC
667	ORF03704	RNA polymerase-associated protein rapa	8.10	2.42	107410.1	1	KL	Cytoplasmic	2-D LC
668	ORF00581	Putative phage portal protein	8.10	1.99	79033.1	1	No related COGs	Cytoplasmic	2-D LC
669	ORF01831	Chromosome partition protein MukE	8.10	9.48	26446.6	1	D	Cytoplasmic	2-D LC
670	ORF02104	Endonuclease VIII (dna glycosylase/ap lyase nei) (dna-(apurinic or apyrimidinic site) lyase nei)	8.10	3.09	31970.6	1	L	Cytoplasmic	2-D LC
671	ORF01057	Insertion sequence from sara17	8.10	8.77	12982.0	1	L	Cytoplasmic	2-D LC
672	ORF03842	Membrane-associated zinc metalloprotease, putative	8.10	4.77	47768.5	1	M	CytoplasmicMembrane	2-D LC
673	ORF03062	Pantothenate kinase	8.10	2.86	36093.0	1	H	Cytoplasmic	2-D LC
674	ORF00028	TRNA (guanine-N1)-methyltransferase	8.10	6.34	30002.9	1	J	Cytoplasmic	2-D LC
675	ORF00040	Hypothetical protein	8.08	23.4	5676.4	1	No related COGs	Unknown	2-D LC
676	ORF02714	Protein YqfB	8.08		12127.9	1	S	Unknown	2-D LC
677	ORF01971	NAD-dependent malic enzyme	6.17	3.36	62918.3	1	C	Unknown	Both
678	ORF03552	Tryptophan synthase, beta subunit	6.16	3.02	42896.6	3	E	Unknown	2-D LC
679	ORF00397	Protein /y1798/	6.15	9.62	22789.2	1	R	Unknown	2-D LC
680	ORF00036	Phospho-2-dehydro-3-deoxyheptonate aldolase	6.15		39331.6	1	E	Cytoplasmic	2-D LC
681	ORF03914	L-carnitine dehydratase/bile	6.14	4.53	43651.9	1	C	Cytoplasmic	2-D LC

		acid-inducible protein F							
682	ORF01040	Protein YgiQ	6.14	4.13	84168.1	1	C	Unknown	2-D LC
683	ORF02196	ModD protein	6.14	4.96	30554.1	1	H	Cytoplasmic	2-D LC
684	ORF03857	Lysine decarboxylase, constitutive	6.14	4.06	80936.1	2	E	Unknown	2-D LC
685	ORF03899	Putative L-2,4-diaminobutyrate decarboxylase	6.14		63875.3	1	E	Unknown	2-D LC
686	ORF02745	YcjX	6.13	3	52794.4	1	R	Cytoplasmic	2-D LC
687	ORF03377	Glycyl-tRNA synthetase, alpha subunit	6.13	6.62	34668.3	1	J	Cytoplasmic	2-D LC
688	ORF02916	O-succinylbenzoate-CoA ligase	6.13		50321.7	1	IQ	Cytoplasmic	2-D LC
689	ORF02094	Cytochrome D ubiquinol oxidase subunit 1	6.13	4.23	58081.5	2	C	CytoplasmicMembrane	2-D LC
690	ORF03725	Acetolactate synthase, large subunit, biosynthetic type	6.13	3.5	62431.3	1	No related COGs	Unknown	2-D LC
691	ORF01709	Alpha-galactosidase	6.13	6.75	50674.2	1	G	Cytoplasmic	2-D LC
692	ORF02416	Formate dehydrogenase, alpha subunit	6.13	3.73	89487.5	1	C	Periplasmic	2-D LC
693	ORF01815	Flagellar assembly protein FliH	6.13	9.96	27640.1	1	N	Unknown	2-D LC
694	ORF02079	Transcriptional regulator, LysR family protein	6.13	7.96	34480.6	1	K	CytoplasmicMembrane	2-D LC
695	ORF03740	D-alanine--D-alanine ligase B	6.13	2.67	32683.1	2	M	Cytoplasmic	2-D LC
696	ORF03168	DNA primase	6.13	3.51	66827.0	1	L	Cytoplasmic	2-D LC
697	ORF03268	Ribonuclease R	6.13	2.7	91535.8	1	K	Cytoplasmic	2-D LC
698	ORF02613	Sucrose-6 phosphate hydrolase	6.13	26.13	12437.4	1	NO related COG	CytoplasmicMembrane	2-D LC
699	ORF00079	Inner membrane protein YpjD	6.13	12.6	28967.6	1	O	CytoplasmicMembrane	2-D LC
700	ORF02557	Aminopeptidase N	6.13	3.09	98643.9	1	E	Cytoplasmic	2-D LC
701	ORF03597	Cell division protein FtsB	6.12	12.28	12747.4	1	O	Unknown	2-D LC
702	ORF01501	ABC transporter	6.12	7.57	26929.0	1	P	Unknown	2-D LC
703	ORF01135	Excinuclease ABC, C subunit	6.12	3.28	69065.8	2	L	Cytoplasmic	2-D LC
704	ORF00012	Protein RecA	6.12		37635.6	1	L	Cytoplasmic	2-D LC
705	ORF00470	Secreted effector protein	6.12	2.1	69409.9	1	No related COGs	Unknown	2-D LC
706	ORF03137	Conserved hypothetical protein	6.12	3.66	40030.5	1	No related COGs	Cytoplasmic	2-D LC
707	ORF02117	Potassium-transporting ATPase A chain	6.12	13.04	14060.4	2	P	CytoplasmicMembrane	2-D LC

708	ORF03605	AAS bifunctional protein	6.12	1.81	79079.4	1	IQ	CytoplasmicMembrane	2-D LC
709	ORF00751	Putative sensory box/ggdef family protein	6.12	2.54	54524.2	1	T	Unknown	2-D LC
710	ORF02570	Issod12, transposase	6.11		6174.4	2	NO related COG	Unknown	2-D LC
711	ORF02995	Sensor kinase DpiB	6.11	3.68	60129.9	1	T	CytoplasmicMembrane	2-D LC
712	ORF03412	UDP-N-acetylglucosamine pyrophosphorylase	6.11	2.41	48715.4	2	M	Cytoplasmic	2-D LC
713	ORF03654	Rrna methylases	6.11	3.64	38535.5	1	J	Unknown	Both
714	ORF01377	Conserved hypothetical protein	6.11	17.35	11056.7	1	No related COGs	Unknown	2-D LC
715	ORF02535	Flagellar biosynthesis protein FlhA	6.11	2.87	75240.5	2	N	CytoplasmicMembrane	2-D LC
716	ORF03254	3'(2'),5'-bisphosphate nucleotidase	6.11	4.88	26910.7	1	G	Unknown	2-D LC
717	ORF02899	DNA gyrase, A subunit	6.11	1.59	96944.7	1	L	Cytoplasmic	2-D LC
718	ORF00548	RecT protein	6.11	4.21	39250.0	1	No related COGs	Cytoplasmic	2-D LC
719	ORF01936	Nucleotide sugar epimerase	6.11	4.78	37043.7	1	M	Unknown	2-D LC
720	ORF00593	Conserved hypothetical protein	6.11	2.73	54959.2	2	No related COGs	Unknown	2-D LC
721	ORF01407	Deoxyguanosinetriphosphate triphosphohydrolase	6.11	3.54	58567.5	2	F	Cytoplasmic	2-D LC
722	ORF02106	Ribosomal-protein-serine acetyltransferase	6.11	8.99	20256.1	1	J	Cytoplasmic	2-D LC
723	ORF00404	Hypothetical protein	6.11	15.66	9159.4	1	No related COGs	Cytoplasmic	2-D LC
724	ORF02578	Methyltransferase	6.11	3.4	79359.7	1	L	Unknown	2-D LC
725	ORF02727	4Fe-4S binding domain protein	6.11	9.47	21460.1	1	C	Cytoplasmic	2-D LC
726	ORF00476	Protein mlc	6.11	3.19	44288.7	1	K	Cytoplasmic	2-D LC
727	ORF02161	Leucyl-tRNA synthetase	6.11	2.56	97159.0	1	J	Cytoplasmic	2-D LC
728	ORF01254	Transposon Tn10 tetd protein	6.11	14.75	14208.2	1	K	Cytoplasmic	2-D LC
729	ORF03428	ATPase rava	6.11	2.98	57841.8	1	R	Cytoplasmic	2-D LC
730	ORF03169	RNA polymerase sigma factor RpoD	6.11	1.64	70169.0	2	K	Cytoplasmic	2-D LC
731	ORF03711	ABC-type multidrug efflux pump	6.10		31430.7	1	PH	CytoplasmicMembrane	2-D LC
732	ORF02857	Hypothetical protein	6.10	37.84	4458.2	1	No related COGs	Unknown	2-D LC

733	ORF02507	Bifunctional aspartokinase/homoserine dehydrogenase II	6.10	1.48	89211.7	1	E	Unknown	2-D LC
734	ORF00119	Conserved hypothetical protein	6.10	5.56	26537.2	1	No related COGs	Unknown	2-D LC
735	ORF00873	2-amino-3-ketobutyrate coenzyme A ligase	6.10	4.27	42774.6	1	H	CytoplasmicMembrane	2-D LC
736	ORF03502	Putative cytoplasmic protein	6.10	4.44	30143.8	1	S	Cytoplasmic	2-D LC
737	ORF01503	Geranyltranstransferase	6.10	2.66	31934.2	1	O	Cytoplasmic	2-D LC
738	ORF01743	Inner membrane protein YjgP	6.10	3.55	40515.7	1	R	CytoplasmicMembrane	2-D LC
739	ORF02684	Biotin carboxylase	6.09	3.15	46560.1	1	I	Unknown	2-D LC
740	ORF02513	RNA methyltransferase, TrmH family, group 2	6.08	4.46	17764.2	1	J	Unknown	2-D LC

APPENDIX B

EDWARDSIELLA ICTALURI PROTEINS IDENTIFIED USING 2-DE

SSP#	ORF #	Protein MW/pI	C.I. %	Protein name	Subcellular location	COG category	COG#	Identified by
2	ORF03829	16088/4.42	100	Flavodoxins	Cytoplasmic	C	COG0716	Both
3	ORF03052	7533.2/7.93	100	Ribosomal protein L7/L12 C-terminal domain	Unknown	J	COG0222	Both
107	ORF02000	18370.8/4.76	100	Glucose-specific phosphotransferase enzyme IIA component	Cytoplasmic	G	COG2190	Both
206	ORF03257	22264.4/4.79	100	Fkbp-type 22 kda peptidyl-prolyl cis-trans isomerase	OuterMembrane	O	COG0545	Both
403	ORF03061	29946.3/5.26	100	Gtpases - translation elongation factors	Cytoplasmic	JE	COG0050	Both
501	ORF02553	40822.9/5.46	100	Outer membrane protein F	OuterMembrane	No Related COG	NO related COG	Both
502	ORF03743	40511.6/4.63	100	Cell division protein FtsZ	CytoplasmicMembrane	C	COG0508	Both
706	ORF03304	57357.8/4.84	100	Chaperonin GroL	Cytoplasmic	O	COG0459	Both
711	ORF02587	38075.3/8.79	100	Outer membrane protein A	OuterMembrane	M	COG2885	Both
804	ORF03304	57357.8/4.84	100	Chaperonin GroL	Cytoplasmic	O	COG0459	Both
807	ORF03223	54698/4.57	100	Transcription elongation factor	Cytoplasmic	K	COG0195	Both
1002	ORF02432	16087/4.8	100	Universal stress protein A	Cytoplasmic	T	COG0589	Both
1003	ORF00975	13529.1/5.06	100	Endoribonuclease L-PSP, putative	Unknown	J	COG0251	Only 2-DE
1006	ORF00889	11580.1/5.04	100	Thioredoxin	Cytoplasmic	OC	COG0526	Both
1008	ORF00507	14792.5/5.61	100	Lactoylglutathione lyase	Unknown	E	COG0346	Only 2-DE
1106	ORF00646	17503.6/4.84	100	Type III secretion low calcium response chaperone lcrh/sycd	Unknown	R	COG0457	Both
1108	ORF02741	17742.2/4.95	100	Thiol peroxidase	Unknown	O	COG2077	Both
1111	ORF03579	12357.4/5.12	100	DNA-binding protein H-NS	Cytoplasmic	R	COG2916	Only 2-DE
1112	ORF01488	48043.6/4.77	100	Trigger factor	Cytoplasmic	O	COG0544	Both
1206	ORF03247	19664/4.99	100	Inorganic pyrophosphatase	Cytoplasmic	C	COG0221	Both

1208	ORF00715	22005.7/5.04	100	Aldolase	Cytoplasmic	G	COG0800	Only 2-DE
1210	ORF02980	22466.9/5.74	100	Tia invasion determinant	OuterMembrane	M	COG3637	Both
1303	ORF01000	49416.4/6.84	100	Acetyl-coa carboxylase, biotin carboxylase	Cytoplasmic	I	COG0439	Only 2-DE
1304	ORF03304	57357.8/4.84	100	Chaperonin GroL	Cytoplasmic	O	COG0459	Both
1404	ORF03061	29946.3/5.26	100	Gtpases - translation elongation factors	Cytoplasmic	JE	COG0050	Both
1409	ORF00703	29686.5/4.99	100	Septum site-determining protein MinD	Cytoplasmic	D	COG2894	Only 2-DE
1411	ORF02980	22466.9/5.74	100	Ttia invasion determinant	OuterMembrane	M	COG3637	Both
1503	ORF02706	40928.8/5.17	100	Outer membrane protein N	OuterMembrane	NO related COG	NO related COG	Both
1507	ORF00232	36497.1/5.03	100	DNA-directed RNA polymerase, alpha subunit	Cytoplasmic	K	COG0202	Both
1604	ORF03414	50011.7/4.94	100	ATP synthase F1, beta subunit	Cytoplasmic	C	COG0055	Both
1701	ORF03304	57357.8/4.84	100	Chaperonin GroL	Cytoplasmic	O	COG0459	Both
1703	ORF01196	17770.6/5.21	100	EvpC	Unknown	NO related COG	NO related COG	Both
1801	ORF03674	68467.3/4.79	100	Chaperone protein Dnak	Cytoplasmic	O	COG0443	Both
1802	ORF03517	70579.9/5	100	Chaperone protein htpg	Cytoplasmic	O	COG0326	Both
1804	ORF03455	51646.5/5.15	100	Glutamine synthetase, type I	Cytoplasmic	E	COG0174	Both
1805	ORF02980	22466.9/5.74	100	Tia invasion determinant	OuterMembrane	M	COG3637	Both
2103	ORF02276	45717.4/5.32	100	Phosphopyruvate hydratase	Cytoplasmic	G	COG0148	Both
2105	ORF01194	19363.8/5.29	100	EvpA	Cytoplasmic	S	COG3516	Both
2108	ORF03262	15260.4/5.29	100	Ribosomal protein S6	Cytoplasmic	J	COG0360	Both
2202	ORF01529	22199/5.21	100	Peroxiredoxin	Cytoplasmic	O	COG0450	Both

2203	ORF00210	22068.8/9.72	100	Ribosomal protein L4/L1 family	Unknown	J	COG0088	Only 2-DE
2206	ORF03102	24436.6/5.35	100	Glutathione S-transferase	Cytoplasmic	O	COG0625	Both
2304	ORF03515	23478.1/5.32	100	Adenylate kinase	Cytoplasmic	F	COG0563	Both
2305	ORF01420	31395.6/5.35	100	Alpha/beta hydrolase fold	Unknown	R	COG1073	Both
2404	ORF03241	32328.2/5.21	100	Malate dehydrogenase, NAD-dependent	Unknown	C	COG0039	Both
2407	ORF03667	34821.1/5.46	100	Transaldolase	Unknown	G	COG0176	Both
2502	ORF00780	35245.1/5.28	100	Glutathione synthase	Unknown	HJ	COG0189	Both
2505	ORF01401	45834.9/5.34	100	Glutamate-1-semialdehyde-2,1-aminomutase	Cytoplasmic	H	COG0001	Both
2507	ORF00872	34791/5.29	100	ADP-L-glycero-D-manno-heptose-6-epimerase	Unknown	MG	COG0451	Only 2-DE
2601	ORF02295	23616.7/4.81	100	GTPases - translation elongation factors	Cytoplasmic	JE	COG0050	Both
2607	ORF01840	28153.4/5.34	100	3-deoxy-D-manno-octulosonate cytidylyltransferase	Cytoplasmic	M	COG1212	Both
2706	ORF02097	43172.6/5.42	100	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase	Cytoplasmic	C	COG0508	Both
2803	ORF02296	77423.2/5.2	100	Translation elongation factor G	Cytoplasmic	J	COG0480	Both
2804	ORF01622	57702.8/5.21	100	Lysyl-trna synthetase	Cytoplasmic	J	COG1190	Both
2813	ORF03079	61194.6/5.33	100	Atpase components of ABC transporters with duplicated atpase domains	Cytoplasmic	R	COG0488	Only 2-DE
3003	ORF00704	10213.5/5.38	100	Cell division topological specificity factor MinE	Cytoplasmic	D	COG0851	Only 2-DE
3101	ORF03543	27900.5/8.98	100	Amino-acid ABC transporter periplasmic component	Periplasmic	E	COG0834	Both
3105	ORF02907	16973.5/5.57	100	Molybdopterin converting factor, subunit 2	Unknown	H	COG0314	Only 2-DE
3106	ORF02291	18179.9/5.5	100	Peptidyl-prolyl cis-trans isomerase B	Cytoplasmic	O	COG0652	Both
3107	ORF00081	18959.4/5.6	100	S-Ribosylhomocysteinase (luxs)	Cytoplasmic	T	COG1854	Only 2-DE
3108	ORF03223	54698/4.57	100	Transcription elongation factor	Cytoplasmic	K	COG0195	Both

3202	ORF02587	38075.3/8.79	100	Outer membrane protein A	OuterMembrane	M	COG2885	Both
3204	ORF01416	19813.8/5.41	100	Ferritin and Dps	Cytoplasmic	L	COG0783	Both
3206	ORF02486	26689.7/5.49	100	Triosephosphate isomerase	Unknown	G	COG0149	Both
3209	ORF02049	26448.6/8.39	100	Uracil phosphoribosyltransferase	Cytoplasmic	F	COG0035	Only 2-DE
3210	ORF01487	22936.5/5.5	100	ATP-dependent Clp protease, proteolytic subunit ClpP	Cytoplasmic	NO	COG0740	Both
3301	ORF03132	25636.8/5.4	100	Purine nucleoside phosphorylase	Cytoplasmic	F	COG0813	Both
3303	ORF03658	27298/5.47	100	Aerobic respiration control protein arca	Cytoplasmic	TK	COG0745	Both
3306	ORF00393	25480.3/5.62	99.98	Transcriptional regulatory protein PhoP	Cytoplasmic	TK	COG0745	Both
3307	ORF01051	32859/7.82	100	Probable N-acetylmuramoyl-L-alanine amidase YbjR	Cytoplasmic	M	COG3023	Both
3403	ORF01880	34536.4/5.59	100	Thioredoxin-disulfide reductase	Unknown	O	COG0492	Both
3404	ORF03835	30581.8/5.54	100	Translation elongation factor Ts	Cytoplasmic	J	COG0264	Both
3406	ORF03831	29907.4/5.57	100	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	Cytoplasmic	E	COG2171	Both
3503	ORF01652	40900.6/5.45	100	Phosphoglycerate kinase	Cytoplasmic	G	COG0126	Both
3506	ORF01652	40900.6/5.45	100	Phosphoglycerate kinase	Cytoplasmic	G	COG0126	Both
3602	ORF02276	45717.4/5.32	100	Phosphopyruvate hydratase	Cytoplasmic	G	COG0148	Both
3603	ORF01874	48696.8/5.47	100	Seryl-tRNA synthetase	Cytoplasmic	J	COG0172	Only 2-DE
3706	ORF03762	53500/6.17	100	Dihydrolipoamide dehydrogenase	Cytoplasmic	C	COG1249	Both
3712	ORF01971	62879/5.57	100	NAD-dependent malic enzyme	Unknown	C	COG0281	Both
3802	ORF03761	55071.2/5.49	100	Pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase	Cytoplasmic	D	COG0206	Both
4001	ORF01998	9055.7/5.61	100	Phosphocarrier protein Hpr	Cytoplasmic	G	COG1925	Both
4002	ORF01508	16136.6/5.65	100	6,7-dimethyl-8-ribityllumazine synthase	Unknown	H	COG0054	Both

4003	ORF01776	10562.4/5.74	100	Antibiotic biosynthesis monooxygenase	Cytoplasmic	S	COG1359	Both
4101	ORF01332	15676.8/5.62	100	Nucleoside diphosphate kinase	Cytoplasmic	F	COG0105	Both
4102	ORF03305	10333.6/5.66	100	Chaperonin GroS	Cytoplasmic	O	COG0234	Both
4103	ORF00081	18959.4/5.6	100	S-Ribosylhomocysteinase (luxs)	Cytoplasmic	T	COG1854	Only 2-DE
4108	ORF03199	10929.6/5.89	100	Protein ElaB	Cytoplasmic	NO related COG	NO related COG	Both
4205	ORF01493	18390.7/5.63	100	Protein /y1016/	Cytoplasmic	S	COG1666	Both
4208	ORF02587	38075.3/8.79	100	Outer membrane protein A	OuterMembrane	M	COG2885	Both
4302	ORF01724	28647.3/6.6	100	Negative regulator of beta-lactamase expression	Cytoplasmic	M	COG3023	Both
4402	ORF01880	34536.4/5.59	100	Thioredoxin-disulfide reductase	Unknown	O	COG0492	Both
4404	ORF00848	25221/5.67	100	Ribonuclease PH	Cytoplasmic	J	COG0689	Only 2-DE
4503	ORF01651	39129.7/5.65	100	Fructose-bisphosphate aldolase, class II	Unknown	G	COG0191	Both
4601	ORF01929	51300.3/5.49	100	6-phosphogluconate dehydrogenase, decarboxylating protein	Unknown	G	COG0362	Only 2-DE
4603	ORF01762	42174.9/5.34	100	3-oxoacyl-[acyl-carrier-protein] synthase 2	CytoplasmicMembrane	IQ	COG0304	Only 2-DE
4604	ORF02276	45717.4/5.32	100	Phosphopyruvate hydratase	Cytoplasmic	G	COG0148	Both
4607	ORF00501	47648/5.61	100	Tyrosyl-tRNA synthetase	Cytoplasmic	J	COG0162	Both
4701	ORF03416	55190.7/5.59	100	ATP synthase F1, alpha subunit	Unknown	C	COG0056	Both
4703	ORF03762	53500/6.17	100	Dihydrolipoamide dehydrogenase	Cytoplasmic	C	COG1249	Both
4706	ORF02735	50777.3/5.74	100	Pyruvate kinase	Cytoplasmic	G	COG0469	Both
4803	ORF02275	60000.8/5.64	100	CTP synthase	Cytoplasmic	F	COG0504	Both
4804	ORF02610	73954/5.67	100	Bifunctional polymyxin resistance arna protein	Cytoplasmic	MG	COG0451	Both
4805	ORF01856	85051.6/5.65	100	Formate acetyltransferase	Cytoplasmic	C	COG1882	Both

4807	ORF02610	73954/5.67	100	Bifunctional polymyxin resistance arna protein	Cytoplasmic	MG	COG0451	Both
4808	ORF02137	63441.9/5.79	100	Glutaminyl-tRNA synthetase	Cytoplasmic	J	COG0008	Both
5001	ORF03176	14512.3/6.82	100	Conserved hypothetical protein	Unknown	S	COG3111	Both
5003	ORF01507	15774.2/5.89	100	Transcription antitermination factor NusB	Cytoplasmic	K	COG0781	Only 2-DE
5004	ORF00649	14631.6/5.92	100	EseE	Cytoplasmic	NO related COG	NO related COG	Both
5006	ORF03413	15169.9/5.95	100	ATP synthase F1, epsilon subunit	Cytoplasmic	C	COG0355	Both
5103	ORF02977	21133.7/6.84	100	Copper/zinc superoxide dismutase	Periplasmic	P	COG2032	Both
5104	ORF02500	17549.1/5.2	100	ATP-dependent protease HslV	Cytoplasmic	O	COG0638	Both
5203	ORF01923	26892.7/5.98	100	Oxygen-insensitive NADPH nitroreductase	Cytoplasmic	C	COG0778	Only 2-DE
5204	ORF01764	25567.1/5.95	100	3-oxoacyl-(acyl-carrier-protein) reductase	Cytoplasmic	QR	COG1028	Both
5302	ORF02267	26208.6/5.91	100	Pyridoxal phosphate biosynthetic protein PdxJ	Cytoplasmic	H	COG0854	Only 2-DE
5303	ORF02095	29553.4/6.07	100	Succinyl-CoA synthetase alpha chain	CytoplasmicMembrane	C	COG0074	Both
5401	ORF00445	33924.6/5.88	100	Phosphoenolpyruvate Phosphomutase	Cytoplasmic	G	COG2513	Both
5403	ORF00880	33910/6.06	100	Branched-chain amino acid aminotransferase	Cytoplasmic	EH	COG0115	Both
5406	ORF01996	34119/6.04	99.95	Cysteine synthase A	Unknown	E	COG0031	Both
5508	ORF00684	35427/6.33	100	Glyceraldehyde-3-phosphate dehydrogenase, type I	Cytoplasmic	Anonymous	Anonymous	Both
5601	ORF01321	44867.9/5.85	100	Cysteine desulfurase IscS	Cytoplasmic	E	COG1104	Both
5605	ORF03032	51501.1/7.68	100	Outer membrane protein TolC	OuterMembrane	MN	COG1538	Only 2-DE
5606	ORF03700	47827.6/6.34	100	Chaperone sura	Periplasmic	O	COG0760	Both
5703	ORF01695	66209/5.98	99.94	Hydrogenase-1 large chain	Periplasmic	C	COG0374	Only 2-DE
5708	ORF01996	34119/6.04	100	Cysteine synthase A	Unknown	E	COG0031	Both

5802	ORF01762	42174.9/5.34	100	3-oxoacyl-[acyl-carrier-protein] synthase 2	CytoplasmicMembrane	IQ	COG0304	Only 2-DE
6003	ORF01863	9380.8/8.09	99.03	Conserved hypothetical protein	Unknown	NO related COG	NO related COG	Only 2-DE
6004	ORF00196	7420.8/6.71	100	Conserved domain protein	Cytoplasmic	K	COG1278	Only 2-DE
6007	ORF00196	7420.8/6.71	100	Conserved domain protein	Cytoplasmic	K	COG1278	Only 2-DE
6101	ORF00458	19476.6/6.07	100	Protein YfbU	Cytoplasmic	S	COG3013	Only 2-DE
6103	ORF02086	18727.3/6.3	100	Peptidoglycan-associated lipoprotein	OuterMembrane	M	COG2885	Both
6104	ORF03057	7922.1/5.04	100	Transcription antiterminator	Unknown	K	COG0250	Both
6106	ORF02583	18779.6/6.3	100	Beta-hydroxyacyl-(acyl-carrier-protein dehydratase FabA	Cytoplasmic	I	COG0764	Only 2-DE
6206	ORF02764	22378/6.24	100	NAD	Cytoplasmic	R	COG2249	Both
6208	ORF03251	23380.2/6.37	100	Methionine-S-sulfoxide reductase	Unknown	O	COG0225	Both
6301	ORF00931	27335.9/6.07	100	Uridine phosphorylase	Cytoplasmic	F	COG2820	Both
6302	ORF02587	38075.3/8.79	100	Outer membrane protein A	Cytoplasmic	M	COG2885	Both
6305	ORF03267	27014.1/6.09	100	RNA methyltransferase, trmh family, group 3	Cytoplasmic	J	COG0566	Only 2-DE
6409	ORF00685	32934.4/6.14	100	Aldose 1-epimerase	Unknown	G	COG0676	Both
6411	ORF00758	25952.3/8.74	100	3-deoxy-8-phosphooctulonate synthase	Cytoplasmic	M	COG2877	Both
6501	ORF01638	40911.7/6.05	100	Glycine cleavage system T protein	Cytoplasmic	E	COG0404	Both
6502	ORF00684	35427/6.33	100	Glyceraldehyde-3-phosphate dehydrogenase, type I	Cytoplasmic	G	COG0057	Both
6505	ORF02422	40022.6/6.19	100	Aspartate-semialdehyde dehydrogenase	Unknown	E	COG0136	Only 2-DE
6508	ORF00766	42289.9/6.45	100	Hypothetical protein	Unknown	NO related COG	NO related COG	Only 2-DE
6511	ORF01336	40678.4/6.35	100	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	Cytoplasmic	M	COG0821	Only 2-DE
6604	ORF02105	48188/6.07	100	Citrate synthase I	Cytoplasmic	C	COG0372	Both

6707	ORF03736	47432.5/6.22	99.99	UDP-N-acetylmuramoylalanine--D-glutamate ligase	Unknown	M	COG0771	Both
6708	ORF01780	59670.2/6.29	100	Fumarate hydratase class I, anaerobic	Unknown	C	COG1838	Only 2-DE
6804	ORF02610	73954/5.67	100	Outer membrane protein A	OuterMembrane	MG	COG0451	Both
6901	ORF01856	85051.6/5.65	100	Formate acetyltransferase	Cytoplasmic	C	COG1882	Both
7002	ORF03260	15743.4/6.3	100	Ribosomal protein L9	Cytoplasmic	J	COG0359	Both
7003	ORF03414	50011.7/4.94	100	ATP synthase F1, beta subunit	Cytoplasmic	C	COG0055	Both
7004	ORF01843	7497.8/6.71	100	Conserved domain protein	Cytoplasmic	K	COG1278	Both
7006	ORF00196	7420.8/6.71	100	Conserved domain protein	Cytoplasmic	K	COG1278	Only 2-DE
7103	ORF02770	20484.4/6.65	100	Protein /y1776/	Unknown	R	COG3150	Only 2-DE
7202	ORF02897	23890.9/6.32	100	Positive response regulator of capsular synthesis	Cytoplasmic	TK	COG2197	Only 2-DE
7203	ORF03837	20752/6.64	100	Ribosome recycling factor	Cytoplasmic	J	COG0233	Both
7205	ORF03447	24295.6/6.45	100	GTP-binding protein	Unknown	R	COG0218	Only 2-DE
7206	ORF03481	22722.6/8.34	100	Thioldisulfide interchange protein DsbA	Periplasmic	OC	COG0526	Only 2-DE
7208	ORF03341	23308.1/6.53	100	Orotate phosphoribosyltransferase	Unknown	F	COG0461	Only 2-DE
7301	ORF01724	28647.3/6.6	100	Negative regulator of beta-lactamase expression	Cytoplasmic	M	COG3023	Both
7303	ORF03834	26295.6/6.61	100	Ribosomal protein S2	Cytoplasmic	J	COG0052	Both
7306	ORF01648	25990.2/6.85	100	Conserved hypothetical protein	Periplasmic	S	COG2968	Both
7309	ORF01305	28196.7/6.56	100	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	Unknown	G	COG0588	Both
7401	ORF00406	31984.4/6.92	100	Hypothetical protein	Unknown	NO related COG	NO related COG	Both
7402	ORF00406	31984.4/6.92	100	Hypothetical protein	Unknown	NO related COG	NO related COG	Both
7404	ORF01724	28647.3/6.6	100	Negative regulator of beta-lactamase expression	Cytoplasmic	M	COG3023	Both
7405	ORF01411	37823.6/7.7	100	ABC transporter, substrate binding protein	Periplasmic	H	COG1840	Only 2-DE

		9						
7501	ORF00684	35427/6.33	100	Glyceraldehyde-3-phosphate dehydrogenase, type I	Cytoplasmic	G	COG0057	Both
7503	ORF00739	41946.6/6.32	100	Aminotransferase, class I and II	Unknown	E	COG1168	Only 2-DE
7506	ORF00684	35427/6.33	100	Glyceraldehyde-3-phosphate dehydrogenase, type I	Cytoplasmic	G	COG0057	Both
7508	ORF01509	40433.8/6.64	100	Riboflavin biosynthesis protein ribd	Unknown	H	COG1985	Only 2-DE
7509	ORF00750	34776.8/6.71	100	Putative 2-hydroxyacid dehydrogenase YcdW	Cytoplasmic	E	COG0111	Only 2-DE
7608	ORF01315	45498/6.73	100	Serine hydroxymethyltransferase	Cytoplasmic	E	COG0112	Both
7611	ORF00890	46905.6/6.75	100	Transcription termination factor Rho	Cytoplasmic	K	COG1158	Both
7705	ORF00890	46905.6/6.75	100	Transcription termination factor Rho	Cytoplasmic	K	COG1158	Both
7710	ORF01351	51732/6.88	99.99	Inosine-5'-monophosphate dehydrogenase	Cytoplasmic	F	COG0516	Both
7804	ORF01185	68077.6/7.22	100	Tonb-dependent vitamin B12 receptor	OuterMembrane	P	COG1629	Only 2-DE
8002	ORF00196	7420.8/6.71	100	Conserved domain protein	Cytoplasmic	K	COG1278	Only 2-DE
8101	ORF01338	22432.6/6.75	100	TPR repeat	Unknown	S	COG2976	Both
8102	ORF03846	18555.7/8.57	100	Beta-hydroxyacyl-(acyl-carrier-protein dehydratase FabZ	Cytoplasmic	I	COG0764	Both
8107	ORF03054	17816.4/9.06	100	50S ribosomal protein L10	Unknown	J	COG0244	Both
8109	ORF01725	20694.8/9.22	100	Monofunctional chorismate mutase	Periplasmic	E	COG1605	Both
8201	ORF02955	27644.2/6.84	100	Mismatch repair ATPase	Unknown	NO related COG	NO related COG	Both
8205	ORF00147	22354.7/9.02	100	Protein YfdX	Unknown	NO related COG	NO related COG	Only 2-DE
8301	ORF03834	26295.6/6.61	100	Ribosomal protein S2	Cytoplasmic	J	COG0052	Both
8303	ORF00985	31138.8/7.16	100	Beta-ureidopropionase	Unknown	R	COG0388	Only 2-DE
8304	ORF01879	23396.2/8.6	100	Leucine-responsive Regulatory protein	Unknown	K	COG1522	Only 2-DE

8305	ORF03543	27900.5/8.98	100	Amino-acid ABC transporter periplasmic component	Periplasmic	E	COG0834	Both
8306	ORF01196	17770.6/5.21	100	EvpC	Unknown	NO related COG	NO related COG	Both
8307	ORF02303	28680.9/8.78	100	Fkbp-type peptidyl-prolyl cis-trans isomerase FkpA	Periplasmic	O	COG0545	Both
8401	ORF00406	31984.4/6.92	100	Hypothetical protein	Unknown	NO related COG	NO related COG	Both
8402	ORF03407	36333.5/8.45	100	Phosphate ABC transporter, phosphate-binding protein PstS	Periplasmic	P	COG0226	Only 2-DE
8404	ORF03730	34731.2/7.05	100	S-adenosyl-methyltransferase MraW	Cytoplasmic	M	COG0275	Only 2-DE
8406	ORF01318	29211.2/7.1	100	Inositol-1-monophosphatase	Cytoplasmic	G	COG0483	Only 2-DE
8408	ORF00996	37450.3/8.74	100	Protein YedY	Unknown	R	COG2041	Both
8409	ORF02587	38075.3/8.79	100	Outer membrane protein A	OuterMembrane	M	COG2885	Both
8504	ORF02587	38075.3/8.79	100	Outer membrane protein A	OuterMembrane	M	COG2885	Both
8505	ORF03654	38512.4/8.34	100	Rrna methylases	Unknown	J	COG0566	Both
8507	ORF03654	38512.4/8.34	100	Rrna methylases	Unknown	J	COG0566	Both
8509	ORF02587	38075.3/8.79	100	Outer membrane protein A	OuterMembrane	M	COG2885	Both
8510	ORF03654	38512.4/8.34	100	Rrna methylases	Unknown	J	COG0566	Both
8601	ORF01315	45498/6.73	100	Serine hydroxymethyltransferase	Cytoplasmic	E	COG0112	Both
8602	ORF00890	46905.6/6.75	100	Transcription termination factor Rho	Cytoplasmic	K	COG1158	Both
8603	ORF02587	38075.3/8.79	100	Outer membrane protein A	OuterMembrane	M	COG2885	Both
8605	ORF01315	45498/6.73	100	Serine hydroxymethyltransferase	Cytoplasmic	E	COG0112	Both
8702	ORF01351	51732/6.88	100	Inosine-5'-monophosphate dehydrogenase	Cytoplasmic	F	COG0516	Both
8704	ORF01745	40546.5/6.28	100	Cytosol aminopeptidase	Cytoplasmic	E	COG0260	Only 2-DE
8705	ORF00888	47148.4/7.75	100	ATP-dependent RNA helicase RhlB	Unknown	LKJ	COG0513	Both

8801	ORF01315	45498/6.73	100	Serine hydroxymethyltransferase	Cytoplasmic	E	COG0114	Both
9004	ORF00102	16482.3/9.12	100	Conserved hypothetical protein	Unknown	NO related COG	NO related COG	Only 2-DE
9007	ORF02877	10429.5/9.34	100	Ribosomal L25p family	Unknown	J	COG1825	Both
9104	ORF01879	23396.2/8.6	100	Leucine-responsive Regulatory protein	Unknown	K	COG1522	Only 2-DE
9106	ORF03054	17816.4/9.06	100	50S ribosomal protein L10	Unknown	J	COG0244	Both
9108	ORF03054	17816.4/9.06	100	50S ribosomal protein L10	Unknown	J	COG0244	Both
9203	ORF03114	24487.7/9.43	100	Toluene tolerance protein Ttg2D	Unknown	N	COG2854	Only 2-DE
9302	ORF02303	28680.9/8.78	100	Fkbp-type peptidyl-prolyl cis-trans isomerase FkpA	Periplasmic	O	COG0545	Both
9307	ORF03415	31735.6/9.05	100	ATP synthase F1, gamma subunit	Unknown	C	COG0224	Both
9309	ORF03055	24705.2/9.63	100	Ribosomal protein L1	Unknown	J	COG0081	Both
9402	ORF02553	40822.9/5.46	100	Outer membrane protein F	OuterMembrane	NO related COG	NO related COG	Both
9403	ORF02587	38075.3/8.79	100	Outer membrane protein A	OuterMembrane	M	COG2885	Both
9405	ORF00307	34017.2/9.22	100	Antigenic protein Et 32	Unknown	NO related COG	NO related COG	Only 2-DE
9701	ORF01982	56535.1/8.6	100	Glucans biosynthesis protein G	Periplasmic	P	COG3131	Both
9703	ORF01982	56535.1/8.6	100	Glucans biosynthesis protein G	Periplasmic	P	COG3131	Both
9705	ORF02740	60215.1/9.1	100	Periplasmic murein peptide-binding protein	Periplasmic	EP	COG0747	Both

APPENDIX C

FLAVOBACTERIUM COLUMNARE PROTEINS IDENTIFIED USING 2-D LC

Serial No	ORF No	Protein name	Σxcorr	Coverage	MW	No of peptide ions	COG category	Subcellular location	Identified by
1	ORF00244	Translation elongation factor Tu	210.33	61.50	43142.9	205	JE	Cytoplasmic	2-DE and 2-D LC
2	ORF02005	Outer membrane protein A	150.26	55.80	50212.6	86	M	OuterMembrane	2-DE and 2-D LC
3	ORF01740	ATP synthase F1, beta subunit	80.18	26.00	54138.4	8	C	Cytoplasmic	2-D LC
4	ORF02366	Hypothetical protein	50.32	33.30	24461.4	27	NO related COG	OuterMembrane	2-D LC
5	ORF00739	1-pyrroline-5-carboxylate dehydrogenase	40.23	12.40	59650.5	18	C	Cytoplasmic	2-DE and 2-D LC
6	ORF00324	Response regulator	40.18	5.10	60289.9	61	TK	Cytoplasmic	2-DE and 2-D LC
7	ORF02110	Methylmalonyl-CoA mutase, N-	38.17	5.00	127709.2	9	I	Cytoplasmic	2-D LC
8	ORF01311	DNA gyrase, A subunit	38.15	4.20	96007.7	6	L	Cytoplasmic	2-D LC
9	ORF02564	Lipoprotein, putative	30.43	21.00	36208.7	6	NO related COG	Unknown	2-D LC
10	ORF01826	Conserved hypothetical protein	30.28	16.30	58975.8	7	NO related COG	Cytoplasmic	2-DE and 2-D LC
11	ORF02009	Xaa-Pro aminopeptidase	30.27	7.80	47092.3	5	E	Unknown	2-D LC
12	ORF02289	Glycine dehydrogenase	30.26	7.90	104143.0	4	E	Unknown	2-D LC
13	ORF02077	Acyl-CoA carboxylase carboxyl Transferase subuni t beta	30.19	15.50	59717.3	12	I	Unknown	2-D LC
14	ORF02335	GldM	30.18	7.60	50668.2	4	NO related COG	Unknown	2-D LC
15	ORF02209	Ribosomal protein S5	30.16	25.90	18231.9	4	J	Unknown	2-D LC
16	ORF02347	Chaperonin GroL	30.15	7.70	57362.4	6	O	Cytoplasmic	2-DE and 2-D LC
17	ORF01066	Major outer membrane protein	30.15	12.30	51409.4	5	Q	Periplasmic	2-D LC

18	ORF00181	Homogentisate 1,2-dioxygenase	28.36	16.60	44897.6	23	Q	Unknown	2-DE and 2-D LC
19	ORF02111	Hypothetical protein	28.18	4.50	157843.5	3	NO related COG	Unknown	2-D LC
20	ORF01649	Adenosylmethionine-8-amino-7-oxononanoate transaminase	28.15	5.00	47345.1	5	H	CytoplasmicMembrane	2-D LC
21	ORF00472	Two-component system sensor histidine kinase	28.14	7.80	59184.5	5	T	CytoplasmicMembrane	2-D LC
22	ORF00257	Putative outer membrane protein probably involved in nutrient binding	28.13	7.00	54026.2	6	NO related COG	Unknown	2-D LC
23	ORF01181	Protein containing tetratricopeptide repeats	26.13	12.20	54907.0	3	R	Cytoplasmic	2-D LC
24	ORF00146	Glucose-6-phosphate isomerase	24.14	10.80	62310.3	4	G	Cytoplasmic	2-D LC
25	ORF02287	Penicillin-binding protein 1C	24.12	4.20	91120.0	3	M	Unknown	2-D LC
26	ORF01487	Aconitate hydratase 2	24.11	1.50	100743.7	4	C	Unknown	2-D LC
27	ORF00695	Translation elongation factor Ts	22.12	14.60	28996.8	4	J	Cytoplasmic	2-DE and 2-D LC
28	ORF02628	Fjo24	22.12	2.70	143387.3	3	NO related COG	OuterMembrane	2-D LC
29	ORF01153	Dihydrolipoyl dehydrogenase	20.38	8.60	50327.5	8	C	Cytoplasmic	2-DE and 2-D LC
30	ORF00676	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	20.34	5.00	55437.6	12	G	Cytoplasmic	2-D LC
31	ORF01408	Conserved hypothetical protein	20.32	11.50	19751.6	28	NO related COG	Cytoplasmic	2-D LC
32	ORF00190	Aspartyl-tRNA synthetase	20.31	4.20	67178.3	11	J	Cytoplasmic	2-DE and 2-D LC
33	ORF01412	Alternative Cytochrome c oxidase subunit 1 (Alternativecytochrome c oxidase polypeptide I	20.29	5.70	66957.4	9	C	CytoplasmicMembrane	2-D LC
34	ORF02144	2-oxoacid dehydrogenase E1 component subunits al pha and beta	20.28	3.80	89844.2	3	C	Cytoplasmic	2-DE and 2-D LC
35	ORF00504	Ornithine--oxo-acid transaminase	20.28	8.00	45236.4	28	E	Cytoplasmic	2-DE and 2-D LC

36	ORF02849	Clp protease	20.27	22.50	24619.3	6	NO	Cytoplasmic	2-DE and 2-D LC
37	ORF02226	Ribosomal protein L3	20.27	20.00	20985.0	10	J	Cytoplasmic	2-D LC
38	ORF01096	Glutamine synthetase	20.23	5.60	81863.4	8	E	Unknown	2-DE and 2-D LC
39	ORF01350	Arginine decarboxylase	20.23	11.40	54401.8	2	E	Periplasmic	2-D LC
40	ORF01646	Pyruvate kinase	20.21	7.30	52565.7	3	G	Cytoplasmic	2-DE and 2-D LC
41	ORF00471	DNA-binding response regulator RprY	20.21	15.00	27433.8	5	TK	Cytoplasmic	2-D LC
42	ORF00187	Outer membrane insertion C- signal domain protein	20.20	9.60	33227.3	6	NO related COG	OuterMembrane	2-D LC
43	ORF01706	FeS assembly protein SufD	20.20	13.20	49571.2	3	R	Cytoplasmic	2-D LC
44	ORF01800	ATP synthase F1, alpha subunit	20.19	5.50	56563.5	3	C	Unknown	2-DE and 2-D LC
45	ORF02336	GldL	20.18	13.30	22395.7	2	NO related COG	Unknown	2-DE and 2-D LC
46	ORF02881	Conserved hypothetical protein	20.18	3.90	34278.7	7	NO related COG	OuterMembrane	2-D LC
47	ORF00226	Conserved hypothetical protein	20.18	3.90	34250.6	7	NO related COG	OuterMembrane	2-D LC
48	ORF02126	3-hydroxyacyl-CoA dehydrogenase	20.18	5.20	88164.9	5	I	Unknown	2-D LC
49	ORF00815	Thiol peroxidase	20.17	11.50	17622.0	5	O	Cytoplasmic	2-D LC
50	ORF00590	Thermostable serine protease, putative	20.17	10.30	55019.5	3	O	Extracellular	2-D LC
51	ORF02321	Conserved hypothetical protein	20.16	12.10	30606.2	3	NO related COG	Unknown	2-DE and 2-D LC
52	ORF00757	GMP synthase	20.16	8.10	56926.6	2	F	Cytoplasmic	2-D LC
53	ORF01629	Glycogen synthase-related protein	20.16	3.40	30358.7	5	G	Cytoplasmic	2-D LC
54	ORF00250	Acyl-CoA dehydrogenase	20.16	11.10	43596.7	4	I	Cytoplasmic	2-DE and 2-D LC

55	ORF02318	D-lactate dehydrogenase	20.16	7.30	108906.2	3	C	Cytoplasmic	2-D LC
56	ORF00745	Methionine aminopeptidase, type I	20.16	15.80	28488.4	4	J	Unknown	2-D LC
57	ORF02446	Peptidase, M16 family	20.15	3.60	110100.5	2	R	Unknown	2-D LC
58	ORF00236	DNA-directed RNA polymerase, beta' subunit	20.15	2.50	162085.1	2	K	Cytoplasmic	2-D LC
59	ORF01831	Secreted aminopeptidase, putative	20.15	10.60	73504.6	2	E	Unknown	2-D LC
60	ORF00779	TonB-dependent outer membrane receptor	20.15	3.90	90272.2	2	P	OuterMembrane	2-D LC
61	ORF01193	Succinyl-CoA synthetase beta chain	20.14	5.50	43095.0	4	C	Cytoplasmic	2-DE and 2-D LC
62	ORF00422	Protein-export membrane protein SecD, putative	20.14	5.40	107552.7	2	N	CytoplasmicMembrane	2-D LC
63	ORF02784	Peroxiredoxin, AhpC/Tsa family	20.14	11.10	24649.8	2	O	Cytoplasmic	2-DE and 2-D LC
64	ORF00332	Acetyl-CoA Acetyltransferase	20.14	9.00	40858.8	8	I	Cytoplasmic	2-DE and 2-D LC
65	ORF01574	Hypothetical protein	20.14	8.20	51113.6	2	NO related COG	Unknown	2-D LC
66	ORF01357	30S ribosomal protein S1	20.14	6.00	65553.0	2	J	Cytoplasmic	2-DE and 2-D LC
67	ORF01434	Secreted peptidase, family M23	20.14	8.50	65221.7	3	M	Unknown	2-D LC
68	ORF00683	Conserved hypothetical protein	20.14	7.00	81817.7	2	NO related COG	Unknown	2-D LC
69	ORF00293	Phenylalanine 4-monooxygenase	20.13	10.10	66290.3	2	E	Cytoplasmic	2-D LC
70	ORF01481	Fibronectin type III domain protein	20.13	1.60	212372.3	2	NO related COG	Unknown	2-D LC
71	ORF00115	Conserved hypothetical protein	20.13	5.40	114705.4	2	NO related COG	Unknown	2-D LC
72	ORF00290	Urocanate hydratase	20.13	4.80	74345.9	3	E	Cytoplasmic	2-DE and 2-D LC
73	ORF01446	Conserved hypothetical protein	20.13	20.40	21027.9	2	NO related	Cytoplasmic	2-D LC

							COG		
74	ORF01552	L-asparaginase	20.13	10.50	38102.7	2	EJ	Cytoplasmic	2-D LC
75	ORF02145	Catalase/peroxidase HPI	20.13	5.20	81883.4	2	P	Unknown	2-D LC
76	ORF01013	Aminotransferase	20.13	11.80	47118.3	2	E	Cytoplasmic	2-D LC
77	ORF00395	Multi-sensor hybrid histidine kinase	20.13	3.20	156256.6	3	T	CytoplasmicMembrane	2-D LC
78	ORF00875	Precorrin-4 C11-methyltransferase/cobalamin biosynthesis protein	20.12	5.20	67906.1	4	H	Unknown	2-D LC
79	ORF00495	Chaperone protein DnaK	20.12	5.70	67725.9	2	O	Unknown	2-DE and 2-D LC
80	ORF01665	Conserved hypothetical protein	20.11	0.00	60068.5	2	NO related COG	Unknown	2-D LC
81	ORF01613	Adenosylhomocysteinase	20.11	5.50	48064.7	4	H	Cytoplasmic	2-D LC
82	ORF01697	Secreted subtilase family protein	18.23	5.40	58669.1	6	O	Extracellular	2-D LC
83	ORF01351	Deoxyhypusine synthase	18.20	12.70	36778.6	5	J	Unknown	2-DE and 2-D LC
84	ORF02635	Short-chain dehydrogenase/reductase family prote in	18.17	16.30	24897.6	6	QR	Cytoplasmic	2-D LC
85	ORF02727	Adenylosuccinate synthetase	18.17	5.90	47111.9	9	F	Cytoplasmic	2-D LC
86	ORF02258	Conserved hypothetical protein	18.17	6.60	80469.2	3	O	Unknown	2-D LC
87	ORF02395	Cation eflux system protein	18.16	11.60	41184.0	6	Q	Unknown	2-D LC
88	ORF01395	NADH-ubiquinone oxidoreductase 75 kda subunit	18.16	18.10	38642.8	3	C	Cytoplasmic	2-D LC
89	ORF02391	Hypothetical protein	18.15	8.30	29112.5	6	NO related COG	Unknown	2-D LC
90	ORF00479	Phosphopantothenoylcysteine decarboxylase/phosphopantothenate--cysteine ligase	18.15	8.20	43777.5	2	H	Unknown	2-D LC
91	ORF01792	Acetyl-CoA carboxylase, biotin carboxylase	18.15	2.90	49934.1	4	I	Cytoplasmic	2-DE and 2-D LC
92	ORF01656	Aminotransferase, class-V	18.14	8.20	44993.3	3	E	Cytoplasmic	2-D LC
93	ORF00507	Conserved hypothetical protein	18.14	23.00	20454.7	2	NO	Unknown	2-D LC

							related COG		
94	ORF00034	two-component system sensor histidine kinase	18.14	4.60	52872.5	3	T	CytoplasmicMembrane	2-D LC
95	ORF02785	Cell shape-determining protein MreB	18.14	9.40	37382.4	2	D	Cytoplasmic	2-D LC
96	ORF00041	Secreted peptidase, family M23	18.13	6.70	44889.3	2	M	Unknown	2-D LC
97	ORF02393	Cation efflux system protein CzcA	18.13	4.60	114602.3	2	P	CytoplasmicMembrane	2-D LC
98	ORF00096	ClpB protein	18.13	5.60	93507.7	5	O	Cytoplasmic	2-D LC
99	ORF02179	TonB-dependent outer membrane receptor	18.13	5.00	91732.3	2	P	OuterMembrane	2-D LC
100	ORF01459	Riboflavin biosynthesis protein RibD	18.12	9.30	37975.5	2	H	Unknown	2-D LC
101	ORF00379	Conserved hypothetical protein	18.11	1.60	57832.3	2	ER	Unknown	2-D LC
102	ORF01815	Membrane protein	18.11	6.10	35677.0	2	NO related COG	CytoplasmicMembrane	2-D LC
103	ORF01477	NlpC/P60 family protein	18.11	2.70	28799.7	3	M	Cytoplasmic	2-D LC
104	ORF02716	UDP-N-acetylmuramate--alanine ligase	16.32	7.20	49570.5	5	M	Cytoplasmic	2-D LC
105	ORF01855	Tetratricopeptide repeat domain protein	16.15	6.00	76468.4	2	R	OuterMembrane	2-D LC
106	ORF02525	Tyrosine-protein kinase ptk	16.15	6.30	89397.8	4	D	CytoplasmicMembrane	2-D LC
107	ORF00742	Snf2 family helicase	16.15	3.10	111108.7	3	KL	OuterMembrane	2-D LC
108	ORF01664	BatA protein	16.14	9.60	37104.5	2	S	CytoplasmicMembrane	2-D LC
109	ORF01228	Glycine cleavage system T protein	16.14	9.70	39487.6	2	E	Cytoplasmic	2-DE and 2-D LC
110	ORF00117	RHS Repeat family	16.13	1.40	423632.7	3	M	Unknown	2-D LC
111	ORF02215	Ribosomal protein L24	16.13	21.40	11161.1	2	J	Unknown	2-D LC
112	ORF01938	Conserved hypothetical protein	16.13	1.50	128405.2	2	D	OuterMembrane	2-D LC
113	ORF00704	TonB-dependent outer membrane receptor, putative	16.12	3.30	104207.6	2	P	OuterMembrane	2-D LC
114	ORF01453	Conserved hypothetical protein	16.11	15.00	24660.5	8	NO	Unknown	2-D LC

							related COG		
115	ORF00661	2-nitropropane dioxygenase	14.16	7.00	34447.2	4	R	Unknown	2-D LC
116	ORF00232	Peptide chain release factor 3	14.16	4.90	60461.8	3	J	Cytoplasmic	2-D LC
117	ORF01433	TonB-dependent receptor plug domain protein	14.15	3.50	94254.1	4	P	OuterMembrane	2-D LC
118	ORF02829	Peptidyl-prolyl cis-trans isomerase C	14.13	5.00	76424.4	2	O	OuterMembrane	2-D LC
119	ORF00114	Conserved hypothetical protein	14.12	0.00	200071.5	4	NO related COG	Unknown	2-D LC
120	ORF02745	Methionine synthase	14.12	4.90	36378.0	2	E	Cytoplasmic	2-D LC
121	ORF01943	Outer membrane protein, putative	14.11	12.40	21830.8	2	M	Unknown	2-D LC
122	ORF02157	Serine hydroxymethyltransferase	12.19	8.50	46543.0	5	E	Cytoplasmic	2-DE and 2-D LC
123	ORF02469	DNA mismatch repair protein MutL	12.14	4.80	69890.1	6	L	Unknown	2-D LC
124	ORF00597	Type II restriction enzyme MthTI	12.14	8.50	32604.8	3	NO related COG	Unknown	2-D LC
125	ORF01611	Geranylgeranylglyceryl phosphate synthase	12.13	20.50	26575.8	3	R	Cytoplasmic	2-D LC
126	ORF01504	Secreted glycyl aminopeptidase, family M61	12.13	7.00	69732.3	2	O	Unknown	2-DE and 2-D LC
127	ORF00202	Ribonucleoside-diphosphate reductase, alpha subunit	12.12	2.70	91738.0	3	F	Cytoplasmic	2-D LC
128	ORF00066	Hypothetical protein	12.10	10.10	34071.0	2	D	Unknown	2-D LC
129	ORF01388	Ribonuclease, Rne/Rng family	12.10	3.50	58626.2	2	J	Cytoplasmic	2-D LC
130	ORF00566	Thioesterase family protein	10.33	12.30	15845.1	12	R	Cytoplasmic	2-D LC
131	ORF02002	Superoxide dismutase [Mn]	10.32	10.40	22295.9	4	P	Unknown	2-DE and 2-D LC
132	ORF02648	Hypothetical protein	10.32	3.50	100370.0	2	NO related COG	Unknown	2-D LC
133	ORF02202	Ribosomal protein S4	10.26	10.40	22865.1	6	J	Cytoplasmic	2-D LC
134	ORF01406	Iron-sulfur binding oxidoreductase	10.26	2.00	102058.6	3	C	Unknown	2-D LC

135	ORF00147	TonB-dependent outer membrane receptor	10.26	1.90	101043.2	4	P	OuterMembrane	2-D LC
136	ORF01963	Leucyl-tRNA synthetase	10.25	3.30	110861.4	2	J	Cytoplasmic	2-D LC
137	ORF00883	Cobalamin biosynthesis protein CobW	10.25	7.50	38888.0	2	R	Cytoplasmic	2-D LC
138	ORF01793	Acetyl-CoA carboxylase, biotin carboxyl carrier protein	10.24	11.00	17709.4	3	I	Unknown	2-DE and 2-D LC
139	ORF01627	Glutamine-fructose-6-phosphate transaminase (isomerizing	10.24	3.10	68044.6	6	M	Cytoplasmic	2-D LC
140	ORF01714	Alcohol dehydrogenase YqhD	10.23	6.50	43298.5	1	C	Cytoplasmic	2-DE and 2-D LC
141	ORF02779	Fructose-bisphosphate aldolase, class II	10.23	6.20	38921.6	4	G	Unknown	2-DE and 2-D LC
142	ORF01312	ATP-dependent Clp protease, ATP-binding subunit ClpC	10.20	2.00	95690.4	2	O	Cytoplasmic	2-D LC
143	ORF02090	ATP-dependent DNA helicase PcrA	10.20	2.70	88710.5	3	L	Unknown	2-D LC
144	ORF00349	Acyl-CoA dehydrogenase	10.20	3.20	41037.8	5	I	Cytoplasmic	2-DE and 2-D LC
145	ORF02720	Phospho-N-acetylmuramoyl-pentapeptide-transferase	10.20	3.20	45692.0	1	M	CytoplasmicMembrane	2-D LC
146	ORF02449	Preprotein translocase, SecA subunit	10.20	1.60	128092.6	1	N	Cytoplasmic	2-D LC
147	ORF02620	Cyclase family protein	10.20	9.10	29182.8	4	R	Unknown	2-D LC
148	ORF00578	Modification methylase DdeI	10.19	4.40	46544.2	3	L	Unknown	2-D LC
149	ORF02801	Lipid-A-disaccharide synthase	10.19	4.80	42255.9	2	M	Unknown	2-D LC
150	ORF00730	YcgL	10.19	4.00	29008.0	5	R	Cytoplasmic	2-D LC
151	ORF00527	Nucleoside diphosphate kinase	10.18	14.40	15110.1	1	F	Cytoplasmic	2-DE and 2-D LC
152	ORF01630	Pantoate--beta-alanine ligase	10.18	5.00	32428.6	2	H	Unknown	2-D LC
153	ORF01689	Endonuclease/Exonuclease/phosphatase family superfamily	10.17	7.40	41330.4	1	NO related COG	Unknown	2-D LC
154	ORF01829	Leucine-rich repeat containing protein	10.16	8.00	35619.8	3	NO related COG	Extracellular	2-D LC

155	ORF01210	Transaldolase	10.16	6.40	17089.7	4	G	Cytoplasmic	2-DE and 2-D LC
156	ORF02424	TonB-dependent outer membrane receptor, putative	10.16	3.90	80434.2	1	P	OuterMembrane	2-D LC
157	ORF01201	Dihydrolipoyl dehydrogenase	10.16	0.00	49327.4	1	C	Cytoplasmic	2-DE and 2-D LC
158	ORF00426	Asparagine synthase (glutamine-hydrolyzing	10.16	5.90	63666.0	1	E	Cytoplasmic	2-D LC
159	ORF01148	PpiA	10.15	4.30	40106.7	3	O	Cytoplasmic	2-D LC
160	ORF02367	Lipoprotein, putative	10.15	6.30	24185.0	1	NO related COG	Unknown	2-D LC
161	ORF02201	DNA-directed RNA polymerase, alpha subunit	10.15	3.90	37420.5	1	K	Cytoplasmic	2-D LC
162	ORF02302	Hypothetical protein	10.15	23.00	15935.9	1	NO related COG	CytoplasmicMembrane	2-D LC
163	ORF01579	Hypothetical protein	10.15	7.60	27231.0	3	NO related COG	Unknown	2-D LC
164	ORF01120	Secreted protein containing PKD domain, putative	10.15	4.60	82622.2	1	NO related COG	Unknown	2-D LC
165	ORF01511	TonB-dependent receptor, putative	10.15	1.70	71016.0	1	P	OuterMembrane	2-D LC
166	ORF01296	ABC-type multidrug/protein/lipid transporter ATP-binding and permease component	10.15	3.90	67105.0	4	Q	CytoplasmicMembrane	2-D LC
167	ORF01473	Putative esterase superfamily	10.15	8.50	32485.0	1	R	Unknown	2-D LC
168	ORF00500	Fucose permease	10.15	4.40	59624.8	1	G	CytoplasmicMembrane	2-D LC
169	ORF00510	Conserved hypothetical protein	10.15	8.30	32843.3	6	NO related COG	Cytoplasmic	2-D LC
170	ORF00511	Conserved hypothetical protein	10.15	8.20	33269.7	6	NO related COG	Cytoplasmic	2-D LC
171	ORF00975	Conserved hypothetical protein	10.15	9.20	15231.8	2	NO related COG	Unknown	2-D LC

172	ORF01471	HtrA protein	10.15	2.00	49755.2	1	O	Periplasmic	2-DE and 2-D LC
173	ORF01603	Hypothetical protein	10.15	23.00	16073.3	1	NO related COG	Unknown	2-D LC
174	ORF01060	Acetylornithine aminotransferase	10.15	7.60	43795.5	2	E	Unknown	2-D LC
175	ORF01341	ATP-dependent chaperone ClpB	10.15	3.90	97696.0	1	O	Cytoplasmic	2-D LC
176	ORF00702	Hypothetical protein	10.15	5.50	27042.3	1	NO related COG	Unknown	2-D LC
177	ORF01168	Hypothetical protein	10.15	8.10	28051.6	1	NO related COG	Unknown	2-D LC
178	ORF00916	ABC transporter, ATP-binding protein	10.15	6.70	61278.9	2	R	CytoplasmicMembrane	2-D LC
179	ORF00420	Peptidase, M56 family protein	10.14	5.70	57867.0	1	NO related COG	CytoplasmicMembrane	2-D LC
180	ORF01106	Hypothetical protein	10.14	10.00	17838.1	5	NO related COG	Cytoplasmic	2-D LC
181	ORF02129	Acyl-CoA dehydrogenase	10.14	1.50	66053.3	5	I	Cytoplasmic	2-D LC
182	ORF01661	Aerotolerance-related protein BatD	10.14	3.30	63326.0	1	NO related COG	Unknown	2-D LC
183	ORF02583	Hypothetical protein	10.14	5.40	49354.3	2	NO related COG	Cytoplasmic	2-D LC
184	ORF00949	Molybdenum cofactor biosynthesis bifunctional pr otein	10.14	6.90	32962.2	2	H	Cytoplasmic	2-D LC
185	ORF02658	YD repeat protein, putative	10.14	0.80	363924.2	1	M	Unknown	2-D LC
186	ORF00396	Glycosyl transferase, group 1	10.14	9.40	46778.0	1	M	Unknown	2-D LC
187	ORF01995	Phosphoenolpyruvate carboxykinase (ATP	10.14	4.80	60297.5	2	C	Cytoplasmic	2-DE and 2-D LC
188	ORF00403	Caax amino protease family	10.14	9.40	35733.6	1	R	CytoplasmicMembrane	2-D LC

189	ORF00882	Cobaltochelatase, CobN subunit	10.14	2.30	142273.4	1	H	Cytoplasmic	2-D LC
190	ORF00142	1-acyl-sn-glycerol-3-phosphate acyltransferase	10.14	12.00	30337.0	2	I	CytoplasmicMembrane	2-D LC
191	ORF01378	6-pyruvoyl tetrahydropterin synthase	10.14	11.30	17061.3	6	H	Unknown	2-D LC
192	ORF00314	Dihydrodipicolinate synthetase	10.14	10.60	34285.6	1	EM	Cytoplasmic	2-D LC
193	ORF01366	Succinyl-CoA synthetase, alpha subunit	10.14	5.20	30070.5	1	C	Unknown	2-DE and 2-D LC
194	ORF01424	Aspartate ammonia-lyase	10.14	7.50	51472.1	1	E	Cytoplasmic	2-D LC
195	ORF00476	Trans-2-enoyl-CoA reductase	10.14	7.80	43235.1	1	S	CytoplasmicMembrane	2-D LC
196	ORF02412	Conserved hypothetical protein	10.14	13.20	15004.3	1	NO related COG	Unknown	2-D LC
197	ORF01457	Modification methylase, HemK family	10.14	10.90	33082.1	2	J	Cytoplasmic	2-D LC
198	ORF00593	Quinolinate synthetase complex, A subunit	10.14	8.20	36515.2	1	H	Cytoplasmic	2-D LC
199	ORF02476	Transmembrane family-2 glycosyl transferase	10.14	8.90	43201.9	2	M	CytoplasmicMembrane	2-D LC
200	ORF00067	Major facilitator superfamily permease	10.14	8.20	46841.4	2	GEPR	CytoplasmicMembrane	2-D LC
201	ORF00213	Transcription termination factor NusA	10.14	4.10	47183.2	1	K	Cytoplasmic	2-D LC
202	ORF02296	Hypothetical protein	10.14	60.00	4636.4	1	NO related COG	Unknown	2-D LC
203	ORF01286	Acetyl-CoA carboxylase, carboxyl transferase, alpha subunit	10.14	4.40	35872.0	1	I	Cytoplasmic	2-DE and 2-D LC
204	ORF00604	Acyltransferase 3	10.14	8.80	41571.2	2	I	CytoplasmicMembrane	2-D LC
205	ORF02159	Lipoprotein, putative	10.14	7.10	45829.8	1	NO related COG	Unknown	2-D LC
206	ORF01297	Leucine dehydrogenase	10.14	5.40	39741.8	1	E	Cytoplasmic	2-DE and 2-D LC
207	ORF00075	AcrB/AcrD/AcrF family membrane transport protein	10.14	3.10	125812.0	1	Q	CytoplasmicMembrane	2-D LC
208	ORF01668	ATPase, MoxR family	10.14	8.70	37629.5	1	R	Cytoplasmic	2-DE and 2-

									D LC
209	ORF02741	Polyprenyl synthetase	10.14	6.70	37461.8	2	H	Cytoplasmic	2-D LC
210	ORF00845	Conserved hypothetical protein	10.14	5.40	39044.7	1	NO related COG	Cytoplasmic	2-D LC
211	ORF02022	Hydrolase YafV	10.14	6.70	29206.3	4	R	Unknown	2-D LC
212	ORF00044	Glucose-1-phosphate thymidylyltransferase	10.14	9.40	36148.0	1	M	Unknown	2-D LC
213	ORF01365	Putative long-chain-fatty-acid--CoA ligase homolog	10.14	5.10	67391.1	1	I	CytoplasmicMembrane	2-D LC
214	ORF02139	Membrane protein, putative	10.14	8.90	24188.8	1	NO related COG	CytoplasmicMembrane	2-D LC
215	ORF00165	Acyl-CoA dehydrogenase	10.14	11.50	43485.5	2	I	Unknown	2-DE and 2-D LC
216	ORF01000	Hypothetical protein	10.13	5.60	46509.6	2	NO related COG	Unknown	2-D LC
217	ORF02819	Cell envelope integrity inner membrane protein TolA, putative	10.13	15.40	17486.1	1	NO related COG	Unknown	2-D LC
218	ORF02485	ISPsy6, transposase	10.13	19.00	15966.3	1	L	Cytoplasmic	2-D LC
219	ORF01158	Lipoprotein, putative	10.13	3.50	57809.8	1	NO related COG	Unknown	2-D LC
220	ORF01070	NorD protein, putative	10.13	3.60	68662.3	1	NO related COG	Unknown	2-D LC
221	ORF01065	Conserved hypothetical protein	10.13	8.10	47835.3	1	NO related COG	Unknown	2-D LC
222	ORF00858	Conserved hypothetical protein	10.13	32.90	7770.2	1	NO related COG	Unknown	2-D LC
223	ORF02234	RNA methyltransferase, TrmH family, group 3	10.13	12.60	27077.1	1	J	Unknown	2-D LC
224	ORF02756	Transmembrane family-2 glycosyl transferase-po ssibly involved in biofilm	10.13	7.00	52407.9	1	M	CytoplasmicMembrane	2-D LC

		formation							
225	ORF02428	Signal recognition particle-docking protein FtsY	10.13	3.80	34399.0	1	N	Unknown	2-D LC
226	ORF00400	Formimidoylglutamase	10.13	5.20	39025.1	1	E	Cytoplasmic	2-D LC
227	ORF01848	Soluble guanylyl cyclase beta subunit	10.13	9.40	20683.5	1	NO related COG	Unknown	2-D LC
228	ORF01362	Major facilitator superfamily permease	10.13	6.90	48803.4	1	R	CytoplasmicMembrane	2-D LC
229	ORF01385	Single-strand binding protein family	10.13	9.10	15077.8	1	L	Unknown	2-D LC
230	ORF01989	Monofunctional biosynthetic peptidoglycan transglycosylase	10.13	12.00	28414.1	2	M	Unknown	2-D LC
231	ORF00164	Conserved hypothetical protein	10.13	9.00	23287.4	2	NO related COG	Cytoplasmic	2-D LC
232	ORF00018	Membrane protein, putative	10.13	3.80	52483.6	1	S	CytoplasmicMembrane	2-D LC
233	ORF01136	LPS biosynthesis protein , RfbU family	10.13	4.40	34191.3	1	M	Unknown	2-D LC
234	ORF01216	Prolipoprotein diacylglyceryl transferase	10.13	7.90	34796.5	1	M	CytoplasmicMembrane	2-D LC
235	ORF01497	Peroxiredoxin Q	10.13	11.40	16713.3	1	O	Unknown	2-D LC
236	ORF01904	Acetylornithine deAcetylase	10.13	4.40	40983.3	1	E	Cytoplasmic	2-D LC
237	ORF01443	DNA-binding protein HU-beta	10.13	17.60	9336.6	2	L	Unknown	2-D LC
238	ORF02678	Glucose-1-phosphate thymidylyltransferase	10.13	11.70	32064.5	2	M	Unknown	2-D LC
239	ORF00080	Hypothetical protein	10.13	8.40	37975.2	1	NO related COG	Unknown	2-D LC
240	ORF02843	Signal peptide peptidase SppA, 67K type	10.13	4.60	66105.1	1	NO	Cytoplasmic	2-D LC
241	ORF01695	Amidophosphoribosyltransferase, putative	10.13	4.00	72442.5	2	F	Cytoplasmic	2-D LC
242	ORF02120	Enoyl-(acyl-carrier-protein) reductase II	10.13	10.90	33803.9	2	R	Cytoplasmic	2-D LC
243	ORF02048	Lipoprotein, putative	10.13	16.20	18543.2	2	NO related COG	Unknown	2-D LC

244	ORF00517	YitL protein	10.13	12.60	32098.7	4	S	Unknown	2-D LC
245	ORF02834	Hypothetical protein	10.13	61.70	5150.2	1	NO related COG	Unknown	2-D LC
246	ORF02414	Conserved hypothetical protein	10.13	19.20	17038.9	1	NO related COG	Unknown	2-D LC
247	ORF01364	Conserved hypothetical protein	10.13	7.10	31970.1	1	R	Unknown	2-D LC
248	ORF00840	Protein containing AAA ATPase central region dom ain	10.13	23.30	15280.5	1	NO related COG	Cytoplasmic	2-D LC
249	ORF01551	Acyltransferase domain protein	10.13	8.20	43930.9	1	NO related COG	Unknown	2-D LC
250	ORF02764	Hypothetical protein	10.13	9.10	24592.0	1	NO related COG	Unknown	2-D LC
251	ORF00385	Glyceraldehyde-3-phosphate dehydrogenase, type I	10.13	3.90	35863.1	1	G	Cytoplasmic	2-DE and 2-D LC
252	ORF00363	AIR synthase related protein, C- domain protein	10.13	2.60	135306. 4	1	F	Unknown	2-D LC
253	ORF01163	Tetraacyldisaccharide 4'-kinase	10.13	8.00	42094.0	2	N	Unknown	2-D LC
254	ORF01140	PolyA polymerase family protein	10.13	3.80	54435.6	1	J	Cytoplasmic	2-D LC
255	ORF01986	Conserved hypothetical protein	10.13	7.20	32019.6	1	R	Unknown	2-D LC
256	ORF01187	Major facilitator superfamily permease	10.13	8.40	45640.1	1	GEPR	CytoplasmicMembrane	2-D LC
257	ORF00824	Hypothetical protein	10.13	4.50	46674.2	1	NO related COG	Unknown	2-D LC
258	ORF02499	Transcriptional regulator, MarR family	10.13	20.40	17692.7	1	KR	Unknown	2-D LC
259	ORF02316	Integrase	10.13	5.10	44008.6	2	L	Cytoplasmic	2-D LC
260	ORF02789	Rod shape-determining protein RodA	10.13	6.20	46690.6	1	D	CytoplasmicMembrane	2-D LC
261	ORF00423	Malate dehydrogenase, NAD-dependent	10.13	9.00	32822.6	1	C	Cytoplasmic	2-DE and 2-D LC
262	ORF00223	Deoxyribose-phosphate aldolase	10.13	12.10	27246.9	1	F	Cytoplasmic	2-D LC

263	ORF00224	Conserved hypothetical protein	10.13	1.90	78781.0	1	NO related COG	OuterMembrane	2-D LC
264	ORF02074	NADH:quinone dehydrogenase	10.13	4.10	48991.3	1	C	Unknown	2-D LC
265	ORF00215	Amino acid permease-associated region	10.13	4.50	51358.8	1	E	CytoplasmicMembrane	2-D LC
266	ORF02793	Hydroxymethylglutaryl-CoA reductase, degradative	10.13	3.00	49520.3	1	I	Cytoplasmic	2-D LC
267	ORF01058	Hypothetical protein	10.13	11.20	31360.6	1	NO related COG	Unknown	2-D LC
268	ORF01450	Transporter	10.13	12.90	27798.4	1	R	CytoplasmicMembrane	2-D LC
269	ORF01273	Hypothetical protein	10.13	1.70	139594.1	1	NO related COG	OuterMembrane	2-D LC
270	ORF01804	ATP synthase F0, A subunit	10.13	4.80	43761.9	1	C	CytoplasmicMembrane	2-D LC
271	ORF02555	Conserved hypothetical protein	10.13	4.80	38999.0	1	NO related COG	Unknown	2-D LC
272	ORF01472	Glyceraldehyde-3-phosphate dehydrogenase, type I	10.13	3.10	53382.3	1	G	Cytoplasmic	2-DE and 2-D LC
273	ORF02148	ThiJ/PfpI domain protein	10.13	13.00	28796.4	1	R	Unknown	2-DE and 2-D LC
274	ORF00752	Asparaginyl-tRNA synthetase	10.13	2.50	55191.4	1	J	Cytoplasmic	2-DE and 2-D LC
275	ORF02405	Fic protein family	10.13	9.00	37735.2	1	S	Unknown	2-D LC
276	ORF01569	Hypothetical protein	10.13	3.70	23951.6	2	NO related COG	Unknown	2-D LC
277	ORF02598	Phospholipase/carboxylesterase family protein	10.13	12.00	24641.6	1	R	Unknown	2-D LC
278	ORF01143	Lipoprotein, putative	10.13	5.60	70424.0	1	NO related COG	OuterMembrane	2-D LC
279	ORF01771	Ribonuclease P protein component	10.13	9.00	17403.4	1	J	Unknown	2-D LC
280	ORF02143	Conserved hypothetical protein	10.13	3.40	110845.5	1	NO related	OuterMembrane	2-D LC

							COG		
281	ORF02032	LAO/AO transport system ATPase	10.13	9.10	40079.4	1	E	Cytoplasmic	2-D LC
282	ORF01295	Conserved hypothetical protein	10.13	12.40	14730.2	1	NO Hits	Cytoplasmic	2-DE and 2-D LC
283	ORF00042	Lipoprotein, putative	10.13	2.70	29857.3	1	NO related COG	Unknown	2-D LC
284	ORF02355	Conserved hypothetical protein	10.13	7.70	27150.7	1	NO related COG	Unknown	2-D LC
285	ORF01144	Oxygen-independent coproporphyrinogen III oxidase	10.13	6.80	52465.9	2	H	Cytoplasmic	2-D LC
286	ORF02432	Competence-damage inducible protein	10.13	8.90	46494.9	2	R	Cytoplasmic	2-D LC
287	ORF00085	OmpA family protein	10.13	2.10	109579.5	1	M	OuterMembrane	2-D LC
288	ORF00212	Protein containing	10.13	14.30	17225.6	1	S	Cytoplasmic	2-D LC
289	ORF01059	Protein containing tetratricopeptide repeats	10.13	5.40	54894.0	1	R	Cytoplasmic	2-D LC
290	ORF01969	TRNA pseudouridine synthase B	10.13	9.90	25954.7	1	J	Unknown	2-D LC
291	ORF02568	PepSY-associated TM helix family	10.13	3.40	82798.9	2	P	CytoplasmicMembrane	2-D LC
292	ORF01117	Conserved hypothetical protein	10.13	10.80	15394.9	1	NO related COG	Unknown	2-D LC
293	ORF02797	Proton/peptide symporter family protein	10.13	7.10	54790.3	2	E	CytoplasmicMembrane	2-D LC
294	ORF01224	MarR family transcriptional regulator protein	10.13	6.00	17260.2	1	K	Cytoplasmic	2-D LC
295	ORF00102	Hypothetical protein	10.13	12.50	17867.2	1	NO related COG	Cytoplasmic	2-D LC
296	ORF00514	23S rRNA (uracil-5--methyltransferase RumA	10.13	5.30	53997.3	1	J	Cytoplasmic	2-D LC
297	ORF01653	Protein containing DUF59	10.13	29.90	12278.1	1	R	Cytoplasmic	2-D LC
298	ORF02056	Conserved hypothetical protein	10.13	6.60	28780.3	2	NO related COG	Unknown	2-D LC

299	ORF02552	Glycosyl transferase, family 2	10.13	5.40	60252.0	1	M	Unknown	2-D LC
300	ORF02015	Fumarate reductase, iron-sulfur protein	10.13	13.00	27687.7	2	C	Unknown	2-D LC
301	ORF01361	ATP-dependent protease La	10.13	2.70	92095.3	1	O	Cytoplasmic	2-D LC
302	ORF00686	Membrane protein, putative	10.13	2.50	124472.3	1	NO related COG	CytoplasmicMembrane	2-D LC
303	ORF00497	Lipoprotein, putative	10.13	5.30	33699.1	1	R	Unknown	2-D LC
304	ORF01015	Hypothetical protein	10.13	54.10	3999.7	1	NO Hits	Unknown	2-D LC
305	ORF01864	Thermolysin	10.13	3.20	97129.3	1	E	Extracellular	2-D LC
306	ORF01272	Hypothetical protein	10.13	2.00	103174.1	1	NO related COG	OuterMembrane	2-D LC
307	ORF00220	Conserved hypothetical protein	10.12	0.80	271595.8	1	NO related COG	Unknown	2-D LC
308	ORF00551	Oxidoreductase	10.12	2.70	54447.3	1	MG	Unknown	2-D LC
309	ORF02024	Gamma-glutamyltransferase	10.12	1.60	62326.1	1	E	Periplasmic	2-D LC
310	ORF01279	Threonyl-tRNA synthetase	10.12	3.40	73923.5	1	J	Cytoplasmic	2-DE and 2-D LC
311	ORF02619	Lipoprotein, putative	10.12	2.80	53576.0	1	NO related COG	OuterMembrane	2-DE and 2-D LC
312	ORF00193	Cyclic nucleotide binding regulatory protein, putative	10.12	11.00	9825.4	3	NO related COG	Cytoplasmic	2-D LC
313	ORF00315	Putative AraC-family transcriptional regulator	10.12	1.80	33140.1	1	K	Unknown	2-D LC
314	ORF00560	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, YrbI family	10.12	12.00	19626.3	1	R	Unknown	2-D LC
315	ORF02362	Acyltransferase domain protein	10.12	1.10	137118.5	2	R	CytoplasmicMembrane	2-D LC
316	ORF00198	Extracellular elastinolytic metalloproteinase	10.12	2.50	101002.8	1	NO related COG	OuterMembrane	2-D LC
317	ORF02298	Transposase	10.12	4.00	32367.3	1	NO related	Unknown	2-D LC

							COG		
318	ORF02837	Conserved domain protein	10.12	10.30	12250.4	1	NO related COG	Cytoplasmic	2-D LC
319	ORF01779	Conserved domain protein	10.12	10.30	12201.4	1	NO related COG	Unknown	2-D LC
320	ORF02003	UvrD/REP family helicase	10.12	1.60	121026.9	1	L	CytoplasmicMembrane	2-D LC
321	ORF00579	Putative two-component system sensor histidine kinase, Putative heat shock protein	10.12	0.80	97470.6	3	L	Unknown	2-D LC
322	ORF02288	Alpha-2-macroglobulin family N- region	10.12	1.30	212165.1	2	R	Unknown	2-D LC
323	ORF02328	Conserved hypothetical protein	10.12	0.70	141579.1	2	R	OuterMembrane	2-D LC
324	ORF01393	NADH dehydrogenase [ubiquinone] flavoprotein 2, precursor	10.12	0.00	20644.6	2	C	Cytoplasmic	2-D LC
325	ORF00086	Hypothetical protein	10.12	11.80	18694.4	1	NO related COG	Unknown	2-D LC
326	ORF01893	Hypothetical protein	10.12	0.00	26982.4	1	NO Hits	Unknown	2-D LC
327	ORF01500	TRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	10.12	4.00	57051.6	1	R	Cytoplasmic	2-D LC
328	ORF01684	NAD(p)-specific glutamate dehydrogenase (nadp-gdh)(nad(p)h-dependent glutamate dehydrogen	10.12	2.90	48692.1	1	E	Cytoplasmic	2-DE and 2-D LC
329	ORF02566	Conserved hypothetical protein	10.12	5.50	54712.5	2	R	Unknown	2-D LC
330	ORF01367	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	10.12	4.50	34004.7	1	M	Cytoplasmic	2-D LC
331	ORF02064	Malate:quinone-oxidoreductase	10.12	2.20	55699.8	1	R	Unknown	2-D LC
332	ORF02848	ATP-dependent Clp protease, ATP-binding subunit ClpX	10.12	5.40	45544.5	1	O	Cytoplasmic	2-D LC
333	ORF01936	Band 7 family protein	10.12	4.60	36693.8	1	O	Unknown	2-D LC
334	ORF01914	S1 RNA binding domain protein	10.12	2.10	79413.8	4	R	Cytoplasmic	2-D LC
335	ORF01853	Two-component system response regulator containi ng LytTR DNA-binding domain	10.12	5.40	27802.9	1	KT	Cytoplasmic	2-D LC

336	ORF00240	Ribosomal protein L1	10.12	3.10	24446.6	1	J	Unknown	2-D LC
337	ORF00927	Protein containing	10.12	2.00	72900.1	2	T	Unknown	2-D LC
338	ORF01717	Protein T24A6.7	10.12	10.20	20641.4	1	S	Unknown	2-D LC
339	ORF01563	IbrB	10.12	7.20	18908.7	1	K	Unknown	2-D LC
340	ORF00218	NADP-dependent malic enzyme	10.12	3.30	84086.0	1	C	Cytoplasmic	2-D LC
341	ORF01560	Hypothetical protein	10.12	15.10	6226.3	1	NO Hits	Unknown	2-D LC
342	ORF00809	Conserved hypothetical protein	10.12	11.80	9675.1	1	NO related COG	Cytoplasmic	2-D LC
343	ORF01313	Ribosomal protein S6 modification protein	10.12	0.00	50313.0	1	HJ	Unknown	2-D LC
344	ORF01859	Cellulase (glycosyl hydrolase family 5), putative	10.12	0.00	60995.6	3	NO related COG	Unknown	2-D LC
345	ORF01485	Hypothetical protein	10.12	0.60	187279.4	1	NO related COG	OuterMembrane	2-D LC
346	ORF02605	DNA polymerase III, beta subunit	10.11	3.50	40972.6	1	L	Unknown	2-DE and 2-D LC
347	ORF02332	Glycosyltransferase, putative	10.11	2.10	56757.6	1	M	Cytoplasmic	2-D LC
348	ORF02259	Hypothetical protein	10.11	0.40	347829.2	1	O	Unknown	2-D LC
349	ORF02378	Conserved hypothetical protein	10.11	6.60	37466.4	2	NO related COG	Unknown	2-D LC
350	ORF01769	CUB domain protein	10.11	0.30	235112.1	1	O	Unknown	2-D LC
351	ORF00371	Outer membrane insertion C- signal domain protein	10.11	2.20	67620.6	2	NO related COG	Unknown	2-D LC
352	ORF02172	TonB-dependent outer membrane receptor	10.11	2.50	90105.0	1	P	OuterMembrane	2-D LC
353	ORF00555	Glutathione peroxidase	10.11	8.30	23411.8	1	O	Periplasmic	2-D LC
354	ORF00182	4-hydroxyphenylpyruvate dioxygenase	10.11	0.00	43990.6	1	ER	Cytoplasmic	2-DE and 2-D LC
355	ORF02265	Peptidase M28	10.11	2.70	56023.7	2	R	Unknown	2-D LC

356	ORF00087	Hypothetical protein	10.11	4.10	45792.4	1	NO related COG	Cytoplasmic	2-D LC
357	ORF01164	Purine nucleoside phosphorylase I, inosine and guanosine-specific	10.11	8.10	30128.4	1	F	Unknown	2-D LC
358	ORF02158	Fumarylacetoacetase	10.11	2.30	47759.8	1	Q	Cytoplasmic	2-D LC
359	ORF00073	Outer membrane efflux protein	10.11	2.30	49963.8	2	MN	OuterMembrane	2-D LC
360	ORF01867	3-oxoacid CoA-transferase, A subunit family	10.11	8.60	25442.1	1	I	Cytoplasmic	2-D LC
361	ORF02053	ABC-type transporter ATP-binding protein	10.11	0.00	35046.3	1	P	Cytoplasmic	2-D LC
362	ORF01020	2-oxoisovalerate dehydrogenase E1 component subu nits alpha and beta	10.11	2.30	74315.5	1	C	Unknown	2-D LC
363	ORF02559	Sugar transferase	10.11	2.40	53888.7	1	M	CytoplasmicMembrane	2-D LC
364	ORF00692	Ribosomal protein L13	10.11	8.60	16619.2	1	J	Unknown	2-D LC
365	ORF02673	Tyrosine-protein kinase	10.11	2.60	93302.6	1	D	CytoplasmicMembrane	2-D LC
366	ORF02788	Penicillin-binding protein 2	10.11	4.20	73772.2	2	M	CytoplasmicMembrane	2-D LC
367	ORF01636	Penicillin-binding protein, putative	10.11	0.00	75783.5	4	M	Unknown	2-D LC
368	ORF00278	Periplasmic copper-binding protein NosD	10.11	4.40	44519.7	3	NO related COG	Periplasmic	2-D LC
369	ORF00608	Exonuclease family protein	10.11	2.00	52500.7	1	L	Cytoplasmic	2-D LC
370	ORF02406	Putative ATP-dependent RNA helicase	10.11	5.70	23954.3	1	LKJ	Unknown	2-D LC
371	ORF02573	Arabinose 5-phosphate isomerase	10.11	4.40	34635.1	1	M	Unknown	2-D LC
372	ORF00077	Hypothetical protein	10.11	5.10	42711.4	1	NO related COG	Cytoplasmic	2-D LC
373	ORF01998	Amino acid transporter, cationic amino acid transporter (CAT) family	10.11	1.20	52208.8	1	E	CytoplasmicMembrane	2-D LC
374	ORF00470	Hypothetical protein	10.11	7.70	15057.5	1	NO related COG	Unknown	2-D LC
375	ORF02667	Conserved hypothetical protein	10.11	2.50	104423.8	1	NO related	Unknown	2-D LC

							COG		
376	ORF00694	Ribosomal protein S2	10.11	4.00	27912.3	1	J	Unknown	2-D LC
377	ORF01888	Conserved hypothetical protein	10.11	3.10	36364.3	2	NO related COG	Unknown	2-D LC
378	ORF00798	Hypothetical protein	10.11	22.00	4647.8	1	NO related COG	Unknown	2-D LC
379	ORF01077	Membrane protein, putative	10.11	7.50	28035.7	1	NO related COG	CytoplasmicMembrane	2-D LC
380	ORF00736	ATPase	10.11	3.20	50384.4	1	O	Unknown	2-D LC
381	ORF00937	OmpA/MotB family outer membrane protein	10.11	1.00	78847.3	1	M	OuterMembrane	2-D LC
382	ORF00035	3-hydroxybutyryl-CoA dehydratase	10.11	7.30	28557.5	1	I	Cytoplasmic	2-D LC
383	ORF00312	Proline racemase	10.11	3.30	37089.2	1	NO related COG	Unknown	2-D LC
384	ORF01645	DNA polymerase IV 2	10.11	3.90	40775.7	1	L	Unknown	2-D LC
385	ORF00576	Methylmalonyl-CoA mutase small subunit	10.11	1.40	56840.6	2	I	Unknown	2-D LC
386	ORF00682	Two-component system sensor protein	10.10	4.40	42205.9	1	T	CytoplasmicMembrane	2-D LC
387	ORF02855	ATP-NAD kinase, putative	10.10	5.80	32939.7	1	R	Unknown	2-D LC
388	ORF01639	OmpA/MotB family outer membrane protein	10.10	4.00	35405.2	1	N	OuterMembrane	2-D LC
389	ORF01244	3-deoxy-D-manno-octulosonate cytidylyltransferase	10.10	6.60	27703.8	1	M	Cytoplasmic	2-D LC
390	ORF00516	Conserved hypothetical protein	10.10	6.40	27151.0	1	NO related COG	CytoplasmicMembrane	2-D LC
391	ORF00944	Major facilitator superfamily MFS_1	10.10	3.90	37702.2	1	NO related COG	CytoplasmicMembrane	2-D LC
392	ORF00935	cAMP-binding domains - Catabolite gene activator and regulatory subunit of cAMP-dependent	10.10	9.20	22596.3	2	T	Cytoplasmic	2-D LC
393	ORF01503	Conserved hypothetical protein	10.10	24.70	8620.8	1	NO	Cytoplasmic	2-D LC

							related COG		
394	ORF00319	LemA protein	10.10	6.40	23239.0	1	S	Unknown	2-D LC
395	ORF02216	Ribosomal protein L14	10.10	7.40	13300.4	1	J	Unknown	2-D LC
396	ORF00786	Two-component system response regulator containi ng LytTR DNA-binding domain	10.10	5.20	29698.9	1	KT	Cytoplasmic	2-D LC
397	ORF01731	S23 ribosomal protein	10.10	20.80	8334.5	1	NO related COG	Unknown	2-D LC
398	ORF01617	Enoyl-CoA hydratase/isomerase family protein	10.10	5.60	27296.2	1	I	Unknown	2-D LC
399	ORF01040	LuxE family acyl-protein synthetase	10.10	3.80	32315.9	1	NO related COG	Cytoplasmic	2-D LC
400	ORF02597	Secreted protein, putative	10.10	6.40	35472.6	1	O	Unknown	2-D LC
401	ORF02131	Copper/zinc superoxide dismutase	10.10	4.60	18413.7	1	P	Periplasmic	2-D LC
402	ORF00550	Hypothetical protein	10.10	7.70	7639.1	1	NO Hits	Unknown	2-D LC
403	ORF02703	Rhs element Vgr protein subfamily, putative	10.10	3.30	23074.7	1	S	Unknown	2-D LC
404	ORF00166	Efflux ABC transporter, permease protein	10.09	3.80	41110.2	1	R	CytoplasmicMembrane	2-D LC
405	ORF02273	Hypothetical protein	10.09	3.70	23331.5	1	NO related COG	Unknown	2-D LC
406	ORF01240	Redox-sensitive transcriptional activator OxyR	10.09	2.60	35612.4	1	K	Cytoplasmic	2-D LC
407	ORF00539	Hypothetical protein	10.09	17.90	4526.1	1	NO Hits	Cytoplasmic	2-D LC
408	ORF02341	Hypothetical protein	10.09	4.90	14313.9	1	NO related COG	Unknown	2-D LC
409	ORF01701	HesB/IscA family scaffold pRotein for iron-sulfu r cluster assembly	10.08	5.50	11987.6	2	S	Unknown	2-D LC
410	ORF02382	3-oxoacyl-[acyl-carrier-protein] synthase III	8.17	6.10	42334.2	1	I	Unknown	2-D LC
411	ORF00238	Ribosomal protein L7/L12	8.15	18.20	12517.3	1	J	Unknown	2-DE and 2-D LC
412	ORF02864	Nitrogen-fixing NifU domain protein	8.15	8.50	34745.4	1	O	Unknown	2-D LC

413	ORF01837	Transcription-repair coupling factor	8.15	0.90	124745.7	1	LK	Unknown	2-D LC
414	ORF01203	Endonuclease I	8.14	5.70	41954.9	1	L	Unknown	2-D LC
415	ORF01031	Conserved hypothetical protein	8.14	0.00	50472.3	4	NO related COG	Unknown	2-D LC
416	ORF00787	Hypothetical protein	8.14	27.70	5668.8	1	NO related COG	Cytoplasmic	2-D LC
417	ORF02584	Conserved hypothetical protein	8.14	24.60	13155.9	2	NO related COG	Unknown	2-D LC
418	ORF02841	SpoU rRNA methylase family protein	8.14	13.00	20159.2	1	J	Cytoplasmic	2-D LC
419	ORF02441	Conserved hypothetical protein	8.13	7.70	30203.5	1	NO related COG	Cytoplasmic	2-D LC
420	ORF00281	Nitrous-oxide reductase (N(2)OR)	8.13	5.20	73169.7	1	C	Periplasmic	2-D LC
421	ORF00341	Outer membrane protein A	8.13	2.40	74837.2	2	M	OuterMembrane	2-DE and 2-D LC
422	ORF02776	RNA methyltransferase, TrmH family	8.13	10.30	27251.5	2	J	Cytoplasmic	2-D LC
423	ORF01223	Band 7 protein	8.13	6.10	65991.6	2	O	Unknown	2-D LC
424	ORF01484	CHU large protein; gliding motility-related protein; putative adhesin AidA-related	8.13	0.70	192948.0	1	NO related COG	Unknown	2-D LC
425	ORF02237	Cysteine protease	8.13	12.10	35636.3	2	NO related COG	Unknown	2-D LC
426	ORF00799	Hypothetical-related protein	8.13	4.00	37639.7	2	G	Unknown	2-D LC
427	ORF00433	ABC transporter, ATP-binding protein	8.13	15.60	25862.8	3	R	CytoplasmicMembrane	2-D LC
428	ORF01475	Membrane protein, putative	8.13	9.90	40641.4	1	NO related COG	CytoplasmicMembrane	2-D LC
429	ORF01222	Conserved hypothetical protein	8.13	0.00	12914.0	3	NO related COG	Unknown	2-D LC

430	ORF02744	Methionine synthase	8.13	2.20	115386.5	1	E	Cytoplasmic	2-D LC
431	ORF00638	Putative phosphate ABC transporter, phosphate-binding component	8.13	5.30	34108.6	1	P	CytoplasmicMembrane	2-D LC
432	ORF02073	Anthranilate synthase component I	8.13	4.20	49576.3	2	EH	Unknown	2-D LC
433	ORF00330	TRNA modification GTPase TrmE	8.13	4.50	51194.6	2	R	Cytoplasmic	2-D LC
434	ORF01394	NADH oxidoreductase (quinone f subunit)	8.13	4.20	50755.6	1	C	Cytoplasmic	2-D LC
435	ORF01011	Conserved hypothetical protein	8.13	11.30	18043.9	2	NO related COG	Unknown	2-D LC
436	ORF01545	Conserved hypothetical protein	8.13	12.80	25466.9	1	NO related COG	Cytoplasmic	2-D LC
437	ORF01303	Reticulocyte binding protein, putative	8.13	2.70	42108.3	1	L	Unknown	2-D LC
438	ORF02138	Conserved hypothetical protein	8.13	9.40	40320.9	1	NO related COG	Unknown	2-D LC
439	ORF00227	Putative outer membrane adhesin like proteiin	8.13	1.50	250778.6	1	NO related COG	Extracellular	2-D LC
440	ORF00169	DNA polymerase III subunit alpha-1	8.12	1.50	172457.7	2	L	Cytoplasmic	2-D LC
441	ORF00062	Type II restriction-modification enzyme	8.12	0.60	145718.8	1	L	Cytoplasmic	2-D LC
442	ORF00214	Translation initiation factor IF-2	8.12	0.00	105954.1	2	J	Cytoplasmic	2-D LC
443	ORF00262	Lipoprotein, putative	8.12	10.20	21672.2	4	NO related COG	Unknown	2-D LC
444	ORF01539	Carbamoyl-phosphate synthase, large subunit	8.12	2.00	105995.1	1	EF	Unknown	2-D LC
445	ORF00646	Kynurenine 3-monooxygenase	8.12	0.00	51846.0	1	HC	Unknown	2-D LC
446	ORF02858	Outer membrane protein assembly complex, YaeT protein	8.12	1.10	98753.4	1	M	OuterMembrane	2-D LC
447	ORF00801	Nitroreductase	8.12	7.10	24263.5	1	C	Unknown	2-DE and 2-D LC

448	ORF01940	TRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	8.12	3.00	70180.8	1	D	Unknown	2-D LC
449	ORF01137	DegT/DnrJ/EryC1/StrS aminotransferase	8.12	6.40	41000.8	3	M	Unknown	2-D LC
450	ORF02676	UDP-glucose 6-dehydrogenase	8.12	3.10	50358.4	1	M	Unknown	2-DE and 2-D LC
451	ORF02823	Acetyltransferase, gnat family	8.12	13.80	18373.0	1	KR	Cytoplasmic	2-D LC
452	ORF00048	Seryl-tRNA synthetase	8.12	1.90	47511.9	1	J	Cytoplasmic	2-D LC
453	ORF00943	Formate dehydrogenase, alpha subunit	8.12	2.00	82311.1	1	C	Periplasmic	2-D LC
454	ORF00747	AcrB/AcrD/AcrF family heavy metal cation efflux protein containing OEP domains	8.12	0.80	159635.0	1	P	CytoplasmicMembrane	2-D LC
455	ORF00103	Conserved hypothetical protein	8.12	2.80	89063.2	1	NO related COG	Unknown	2-D LC
456	ORF02232	TonB-dependent outer membrane receptor	8.12	1.20	115167.0	1	P	OuterMembrane	2-D LC
457	ORF02570	ABC transporter, ATP-binding protein	8.12	10.60	27741.9	1	R	Cytoplasmic	2-D LC
458	ORF02639	ABC transporter, ATP-binding protein	8.12	3.90	29046.3	1	N	Cytoplasmic	2-D LC
459	ORF01354	Aspartate carbamoyltransferase	8.12	6.50	34225.4	1	F	Cytoplasmic	2-D LC
460	ORF00258	RagA protein, putative	8.12	1.70	117966.7	2	P	OuterMembrane	2-D LC
461	ORF00266	Putative TonB-dependent receptor	8.11	2.70	80838.0	1	P	OuterMembrane	2-D LC
462	ORF02095	Signal recognition particle protein	8.11	4.20	49351.0	1	N	Cytoplasmic	2-D LC
463	ORF00836	Tetracycline resistance element mobilization regulatory protein RteC	8.11	0.00	32690.4	1	NO related COG	Unknown	2-D LC
464	ORF00768	B-glycosyltransferase, glycosyltransferase family 2 protein	8.11	5.40	34684.0	1	M	Unknown	2-D LC
465	ORF00649	Propeptide, peptidase M4 and M36	8.11	2.50	76272.2	1	NO related COG	Unknown	2-D LC
466	ORF01812	Conserved hypothetical protein	8.11	5.00	23841.1	1	NO related COG	Unknown	2-D LC
467	ORF01733	Cytochrome c551 peroxidase	8.11	3.80	38292.3	1	P	Unknown	2-D LC

468	ORF01283	Conserved hypothetical protein	8.11	2.30	34401.5	3	R	Unknown	2-D LC
469	ORF01089	Putative transcriptional regulator	8.11	3.40	51920.9	1	R	Cytoplasmic	2-D LC
470	ORF02536	UDP-N-acetylglucosamine 2-epimerase	8.11	3.70	42420.3	1	M	Cytoplasmic	2-D LC
471	ORF01690	TonB-dependent outer membrane receptor	8.11	1.70	105170.8	1	P	OuterMembrane	2-D LC
472	ORF00348	Anhydro-N-acetylmuramic acid kinase	8.11	1.70	40612.9	1	S	Cytoplasmic	2-D LC
473	ORF01843	Hypothetical protein	8.11	27.00	4491.5	1	NO Hits	Unknown	2-D LC
474	ORF00178	Transcription elongation factor GreA	8.11	8.20	18878.6	1	K	Cytoplasmic	2-DE and 2-D LC
475	ORF02236	ATPase, AAA family	8.11	2.40	47712.2	1	L	Unknown	2-D LC
476	ORF02599	Conserved hypothetical protein	8.11	10.80	12257.4	1	GEPR	CytoplasmicMembrane	2-D LC
477	ORF00203	Ribonucleoside-diphosphate reductase, beta subunit	8.11	7.40	37885.0	1	F	Cytoplasmic	2-D LC
478	ORF00263	Lysyl-tRNA synthetase	8.11	1.20	64060.0	1	J	Cytoplasmic	2-DE and 2-D LC
479	ORF00609	TRNA delta(2-isopentenylpyrophosphate transferase	8.11	4.90	35788.5	2	J	Cytoplasmic	2-D LC
480	ORF02389	Phenylalanine and histidine ammonia-lyase	8.11	0.00	35768.6	1	E	Cytoplasmic	2-D LC
481	ORF01861	Integral membrane protein	8.11	1.20	118742.6	1	NO related COG	CytoplasmicMembrane	2-D LC
482	ORF00475	Hemagglutinin, putative	8.10	4.60	29591.2	2	NO related COG	Unknown	2-D LC
483	ORF00528	Conserved hypothetical protein	8.10	2.50	62219.8	1	D	Unknown	2-D LC
484	ORF02672	Polysaccharide export outer membrane protein	8.10	7.70	29413.2	1	M	Cytoplasmic	2-D LC
485	ORF00606	Hypothetical protein	8.10	24.10	6838.0	1	NO related COG	Cytoplasmic	2-D LC
486	ORF01105	Conserved hypothetical protein	8.10	3.80	23189.7	1	R	Cytoplasmic	2-D LC
487	ORF01212	Short-chain dehydrogenase/reductase family prote in	8.10	2.60	29479.1	1	QR	Cytoplasmic	2-D LC

488	ORF01142	Cell-division ATP-binding protein	8.10	5.30	25338.1	1	D	Cytoplasmic	2-D LC
489	ORF00171	Riboflavin biosynthesis protein RibF	8.09	5.20	33247.7	1	H	Cytoplasmic	2-D LC
490	ORF01035	Hemagglutinin	8.09	4.60	17678.6	2	N	Unknown	2-D LC
491	ORF00574	Septum formation initiator subfamily	8.08	9.20	13714.6	1	S	Cytoplasmic	2-D LC
492	ORF02042	Hypothetical protein	6.14	2.70	80623.7	2	P	OuterMembrane	2-D LC
493	ORF00231	Sensory transduction histidine kinase, putative	6.13	5.50	64853.9	1	T	CytoplasmicMembrane	2-D LC
494	ORF02060	Mannosyltransferase	6.13	5.10	43014.4	1	M	Cytoplasmic	2-D LC
495	ORF01402	NADH dehydrogenase i chain m	6.13	2.10	53357.5	1	C	CytoplasmicMembrane	2-D LC
496	ORF00451	Putative RNA methylase family family	6.13	4.90	44276.6	2	L	Unknown	2-D LC
497	ORF01872	Collagenase	6.13	6.50	46043.5	1	O	Cytoplasmic	2-D LC
498	ORF01390	NADH dehydrogenase i, b subunit	6.13	5.50	20103.2	1	C	Unknown	2-D LC
499	ORF01370	UDP-3-0-acyl N-acetylglucosamine deacetylase	6.13	5.00	51143.1	1	M	Unknown	2-D LC
500	ORF02664	Amidinotransferase family protein	6.12	3.30	34485.8	1	NO related COG	Cytoplasmic	2-D LC
501	ORF02212	Ribosomal protein S8	6.12	11.40	14722.1	1	J	Unknown	2-D LC
502	ORF00534	Prolyl-tRNA synthetase	6.12	1.40	55885.4	1	J	Cytoplasmic	2-DE and 2-D LC
503	ORF02094	Prolyl endopeptidase	6.12	1.40	79598.6	3	E	Periplasmic	2-D LC
504	ORF01422	Membrane protein	6.12	0.90	90975.1	1	R	CytoplasmicMembrane	2-D LC
505	ORF00611	DNA gyrase subunit A	6.12	1.10	103195.9	1	L	Cytoplasmic	2-D LC
506	ORF01851	Cyanobacterial phytochrome B	6.11	2.00	40347.7	1	T	CytoplasmicMembrane	2-D LC
507	ORF01898	Crispr-associated protein, Csn1 family	6.11	1.30	172670.0	1	S	Unknown	2-D LC
508	ORF01879	Transcriptional regulator, AraC family protein	6.11	1.20	66738.8	2	R	OuterMembrane	2-D LC
509	ORF00777	Lipoprotein, putative	6.11	4.30	42261.5	1	O	Unknown	2-D LC

510	ORF00078	Hypothetical protein	6.11	4.50	34305.8	1	NO related COG	Unknown	2-D LC
511	ORF02436	Lipoamide acyltransferase component of branched-chain alpha-keto aciddehydrogenase complex	6.11	9.80	45405.7	2	C	Cytoplasmic	2-DE and 2-D LC
512	ORF00430	ATP-dependent rna helicase, dead/deah box family	6.11	3.30	68182.7	1	LKJ	Cytoplasmic	2-D LC
513	ORF02106	Hypothetical protein	6.11	39.60	5515.6	1	NO related COG	Unknown	2-D LC
514	ORF01342	DNA polymerase III subunit gamma/tau	6.10	9.00	16839.2	1	J	Cytoplasmic	2-D LC
515	ORF02141	Peptidase, M28 family	6.10	1.50	44604.3	1	R	Extracellular	2-D LC
516	ORF00956	Molybdopterin biosynthesis MoeA protein	6.10	2.10	42858.8	1	H	CytoplasmicMembrane	2-D LC
517	ORF01061	Conserved hypothetical protein	6.10	0.00	65001.9	1	NO related COG	Unknown	2-D LC
518	ORF00895	Conserved hypothetical protein	6.10	4.10	37145.9	3	NO related COG	Unknown	2-D LC
519	ORF00693	Ribosomal protein S9	6.10	11.70	14330.7	2	J	Unknown	2-D LC
520	ORF01085	Conserved hypothetical protein	6.10	12.00	13899.0	2	NO related COG	Cytoplasmic	2-D LC
521	ORF01842	Methionyl-tRNA synthetase	4.14	3.80	78063.4	1	J	Cytoplasmic	2-DE and 2-D LC
522	ORF02371	3-oxoacyl-[acyl-carrier-protein] synthase	4.14	5.30	43175.9	1	IQ	Cytoplasmic	2-D LC
523	ORF01129	Conserved hypothetical protein	4.13	6.20	37620.9	1	P	Unknown	2-D LC
524	ORF00325	Conserved hypothetical protein	4.13	9.20	16409.2	1	NO related COG	Cytoplasmic	2-D LC
525	ORF02645	Transposase, IS4	4.13	21.00	6965.1	1	NO related COG	Unknown	2-D LC
526	ORF00295	Conserved hypothetical protein	4.13	5.60	20464.1	1	NO related	CytoplasmicMembrane	2-D LC

							COG		
527	ORF01933	Glutaminyl-tRNA synthetase	4.12	2.30	65257.2	2	J	Unknown	2-DE and 2-D LC
528	ORF01197	Excinuclease ABC, A subunit	4.12	2.10	105686.0	1	L	Unknown	2-D LC
529	ORF00788	Type III restriction enzyme, res subunit family	4.12	3.30	59660.6	1	L	Unknown	2-D LC
530	ORF00796	ABC transporter domain protein	4.12	2.70	62582.5	1	R	Cytoplasmic	2-DE and 2-D LC
531	ORF00093	Conserved hypothetical protein	4.11	4.40	43994.9	1	NO related COG	Unknown	2-D LC
532	ORF00945	NarK2 protein	4.11	5.10	30308.5	1	P	CytoplasmicMembrane	2-D LC
533	ORF02655	ISSod13, transposase	4.11	4.10	42546.1	1	L	Cytoplasmic	2-D LC
534	ORF00129	ISSod13, transposase	4.11	5.00	34552.1	1	NO related COG	Cytoplasmic	2-D LC
535	ORF01316	Protein Nmul_A1510	4.11	2.70	60451.0	1	S	Unknown	2-D LC
536	ORF01902	Argininosuccinate lyase	4.10	3.50	49158.4	1	E	Cytoplasmic	2-D LC
537	ORF00681	Sensory transduction protein LytT	4.10	2.90	27936.2	1	KT	Cytoplasmic	2-D LC
538	ORF00922	Secreted aminopeptidase	4.10	2.40	82317.3	1	E	Cytoplasmic	2-D LC
539	ORF00629	Membrane protein, putative	4.10	3.60	47245.3	1	R	CytoplasmicMembrane	2-D LC
540	ORF00726	Aspartate aminotransferase A	2.14	6.30	43487.6	2	E	Unknown	2-DE and 2-D LC
541	ORF00541	Lipoprotein, putative	2.13	0.00	61397.5	1	NO related COG	Unknown	2-D LC
542	ORF00600	Probable 3-hydroxybutyryl-CoA dehydrogenase	2.13	12.10	32362.5	1	I	Cytoplasmic	2-D LC
543	ORF01889	DNA processing chain A	2.13	9.60	41296.4	1	LN	Cytoplasmic	2-D LC
544	ORF02171	Putative transcriptional regulator, MarR family	2.12	0.00	23244.0	2	K	Unknown	2-D LC
545	ORF00718	Peptidase U32	2.11	2.20	71375.5	1	O	Unknown	2-D LC

546	ORF00043	Secreted protein containing tetratricopeptide re peats	2.11	0.00	52853.9	1	R	Unknown	2-D LC
547	ORF01840	Carboxypeptidase	2.11	2.00	33686.9	2	S	Unknown	2-D LC
548	ORF00587	Hypothetical protein	2.08	12.00	5625.7	1	NO Hits	Unknown	2-D LC

APPENDIX D

FLAVOBACTERIUM COLUMNARE PROTEINS IDENTIFIED USING 2-DE

Spot #	SSP #	ORF #	Protein MW/pI	C.I. %	Protein name	Peptide count	Subcellular location	COG category	Identified by
1	1601	ORF00011	51419.6/4.96	100	Peptidase, M20/M25/M40 family	26	Unknown	E	2-DE
2	8302	ORF00027	38302.7/6.03	100	Uroporphyrinogen decarboxylase	14	Cytoplasmic	H	2-DE
3	2004	ORF00040	14894.4/5.19	100	Protein containing	12	Cytoplasmic	NO related COG	2-DE
4	2601	ORF00063	42911/5.13	100	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase	21	Cytoplasmic Membrane	C	2-DE
5	8402	ORF00165	43457.8/5.84	100	Acyl-CoA dehydrogenase	20	Unknown	I	2-DE and 2-D LC
6	2005	ORF00178	18867/5.06	100	Transcription elongation factor GreA	14	Cytoplasmic	K	2-DE and 2-D LC
7	8503	ORF00181	44869.4/6.02	100	Homogentisate 1,2-dioxygenase	18	Unknown	Q	2-DE and 2-D LC
8	2401	ORF00182	43963.1/5.1	100	4-hydroxyphenylpyruvate dioxygenase	16	Cytoplasmic	ER	2-DE and 2-D LC
9	5801	ORF00190	67135.8/5.41	100	Aspartyl-tRNA synthetase	24	Cytoplasmic	J	2-DE and 2-D LC
10	7204	ORF00211	30920.8/5.79	100	Universal stress protein family protein	18	Cytoplasmic	T	2-DE
11	7803	ORF00225	73367.3/7.08	100	OmpA/MotB family outer membrane protein	21	OuterMembrane	M	2-DE
12	8801	ORF00225	73367.3/7.08	100	OmpA/MotB family outer membrane protein	29	OuterMembrane	M	2-DE
13	1	ORF00238	12509.7/4.59	100	Ribosomal protein L7/L12	6	Unknown	J	2-DE and 2-D LC
14	2001	ORF00239	17992.6/5.25	100	Ribosomal protein L10	7	Cytoplasmic	J	2-DE
15	3001	ORF00239	17992.6/5.25	100	Ribosomal protein L10	11	Cytoplasmic	J	2-DE
16	3002	ORF00239	17992.6/5.25	100	Ribosomal protein L10	13	Cytoplasmic	J	2-DE
17	3501	ORF00244	43115.9/5.14	100	Translation elongation factor Tu	22	Cytoplasmic	JE	2-DE and 2-D LC
18	8404	ORF00250	43569/6.05	100	Acyl-CoA dehydrogenase	19	Cytoplasmic	I	2-DE and 2-D LC
19	5508	ORF00254	50081.1/5.5	100	Cystathionine beta-synthase	18	Cytoplasmic	E	2-DE

20	2702	ORF00263	64019.4/5.14	100	Lysyl-tRNA synthetase	15	Cytoplasmic	J	2-DE and 2-D LC
21	4401	ORF00283	48119.6/5.5	100	Glutamate-1-semialdehyde-2,1-aminomutase	17	Unknown	H	2-DE
22	8805	ORF00290	74298.9/5.95	100	Urocanate hydratase	24	Cytoplasmic	E	2-DE and 2-D LC
23	8806	ORF00296	67323.7/6.04	100	Transcription termination factor Rho	20	Cytoplasmic	K	2-DE
24	7704	ORF00324	60251.8/5.74	100	Response regulator	22	Cytoplasmic	TK	2-DE and 2-D LC
25	6401	ORF00332	40832.9/5.49	100	acetyl-CoA acetyltransferase	15	Cytoplasmic	I	2-DE and 2-D LC
26	6804	ORF00341	74790.9/5.79	100	Outer membrane protein A	21	OuterMembrane	M	2-DE and 2-D LC
27	7401	ORF00349	41011.7/5.71	100	Acyl-CoA dehydrogenase	23	Cytoplasmic	I	2-DE and 2-D LC
28	8301	ORF00385	35840.8/5.85	100	Glyceraldehyde-3-phosphate dehydrogenase, type	19	Cytoplasmic	G	2-DE and 2-D LC
29	2202	ORF00423	32801.9/5.08	100	Malate dehydrogenase, NAD-dependent	11	Cytoplasmic	C	2-DE and 2-D LC
30	6205	ORF00482	31839.9/5.75	100	Dihydrodipicolinate synthase	16	Unknown	EM	2-DE
31	5	ORF00494	13996.2/4.74	100	Endoribonuclease L-PSP, putative	6	Unknown	J	2-DE
32	1801	ORF00495	67684.6/4.86	100	Chaperone protein DnaK	29	Unknown	O	2-DE and 2-D LC
33	6403	ORF00504	45208.3/5.64	100	Ornithine--oxo-acid transaminase	22	Cytoplasmic	E	2-DE and 2-D LC
34	2003	ORF00518	12662.4/5.1	100	Conserved hypothetical protein	8	Unknown	NO related COG	2-DE
35	5001	ORF00527	15100.6/5.36	100	Nucleoside diphosphate kinase	10	Cytoplasmic	F	2-DE and 2-D LC
36	4801	ORF00532	67017.5/5.26	100	GTP-binding protein TypA/BipA	26	Cytoplasmic	N	2-DE
37	3803	ORF00532	67017.5/5.26	100	GTP-binding protein TypA/BipA	20	Cytoplasmic	N	2-DE
38	6606	ORF00534	55850.2/5.55	100	Prolyl-tRNA synthetase	25	Cytoplasmic	J	2-DE and 2-D LC
39	5702	ORF00534	55850.2/5.55	100	Prolyl-tRNA synthetase	20	Cytoplasmic	J	2-DE and 2-D LC

40	4001	ORF00558	16891.7/6.73	100	Hypothetical protein	9	Unknown	NO related COG	2-DE
41	7101	ORF00572	27686.3/5.77	100	Triose-phosphate isomerase	11	Unknown	G	2-DE
42	4302	ORF00640	30450.1/5.52	100	TonB	12	Unknown	M	2-DE
43	5302	ORF00640	30450.1/5.52	100	TonB	12	Unknown	M	2-DE
44	7501	ORF00652	39991.2/5.74	100	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	21	Cytoplasmic	J	2-DE
45	1201	ORF00695	28978.9/4.91	100	Translation elongation factor Ts	20	Cytoplasmic	J	2-DE and 2-D LC
46	1204	ORF00695	28978.9/4.91	100	Translation elongation factor Ts	24	Cytoplasmic	J	2-DE and 2-D LC
47	1002	ORF00699	13841.2/5.36	100	Conserved hypothetical protein	6	Cytoplasmic	NO related COG	2-DE
48	5403	ORF00726	43460.1/5.49	100	Aspartate aminotransferase A	14	Unknown	E	2-DE and 2-D LC
49	7706	ORF00739	59613.2/5.78	100	1-pyrroline-5-carboxylate dehydrogenase	15	Cytoplasmic	C	2-DE and 2-D LC
50	7601	ORF00739	59613.2/5.78	100	1-pyrroline-5-carboxylate dehydrogenase	24	Cytoplasmic	C	2-DE and 2-D LC
51	6702	ORF00739	59613.2/5.78	100	1-pyrroline-5-carboxylate dehydrogenase	19	Cytoplasmic	C	2-DE and 2-D LC
52	5602	ORF00752	55157/5.51	100	Asparaginyl-tRNA synthetase	24	Cytoplasmic	J	2-DE and 2-D LC
53	8703	ORF00763	61877.9/6.1	100	CTP synthase	22	Cytoplasmic	F	2-DE
54	5802	ORF00796	62544/5.39	100	ABC transporter domain protein	17	Cytoplasmic	R	2-DE and 2-D LC
55	1105	ORF00801	24248.2/4.95	100	Nitroreductase	13	Unknown	C	2-DE and 2-D LC
56	105	ORF00807	23568.7/4.65	100	Antioxidant, AhpC/Tsa family	15	Cytoplasmic	O	2-DE
57	104	ORF00807	23568.7/4.65	100	Antioxidant, AhpC/Tsa family	18	Cytoplasmic	O	2-DE
58	9501	ORF00901	46452.9/6.35	100	Peptidase T	14	Cytoplasmic	E	2-DE
59	8607	ORF00982	52337.5/6.19	100	Inosine-5'-monophosphate dehydrogenase	25	Unknown	F	2-DE

60	3302	ORF01021	24907.6/5.31	100	Iron/ascorbate-dePendent oxidoreductase family protein	14	Cytoplasmic	R	2-DE
61	7901	ORF01096	81812.8/5.78	100	Glutamine synthetase	36	Unknown	E	2-DE and 2-D LC
62	3101	ORF01119	31632.2/5.16	100	Nicotinate-nucleotide pyrophosphorylase	4	Cytoplasmic	H	2-DE
63	6304	ORF01124	36443/5.77	100	Aspartate-semialdehyde dehydrogenase	21	Unknown	E	2-DE
64	9603	ORF01153	50296.3/6.38	100	Dihydrolipoyl dehydrogenase	10	Cytoplasmic	C	2-DE and 2-D LC
65	7201	ORF01161	29727.8/5.74	100	Conserved hypothetical protein	22	Cytoplasmic	R	2-DE
66	2402	ORF01193	43068/5.08	100	Succinyl-CoA synthetase beta chain	26	Cytoplasmic	C	2-DE and 2-D LC
67	8601	ORF01201	49296.6/5.79	100	Dihydrolipoyl dehydrogenase	17	Cytoplasmic	C	2-DE and 2-D LC
68	2101	ORF01210	17078.8/5.53	100	Transaldolase	10	Cytoplasmic	G	2-DE and 2-D LC
69	4303	ORF01228	39462.8/5.33	100	Glycine cleavage system T protein	14	Cytoplasmic	E	2-DE and 2-D LC
70	9701	ORF01261	51197.1/6.67	100	Glycyl-tRNA synthetase	23	Cytoplasmic	J	2-DE
71	9702	ORF01261	51197.1/6.67	100	Glycyl-tRNA synthetase	24	Cytoplasmic	J	2-DE
72	7804	ORF01279	73877.7/5.87	100	Threonyl-tRNA synthetase	24	Cytoplasmic	J	2-DE and 2-D LC
73	5301	ORF01286	35849.3/5.41	100	Acetyl-CoA carboxylase, carboxyl transferase, alpha subunit	15	Cytoplasmic	I	2-DE and 2-D LC
74	8504	ORF01293	46569.8/6.05	100	2-amino-3-ketobutyrate coenzyme A ligase	12	Unknown	H	2-DE
75	1005	ORF01295	14721.2/4.89	100	Conserved hypothetical protein	16	Cytoplasmic	NO Hits	2-DE and 2-D LC
76	8401	ORF01297	39717.3/5.84	100	Leucine dehydrogenase	14	Cytoplasmic	E	2-DE and 2-D LC
77	7402	ORF01297	39717.3/5.84	100	Leucine dehydrogenase	13	Cytoplasmic	E	2-DE and 2-D LC
78	7301	ORF01297	39717.3/5.84	100	Leucine dehydrogenase	14	Cytoplasmic	E	2-DE and 2-D LC
79	1301	ORF01351	36754.7/4.84	100	Deoxyhypusine synthase	13	Unknown	J	2-DE and 2-D LC

80	2803	ORF01357	65513.1/5.08	100	30S ribosomal protein S1	30	Cytoplasmic	J	2-DE and 2-D LC
81	2802	ORF01357	65513.1/5.08	100	30S ribosomal protein S1	31	Cytoplasmic	J	2-DE and 2-D LC
82	4202	ORF01366	30051.5/5.49	100	Succinyl-CoA synthetase, alpha subunit	10	Unknown	C	2-DE and 2-D LC
83	5202	ORF01366	30051.5/5.49	100	Succinyl-CoA synthetase, alpha subunit	12	Unknown	C	2-DE and 2-D LC
84	5204	ORF01366	30051.5/5.49	100	Succinyl-CoA synthetase, alpha subunit	13	Unknown	C	2-DE and 2-D LC
85	3104	ORF01368	20787.6/5.28	100	Translation elongation factor P	10	Cytoplasmic	J	2-DE
86	8505	ORF01471	49725.1/8.31	100	HtrA protein	17	Periplasmic	O	2-DE and 2-D LC
87	9402	ORF01471	49725.1/8.31	100	HtrA protein	21	Periplasmic	O	2-DE and 2-D LC
88	8602	ORF01472	53349.8/5.95	100	Glyceraldehyde-3-phosphate dehydrogenase, type I	20	Cytoplasmic	G	2-DE and 2-D LC
89	8605	ORF01472	53349.8/5.95	100	Glyceraldehyde-3-phosphate dehydrogenase, type	21	Cytoplasmic	G	2-DE and 2-D LC
90	4502	ORF01476	43181.1/5.8	100	Hypothetical protein	14	Unknown	NO related COG	2-DE
91	5506	ORF01476	43181.1/5.8	100	Hypothetical protein	12	Unknown	NO related COG	2-DE
92	6605	ORF01491	51694.2/6.62	100	PpiC-type peptidyl-prolyl cis-trans isomerase	25	Periplasmic	O	2-DE
93	4802	ORF01504	69688.4/5.55	100	Secreted glycyl aminopeptidase, family M61	22	Unknown	O	2-DE and 2-D LC
94	7701	ORF01646	52532.5/5.73	100	Pyruvate kinase	20	Cytoplasmic	G	2-DE and 2-D LC
95	6202	ORF01668	37606.1/5.63	100	ATPase, MoxR family	26	Cytoplasmic	R	2-DE and 2-D LC
96	7503	ORF01671	44532.4/5.92	100	3-oxoacyl-[acyl-carrier-protein] synthase 2	14	Cytoplasmic	IQ	2-DE
97	8502	ORF01671	44532.4/5.92	100	3-oxoacyl-[acyl-carrier-protein] synthase 2	16	Cytoplasmic	IQ	2-DE
98	6501	ORF01684	48661.4/5.58	100	NAD(p-specific glutamate dehydrogenase (nadp-gdh)(nad(p)h-dependent glutamate dehydrogenase	22	Cytoplasmic	E	2-DE and 2-D LC

99	4501	ORF01684	48661.4/5.58	100	NAD(p-specific glutamate dehydrogenase (nadp-gdh)(nad(p)h-dependent glutamate dehydrogenase	28	Cytoplasmic	E	2-DE
100	5507	ORF01684	48661.4/5.58	100	NAD(p-specific glutamate dehydrogenase (nadp-gdh)(nad(p)h-dependent glutamate dehydrogenase	28	Cytoplasmic	E	2-DE
101	5503	ORF01684	48661.4/5.58	100	NAD(p-specific glutamate dehydrogenase (nadp-gdh)(nad(p)h-dependent glutamate dehydrogenase	28	Cytoplasmic	E	2-DE
102	9401	ORF01686	41760.4/6.27	100	cystathionine beta-lyase	11	Cytoplasmic	E	2-DE
103	4601	ORF01687	56251.3/5.31	100	Piperideine-6-carboxylate dehydrogenase	22	Cytoplasmic	C	2-DE
104	2301	ORF01694	34096.6/5.13	100	PfkB family carbohydrate kinase	15	Unknown	G	2-DE
105	2303	ORF01694	34096.6/5.13	100	PfkB family carbohydrate kinase	15	Unknown	G	2-DE
106	4702	ORF01702	53903.9/5.34	100	FeS assembly protein SufB	17	Unknown	R	2-DE
107	1205	ORF01704	27792.4/5.04	100	FeS assembly ATPase SufC	10	CytoplasmicMembrane	R	2-DE
108	2201	ORF01704	27792.4/5.04	100	FeS assembly ATPase SufC	14	CytoplasmicMembrane	R	2-DE
109	3401	ORF01714	43271.2/5.14	100	Alcohol dehydrogenase YqhD	26	Cytoplasmic	C	2-DE and 2-D LC
110	1401	ORF01714	43271.2/5.14	100	Alcohol dehydrogenase YqhD	11	Cytoplasmic	C	2-DE and 2-D LC
111	2304	ORF01714	43271.2/5.14	100	Alcohol dehydrogenase YqhD	21	Cytoplasmic	C	2-DE and 2-D LC
112	3003	ORF01747	14369.2/5.3	100	Conserved hypothetical protein	12	Unknown	NO related COG	2-DE
113	8403	ORF01791	47643.8/8.61	100	Conserved hypothetical protein	18	Unknown	E	2-DE
114	8603	ORF01792	49902/5.93	100	Acetyl-CoA carboxylase, biotin carboxylase	25	Cytoplasmic	I	2-DE and 2-D LC
115	8501	ORF01792	49902/5.93	100	Acetyl-CoA carboxylase, biotin carboxylase	22	Cytoplasmic	I	2-DE and 2-D LC
116	101	ORF01793	17698.3/4.59	100	Acetyl-CoA carboxylase, biotin carboxyl carrier protein	10	Unknown	I	2-DE and 2-D LC

117	4701	ORF01800	56528.8/5.44	100	ATP synthase F1, alpha subunit	22	Unknown	C	2-DE and 2-D LC
118	5601	ORF01800	56528.8/5.44	100	ATP synthase F1, alpha subunit	23	Unknown	C	2-DE and 2-D LC
119	2501	ORF01818	46602.3/5.02	100	Phosphoribosylamine--glycine ligase	15	Unknown	F	2-DE
120	5101	ORF01824	20888.5/5.46	100	DTDP-4-dehydrorhamnose 3,5-epimerase	11	Unknown	M	2-DE
121	7707	ORF01826	58939.4/5.48	100	Conserved hypothetical protein	23	Cytoplasmic	NO related COG	2-DE and 2-D LC
122	7705	ORF01826	58939.4/5.48	100	Conserved hypothetical protein	20	Cytoplasmic	NO related COG	2-DE and 2-D LC
123	8704	ORF01830	58888.1/6.02	100	Fumarate hydratase class I, anaerobic	29	Unknown	C	2-DE
124	5901	ORF01842	78014.9/5.41	100	Methionyl-tRNA synthetase	29	Cytoplasmic	J	2-DE and 2-D LC
125	1004	ORF01844	19766.8/4.97	100	Nonheme iron-containing ferritin	12	Cytoplasmic	P	2-DE
126	4203	ORF01911	36633.4/5.33	100	Hypothetical protein	17	Cytoplasmic	T	2-DE
127	6803	ORF01933	65216.7/5.65	100	Glutaminyl-tRNA synthetase	24	Unknown	J	2-DE and 2-D LC
128	1003	ORF01985	14883.3/4.9	100	Conserved hypothetical protein	8	Unknown	J	2-DE
129	7502	ORF01993	51006.1/5.83	100	Saccharopine dehydrogenase	17	Unknown	E	2-DE
130	2701	ORF01995	60260/4.98	100	Phosphoenolpyruvate carboxykinase (ATP	29	Cytoplasmic	C	2-DE and 2-D LC
131	2103	ORF02002	22281.6/5.21	100	Superoxide dismutase [Mn]	8	Unknown	P	2-DE and 2-D LC
132	1501	ORF02005	50181.4/4.9	100	Outer membrane protein A	20	OuterMembrane	M	2-DE and 2-D LC
133	9804	ORF02014	74106/6.29	100	Succinate dehydrogenase flavoprotein subunit	20	Cytoplasmic	C	2-DE
134	9801	ORF02014	74106/6.29	100	Succinate dehydrogenase flavoprotein subunit	27	Cytoplasmic	C	2-DE
135	2801	ORF02047	71591.5/5.02	100	Hsp90 protein	20	Cytoplasmic	O	2-DE
136	3201	ORF02062	29378.2/5.26	100	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	19	Cytoplasmic	E	2-DE

137	5404	ORF02127	41950.3/5.49	100	3-ketoacyl-CoA thiolase	15	Cytoplasmic	I	2-DE
138	4901	ORF02144	89788.7/5.29	100	2-oxoacid dehydrogenase E1 component subunits alpha and beta	32	Cytoplasmic	C	2-DE and 2-D LC
139	2104	ORF02148	28778.7/5.73	100	ThiJ/PfpI domain protein	11	Unknown	R	2-DE and 2-D LC
140	2502	ORF02157	46513/5.04	100	Serine hydroxymethyltransferase	18	Cytoplasmic	E	2-DE and 2-D LC
141	1001	ORF02176	19989/5.85	100	Secreted protein	9	OuterMembrane	NO related COG	2-DE
142	1402	ORF02198	46028.3/4.97	100	Phosphopyruvate hydratase	14	Cytoplasmic	G	2-DE
143	1403	ORF02198	46028.3/4.97	100	Phosphopyruvate hydratase	14	Cytoplasmic	G	2-DE
144	6404	ORF02199	40955.3/5.68	100	Carbamoyl-phosphate synthase, small subunit	14	Unknown	EF	2-DE
145	2902	ORF02228	79136.9/5.06	100	Translation elongation factor G	36	Cytoplasmic	J	2-DE
146	2102	ORF02269	21358.9/5.04	100	Adenylate kinase	14	Cytoplasmic	F	2-DE
147	1006	ORF02270	20202.4/5.04	100	Hypoxanthine phosphoribosyltransferase	10	Cytoplasmic	F	2-DE
148	1203	ORF02321	30587.3/4.95	100	Conserved hypothetical protein	13	Unknown	NO related COG	2-DE and 2-D LC
149	1008	ORF02321	30587.3/4.95	100	Conserved hypothetical protein	13	Unknown	NO related COG	2-DE and 2-D LC
150	1202	ORF02321	30587.3/4.95	100	Conserved hypothetical protein	15	Unknown	NO related COG	2-DE and 2-D LC
151	2302	ORF02334	38490.6/6.78	100	GldN	17	Unknown	NO related COG	2-DE
152	3105	ORF02336	22381.3/5.31	100	GldL	9	Unknown	NO related COG	2-DE and 2-D LC
153	1007	ORF02346	9795.2/5.01	100	Chaperonin GroS		Cytoplasmic	O	2-DE
154	1702	ORF02347	57327/4.94	100	Chaperonin GroL	29	Cytoplasmic	O	2-DE and 2-D LC
155	103	ORF02411	20395.6/4.71	100	Co-chaperone GrpE	12	Cytoplasmic	O	2-DE
156	4301	ORF02426	34182/5.35	100	Thioredoxin-disulfide reductase	12	Cytoplasmic	O	2-DE

157	2602	ORF02436	45377.8/5.04	100	Lipoamide acyltransferase component of branched-chain alpha-keto aciddehydrogenase complex	21	Cytoplasmic	C	2-DE and 2-D LC
158	2603	ORF02436	45377.8/5.04	100	Lipoamide acyltransferase component of branched-chain alpha-keto aciddehydrogenase complex (Dihydro	22	Cytoplasmic	C	2-DE and 2-D LC
159	301	ORF02471	29300.5/4.54	100	TPR domain protein, putative	9	Unknown	R	2-DE
160	7202	ORF02498	33765.3/5.77	98.853	Cyclophilin/fkbp-type peptidyl-prolyl cis-trans isomerase	8	Periplasmic	O	2-DE
161	6203	ORF02498	33765.3/5.77	100	Cyclophilin/fkbp-type peptidyl-prolyl cis-trans isomerase	16	Periplasmic	O	2-DE
162	7203	ORF02498	33765.3/5.77	100	Cyclophilin/fkbp-type peptidyl-prolyl cis-trans isomerase	15	Periplasmic	O	2-DE
163	3901	ORF02512	80651/5.18	100	Isocitrate dehydrogenase, NADP-dependent	29	Unknown	C	2-DE
164	401	ORF02605	40946.9/4.65	100	DNA polymerase III, beta subunit	13	Unknown	L	2-DE and 2-D LC
165	3301	ORF02616	36317.4/5.18	100	Phosphoribosylaminoimidazole-succinocarboxamide synthase	19	Cytoplasmic	F	2-DE
166	8606	ORF02619	53542.8/6.35	100	Lipoprotein, putative	19	OuterMembrane	NO related COG	2-DE and 2-D LC
167	8604	ORF02619	53542.8/6.35	100	Lipoprotein, putative	25	OuterMembrane	NO related COG	2-DE and 2-D LC
168	6303	ORF02631	37430.6/5.61	100	Pyruvate dehydrogenase E1 component, alpha subunit	15	Cytoplasmic	C	2-DE
169	7603	ORF02676	50326.9/5.82	100	UDP-glucose 6-dehydrogenase	21	Unknown	M	2-DE and 2-D LC
170	2002	ORF02712	15879.4/5	100	Protein containing GatB/Yqey domain	10	Unknown	S	2-DE
171	3704	ORF02714	50050.4/5.28	100	Cell division protein FtsA	20	Cytoplasmic	D	2-DE
172	3503	ORF02763	46787.9/5.23	100	Methionine adenosyltransferase	15	Cytoplasmic	H	2-DE
173	9202	ORF02770	33633.6/6.34	100	Electron transfer flavoprotein subunit alpha	15	Unknown	C	2-DE
174	1101	ORF02771	26472.8/4.92	100	Electron transfer flavoprotein subunit beta	18	Cytoplasmic	C	2-DE
175	5303	ORF02779	38897.7/5.5	100	Fructose-bisphosphate aldolase, class II	13	Unknown	G	2-DE and 2-D LC

176	6301	ORF02779	38897.7/5.5	100	Fructose-bisphosphate aldolase, class II	19	Unknown	G	2-DE
177	8701	ORF02782	56378.4/6.01	100	Bifunctional purine biosynthesis protein PurH	22	Unknown	F	2-DE
178	4101	ORF02784	24634.6/5.53	100	Peroxiredoxin, AhpC/Tsa family	17	Cytoplasmic	O	2-DE and 2-D LC
179	6102	ORF02784	24634.6/5.53	100	Peroxiredoxin, AhpC/Tsa family	14	Cytoplasmic	O	2-DE and 2-D LC
180	6101	ORF02784	24634.6/5.53	100	Peroxiredoxin, AhpC/Tsa family	18	Cytoplasmic	O	2-DE and 2-D LC
181	6302	ORF02825	37326.5/5.61	100	Fructose-1,6-bisphosphatase	13	Cytoplasmic	G	2-DE
182	1104	ORF02849	24603.2/5.87	100	Clp protease	10	Cytoplasmic	NO	2-DE and 2-D LC

APPENDIX E

PATHWAYS SIGNIFICANTLY REPRESENTED IN *FLAVOBACTERIUM COLUMNARE* PROTEIN DATASET

Pathway name	No. of proteins	Total no. of entities	*P* value	Classification
Folate biosynthesis	20	162	2.25E-9	Metabolism of Cofactors and Vitamins
Purine metabolism	24	289	1.44E-7	Nucleotide Metabolism
Benzoate degradation via CoA ligation	14	156	2.98E-5	Xenobiotics Biodegradation and Metabolism
Citrate cycle (TCA cycle)	10	82	3.19E-5	Carbohydrate Metabolism
Aminoacyl-tRNA synthetases	10	83	3.54E-5	Translation
Pyrimidine metabolism	15	204	1.55E-4	Nucleotide Metabolism
Oxidative phosphorylation	10	100	1.76E-4	Energy Metabolism
Ethylbenzene degradation	8	75	5.12E-4	Xenobiotics Biodegradation and Metabolism
Propanoate metabolism	11	136	5.42E-4	Carbohydrate Metabolism
Valine, leucine and isoleucine degradation	10	116	5.89E-4	Amino Acid Metabolism
Pyruvate metabolism	12	163	7.13E-4	Carbohydrate Metabolism
Glutamate metabolism	9	102	9.38E-4	Amino Acid Metabolism
Alanine and aspartate metabolism	9	104	1.08E-3	Amino Acid Metabolism
Selenoamino acid metabolism	9	105	1.15E-3	Metabolism of Other Amino Acids
Limonene and pinene degradation	11	150	1.23E-3	Biosynthesis of Secondary Metabolites
Glycolysis / Gluconeogenesis	11	151	1.30E-3	Carbohydrate Metabolism
Glycine, serine and threonine meta	12	191	2.79E-3	Amino Acid Metabolism
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	5	41	3.29E-3	Glycan Biosynthesis and Metabolism
1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT)	6	63	4.61E-3	Xenobiotics Biodegradation and Metabolism
Ubiquinone biosynthesis	8	115	7.77E-3	Metabolism of Cofactors and Vitamins
Butanoate metabolism	9	172	2.76E-2	Carbohydrate Metabolism
One carbon pool by folate	5	72	3.33E-2	Metabolism of Cofactors and Vitamins
Caprolactam degradation	5	72	3.33E-2	Xenobiotics Biodegradation and Metabolism
Histidine metabolism	8	150	3.35E-2	Amino Acid Metabolism
Lipopolysaccharide biosynthesis	7	124	3.49E-2	Glycan Biosynthesis and Metabolism
Lysine degradation	9	180	3.56E-2	Amino Acid Metabolism
Biotin metabolism	4	53	4.25E-2	Metabolism of Cofactors and Vitamins
Lysine biosynthesis	6	104	4.50E-2	Amino Acid Metabolism