Whole Proteome Approach to Delineate Leptospiral Pathogenesis

by

Azad Eshghi
B.Sc, Brock University, 2006

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Supervisory Committee

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Abstract

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The study of leptospiral pathogenesis is hampered by the lack of efficient mutagenesis methodologies. Thus research has focused on alternative approaches including genome sequencing, comparative genomics, transcriptomics and proteomics. In this thesis a comparative proteomic approach was used to identify leptospiral proteins with a potential role in the leptospiral infection process. Identification of proteins was followed by characterization of target proteins with potential roles in the infection process and ultimately led to the identification of a novel leptospiral virulence factor.

Specifically, comparative proteomics using isobaric tags for relative and absolute quantitation complemented with two-dimensional gel electrophoresis were used for mass spectrometry-based protein identification and quantitation. These methodologies were utilised to identify and quantitate leptospiral proteins altered in expression in response to growth media limited in iron supply and/or supplemented with serum. These conditions were designed to mimic a subset of variables encountered by the bacteria within the host. These experiments led to the identification of five proteins with potentially novel roles in the leptospiral infection process.

One of these proteins was further characterized as a periplasmic catalase, KatE. Using insertion mutagenesis it was demonstrated that KatE enhances extracellular H_2O_2 resistance and is required for virulence in guinea pigs and hamsters.

Proteomic analyses also led to the identification of glutamic acid methylation of a protein that was further characterised to be surface exposed and expressed during leptospiral colonization of hamster liver and kidneys. This was the first description of glutamic acid methylation of a surface exposed protein in *Leptospira*.

Table of Contents

Supervisory Committee ii
Abstract iii
Table of Contents v
List of Tables viii
List of Figures ix
Acknowledgments x
Abbreviations xi
Chapter 1 Introduction 1
1.1.1 General background on *Leptospira*: 1
1.2.1 Genomic approaches to the study of pathogenesis 4
1.2.2 Signature-tagged mutagenesis 5
1.2.3 Transposon site hybridization (TraSH) 6
1.2.4 Random transposon insertion: identification of genes required for leptospiral virulence 7
1.2.5 Leptospiral genome sequence and insights into virulence 8
1.2.6 Comparative microarray studies 9
1.3.1 Mass spectrometry-based proteomics 11
1.3.2 Ionization: Matrix assisted laser desorption ionization (MALDI) 12
1.3.3 Ionization: Electrospray ionization (ESI) 13
1.3.4 Analyzer: Time of flight analyzer (TOF) 14
1.3.5 Analyzer: Quadruple ion trap (QIT) 14
1.3.6 Analyzer: Fourier transform ion cyclotron resonance (FT-ICR) 16
1.3.7 Analyzer: Linear quadrupole ion trap 17
1.3.8 Fragmentation 18
1.3.9 Protein identification via mass spectrometry 19
1.3.10 Peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS) 20
1.3.11 Protein quantitation via mass spectrometry 21
1.3.12 Two-dimensional gel electrophoresis 21
1.3.13 Isobaric tags for relative and absolute quantitation (iTRAQ) 22
1.3.14 Selected reaction monitoring (SRM) 23
1.4.1 Overview of proteomic studies that have been conducted within *Leptospira* to date 23
1.4.2 Comparative proteomics of OMPs *in vitro* 24
1.4.3 Characterization of proteins identified through proteomic studies 27
1.4.4 Current understanding of proteins that contribute to leptospiral pathogenesis 28
1.5.1 Summary of results presented in this thesis 29
Chapter 2 Global proteome analysis of *Leptospira interrogans* 31
2.1.1 Abstract 32
2.2.1 Introduction 33
2.3.1 Materials and methods 37

2.3.2 *Leptospira* and culture conditions 37
2.3.3 Protein extraction and sample preparation 37
2.3.4 iTRAQ analysis 38
2.3.5 iTRAQ experimental design 39
2.3.6 Liquid chromatography-electrospray ionization tandem mass spectrometry 39
2.3.7 iTRAQ data analysis 42
2.3.8 2DGE experiments 44
2.3.9 Matrix-assisted laser desorption ionization time of flight mass spectrometry 45
2.3.10 Computer database analysis 46
2.3.11 Recombinant protein expression and purification 46
2.3.12 Antibodies 49
2.3.13 Immunoblot analyses 49
2.3.14 Serum samples 51
2.3.15 Enzyme-linked immunosorbent assay (ELISA) 51
2.3.16 Immunofluorescence microscopy 52
2.4.1 Results and discussion 54
2.4.2 Overview of global proteome analyses 54
2.4.3 Assignment of functional categories 58
2.4.4 Energy production and metabolism 64
2.4.5 Chaperones and protein synthesis 65
2.4.6 Flagellar and chemotaxis proteins 66
2.4.7 Confirmed/potential virulence factors 67
2.4.8 Verification of protein expression during infection 71
2.4.9 Confirmation of upregulation of protein expression in *L. interrogans* grown in – Fe/FBS medium 75
2.4.10 Conclusions 75
2.5.1 Acknowledgements 78
Chapter 3 *Leptospira interrogans* catalase *katE* enhances resistance to extracellular H_2O_2 and is required for virulence 79
3.1.1 Abstract 80
3.2.1 Introduction 81
3.3.1 Methods 85
3.3.2 *Leptospira* strains and culturing conditions 85
3.3.3 Recombinant protein expression and antiserum production 85
3.3.4 Catalase assay methodology 85
3.3.5 Catalase activity assay 86
3.3.6 Bacterial oxidative stress conditions and inhibition assays 86
3.3.7 Bacterial viability assays 87
3.3.8 Protein fractionation 88
3.3.9 Immunoblot analyses 88
3.3.10 Construction of *Leptospira* insertion mutants 89
3.3.11 Animal Infections 89
3.4.1 Results 91
3.4.2 Recombinant KatE displays catalase activity 91
3.4.3 KatE expression remains unchanged during H_2O_2-induced oxidative stress 91
3.4.4 KatE is localised to the periplasmic space 95

3.4.5 *katE* mutant *Leptospira* do not display H_2O_2 hydrolysis activity 95
3.4.6 *katE* enhances *L. interrogans* viability under oxidative stress 98
3.4.7 *katE* is required for *L. interrogans* virulence 98
3.5.1 Discussion 102
3.6.1 Acknowledgments 108
Chapter 4 Differential methylation and *in vivo* expression of the surface-exposed *Leptospira interrogans* outer membrane protein OmpL32 109
4.1.1 Abstract 110
4.2.1 Introduction 111
4.3.1 Methods 114
4.3.2 *Leptospira* and culture conditions 114
4.3.3 Two-dimensional gel electrophoresis (2DGE) and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) experiments 114
4.3.4 Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) 114
4.3.5 Bioinformatic analyses 116
4.3.6 Recombinant protein expression and purification 118
4.3.7 Antibodies 121
4.3.8 Immunoblot analysis 121
4.3.9 Immunofluorescence assay 122
4.3.10 Immunofluorescence of tissue sections 123
4.4.1 Results 125
4.4.2 *L. interrogans* cells differentially express isoforms of a putative OMP 125
4.4.3 LIC11848 is differentially methylated on glutamic acid residues 129
4.4.4 Correlation between the observed LIC11848 protein methylation pattern and predicted B cell epitope locations 130
4.4.5 LIC11848 is a surface exposed protein 133
4.4.6 *Leptospira* express LIC11848 during colonization of hamster kidneys and liver 136
4.5.1 Discussion 138
4.6.1 Acknowledgements 144
Chapter 5 Conclusions 145
Bibliography 153
Appendix A Supporting Information 176
Appendix B Supporting information 177

List of Tables

Table 1: ORF-specific primers used to amplify fragments for recombinant expression.. 48
Table 2: Proteins exhibiting altered expression levels in *L. interrogans* in response to *in vivo*-like conditions (exposure to –Fe/FBS) as determined by 2DGE. 59
Table 3: Proteins exhibiting altered expression levels in *L. interrogans* in response to *in vivo*-like conditions (exposure to –Fe/FBS) as determined by iTRAQ. 60
Table 4: Proteins exhibiting altered expression levels in *L. interrogans* in response to *in vivo*-like conditions (exposure to –Fe/FBS) as determined by immunoblot analyses. 76
Table 5: Quantitative immunoblot analyses of KatE expression levels in response to H_2O_2 exposure. 94
Table 6: Primers used to amplify LIC11848..... 120
Table 7: Mass to charge ratios used to identify LIC11848 isoforms via peptide mass fingerprinting. 128
Table 8: Predicted B cell epitopes present within LIC11848 protein (OmpL32) and correlation with observed methylation profile. 132

List of Figures

Figure 1: iTRAQ experimental design. 40
Figure 2: Mass spectra of iTRAQ reporter ion m/z peak intensities used for peptide quantitation and of m/z peaks used for peptide sequence identification. 55
Figure 3: Mass spectra of iTRAQ reporter ion m/z peak intensities used for peptide quantitation and of m/z peaks used for peptide sequence identification. 56
Figure 4: Reactivity of antibodies in human serum to recombinant *L. interrogans* proteins as determined by ELISA. 72
Figure 5. *In situ* expression of *L. interrogans* proteins in golden Syrian hamster liver tissue. 74
Figure 6: Recombinant KatE displays catalase activity. 92
Figure 7: KatE expression remains unchanged during H_2O_2-induced oxidative stress. ... 93
Figure 8: KatE localizes to the periplasmic space. 96
Figure 9: *katE* mutant *Leptospira* do not degrade H_2O_2. 97
Figure 10: KatE enhances resistance to extracellular H_2O_2. 99
Figure 11: *katE* mutant *Leptospira* show attenuated virulence in guinea pigs and in hamsters. 100
Figure 12: Comparative proteome analysis of *L. interrogans* exposed to differing growth conditions. 127
Figure 13: Differential glutamic acid methylation of LIC11848 protein 131
Figure 14: Immunofluorescence microscopy identifies LIC11848 protein as a surface-exposed, outer membrane protein. 135
Figure 15: *Leptospira* express LIC11848 protein during colonization of hamster kidneys and liver as evidenced by immunofluorescence microscopy. 137

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Abbreviations

2DGE	Two-dimensional gel electrophoresis
CRM	Charged residue model
ECD	Electron capture dissociation
ESI	Electrospray ionization
ETD	Electron transfer dissociation
FT-ICR	Fourier transforms ion cyclotron resonance
ICR	Ion cyclotron resonance
IRMPD	Infrared multiphoton dissociation
IEF	Isoelectric focusing
IEM	Ion evaporation model
iTRAQ	Isobaric tags for relative and absolute quantitation
MALDI	Matrix assisted laser desorption
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
pI	Isoelectric point
PMF	Peptide mass finger printing
QIT	Quadruple ion trap
SRM	Selected reaction monitoring
STM	Signature tagged mutagenesis

TraSH	Transposon site hybridization
wt	Wild type

Chapter 1
Introduction

1.1.1 General background on *Leptospira*:

Leptospira belong in the domain bacteria, phylum *Spirochaetes*, as determined by 16S ribosomal RNA sequencing (Paster *et al.*, 1991). Other spirochetes include the *Treponema*, *Borrelia*, *Leptonema* and *Serpula* species. The spirochetes are morphologically similar in their spiral cell shape and microscopic analysis of *Leptospira* reflects this general morphology. Phase contrast micrographs reveal *Leptospira* to be 0.1 µm in diameter, and to range anywhere from 6-20 µm in length (Holt, 1978) with a coil amplitude of 0.1-0.15 µm and a wavelength of 0.5 µm (Faine *et al.*, 1999). Besides similar 16S ribosomal RNA sequences and morphology, *Leptospira* share very little commonalities with the other spirochete species. For example sequencing of the *Leptospira interrogans* genome (Ren *et al.*, 2003) revealed a genome totalling ~4.7 mega base pairs compared to ~1.0 and ~1.5 mega base pairs for *Treponema* (Fraser *et al.*, 1998) and *Borrelia* (Fraser *et al.*, 1997), respectively. Another distinct feature to *Leptospira* is their Gram negative-like outer membrane composition due to the presence of lipopolysaccharide (LPS) (Vinh *et al.*, 1986) and LPS encoding genes (Ren *et al.*, 2003). The relatively large genome of *Leptospira* correlates with it's biology as various strains are able to survive as saprophytes and as pathogens in a variety of land and marine mammals. As saprophytes, *Leptospira* exist mainly in stagnant fresh water sources in tropical climates, and as pathogens serodiagnosis reveals worldwide prevalence of *Leptospira* in humans and other animals with a higher concentration in regions with tropical climates.

DNA relatedness experiments on *Leptospira* have led to the identification of 303 strains revealing diversity in this genre (Brenner *et al.*, 1999). Other species and strains have been discovered since and the list of leptospiral strains will likely continue to expand with time. Pathogenic species include *interrogans* (91 strains), *santarosi* (65 strains), *borgpetersenii* (49 strains), *kirschneri* (29 strains), *noguchi* (20 strains), *alexanderi* (6 strains) and genome species 1 (2 strains) as determined by DNA relatedness (Brenner *et al.*, 1999). Two other pathogenic species have been suggested, namely *weilii* (Corney *et al.*, 2008) and *wolfii* (Slack *et al.*, 2008). Pathogenic species of *Leptospira* are believed to be maintained in rats (Middleton, 1929; Noguchi, 1917; Noguchi, 1919a) and mice (Stavitsky & Green, 1945; Yager *et al.*, 1953) as these organisms serve as reservoir hosts capable of supporting a high burden of leptospiral load while remaining asymptomatic. The target organs are the kidneys (Moulton & Howarth, 1957; Noguchi, 1919b; Noguchi, 1919c; Noguchi & Kligler, 1920), liver (Noguchi, 1919b; Noguchi, 1919c; Noguchi & Kligler, 1920) and to a lesser extent the lungs (Noguchi, 1919b; Noguchi, 1919c; Noguchi & Kligler, 1920). *Leptospira* have been detected in the urine of maintenance hosts (Merien *et al.*, 1992; Thiermann, 1977; Thiermann, 1981) and the mode of transmission is thought to occur via contamination of stagnant water supplies via urinary shedding of leptospires by maintenance hosts and eventual transmission of the bacteria to susceptible hosts (Jansen & Schneider, 2011; Thiermann & Frank, 1980).

Susceptible hosts display a variety of disease symptoms either inclusive or individual, including fever (Noguchi, 1919b; Noguchi & Kligler, 1920), jaundice in various organs and tissues (Noguchi, 1919b; Noguchi & Kligler, 1920; Ristow *et al.*,

2007), pulmonary haemorrhage (Noguchi, 1919b; Noguchi & Kligler, 1920) and lesions in kidneys and the liver (Noguchi, 1919b; Noguchi & Kligler, 1920; Reilly, 1970; Ristow *et al.*, 2007). While non-human animals serve as the main hosts, humans can serve as "accidental" hosts and the most recent WHO statistics report more than 500,000 severe human leptospirosis cases per year with mortality rates greater than 10% (WHO, 1999). However, the total number of worldwide infections is expected to be grossly underestimated, due to inefficient diagnosis resulting from extensive serological diversity among pathogenic *Leptospira* species and an array of disease symptoms (Bharti *et al.*, 2003; Heron *et al.*, 1997; Monsuez *et al.*, 1997; O'Neil *et al.*, 1991). Although leptospirosis can be effectively treated with tetracyclines and β-lactam/cephalosporin antibiotics in the early stages of the disease (Bharti *et al.*, 2003), accurate disease diagnosis at this stage of infection is rarely achieved. Leptospirosis control is further hindered by the lack of an effective vaccine.

The history of research on *Leptospira* has been broad in range. Early research focused on disease symptoms and progressed to attempts at the development of vaccines for the purpose of protection against leptospirosis. The macroscopic agglutination test (MAT) has been the main method of serodiagnosis and has been used in numerous studies demonstrating *Leptospira* colonization of a variety of animal species worldwide (Adler *et al.*, 1981; Adler *et al.*, 1982; Antoniadis & Papapanagiotou, 1979; Ballard *et al.*, 1984; Flint *et al.*, 1986; Hartman *et al.*, 1984; Hunter *et al.*, 1988; Terpstra *et al.*, 1980). Recent improvements in DNA extraction from serum have made PCR a viable option for diagnosis of leptospiral colonization (Villumsen *et al.*). A greater understanding of the *Leptospira* infection process is required and research focusing on the molecular

components utilized by *Leptospira* to establish infection will permit the identification of novel virulence factors that may facilitate vaccine design and the development of novel diagnostics. Recent molecular approaches have utilized genetic and proteomic approaches to decipher leptospiral pathogenesis. An overview of these methodologies and their utilization in the study of leptospiral pathogenesis is provided in the following sections.

1.2.1 Genomic approaches to the study of pathogenesis

Identification of bacterial virulence genes can be achieved through various genetic methodologies. For genetically modifiable organisms the gold standards are signature-tagged mutagenesis (STM) and transposon site hybridization (TraSH) where genes required for infection are identified in an animal model. For fastidious organisms lacking the machinery for complex genetic manipulation such as recombination, random transposon mutagenesis is an option. Other methodologies such as *in vivo* expression technology (IVET) and microarrays can be used to detect expression of genes in the animal only or to quantitate bacterial RNA levels in an animal compared to *in vitro* grown bacteria, respectively. Lastly, for bacteria with sequenced genomes, comparison of pathogenic to saprophytic strains allows identification of genes unique to the pathogen. The following sections give a brief overview of genetic methodologies applicable to the study of bacterial pathogenesis followed by their application specifically to the study of leptospiral pathogenesis. Although STM and TraSH have not been applied to *Leptospira*, these methodologies hold potential for enhancing the current random transposon insertion mutation system used to generate leptospiral mutants into a more efficient methodology.

Thus a description of these techniques and their potential application to leptospiral genetics is also included in the following sections.

1.2.2 Signature-tagged mutagenesis

First described in 1995 (Hensel *et al.*, 1995), signature-tagged mutagenesis (STM) uses a unique sequence of oligonucleotides to tag transposon mutated bacteria with the purpose of identifying virulence genes. The process requires synthesis of unique sequences of 40 base pair (bp) oligonucleotides, flanked on both sides by common 20 bp oligo-arms, which end in a restriction enzyme recognition sequence. The arms allow PCR amplification of the tags. The tags are then ligated into a transposon element that itself resides on a suicide vector. The vector is then used to transform *E. coli* and delivered to the target bacteria via conjugation. This results in single, near random and stable transposon integration events and the loss of the vector in the recipient bacteria.

Transposon tagged bacteria are then arrayed on 96 well microplates and replica-DNA blotted on two separate membranes. Bacteria from individual 96 well plates are pooled, a sample removed for DNA extraction (input pool), and injected into mice. Following appropriate infection times bacteria are recovered by laboratory media plating of tissue homogenates. Ten thousand colonies are pooled and used for DNA extraction (output pool). Input and output DNA samples are used in two separate PCR reactions (using the 20 bp oligo-sequences flanking the tag as primers) using a radiolabeled deoxynucleotide triphosphate to amplify and label the unique tags. The common regions are removed via restriction digestion and the labeled tags used to probe previously generated DNA blots. Reactivity detected with the input but not with the output pool

identifies genes required for infection. Genes are identified by sequencing of regions flanking the transposon.

This methodology can be utilized with any bacteria with an established transposon mutagenesis system (Lu *et al.*, 1994). Transposon mutagenesis has been established in *Leptospira* and STM holds immense potential for enhancing insertion inactivation to a more efficient methodology. However, random transposon mutagenesis in *Leptospira* is far from a perfect system due to limitations in low transformation efficiencies and a slow growth rate in solid media (up to a month before visible colonies appear). It follows that the largest insertion mutant library created to date in *Leptospira* consists of 551 distinct mutants (Murray *et al.*, 2009a) representing 14.7% of the potential 3728 open reading frames. These limitations would manifest even if STM were used for creating insertion mutants.

1.2.3 Transposon site hybridization (TraSH)

Similar to STM, TraSH is a method that can be used to identify virulence genes via hybridization of labeled RNA to a DNA microarray (Sassetti *et al.*, 2001). Pooled bacterial transposon mutants are used in infection experiments and recovered mutants are subjected to DNA extraction. Chromosomal DNA is then subjected to restriction digestion with an enzyme predicted to be a frequent cutter. Digested DNA is ligated (on both ends) to common adapters and used in PCR reactions with primers specific to the adapters and the transposon, thus amplifying flanking regions of the transposon. Amplicons are *in vitro* transcribed, producing labeled RNA that is used to probe DNA microarrays containing fragments of DNA representing every open reading frame in the genome. Reactivity with the input but not output pools (see STM section for definition of

input and output pools) identifies genes required for the infection process. Like STM, this method has yet to be established in *Leptospira* and has the potential to advance insertion mutation in *Leptospira* to a more efficient methodology. The limitations discussed in the STM section would be applicable to TraSH as well.

1.2.4 Random transposon insertion: identification of genes required for leptospiral virulence

Transposons are defined as genetic elements containing terminal inverted repeats. Transposase enzymes rearrange transposons within and between genomes using inverted repeat sequences. Transposons and their respective transposase enzymes can be incorporated into suicide vectors with the goal of engineering a construct containing a promoter driven transposase flanked by a selectable marker (usually antibiotic resistance gene) that has been inserted into the transposon and is therefore flanked by the inverted repeats. Transformation of the engineered suicide vector into the target bacteria would ideally lead to incorporation of the antibiotic resistance gene randomly within the genome. Selection on antibiotics and sequencing of regions flanking the transposon would identify genomic regions of insertion. Transformants would then be used in infection experiments to identify virulence associated genes. Due to it's simplicity, the Himar1 transposon element (Hayes, 2003) has been engineered to incorporate kanamycin resistance on a suicide vector that transforms into *Leptospira* resulting in insertion mutants (Bourhy *et al.*, 2005).

To date this methodology has been used to successfully identify six virulence factor-encoding genes in *Leptospira*, including *la0222* in *L. interrogans* serovar Lai which encodes the outer membrane lipoprotein Loa22 (Ristow *et al.*, 2007), *la2613* in serovar Lai which encodes the flagella motor switch protein FliY (Liao *et al.*, 2009), an

orthologue to serovar Lai *lb186* in *L. interrogans* serovar Manilae which encodes the heme oxygenase HemO (Murray *et al.*, 2009b), two genes which encode LPS biosynthesis proteins in serovar Manilae, with one being orthologous to serovar Lai *la1641* and the other having no observed serovar Lai orthologue (Murray *et al.*, 2010) and *la3976* in serovar Lai encoding an invasion-associated protein A InvA (Luo *et al.*, 2011).

1.2.5 Leptospiral genome sequence and insights into virulence

Whole genome random sequencing (Fleischmann *et al.*, 1995) is a robust method for genome sequencing and has been used to sequence spirochete genomes (Fraser *et al.*, 1997; Fraser *et al.*, 1998; Ren *et al.*, 2003). In this methodology genomic DNA is sheared to create two libraries; a library of small fragments of ~2 kilo base pairs (Kbp) and a library of large fragments of ~15-20 Kbp. Random sequencing of each library is then conducted in sufficient amounts to cover the libraries by a factor of 6. Sequence fragments are then assembled, repeat regions identified and gaps closed via primer walking. In the last step the genome sequence is annotated for open reading frames.

Whole genome random sequencing in *Leptospira* (Ren *et al.*, 2003) has revealed a number of genes with sequence homology to genes shown to be involved in virulence in other bacteria. Specifically, the authors identified the 4 genes *mce* (Chitale *et al.*, 2001), *atsE* (Murooka *et al.*, 1978), *mviN* (Rudnick *et al.*, 2001) and *invA* (Gaywee *et al.*, 2002), potentially involved in attachment and invasion of eukaryotic cells. It follows that a recent study has demonstrated the requirement of *invA* for leptospiral pathogenesis in hamsters and for infection of macrophages (Luo *et al.*, 2011), providing a tangible

measure that reflects the advantage of using genome sequencing to study leptospiral pathogenesis.

1.2.6 Comparative microarray studies

As the name implies micron sized wells in arrays can be used for large scale quantitation of molecules. With respect to mRNA quantitation, a microarray is generated containing small fragments of a genome in individual micro wells. The array is designed in a manner where each micro well contains multiple copies of a unique open reading frame. DNA microarrays can be generated from cDNA or more commonly ORF fragments generated from plasmid preps containing individual fragments of restriction enzyme-digested genomic DNA. The array is designed in a manner where the sequence of the DNA and its position in the microarray are known. Once a DNA microarray for a genome has been prepared, RNA from experimental samples can be used to generate labeled cDNA using poly dTTP or random poly dNTPs, the latter being applicable to microbial mRNA. There are various methods used to label cDNA, a common method includes use of fluorophore labeled dNTPs in cDNA synthesis reactions. The labeled cDNA referred to as the probe is then applied to the DNA microarray where complementary sequences hybridize while non-complementary sequences are washed away in subsequent steps. Quantitation is then achieved via excitation of complemented fluorophore labeled probes.

Comparative quantitative mRNA studies have contributed valuable data for delineating leptospiral response to environmental cues mimicking host conditions and to identifying potential virulence associated genes. For example, experimental RNA from *Leptospira* exposed to temperatures mimicking that outside and within the host (20 °C or

30 °C and 37 °C or 39 °C, respectively) has been used in comparative quantitative microarray experiments (Lo *et al.*, 2006). These experiments revealed altered expression of various genes in response to a temperature shifts highlighting the effect of temperature on gene regulation in *Leptospira*. Using clusters of orthologous groups (COGs) various genes with potential roles in leptospiral pathogenesis such as those involved in chemotaxis and motility, export of outer membrane proteins and transcription factors were identified and shown to be altered in expression.

Comparative quantitative RNA experiments have also been used to study the effects of serum (Patarakul *et al.*, 2010) and iron limitation (Lo *et al.*, 2010) on gene expression in *Leptospira*. In the host, free iron is sequestered by hemoglobin in red blood cells and it follows that bacteria face limited iron supply in the host environment. It is not surprising that bacteria have evolved various genes that serve in iron acquisition and many of these genes are regulated transcriptionally by the product of the ferric uptake regulator (*fur*) gene. *Leptospira* hypothetically encode four genes orthologous to *fur* and a transposon mutant in one orthologue, *la1857* in serovar Lai, displays altered transcription of various genes including increased transcription of a putative catalase potentially involved in H_2O_2 detoxification (Lo *et al.*, 2010). It follows that mutant *la1857* displays 8 fold higher resistance to extracellular H_2O_2 compared to *wt* Lai suggesting a possible role for the putative catalase in oxidative stress resistance initiated by iron starvation such as that encountered in the host. In addition to these findings iron limitation of *wt* Lai altered transcription of 16 genes not encoded by the saprophyte *biflexa* suggesting a potential role for these genes in pathogenesis.

Comparative mRNA quantitation has also been conducted on the leptospiral response during exposure to innate immune cells (Xue *et al.*, 2010). Specifically, these experiments demonstrated that *Leptospira* respond to macrophage-derived cells by decreasing transcription of various outer membrane protein encoding genes namely *ompL1*, *lipL32*, *lipL41*, *lipL48* and *ompL47*. Assuming this correlates with a decreased expression of outer membrane proteins, the effect of this response could significantly reduce the extent of the immune response to *Leptospira* due to decreased antigen recognition by the host immune response. Another important finding from these experiments was increased transcription of a putative catalase gene (*katE*) suggesting a potential role for this gene in resistance to oxidative stress, exerted by immune cells against *Leptospira*.

While comparative microarray studies have advanced the study of leptospiral pathogenesis a limitation to this approach is that quantitation of transcripts does not always show a direct correlation to protein expression (Lo *et al.*, 2009). Proteins are the main components utilized by bacteria to establish infection thus comparative protein experiments provide a more definitive picture of the components potentially utilized during pathogenesis.

1.3.1 Mass spectrometry-based proteomics

Before proceeding to a review of proteomic literature on *Leptospira* it is helpful to introduce mass spectrometry based proteomics as this approach is by far the most widely used approach towards proteome studies in *Leptospira* and the central methodology utilised in this thesis. The following sections will provide a detailed review of mass spectrometers and their use in protein identification and quantitation.

The field of mass spectrometry is rapidly evolving and discussion of every known mass spectrometer is beyond the scope of this thesis. The discussion here will be limited only to those mass spectrometers most popularly applied to the study of proteins. The mass spectrometer is a very complex piece of equipment but its basic principal components can be broken down into three main parts. The first component involves ionization where the analyte is ionized and sublimated, the second component includes an analyzer where the ion is analysed based on mass to charge ratio and the third component contains a detector where the gaseous ion is detected via an ion detector. While most mass spectrometers share similar detectors, ionizers and analyzers differ and define the type of mass spectrometer.

1.3.2 Ionization: Matrix assisted laser desorption ionization (MALDI)

The laser desorption ionization (Karas & Hillenkamp, 1988) uses electromagnetic radiation in the far UV range to ionize the analyte in the presence of a matrix (Hill *et al.*, 1991). The use of electromagnetic radiation in the 300-400 GHz frequency range provides enough energy for sublimation of macromolecules such as proteins and carbohydrates. Electromagnetic radiation in the 300-400 GHz frequency in the presence of a matrix does not contain the necessary energy to disrupt covalent bonds thus macromolecules remain intact during the ionization process. Ionization and sublimation both require the presence of a matrix that acts as a source of hydrogen protons and energy transfer medium during irradiation (Zenobi & Knochenmuss, 1998). Matrices are usually acidic compounds and able to absorb electromagnetic radiation in the UV range (Zenobi & Knochenmuss, 1998). Common matrices include 2,5-di-hydroxy benzoic acid (Karas

et al., 1990), sinapic acid (Beavis & Chait, 1989) and α-cyano-4-hydroxy-cinnamic acid (Beavis *et al.*, 1992).

MALDI can be used to ionize both micro and macro molecules with an appropriate matrix (Andersen *et al.*, 1996). A second benefit is that ionization prominently occurs with the transfer of a single proton resulting in single charged ions, thereby reducing spectral complexity downstream. A limitation to this technique is that certain analytes ionize better than others resulting in biased spectra when analysing complex samples.

1.3.3 Ionization: Electrospray ionization (ESI)

In ESI (Fenn *et al.*, 1989) the analyte is ionised by a high voltage and moves from a liquid to a gas phase. This is achieved by mixing the analyte in a solvent containing both aqueous and organic solvents to dissolve polar and non-polar analytes and application of the mixture to a micro tip capillary. Application of a high voltage to the tip results in spraying of the mixture into charged microdroplets (Andersen *et al.*, 1996). The mechanism leading to the production of gas phase analytes from the microdroplets has yet to be experimentally demonstrated. Two theories predict the aforementioned mechanism; the ion evaporation model (IEM) (Iribarne & Thomson, 1976) and the charged residue model (CRM) (Schmelzeisen-Redeker *et al.*, 1989). IEM states that as the radius of a droplet reaches a certain value the field strength at the surface of the droplet becomes strong enough for the ionization of the analyte. The CRM suggests that microdroplets undergo evaporation and fission resulting in smaller “progeny” droplets that eventually evaporate and leave the remaining charge with the analyte now in gas phase.

An advantage of using ESI is the gentle ionization process that allows analyses of non-covalent interactions such as protein complexes (Smith & Light-Wahl, 1993). One disadvantage is that this method of ionization results in multiply charged analytes leading to complex spectra (Andersen *et al.*, 1996).

1.3.4 Analyzer: Time of flight analyzer (TOF)

Time of flight analyzers (TOF) are relatively simple in design compared to other MS analyzers. TOF analyzers measure the time of flight of a given analyte from ionization to detection. Knowing the distance travelled in the flight chamber and the force (Voltage) used for acceleration it is possible to calculate the mass of the analyte. Hence, analyte mass can be measured using the equation $m = q(tk^{-1})^2$ where;

m = mass

q = ion charge

t = time

k = proportionality constant representing an instrument's characteristics and settings (length of flight path and Voltage used in acceleration)

Hence the time taken from entering the flight tube until detection under a known Voltage with a known distance travelled to the ion detector and a known charge of the analyte can be used to calculate mass.

TOF analyzers can be combined with both MALDI and ESI ionization, with MALDI-TOF being the most common combination.

1.3.5 Analyzer: Quadruple ion trap (QIT)

The quadruple ion trap is a mass analyzer (Paul & Steinwedel, 1960) that utilizes four hyperbolic shaped electrodes to trap ions in three dimensions (Payne & Glish, 2005).

The electrodes are positioned in a spherical fashion with the distance from opposing electrodes defining the diameter of the spherically shaped trap. One of these electrodes allows ions to enter the trap from an ionization source through entrance holes while the opposing electrode allows ejection of ions through exit holes leading to the ion detector. A combination of alternating and direct currents is used to maintain ions within the trap (Payne & Glish, 2005). The trajectory of ion flight path requires complex mathematics and physics beyond the scope of this thesis and the reader should access the following reference (March & Londry, 1995) for a detailed theory. The mass to charge ratio of the ions in combination with the applied current results in periodic motion and thus each *m/z* has a unique periodic motion referred to as secular frequency (Payne & Glish, 2005). The current can be manipulated to resonate with specific secular frequencies thus altering the kinetic energy of an ion, ultimately leading to ejection of the ion through the exit hole and onto the ion detector. This type of ejection is most commonly used to eject macromolecules such as proteins and is termed resonance ejection (Payne & Glish, 2005).

The main advantage of QIT is that in a distribution of high and low abundance ion species, ion species of low abundance can be trapped while those in high abundance can be ejected using respective secular frequencies. This allows for accumulation of the previously low abundance ion species increasing detection limits and thereby sensitivity of the QIT analyzer relative to the TOF analyzer. A drawback to using these analyzers is that in complex analyte samples the trap can become overloaded with ions leading to reduced resolution (defined as the dimensionless ratio of the mass of a peak divided by the peak width, with the peak width being measured at half the peak height). Increasing

the number of ions in the trap increases repulsion forces between ions, disrupting the ability of the analyzer to deliver ions to the analyzer in a discrete packet (Payne & Glish, 2005). Hence, complex analyte samples must be separated prior to ionization.

1.3.6 Analyzer: Fourier transform ion cyclotron resonance (FT-ICR)

The application of Fourier transform to ion cyclotron resonance mass spectrometry (Marshall *et al.,* 1998) has made these instruments the most powerful analyzers in both resolution and mass accuracy. The ICR is a trapping analyzer similar to QIT analyzers (section 1.3.5) with two differences. The first difference is the trapping of ions by a magnetic force compared to a current in QIT and the second is trapping of ions in two dimensions as opposed to three dimensions in QIT. The analyzer in an ICR is composed of six plates arranged to form a cube with one side of the cube containing slits where ions can enter from the ionization source. Two of the plates are perpendicular to the magnetic field and it is at these plates where ions are trapped in a two dimensional field. The magnetic force causes ions to traverse the two dimensional field in a cyclotron motion. The angular velocity of these ions has mathematical relation to both the magnetic force applied and a cyclotron frequency that is unique to each *m/z* (Payne & Glish, 2005). The other four plates in the trapping chamber are positioned in a fashion where two opposing plates serve as detection plates while the other two opposing plates serve as excitation plates. The excitation plates are used to deliver frequencies that span the cyclotron frequencies of the ions within the trap raising the kinetic energy of the ions which translates to larger radius of orbit (Payne & Glish, 2005). When this orbit is large enough positively charge ions approach the detection plate close enough to attract electrons. Since ions are in orbit they will pass each detector continuously over time

creating an oscillating current at the same frequency of the cyclotron frequency of the ion (Payne & Glish, 2005). The oscillating current is then detected by an external circuit (detector) and Fourier transform applied to the oscillating current to calculate the cyclotron frequency of the ion which can then be used to calculate the *m/z* of the ion (Payne & Glish, 2005).

FT-ICR has the same benefits of QIT with even better sensitivity and resolution. Similarly the same drawbacks that apply to QIT also apply to FT-ICR.

1.3.7 Analyzer: Linear quadrupole ion trap

The linear quadrupole mass analyzer (Paul & Steinwedel, 1960) utilizes the same principles of QIT (section 1.3.5) except that the poles are parallel in linear analyzers. A combination of AC and DC current along the poles accelerates the ion (Hager, 2002) introduced from an ionization source. The majority of mass spectrometers utilize quadrupole analyzer in tandem triplicate (triple quadrupole mass spectrometry). Ions of specific *m/z* are accelerated in the first quadrupole in a vacuum and directed into the second quadrupole containing an inert gas, resulting in collision of entering ions with inert gas molecules causing fragmentation via a process of collision induced dissociation (section 1.3.8). Fragments of specific *m/z* are further accelerated in the third quadrupole and directed at the ion detector for detection. In some mass spectrometers the third quadrupole is replaced with a TOF analyzer (section 1.3.4) termed as quadrupole time of flight mass spectrometry.

Linear quadrupole mass spectrometry has the same advantage of high sensitivity as QIT and suffers from the same disadvantage of the requirement for separation of ions prior to ionization. However, more ions are required to reduce resolution in linear traps

since the volume of the trap in a linear quadrupoles is greater than those in three dimensional QIT, reducing the effects of coulumbic repulsion between ions (Hager, 2002).

1.3.8 Fragmentation

Determining the sequence of peptides requires fragmentation and various methods have been developed to achieve fragmentation in a manner that results in comprehensive mass spectra. In collision induced dissociation (CID), the most common method used for peptide fragmentation, ions with kinetic energy are subjected to collision with inert atoms such helium, argon or nitrogen in gas phase. Collisions result in transfer of energy leading to disruption of covalent bonds between atoms. When ionized peptides are exposed to CID covalent bond breaks occur between peptide bonds and/or within amino acids such as those between alpha carbons and functional groups. To reduce spectral complexity for downstream spectral analyses, collision energy can be manipulated to bias peptide bond disruption. The collision energy is directly proportional to the number and type of inert gas atoms present (pressure), to the the force used to accelerate ions and to the time collision is allowed to proceed. Thus manipulation of instrument settings modifying the aforementioned variables can lead to predominant fragmentation at peptide bonds, reducing spectral complexity.

Disruption of peptide bonds leads to fragment product b ions if the charge is carried on the C-terminus of the peptide or y ions if the charge is carried on the N-terminus of the peptide (Johnson *et al.*, 1987). Detection of these ions results in mass spectra that can be used to identify a given peptide both from the C and N-terminus using b and y ions, respectively. Further cleavage of b and y ions does occur producing a and z

ions, respectively, and these resulting fragment ions can be used to further confirm peptide sequence.

In electron capture dissociation (ECD) (McLafferty *et al.*, 2001; Zubarev *et al.*, 1998) a multiply charged peptide gains an electron resulting in an odd electron compound that releases excess electrical potential energy in the form of bond disruption. This form of bond disruption leads to significantly different peptide fragment ions compared to CID (Cooper, 2005) and has the advantage of retaining post-translational modifications (Creese & Cooper, 2008; Mirgorodskaya *et al.*, 1999; Renfrow *et al.*, 2005; Shi *et al.*, 2001; Woodling *et al.*, 2007).

Similar to ECD, another method of peptide fragmentation electron transfer dissociation (ETD) utilizes an anion radical as an electron source that transfers electrons to a multiply charged peptide causing fragmentation (Syka *et al.*, 2004). Fragment ions from ETD are generated mainly from disruption of backbone peptide bonds making this method of fragmentation also useful for detection of post-translational modifications (Chi *et al.*, 2007).

The least commonly used type of fragmentation, infrared multiphoton dissociation (IRMPD), uses infrared radiation to increase vibrational energy within peptide bonds ultimately leading to bond disruption and thus fragmentation (Little *et al.*, 1994).

1.3.9 Protein identification via mass spectrometry

The most widely used method for identifying proteins is bottom up mass spectrometry where representative peptides from a given protein are used for identification. Peptides are derived via protease digestion with (but not limited to) trypsin, due to its specific hydrolysis at carbonyl arginine and lysine residues. Using

proteases with known hydrolytic properties allows *in silico* prediction of peptide masses from proteomes. Hence, this approach is most useful for organisms whose genomes have been sequenced and their complement proteome predicted and/or confirmed. For organisms without a sequenced genome it is possible to use a database obtained from an organism close in genetic homology to the organism under study, though proteome coverage will likely be sacrificed due to genetic differences. Proteins can be identified by peptide mass finger printing (PMF) using peptide masses alone or by tandem mass spectrometry to determine the amino acid sequence of a single peptide or set of peptides.

1.3.10 Peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS)

Use of PMF is limited to identification of single proteins since assignment of peptides of similar masses would become ambiguous if more than one protein is being identified. In this methodology a protein is subject to protease digestion and resulting peptides used to obtain a mass spectra via mass spectrometry. A protein is identified via alignment of the experimental peptide spectra with a database containing the corresponding theoretical proteome.

In tandem mass spectrometry (MS/MS) fragment ions b, y, a and z *m/z* rather than whole peptide *m/z* are used to search a given database for the purpose of protein identification. Thus MS/MS can be used to identify multiple proteins at once with a limiting factor being overwhelming the mass spectrometer with too many peptides. Use of high performance liquid chromatography to separate a complex peptide mixture either prior to MS/MS analysis (off-line) or directly in to the MS/MS analyzer (in-line) remedies overwhelming of the mass spectrometer and facilitates identification of multiple proteins from a single complex peptide mixture.

1.3.11 Protein quantitation via mass spectrometry

Comparative quantitative proteomic studies like any other quantitative studies require multiple experimental replicates for any statistically relevant measurements to be taken. When using mass spectrometry to quantitate proteins multiple measurements increase both time and cost. Various methodologies have been devised to address these issues by either quantitating individual proteins via quantitative two-dimensional gel electrophoresis prior to their identification via mass spectrometry or using various isotopic and isobaric labelling of proteins or peptides, respectively, to combine multiple experimental samples into a single mass spectral analysis. A detailed review of each methodology including theoretical background, pros and cons is provided in the following sections.

1.3.12 Two-dimensional gel electrophoresis

The principle behind two-dimensional gel electrophoresis (2DGE) (Rabilloud *et al.*, 2010; Raymond & Aurell, 1962; Valledor & Jorrin, 2011) entails separation of proteins based on isoelectric point (pI) via isoelectric focusing (IEF) in the first dimension, followed by separation based on molecular weight via SDS-PAGE in the second dimension. The goal is to separate proteins into individual spots for accurate quantitation. While SDS-PAGE is a reproducible method of separating proteins there was extensive variability in IEF until the advent of immobilized IEF strips (Choe & Lee, 2000). These strips provide a supportive lattice usually in the form of a plastic polymer where a pH gradient is established in a low percentage acrylamide solid support. The immobilized pH gradient allows reproducible separation of proteins resulting in accurate downstream quantitation and identification of protein spots.

Quantitation can be achieved qualitatively by eye via Coomassie Brilliant Blue staining or quantitatively via fluorescent protein stains compatible with MS such as SYPRO Ruby (Berggren *et al.*, 1999) or Cy dyes (Alban *et al.*, 2003). Cy dyes excite and emit at three distinct wavelengths and afford the advantage of separating proteins from up to three experimental samples in one 2DGE experiment. Protein spots of interest are then excised and used for in-gel trypsin digestion and resulting peptides used to identify the protein either by peptide mass fingerprinting or tandem mass spectrometry.

1.3.13 Isobaric tags for relative and absolute quantitation (iTRAQ)

Use of isobaric tags (Ross *et al.*, 2004) enables mass spectrometry analyses of up to 8 experimental samples at once. The isobaric tag is composed of a reporter group based on N-methylpiperazin, a carbonyl balancer group and a primary amine reactive NHS ester group. Reporter groups are isotopically modified giving rise to reporter groups of identical molecular structure that differ in mass with masses of 113.1, 114.1, 115.1, 116.1, 117.1, 118.1, 119.1, and 121.1 Daltons. Balance groups are similarly isotopically modified yielding a mass range of 24, 26, 27, 28, 29, 30, 31 and 32 Daltons that when covalently bonded to reporter groups result in 8 tags that are identical in structure and mass but not in isotope composition. To perform quantitative proteomics using iTRAQ, protein from up to 8 different experimental conditions are subject to enzymatic digestion and resulting peptides reacted with one of eight isobaric tags. The labelled peptides from all experimental groups are combined and used as a single sample for high performance liquid chromatography tandem mass spectrometry (LC-MS/MS).

Identical molecular structure of the isobaric tags results in the same LC elution times of peptides with exact amino acid sequences from different experimental groups.

Eluted isobaric labelled peptides of identical amino acid sequence and thus identical *m/z* enter the mass spectrometer at the same time and are subject to CID where covalent bonds between the reporter and balance group are disrupted. Since reporter groups differ in *m/z* they can be separated following CID and upon detection give rise to distinct peaks in mass spectra. The intensities of these peaks are then used to make a relative quantitation of the peptides and thus proteins from respective experimental conditions.

1.3.14 Selected reaction monitoring (SRM)

Selective reaction monitoring (Schmidt *et al.*, 2008), also referred to as multiple reaction monitoring (MRM) (Kuzyk *et al.*, 2009), utilizes isotopically labelled synthetic peptide standards to measure absolute peptide and protein concentrations. For a complex protein sample such as total protein from an organism, a peptide atlas is formed by identifying all detectable peptides (proteotypic peptides) via LC-MS/MS. Proteotypic peptides representing proteins of interest are then used to design isotopically labelled synthetic peptides. Synthetic peptides of known concentrations are then used to generate standard curves against MS spectral intensities. Spectral intensities of proteotypic peptides from experimental samples can be calculated using counterpart synthetic peptide standard curves. When working with cell cultures this methodology can be used to quantify absolute protein numbers expressed per cell (Malmstrom *et al.*, 2009).

1.4.1 Overview of proteomic studies that have been conducted within *Leptospira* to date

Genomic methodologies for identifying leptospiral genes involved in pathogenesis are limited to random transposon insertion. Lack of efficiency in this system has forced researchers into utilising other genomic and transcriptomic methodologies such as comparative genomics and microarrays to identify potential virulence genes.

Comparative analyses have been further extended to proteins to eliminate the observed discrepancy between mRNA and protein levels. With respect to proteomic approaches in the study of pathogenesis, outer membrane proteins are ideal targets as these proteins are exposed to the host environment and likely involved in various steps of pathogenesis such as attachment, dissemination and/or immune evasion. A second benefit to the study of outer membrane proteins is that these proteins serve as ideal antigens for potential vaccine candidates as they are immediately exposed to the host immune system. The majority of proteomic studies within the leptospiral research field have focused on leptospiral outer membrane proteins and are reviewed in the following sections.

1.4.2 Comparative proteomics of OMPs *in vitro*

In general these studies followed the basic principle of leptospiral culturing under conditions mimicking the host environment followed by comparative proteomic analysis to detect changes in protein expression.

As *Leptospira* can transmit from the external environment to a host they must be able to adapt to a complex set of changing variables between these environments. Some of these changes include limited iron in the host, temperature changes, direct contact with host factors and altered osmolarity. The effects on outer membrane protein expression changes in *Leptospira* grown in limited iron, in altered temperature and in complement-inactivated bovine serum have been demonstrated by Cullen and coworkers (Cullen *et al.*, 2002). In this study 2DGE and MS analysis identified differential expression of previously identified outer membrane proteins LipL36 (Nally *et al.*, 2001b), LipL41 (Nally *et al.*, 2001b), LipL48 (Haake & Matsunaga, 2002) and various LipL32 isoforms. Additional MS/MS analysis identified eight outer membrane proteins pL18, pL21, pL22,

pL24, pL45, pL47/49, pL50, and pL55. This study contributed to leptospiral research by identifying LipL36, LipL41, LipL48 and LipL32 as potential targets for leptospiral pathogenesis and identified eight novel proteins localised in the outer membrane of *Leptospira* (Cullen *et al.*, 2002).

The effects of temperature shift from 30 °C to 37 °C on leptospiral outer membrane protein expression has also been analyzed using both 2DGE-MS/MS and iTRAQ-LC-ESI-MS/MS (Lo *et al.*, 2009). In this study both microarray and proteomic studies were used to analyse temperature shift response in *Leptospira*. These analyses identified a subset of genes whose expression was controlled post-transcriptionally as changes in expression were detected at the protein level but not at the RNA level. Additionally, this study identified decreased expression of 66 proteins and increased expression of 27 proteins in response to a temperature shift providing the most comprehensive list of leptospiral proteins displaying altered expression in response to a temperature shift.

Another significant difference between the external and host environment is osmotic pressure. To test the effects on protein expression in response to changes in osmolarity, *Leptospira* were exposed to altered osmotic pressures induced by varying either sodium chloride, potassium chloride, or sodium sulphate concentrations during culturing (Matsunaga *et al.*, 2005; Matsunaga *et al.*, 2007b). Altering the concentration of salts to mimic physiological osmolarity induced expression of immunoglobulin like proteins LigB, LigA and a haemolysin Sph2 and caused release of LigA (Matsunaga *et al.*, 2005) and Sph2 (Matsunaga *et al.*, 2007b) into the extracellular medium. These

studies highlighted the importance of osmolarity in activating a leptospiral response and identified 3 proteins with a potential role in leptospiral pathogenesis.

With respect to the study of leptospiral pathogenesis another useful proteomic comparison is that of a virulent strain maintained in an animal and one attenuated in virulence due to long term passage in culture media. Proteins displaying altered expression in the virulent strain serve as targets likely required for virulence. Evidence supporting differences between virulent and attenuated leptospiral strains has been provided previously (Nally *et al.*, 2005a), showing altered lipopolysaccharide and protein content between the strains. Further, 2DGE immunoblots with both monospecific antibodies and convalescent rat serum (the latter combined with MS/MS for identification purposes) comparing outer membrane protein fractions from *in vitro* cultivated *Leptospira* and *Leptospira* collected from rat urine identified increased expression of the previously identified virulence factor Loa22 and the outer membrane lipoprotein Lipl32 and decreased expression of the outer membrane protein OmpL1 and outer membrane lipoproteins LipL41 and LipL21 (Nally *et al.*, 2007). Altered expression of outer membrane proteins between virulent and avirulent *Leptospira* expanded the list of outer membrane proteins potentially involved in leptospiral pathogenesis.

The future of comparative proteomics holds immense potential for deciphering leptospiral pathogenesis given the recent advance in the development of a peptide atlas for the *Leptospira* proteome (Malmstrom *et al.*, 2009). The Malmstrom study utilized a directed mass spectrometry approach (Schmidt *et al.*, 2008) on a whole leptospiral proteome extract generating a peptide atlas containing 18,303 unique peptides corresponding to the identification of 2,221 proteins, a 61% coverage of the predicted *L.*

interrogans ORFs. Combined with an SRM approach the absolute number of 19 proteins was determined using 32 unique isotope labelled peptides. These isotope labelled peptides were then used as calibration points to estimate the total number of proteins within cells for 1,095 other proteins using an average of three peptide spectral counts. Thus the peptide atlas provided by this study can be used by researchers in future comparative proteomic studies to focus on proteotypic peptide quantitation using a few isotopically labelled peptides as calibrants. Additionally this approach would significantly increase protein coverage and thus the probability of identifying proteins potentially required for leptospiral pathogenicity.

1.4.3 Characterization of proteins identified through proteomic studies

Various surface exposed proteins have been identified in *Leptospira* and *in vitro* studies have revealed a potential role for some of these proteins in the infection process. A brief summary of these proteins includes; LcpA shown to bind C4BP, a protein involved in the complement system (Barbosa *et al.*, 2010), Lfha a factor H-binding protein (Verma *et al.*, 2006) and LigA (Matsunaga *et al.*, 2003) and LigB (Matsunaga *et al.*, 2003) whose heterologous expression in the saprophyte *L. biflexa* increases binding to culture cells and fibronectin (Figueira *et al.*, 2011). Other surface exposed proteins shown to be potentially involved in attachment include OmpL37, demonstrated to bind skin and vascular elastin (Pinne *et al.*, 2011) and LenA (Stevenson *et al.*, 2007; Verma *et al.*, 2010), Lsa21 (Atzingen *et al.*, 2008), Lsa63 (Vieira *et al.*, 2010) and LipL53 (Oliveira *et al.*, 2010) that exhibit binding to extracellular matrix proteins. Collectively these studies provide evidence for a potential role of these surface exposed proteins as immune modifiers and as attachment proteins thus suggesting a role for these proteins in

the leptospiral infection process. Future studies utilizing insertion mutants in genes encoding the above listed proteins in infection studies in hamsters would definitively define the role these proteins play in leptospiral pathogenesis.

1.4.4 Current understanding of proteins that contribute to leptospiral pathogenesis

As discussed in section 1.2.4, random transposon mutation studies have led to the discovery of six genes required for the leptospiral infection process including *la0222* encoding the outer membrane lipoprotein Loa22 (Ristow *et al.*, 2007), *la2613* encoding the flagella motor switch protein FliY (Liao *et al.*, 2009), *lb186* encoding the heme oxygenase HemO (Murray *et al.*, 2009b), two genes which encode LPS biosynthesis proteins (Murray *et al.*, 2010) and *la3976* encoding an invasion-associated protein A (InvA) (Luo *et al.*, 2011). Besides their requirement for infection little is known about the role these proteins play in pathogenesis, with the exception of *invA* (Luo *et al.*, 2011). Specifically, Luo et al., have shown that recombinant InvA possesses hydrolase activity using dinucleoside oligophosphates as substrates. The authors suggest that this protein is important for detoxification of oxidized dinucleosides that could arise during oxidative stress conditions occurring within host immune cells. In support of this theory the authors demonstrated that InvA was expressed only during the late infection stage of macrophages and that *invA Leptospira* insertion mutants were unable to survive within macrophages compared to *wt Leptospira*. As already discussed these mutants were also deficient in their ability to establish infection of hamsters.

The above discussed leptospiral proteins involved in pathogenesis were not identified at the commencement of my work for this thesis. Additionally, previous proteomic studies by other researchers focused on gel based approaches and on outer

membrane proteins. Thus the main goal of this thesis project was to build upon previous studies on leptospiral pathogenesis using comparative quantitative proteomic approaches and to expand these studies to include all leptospiral proteins using a novel (in leptospiral research) iTRAQ approach.

1.5.1 Summary of results presented in this thesis

The work presented in this thesis builds on previous proteomic studies and has advanced our understanding of *Leptospira* pathogenesis in three ways.

1. A global proteomic approach identified five proteins with potentially novel roles in leptospiral pathogenesis.
2. One of these proteins was determined through further characterization to be a catalase required for leptospiral virulence in hamsters and guinea pigs.
3. Discovery of a novel surface exposed protein that displayed differential glutamic acid methylation.

As reviewed in sections 1.4.2-1.4.4, the majority of proteomic studies on *Leptospira* have focused on outer membrane proteins as these proteins are in direct contact with host factors and thus attractive candidate virulence factors. An obvious limitation to this approach is that various proteins that contribute to pathogenesis but are not localized to the outer membrane will not be identified. At the commencement of this thesis iTRAQ was a relatively novel technology not applied to the field of leptospiral proteomics and held value for a comparative whole proteome approach to study and identify potential pathogenesis-related leptospiral proteins. Thus iTRAQ was utilized to conduct comparative quantitative total protein experiments on *Leptospira* cultured under various

conditions meant to replicate a subset of variables encountered within the host. The results of these experiments are the subject of Chapter 2 within this thesis.

The iTRAQ results combined with COGs and data from other previously published studies investigating bacterial pathogenesis suggested a number of protein targets with potential roles in leptospiral pathogenesis. One of these proteins, predicted to be a catalase KatE, was further characterized for function, cellular localization and requirement for both oxidative stress resistance *in vitro* and pathogenesis in the hamster and guinea pig models of infection. The results from these experiments are presented within Chapter 3 of this thesis.

The iTRAQ experiments were also complemented with gel based proteomics using 2DGE. In addition to confirming quantitation values for various proteins an unexpected finding from these experiments was identification of glutamic acid methylation of a putative outer membrane. This protein was further characterized and tested for outer membrane localization and expression during colonization of hamster liver and kidneys. These results are the topic of Chapter 4 of this thesis.

In the 5th and final chapter of this thesis the experiments presented in chapters 2-4 are briefly discussed in the context of leptospiral research. The main focus of chapter 5, however, will be on the significance of these experiments and their limitations and suggestion of future experiments that can expand both our knowledge of leptospiral pathogenesis and the field of study itself.

Chapter 2
Global proteome analysis of *Leptospira interrogans*

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Figure 5 work performed and figure generated by Dr. Richard L. Zuerner
Dr. Paul A. Cullen assisted with the proteomic data analyses
Dr. Laura Cowen developed statistical analyses of iTRAQ data

2.1.1 Abstract

Comparative global proteome analyses were performed on *Leptospira interrogans* serovar Copenhageni grown under conventional *in vitro* conditions and those mimicking *in vivo* conditions (iron limitation and serum presence). Proteomic analyses were conducted using iTRAQ and LC-ESI-tandem mass spectrometry complemented with two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry. A total of 563 proteins were identified in this study. Altered expression of 65 proteins, including upregulation of the *L. interrogans* virulence factor Loa22 and 5 novel proteins with homology to virulence factors found in other pathogens, was observed between the comparative conditions. Immunoblot analyses confirmed upregulation of 5 of the known or putative virulence factors in *L. interrogans* exposed to the *in vivo*-like environmental conditions. Further, ELISA analyses using serum from patients with leptospirosis and immunofluorescence studies performed on liver sections derived from *L. interrogans*-infected hamsters verified expression of all but one of the identified proteins during infection. These studies, which represent the first documented total proteome analysis of *Leptospira*, demonstrated proteome alterations under conditions that mimic *in vivo* infection and allowed the identification of novel putative *L. interrogans* virulence factors.

2.2.1 Introduction

Many pathogenic bacteria establish infection by altering the expression of their proteome (defined as the entire complement of proteins expressed by a genome, cell, tissue or organism (Wasinger *et al.*, 1995)) in response to environmental cues such as iron availability and the presence of host factors. Pathogenic bacteria use various outer membrane proteins (OMPs) to detect environmental changes, which in turn leads to the activation of signal transduction pathways, alteration of the proteome and induction of conditions that favour pathogen survival (Braun & Braun, 2002). Paradoxically, the same OMPs that facilitate pathogen survival can also lead to pathogen demise if detected by the host immune system (Nart *et al.*, 2007). Therefore, the regulation of bacterial OMPs and subcellular proteins involved in OMP regulation is crucial for the survival of the invading bacteria and for their ability to establish infection.

Multiple reports have established that *Leptospira* respond to altered environmental conditions at both the transcriptome (Lo *et al.*, 2006; Qin *et al.*, 2006) and proteome levels (Artiushin *et al.*, 2004; Cullen *et al.*, 2002; Cullen *et al.*, 2005; Guerreiro *et al.*, 2001; Haake & Matsunaga, 2002; Matsunaga *et al.*, 2007a; Monahan *et al.*, 2008; Nally *et al.*, 2001a; Nally *et al.*, 2001b; Nally *et al.*, 2007; Sakolvaree *et al.*, 2007; Velineni *et al.*, 2006). Genome-wide transcriptional analyses have previously been conducted on *L. interrogans* serovar Lai exposed to temperature shifts from environmental/laboratory conditions to physiological conditions using DNA microarrays and real-time reverse transcription-PCR. These studies demonstrated regulation of genes whose encoded products can be classified based upon various functional groups, including energy production, protein export, heat shock protection and chaperone activity

(Lo *et al.*, 2006; Qin *et al.*, 2006). At the protein level, previous gel-based proteomic analyses conducted on *L. interrogans* have collectively: 1) identified and determined cellular location for a diverse repertoire of *L. interrogans* proteins (Cullen *et al.*, 2005; Haake & Matsunaga, 2002; Monahan *et al.*, 2008; Sakolvaree *et al.*, 2007) 2) demonstrated the immunoreactive potential of some of these identified proteins (Artiushin *et al.*, 2004; Guerreiro *et al.*, 2001; Sakolvaree *et al.*, 2007); and 3) revealed that *L. interrogans* display unique protein expression profiles within different microenvironments (Cullen *et al.*, 2002; Matsunaga *et al.*, 2007a; Nally *et al.*, 2001a; Nally *et al.*, 2001b; Nally *et al.*, 2007; Velineni *et al.*, 2006). In particular, immunoblot analysis of outer membrane protein extracts of *L. interrogans* cultured under conditions of iron limitation, using sera collected from patients with leptospirosis, revealed expression of four immunoreactive OMPs of approximate molecular masses of 50, 55, 60 and 64 kDa (Velineni *et al.*, 2006). Additionally, comparative proteomic analysis of *L. interrogans* cultured at temperatures above 30 ˚C in medium containing 10% fetal bovine serum (FBS) and depleted of iron (-Fe) revealed altered regulation of various OMPs and cleavage of the lipoprotein LipL32 into various isoforms (Cullen *et al.*, 2002).

In the present study we have extended the above-mentioned gel-based proteomic investigations by performing both non-gel-based and gel-based proteomic analyses on an organismal level for *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (henceforth referred to as *L. interrogans*). Comparative analyses were conducted on total protein extracted from bacteria that had been grown under conventional *in vitro* conditions and those mimicking *in vivo* conditions (iron limitation and serum presence, defined as *in vivo*-like conditions). These comparative culture conditions selected for

identification of proteins that were differentially expressed upon exposure to host components (serum) and iron limitation independent of proteomic changes induced by a temperature shift. These studies provide the first comprehensive, comparative and quantitative analysis of *Leptospira* total proteome alterations in response to environmental conditions that mimic those found within the host.

In the non-gel-based proteomic approach, isobaric tags for relative and absolute quantitation (iTRAQ, for a review see (Aggarwal *et al.*, 2006)) and liquid chromatography-electrospray ionization (LC-ESI) tandem mass spectrometry were utilized. This approach combines the ability of iTRAQ to facilitate simultaneous protein abundance determination between different experimental conditions with the specificity of mass spectrometry for protein identification. Briefly, in iTRAQ proteins derived from a complex mixture are first digested by trypsin into peptides and an isobaric tag is covalently added onto primary amines within the peptides at N-termini and lysine side chains (Ross *et al.*, 2004). Tandem mass spectrometry analysis of the labelled peptides allows for quantitation of the isobaric tags and, accordingly, the peptide to which the tag is coupled. The isobaric nature of the tags ensures that the tagged peptides originating from the comparative conditions are treated as equal for separation purposes but can be accurately quantitated during tandem mass spectrometry analysis. This approach offers several advantages over traditional proteomic methodologies. First, the reduced spectral complexity associated with the isobaric tags used in iTRAQ allows quantitation determination via comparison of reporter ion peak intensities during collision induced dissociation (Ross *et al.*, 2004). Second, iTRAQ/LC/ESI-tandem mass spectrometry allows accurate identification and quantitation of all proteins within a complex sample,

irrespective of molecular weight or isoelectric point (Aggarwal *et al.*, 2006; Washburn *et al.*, 2001), thus facilitating analysis of a diverse set of proteins with various functional roles, divergent cellular locations and differing abundance (Cong *et al.*, 2006). Finally, iTRAQ permits simultaneous proteomic analysis of multiple samples (Ross *et al.*, 2004), thereby facilitating statistical verification of changes in protein expression between different environmental conditions (Gan *et al.*, 2007). This methodology has been successfully used to analyze the proteomic profiles of *Halobacterium salinarum* NRC-1 (Leuko *et al.*, 2009) and *Cyanobacteria* (Pandhal *et al.*, 2009) in relation to salt tolerance, *Nostoc punctiforme* ATCC 29133 during different nitrogen fixation stages (Ow *et al.*, 2009), *Mycobacterium ulcerans* during mycolactone biosynthesis (Tafelmeyer *et al.*, 2008) and *Escherichia coli* grown in either Lauria Broth media or fresh milk (Lippolis *et al.*, 2009), thus displaying the effectiveness of this methodology for analysis of bacterial proteomes.

To complement and confirm our proteomic findings obtained via the non-gel-based proteomic approach, we conducted simultaneous gel-based proteomic analyses using two-dimensional gel electrophoresis (2DGE) and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Cullen *et al.*, 2002; Drummelsmith *et al.*, 2007; Zeaiter *et al.*, 2008). Combined, the iTRAQ and 2DGE experiments identified a total of 563 expressed leptospiral proteins. Altered expression levels were observed for 65 of these proteins, including 6 proteins that have been previously shown to play a direct role in the virulence of either *L. interrogans* or other pathogenic bacteria.

2.3.1 Materials and methods

2.3.2 *Leptospira* and culture conditions

Leptospira interrogans serovar Copenhageni strain Fiocruz L1-130 is a clinical isolate originating from Salvador, Brazil (Ko *et al.*, 1999) that was kindly provided by Dr. David A. Haake (Veterans Affairs Greater Los Angeles Health Care System, Los Angeles, California). Prior to protein extraction for proteomic analysis, bacteria were passaged *in vitro* a maximum of six times via cultivation in Ellinghausen and McCullough medium (Ellinghausen & McCullough, 1965) as modified by Johnson and Harris (Johnson & Harris, 1967) (EMJH) at 29.5 °C. Bacteria were diluted in phosphate buffered saline (PBS) containing 5 mM $MgCl_2$ to allow accurate enumeration using a Petroff Hauser counting chamber (Fisher Scientific, Ottawa, Ontario) and a dark field microscope (Nikon ECLIPSE 50i, Mississauga, Ontario). For media shift experiments cultures were grown to a density of 1 x 10^8 cells/mL, centrifuged at 8,500 x *g* and resuspended at the same concentration in either (1) fresh EMJH media pre-incubated at 37 °C or (2) EMJH medium depleted of iron by overnight incubation with 0.4 mM 2,2-dipyridyl (Cullen *et al.*, 2002) (Sigma, Oakville, Ontario, Canada) and supplemented with fetal bovine serum (FBS; Sigma) to a final concentration of 10% (–Fe/FBS media) at 37 °C. Cultures were then incubated for an additional 72 h at 37 °C prior to harvest.

2.3.3 Protein extraction and sample preparation

Subsequent to harvest**,** bacteria were washed twice with repeated steps of centrifugation at 9,500 x *g* and resuspended in phosphate buffered saline (PBS) supplemented with 5 mM $MgCl_2$ (Cullen *et al.*, 2002). Pellets obtained from the comparative conditions were lyophilized, weighed and resuspended to equal dry mass per

volume. For iTRAQ and immunoblotting analyses, total protein was prepared according to the following protocol: pellets were resuspended in 0.1% SDS, lysed via sonication (Sonicator 3000, Masonix, Farmindale, New York, USA) using 4 Watts of continuous power for a total of 3 x 30 s on ice, centrifuged at 16,000 x *g* to remove debris and subjected to overnight acetone precipitation at – 20 °C. Protein precipitates were centrifuged at 10,000 x *g* (30 min at 4 °C), excess acetone was removed and the resulting pellets were resuspended in 1 mL 100 mM triethylammonium bicarbonate/4M urea/0.2% sodium dodecyl sulphate and subjected to a second acetone precipitation. For 2DGE experiments, pellets were resuspended to a concentration of 2.5 mg/mL of dry weight in IEF buffer (5 M urea, 2 M thiourea, 2 mM tri-butyl phosphine (Sigma), 2% CHAPS (Sigma), 1% carrier ampholytes (Bio-Rad Laboratories, Mississauga, Ontario), 2% 3-(Decyldimethylammonio)propanesulfonate inner salt (Sigma), 40 mM TRIS-HCl and 0.002% bromphenol blue). Lysis was achieved via sonication followed by the addition of 150 U of benzonase (Sigma) per mL of lysate and incubation at room temperature for 20 min. The resulting lysate was centrifuged at 16,000 x *g* to remove debris and the isolated supernatant was used in IEF experiments.

2.3.4 iTRAQ analysis

Total protein samples were submitted to the University of Victoria-Genome BC Proteomics Centre for iTRAQ analysis. Trypsin digestion and isobaric tag labelling were performed according to the manufacturer's instructions (Applied Biosystems, Foster City, California, USA).

2.3.5 iTRAQ experimental design

To control for biological, technical and experimental variations, two independent iTRAQ experiments were performed using the experimental strategy developed by (Gan *et al.*, 2007). Figure 1 shows an overview of the iTRAQ experimental design used to analyze the *L. interrogans* protein samples. Biological variation was controlled for at the culture level by using pooled cultures, where A1 and B1 each represent 75 independent cultures and A2 and B2 each represent 25 independent cultures. Following tryptic digestion of all four samples (A1, A2, B1, B2), samples A1 and B1 were split into three equal volumes, and the resulting 8 samples were labelled with appropriate isobaric tags as depicted in Figure 1. Replicate samples (2 x A1 and 2 x B1) in the first iTRAQ experiment (iTRAQ1) controlled for technical variation within the same iTRAQ experiment and analysis of A1 and B1 in the second iTRAQ experiment (iTRAQ2) controlled for experimental variation between independent iTRAQ experiments. Lastly, comparison of the experimental replicates A1 to A2 and B1 to B2 in iTRAQ2 allowed evaluation of variation introduced via the protein extraction steps.

2.3.6 Liquid chromatography-electrospray ionization tandem mass spectrometry

Mass spectrometry was performed by the University of Victoria-Genome BC Proteomics Centre (Dean *et al.*, 2007). Briefly, LC-MS/MS analysis was performed using an integrated Famos autosampler, SwitchosII switching pump, and UltiMate micro pump (LC Packings, Amsterdam, The Netherlands) system with an Hybrid Quadrupole-TOF LC-MS/MS Mass Spectrometer (QStar Pulsar i) equipped with a nano-electrospray ionization source (Proxeon, Odense, Denmark) and fitted with a 10 μm fused-silica emitter tip (New Objective, Woburn, Massachusetts). Chromatographic separation was

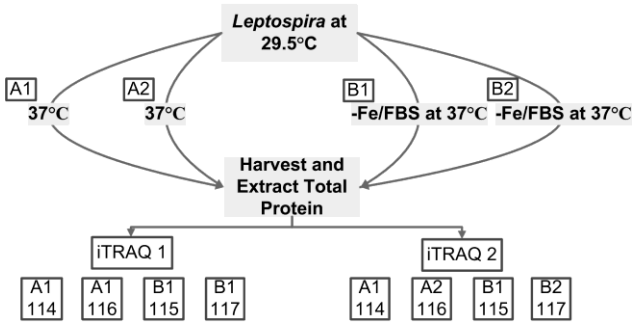

Figure 1: iTRAQ experimental design.
Pooled biological samples were used to control for biological variation by combining 75 independent cultures in samples A1 (shifted to 37 °C) and B1 (shifted to 37 °C in –Fe/FBS medium) and 25 independent cultures in samples A2 (shifted to 37 °C) and B2 (shifted to 37 °C in –Fe/FBS medium). To control for technical variation within a single iTRAQ experiment and experimental variation between two iTRAQ experiments, samples A1 and B1 were split into 3 equal volumes subsequent to tryptic digestion but prior to isobaric tag labelling. Comparison of A1 to A1 and B1 to B1 in iTRAQ1 and A1 to A2 and B1 to B2 within iTRAQ2 controlled for technical variation within the same iTRAQ experiment, while comparison of A1 and B1 between iTRAQ1 and iTRAQ2 controlled for experimental variation between separate iTRAQ experiments. Numbers 114, 115, 116 and 117 correspond to the isobaric tags used for labelling the samples. The overall experimental strategy was adapted from (Gan *et al.*, 2007).

achieved on a 75 μm x 15 cm C18 PepMap Nano LC column (3 μm, 100 Å, LC Packings) and a 300 μm x 5 mm C18 PepMap guard column (5 μm, 100 Å, LC Packings) was in place before switching inline with the analytical column and the MS. The mobile phase (solvent A) consisted of water/acetonitrile (98:2 (v/v)) with 0.05% formic acid for sample injection and equilibration on the guard column at a flow rate of 100 μL/min. A linear gradient was created upon switching the trapping column inline by mixing with solvent B which consisted of acetonitrile/water (98:2 (v/v)) with 0.05% formic acid and the flow rate was reduced to 200 nL/min for high resolution chromatography and introduction into the mass spectrometer.

Samples were brought up to 20 μL with 5% ACN and 3% FA and transferred to autosampler vials (LC Packings). Ten microliters of sample were injected in 95% solvent A (10 mM KPO_4 (pH 2.7), 25% ACN) and allowed to equilibrate on the trapping column for 10 min to wash away any contaminants. Upon switching inline with the MS, a linear gradient from 95% to 40% solvent A developed for 40 min and in the following 5 min the composition of mobile phase was increased to 20% A before decreasing to 95% A for a 15 min equilibration before the next sample injection. MS data was acquired automatically using Analyst QS 1.0 software Service Pack 8 (ABI MDS SCIEX, Concord, Canada). An information-dependent acquisition method consisting of a 1 s TOFMS survey scan of mass range 400-1200 amu and two 2.5 s product ion scans of mass range 100-1500 amu was performed. The two most intense peaks over 20 counts with charge state 2-5 were selected for fragmentation and a 6 amu window was used to prevent the peaks from the same isotopic cluster from being fragmented again. Once an ion was selected for MS/MS fragmentation it was put on an exclude list for 180 s. Curtain

gas was set at 23, nitrogen was used as the collision gas and the ionization tip voltage used was 2700 V.

If the observed A_{215} was greater than 0.1 for any fraction collected during the strong cation exchange 2.5 h gradient, then 95-50% solvent A was used to compensate for the higher peptide concentration in that fraction. Data files were processed using the Protein Pilot software (Version 2.0.1, Applied Biosystems).

2.3.7 iTRAQ data analysis

Data analysis was first conducted by exporting all data sets from Protein Pilot to Microsoft Excel (Office 2007, Microsoft, Seattle, Washington, 2007). These were then converted to text files to be further analyzed using Statistical Analysis System (SAS) software Version 9.1 of the SAS System for Windows (Copyright © 2003 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, North Carolina, USA)

To be considered as significantly altered in expression, a protein and it's representative peptides must have met the following criteria: 1) the protein had to demonstrate a database match with a leptospiral protein; 2) the protein had to be represented by at least two peptides of differing amino acid sequence (Aggarwal *et al.*, 2006; Chong *et al.*, 2006; Wiese *et al.*, 2007); 3) the test to determine if the ratio of protein expression equals 1 had to have a p-value less than 0.05 for at least 2 of the 4 possible comparisons (eg. for iTRAQ1; 115:114, 115:116, 117:114 and 117:116); and 4) experimental and non-experimental 95% confidence intervals for a given protein ratio must not have overlapped. Confidence intervals for the average protein ratios were

calculated on the $\log_{10}$ scale and then back-transformed to the linear scale (the same scale as the original ratio).

To obtain an estimate of the average peptide ratio for a protein within a replicate experiment (replicate *i*), Protein Pilot calculates a weighted average of the $\log_{10}$ peptide ratios as

$$\bar{x}_i = \frac{\sum_{j=1}^{n} w_j x_{ij}}{\sum_{j=1}^{n} w_j}$$

where

n is the number of peptides used to identify a protein

x_{ij} is the $\log_{10}$ ratio of peptide *j* for replicate *i*; *i*=1,2,..,N, *j*=1,2,..,n

w_j is the weight given to peptide *j* where $w_j = 1/\%Error_j$

and the percent error for each peptide ratio is formulated by Protein Pilot as

$$\%Error_j = \sqrt{\left(\frac{ErrorA}{AreaA}\right)^2 + \left(\frac{ErrorB}{AreaB}\right)^2}$$

where AreaA and AreaB are the areas for each peak in the ratio of experimental to non-experimental peptides. ErrorA and ErrorB are the mass spectrometry measurement errors for AreaA and AreaB, respectively.

As previously discussed, iTRAQ experiments were designed to control for biological, experimental, and technical variation (Figure 1) resulting in four iTRAQ replicate experiments. This provides up to four estimates of the average peptide ratio for each protein. These estimates were averaged to obtain the grand mean of the $\log_{10}$ peptide ratio which is calculated as $\bar{x}_\cdot = \sum_{i=1}^{N} \frac{\bar{x}_i}{N}$, where, for the experimental ratios, N is the

number of replicate experiments that satisfied criteria 1, 2, and 3 above. For the non-experimental ratios, N is the number of replicate experiments that satisfied criteria 1 and 2 above. Thus N varied between 2 and 4 depending on the protein.

As each replicate experiment provides an estimate of the average peptide ratio, the variation among these estimates (the empirical variance) is used to obtain the standard error of the grand mean (1987). The estimate of the empirical variance of the grand mean $\log_{10}$ peptide ratio is calculated as $\hat{Var}(\bar{x}.) = \frac{1}{N}\sum_{i=1}^{N}\frac{(\bar{x}_i - \bar{x}.)^2}{N-1}$.

Finally, a 95% confidence interval for the average $\log_{10}$ peptide ratio for a protein is obtained using $\bar{x}. \pm t_{N-1,0.025}\sqrt{\hat{Var}(\bar{x}.)}$ where $t_{N-1,0.025}$ is the critical value of the Student *t* distribution with *N-1* degrees of freedom that corresponds with a 95% confidence level. The upper and lower levels of the confidence interval were back transformed from $\log_{10}$ space into linear space to obtain 95% confidence intervals for the average peptide ratio. All calculations were automated by writing custom scripts in SAS available from the authors.

2.3.8 2DGE experiments

Supernatants from protein extraction experiments were split into three equal volumes and run in triplicate for a total of 6 IEF and SDS-PAGE experiments (3 replicates for *L. interrogans* in unmodified culture media and 3 replicates for *L. interrogans* in –Fe/FBS medium) conducted concurrently to limit experimental and technical variation. Isoelectric focusing was conducted using the Ettan IPGphor isolectric focusing system (Amersham Bioscience, Piscatway, New Jersey, USA) and non-linear pH 3-11 immobilized pH gradient isoelectric focusing strips (Bio-Rad

Laboratories). The run parameters included overnight hydration of the strips with sample in IEF buffer at room temperature under a gradient voltage to a maximum of 100 V. Isoelectric focusing run parameters included gradient voltage to 3500 V over 12 h and a hold at 3500 V for 18 h. The strips were then incubated at room temperature for 20 min in equilibration buffer (6 M urea, 2% SDS, 0.36 M TRIS-HCl, 20% glycerol, 5 mM tri-butyl phosphine and 2.5% acrylamide) and subsequently used in SDS-PAGE experiments. SDS-PAGE was conducted on an Etta Dalt Twelve system (Amersham) using 8 – 16% gradient acrylamide gels. Gels were subsequently stained with colloidal Coomassie Blue G-250, and proteins that reproducibly exhibited differential protein expression levels between the experimental conditions were cored for in-gel tryptic digest(Cullen *et al.*, 2002). Core digests were C8 ZipTip purified (Bio-Rad Laboratories), manually spotted on MALDI plates and overlaid with α-cyano-4-hydroxycinnamic acid (Sigma) at a concentration of 10 mg/mL in 50% acetonitrile/0.1% trifluoroacetic acid for MALDI-TOF mass spectrometry analyses.

2.3.9 Matrix-assisted laser desorption ionization time of flight mass spectrometry

MALDI-TOF MS (Voyager-DE STR; Applied Biosystems) was conducted in reflector mode under high voltage settings. Laser intensities ranged from 2500 to 2800 Hz depending on the observed signal-to-noise ratios in the mass spectra. A three point calibration was performed under reflector mode using an angiotensin tryptic digest calibration mix (Sigma). Prior to database analysis, mass spectra were noise filtered, baseline corrected, two point internally calibrated using trypsin m/z peaks 841.51 and 2210.1 and, depending on signal-to-noise ratios, the detection level was adjusted between 2 to 15%. Peptide mass fingerprint searches were conducted using the Mascot peptide

mass fingerprinting database (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF) and search parameters were set as follows: taxonomy was set to "other bacteria" or "all entries" for identification of leptospiral proteins and protein contaminants, respectively; trypsin was selected as the enzyme; the number of missed cleavage sites was set to one; variable modifications were set to carbamidomethyl and methionine oxidation; mass tolerance was set between 10 – 50 ppm; and mass values were set to monoisotopic. Proteins with significant scores and at least four representative peptides were considered for further computer database analyses.

2.3.10 Computer database analysis

Proteins were annotated and accession numbers were assigned using the ExPASy proteomics server (http://ca.expasy.org/). Proteins deemed as significantly altered in expression were further confirmed using an annotated leptospiral genome database operated and maintained by Dr. T. Seemann in collaboration with the Victorian Bioinformatics Consortium at Monash University, Clayton, Victoria, Australia. The cellular localization of proteins was predicted with PSORTb v.2.0 (http://www.psort.org/psortb/index.html) using the Gram-negative algorithm, while LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/), SpLip (http://jcslab.vbi.vt.edu/splip/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) bioinformatic tools were utilized to predict lipoproteins and signal peptides.

2.3.11 Recombinant protein expression and purification

All recombinant proteins were prepared as follows. Open reading frames (ORFs) corresponding to LIC12032, LIC13166, LIC11966 and LIC12575 were PCR amplified

from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genomic DNA, while LIC13050 was amplified from *L. interrogans* serovar Copenhageni strain RJ15958 genomic DNA. Primer pairs used in the amplifications are listed in Table 1. The LIC13050 PCR product was ligated first into the donor vector pDONR201 followed by the expression vector pDEST17 (Gateway Technology, Invitrogen, Carlsbad, California, USA). Amplicons corresponding to LIC12032, LIC13166, LIC11966 and LIC12575 were ligated first into the cloning vector pJET1 (CloneJet, Fermentas, Ontario, Canada), digested with *Nde*I and either *Hind*III (LIC12032), *Eco*RI (LIC13166) or *Xho*I (LIC11966 and LIC12575) followed by ligation into a similarly digested pET28a expression vector (Novagen, Gibbstown, New Jersey, USA). The sequence and reading frame of the expression constructs were verified by DNA sequencing with vector-specific primers. The LIC13050/pDEST17 construct was transformed into the *E. coli* expression strain BL21-AI™ and all other constructs were transformed into the *E. coli* expression strain BL21 Star™ (DE3) (both from Invitrogen). Expression of the constructs and purification of the resulting six-histidine-tagged recombinant proteins were performed as follows. Recombinant expression was induced during log-phase growth using final concentrations of 1 mM isopropyl β-D-thiogalactopyranoside for LIC11966, LIC12575, LIC12032 and LIC13166 and 0.2% L-arabinose for LIC13050. Bacteria were harvested by centrifugation and lysed via sonication (3 x 20 s) in the presence of a protease inhibitor cocktail (Catalogue number 539134; Calbiochem, San Diego, California, USA). For LIC11966, LIC12575 and LIC13050, insoluble recombinant protein preparations were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (QIAGEN, Mississauga, Ontario, Canada) as previously described (Cameron *et al.*, 2000). For

Table 1: ORF-specific primers used to amplify fragments for recombinant expression.

ORF	*Sense primer	*Antisense primer	Size (bp)	Portion of ORF (bp)
LIC13050	5'-CAGGAAGATCTGGATGAAAG	5'-TTATTTTTTTGTAGGTTGAGTAGTAGT	1114	70-1114
LIC12032	5'-CTAGACCATATGATGAGTAGAAAAACCCTTACTAC	5'-GTCAGAAGCTTTTAAACT CGACTCAGAGCAAG	1470	1-1470
LIC13166	5'-CTAGACCATATGAACAATCAGGGCGGTAATCAG	5'-GTCAGGAATTCTTAAGGT CTAACCGAAATCAC	864	82-864
LIC11966	5'-CTAGACCATATGATGAAAAAA CATTCTATCAGTAAAATC	5'-GTCAGCTCGAGTTATTGA GAAGCGTATTCTTTCGC	504	1-504
LIC12575	5'-CTAGACCATATGTTAGAATATG CTTATAAACATAGAG	5'-GTCAGCTCGAGTCATTTAGCGAGATAATAGCGCAAC	604	943-1518

***** Underlined nucleotides represent incorporated restriction sites

LIC12032 and LIC13166, soluble recombinant protein preparations were purified using Ni-NTA agarose beads according to the ProBond native purification protocol (Invitrogen). Briefly, bacterial lysates (see above) were centrifuged at 10,000 x *g* at 4 °C for 15 min and the supernatant applied to Ni-NTA agarose beads (QIAGEN) in a 50 mL conical tube. The supernantant-Ni-NTA agarose bead suspension was incubated overnight at 4 °C on a hematology/chemistry mixer 346 (Fisher Scientific) followed by centrifugation at 200 x *g* at 4 °C and removal of the supernatant. Beads were washed twice with wash buffer (60 mM NaH_2PO_4, 500 mM NaCl and 20 mM imidazole, pH 8.0) and the protein was eluted with elution buffer (identical to the wash buffer except containing 250 mM imidazole). The expressed recombinant proteins were dialyzed against PBS and quantitated using the BCA Protein Assay kit (Pierce, Rockford, Illinois, USA).

2.3.12 Antibodies

Polyclonal rabbit antiserum was prepared against each of LIC12032, LIC13166, LIC11966, and LIC12575 by ImmunoPrecise Antibodies (Victoria, British Columbia, Canada). Monoclonal antibody 1H8 against FlaA1 has been described previously (Trueba *et al.*, 1992). Mouse antiserum specific for Loa22 (LIC10191) was kindly provided by Drs. Nobuo Koizumi and Haruo Watanabe (National Institute of Infectious Diseases, Tokyo, Japan). LIC13050-specific rabbit antiserum was a gift from Dr. David A. Haake (Veterans Affairs Greater Los Angeles Health Care System, Los Angeles, California).

2.3.13 Immunoblot analyses

Bacteria were grown and shifted as described in the *Leptospira* and culture conditions section. Cultures were harvested at 10,000 x *g*, washed twice with wash buffer

(PBS and 5 mM $MgCl_2$), split into duplicate samples and lyophilized. Equal dry weights were resuspended to a concentration of 7.5 mg/mL with sample buffer (100 mM Tris pH 6.8, 8 M urea, 2% SDS w/v, 10% glycerol v/v and 100 mM DTT) and sonicated (Sonicator 3000) at 6 W for 30 s continuous bursts on ice until a homogeneous mixture was obtained. Samples were centrifuged at 16,000 x *g* and the supernatant was removed and heated at 40 °C for 30 min. Ten microliters of protein sample for LIC13166, LIC12032, LIC11966 and LIC10191 and 30 µL of protein sample for LIC12575 and LIC13050 were separated on 15% sodium dodecyl sulfate-polyacrylamide gels. High-range molecular mass markers (Spectra Broad Range Protein Ladder; Fermentas, Burlington, Ontario, Canada) were used as standards. Proteins were electrophoretically transferred to Immobilon-polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, Massachusetts, USA). Immunoblots were blocked with 2.5% milk powder in Tris-buffered saline, pH 7.4, with 0.05% Tween 20 (TBST) for 1 h at room temperature. Membranes were washed 2 x 5 min with TBST followed by incubation for 90 min at room temperature with a 1:2000 dilution of LIC10191-specific mouse antiserum, a 1:2000 dilution of rabbit antiserum specific for LIC13050, LIC12032, LIC13166 and LIC11966, or a 1:1000 dilution of rabbit antiserum specific for LIC12575, all diluted in 2.5% milk powder/TBST. To normalize the samples, a 1:2000 dilution of anti-FlaA1 1H8 monoclonal antibody was included within each of these incubations. FlaA1 was demonstrated through iTRAQ analyses to remain unchanged between the comparative conditions and has been used elsewhere as a normalization control. Membranes were washed with TBST 4 x 5 min, incubated with a 1:10,000 dilution of anti-mouse IgG (H+L) IRDye700 conjugate for LIC13166, anti-mouse IgG (H+L) IRDye800 conjugate

for LIC13050, LIC12032, LIC11966, LIC12575 and LIC10191, and anti-rabbit IgG (H+L) IRDye800 conjugate for LIC13166, LIC13050, LIC12032, LIC11966 and LIC12575 (IRDye conjugates were purchased from Rockland Immunochemicals for Research, Gilbertsville, Pennsylvania, USA) in 2.5% milk powder/TBST for 60 min at room temperature followed by washing 4 x 5 min with TBST. Image analyses were performed with an Odyssey infrared imaging system equipped with Version 2.1 software (LI-COR Bioscience, Lincoln, Nebraska, USA).

2.3.14 Serum samples

Serum samples collected from patients with severe leptospirosis were a kind gift from Dr. Albert I. Ko (Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil). Samples were obtained from the collection of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) and have been described elsewhere(Van Voorhis *et al.*, 2003). A pooled sample was prepared by combining 5 µL of serum collected from each of 15 individuals with laboratory-confirmed leptospirosis. Normal human serum was obtained from Lonza (Basel, Switzerland). Approval was obtained from the Human Subjects Institutional Review Board of the University of Victoria, as well as the Oswaldo Cruz Foundation.

2.3.15 Enzyme-linked immunosorbent assay (ELISA)

Unless otherwise stated, all incubations were performed at 37 °C in a humidified chamber. Ninety-six-well plates (Nunc-Immuno Maxisorp; Sigma) were coated in triplicate overnight with 100 ng per well of rLIC13050, rLIC12032, rLIC13166, rLIC11966 or rLIC12575 in 50 mM sodium carbonate-bicarbonate buffer (pH 9.6). Plates were washed with PBS, pH 7.4 with 0.05% Tween 20 (PBST) and blocked with

200 µL per well of 3% bovine serum albumin (BSA; Sigma)/PBST for 60 min. Plates were washed with PBST and 100 µL of a 1:500 dilution of pooled laboratory-confirmed leptospirosis sera or normal human sera prepared in 3% BSA/PBST was added to wells containing recombinant proteins and to wells lacking recombinant protein (for background control) and incubated for 60 min. Plates were washed with PBST and 100 µL of a 1:3000 dilution of goat anti-human IgG (Fab specific) peroxidase conjugate (Sigma) prepared in 3% BSA/PBST was added. Following a final incubation for 60 min, plates were washed with PBST and developed for 10 min at room temperature with 100 µL of tetra-methylbenzidine-H_2O_2 substrate (TMB; Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA). Absorbance was measured at 600 nm on a Synergy HT microplate reader (BioTek, Vermont, USA). Values were background subtracted and statistical analyses were performed using the Student's *t* test.

2.3.16 Immunofluorescence microscopy

Paraffin-embedded, formalin-fixed (PEFF) tissue samples from golden Syrian hamsters (*Mesocricetus auratus*) infected with *L. interrogans* serovar Pomona strain 11000-74A were analyzed by indirect immunofluorescence microscopy as described previously (Matsunaga *et al.*, 2006; Zuerner *et al.*, 2008). Briefly, paraffin was removed from 4 µm-thick sections of PEFF samples adhered to glass slides using standard procedures, then blocked with 10% normal horse serum. Sections were incubated overnight at 4 °C with rabbit antibodies prepared to either LipL21 (Cullen *et al.*, 2003) or LIC13050. Slides were washed with PBS and then incubated for 1 h at room temperature in the dark with goat anti-rabbit Alexa 488-conjugated secondary antibody (Molecular Probes, Eugene, Oregon, USA). After washing the slides with PBS, the samples were

counterstained with DAPI (4', 6-diamidino-2-phenylindole, 1.5 mg/mL), and coverslips mounted using SlowFade Light antifade (Molecular Probes). Stained tissue sections were examined with a Nikon Eclipse E800 microscope (Nikon Instruments Inc., Melville, New York, USA) using a 100 x Pan Fluor objective. Separate images captured with a Spot RT color CCD camera (Diagnostic Instruments Inc., Sterling Heights, Michigan, USA) using B-2A and UV-2E/C filters were merged using Spot Advanced Software (Diagnostic Instruments, Inc.). Use of serial tissue sections enabled analysis of adjacent sections incubated with different primary antibodies.

2.4.1 Results and discussion

2.4.2 Overview of global proteome analyses

In this study global proteome analyses were conducted to directly investigate the differential proteomic profiles that result upon exposure of *L. interrogans* to environmental conditions that mimic those found within the host. Comparative conditions consisted of growth of *L. interrogans* at 37 °C in either unmodified culture media or culture media that had been depleted of iron and supplemented with 10% FBS (-Fe/FBS medium representative of *in vivo*-like conditions). Although it should be stressed that these conditions are entirely *in vitro* in nature, these analyses revealed two findings. First, they provided a novel global analysis of the *L. interrogans* proteome. And second, the comparative conditions allowed identification of the complete repertoire of proteins that are differentially expressed in specific response to limited iron availability and the presence of serum factors, two conditions that represent, at least in part, the environment present within a host.

Two independent yet complementary quantitative global proteomic analytical techniques were used to investigate the proteomes isolated from the comparative growth conditions. Proteomes were first analyzed using iTRAQ combined with LC-ESI tandem mass spectrometry. This approach allows direct determination of relative protein abundance levels between comparative conditions. Representative MS/MS spectra for peptides identified from an endoflagellar filament core protein (LIC11890) and a probable aconitate hydratase (LB327) representing proteins that are up- (Figure 2) and down-regulated (Figure3) in *L. interrogans* grown in –Fe/FBS culture medium,

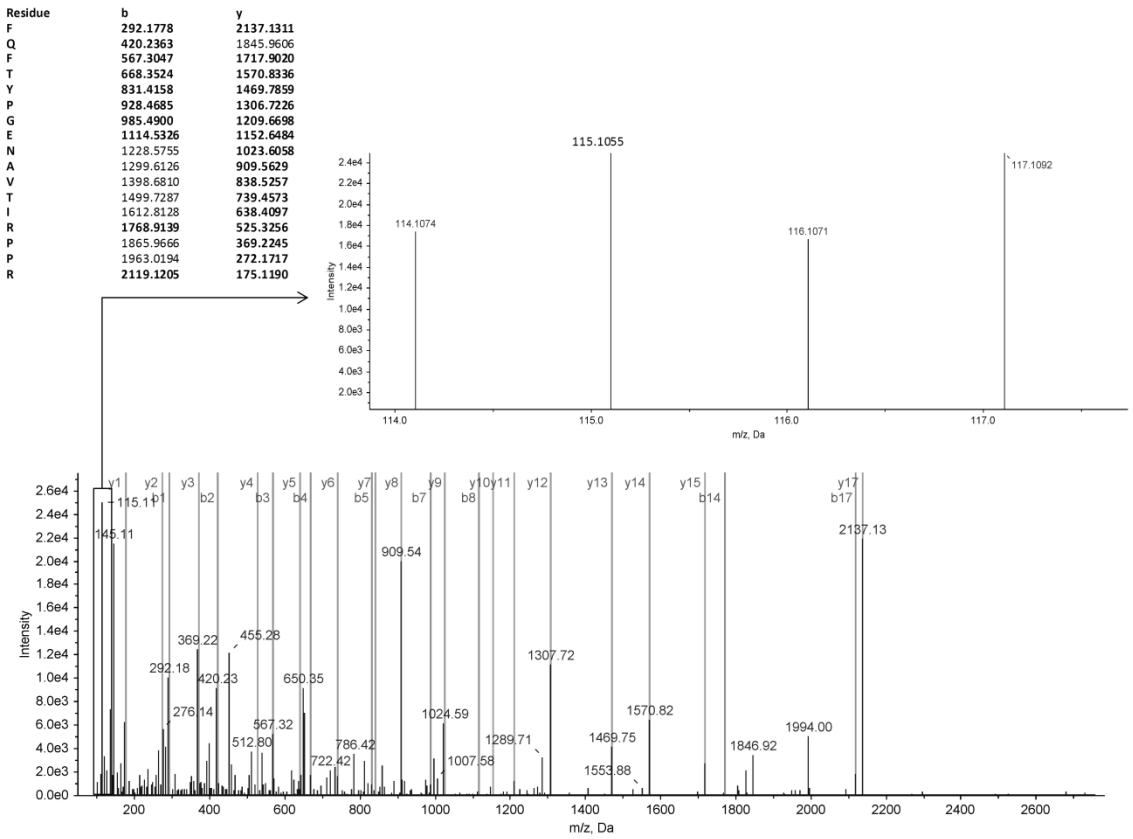

Figure 2: Mass spectra of iTRAQ reporter ion m/z peak intensities used for peptide quantitation and of m/z peaks used for peptide sequence identification.

Upon collision induced dissociation of gated peptides of a given precursor mass, reporter ions are released and their relative intensities used for quantitation. Representative MS/MS spectrum for a peptide identified and quantitated from an endoflagellar filament core protein (LIC11890) suggests up-regulation in response to growth in –Fe/FBS medium.

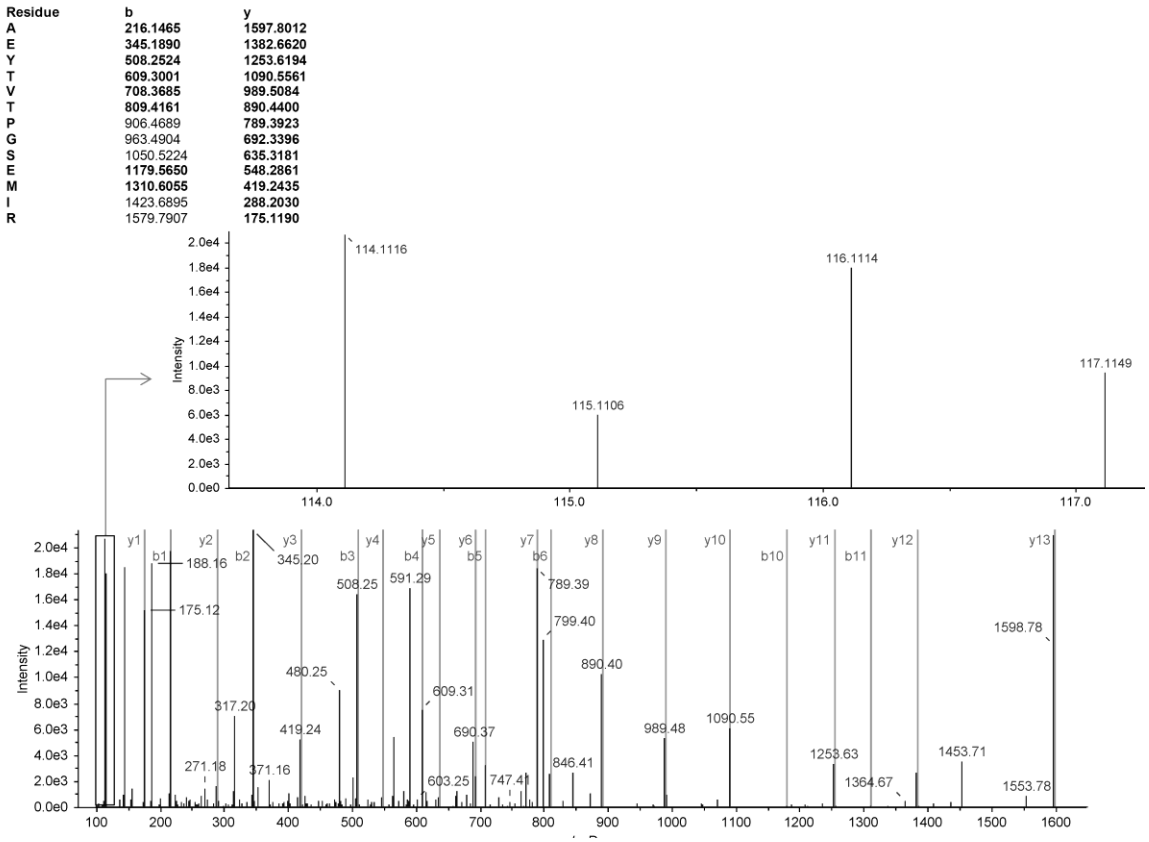

Figure 3: Mass spectra of iTRAQ reporter ion m/z peak intensities used for peptide quantitation and of m/z peaks used for peptide sequence identification.

Upon collision induced dissociation of gated peptides of a given precursor mass, reporter ions are released and their relative intensities used for quantitation. Representative MS/MS spectrum for a peptide identified and quantitated from a probable aconitate hydratase (LB327) suggests down-regulation in response to growth in –Fe/FBS medium.

respectively. The peaks of iTRAQ signature ions (114 and 116 for proteins isolated from *L. interrogans* grown in unmodified culture media and 115 and 117 for proteins isolated from *L. interrogans* grown in –Fe/FBS culture media) are shown as inserts. To reduce the possibility of both false positive protein identification and variation in protein quantitation, biological, technical and experimental variations were controlled for by designing and analyzing iTRAQ experiments as outlined in Figure 1 (Aggarwal *et al.*, 2006; Chong *et al.*, 2006; Gan *et al.*, 2007; Ross *et al.*, 2004; Wiese *et al.*, 2007). Briefly, biological variation was controlled for by extracting protein from pooled independent culture conditions while technical variation in our sample processing was controlled for by conducting analyses on two independent pooled samples for each culture condition. Variability within the same iTRAQ experiment and between separate iTRAQ experiments was controlled for by conducting analyses on samples A1 and B1 twice in iTRAQ1 and once in iTRAQ2. Statistical analyses were subsequently performed to determine proteins that were altered in expression levels under –Fe/FBS culture conditions within a 95% confidence interval. This method of analysis allowed for determination of statistically significant altered expression levels from "control" conditions, regardless of the observed fold change. Collectively this strategy for experimental design and statistical analysis allowed for stringent and sensitive determination of altered protein expression levels in response to the *in vivo*-like environmental conditions. To further increase confidence in assigning protein expression levels we also performed gel-based proteomics via 2DGE combined with MALDI-TOF mass spectrometry.

Together iTRAQ and 2DGE analyses identified 563 *L. interrogans* proteins (see Tables 2, 3, S1, S2, S3 and Figures S1, S2, S3, supplementary tables and figures can be accessed via web link in appendix A). This corresponds to approximately 15% of the total protein-expressing ORFs of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (563/3728 total) (Nascimento *et al.*, 2004) and, to our knowledge, represents the highest global proteomic coverage achieved to date in *Leptospira*. Of the identified proteins 65 demonstrated statistically significant alterations in expression levels between the two experimental conditions tested (see Tables 2 and 3). The highly sensitive iTRAQ analyses identified 62 proteins that were altered in expression (Table 3) while the 2DGE analyses identified a narrower range of 6 proteins altered in expression levels (Table 2 and Figure S3), a finding consistent with the reduced experimental sensitivity associated with 2DGE technology. The average fold change in expression levels as determined by iTRAQ analyses ranged from -5.863 to 2.731 (Table 3), which compares with other investigations focused on comparative global proteome analyses (Cong *et al.*, 2006; Glen *et al.*, 2008; Liu *et al.*, 2007).

2.4.3 Assignment of functional categories

To evaluate the global protein expression changes in *L. interrogans* in response to a shift to –Fe/FBS medium, proteins altered in expression were assigned to 9 distinct categories based upon their database-assigned functionality (Tables 2 and 3). *Leptospira interrogans* shifted to –Fe/FBS medium altered the expression of proteins with predicted functional roles corresponding to energy production and metabolism (18), protein synthesis (17), unknown function (9), confirmed/potential virulence factors (6), chaperones and post-translational regulation (5), transcription (4), chemotaxis and

Table 2: Proteins exhibiting altered expression levels in *L. interrogans* in response to *in vivo*-like conditions (exposure to –Fe/FBS) as determined by 2DGE.

Accession#	Protein	ORF	Protein expression
Energy Production and Metabolism (1)			
Q72VD7	Electron transfer flavoprotein beta-subunit EtfB	LIC10361	Downregulated
Confirmed/Potential Virulence Factors (2)			
Q72MY9	Putative glycoside hydrolase	LIC13050	Upregulated
Q72MM7	Putative coagulase	LIC13166	Upregulated
Chaperones and Post-translational Regulation (2)			
CH10_ LEPIC	GroES	LLIC11336	Downregulated
Q72QA2	IbpA-1	LIC12210	Downregulated
Chemotaxis and Motility (1)			
Q72R58	Periplasmic flagellin FlaB1	LIC11890	Upregulated

Table 3: Proteins exhibiting altered expression levels in *L. interrogans* in response to *in vivo*-like conditions (exposure to –Fe/FBS) as determined by iTRAQ.

		iTRAQ 1			**iTRAQ 2**			
Protein	**ORF**	**C***	**E***	**FC****	**C***	**E***	**FC****	**OFC**
Energy Production and Metabolism (18)								
Bifunctional dihydrolipoyllysine-residue acetyltransferase/dihydrolipoyllysine-residue succinyltransferase	LIC1_SPN3184	NS	NS	NS	0.905 - 1.239	1.507 - 1.74	1.384	1.384
Cytochrome C oxidase subunit II	LIC10208	0.971 - 1.123	1.221 - 1.395	1.214	NS	NS	NS	1.214
Transketolase, C-terminal subunit	LIC11355	NS	NS	NS	0.872 - 1.275	1.369 - 1.562	1.209	1.209
Cytochrome C oxidase, subunit I	LIC10209	0.996 - 1.064	1.185 - 1.29	1.196	NS	NS	NS	1.196
Nucleoside-diphosphate kinase	LIC13326	0.977 - 1.042	0.863 - 0.945	-1.072	NS	NS	NS	-1.072
Acyl carrier protein	LIC20065	0.922 - 1.39	0.629 - 0.911	-1.076	NS	NS	NS	-1.076
Methionine adenosyl transferase	LIC11354	1.106 - 1.107	0.708 - 0.942	-1.107	NS	NS	NS	-1.107
Ketol-acid reductoisomerase	LIC13393	0.718 - 1.557	0.6 - 0.709	-1.041	0.942 - 1.236	0.649 - 0.804	-1.209	-1.125
Sugar pyridoxal-phosphate-dependent aminotransferase	LIC12198	1.011 - 1.155	0.707 - 0.859	-1.187	NS	NS	NS	-1.187
Thiosulfate sulfurtransferase	LIC11115	0.712 - 1.524	0.52 - 0.595	-1.229	0.923 - 1.332	0.553 - 0.758	-1.286	-1.257
Succinyl-CoA synthetase, alpha subunit	LIC12574	1.027 - 1.095	0.685 - 0.805	-1.270	NS	NS	NS	-1.270
Acyl dehydratase	LIC12739	NS	NS	NS	0.762 - 1.936	0.362 - 0.634	-1.310	-1.310
NADH dehydrogenase (ubiquinone), E chain	LIC12745	0.838 - 1.36	0.413 - 0.759	-1.402	NS	NS	NS	-1.402

Isopropylmalate Isomerase	LIC11821	NS	NS	NS	0.606 - 1.823	0.376 - 0.43	-1.434	-1.434
Nitrogen regulatory protein PII	LIC10440	0.848 - 1.279	0.501 - 0.568	-1.526	NS	NS	NS	-1.526
Electron transfer flavoprotein, alpha subunit	LIC10360	0.949 - 1.093	0.47 - 0.502	-1.918	0.956 - 1.245	0.541 - 0.695	-1.429	-1.674
Electron transfer flavoprotein, beta subunit	LIC10361	1.015 - 1.065	0.446 - 0.497	-2.043	NS	NS	NS	-2.043
Aconitate hydratase	LIC20249	0.98 - 1.043	0.415 - 0.423	-2.314	0.85 - 1.317	0.376 - 0.455	-1.942	-2.128
Protein Synthesis (17)								
50S Ribosomal protein L7/L12	LIC10752	0.81 - 1.355	0.69 - 0.778	-1.055	0.932 - 1.223	0.733 - 0.899	-1.075	-1.065
50S Ribosomal protein L4	LIC12872	0.941 - 1.15	0.751 - 0.84	-1.139	NS	NS	NS	-1.139
50S Ribosomal protein L2	LIC12870	0.962 - 1.149	0.751 - 0.858	-1.140	NS	NS	NS	-1.140
50S Ribosomal protein L3	LIC12873	NS	NS	NS	0.883 - 1.446	0.561 - 0.821	-1.151	-1.151
Ribosomal protein S12	LIC10755	NS	NS	NS	0.913 - 1.169	0.702 - 0.802	-1.178	-1.178
50S Ribosomal protein L15	LIC12854	NS	NS	NS	0.87 - 1.573	0.474 - 0.731	-1.263	-1.263
30S Ribosomal Protein S13	LIC12849	0.943 - 1.098	0.706 - 0.73	-1.291	1.077 - 1.317	0.536 - 0.858	-1.238	-1.265
Protein-synthesizing GTPase complex, EF-Tu component	LIC12875	0.952 - 1.08	0.717 - 0.746	-1.284	NS	NS	NS	-1.284
50S Ribosomal protein L10	LIC10751	0.834 - 1.423	0.499 - 0.635	-1.360	0.911 - 1.48	0.519 - 0.787	-1.228	-1.294
30S Ribosomal protein S17	LIC12864	1.009 - 1.027	0.713 - 0.755	-1.339	NS	NS	NS	-1.339
30S Ribosomal protein S1	LIC12447	NS	NS	NS	0.68 - 1.91	0.362 - 0.536	-1.355	-1.355
50S Ribosomal protein L16	LIC12866	0.836 - 1.246	0.539 - 0.57	-1.478	0.953 - 1.12	0.698 - 0.765	-1.262	-1.370
50S Ribosomal protein L11	LIC10749	0.776 - 1.387	0.5 - 0.568	-1.404	NS	NS	NS	-1.404
50S Ribosomal protein L22	LIC12868	0.833 - 1.243	0.552 - 0.595	-1.428	NS	NS	NS	-1.428
30S Ribosomal protein S3	LIC12867	1.011 - 1.034	0.667 - 0.699	-1.432	NS	NS	NS	-1.432
50S Ribosomal protein L1	LIC10750	1.024 - 1.039	0.65 - 0.7	-1.437	NS	NS	NS	-1.437
50S Ribosomal protein L29	LIC12865	NS	NS	NS	1.093 - 1.404	0.296 - 0.508	-2.082	-2.082

Unknown Function (9)								
Protein of unknown function	LIC10314	NS	NS	NS	1.035 - 1.039	1.116 - 1.341	1.180	1.180
Protein of unknown function	LIC13123	0.975 - 1.066	1.198 - 1.247	1.169	NS	NS	NS	1.169
Protein of unknown function	LIC10672	NS	NS	NS	0.858 - 1.323	1.341 - 1.557	1.164	1.164
Protein of unknown function	LIC10027	0.895 - 1.138	1.297 - 1.315	1.158	NS	NS	NS	1.158
Protein of unknown function	LIC13314	NS	NS	NS	1.044 - 1.063	1.077 - 1.265	1.108	1.108
Protein of unknown function	LA0268	NS	NS	NS	0.796 - 1.329	0.678 - 0.767	-1.073	-1.073
Protein of unknown function	LIC11784	NS	NS	NS	0.803 - 1.483	0.405 - 0.542	-1.571	-1.571
Protein of unknown function	LIC13183	NS	NS	NS	1.085 - 1.317	0.308 - 0.461	-2.220	-2.220
Conserved protein of unknown function	LIC11059	NS	NS	NS	1.205 - 1.304	0.099 - 0.187	-5.863	-5.863
Confirmed/ Potential Virulence Factors (5)								
Putative glycoside hydrolase	LIC13050	NS	NS	NS	0.538 - 2.069	5.271 - 5.441	2.731	2.731
Putative Erp Y-like lipoprotein	LIC11966	NS	NS	NS	0.889 - 1.176	1.975 - 2.063	1.755	1.755
OmpA-family lipoprotein (Loa22)	LIC10191	0.908 - 1.141	1.267 - 1.317	1.152	NS	NS	NS	1.152
Catalase	LIC12032	0.956 - 1.114	1.197 - 1.29	1.151	NS	NS	NS	1.151
TolC-like protein	LIC12575	0.8 - 1.325	1.378 - 1.512	1.122	NS	NS	NS	1.122
Chaperones and Postranslational Regulation (3)								
Sensor histidine kinase and response regulator of a two component complex	LIC11709	NS	NS	NS	0.786 - 1.366	0.471 - 0.688	-1.333	-1.333
Endopeptidase Clp, ATP-dependent proteolytic subunit	LIC12017	0.905 - 1.136	0.379 - 0.393	-2.313	NS	NS	NS	-2.313
Preprotein translocase, SecA subunit	LIC11944	0.931 - 1.127	0.328 - 0.408	-2.485	NS	NS	NS	-2.485
Transcription (4)								

Transcription termination factor Rho	LIC12636	NS	NS	NS	0.845 - 1.254	0.757 - 0.809	-1.049	-1.049
Transcription elongation factor	LIC12706	NS	NS	NS	0.906 - 1.157	0.498 - 0.591	-1.631	-1.631
Two-Component Response Regulator Transcriptional Regulator Protein	LIC13088	0.942 - 1.119	0.49 - 0.572	-1.733	NS	NS	NS	-1.733
Response Regulator	LIC20254	1.055 - 1.061	0.478 - 0.561	-1.825	NS	NS	NS	-1.825
Chemotaxis and Motility (4)								
Methyl-accepting chemotaxis protein	LIC11523	0.858 - 1.343	1.593 - 1.952	1.409	NS	NS	NS	1.409
Endoflagellar filament core protein	LIC11890	0.996 - 1.029	1.293 - 1.356	1.303	NS	NS	NS	1.303
Methyl-accepting chemotaxis protein	LIC12921	0.954 - 1.115	1.162 - 1.242	1.111	1 - 1.018	1.064 - 1.181	1.111	1.111
Response regulator receiver domain	LIC11526	0.724 - 1.557	0.51 - 0.659	-1.176	NS	NS	NS	-1.176
Cell Shape (1)								
Actin-like ATPase involved in cell morphogenesis	LIC11258	0.938 - 1.078	0.512 - 0.532	-1.787	1.049 - 1.137	0.547 - 0.712	-1.467	-1.627
DNA Regulation (1)								
RecA recombinase	LIC11745	NS	NS	NS	0.725 - 1.51	0.592 - 0.683	-1.090	-1.090

C: Control

E: Experimental

FC: Fold change

OFC: Overall fold change

NS: Not significant. Protein quantitation was deemed not significant if any of the following conditions were found: proteins represented by less than two peptides; proteins displaying a *p* value greater than 0.05; or proteins having an error factor of greater than two or overlapping 95% confidence values for their respective average ratios.

* 95% confidence interval for a given protein ratio

** Value was obtained by averaging absolute high and low control and experimental protein ratios in log space. Values were then transformed into linear space and experimental values divided by control values.

motility (4), cell shape (1) and DNA repair (1). The broad changes in observed protein expression levels are consistent with a previous genome-wide transcriptional analysis performed on *L. interrogans* serovar Lai strain Lai in response to a temperature shift, which revealed altered expression of 106 genes (Qin *et al.*, 2006). However, whereas overall upregulation was observed in transcript levels of chaperones and protein synthesis machinery in response to a temperature shift, in our studies we observed a general trend towards downregulation of proteins belonging to the same functional categories in response to a shift to –Fe/FBS medium.

The following sections describe in detail the proteins identified via our proteomic analyses to show altered expression levels in response to the *in vivo*-like growth conditions compared to control *in vitro* conditions. Proteins are discussed in the context of leptospiral pathogenesis within the selected functional categories of energy production and metabolism, chaperones and protein synthesis, motility and chemotaxis, and confirmed/potential virulence factors.

2.4.4 Energy production and metabolism

In our studies numerous proteins mediating key steps of oxidative phosphorylation, amino acid biosynthesis and nucleotide metabolism were downregulated in response to a shift to –Fe/FBS media (Figure S4). Downregulation of proteins involved in energy production and metabolism was in accordance with the downregulation of 17 proteins involved in protein synthesis. Experimentally, *L. interrogans* shifted to –Fe/FBS medium displayed slower growth rates and generation times. It follows that the observed downregulation of energy production and metabolic

processes correlates well with the slower generation time in –Fe/FBS medium. As iron is an essential cofactor for many enzymes that are in turn required for cell viability (Louvel *et al.*, 2006), bacteria have evolved various mechanisms for sensing changes in iron concentration within their environment and for iron acquisition via siderophore expression. Hence, a rationale for the reduced energetic and metabolic processes may have been the inability to acquire necessary iron for optimal functioning of these processes, thereby resulting in downregulation of the proteins involved in these pathways when the *L. interrogans* were shifted to the –Fe/FBS medium.

2.4.5 Chaperones and protein synthesis

The stress response pathway includes the alteration of expression of chaperones, a group of proteins with broad molecular functions ranging from protein repair and regulation to protein export (for a review see(Cheng Vollmer & Van Dyk, 2004)). In our studies, shifting *L. interrogans* to –Fe/FBS media resulted in downregulation of 3 chaperones, namely small heat shock protein IbpA-1, GroES and ClpA-1 (Tables 2 and 3). Downregulation of chaperone expression fits with the overall trend towards decreased protein synthesis as there would be fewer proteins requiring repair, regulation and export to non-cytoplasmic locations.

As previously stated, we also observed downregulation of 17 proteins involved in protein synthesis in –Fe/FBS medium (Table 3). This number is likely an underestimation of the actual number of downregulated proteins mediating protein synthesis, due to technical limitations associated with conducting proteome analyses on a complex sample. In *L. interrogans,* genes encoding ribosomal proteins appear to be organized in operons of varying sizes, and in select regions of the genome these operons are tandemly

organized. As expression generally occurs at the operon level rather than the individual gene level, detection of downregulation of individual protein products in our studies suggests downregulation of the operon as a whole. The downregulated proteins we detected comprised 16 ribosomal proteins and the GTPase complex of the EF-Tu component TufB, which facilitates aminoacyl-tRNA binding to the A-site of ribosomes during translation. As translational machinery was downregulated in –Fe/FBS medium it is likely that downregulation of proteins involved in energy production, metabolism, export and chaperones was occurring, at least partially, at the translational level. Combined, these results correlate well with the slower growth rate observed in the –Fe/FBS medium.

2.4.6 Flagellar and chemotaxis proteins

An important step in the infection process is the use of chemotactile and flagellar proteins for traversal of host barriers resulting in dissemination to deeper tissues (Dons *et al.*, 2004; Williams *et al.*, 2007). Directional changes and swimming behaviour of motile bacteria is a complex process involving the regulation of an extensive number of proteins at both the translational and post-translational levels in response to altered environmental conditions (for a review see (Charon & Goldstein, 2002)). Traversal of host barriers is not a random event, as previously shown by motile *Treponema denticola* mutants that do not express the chemotaxis protein histidine kinase CheA and/or the chemoreceptors DmcA or DmcB. These mutants show only 2-30% penetration rates of epithelial cell layers when compared to the wild type (Lux *et al.*, 2001). It has also previously been observed that the swimming behaviour of motile pathogens is a specific result of differential concentration gradients of attractants and repellents. In particular virulent, but not avirulent, *Leptospira*

demonstrate strong chemo-attractant behaviour towards haemoglobin, as shown by movement of virulent *Leptospira* towards haemoglobin but not distilled water in a chemotaxis assay utilizing semisolid medium-filled U-shaped polypropylene tubes (Yuri *et al.*, 1993).

In contrast to the overall trend towards downregulation observed for the majority of the aforementioned leptospiral proteins, proteins involved in motility were generally observed to be upregulated in *L. interrogans* shifted to –Fe/FBS medium (Table 3). Directed motility and chemotaxis are central to spirochete virulence (Lux *et al.*, 2000), and thus the observed upregulation of flagellar proteins in –Fe/FBS medium parallels the experimental conditions that mimic an *in vivo* environment. The sole chemotaxis protein that was downregulated corresponded to the two-component response regulator CheY which is involved in facilitating directional changes in motile bacteria in response to chemotactile stimuli (Motaleb *et al.*, 2005; Park *et al.*, 2004).

2.4.7 Confirmed/potential virulence factors

Through our comparative proteomic experimental approach we observed upregulation of 6 proteins that fit within the confirmed/potential virulence factor category (Tables 2 and 3). One of these proteins (Loa22) has been previously identified in *L. interrogans* as a virulence factor. The remaining 5 upregulated proteins within this category share significant sequence similarity with proteins shown to play key roles in the pathogenesis of other organisms, including coagulase, catalase, TolC, glycoside hydrolase and the outer surface lipoprotein ErpY. The following sections describe each of these upregulated leptospiral proteins within the functional context of pathogenesis of either *L. interrogans* for previously characterized proteins or other pathogens for

previously uncharacterized proteins. In addition, the results of experiments conducted to independently confirm upregulation of protein expression and verify protein expression during the course of infection are included. Discussion of the putative ErpY-like lipoprotein is excluded as the immunoblot analysis did not confirm upregulation of this protein under the tested *in vivo*-like environmental conditions (see below).

Loa22

Previous gel-based analyses investigating the outer membrane proteome of *L. interrogans* serovar Copenhageni strain RJ16441 during infection of Hartley male guinea pigs revealed that the *L. interrogans* surface lipoprotein Loa22 is expressed during infection (Koizumi & Watanabe, 2003). Furthermore, *Himar1* transposon mutagenesis of the gene encoding Loa22 rendered *L. interrogans* serovar Lai strain Lai avirulent in Hartley male guinea pig and Golden Syrian male hamster models of leptospirosis and Loa22 genetic complementation in these mutants restored virulence (Ristow *et al.*, 2007). Combined, these studies definitively show that Loa22 is essential for *L. interrogans* virulence. In our studies, we detected a 1.152-fold upregulation of Loa22 upon a shift to –Fe/FBS media (Table 3). To our knowledge, this is the first documented report of regulation of Loa22 expression in response to defined stimuli that mimic infection, namely iron limitation and the presence of serum.

Putative coagulase

A noteworthy observation arising from the 2DGE analyses was the upregulation of a protein in response to the –Fe/FBS medium that shared amino acid sequence similarity with a region of the *Staphylococcus aureus* surface protein coagulase (Table 2; 53% similarity/26% identity over 128 amino acids). In *S. aureus*, this protein has been

shown to contribute to bacterial pathogenesis in two ways. First, it plays a pivotal role in the development of blood-borne infection through binding of the blood factor prothrombin to the bacterial surface, which upon activation to thrombin results in subsequent conversion of fibrinogen to fibrin. This proteolytic cascade results in the formation of fibrin clots on the bacterial surface (Wolz *et al.*, 1996), thus causing the bacteria to become refractory to neutrophil phagocytosis (Aarestrup *et al.*, 1994) and promoting evasion of the host immune response. Second, in *S. aureus* the coagulase protein further contributes to pathogenesis by aiding invasion of deeper tissues via degradation of the host fibrinogen barriers to fibrin. In support of this proposed functional role, expression of the *S. aureus* coagulase has been associated with haematogenous pulmonary infection in mice (Sawai *et al.*, 1997).

Putative catalase

An additional obstacle that pathogenic bacteria must overcome to establish infection is host-induced oxidative stress and the release of reactive oxygen species. Catalase and peroxidase activity and H_2O_2 detoxification have previously been established in *Leptospira* (Corin *et al.*, 1978; Rao *et al.*, 1964). In our studies we have detected 1.151-fold upregulation of a catalase (KatE) in response to growth of *L. interrogans* in –Fe/FBS media (Table 3). In *L. interrogans* the gene encoding this protein resides in a putative two-component operon with a gene encoding the ankyrin-like protein AnkB, a genetic organization that is mirrored in the pathogen *Pseudomonas aeruginosa*. In *P. aeruginosa* KatB and AnkB, which localize to the periplasmic space and inner membrane, respectively, physically interact and form an antioxidant scaffolding that

functions as a protective lattice network for optimal H_2O_2 degradation and protection from reactive oxygen species, thus promoting bacterial survival.

TolC-like outer membrane protein

During the infection process *Leptospira* specifically target the kidneys and liver and can attain bacterial loads as high as 10^9 bacteria per gram of guinea pig liver (Faine *et al.*, 1999). To ensure bacterial survival *Leptospira* must possess efficient efflux systems for removal of potentially toxic compounds. A canonical example of an efflux system is the *Escherichia coli* AcrAB-TolC system belonging to the resistance-nodulation-cell division superfamily of efflux systems (Fralick, 1996; Saier *et al.*, 1994). The TolC protein of this complex is an OM porin that many Gram-negative transport systems require for a functional efflux system (Paulsen *et al.*, 1997). Studies utilizing TolC mutants have revealed increased sensitivity to bile salts in *Salmonella enteric* (Ramos-Morales *et al.*, 2003) and *Vibrio cholera* (Bina & Mekalanos, 2001). The critical importance of TolC in spirochete pathogenesis has recently been established in *B. burgdorferi* where a TolC$^-$ mutant was unable to establish infection in mice (Bunikis *et al.*, 2008). In the present study a TolC-like protein was observed to be upregulated 1.122-fold in *L. interrogans* grown in –Fe/FBS medium (Table 3). Interestingly, previous investigations conducted on the outer membrane proteome of *Leptospira* have failed to detect this protein (Cullen *et al.*, 2005; Monahan *et al.*, 2008; Nally *et al.*, 2005b; Nally *et al.*, 2007).

Putative glycoside hydrolase

To reach their target organs *Leptospira* must be capable of traversal and invasion of host tissue barriers. Indeed, *L. interrogans* have been shown to efficiently traverse

epithelial cell barriers *in vitro* using a transwell assay procedure (Barocchi *et al.*, 2002). One of the preliminary steps in traversal of host barriers is the degradation of mucin barriers. In the mammalian reovirus pathogen the σ1 coat protein has been shown to possess a glycoside hydrolase activity that functions to degrade mucin, thus facilitating infection of Madin Darby canine kidney (MDCK) cells (Bisaillon *et al.*, 1999). The requirement for glycoside hydrolases in establishing infection has also been shown in *Streptococcus pneumoniae* where a SP2159 (coding for a family 98 glycoside hydrolase (Higgins *et al.*, 2009)) mutant displayed attenuated virulence in a murine model of infection (Hava & Camilli, 2002). In our studies we have detected 2.731-fold upregulation of a putative glycoside hydrolase predicted to contain a bacterial fibronectin type III (Fn3) domain (38% similarity/20% identity over 320 amino acids) in response to our *in vivo*-like conditions (Table 3). In bacteria, extracellular glycoside hydrolases have been observed to contain fibronectin type III domains (Gilkes *et al.*, 1991). Indeed, in *L. interrogans* this protein has been shown to localize to the bacterial surface through a previous study examining the subset of outer membrane proteins (Cullen *et al.*, 2005).

2.4.8 Verification of protein expression during infection

To verify expression of the confirmed/potential virulence factors during infection two independent experimental approaches were utilized. In the first approach the antibody reactivity of serum samples collected from patients with leptospirosis was tested against the panel of proteins identified through the proteomic analyses (excluding Loa22 which has previously been well established to be expressed during infection) (Ristow *et al.*, 2007). For these studies recombinant versions of each of the virulence factors were

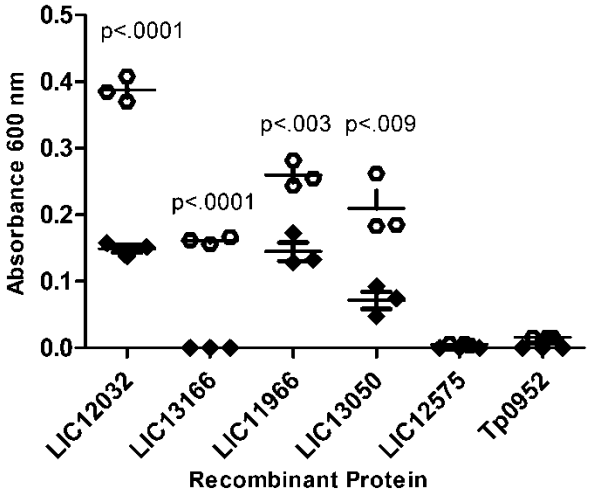

Figure 4: Reactivity of antibodies in human serum to recombinant *L. interrogans* proteins as determined by ELISA.

A 1:500 dilution of pooled serum samples collected from each of 15 individuals with laboratory-confirmed leptospirosis (open circles) and normal human serum (closed diamonds) were tested against the indicated recombinant *L. interrogans* proteins as well as a negative control recombinant *Treponema pallidum* protein (Tp0952) in triplicate. Reactivity was measured via absorbance at 600 nm and background subtracted values (no recombinant protein) were used in two-tailed *t* test calculations to determine statistical significance.

heterologously produced and subsequently analyzed via ELISA for specific reactivity with pooled serum from patients with leptospirosis (n=15). As shown in Figure 4, the pooled serum samples from patients with leptospirosis demonstrated significantly higher reactivity against 4 of the 5 recombinant *L. interrogans* proteins ($p<0.009$) compared to normal human serum, while no significant reactivity was observed against an irrelevant spirochete recombinant protein (Tp0952). The presence of antibodies against these leptospiral proteins in serum samples collected from natural leptospirosis infections is indicative of expression of the proteins during the course of infection. No reactivity was observed against the TolC-like protein (LIC12575), a finding which indicates either lack of expression *in vivo* or lack of reactivity against the portion of the recombinant protein that was produced (amino acids 315 to 506 out of 525 total). Antibody reactivity against the putative catalase (LIC12032), the ErpY-like lipoprotein (LIC11966) and the putative coagulase (LIC13166) was also detected in line blot experiments using sera from rabbits infected with virulent *L. interrogans* serovar Pomona (data not shown), suggesting *in vivo* expression of these proteins during infection in rabbits.

The second approach utilized immunofluorescence microscopy performed on liver sections prepared from *L. interrogans* serovar Pomona-infected hamsters. Fixed tissue sections were probed with primary rabbit polyclonal antibody against the panel of proteins identified (with the exception of Loa22, as the ORF coding for this protein has not been found in Pomona) and LipL21 (Figure 5 a) as a positive control. Preimmune rabbit serum was used as a negative control in these studies (Figure 5 c). Fluorescence reactivity indicated expression of the putative glycoside hydrolase (LIC13050) in liver sections of Pomona-infected hamsters (Figure 5 b). Lack of reactivity against the other

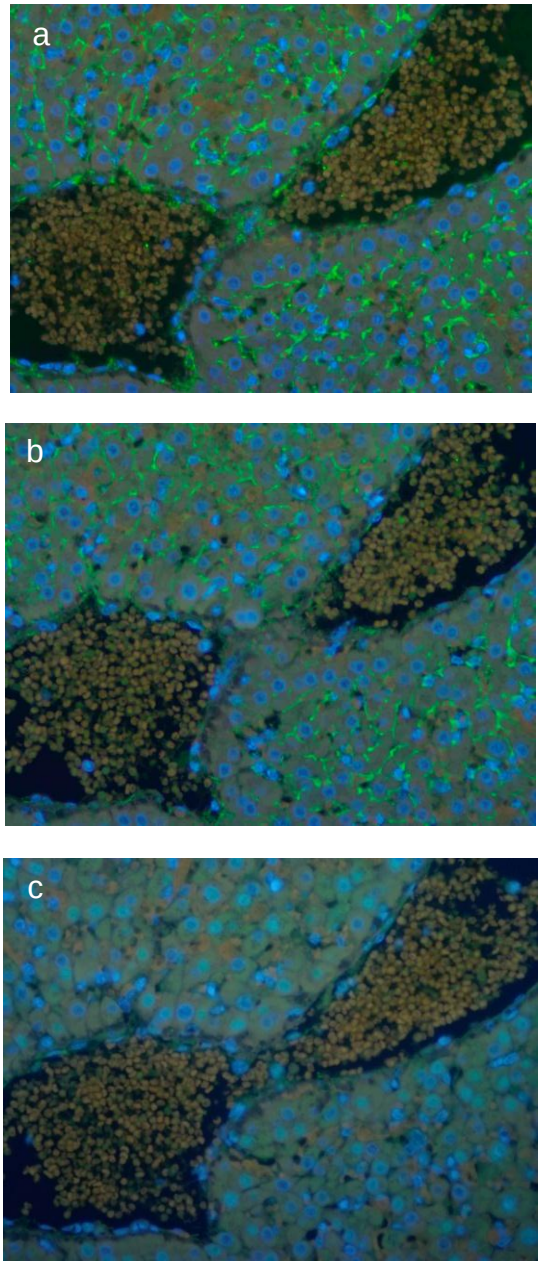

Figure 5. *In situ* expression of *L. interrogans* proteins in golden Syrian hamster liver tissue. Immunofluorescence microscopy was used to evaluate expression of selected *L. interrogans* proteins during infection. Shown are PEFF tissue sections prepared from hamsters infected with *L. interrogans* serovar Pomona and probed with primary polyclonal antisera raised against LipL21 (a) and LIC13050 (b). A negative control using preimmune rabbit serum (c) is shown. In the liver, *L. interrogans* are found predominantly in the intracellular junctions (a and b), consistent with previous findings. Fluorescence reactivity was detected for the positive control LipL21(a) and the putative glycoside hydrolase (LIC13050) (b), while no fluorescence was observed for the negative control (c) or the 4 other proteins tested (LIC12032, LIC13166, LIC11966 and LIC12575-data not shown).

four proteins may have been due to a lack of expression of these proteins in serovar Pomona.

2.4.9 Confirmation of upregulation of protein expression in *L. interrogans* grown in –Fe/FBS medium

To confirm our iTRAQ quantitation results we performed immunoblot analysis to determine the level of expression of each of the identified proteins within the virulence factor category in response to growth within –Fe/FBS medium (Table 4). In concordance with our iTRAQ results, immunoblot analyses verified upregulation of expression for 5 of the 6 proteins within the virulence factor category upon growth of *L. interrogans* in –Fe/FBS media. Specifically, after normalizing the results against FlaA1 expression, fold changes of 1.6, 1.4, 1.3, 1.0 and 1.0 were obtained for the TolC-like outer membrane protein, the putative glycoside hydrolase, Loa22, the putative catalase and the putative coagulase, respectively. Overall the increased expression levels observed by immunoblot analyses for these proteins were similar to the results obtained via the 2DGE and iTRAQ experiments (refer to Tables 2 and 3, respectively), although the fold changes obtained from each of the approaches differed. The only protein for which the immunoblot analyses did not confirm the upregulation observed in the iTRAQ analyses was the putative ErpY-like lipoprotein, which showed a 1.3-fold downregulation upon growth of *L. interrogans* in –Fe/FBS medium.

2.4.10 Conclusions

In this study we have identified leptospiral proteins with potential roles in the infection process by analyzing *L. interrogans* global proteome expression using an *in vitro* environment designed to mimic *in vivo* infection. Our study revealed that *L. interrogans* undergo a complex change in protein expression profiles in response to a

Table 4: Proteins exhibiting altered expression levels in *L. interrogans* in response to *in vivo*-like conditions (exposure to –Fe/FBS) as determined by immunoblot analyses.

Accession#	Protein	ORF	Control*	Experimental*	Fold Change
Q72PA0	TolC-like protein	LIC12575	9.9	15.6	1.6
Q72MY9	Putative glycoside hydrolase	LIC13050	3.1	4.2	1.4
Q72VV5	OmpA-family lipoprotein (Loa22)	LIC10191	18.3	23.2	1.3
Q72QS7	Catalase	LIC12032	6.3	6.4	1.0
Q72MM7	Putative coagulase	LIC13166	41.5	41.7	1.0
Q72QY9	Putative ErpY-like lipoprotein	LIC11966	12.3	9.4	-1.3

*Average values (n=2).

shift to the *in vivo*-like environment of –Fe/FBS medium. Specifically, proteins involved in the process of protein synthesis were downregulated, reflecting the general trend towards downregulation of proteins involved in energy production, metabolism and regulation. A shift to –Fe/FBS media also caused upregulation of the majority of proteins involved in motility, an expected response since motility is required for infection in all pathogenic spirochetes. Lastly, our proteomic studies also revealed that, in response to iron limitation and the presence of serum factors, *L. interrogans* upregulate a number of known and potential virulence factors, as determined by iTRAQ and 2DGE analyses. These include the previously identified outer membrane lipoprotein Loa22 and 5 novel potential virulence factors that exhibit sequence similarity to proteins shown to play key functional roles in other pathogens, including the outer surface lipoprotein ErpY, coagulase, catalase, TolC and glycoside hydrolase. Immunoblot analyses verified upregulation for 5 of these 6 proteins between the comparative conditions, and immunological experiments verified *in vivo* expression of all but one of the identified protein. Future characterization of these leptospiral proteins will significantly advance our understanding of this important pathogen, and future proteomic analyses can expand on this study by investigating the global *L. interrogans* proteome response to additional environmental conditions that are representative of the environment found within a host. Moreover, the described "infection proteomics" approach can be applied to study the molecular mechanisms of infection in other pathogens. The advantage of this approach is that it examines the organism at the phenotypic level, confirming expression of proteins previously designated as hypothetical and incorporating the myriad complexity that is encoded at both the post-transcriptional and post-translational levels

2.5.1 Acknowledgements

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Chapter 3
Leptospira interrogans catalase *katE* enhances resistance to extracellular H_2O_2 and is required for virulence

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Unpublished data

Figure 9 and 11 and Manilae insertion mutant strain generation and infection experiments performed by Dr. Kristal Lourdault in Dr. Mathieu Picardeau`s lab
Animal work involving strain Pomona performed by Drs. Amporn Srikram and Rasana W. Sermswan
Pomona insertion mutant generated by Drs. Gerald Murray and Ben Adler
Brendan Snarr optimised the catalase assay conditions
Dr. Richard L. Zuerner conducted work on this project but not included in this thesis

3.1.1 Abstract

Compared to saprophytic strains, pathogenic *Leptospira* are likely to encounter higher concentrations of reactive oxygen species induced by the host immune response. In support of this, previous studies have suggested a more robust oxidative stress resistance system exists within pathogenic *Leptospira*. In this study we demonstrate that catalase (KatE) expression enhances extracellular H_2O_2 resistance and show that it is required for virulence of *Leptospira interrogans* in guinea pigs and hamsters.

Comparison of survival rates of pathogenic *L. interrogans* with that of the saprophytic *L. biflexa,* during H_2O_2-induced oxidative stress, revealed the pathogens survival rate to be up to ~50 fold higher, and that the enhanced viability was predominantly catalase-dependent. Insertion mutants in the gene encoding catalase (*katE*) drastically diminished viability of pathogenic *Leptospira* to extracellular H_2O_2. In addition to characterizing the global leptospiral oxidative stress resistance response, we also initiated characterization of KatE, the only annotated catalase found within pathogenic *Leptospira* species, including defining the function and cellular location. Catalase assays performed with recombinant KatE confirmed specific catalase activity, and protein fractionation experiments localized KatE to the bacterial periplasmic space. Infection trials performed in guinea pigs and hamsters with *katE* insertion mutants showed this gene is required for leptospiral survival within a mammalian host.

3.2.1 Introduction

Gross examination of kidneys and lungs harvested from *Leptospira*-infected guinea pigs has revealed inflammation and haemorrhage (Ristow *et al.*, 2007). It is currently not known whether such tissue damage is caused by *Leptospira* activity or the host immune response to infection. However, many bacterial infections induce a host-mediated immune response that generates an oxidative burst resulting in tissue damage. Specifically, histological analysis of *Helicobacter pylori*-infected gastric epithelia has revealed increased levels of reactive oxygen species (ROS) that correlate with the level of tissue damage in the form of inflammation, erosion and haemorrhage (Davies *et al.*, 1994). In general, oxidative damage has been demonstrated to occur on proteins, lipids and DNA (Imlay & Linn, 1986; Nathan & Shiloh, 2000), and host DNA damage due to oxidative burst has been shown to contribute to inflammation (Evans *et al.*, 2004). The release of ROS in response to *Leptospira* has been previously assessed *in vitro* where isolated rat Kupffer cells have been demonstrated to release reactive oxygen species in the presence of *Leptospira* (Marangoni *et al.*, 2006). *In vivo*, it has been observed that cattle diagnosed with leptospirosis display elevated levels of serum oxidative stress biomarkers (Erdogan *et al.*, 2008). Together these studies suggest an oxidative burst that would serve to defend against *Leptospira* tissue colonization but, depending on the magnitude of the generated response, could also lead to the detrimental situation of generating significant tissue damage.

Reactive oxygen species are antimicrobial compounds that are generated from free radicals of molecular oxygen (superoxide) through several different mechanisms. Reactive oxygen species can be generated innately by hemoglobin (Jiang *et al.*, 2007;

Kawano *et al.*, 2002). Hemoglobin can produce superoxide (see below for a discussion of antimicrobial activity of ROS) from molecular oxygen when it is oxidised to its ferric form, methemoglobin, which possesses pseudoperoxidase activity (Alayash, 1999; Jiang *et al.*, 2007; Kawano *et al.*, 2002). As production of superoxide by methemoglobin can be toxic to the host the concentration of this protein is kept in check by erythrocytes through hemoglobin sequestration (Jiang *et al.*, 2007). As iron is an element that is required by all life forms, various bacterial pathogens have evolved virulence factors that cause cell lysis to obtain iron sequestered by haemoglobin. However, through this process bacterial pathogen associated molecular patterns, especially proteases, also activate the pseudoperoxidadic activity of methemoglobin, thus exposing the pathogen to toxic ROS (Jiang *et al.*, 2007). Immune cells such as neutrophils and macrophages (Nathan & Shiloh, 2000) can also produce ROS via the NADPH phagocyte oxidase pathway, which utilizes both membrane and cytosolic proteins to generate oxygen radicals from molecular oxygen (Babior *et al.*, 2002; Vignais, 2002). The oxygen radical can then be used to generate other ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals and hypochlorous acid.

The molecular mechanism(s) underlying killing of microorganisms via ROS by resident immune cells is a subject of debate (Fang, 2004). It has generally been accepted that the main target of ROS is bacterial DNA that when sufficiently damaged would result in loss of bacterial viability. However, recent evidence in *Salmonella typhimurium* demonstrates that the periplasmic superoxide dismutase (SodC) and not the cytoplasmic versions SodA and SodB are required for resistance to extracellular ROS, thus suggesting a target other than cytoplasmic components for ROS damage (Craig & Slauch, 2009).

Outer membrane and protein damage due to ROS has been demonstrated in *Borrelia* (Boylan *et al.*, 2008) and could represent one mechanism leading to loss of spirochete viability during the course of infection.

Microbial pathogens have evolved various mechanisms to counter the effects of host generated ROS including disruption of ROS delivery to the phagosome (Vazquez-Torres *et al.*, 2000), inhibition of ROS production (Cowley *et al.*, 1996), active DNA repair systems (Imlay & Linn, 1986; Suvarnapunya *et al.*, 2003) and enzymatic detoxification of ROS (Bishai *et al.*, 1994; Fang *et al.*, 1999; Wilson *et al.*, 1995). Microbial enzymes utilized for ROS detoxification include catalase (Chelikani *et al.*, 2004) and peroxidase (Rodriguez-Lopez *et al.*, 2001) which serve to degrade hydrogen peroxide, and superoxide dismutase which detoxifies superoxide anions (Tainer *et al.*, 1983). Catalases, also referred to as hydroperoxidases, are antioxidant enzymes (E.E. 1.11.1.6;H_2O_2:H_2O_2 oxidoreductase) (Tondo *et al.*) utilized for degradation of H_2O_2 into H_2O and O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$) (Chelikani *et al.*, 2004). Sequence and structural data have been used to classify catalases into three categories (Klotz *et al.*, 1997; Nicholls *et al.*, 2000). The class 1 catalases are heme-containing monofunctional enzymes (Klotz *et al.*, 1997), class 2 catalases are heme-containing bifunctional catalase-peroxidase enzymes and class 3 catalases include non-heme-containing enzymes (Nicholls *et al.*, 2000). In addition to these enzymes, several other heme-containing proteins such as chloroperoxidases and myoglobin also display low levels of catalase activity (Klotz *et al.*, 1997).

Various studies on pathogenic bacteria have revealed the importance of catalases for optimal detoxification of H_2O_2 (Elkins *et al.*, 1999; Howell *et al.*, 2000), survival in

macrophages (Das & Bishayi, 2009), resistance to phagocyte mediated killing (Srinivasa Rao *et al.*, 2003) and virulence (Bishai *et al.*, 1994; Steele *et al.*, 2010; Tondo *et al.*; Wilson *et al.*, 1995). Previous work on enzymatic detoxification of ROS in *Leptospira* has revealed that pathogenic *L. interrogans* displays catalase activity while the saprophytic *L. biflexa* displays predominantly peroxidadic activity (Corin *et al.*, 1978), despite the fact that bioinformatic analysis reveals a KatG homolog is present within *L. biflexa* (Xue *et al.*, 2010). *L. interrogans* can degrade H_2O_2 at concentrations that are 50 fold higher than those tolerated by *L. biflexa* (Corin & Cox, 1980), consistent with a greater susceptibility of *L. biflexa* to H_2O_2 mediated killing (Murgia *et al.*, 2002). This suggests *L. interrogans* may have evolved an extensive H_2O_2 detoxification system which is absent from *L. biflexa*.

Previously, we showed KatE was upregulated by *L. interrogans* serovar Copenhageni when exposed to "*in vivo*-like" conditions represented by bacterial growth in medium depleted of iron and containing serum (Eshghi *et al.*, 2009). This finding has been supported at the transcriptional level where *katE* transcript levels were shown to be increased in response to an increase in temperature and the presence of serum (Lo *et al.*, 2010; Xue *et al.*, 2010). These previous results together with the potential exposure of *Leptospira to* extracellular ROS during the infection process prompted further characterization of the leptospiral response to ROS. In this study we demonstrate that KatE is a periplasmic catalase that enhances resistance of *L. interrogans* to extracellular oxidative stress conditions and is required for virulence in guinea pigs and hamsters.

3.3.1 Methods

3.3.2 *Leptospira* strains and culturing conditions

Leptospira interrogans serovar Copenhageni strain Fiocruz L1-130 was originally isolated from a clinical sample in Salvador, Brazil (Ko *et al.*, 1999). *L. interrogans* serovar Pomona was obtained from the collection of the Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil, *L. interrogans* serovar Manilae was provided by Dr. N. Koizumi, National Institute of Infectious Diseases, Tokyo, Japan and *L. biflexa* serovar Patoc strain Patoc1 (designated Paris strain) was originally obtained from a fresh water stream (Babudieri, 1961) and is stored and maintained in the National Reference Center of *Leptospira* (Institute Pasteur, Paris, France). All strains were cultured aerobically in Ellinghausen and McCullough (Ellinghausen & McCullough, 1965) as modified by Johnson and Harris (Johnson & Harris, 1967) (EMJH) medium at 29.5 °C.

3.3.3 Recombinant protein expression and antiserum production

Expression of recombinant KatE (Eshghi *et al.*, 2009) and rabbit antiserum production against KatE (Eshghi *et al.*, 2009), LipL32 (Haake *et al.*, 2000) and FlaA1(Cullen *et al.*, 2005) have been described elsewhere.

3.3.4 Catalase assay methodology

Catalase activity was measured using an assay (Cayman Chemical Company, Ann Arbor, MI, USA) that measures the peroxidadic activity of catalase where methanol is used as an electron donor to decompose H_2O_2 into formaldehyde and water (Johansson & Borg, 1988; Wheeler *et al.*, 1990). All reagents, incubations and measurements were conducted at room temperature, and the assay was performed according to the manufacturer's instructions. Recombinant KatE was added at final concentrations ranging

from 0.23 ng/µl to 15 ng/µl. Catalase (KatE) inhibition experiments were conducted by adding 3-amino-1,2,4-triazol (ATZ) (USB Corporation, Cleveland, OH, USA) to the assay buffer (100 mM potassium phosphate, pH 7.0) at a final concentration of 100 mM, prior to conducting the catalase assay. The formaldehyde control assay was performed concurrently as described by the manufacturer's protocol. Catalase and formaldehyde standard assays were conducted using duplicate samples. Data are reported as the mean absorbance at 540 nm for one experiment. The experiment was performed a total of three times with comparable results.

3.3.5 Catalase activity assay

Leptospira wt and mutant strains were pelleted and exposed to 500 µl of 10M H_2O_2. Cultures were monitored qualitatively for effervescence.

3.3.6 Bacterial oxidative stress conditions and inhibition assays

Leptospira interrogans serovars Copenhageni, Manilae, Pomona, Manilae *katE* mutant (m69), Pomona *katE* mutant (P3) and *Leptospira biflexa* serovar Patoc1 were enumerated using a Petroff Hauser counting chamber (Fisher Scientific, Ottawa, ON, Canada) and an ECLIPSE 50i dark field microscope (Nikon, Mississauga, ON, Canada). Bacteria were grown to >4 x 10^8 bacteria/ml and diluted to 3 x 10^8 bacteria/ml in either EMJH media, EMJH media containing H_2O_2 at a final concentration of 0.1 mM, 1 mM, or 10 mM, or EMJH media containing a final concentration of 100 mM ATZ and either no H_2O_2 or a final concentration of 0.1 mM, 1 mM, or 10 mM H_2O_2. Samples were incubated for 1 h at 37 ºC. Bacterial oxidative stress assays were also conducted, as described above, without ATZ for 10 min (exposure for 30 and 60 min was also tested with similar results) at 37°C. A total of 5 x 10^9 L1-130 from each experimental group (no

H_2O_2 exposure, 0.1, 1 and 10 mM H_2O_2 exposure conditions) were collected by centrifugation at 2 000 x *g*, and the bacteria were flash frozen in liquid nitrogen and stored at -20 ºC for subsequent immunoblot analyses (described in the immunoblot analyses section).

3.3.7 Bacterial viability assays

Triplicate samples of *Leptospira* exposed to the above listed conditions were aliquoted in sterile FALCON 96 well flat bottom polystyrene microplates (Becton Dickinson, Franklin Lakes, NJ, USA) (250 µl/well). To each well 50 µl of an alamarBlue (Biosource, Camarillo, CA, USA) working solution, consisting of 0.4 ml alamarBlue stock solution plus 9.6 ml 0.1 M potassium phosphate buffer pH 7.4, were added and the microplate was incubated at 30 ºC in a humidified HYBAID chamber (ThermoFisher Scientific). Chromogenic shift was measured via fluorescence using a 530/25 nm filter for excitation and a 590/20 nm filter for emission in a Synergy HT microplate reader (BioTek) at 0, 2 and 4 hours post-incubation. As a control for contamination and variability within the alamar blue assay, solutions used in the oxidative stress assay were aliquoted as described above, without *Leptospira.* Percent survival was calculated at 2 and 4 h by first subtracting fluorescence measured at 0 h and then by subtracting any increased fluorescence observed in the control respective H_2O_2/ATZ media solutions. The resulting fluorescence values for *Leptospira* exposed to 0.1, 1 and 10 mM H_2O_2 were divided by fluorescence values obtained from *Leptospira* in EMJH alone. A similar calculation was applied to *Leptospira* exposed to the same conditions containing ATZ. Leptospiral viability was confirmed via dark field microscopy where motile bacteria were deemed as viable and non-motile bacteria as non-viable. Data are representative of a

single experiment with three replicates. Experiments were repeated at least two times with similar results.

3.3.8 Protein fractionation

Leptospira protein fractionation was performed as previously described (Haake *et al.*, 1991; Pinne & Haake, 2009; Zuerner *et al.*, 1991) except for the modification that fractionation was performed on 10^{11} cells at a concentration of 5 x 10^9 bacteria/ml.

3.3.9 Immunoblot analyses

Flash frozen *Leptospira* that had been exposed to oxidative stress conditions were resuspended in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) sample loading buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 20% glycerol) and boiled at 100 ºC with intermittent vortexing. Protein samples were subjected to SDS-PAGE using 15% polyacrylamide gels and transferred to Immobilon polyvinylidene fluoride (PVDF) transfer membranes (Millipore, Billerica, MA, USA) as previously described (Eshghi *et al.*, 2009) with the following modifications. Membranes were blocked overnight in 5% milk powder (w/v), 0.05% Tween 20 (v/v), in Tris-buffered saline (137 mM NaCl, 2.68 mM KCl, 24.8 mM Trizma base) (5% MP-TBS-T) followed by 2 x 5 min washes in 0.05% Tween 20 (v/v) in TBS (TBS-T). Membranes were then incubated with primary rabbit antiserum (anti-KatE, anti-FlaA1 or anti-LipL32) at a 1:2000 dilution in 5% MP -TBS-T for 2 h on a rotating shaker (Stovall) at room temperature, followed by 2 rinses with 50 ml TBS-T and 4 x 5 min washes with TBS-T with shaking at room temperature. Secondary anti-rabbit IgG (H&L) (Goat) IRDye 800 conjugate antibody (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) was added to the membranes at a dilution of 1:10,000 in 5%

MP-TBS-T and incubated for 1 h with shaking at room temperature, followed by 2 rinses with 50 ml TBS-T and 2 x 5 min washes with TBS-T. Image analyses were performed with an Odyssey infrared imager using Version 2.1 software (LI-COR Bioscience, Lincoln, NE, USA).

3.3.10 Construction of *Leptospira* insertion mutants

Random insertion mutagenesis was carried out in *L. interrogans* serovar Manilae strain L495 and serovar Pomona strain PO-06-047 with a kanamycin-resistant *Himar1* transposon as previously described (Murray *et al.*, 2009a). Among the kanamycin transformants, we identified a mutant (mutant 69) with an insertion into *katE* (LA1859) at position 1829815 in the large chromosome (nucleotide position based on the genome of *L. interrogans* serovar Lai strain 56601).

Genomic DNA was extracted from a pellet of 20-ml cultures by using the QIAamp DNA blood minikit (Qiagen, Inc., Valencia, CA). Confirmation of genotype was performed by using PCR with primers 69a (5'-GATACGGAAAGAGATCCGAG-3') and 69b (5'-ATGATCTGAACACAAAACTTC-3'), which are located in the flanking sequences of the insertion site of the transposon, and Southern blots of *Eco*RV-digested DNA probed for hybridization with the kanamycin-resistant cassette.

3.3.11 Animal Infections

L. interrogans strains were tested for virulence in the gerbil and hamster models of acute leptospirosis. Groups of four 28-day-old male gerbils (Charles River Laboratories, http://www.criver.com) were inoculated intraperitoneally with 10^8 leptospires. Groups of eight 28-day-old male hamsters (Janvier, http://www.janvier-europe.com) were inoculated intraperitoneally with 10^6 leptospires. Animals were

monitored daily for clinical signs of leptospirosis (i.e., prostration, jaundice, etc.) and survival for up to 21 days post infection. Protocols for animal experiments were prepared according to the guidelines of the Animal Care and Use Committees of the Institut Pasteur.

3.4.1 Results

3.4.2 Recombinant KatE displays catalase activity

To determine whether KatE (LIC12032) displays catalase activity, soluble recombinant KatE (rKatE) was expressed, purified and utilized in a catalase-specific assay (Figure 6). Peroxidases do not utilize methanol and formaldehyde to decompose H_2O_2 (Johansson & Borg, 1988), and therefore any observed activity can be specifically deemed to result from catalase activity. Absorbance increased proportionally to rKatE concentration, indicating production of formaldehyde from methanol and H_2O_2 (Figure 6a). Inclusion of 100 mM ATZ, a catalase inhibitor (Margoliash & Schejter, 1962), in the assay diminished the observed proportionality of absorbance to rKatE concentration (Figure 6a) but did not diminish the proportionality of absorbance due to formaldehyde concentration (Figure 6b).

3.4.3 KatE expression remains unchanged during H_2O_2-induced oxidative stress

To test whether KatE is expressed during exposure of *Leptospira* to H_2O_2-induced oxidative stress, bacteria were exposed to H_2O_2 concentrations ranging from 0 to 10 mM. Total cellular protein was separated by SDS-PAGE and subjected to immunoblot analysis using antisera specific for KatE and FlaA1 (flagellar filament outer layer protein A, used as a loading control). KatE- and FlaA1-specific antisera reacted with bands having molecular masses consistent with the 54.7 kDa and 34.9 kDa predicted molecular masses of the native proteins, respectively (Figure 7). Quantitative immunoblot analyses of KatE expression in response to varying H_2O_2 concentrations (normalized to FlaA1)

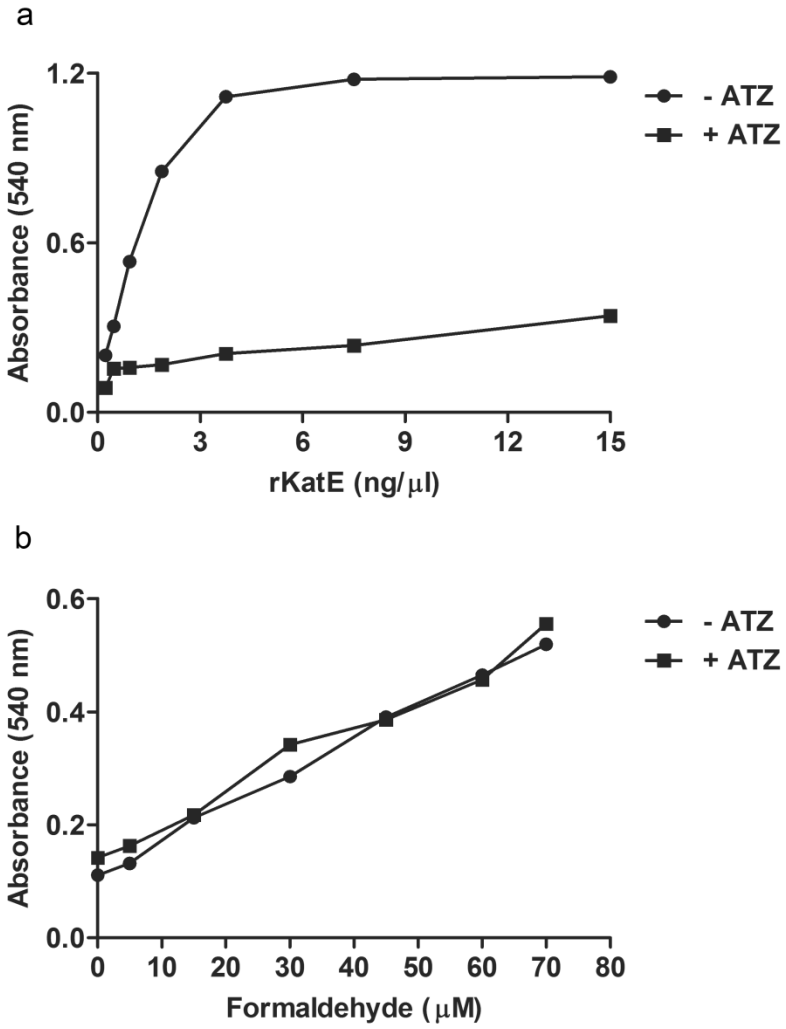

Figure 6: Recombinant KatE displays catalase activity.
Recombinant KatE (rKatE) was analyzed in a catalase assay in the presence or absence of the specific catalase inhibitor ATZ (100 mM). An increase in the formaldehyde concentration, measured at 540 nm, is indicative of catalase activity. a) Increased absorbance with increasing rKatE concentration in the absence of ATZ. No correlation was observed between absorbance and rKatE in the presence of ATZ. b) ATZ did not alter the reaction of formaldehyde standards with chromogen-producing compounds. Data are reported as the mean from duplicate absorbance values at 540 nm for a single experiment.

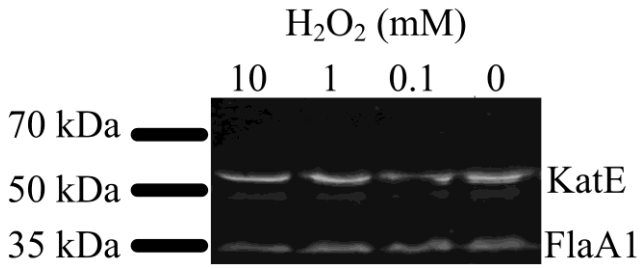

Figure 7: KatE expression remains unchanged during H_2O_2-induced oxidative stress.
Leptospira were exposed to 0, 0.1, 1 or 10 mM H_2O_2 and immunoblots performed on total protein. Higher bands represent KatE and lower bands represent FlaA1, the latter being used as a loading control. Eight replicate densitometric values were used for statistical analyses.

Table 5: Quantitative immunoblot analyses of KatE expression levels in response to H_2O_2 exposure.

	H_2O_2 concentration (mM)			
	0	0.1	1	10
Relative fluorescence*	20.50 ± 7.98	22.01 ± 7.35	22.80 ± 9.74	23.11 ± 5.38

* FlaA1 was used as a loading control to normalize KatE fluorescence values.

showed KatE expression levels were unchanged when cells were exposed to increasing H_2O_2 concentrations (Table 5).

3.4.4 KatE is localised to the periplasmic space

Total cellular *L. interrogans* protein was fractionated to yield a protoplasmic cylinder (PC) containing cytoplasmic and inner membrane proteins, an aqueous phase (AQ) containing periplasmic proteins and a detergent phase (DET) containing outer membrane proteins (Haake *et al.*, 1991; Pinne & Haake, 2009; Zuerner *et al.*, 1991). Protein fractions were separated via SDS-PAGE and subjected to immunoblot analysis. Antiserum to FlaA1 displayed reactivity with the PC fraction and minor reactivity with the DET fraction but not with the AQ fraction (Figure 8). KatE-specific antiserum displayed reactivity to a band migrating at 52-55 kDa in both the AQ and PC phases (Figure 8) but no band was observed in the DET phase. Antiserum to LipL32 displayed reactivity with a protein band of approximately 30 kDa in all phases.

3.4.5 *katE* mutant *Leptospira* do not display H_2O_2 hydrolysis activity

To determine the degree of catalase activity existing within the mutant m69 strain in comparison to the *wt* Manilae strain the bacteria were tested in a catalase activity assay. In this assay *wt* and m69 strains were aliquoted into separate test tubes and H_2O_2 added to a concentration of 10M. Catalases degrade H_2O_2 into H_2O and O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$) (Chelikani *et al.*, 2004) and the generation of O_2 can be qualitatively observed in broth culture as effervescence. In our experiments O_2 generation post H_2O_2 exposure was observed in the *wt* but not m69 broth culture (Figure 9).

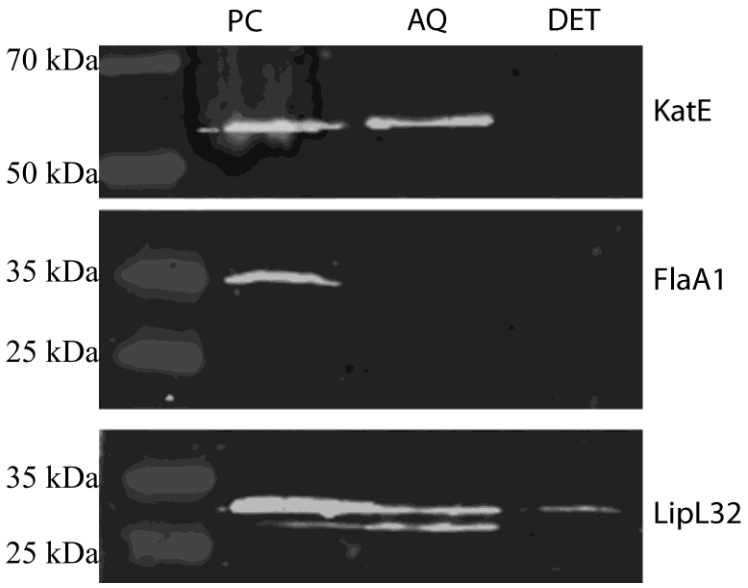

Figure 8: KatE localizes to the periplasmic space.
Immunoblot analyses were conducted on *L. interrogans*-fractionated protein samples. Antiserum to FlaA1 was used as a control to determine fraction purity, with reactivity being limited to the protoplasmic cylinder (PC) phase (cytoplasmic and inner membrane proteins) as previously demonstrated (Pinne & Haake, 2009) and minor reactivity in the detergent fraction. Antiserum to KatE showed reactivity in both PC and aqueous (AQ) (periplasmic proteins) phases, but not with proteins in the detergent (DET) (outer membrane protein) phase. Antiserum to LipL32 displayed reactivity with all fractions.

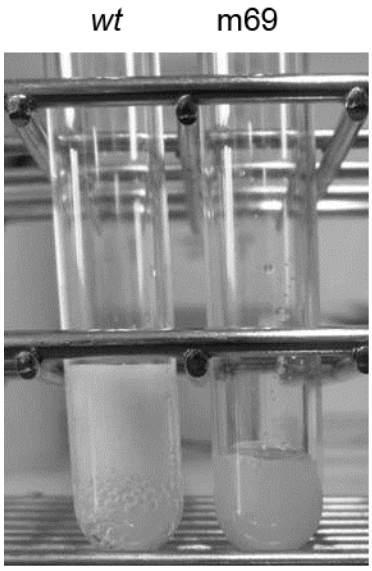

Figure 9: *katE* mutant *Leptospira* do not degrade H_2O_2.
Leptospira katE mutant m69 and *wt* Manila serovar in broth culture were exposed to H_2O_2. Effervescence was observed post H_2O_2 exposure in *wt* broth cultures but not in m69.

3.4.6 *katE* enhances *L. interrogans* viability under oxidative stress

Leptospira strains were exposed to various H_2O_2 concentrations (10, 1 and 0.1 mM) and tested for viability using an AlamarBlue survival assay, over a four hour time frame. In this assay viable bacteria are able to reduce the non-fluorescent compound resazurin to the fluorescent compound resorufin (Nakayama *et al.*, 1997), and this fluorescence can be measured spectrophotometrically and the resulting values used to determine percent survival (see section 3.3.7 for a description of calculations). Wild type *L. interrogans* strains reduced resazurin to resorufin after exposure to 0.1, 1 and 10 mM H_2O_2, displaying 85-100% survival at 0.1 and 1 mM H_2O_2 and 30-50% survival at 10 mM H_2O_2 (Figure 10a, c and e). *L. biflexa* displayed survival rates of ~40, 10 and 2% (Figure 10b) and mutant *interrogans* strains showed survival rates of ~10, 5 and 2% (Figure 10d and f) at 0.1, 1 and 10 mM H_2O_2, respectively.

L. interrogans, mutant and *wt* strains, and *L. biflexa* strain Patoc1 were also exposed to H_2O_2-induced oxidative stress under conditions in which total catalase activity was inhibited with 100 mM ATZ. Inhibition of catalase activity at 10, 1 and 0.1 mM H_2O_2 severely reduced viability in *wt interrogans* strains (Figure 10a, c and e). A less pronounced reduction in survival was observed after catalase inhibition and H_2O_2 exposure in *L. biflexa* and mutant *interrogans* strains (Figure 10b, d and f).

3.4.7 *katE* is required for *L. interrogans* virulence

To test the requirement for *katE* in leptospiral virulence, Himar 1 *katE* insertion mutant Manilae (m69) and Pomona (P3) were generated and used in infection experiments. *Leptospira* were intraperitoneally injected into guinea pigs (n = 4) and hamsters (n = 10) using 10^6 *wt* Manilae or 10^6 mutant m69. By day 10 post injection all

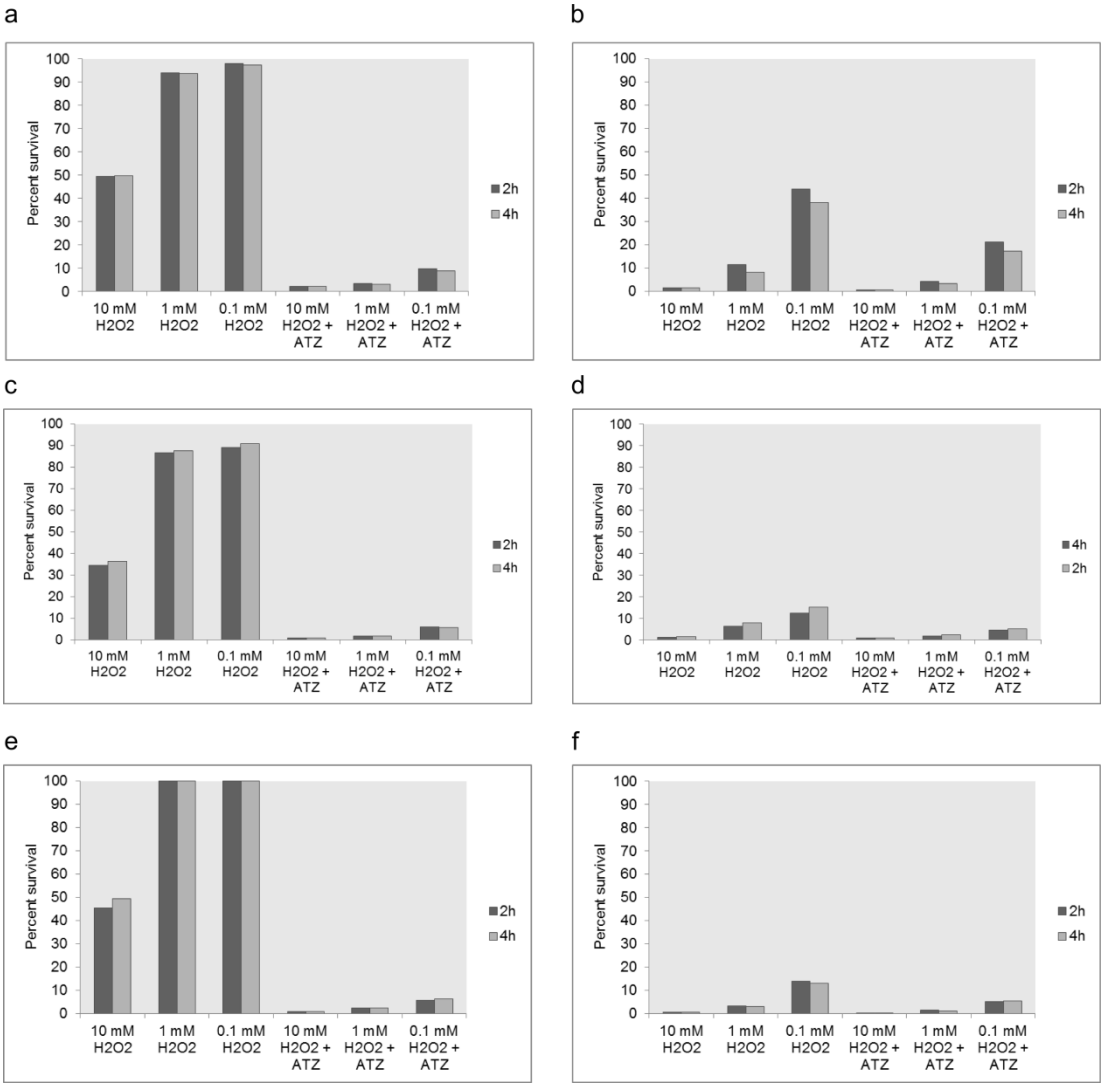

Figure 10: KatE enhances resistance to extracellular H_2O_2.
Leptospiral strains were exposed to oxidative stress at 10 mM, 1 mM, 0.1 mM or no H_2O_2 for 1 hour at 37ºC and subsequently shifted to 29.5ºC with or without inhibition of catalase activity using 100 mM 3-amino-1,2,4-triazole (ATZ). Survival was measured using an AlamarBlue assay and percent survival was calculated from fluorescence values measured at 2 and 4 hours. *L. interrogans* serovar Copenhageni (a) *L. biflexa* serovar Patoc (b*) L. interrogans* serovar Pomona (c) *L. interrogans* serovar Pomona *katE* mutant P3 (d) *L. interrogans* serovar Manilae (e) *L. interrogans* serovar Manilae *katE* mutant m69 (f). Results are shown as means from triplicate values from a single experiment.

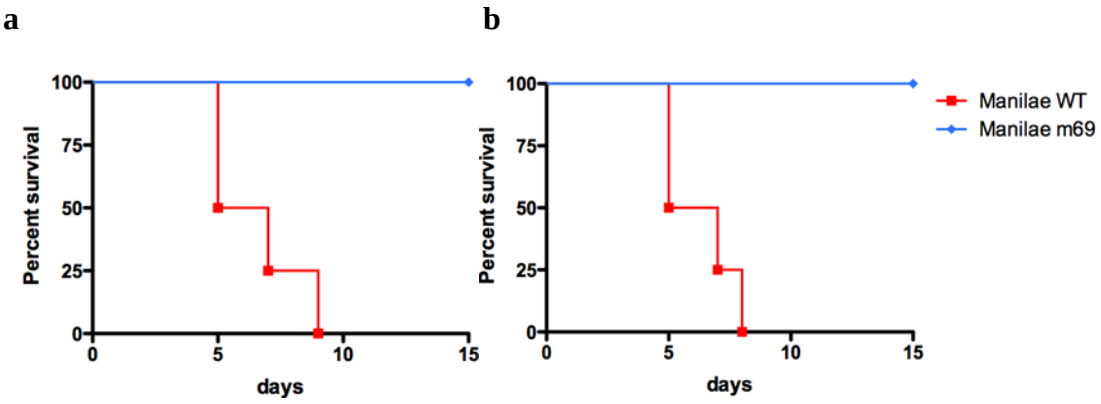

Figure 11: *katE* mutant *Leptospira* show attenuated virulence in guinea pigs and in hamsters. Guinea pigs (n = 4) (a) and hamsters (n = 10) (b) were intraperitoneally injected with 10^6 *wt* Manilae or 10^6 m69 *katE* mutant Manila. All animals infected with wt serovar displayed disease symptoms and died by day 9 of infection. Animals infected with mutant m69 did not display disease symptoms and survived past day 15.

guinea pigs and hamsters injected with the *wt* strain had manifested disease symptoms and died whereas all guinea pigs and hamsters injected with mutant m69 survived to 15 days post injection (Figure11), without disease manifestation. Similarly, preliminary experiments using Pomona *katE* mutants suggest these mutants are also attenuated in the hamster model of infection. In these experiments 10^4 of either *katE* mutant or *wt* Pomona were used to infect groups of 10 hamsters. After 15 days post infection all 10 hamsters infected with *wt* Pomona died compared to 4 hamsters in the group infected with the *katE* mutant strain. However, PCR analysis of the mutant *katE* Pomona strain used in the above described infection trials suggested a population of several mutants with *katE* mutant being the predominant strain as suggested by amplification intensity observed on an ethidium bromide stained agarose gel. A pure strain of Pomona *katE* mutant has recently been obtained that will subsequently be used in hamster infection trials to confirm the initially observed attenuation in the 6 hamsters that survived past day 15.

3.5.1 Discussion

This study investigated the response of *Leptospira* species to oxidative stress conditions and characterized the role of the *L. interrogans* H_2O_2-detoxyifying enzyme KatE in mediating protection from oxidative damage and its requirement for leptospiral virulence. We showed that *L. interrogans* serovars are resistant to higher H_2O_2 concentrations than the saprophyte *L. biflexa* serovar Patoc strain Patoc1, a finding which is in agreement with a previous report by Murgia et al. documenting a lower susceptibility of pathogenic *L. interrogans* to H_2O_2 toxicity (Murgia *et al.*, 2002). Additionally, we demonstrated that leptospiral resistance to an H_2O_2-rich environment is enhanced upon expression of KatE, and that loss of KatE expression leads to attenuation of leptospiral virulence within an animal model of infection.

Through insertion inactivation, six other leptospiral genes have been shown to display attenuation in the animal model of infection. These included genes encoding an outer membrane lipoprotein Loa 22 (Ristow *et al.*, 2007), a heme oxygenase HemO (Murray *et al.*, 2009b), a flagella motor switch protein FliY (Liao *et al.*, 2009), two enzymes involved in LPS biosynthesis (Murray *et al.*, 2010) and a nudix hydrolase InvA protein (Luo *et al.*, 2011). Identification of KatE as a virulence factor in *Leptospira* adds to the broad range of enzyme activity seemingly required for leptospiral virulence. Additionally, as KatE significantly enhances resistance to extracellular H_2O_2 the identification of this enzyme as a virulence factor highlights the importance of ROS resistance for leptospiral pathogenesis.

The importance of ROS detoxifying enzymes for viability of various pathogens

both *in vitro* and during infection have previously been demonstrated (Bishai *et al.*, 1994; Das & Bishayi, 2009; Fang *et al.*, 1999; Howell *et al.*, 2000; Srinivasa Rao *et al.*, 2003; Wilson *et al.*, 1995). Specifically, in *Pseudomonas aeruginosa* the KatB catalase colocalizes to the periplasmic space with the ankyrin-like protein AnkB, and together these proteins work to optimally detoxify extracellular H_2O_2 (Howell *et al.*, 2000). *Edwardseilla tarda*, a gastrointestinal pathogen of humans, utilizes a periplasmic KatB to detoxify H_2O_2, and this protective bacterial response has been demonstrated to be necessary for resistance to phagocyte mediated killing (Srinivasa Rao *et al.*, 2003). Similarly, the capability of *Staphylococcus aureus* to resist phagocyte-mediated killing also depends, at least in part, upon catalase expression (Das & Bishayi, 2009). Finally, the requirement of catalase for full bacterial virulence *in vivo* has been demonstrated using the *Mycobacterium bovis* guinea pig infection model, where it was shown that an *M. bovis* strain that had lost catalase activity was significantly less virulent than a KatG catalase-complemented strain (Wilson *et al.*, 1995). These studies provide evidence that catalase is a ROS detoxifying enzyme which is necessary for establishing infection and ensuring bacterial survival within the host environment.

In a previous quantitative proteomic study (Eshghi *et al.*, 2009) we detected increased expression of KatE in L1-130 shifted to media that had been depleted of iron and supplemented with 10% fetal bovine serum to mimic conditions encountered within the host. Similar quantitative proteomic studies demonstrated increased expression of KatE in response to a shift in temperature of L1-130 from 29.5 ºC to 37 ºC (A. Eshghi and C.E. Cameron, data not shown). These observations are in agreement with findings demonstrating increased transcription of *katE* in response to elevated temperatures and/or

the presence of serum (Xue *et al.*, 2010). Interestingly, Lo et al. did not observe an increase in *katE* expression within wild-type *Leptospira* under iron-limiting conditions (Lo *et al.*, 2010), and similarly in this study we did not observe an increase in KatE expression upon exposure of wild-type *Leptospira* to hydrogen peroxide-rich conditions. Combined, these studies suggest temperature and serum as environmental cues resulting in altered KatE expression at both the transcript and protein levels.

The observed increase in KatE expression in response to host-mimicking conditions suggests that, similar to other bacterial pathogens, KatE may play a role in establishment and maintenance of *Leptospira* infection within a host. As a first step in the characterization of the *Leptospira* KatE, *in vitro* catalase assays were performed using recombinant KatE (rKatE) to verify the bioinformatic identification of LIC12032 as a catalase. The observation that increasing concentrations of rKatE correlated with an increased production of formaldehyde from methanol and H_2O_2 verified rKatE displayed peroxidatic activity. Peroxidatic activity using aliphatic alcohols as an electron source has only been observed in catalases, thus the results confirm that rKatE is indeed a catalase.

The cellular location of KatE was also ascertained via immunoblot analysis of fractionated *L. interrogans* serovar Copenhageni. The results showed that KatE fractionates with both the aqueous phase and the protoplasmic cylinder, suggesting this protein localizes to the periplasmic space and to the inner membrane and/or the cytoplasm. Cytoplasmic catalases are utilized for detoxification of endogenous H_2O_2 produced from energy generation in the electron transport chain, while periplasmic and extracellular catalases are utilized for detoxification of exogenous H_2O_2 encountered during the oxidative burst response produced by host immune cells (Naclerio *et al.*, 1995;

Steele *et al.*, 2010). In this study we did not assess extracellular presence of KatE thus the periplasmic local of KatE may not be a complete picture of leptospiral resistance to extracellular H_2O_2. Regardless of KatE secretion, a periplasmic location of residence may contribute to *Leptospira* infection by protecting leptospiral cytoplasmic and periplasmic components, such as proteins and lipids, against a host-derived oxidative burst response, and is consistent with a previous observation that establishes the importance of periplasmic and not cytoplasmic superoxide dismutase for extracellular ROS resistance (Craig & Slauch, 2009).

Further evidence supporting KatE mediated extracellular H_2O_2 resistance comes from a previous study which utilized microarray analyses to investigate iron-responsive genes, and specifically included transcriptional analyses of the level of expression of the region of the leptospiral genome corresponding to *la1857-la1859* (*lic12034-lic12032*) (Lo *et al.*, 2010). These investigations identified a putative peroxide stress regulator PerR homolog encoded by the *la1857* (*lic12034*) gene, upstream of *katE*. Their investigations further showed that a *la1857* mutant resulted in a 4.3-fold increase in *katE* expression and an 8-fold increase in resistance to H_2O_2 exposure. Use of *katE* mutants in catalase activity and oxidative stress resistance assays in our study validate these previous studies and lead to the conclusion that KatE expression by *Leptospira* significantly enhances the ability to survive extracellular H_2O_2 toxicity.

A previous study which investigated *Leptospira* catalase activity demonstrated that *L. interrogans* lysates can detoxify H_2O_2 concentrations that are 50 fold higher than those detoxified by *L. biflexa* lysates (Corin *et al.*, 1978). It is important to note that the observed 50 fold higher detoxification of H_2O_2 observed in pathogenic *Leptospira* in the

Corin et al. study measured enzyme activity in whole lysates. In contrast our study, which measured bacterial survival, suggested a 2-50 fold higher resistance in *interrogans* compared to *biflexa* depending on the concentration of extracellular H_2O_2. From their results, Corin et al., concluded that *L. interrogans* possesses this highly efficient H_2O_2 detoxification system to detoxify endogenous rather than exogenous H_2O_2 based on the following two observations. First, *L. interrogans* contains cytochrome d, a heme containing enzyme capable of catalyzing H_2O_2 to water and oxygen, while *L. biflexa* does not (Corin *et al.*, 1978). Second, the extracellular locale of pathogenic *Leptospira* would preclude exposure to high concentrations of extracellular H_2O_2, as would be encountered within a phagosome. However, a study in a zebrafish embryo system has demonstrated that *L. interrogans* remains in host immune cells for up to 48 hours and may use these cells as a method for dissemination to different tissues (Davis *et al.*, 2009). This observation has been confirmed *in vitro* through studies demonstrating *Leptospira* can survive in macrophages (Luo *et al.*, 2011; Toma *et al.*, 2011). It is therefore plausible that intracellular *Leptospira* may become exposed to high concentrations of exogenous H_2O_2 and other ROS while residing within host immune cells. While we have not tested the capacity of *katE* mutants to survive within macrophages we have demonstrated their inability to establish infection in guinea pigs or hamsters. *In vitro* experiments utilizing *katE* mutants and macrophage invasion assays would assess the role of *katE* in the intracellular viability of *Leptospira* and provide a plausible explanation for the inability of *katE Leptospira* mutants to establish infection in animals.

In summary, this study has served to advance our understanding of leptospiral pathogenesis by demonstrating that protection of pathogenic *Leptospira* to peroxide-

mediated oxidative stress conditions is enhanced by the protein KatE. Further, we have shown that elimination of this enzyme correlates with loss of bacterial survival *in vivo*, demonstrating the essential nature of this enzyme for leptospiral virulence and allowing its designation as the seventh leptospiral virulence factor to be identified in *Leptospira* to date.

3.6.1 Acknowledgments

The authors would like to thank Drs. David A. Haake and N. Koizumi for their generous gifts of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 and serovar Manilae, respectively.

Chapter 4
Differential methylation and *in vivo* expression of the surface-exposed *Leptospira interrogans* outer membrane protein OmpL32

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Figure 15 work performed and figure generated by Ami Frank in Dr. Richard Zuerner's laboratory
Dr. Marija Pinne provided technical support for IFA
Dr. David A. Haake suggested and assisted with bioinformatic analyses suggesting outer membrane localization

4.1.1 Abstract

Recent studies have revealed that bacterial protein methylation is a widespread post-translational modification that is required for virulence in selected pathogenic bacteria. In particular, altered methylation of outer membrane proteins has been shown to modulate the effectiveness of the host immune response. In this study, two-dimensional gel electrophoresis combined with matrix-assisted laser desorption/ionization-time of flight mass spectrometry identified a *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 protein, corresponding to open reading frame (ORF) LIC11848, which undergoes extensive and differential methylation of glutamic acid residues in response to altered environmental conditions. Immunofluorescence microscopy implicated LIC11848 as a surface-exposed outer membrane protein, prompting the designation OmpL32. Indirect immunofluorescence microscopy of liver and kidney sections revealed expression of OmpL32 during colonization of these organs. Identification of methylated surface-exposed outer membrane proteins, such as OmpL32, provides a foundation for delineating the role of this post-translational modification in leptospiral virulence.

4.2.1 Introduction

Understanding pathogenicity mechanisms surrounding the leptospiral infection process has been hampered by the lack of targeted genetic tools available to leptospiral researchers, such as signature tag mutagenesis (STM) (Hensel *et al.*, 1995) and transposon site hybridization (TraSH) (Sassetti *et al.*, 2001). Random transposon mutagenesis, while not as robust and efficient as STM and TraSH, has been successfully employed to identify virulence factor-encoding genes of leptospiral pathogens (Bourhy *et al.*, 2005). To date five genes encoding virulence factors have been identified in this manner, including *la0222* in *L. interrogans* serovar Lai which encodes the outer membrane lipoprotein Loa22 (Ristow *et al.*, 2007), *la2613* in serovar Lai which encodes the flagella motor switch protein FliY (Liao *et al.*, 2009), an orthologue to serovar Lai *lb186* in *L. interrogans* serovar Manilae which encodes the heme oxygenase HemO (Murray *et al.*, 2009b), and two genes which encode LPS biosynthesis proteins in serovar Manilae, with one being orthologous to serovar Lai *la1641* and the other having no observed serovar Lai orthologue (Murray *et al.*, 2010). In addition to genetic approaches various proteomic approaches have been utilized to study *Leptospira* species and identify potential leptospiral virulence factors. Such proteomic studies have elucidated the subcellular location for various proteins (Beck *et al.*, 2009; Cullen *et al.*, 2005; Haake & Matsunaga, 2002; Monahan *et al.*, 2008; Pinne & Haake, 2009; Sakolvaree *et al.*, 2007), identified immunoreactive proteins (Artiushin *et al.*, 2004; Guerreiro *et al.*, 2001; Sakolvaree *et al.*, 2007), quantitated absolute protein numbers per bacterium (Malmstrom *et al.*, 2009), provided evidence for differential protein expression in response to changes in microenvironments (Cullen *et al.*, 2002; Eshghi *et al.*, 2009; Lo *et al.*, 2009; Matsunaga *et al.*, 2007a; Nally *et al.*, 2001a; Nally *et al.*, 2001b; Nally *et al.*, 2007;

Velineni *et al.*, 2006), contributed to the identification of the first confirmed leptospiral virulence factor, Loa22 (Nally *et al.*, 2007) and revealed diverse and extensive post-translational modifications (PTMs) (Cao *et al.*, 2009)

Of particular interest, a recent global proteomic analysis conducted of *L. interrogans* serovar Lai identified a total of 155 methylated proteins, including predicted and confirmed outer membrane proteins; OmpA-family protein (LA0056), outer membrane efflux protein (LA0957), putative outer membrane protein (LA2244) and LipL32 (LA2637) (Cao *et al.*, 2009), suggesting a widespread role for methylation in *L. interrogans* protein function and/or regulation. In the context of virulence, methylation has been shown to be an essential PTM in various bacterial pathogens. Using genetic approaches, native methylation has been shown to be required for regulation of transcription and phase variation of outer membrane components (Deitsch *et al.*, 2009) and for maintenance of virulence (Giacomodonato *et al.*, 2009). Methylation of glycolipids has been shown to be essential for *Mycobacterium avium* virulence in mice (Krzywinska *et al.*, 2005), while methylation of proteins has been shown to alter the antigenicity of the outer membrane protein OmpB from *Rickettsia typhi* (Chao *et al.*, 2008) and to alter both the antigenicity of and host T cell-mediated immune response against the heparin-binding hemagglutinin from *Mycobacterium tuberculosis* (Parra *et al.*, 2004; Temmerman *et al.*, 2004). In addition, methylation has been demonstrated to be essential for functional type III secretion, and thus virulence, in *Yersinia pseudotuberculosis* (Garbom *et al.*, 2004; Garbom *et al.*, 2007) and methylation of the surface protein OmpB in *Rickettsia prowazaki* has been suggested to be central to the pathogenesis of this bacterium (Chao *et al.*, 2004; Chao *et al.*, 2007). Collectively these

investigations highlight the importance of methylation of outer membrane surface components in bacterial virulence.

The present study describes the proteomic identification of the novel *L. interrogans* outer membrane protein OmpL32 (LIC11848). Immunological analyses confirmed exposure of OmpL32 on the leptospiral surface and expression of this protein during the course of bacterial infection. Proteomic analysis revealed differential and extensive glutamic acid methylation in response to exposure to varying environmental conditions. The potential implications of this PTM are discussed within the context of leptospiral virulence.

4.3.1 Methods

4.3.2 *Leptospira* and culture conditions

Leptospira interrogans serovar Copenhageni strain Fiocruz L1-130 is a clinical isolate originating from Salvador, Brazil (Ko *et al.*, 1999). *Leptospira interrogans* serovar Pomona type kennewicki strain RM-211 is an isolate from a swine abortion case (Thiermann *et al.*, 1984).Cultures were maintained in Ellinghausen and McCullough (Ellinghausen & McCullough, 1965) as modified by Johnson and Harris (Johnson & Harris, 1967) (EMJH) media at 29.5°C. Bacterial enumeration and media shift experiments were performed as previously described (Eshghi *et al.*, 2009).

4.3.3 Two-dimensional gel electrophoresis (2DGE) and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) experiments

L. interrogans was harvested at 8,500 *g* and washed twice with phosphate buffered saline (PBS) supplemented with 5 mM $MgCl_2$. Cell pellets were lyophilized, weighed and resuspended to a concentration of 2.5 mg ml^{-1}. Two dimensional gel electrophoresis, staining of gels with colloidal Coomassie Brilliant Blue and MALDI-TOF MS experiments were performed as previously described (Eshghi *et al.*, 2009).

4.3.4 Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

Trypsin digests were performed with Genomic Solutions ProGest (DigiLab,Inc. Holliston, MA, USA). Briefly, gel slices were manually cut into 1 mm cubes and transferred to a Genomics Solutions ProGest perforated digestion tray. The gel pieces were de-stained (50/45/5 v/v methanol/water/acetic acid) prior to reduction and alkylation with 10 mM dithiothreitol and 100 mM iodoacetamide, respectively (Sigma, Oakville, Ontario, Canada). Modified sequencing grade porcine trypsin solution (20 ng μl^{-1},

Promega, Madison,WI, USA) was added to the gel slices at an enzyme/protein ratio of 1:50. Proteins were digested for 5 h at 37 °C prior to collection of resulting peptides and acid extraction of the gel slices (50/40/10 v/v acetonitrile/water/formic acid). Samples were then speed vac centrifuged to dry and stored at -20 ˚C until analyzed by LC-ESI-MS/MS. An Ultimate Nano-HPLC (LC-Packings/Dionex, Oakville, ON, Canada) coupled to an Applied Biosystems/MDS Sciex QSTAR Pulsar I Hybrid Quadrupole-TOF LC-MS/MS Mass Spectrometer (AB Sciex, Concord, ON, Canada) was used to perform the LC-MS/MS analyses. The nano-column was connected to a 20 µm I.D. emitter tip (New Objective Inc., Woburn, MA, USA) positioned at the orifice plate of the mass spectrometer with spray established by applying a tip voltage of 1900 V to the pre-nano column via a platinum wire nano-tee high voltage connection.

The lyophilised samples were rehydrated in 40 µl 2% v/v acetonitrile/0.1% formic acid and 15 µl of each sample was injected using a FAMOS Autosampler (LC-Packings/ Dionex, Oakville, ON, Canada) for each analysis. The samples were concentrated/de-salted using a SwitchOSII loading pump (LC-Packings/ Dionex) with 98% solvent A (2% v/v acetonitrile, 0.1% formic acid) at a rate of 30 $\mu l min^{-1}$ for 10 min over an Agilent Zorbax C18-SB trap column (5 X 0.3 mm) (Agilent Technologies, Mississauga, ON, Canada) to protect the in-house prepared nano-analytical column Magic C_{18}AQ resin, 150 mm x 75 µm column diameter (Michrom Bioresources Inc, Auburn, CA, USA). After the 10 min loading period the trap column was switched in-line with the nano-column gradient flow. The following 1 h HPLC analytical separation was performed at 300 nl min^{-1} (33 min linear gradient from 5-60% solvent B (98% v/v acetonitrile, 0.1% formic

acid), 2 min linear gradient from 60-75% solvent B, 5% solvent B over 3 min and re-equilibrated 5% B solvent for 12 min before the next injection).

Mass spectra analyses were acquired collecting a 1sec TOFMS survey scan 400-1200 m/z followed by two 2.5 sec product ion scans in the 100-1500 m/z mass range. MS/MS spectra were acquired in a data dependent manner selecting the top 2 most intense eluting ions in the 400-1,200 *m/z* range with a 2^+ to 4^+ charge state greater than 20 counts. Following selection for MS/MS analysis, precursor ions were excluded from selection for MS/MS analysis for 180 sec. A rolling collision energy for fragmentation was selected based on the precursor ion mass using the following formula; 0.05 m/z + 5 V. Known keratin tryptic and trypsin autolysis product masses were excluded to prevent these contaminant ions from being selected for fragmentation.

Mass spectrometer parameters used were as follows: Declustering potential setting of 50, Focusing Potential setting of 220, Curtain gas setting of 25 and CAD gas setting of 5 with Nitrogen in the collision cell.

4.3.5 Bioinformatic analyses

Peptide mass fingerprint (PMF) searches were conducted as previously described (Eshghi *et al.*, 2009) with the following change: no variable modifications were selected. PMFs were further analyzed for potential post-translational modifications using the ExPASy Proteomics Server FindMod tool (Wilkins *et al.*, 1999) (http://ca.expasy.org/tools/findmod/). Predictions were conducted with the following parameter settings: ion mode was set to $[M+H]^+$, mass was set to monoisotopic and mass tolerance was set to 10 ppm.

MS/MS data were searched in Mascot script against the *L. interrogans* proteome in UniProtKB annotation (http://www.uniprot.org/uniprot/?query=leptospira+interrogans+serovar +copenhageni&sort=score&format=*) that was maintained in-house at the University of Victoria-Genome BC Proteomics Centre. Search parameters used were as follows: taxonomy was set to *Leptospira*, enzyme was set to trypsin allowing for 1 missed cleavage site, variable modifications were set to carbamedomethyl (C), deamidated (NQ), methyl (DE) and oxidation (M). Peptide and MS/MS tolerances were set to 0.6 and 0.3 Da respectively, monoisotope mass was selected, peptide charge was set to 1+, 2+ and 3+ and ESI-QUAD-TOF was the selected instrument. Glutamic acid methylation were also confirmed via manual *de novo* sequencing in PEAKS (PEAKS, Waterloo, ON, Canada) (Ma *et al.*, 2003) using the user input sequence for a given peptide spectra. To be classified as a methylated peptide spectral ions representing the methylated glutamic acid residue and the N and C-terminal residues adjacent to the methylated glutamic acid residues had to be present. The only exception to this rule was when the methylated glutamic acid residue was in the second residue position from the N-terminus of a given peptide, in which case spectral ions representing the glutamic acid residue and the C-terminal residue adjacent to the glutamic acid residue had to be present (b1 ions are not commonly detected in peptide spectra). Annotated spectra were exported as .svg files to easily search spectra and are available in Supplementary figures 1-4.

For prediction of subcellular localization, the LIC11848 amino acid sequence was analyzed for the presence of a putative signal peptide sequence using LipoP 1.0 (Rahman *et al.*, 2008) (http://www.cbs.dtu.dk/services/ LipoP/), SpLip (Setubal *et al.*, 2006) and

SignalP (Emanuelsson *et al.*, 2007) (http://www.cbs.dtu.dk/services/SignalP/) signal peptide prediction programs. Secondary structure analyses for prediction of beta sheets was conducted using GOR (Kloczkowski *et al.*, 2002; Sen *et al.*, 2005) (http://pbil.ibcp.fr/htm/index.php) and homology predictions to Gram-negative outer membrane proteins was conducted using OMPdb (Tsirigos *et al.*, 2010) (http://aias.biol.uoa.gr/OMPdb/index.php). Transmembrane domain prediction was conducted using the hidden Markov model outer membrane β-barrel protein prediction program PRED-TMBB (Bagos *et al.*, 2004) (http://biophysics.biol.uoa.gr/PRED-TMBB/). B cell epitope predictions were made using the BCPreds B cell epitope prediction server (El-Manzalawy *et al.*, 2008), using the search parameter "fixed length epitope prediction" and epitope lengths of 12, 14, 20 or 22 amino acids (http://ailab.cs.iastate.edu/bcpreds/predict.html).

4.3.6 Recombinant protein expression and purification

Open reading frame LIC11848 was PCR amplified from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genomic DNA. Primer pairs used are listed in Table 6. The LIC11848 amplicon was ligated first into the cloning vector pJET1 (CloneJet, Fermentas, Burlington, ON, Canada) and digested with *Nde*I and *Xho*I followed by ligation into a similarly digested pET28a expression vector (Novagen, Gibbstown, NJ, USA). The sequence and reading frame of the expression construct were verified by DNA sequencing with vector-specific primers. The LIC11848/pET28a construct was transformed into the *E. coli* expression strain BL21 Star™ (DE3) (Invitrogen, Carlsbad, CA, USA). Bacteria were grown at 37°C in a shaking incubator and recombinant expression was induced at an OD 600 of 1.0 using a final concentration of 0.4 mM

isopropyl β-D-thiogalactopyranoside (Invitrogen). The incubation temperature was then reduced to 16°C and bacteria grown overnight.

Soluble recombinant protein was purified using the following methodology. Bacteria were harvested by centrifugation at 3,000 *g*, resuspended in binding buffer (60 mM NaH_2PO_4, 500 mM NaCl and 20 mM imidazole, pH 8.0) and lysed via sonication (3 x 30 sec) in the presence of a protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; 100 mM AEBSF, 80 µM Aprotinin, 5 mM Bestatin, 1.5 mM E-64, 2 mM Leupeptin, 1 mM Pepstatin A) (Calbiochem, San Diego, CA, USA). The lysate was subsequently centrifuged at 20,000 *g* at 4˚C for 30 min and the supernatant filtered through a 0.45 µm filter. Purification was performed on an ÄKTAprime™ plus Fast Protein Liquid Chromatography system (GE Healthcare, Baie d'Urfe, QC, Canada) fitted with a 1 ml HisTrap™ HP Column (GE Healthcare). All steps were performed at a flow rate of 1 $mlmin^{-1}$ and a pressure limit of 0.3 MPa. The run parameters included a 12 ml equilibration step with binding buffer, application of 30 ml of the filtered supernatant (protein sample), a 40 ml wash with binding buffer, a gradient elution of 0-100% elution buffer (60 mM NaH_2PO_4, 500 mM NaCl and 500 mM imidazole, pH 8.0) over a 50 ml volume with fractions collected at 0.5 ml increments. Fractions containing recombinant OmpL32 (rOmpL32) (identified via absorbance at 280 nm readings of fractions in combination with SDS-PAGE) were then combined and concentrated using a 10 K Amicon Ultra-4 Centrifugal Filter Unit (Millipore, Etobicoke, ON, Canada).

Desalting was achieved in HEPES buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4), over 90 ml, via an ÄKTAprime™ plus Fast Protein Liquid Chromatography apparatus fitted with a Hiload^{TM} 16/60 Superdex 75 prep grade gel

Table 6: Primers used to amplify LIC11848.

ORF	*Sense primer	*Antisense primer	Size (bp)	Portion of ORF (bp)
LIC11848	5'-CTAGACCATATGTCCGGATCCGATCAAAAATC	5'-GTCAGCTCGAGTT AGTAGCGGAGGGAATCC	693	70-762

*Underlined nucleotides represent incorporated restriction sites

filtration column (GE Healthcare). Purified rOmpL32 was quantitated using a BCA Protein Assay (Pierce, Rockford, IL, USA).

4.3.7 Antibodies

Polyclonal rabbit antiserum was prepared against recombinantly expressed LIC11848 (rLIC11848) by ProSci Inc. (Poway, CA, USA). Polyclonal rabbit antisera against OmpL36 (Eshghi *et al.*, 2009), FlaA1 (Cullen *et al.*, 2005) and LipL32 (Haake *et al.*, 2000) have been described previously.

4.3.8 Immunoblot analysis

Approximately 1 x 10^8 *Leptospira* were harvested at 10,000 x *g* and washed twice with wash buffer (PBS and 5 mM $MgCl_2$). The pellet was resuspended in SDS-PAGE loading buffer, boiled for 10 min and separated on 15% sodium dodecyl sulfate-polyacrylamide gel. Proteins were electrophoretically transferred to Immobilon-polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Immunoblots were blocked with 2.5% milk powder in Tris-buffered saline, pH 7.4, with 0.05% Tween 20 (TBST) for 1 h at room temperature. Membranes were washed 2 x 5 min with TBST followed by incubation for 90 min at room temperature with a 1:1,500 dilution of rLIC11848-specific rabbit antiserum or preimmune serum in 2.5% milk powder/TBST. The membrane was washed with TBST 4 x 5 min, incubated with a 1:5,000 dilution of goat anti-rabbit IgG horseradish peroxidase (HRP; Sigma) conjugate secondary antibody in 2.5% milk powder/TBST for 60 min at room temperature followed by rinsing twice and washing 1 x 15 min and 3 x 5 min with TBST. Enhanced chemiluminescence reagent (GE Healthcare) was applied as described by the manufacturer's protocol with the modification of incubating the membrane with the

reagent for one min. The membrane was exposed to X-ray film for 1 min and developed with a Kodak X-OMAT 2000A processor (Carestream Health, Inc., Toronto, ON, Canada).

4.3.9 Immunofluorescence assay

Immunofluorescence assays (IFA) were conducted as previously described (Cullen *et al.*, 2005; Pinne & Haake, 2009; Pinne & Haake, 2011). Briefly, *L. interrogans* was harvested at 2,000 *g* to maintain outer membrane integrity, resuspended in PBS 5 mM $MgCl_2$ to a density of 5 x 10^8 cellsml^{-1}, applied in 1 ml aliquots to NUNC 4 well Lab-Tek™ II Chamber Slides™ (Thermo Fisher Scientific, Rochester, NY, USA) and incubated at 29.5˚C for 80 min for the purpose of adhering cells to the glass slides. All analyses were done in duplicate. Chamber slides were carefully aspirated and bacteria fixed using 1 ml per well of 2% (w/v) paraformaldehyde in PBS for 40 min at 29.5 ˚C. For the purpose of demonstrating an intact outer membrane, replicate chamber slides were permeabilized with 100% ice cold methanol for 20 min at -20 ˚C. Chamber slides were then aspirated and blocked with 1 ml of EMJH for 90 min at 29.5 ˚C. Rabbit antiserum specific for OmpL36, rLIC11848 and FlaA1 were diluted 1:200, 1:100 and 1:600, respectively, and preimmune serum was diluted 1:100 in EMJH. Sera at the specified dilutions were added in 1 ml volumes to chamber slides and incubated for 60 min at 29.5˚C. Chamber slides were washed 3 times with PBS. Alexa Fluor® 568 goat anti-rabbit IgG (H+L) (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI) stain (Invitrogen) were diluted to 1:1500 and 0.25 µgml^{-1}, respectively, in EMJH and applied to chamber slides in 1 ml volumes followed by a 45 min incubation at 29.5 ˚C in the dark. Chambers were then washed twice with PBS and once with water. Chambers were

removed and glass slides were air dried in the dark. ProLong® Gold antifade reagent (Invitrogen) diluted 1:3 in PBS (20 µl) was added to each slide, cover slips were mounted and slides were incubated at room temperature overnight in the dark.

Fluorescence was achieved with an Eclipse 80i microscope fitted with an X-Cite 120 Illuminator and a DS-U1 camera (all from Nikon Canada Inc., Mississauga, ON, Canada). Images were processed using ACT-2U imaging software (Excel Technologies, Inc., Enfield, CT, USA).

4.3.10 Immunofluorescence of tissue sections

Immunofluoresence of tissue sections was conducted as previously described (Matsunaga *et al.*, 2006). All animal studies were approved by the local institutional review boards and conducted in accordance with standard accepted principles. Briefly, Golden Syrian hamsters were inoculated with *L. interrogans*, serovar Pomona type kennewicki strain RM-211. Moribund hamsters were euthanized, and liver and kidney tissues were removed, fixed in 10% buffered zinc formalin, and paraffin embedded. Serial 4 µm sections of hamster tissue were cut. Paraffin was removed from sections with xylene and ethanol, using standard procedures. Non-specific staining of tissue sections was blocked using 10% normal goat serum in PBS at room temperature for 60 min, prior to incubation overnight at 4 ºC with primary antibody. Anti-rLIC11848, anti-LipL46 (Matsunaga *et al.*, 2006), and anti-LipL32 (Haake *et al.*, 2000) antisera were used at a 1:50, 1:100 and 1:200 dilution, respectively. Normal goat sera block was used as a negative control (no primary antibody) on all sections from both infected and uninfected hamsters. Sections were washed with PBS to remove unbound antibody, and then incubated for 60 min at room temperature in the dark with a 1:5000 dilution of Alexa

Fluor 488 $(Fab')_2$ goat anti-rabbit secondary antibody and 0.4 µg/ml DAPI (Invitrogen). Slides were mounted with ProLong® Gold antifade reagent (Invitrogen). All images were captured on a Spot RT color CCD camera mounted on a Nikon Eclipse E800. All immunohistochemistry images were captured under the same exposure conditions.

4.4.1 Results

4.4.2 *L. interrogans* cells differentially express isoforms of a putative OMP

To detect changes in protein expression resulting from exposure to varying environmental conditions, *L. interrogans* cells were grown at 37˚C in EMJH media, in EMJH media depleted of iron, in EMJH media supplemented with 10% fetal bovine serum (FBS) or in EMJH media supplemented with 10% FBS and depleted of iron. Total protein was separated by 2DGE, gels were stained with colloidal coomassie stain and selected protein spots were subjected to trypsin digestion and subsequent matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Comparative analysis of *Leptospira* grown under these varied culture conditions, using conventional growth at 37°C in normal EMJH medium as the comparator, revealed altered expression of numerous proteins, as previously reported (Eshghi *et al.*, 2009). One protein, identified through this study as corresponding to the ORF LIC11848, was deemed to be of particular interest due to the following observation. While the total amount of LIC11848 protein remained unchanged between the comparative conditions, as evidenced by iTRAQ quantitative proteomic analysis (Eshghi *et al.*, 2009), comparative 2DGE analysis revealed the presence of multiple LIC11848 isoforms that displayed altered intensity and differed in their respective isoelectric points over the pI range of 3.5-7.5 (Figure 12).

Due to the complexity associated with analysis of the comparative 2DGE patterns, a brief description of how the different LIC11848 isoforms were identified is provided. A visual identification of similar LIC11848 isoforms was made by locating protein spots that migrated to the same location in each of the comparative growth conditions. This allowed for the designation of representative spots that constituted each of the identified

isoforms and corresponded to spot 1 (deemed to be the same as spots 4, 8 and 11, indicated by red circles in Figure 12 panels a, b, c and d, respectively), spot 3 (deemed to be the same as spots 6, 10 and 13, indicated by blue circles in Figure 12 panels a, b, c and d, respectively), and spot 2 (deemed to be the same as spots 5, 9, and 12, indicated by green circles in Figure 12 panels a, b, c and d, respectively). The isoform represented by spot 7, was unique to growth of *Leptospira* in conditions of EMJH supplemented with 10% serum (Figure 12d, indicated by a black circle). Representative spots 1, 3, and 7, as well as spots 6 and 13 to ensure accurate prediction of identical isoforms, were subjected to in-gel tryptic digestion, the resulting peptides were analyzed via MALDI-TOF MS analysis, and peptide mass fingerprints (PMF) were used to perform MASCOT database searches to allow protein identification.

The mass to charge (*m/z*) ratios, peptide coverage and expect values obtained for each of the representative spots (1, 3, 6, 7 and 13) used for identification via PMF are summarized in Table 7. A large number of *m/z* ratios were obtained for each of the spots, with 34, 37, 22, 40, and 42 *m/z* ratios obtained for spots 1, 3, 6, 7 and 13, respectively. The high degree of peptide mass coverage obtained for each of the representative spots allowed for a definitive identification of each of the five protein spots as LIC11848. Spot 2, which is representative of spots 5, 9 and 12, was definitively identified as a LIC11848 isoform through subsequent MS/MS analyses (see below).

Two additional observations could be made from these comparative analyses: (1) different isoforms displayed altered relative intensities within a particular growth condition (compare spots 1, 2 and 3 within Figure 12a) and (2) the same isoform

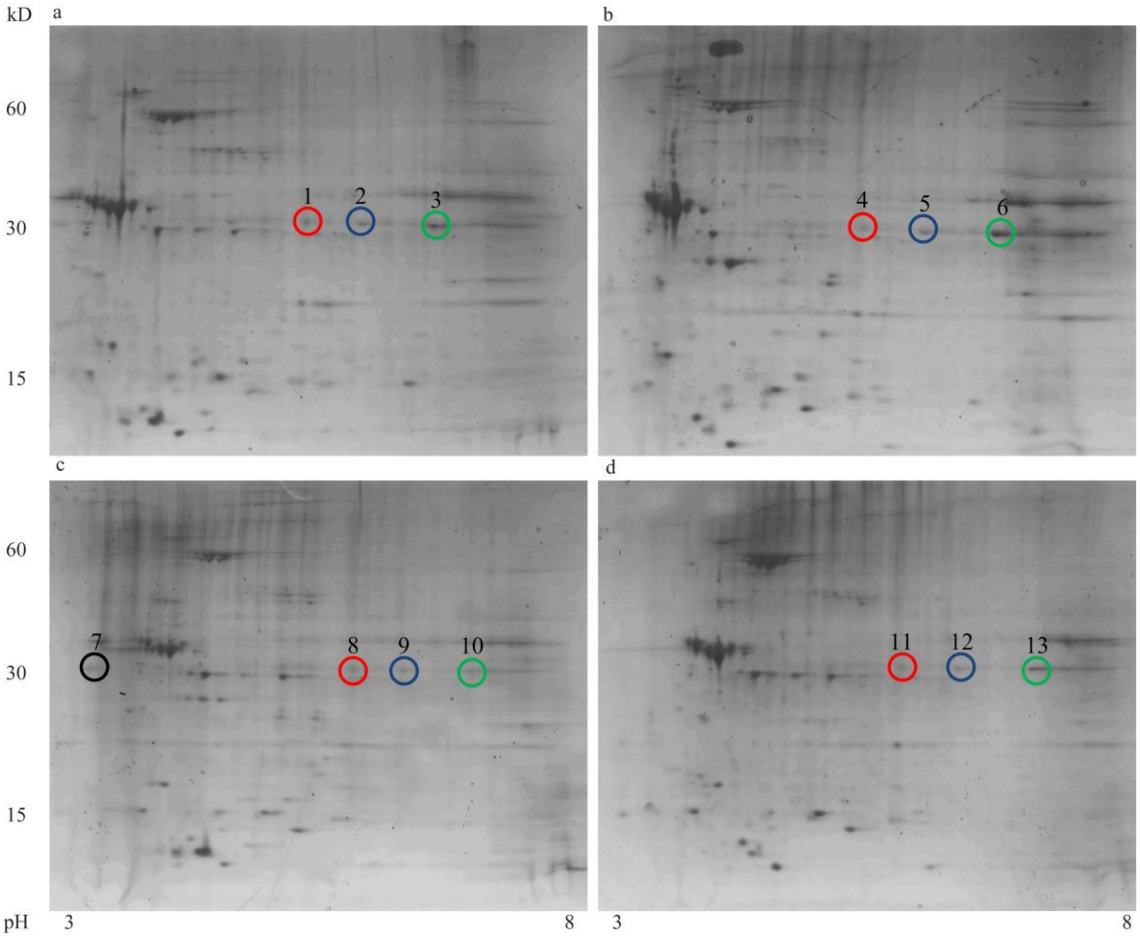


Figure 12: Comparative proteome analysis of *L. interrogans* exposed to differing growth conditions. Total protein from *L. interrogans* was separated using 2DGE following growth of *L. interrogans* in the following comparative conditions: 37˚C in EMJH (a), EMJH supplemented with 10% FBS and depleted of iron (b), EMJH supplemented with 10% FBS (c) and EMJH depleted of iron (d). Representative protein spots selected for subsequent proteomic analyses via MALDI-TOF-MS and LC-ESI-MS/MS are indicated. Identified identical isoforms arising within the different growth conditions are distinguished by different coloured circles, refer to text for further description.

Table 7: Mass to charge ratios used to identify LIC11848 isoforms via peptide mass fingerprinting. The shown mass to charge ratios are from the representative protein spots used for analysis (spots 1, 3, 6, 7 and 13 shown in Figure 12).

Spot 1		Spot 3			Spot 6	
832.3212	1952.8594	832.3169	1844.9249	2973.4056	631.3890	2299.9677
842.5100	1962.0001	842.5100	2043.6810	3338.7352	842.5100	2313.9853
919.5018	1976.7171	919.5029	2056.8903	3348.5966	919.5006	2973.4115
930.4430	1993.0298	930.4427	2072.0175		930.4422	3031.4911
1032.5733	1994.0212	962.4975	2088.8989		1107.5987	3136.4754
1045.5692	2072.0172	978.4905	2102.9141		1569.7536	
1277.7134	2088.9043	1040.5440	2104.8891		1732.7744	
1475.7497	2102.9234	1045.5717	2118.8951		1765.8016	
1569.7519	2104.8998	1107.6002	2211.1040		1779.8127	
1583.7769	2132.0279	1552.4505	2225.1128		1830.9167	
1616.8527	2211.1040	1569.7521	2283.0784		1844.9270	
1716.8573	2225.1156	1583.7634	2299.9780		1959.0148	
1732.7766	2300.0254	1623.8061	2313.9857		2043.6550	
1765.8038	2314.0548	1732.7699	2502.1729		2072.0161	
1779.8173	2807.3192	1765.7988	2807.3148		2088.9071	
1830.9226	2839.3222	1779.8081	2811.3103		2104.8891	
1844.9414	2921.3607	1830.9144	2839.2961		2118.8977	
Spot 7		**Spot 13**				
832.3210	1844.9326	842.5100	2072.0292		**Seq. Cov.**	**Expect**
842.5100	2043.7320	919.5041	2088.9070	**Spot 1**	38%	0.017
919.5020	2072.0203	930.4461	2102.9196	**Spot 3**	47%	2.30×10^{-8}
927.4976	2088.9041	933.5170	2104.9008	**Spot 6**	44%	7.30×10^{-10}
930.4434	2102.9155	962.5057	2118.9171	**Spot 7**	47%	5.80×10^{-08}
933.5084	2104.8990	978.4885	2211.1040	**Spot 13**	49%	5.80×10^{-11}
942.4468	2118.9110	1040.5510	2225.1193			
962.5015	2211.1040	1107.6042	2274.2349			
978.4882	2225.1167	1259.6697	2283.0863			
1040.5461	2239.1268	1422.7305	2299.9801			
1045.5687	2299.9817	1552.4508	2313.9935			
1107.5973	2314.0081	1567.7494	2485.1051			
1479.7991	2502.1780	1569.7562	2501.1233			
1505.6913	2807.3174	1583.7750	2502.1744			
1553.4145	2839.3059	1732.7752	2807.3245			
1567.7539	1690.7616	1746.7922	2973.4320			
1569.7556	1732.7756	1765.8082	3136.4830			
1583.7706	1765.8051	1779.8206	3338.7565			
1623.7441	1779.8168	1786.9269	1959.0272			
1639.9436		1830.9255	2043.6430			
1830.9210		1844.9327	2056.9017			

Type of search: peptide mass fingerprint, enzyme: trypsin, mass values: monoisotopic, protein mass: unrestricted, peptide mass tolerance: ± 10 ppm, max missed cleavages: 1, database: NCBI nr, taxonomy: other bacteria

displayed altered relative intensities between growth conditions (compare spots 1, 4, 8 and 11 in Figure 12 a, b, c and d, respectively). In the context of these observations, it is important to note that the total amount of LIC11848 protein remained unchanged between the comparative growth conditions as determined by iTRAQ analysis (Eshghi *et al.*, 2009).

4.4.3 LIC11848 is differentially methylated on glutamic acid residues

To identify a plausible cause for the observed pI shift in LIC11848, PMF data were further analyzed using the ExPASy FindMod tool (Wilkins *et al.*, 1999) to predict potential post-translational modifications. This analysis predicted methylation of glutamic acid residues within four peptides for spot 1, five peptides for spot 3, three peptides for spot 6, six peptides for spot 7, and eight peptides for spot 13. The number of predicted methylated peptides was proportional to the number of *m/z* ratios used to conduct searches, with a higher degree of peptide coverage correlating with an increased number of methyl-ester adducts being identified. To confirm these *in silico* predictions, tryptic digests of LIC11848 protein from representative spots 1, 2, 6 and 7 (Figure 12 panels a-c) from replicate second dimension gels were subjected to on-line high-performance liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). These analyses confirmed *in silico* predictions and revealed glutamic acid methylation of four peptides in spot 1 (Figure 13a, Supplementary figure 1), seven peptides in spot 2 (Figure 13b, Supplementary figure 2), five peptides in spot 7 (Figure 13c, Supplementary figure 3) and eight peptides from spot 6 (Figure 13d, supplementary figure 4). The glutamic acid residue at amino acid residue position 54 showed

methylation in all four spots (Figure 13 and Supplementary figure 1-4). Similar to the PMF analyses, the number of methyl-esters identified was proportional to the number of *m/z* values present in the MS/MS searches, with a higher peptide coverage correlating with identification of more extensive methylation. Of particular note was the observation that methyl group location varied among glutamic acid residues within identical peptides identified between comparative growth conditions. For example, the peptide spanning amino acids 105-116 (VEVYDFIRDEER) was represented in all isoforms, with three of four isoforms displaying methylation on the most N-terminal glutamic acid residue (residue 2, counting from the N-terminus of the peptide; Figure 13b-d) and one of four isoforms displaying methylation on the most C-terminal glutamic acid (residue 11, counting from the N-terminus of the peptide; Figure 13a).

4.4.4 Correlation between the observed LIC11848 protein methylation pattern and predicted B cell epitope locations

Comparison of the identified LIC11848 methylated peptides with B cell epitopes predicted using the BCPreds online prediction tool revealed all 11 methylated glutamic acid residues to be contained within regions of the protein predicted as possible B cell epitopes (Table 8). Further, 4 of the predicted B cell epitopes correlated with methylated peptides contained within all 4 of the protein spots subjected to methylation profile analysis (predicted epitopes 43-54, 89-110, 103-122, and 173-192). With the exception of two predicted B cell epitopes, the scores for these analyses were highly significant ($\geq$0.926).

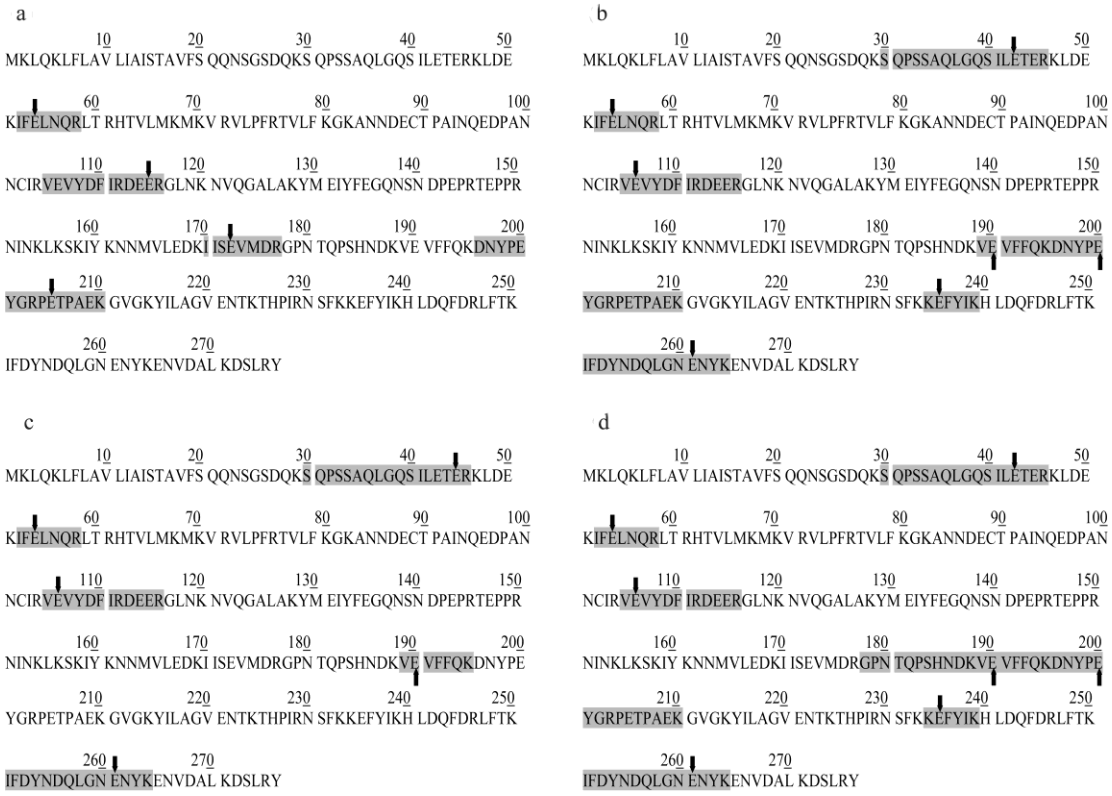


Figure 13: Differential glutamic acid methylation of LIC11848 protein
Glutamic acid methyl ester peptides detected from representative protein spots 1 (a), 2 (b), 7 (c) and 6 (d). Peptides containing methyl ester glutamic acids are highlighted and the methylated glutamic acids are indicated by arrowheads. Spectral evidence can be accessed in Supplementary figures 1-4.

Table 8: Predicted B cell epitopes present within LIC11848 protein (OmpL32) and correlation with observed methylation profile.

Predicted B cell epitope*	Probability	Predicted epitope length (amino acids)	Amino acid range within OmpL32	Methylated amino acid (residue number)	Protein spot
ET**E**RKLDEKIFE	0.28	12	43-54	43, 45, 54	1, 2, 6, 7
CTPAINQEDPANN CIRV**E**VYDF	0.992	22	89-110	106	1, 2, 6, 7
IRV**E**VYDFIRDE**E** RGLNKNV	0.959	20	103-122	106, 115	1, 2, 6, 7
EVMDRGPNTQPS HNDKV**E**VF	0.961	20	173-192	173, 190	1, 2, 6, 7
FQKDNYP**E**YGRP **E**TPAEKGVGK	0.996	20	195-214	200, 205,	1, 2, 6
SFKK**E**FYIKHLDQ F	0.741	14	231-244	235	2, 6
TKIFDYNDQLGN**E** NYKENVDAL	0.926	22	249-270	261	2, 6, 7

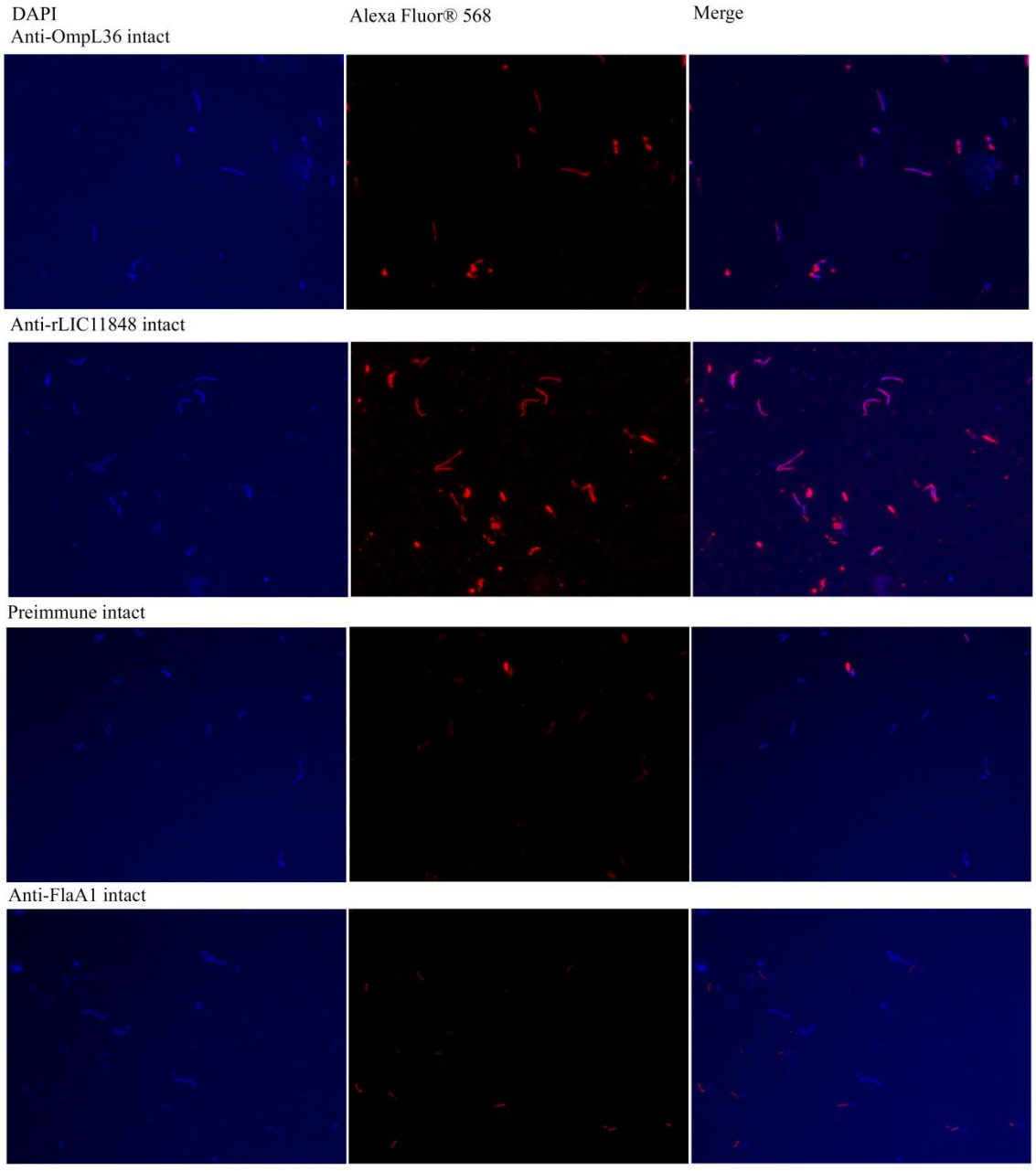
*bolded font = methylated glutamic acid residues

4.4.5 LIC11848 is a surface exposed protein

To predict the cellular location of LIC11848 the amino acid sequence was analyzed using the signal peptide prediction tools LipoP 1.0 (Rahman *et al.*, 2008) and SignalP 3.0 (Emanuelsson *et al.*, 2007). Both tools predicted the presence of an N-terminal SpI signal peptide, with LipoP predicting a potential cleavage site between serine 20 and glutamine 21 and SignalP predicting a potential cleavage site between glycine 24 and serine 25, a finding which is consistent with a potential outer membrane locale for this protein. Secondary structure analysis conducted using GOR predicted the secondary structure composition of the protein to be 15% beta sheet. To further assess the predicted location of LIC11848 the amino acid sequence was searched against the OMPdb database for Gram-negative bacterial beta-barrel-containing outer membrane proteins (Tsirigos *et al.*, 2010). This search identified significant homology between LIC11848 and the C-terminal residues 633-752 from the *Bacteroides fragilis* outer membrane receptor (OMR-TonB Dependent Receptor) family (27.13% sequence identity and 44.97% sequence similarity over 129 amino acids). Further evidence suggesting membrane association for this protein was obtained via identification of 9 transmembrane domains as predicted by PRED-TMBB analysis, although the generated score for this analysis of 3.020 was slightly higher than the outer membrane protein threshold cut-off value of 2.965 for this prediction program.

To experimentally evaluate whether LIC11848 protein is present on the surface of *L. interrogans*, immunofluorescence assays (Cullen *et al.*, 2005; Pinne & Haake, 2009; Pinne & Haake, 2011) were conducted using intact and methanol-permeabilized *L. interrogans* with rLIC11848-specific serum, serum specific for the known surface-

a


DAPI
Alexa Fluor® 568
Merge
Anti-OmpL36 intact
Anti-rLIC11848 intact
Preimmune intact
Anti-FlaA1 intact

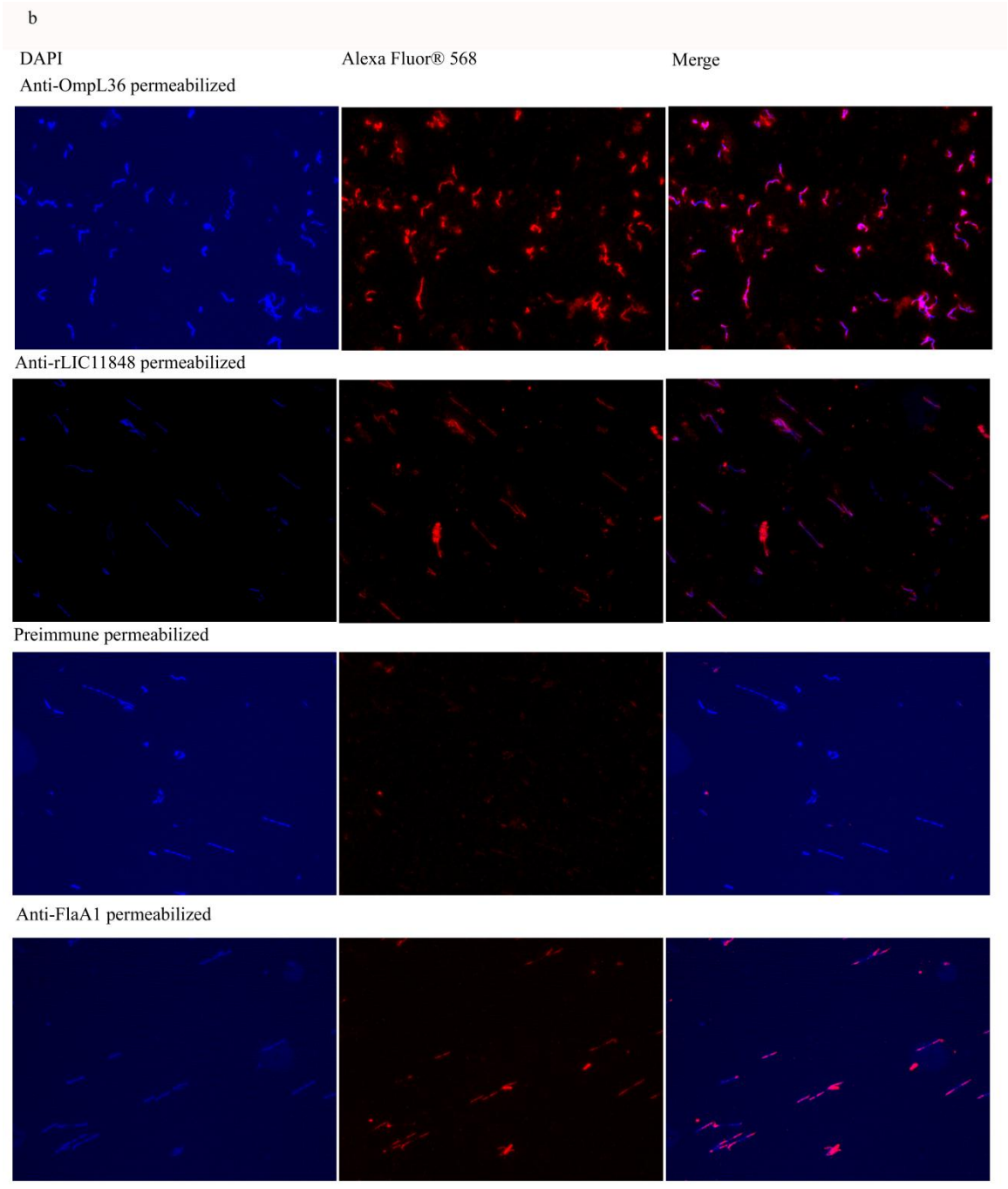


Figure 14: Immunofluorescence microscopy identifies LIC11848 protein as a surface-exposed, outer membrane protein.
Intact (a) and methanol–permeabilized (b) *L. interrogans* were probed with rabbit preimmune serum or rabbit serum specific for either OmpL36 (outer membrane protein positive control), rLIC11848, or FlaA1 (periplasmic negative control protein). Observed fluorescence corresponds to either Alexa Fluor 568-labelled leptospires or DAPI-stained leptospiral DNA. Merged images reveal overlap of DAPI-stained and Alexa Fluor 568-labelled *Leptospira*.

exposed protein OmpL36 (positive control) (Pinne & Haake, 2009) and serum specific for the periplasmic endoflagellar protein FlaA1 (negative control). Immunofluorescence microscopy revealed reactivity to intact *L. interrogans* with serum specific for rLIC11848 and OmpL36 but not with preimmune serum or serum specific for FlaA1 (Figure 14a). Immunofluorescence was also detected in permeabilized *Leptospira* using serum specific for LIC11848, OmpL36 and FlaA1 but not preimmune serum (Figure 14b). The specificity of the rLIC11848-specific antiserum was tested via immunoblot analysis. This analysis revealed reactivity to a protein band migrating at a molecular weight corresponding with the 32kDa predicted molecular mass for LIC11848 (Supplementary figure 5). Immunoblot analysis using preimmune serum showed an absence of reactivity (data not shown).

4.4.6 *Leptospira* express LIC11848 during colonization of hamster kidneys and liver

To assess expression of LIC11848 during the infection process immunofluorescence microscopy was conducted on kidney and liver sections of hamsters infected with *Leptospira*. Use of antiserum specific for LipL32 (positive control) or rLIC11848 in these experiments resulted in leptospiral fluorescence in both kidney and liver sections of hamsters infected with *Leptospira* (Figure 15). Although leptospires were observed throughout the tissue of infected hamsters, the level of LIC11848 protein expression was reduced in comparison to the highly conserved and abundant outer membrane lipoprotein, LipL32. This observation was in agreement with absolute protein numbers per *Leptospira* previously described (Malmstrom *et al.*, 2009), and more similar in protein expression levels to LipL46 (data not shown) in serially sectioned tissue. In kidney sections, the spirochetes are mainly observed disseminated in the blood vessels

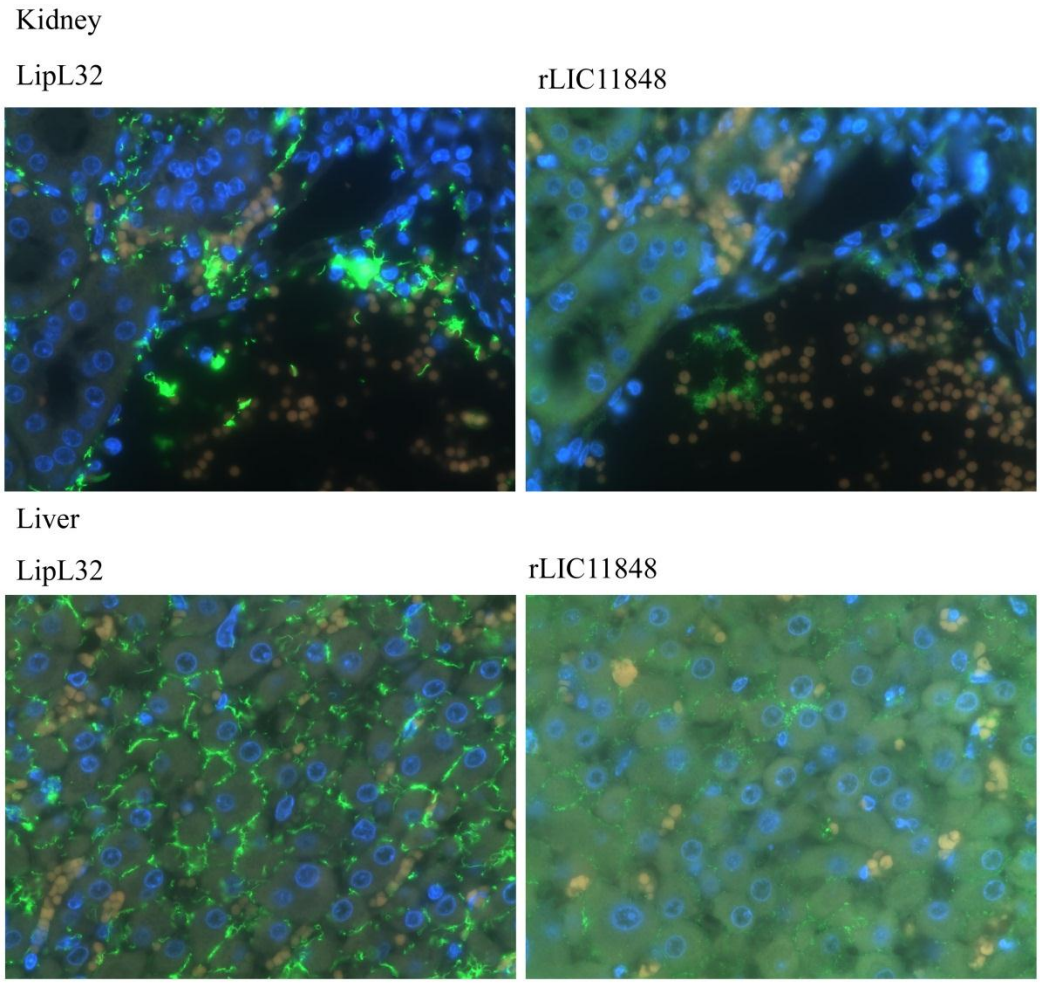


Figure 15: *Leptospira* express LIC11848 protein during colonization of hamster kidneys and liver as evidenced by immunofluorescence microscopy.
Leptospira-infected tissue samples from Syrian hamsters were probed with LipL32 (positive control) or rLIC11848 antisera and viewed rLIC11848 by indirect fluorescence microscopy (Matsunaga *et al.*, 2006). Fluorescence (green areas) was detected in both kidney and liver tissue sections but not in healthy uninfected tissue sections (data not shown).

and in the interstitium between tubules. In liver sections, the leptospires were observed within the intercellular spaces between hepatocytes. Use of antiserum specific for rLIC11848 on healthy (not infected with Leptospira) hamster kidney and liver sections did not result in fluorescence (data not shown).

4.5.1 Discussion

The results presented herein enhance our understanding of *Leptospira interrogans* biology and serve as a platform for the study of leptospiral virulence. In this study, proteome analyses conducted on *L. interrogans* grown under the varying environmental conditions of media depleted in iron, media supplemented with 10% serum or media depleted of iron and containing 10% serum (*in vivo*-like growth conditions) identified the novel protein LIC11848. Immunofluorescence experiments revealed reactivity of serum specific for the 32 kDa LIC11848 protein product with intact *L. interrogans*, thus providing evidence for exposure of this protein on the surface of viable *L. interrogans*. This finding, combined with the bioinformatic predictions that this protein possesses a cleavable SPI signal sequence and may have a propensity to form a β-barrel structure, prompted our designation of this protein as OmpL32. The present study also demonstrated expression of OmpL32 during leptospiral infection of the kidneys and liver of hamsters by immunohistochemistry, correlating with a prior report of OmpL32 expression in virulent *Leptospira* freshly cultured from the liver and kidney of infected hamsters (Vieira *et al.*, 2009). The level of OmpL32 expression compared to LipL32 *in vivo* was in agreement with a previous proteomic study that demonstrated an 11 fold higher expression level of LipL32 (32,190 copies per cell) compared to OmpL32 (2,740 copies per cell) (Malmstrom *et al.*, 2009) in *Leptospira* grown under laboratory

conditions. Finally, and most importantly, proteomic analysis of multiple isoforms of OmpL32 revealed the interesting observation of differential glutamic acid methylation within this surface exposed protein.

To date, glutamic acid carboxymethylation in bacteria has been discussed within the context of chemotaxis (Ahlgren & Ordal, 1983; Kehry *et al.*, 1984; Kleene *et al.*, 1977; Nishiyama *et al.*, 1999). Specific carboxymethylation on glutamic acid residues serves to modulate protein function from an active to an inactive conformation or vice versa. Activation via methylation results in conformational changes of the methylated protein leading to activation of downstream proteins that eventually activate flagella for directional motility (Clarke, 1993). The discovery that OmpL32 displays differential methylation upon exposure to altered environmental conditions suggests a related methylation-induced regulation may occur with this leptospiral protein. However, two observations provide evidence suggesting that OmpL32 does not play a role in bacterial chemotaxis. First, the OmpL32 amino acid sequence does not reveal homology to proteins involved in either chemotaxis or motility, which in general are highly homologous to one another. And second, glutamic acid methylation of chemotaxis proteins has been demonstrated to occur on only a few targeted amino acid residues (Rice & Dahlquist, 1991), which is in distinct contrast to the widespread methylation pattern observed for OmpL32. These observations suggest that the glutamic acid-specific methylation pattern detected in OmpL32 has an alternative functional role.

Multiple studies have established a requirement for methylation in bacterial pathogenesis. A requirement for methylation has been demonstrated in *Y. pseudotuberculosis* where an insertion mutant deficient in the VagH protein is avirulent

in mice (Garbom *et al.*, 2004). In *Y. pseudotuberculosis* the VagH protein is homologous to the *Escherichia coli* HemK protein (Garbom *et al.*, 2007), an N^5-methyltransferase (Heurgue-Hamard *et al.*, 2002; Nakahigashi *et al.*, 2002). The *Y. pseudotuberculosis vagH* insertion mutant demonstrated repression of secretion of the virulence determinant YopD via the type III secretion system (Garbom *et al.*, 2007). Additionally, this mutant displayed characteristics similar to a type III secretion mutant during pathogenesis suggesting a role for protein methylation in type III secretion function (Garbom *et al.*, 2007). Within the context of outer membrane protein methylation and bacterial pathogenesis, a link has been demonstrated in the bacterial pathogen *R. prowazekii*. The avirulent Madrid E strain has a null mutation in the methyltransferase-encoding *rp027* gene (Chao *et al.*, 2007), and consequently shows hypomethylation of the immunodominant outer membrane protein B (OmpB). In comparison, the virulent *R. prowazekii* Breinl strain, which expresses the Rp027 protein product, exhibits hypermethylation of OmpB (Chao *et al.*, 2004), suggesting a role in virulence for outer membrane protein methylation in *R. prowazekii* (Chao *et al.*, 2007).

Methylation of bacterial outer membrane proteins could contribute to virulence in at least two ways. First, methylation could potentially be used by bacteria to regulate protein function analogous to that seen for chemotaxis proteins (Wadhams & Armitage, 2004) and the *Y. pseudotuberculosis* type III secretion system (Garbom *et al.*, 2007). In this scenario, methylation could act to regulate the function of leptospiral virulence factors present on the spirochetal surface by providing a post-translationally-controlled switch between an active/inactive state, and in this way ensure optimal timing of virulence protein function within the infection process. Also, the presence or absence of

protein methylation could be critical for real-time phase variation as a method of altering the antigenicity of bacterial outer membrane proteins, thereby modulating the host immune response recognition of these proteins. Support for the existence of effects on antigenicity and their influence on cellular and humoral immune responses to bacterial pathogens comes from studies conducted within the pathogens *M. tuberculosis* and *Rickettsia typhi*. In *M. tuberculosis*, correlation of the level of protein methylation and host immune response modulation has been demonstrated (Temmerman *et al.*, 2004), with native methylation of the surface-exposed, *M. tuberculosis* heparin-binding hemagglutinin protein being required for an effective T-cell mediated immune response. The effect of protein methylation on the host humoral immune response has been studied in *R. typhi* using methylated and non-methylated protein fragments of the outer membrane protein OmpB (Chao *et al.*, 2008). In this study experiments conducted using patient sera in an ELISA-based format revealed higher titers to the methylated OmpB fragment when compared to the unmethylated fragment. Together these studies provide evidence that methylation of surface-exposed proteins in bacteria directly affects pathogen recognition by the host immune response.

In order for OmpL32 to function in a methylation-dependent, immune evasion capacity within *Leptospira*, the protein must be present on the surface of the bacterium. Immunofluorescence microscopy experiments confirmed surface exposure of this protein in *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. The presentation of this protein on the leptospiral surface would facilitate direct recognition by the host immune response. The capacity of the bacterium to differentially methylate OmpL32 could contribute to the ability of this bacterium to persist in the presence of the generated host

immune response, and thus establish a chronic infection. In this scenario OmpL32 methylation could serve to modulate the level of the host immune response in a manner similar to that documented for *M. tuberculosis* (Temmerman *et al.*, 2004) and *R. typhi* (Chao *et al.*, 2008). In support of this we have observed altered intensity of identical OmpL32 isoforms between comparative growth conditions, as well as different isoforms within an individual growth condition, suggesting that the extent of methylation differs depending upon the conditions under which *L. interrogans* are grown. Further, the direct identification of multiple different locations of methylation on amino acid residues within OmpL32 provides an additional indication that this process may represent a novel mechanism that would alter the surface of the bacterium. A definitive answer as to whether *Leptospira* alter the number of methylated residues within OmpL32 upon exposure to a host environment must await further investigation. However, in this study we have provided a foundation for future studies by showing that an outer membrane protein is methylated, that the methylation pattern of this protein varies resulting in multiple isoforms and that the amount of each of these isoforms is altered in response to varying growth conditions. An alternate hypothesis arising from these observations is whether the position of methylation alone rather than the absolute number of methylations leads to altered recognition by the host.

Consistent with a purported functional role of differential methylation of OmpL32 in evasion of a host-generated humoral immune response, the entire repertoire of differentially methylated glutamic residues observed within OmpL32 in our study are located within predicted OmpL32-specific B cell epitopes. Also of note is the observation that the gene encoding OmpL32 (*lic11848*) resides in a putative three

component operon with a gene encoding a hypothetical protein (*lic11849*) and a gene encoding a putative methyltransferase (*lic11850*). Since co-expressed proteins are routinely functionally linked, this is suggestive that OmpL32 serves as a methylation substrate for the downstream putative methyltransferase. Combined, these observations suggest that all the required components for a rapid and effective immune evasion strategy focused around OmpL32 may be in place. In this model, exposure to altered environmental conditions would promote methylation of OmpL32 by the simultaneously expressed methyltransferase on residues contained within B cell epitopes, thus leading to an altered immune recognition of this protein on the leptospiral surface.

In summary, the current study has identified a novel *L. interrogans* protein designated OmpL32 which we have shown to be surface-exposed, expressed during the course of infection and differentially methylated on glutamic acid residues in response to altered growth conditions. Further investigation into the methylation status of leptospiral surface-exposed proteins and the potential role this post-translational modification plays in the leptospiral infection process is warranted and may lead to the identification of a novel immune evasion strategy in existence within *Leptospira*.

4.6.1 Acknowledgements

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Chapter 5
Conclusions

The main objective of the work described in this thesis was to expand our understanding of the leptospiral infection process. To achieve this goal both gel and non-gel based proteomic approaches were utilized for the purpose of conducting comparative quantitative proteomic experiments on *Leptospira* grown under *in vitro* conditions designed to mimic a subset of the variables encountered by bacteria within the host. The hypothesis was that *Leptospira* will alter expression of proteins required for survival under conditions mimicking infection. Thus identification of these proteins via iTRAQ complemented with 2DGE would serve as a foundation for the discovery of new virulence-associated proteins.

Chapter 2 presents the data from the above described proteomic approaches and the original publication can be accessed from the publishers website using the following reference (Eshghi *et al.*, 2009). Among altered expression of various proteins involved in housekeeping processes such as metabolism, genome maintenance and protein expression, as examples, five proteins were suggested as having potentially novel roles in the leptospiral infection process as suggested by sequence similarity and by evidence provided in studies describing orthologues of these proteins as being virulence-associated (see chapter 2 for a discussion of these studies). The potential role of these proteins in the leptospiral infection process is discussed in detail in chapter 2, section 2.4.7. Thus the significance of this study was the identification of five proteins with potentially novel roles in the leptospiral infection process; a putative glycoside hydrolase, a putative coagulase, a putative ErpY-like protein, a putative catalase KatE and a TolC-like protein.

The results from the above study require further investigation to definitively determine the role of the potential virulence associated proteins in the leptospiral infection process. Ideally, to determine the necessity of these proteins for virulence, *Leptospira* strains mutated in their respective ORFs would have to be generated using random Himar1 transposon insertion mutagenesis (Murray *et al.*, 2009a) and subsequent infection trials in animals performed to assess the requirement of these proteins for survival within an animal host.

While proteomics has been successfully demonstrated to complement genetic approaches in the study of leptospiral pathogenesis it is important to be aware of limitations inherent to this methodology, especially for the purpose of implementing realistic objectives. A central limitation to the approach utilised in this thesis was that proteins whose function cannot be defined based on COGs and yet may have an unknown function required for infection will not be identified as potential virulence-associated proteins. This is a significant limitation as the majority of leptospiral ORFs identified via genome sequencing (Nascimento *et al.*, 2004; Ren *et al.*, 2003) cannot be assigned a function based on orthology. Another limitation reflecting the opposing spectrum to the point made above is identification of false positive targets that are differentially regulated but are not in reality required for virulence. A classic example highlighting this limitation is the enormous number of publications, in the last decade, that focused on the study of the most abundant pathogen-specific leptospiral outer membrane-associated lipoprotein, LipL32. A recent study using an insertion mutant in the ORF encoding LipL32 has been used in infection trials and all animals challenged with this mutant survived (Murray *et al.*, 2009c), thus negating years of experiments on a potential role for LipL32 in

leptospiral survival within the host. Another noteworthy limitation to this approach is that proteins or enzymes whose function is controlled by variables other than expression levels that maintain a role in infection will be neglected.

In addition to inherent limitations discussed above, technical short-comings also affect the efficiency of a proteomic approach. Dynamic range is one example of a technical limitation where identification is biased towards highly expressed proteins whose signal overwhelms low abundance proteins resulting in reduced proteome coverage. However, the issue of dynamic range can at least be partially remedied using the directed proteomics approach (Domon & Aebersold, 2010) generating a peptide atlas of proteotypic peptides. This approach has its limitation in that quantitation is limited to SRM which can become costly depending on the number of proteins quantitated. Another technical limitation is the complex issue of maintaining membrane and cytoplasmic proteins soluble in the same solution while ensuring compatibility of the compounds used in the solution with mass spectrometers. These technical limitations are reflected in the protein coverage of approximately 15% reported in chapter 2. Thus comparative proteomics used for the purpose of identifying potential virulence associated proteins should not be received as inclusive of all virulence associated proteins.

As already discussed, a global comparative proteomic approach led to the identification of 5 proteins with potential roles in the infection process. One of these proteins was annotated as a catalase, KatE, which was further characterized and demonstrated to be required for leptospiral virulence in animals, as discussed in chapter 3. The significance of this work was twofold in that a novel leptospiral virulence protein was identified and second, the use of proteomics for the identification of virulence factors

was validated thus adding merit to the use of comparative proteomics for the study of leptospiral pathogenesis.

Future experiments focusing on extending the above study should concentrate on the mechanism of virulence disruption observed in *katE* mutants. Previous studies have established that *Leptospira* are capable of phagocytic uptake and able to retain viability intracellularly (Luo *et al.*, 2011; Toma *et al.*, 2011) and disseminate via intracellular "transport" by resident macrophages in zebra fish embryos (Davis *et al.*, 2009). A potential obstacle that *Leptospira* may have to overcome to maintain an intracellular lifestyle in host immune cells is resistance to toxic concentrations of various compounds used by immune cells in bacterial killing including reactive oxygen species such as H_2O_2. As demonstrated in chapter 3 *katE* is required for resistance to extracellular H_2O_2 and it follows that an attractive starting point would be to test the ability of *katE* mutant *Leptospira* to invade and remain viable in mammalian macrophage cells, *in vitro*. An inability to remain viable post exposure to mammalian macrophage cell lines would suggest that a similar scenario could occur during the infection process and would suggest a mechanism of clearance of *katE* deficient *Leptospira* by the host.

Currently, complementation is a very difficult methodology in *Leptospira* with a very low success rate. To definitively assess the requirement for a gene in virulence it necessitates complementing a mutant with the *wt* version of the gene and subsequently observing gain of virulence in the model of infection. Thus a limitation to the experiments presented in chapter 3 was the lack of a complemented *katE* mutant for assessment of gain of virulence in the animal model of infection. This shortcoming is currently being partially remedied by utilizing an independent *katE* mutant in a different

interrogans serovar Pomona in infection trials by an independent laboratory. Preliminary results hold promise as this Pomona *katE* mutant strain also displays attenuated virulence in hamsters. However, this mutant was determined to not be a pure culture and instead was found to be a mixed mutant culture with the *katE* mutant being the predominant mutant as determined by PCR amplification experiments. We have recently been successful at isolating a pure Pomona *katE* mutant which will be used to repeat the infection trials for the purpose of testing this mutant for attenuated virulence. As a second level of control a strain containing an insertion mutant in the gene upstream of *katE* has been used in infection trials and this mutant does not display attenuated virulence (data not shown) adding merit to the reality that the insertion within *katE* is responsible for the observed attenuation in virulence. Lastly, a limitation inherent to transposon mutagenesis is the polar effect observed in bacteria due to the efficient organization of genes where promoters, operators or terminators for a given gene are present within another gene. Thus disruption of one gene has a high probability of altering the expression of another gene. Complementation eliminates the ambiguity that can arise if the expression of more than one gene is being altered due to mutagenesis.

In addition to leading to the identification of a novel leptospiral virulence factor, an unexpected finding from the proteomic experiments was the discovery of differential glutamic acid methylation of a protein identified as a putative outer membrane protein. At the time of the discovery outer membrane protein methylation had not been documented in *Leptospira* but has recently been validated (Cao *et al.*, 2009). The results described in chapter 4 of this thesis demonstrate the occurrence of glutamic acid methylation of a protein that was also demonstrated to be surface exposed and expressed during

colonization of hamster liver and kidneys. The significance of this work could be realized in future studies assessing the role of this PTM in controlling outer membrane protein function and/or altering antigenic variation in *Leptospira*. Future studies should focus on extending the list of methylated outer membrane proteins via mass spectrometry based proteomics. The gold standard for identification of PTM via mass spectrometry is based on validating mass spectra with synthetic peptides design to contain identical amino acid sequence and modifications of the MS/MS identified PTM containing peptide. In all instances using soft fragmentation approaches such as electron capture dissociation or alternatively electron transfer dissociation would be the ideal approach for identification of PTMs. The synthetic peptide is then subjected to MS/MS fragmentation, in the same manner initially used to identify the peptide containing the modification, and resulting mass spectrum used to confirm the presence of the identified PTM. Upon identification of global outer membrane protein methylation subsequent studies could focus on the effect of this PTM on the antigenicity of outer membrane proteins and the resulting effects on the host immune response upon altered methylation patterns. A simple method of testing antigenicity at the antibody level is to raise antibodies against a given synthetic peptide sequence. Synthesis of a synthetic peptide identical in sequence but containing methylated residues could then be used to measure the binding capacity of the previously generated antibody. Altered binding capacity would indicate altered antigenicity due to methylation.

These studies could then be further extended to identify methyl transferases that are responsible for outer membrane protein methylation. A plausible approach for identifying such proteins would be to purify methyl transferases from a whole cell lysate

over columns with covalently linked S-adenosyl-L-homocysteine (SAH) as the majority of methyl transferases have a binding capacity for this compound. Tandem mass spectrometry would identify eluted fractions of SAM binding proteins. Insertion mutants of genes encoding these proteins would be required to assess the extent of or lack of OMP methylation in these mutants. Further, these mutants can then be used in *in vitro* assays to determine antibody and cell mediated immune responses to these mutants. Lastly, testing these mutants in infection trials would determine the requirement for outer membrane protein methylation for leptospiral virulence.

A limitation to the experiments discussed in chapter 4 was a lack of an independent methodology for the purpose of confirming surface exposure of OmpL32. Identification of surface exposed proteins should be performed using more than one methodology (Pinne & Haake, 2009; Pinne & Haake, 2011). Other methodologies including detergent fractionation, surface biotinylation and surface proteinase k digestions were attempted but technical limitations in these experiments failed to confirm or negate immunofluorescence microscopy results.

The work in this thesis has contributed to advancing leptospiral research in a number of ways. Global proteomics identified five proteins a glycoside hydrolase, a putative coagulase, an ErpY-like protein, a TolC-like protein and a putative catalase KatE with potentially novel roles in the leptospiral infection process. Further characterization of KatE as a catalase and use of *katE* insertion mutants suggested attenuated virulence in the animal model of infection. This finding represents only the seventh virulence factor to be identified in *Leptospira* to date and suggests ROS resistance is a requirement for fending off the host immune system and, ultimately, for pathogenesis. Results also led to

the discovery of outer membrane protein methylation and of a novel surface exposed protein. Only one other study in *Leptospira* has reported protein methylation thus these discoveries have opened a potentially novel area of study in leptospiral research. Collectively, the results reported in this thesis have significantly advanced leptospiral research and the objective of using a global proteomic approach to study leptospiral pathogenesis was satisfied by identifying a novel leptospiral virulence factor KatE.

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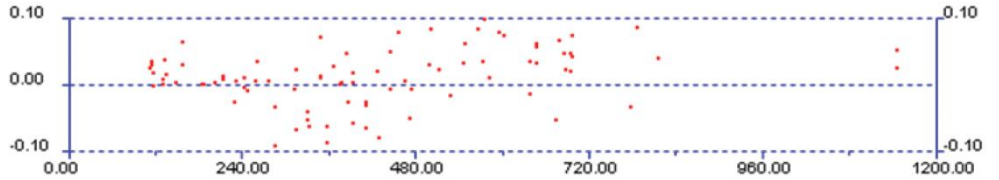
Appendix A
Supporting Information

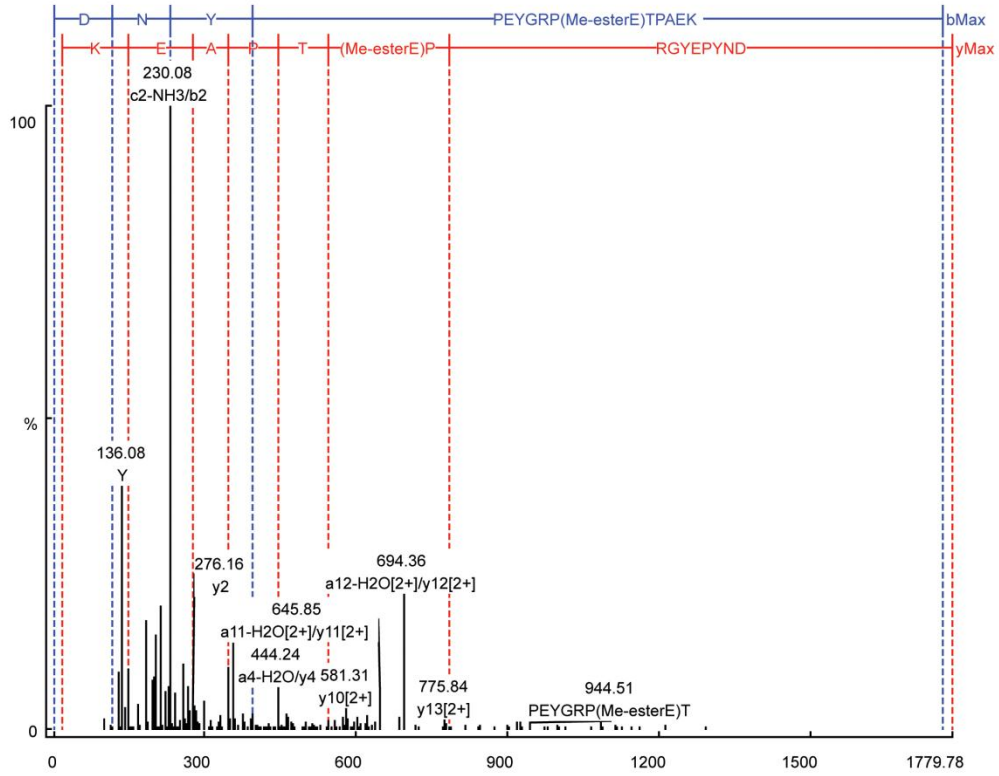
http://pubs.acs.org/doi/suppl/10.1021/pr9004597

Appendix B
Supporting information

Peptide: DNYPEYGRP(Me-EsterE)TPAEK

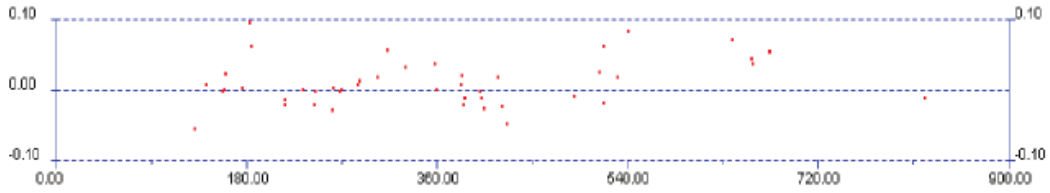
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1	116.0526			133.0782		D						15
2	230.0831	212.0753	202.0856	247.0962		N						14
3	393.1456	375.1325	365.1751	410.1430	136.0802	Y					775.8410	13
4						P					694.3625	12
5		601.3010			102.0493	E					645.8494	11
6					136.0802	Y			1144.5...	1145.6...	581.3060	10
7						G					499.8474	9
8					129.1039	R					471.2020	8
9						P	785.4899				393.1456	7
10					116.0526	(Me-est...				672.2731		6
11						T	545.3270	527.2663				5
12						P	444.2385	426.2557	427.1405			4
13						A	347.2068	329.1412	330.1132	331.1030		3
14					102.0493	E	276.1617	258.1513	259.1631			2
15						K	147.1161	129.1039	130.0944	131.1244		1

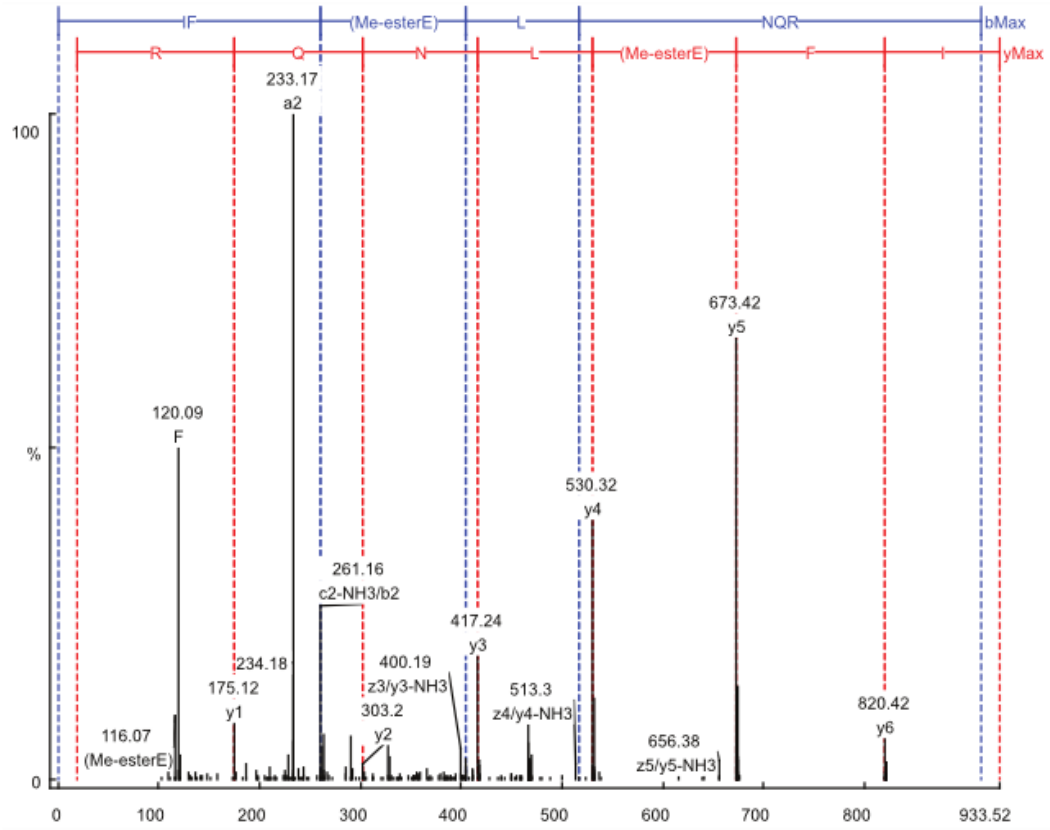




Peptide:IF(Me-EsterE)LNQR

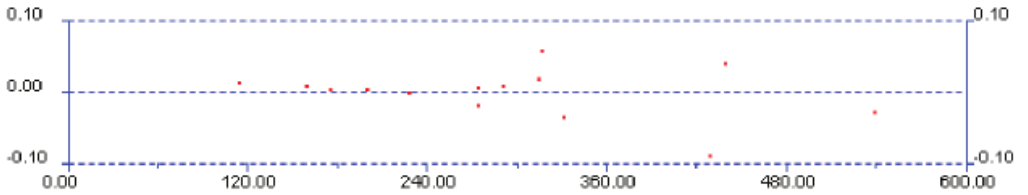
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				131.0631		I						7
2	261.1637	243.1285	233.1659		120.0861	F	820.4206					6
3	404.1920	386.1965		421.2226	116.0718	(Me-esterE)	673.4189		656.3805	657.3753		5
4	517.2840		489.2999			L	530.3238		513.3036			4
5						N	417.2396		400.1923	401.1837		3
6					101.0736	Q	303.1961	285.1761	286.1640			2
7						R	175.1236	157.1079	158.0938	159.1164		1

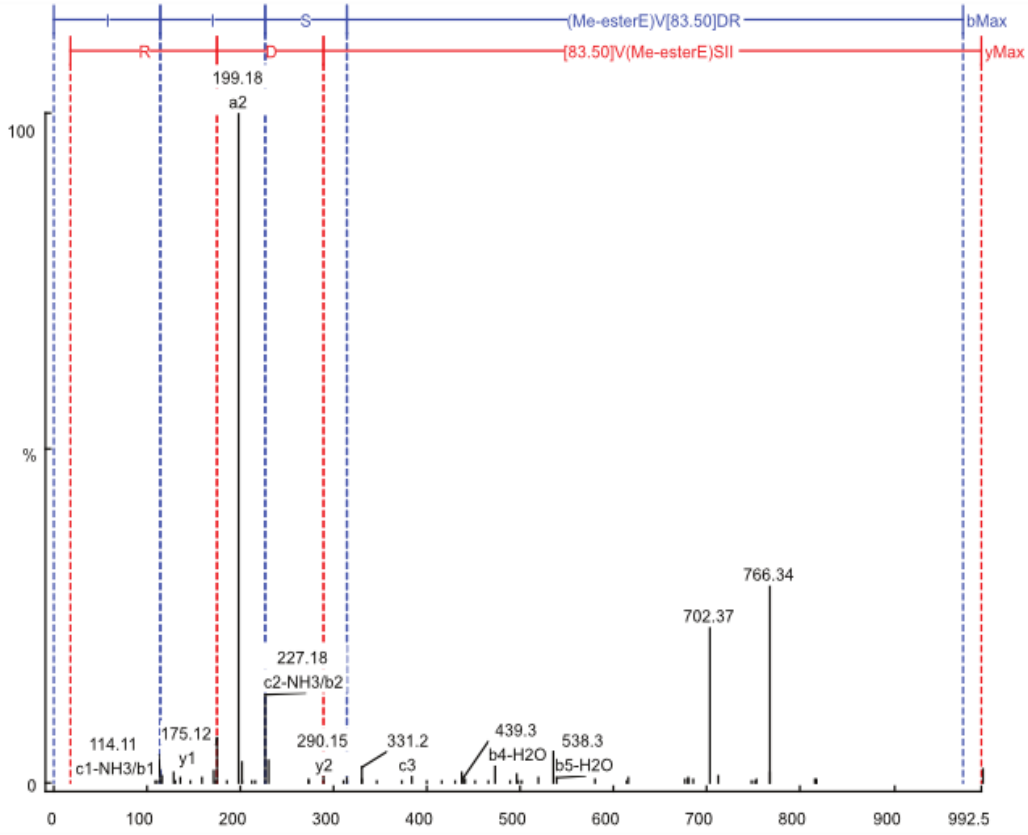




Peptide: IIS(Me-EsterE)V(Methionine oxidation with neutral loss = 83.50)DR

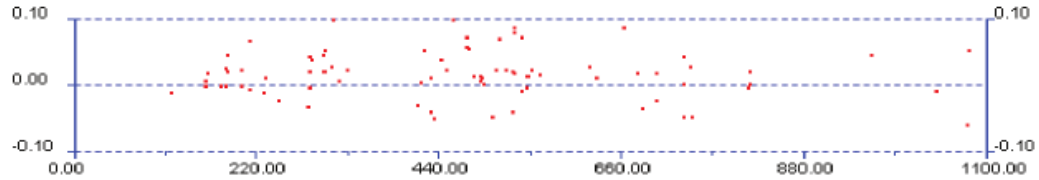
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1	114.1050					I						8
2	227.1750		199.1850			I						7
3	314.2260			331.2000		S						6
4		439.2960	429.1810		116.0720	(Me-est...						5
5		538.2960				V						4
6						[83.50]						3
7						D	290.1550		273.1010	274.1260		2
8						R	175.1240			159.1000		1

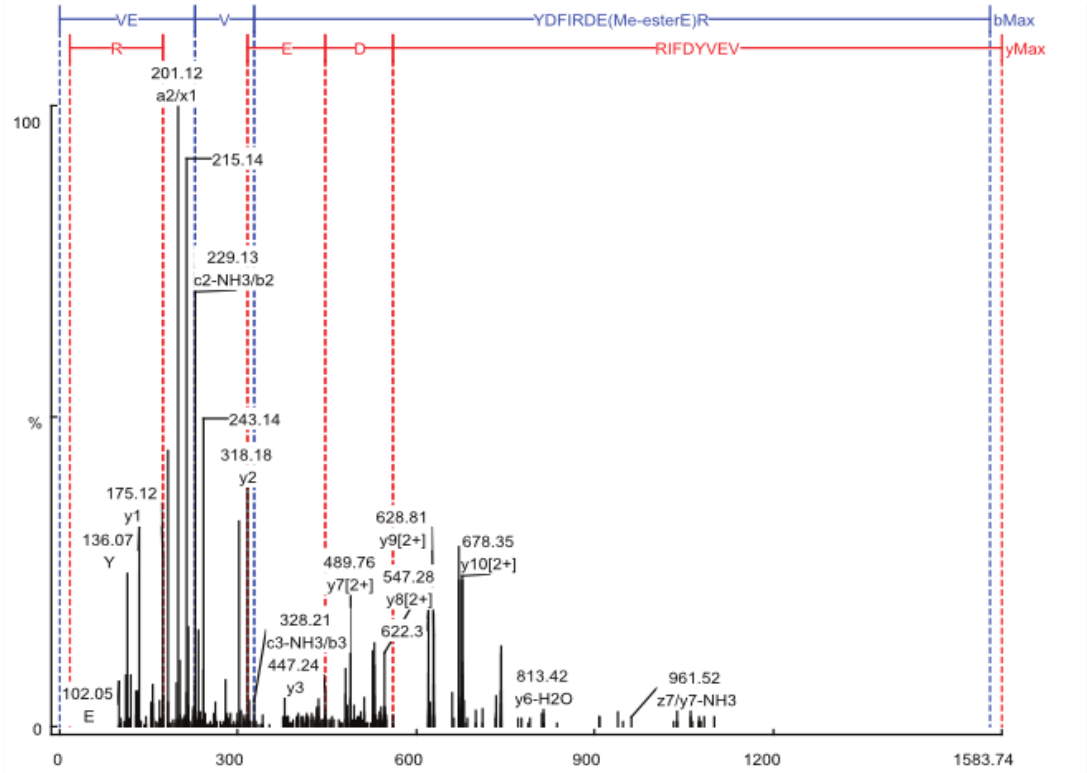




Peptide:VEVYDFIRDE(Me-EsterE)R

#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				117.0931		V						12
2	229.1298	211.1018	201.1227	246.1222	102.0538	E					742.8800	11
3	328.2109	310.2050	300.2124			V					678.3487	10
4		473.2964		508.3019	136.0750	Y					628.8076	9
5						D			1076.4...	1077.5...	547.2771	8
6					120.0763	F			961.5198		489.7639	7
7						I		813.4194	814.4250	815.4058	416.2194	6
8				1039.5...	129.1090	R			701.3401	702.2975		5
9						D	562.2626	544.2335	545.2332			4
10					102.0538	E	447.2435		430.1519			3
11					116.0718	(Me-est...	318.1847	300.2124	301.1718	302.2025		2
12					129.1090	R	175.1177	157.1079	158.0994	159.1108		1

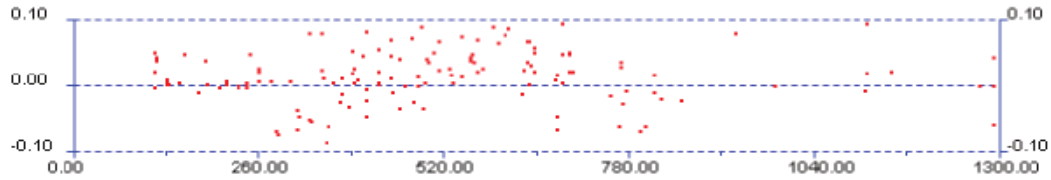


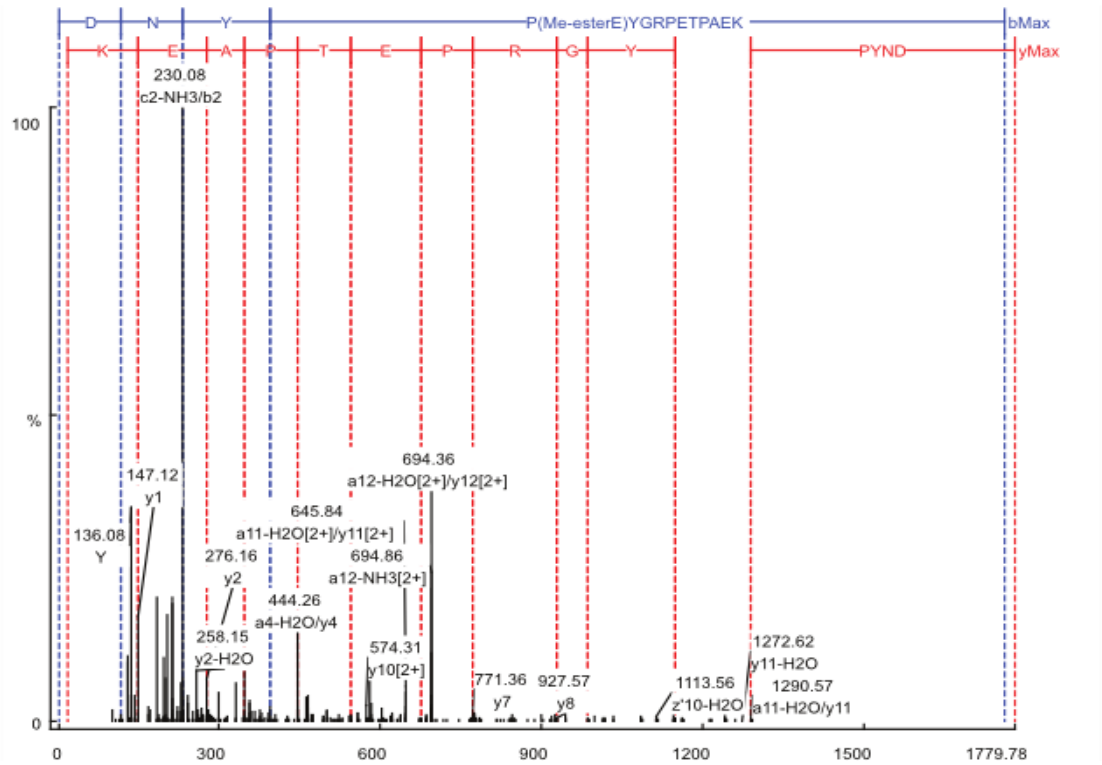


Supplementary Figure 1. Tandem mass spectral evidence of glutamic acid methylation in LIC11848 protein isoform 1. MS/MS analysis identified glutamic acid methylation of four peptides. For each peptide the identified peptide sequence and position of methylesterification is provided followed by ion coverage and the mass spectrum.

Peptide: DNYP(Me-EsterE)YGRPETPAEK

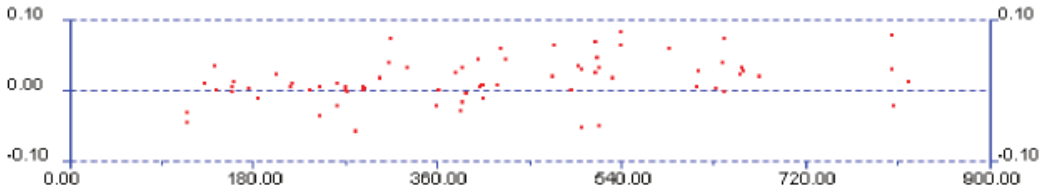
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1	116.0766			133.0628		D						15
2	230.0763	212.0688	202.0793	247.1523		N						14
3	393.1456	375.1412	365.1496	410.1611	136.0802	Y					775.8659	13
4						P					694.3625	12
5					116.0766	(Me-est...	1290.5...	1272.6...			645.8381	11
6				813.3304	136.0802	Y	1147.5...				574.3134	10
7						G	984.5113				492.7603	9
8					129.1039	R	927.5690				464.2449	8
9						P	771.3611	753.3629			386.1614	7
10					102.0538	E	674.3455					6
11						T	545.3061	527.3073	528.2811			5
12						P	444.2573	426.2373	427.2235	428.2385		4
13						A	347.2152	329.2626	330.1132	331.1111		3
14					102.0538	E	276.1617	258.1513	259.1487	260.1552		2
15						K	147.1161	129.1039	130.0893	131.0938		1

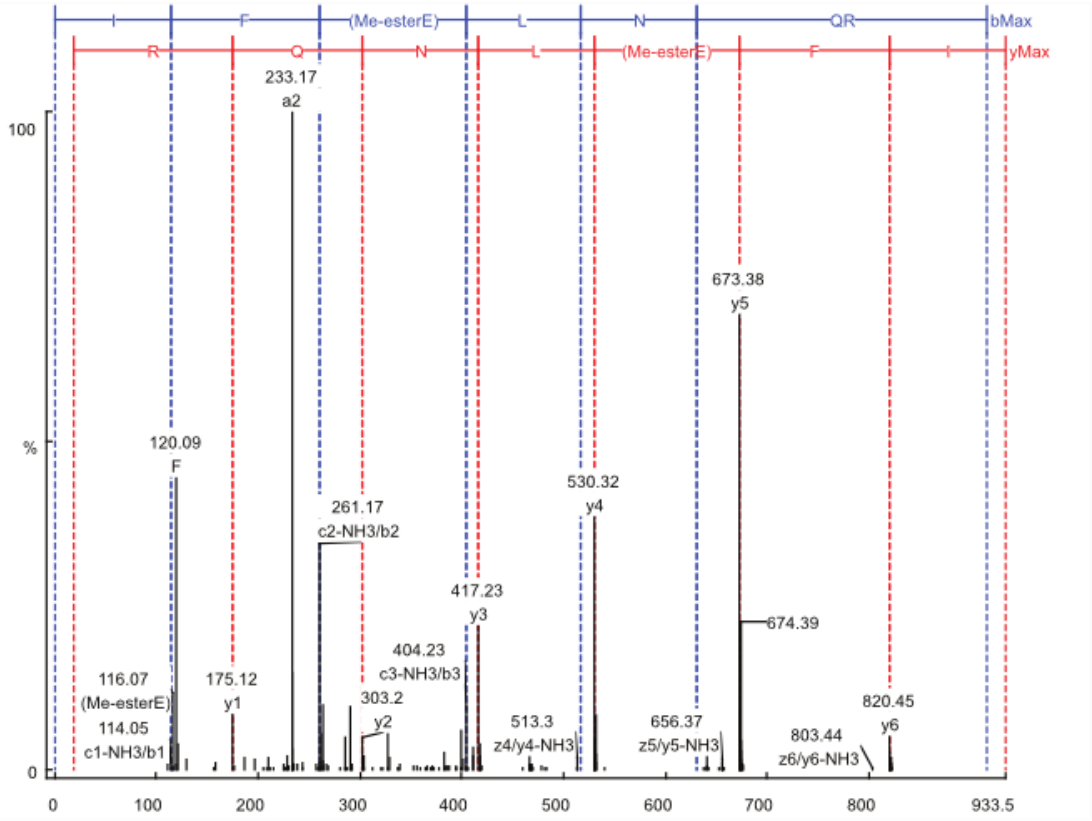




Peptide: IF(Me-EsterE)LNQR

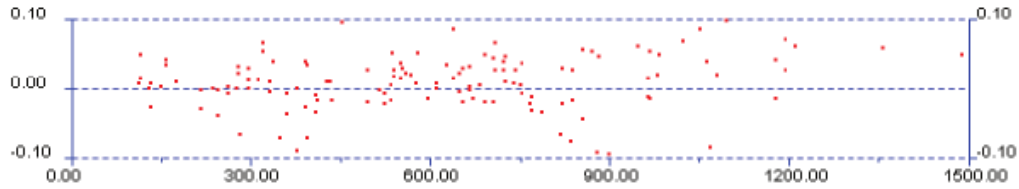
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1	114.0474			131.1295		I						7
2	261.1709	243.1146	233.1659	278.1300	120.0861	F	820.4461	802.5000	803.4356	804.3844		6
3	404.2279	386.2052	376.2481	421.3050	116.0718	(Me-est...	673.3842	655.3749	656.3690	657.3639		5
4	517.3347	499.2391	489.3098			L	530.3238	512.3639	513.3036	514.3251		4
5	631.3499	613.3409				N	417.2304	399.2557	400.2012	401.2015		3
6					101.0601	Q	303.1961	285.1685	286.1564	287.1536		2
7						R	175.1236	157.1079	158.0994	159.1052		1

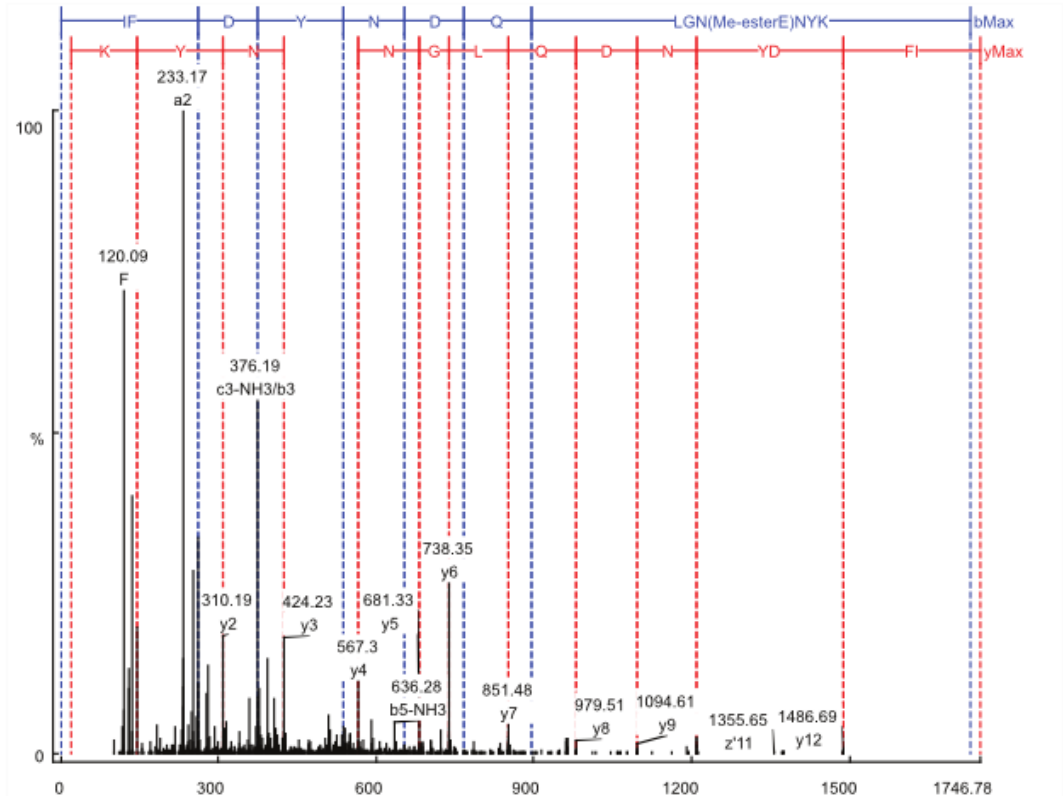




Peptide: IFDYNDQLGN(Me-EsterE)NYK

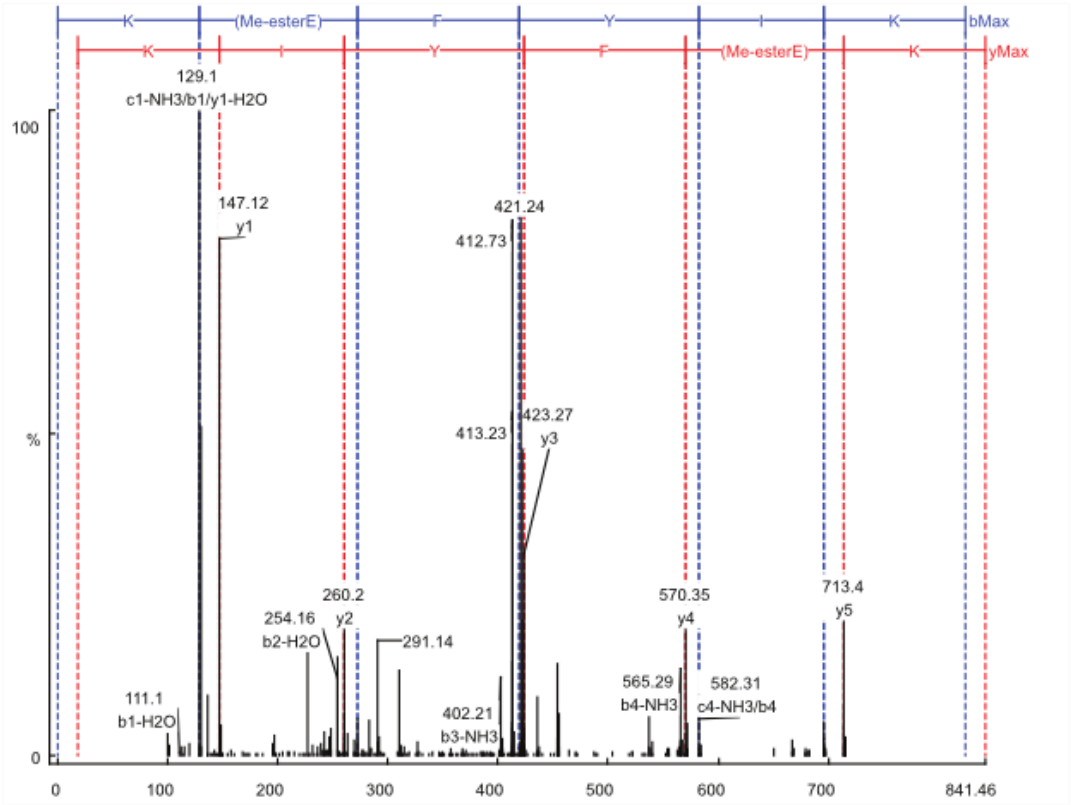
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				131.0938		I						14
2	261.1637	243.1494	233.1659	278.1226	120.0861	F						13
3	376.1875	358.1411	348.1220	393.1456		D	1486.6...					12
4	539.2695	521.2186	511.2536	556.3010	136.0750	Y				1355.6...		11
5	653.2749	635.3698	625.3328	670.3079		N	1208.6...		1191.6...	1192.5...		10
6	768.2910	750.3044	740.3527	785.3149		D	1094.6...			1078.5...		9
7	896.2843	878.2762	868.4397		101.0691	Q	979.5059	961.4645	962.4747	963.5131		8
8			981.5177			L	851.4829	833.3403	834.3839	835.4281		7
9						G	738.3509	720.3731	721.3434	722.3623		6
10						N	681.3269	663.3144	664.3260	665.2924		5
11					116.0718	(Me-est...	567.2992	549.3039	550.2664	551.2821		4
12						N	424.2315	406.1768	407.1846	408.1756		3
13					136.0750	Y	310.1893	292.1806	293.1805	294.1515		2
14						K	147.1161	129.1039	130.0944	131.0938		1





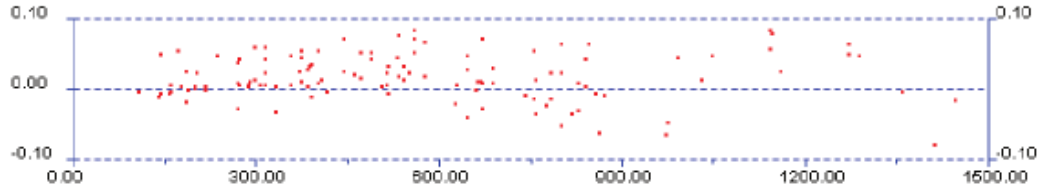
Peptide: K(Me-EsterE)FYIK

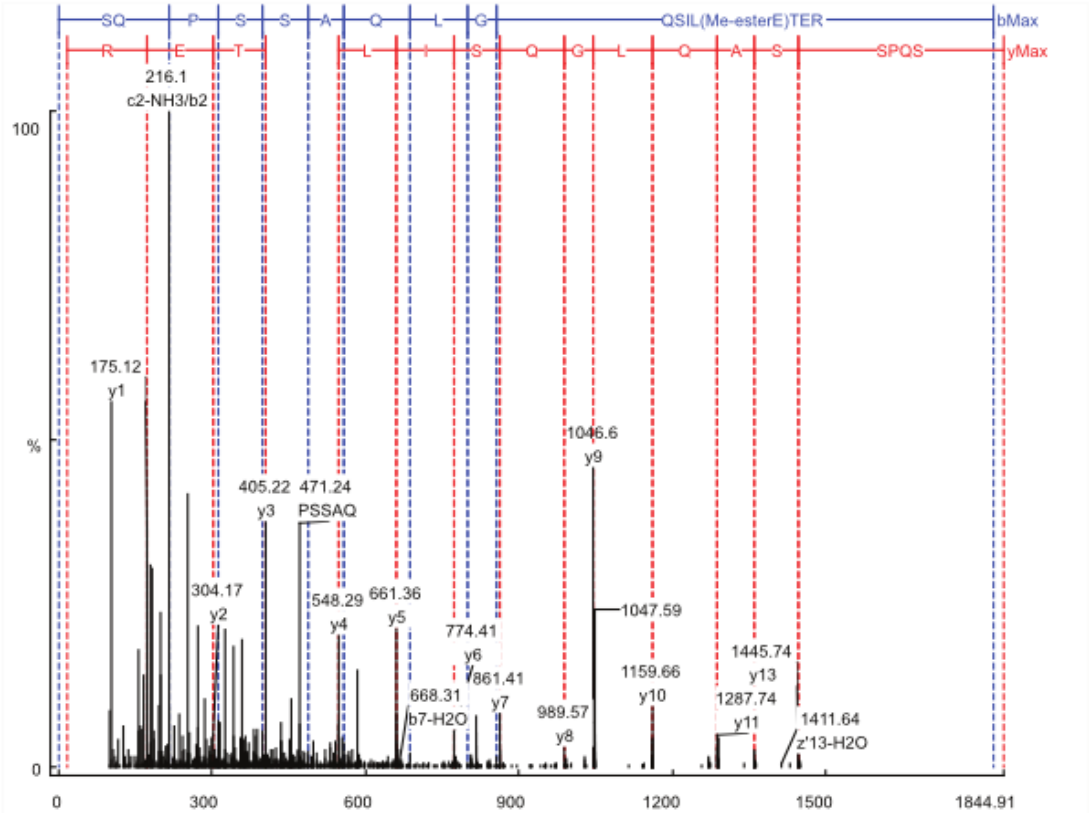
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1	129.1039	111.0982	101.1050			K						6
2	272.1576	254.1595	244.1732	289.1910	116.0766	(Me-est...	713.3965	695.3740	696.3862	697.3755		5
3	419.2379	401.1926	391.2017	436.1972	120.0861	F	570.3533	552.3721		554.3138		4
4	582.3069	564.2746	554.3138		136.0802	Y	423.2671	405.2063				3
5	695.3740		667.3886			I	260.1984	242.1833	243.1772	244.1732		2
6						K	147.1161	129.1039	130.0893	131.0886		1



Peptide: SQPSSAQLGQSIL(Me-EsterE)TER

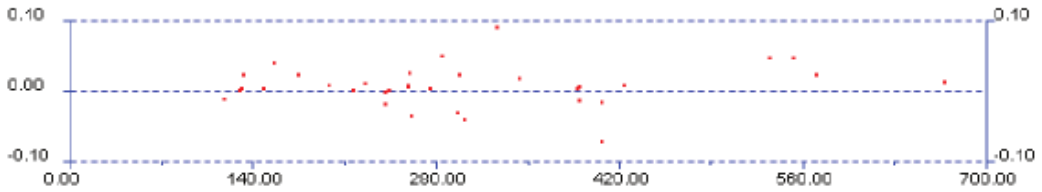
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				105.0641		S						17
2	216.1033	198.0906	188.1032	233.1727	101.0691	Q						16
3	313.1747	295.1548	285.1610	330.1457		P						15
4	400.1923	382.1998	372.2001			S						14
5	487.2689	469.2573	459.2402	504.2461		S	1445.7...					13
6	558.3233	540.2750	530.3032	575.2975		A	1358.7...					12
7	686.3206	668.3110	658.3137		101.0691	Q	1287.7...		1270.7...	1271.7...		11
8	799.4189			816.3871		L	1159.6...	1141.6...	1142.6...	1143.6...		10
9	856.4113	838.4486				G	1046.5...		1029.5...			9
10					101.0691	Q	989.5717	971.4513	972.4528			8
11						S	861.4064		844.5058			7
12						I	774.4125	756.3908	757.4219			6
13						L	661.3624	643.3007	644.3535	645.3733		5
14					116.0718	(Me-est...	548.2901	530.3032	531.2592	532.3190		4
15						T	405.2242	387.2318	388.1718	389.2186		3
16					102.0718	E	304.1680	286.1564	287.1460	288.1449		2
17						R	175.1236	157.1023	158.0994	159.0883		1

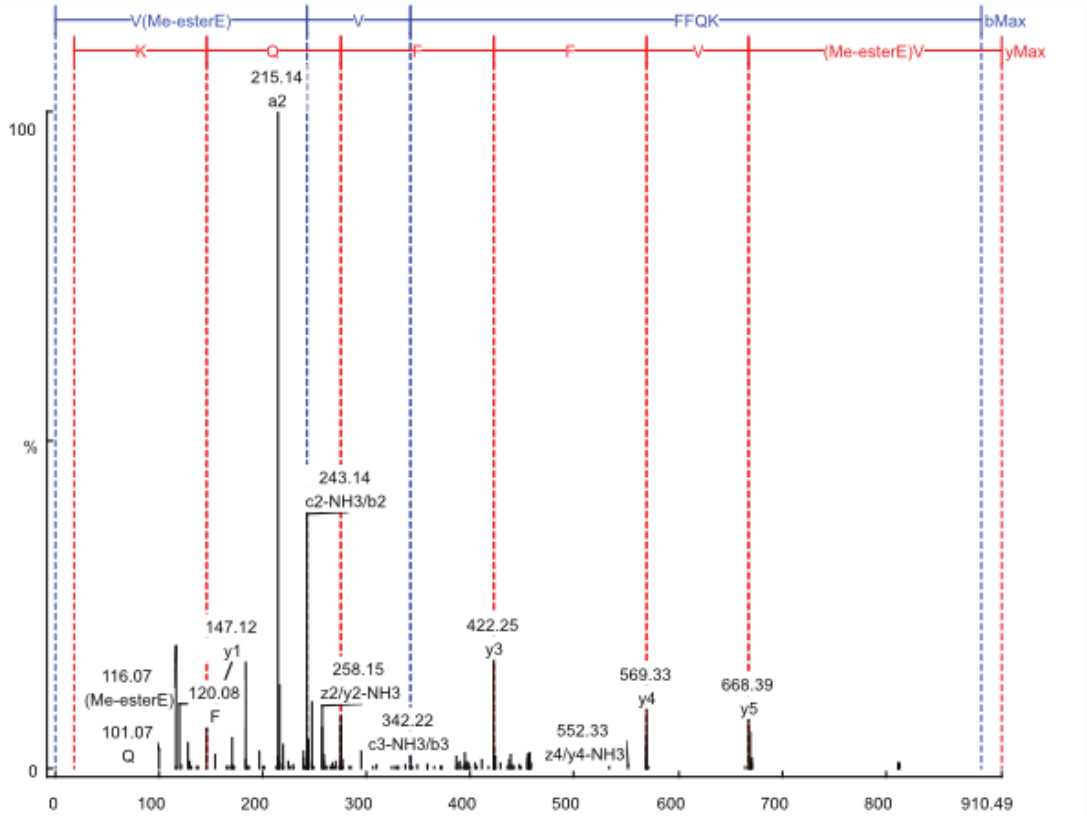




Peptide: V(Me-EsterE)VFFQK

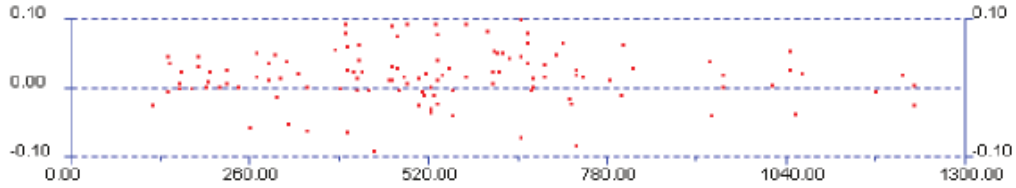
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				117.0931		V						7
2	243.1355	225.1356	215.1402	260.1264	116.0718	(Me-est..						6
3	342.2200					V	668.3917					5
4					120.0812	F	569.3308		552.3302			4
5					120.0812	F	422.2489		405.1973	406.1409		3
6					101.0691	Q	275.1764	257.1701	258.1513	259.1702		2
7						K	147.1161	129.1039	130.0893	131.1091		1

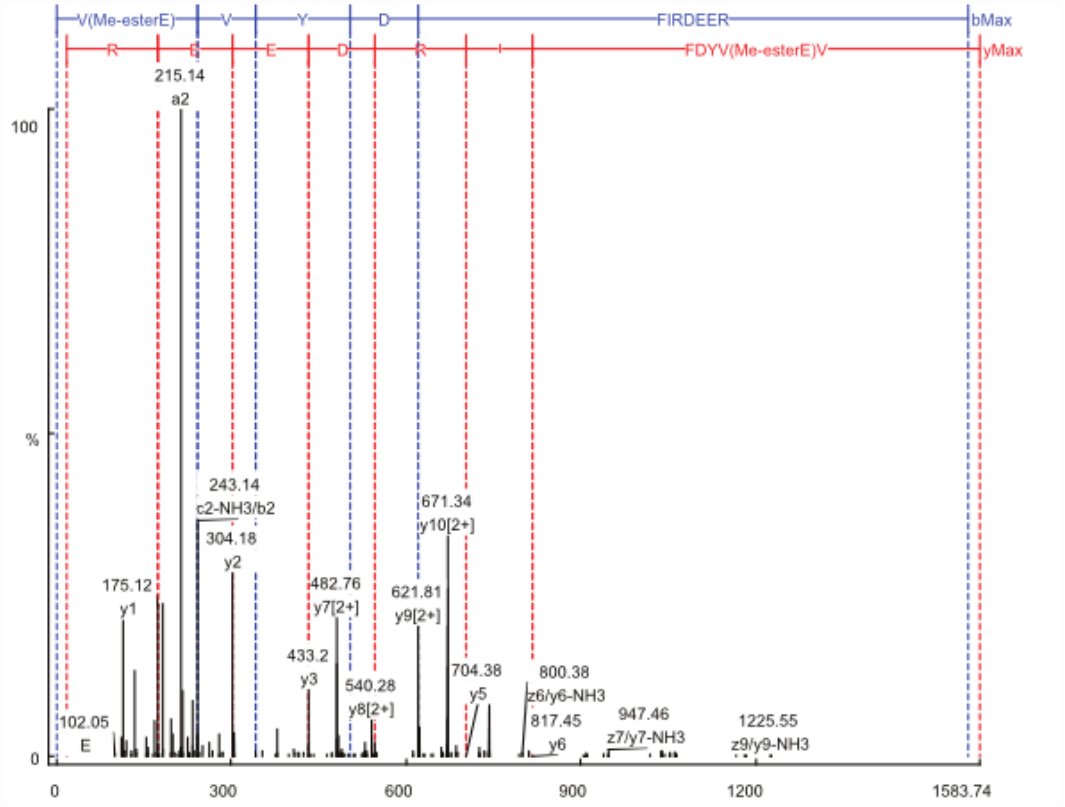




Peptide: V(Me-EsterE)VYDFIRDEER

#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				117.0786		V						12
2	243.1355	225.1289	215.1402	260.1048	116.0718	(Me-est...					742.8678	11
3	342.2035		314.1545			V					671.3364	10
4	505.2786	487.3477		522.2581	136.0802	Y			1225.5...	1226.5...	621.8121	9
5	620.3443			637.3620		D			1062.5...		540.2750	8
6					120.0812	F			947.4597	948.4763	482.7587	7
7						I	817.4456		800.3780	801.4513	409.2309	6
8				1053.5...	129.1141	R	704.3801		687.3379	688.3208		5
9				1168.5...		D	548.2587	530.3135		532.2161		4
10					102.0493	E	433.2018	415.1915	416.1921	417.2396		3
11					102.0493	E	304.1758	286.1866	287.1460			2
12					129.1141	R	175.1177	157.1079	158.0994	159.1164		1

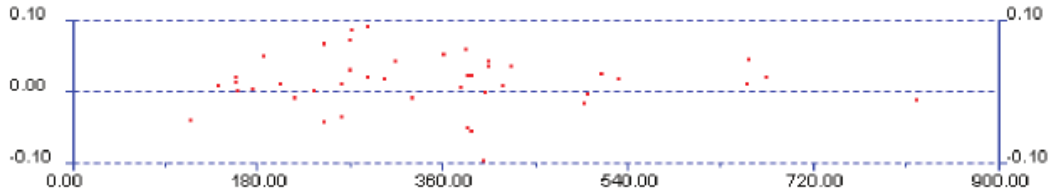


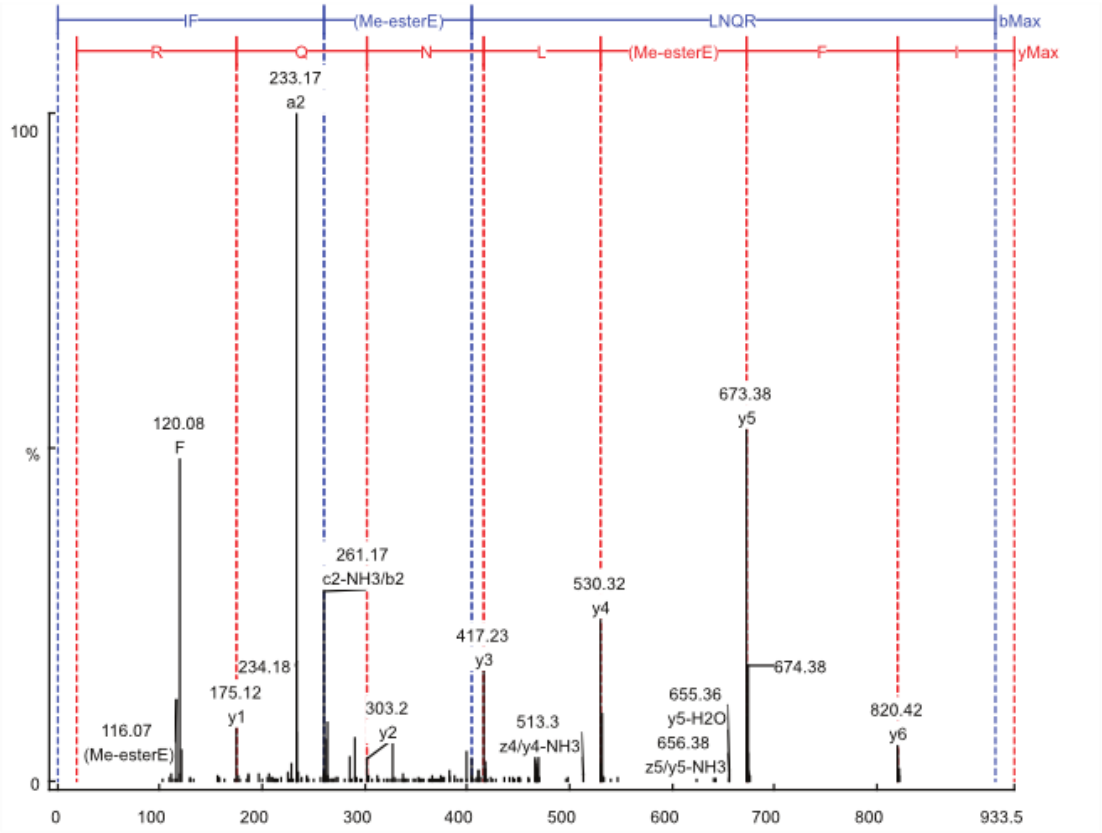


Supplementary Figure 2. Tandem mass spectral evidence of glutamic acid methylation in LIC11848 protein isoform 2. MS/MS analysis identified glutamic acid methylation of seven peptides. For each peptide the identified peptide sequence and position of methylesterification is provided followed by ion coverage and the mass spectrum.

Peptide: IF(Me-EsterE)LNQR

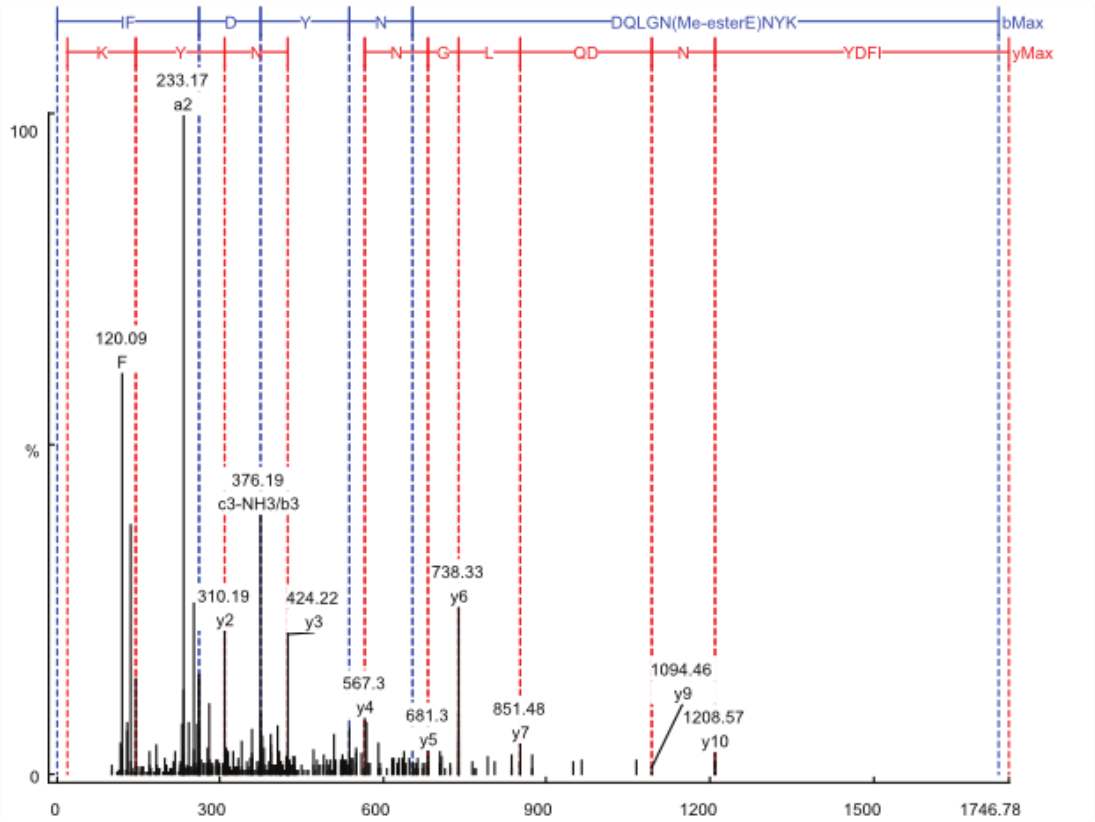
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1						I						7
2	261.1709	243.1077	233.1659		120.0812	F	820.4206					6
3	404.2548	386.1526	376.2308		116.0718	(Me-est...	673.3842	655.3635	656.3805			5
4		499.2889				L	530.3238		513.3036			4
5						N	417.2304	399.1130	400.1923			3
6					101.0556	Q	303.1961	285.2590	286.1715			2
7						R	175.1236	157.1303	158.1050	159.0939		1





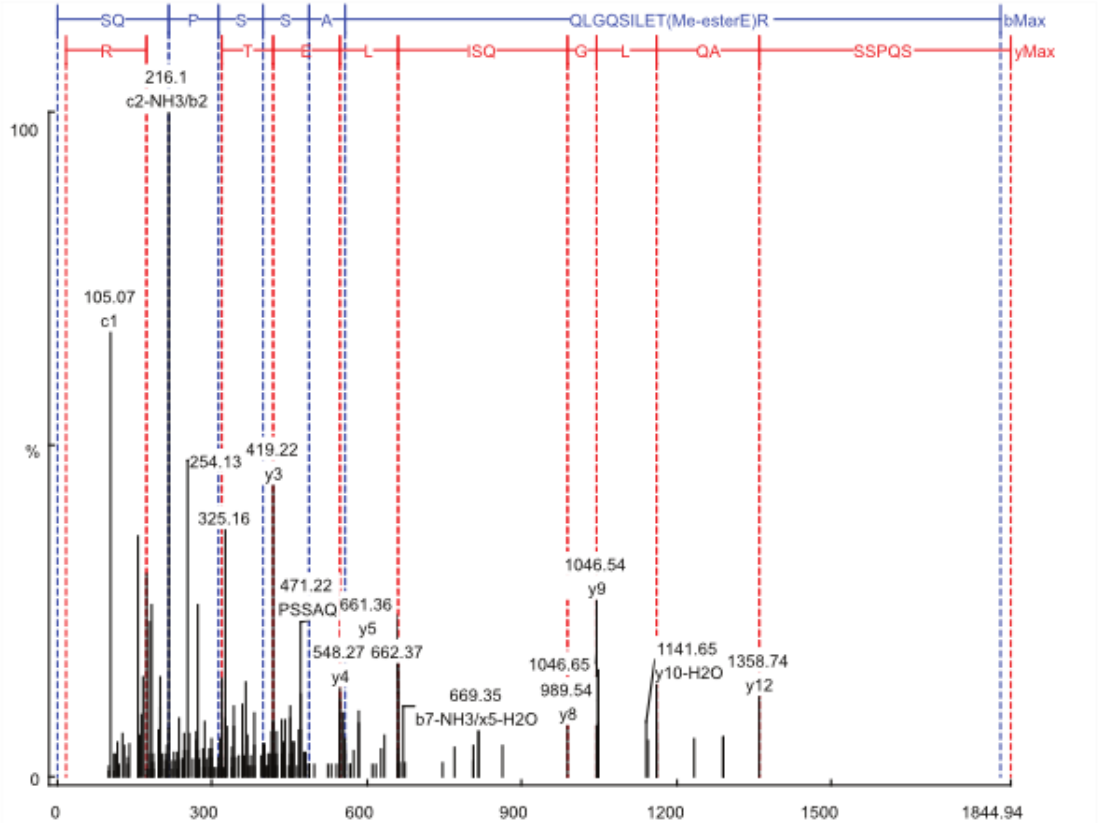
Peptide: IFDYNDQLGN(Me-EsterE)NYK

#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1						I						14
2	261.1637	243.1355	233.1727	278.1226	120.0861	F						13
3	376.1875	358.1242	348.1886	393.1279		D						12
4	539.2902	521.1982	511.3242		136.0802	Y						11
5	653.3091					N	1208.5...					10
6						D	1094.4...					9
7					101.1095	Q						8
8						L	851.4829		834.3710			7
9						G	738.3267					6
10						N	681.3036	663.2685	664.3835			5
11					116.0766	(Me-est...	567.2992	549.2830	550.3083			4
12						N	424.2223		407.1936	408.1665		3
13					136.0802	Y	310.1893	292.1577				2
14						K	147.1161	129.1090	130.0944			1



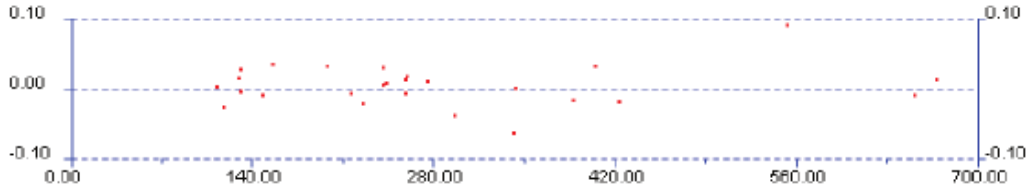
Peptide: SQPSSAQLGQSILET(Me-EsterE)R

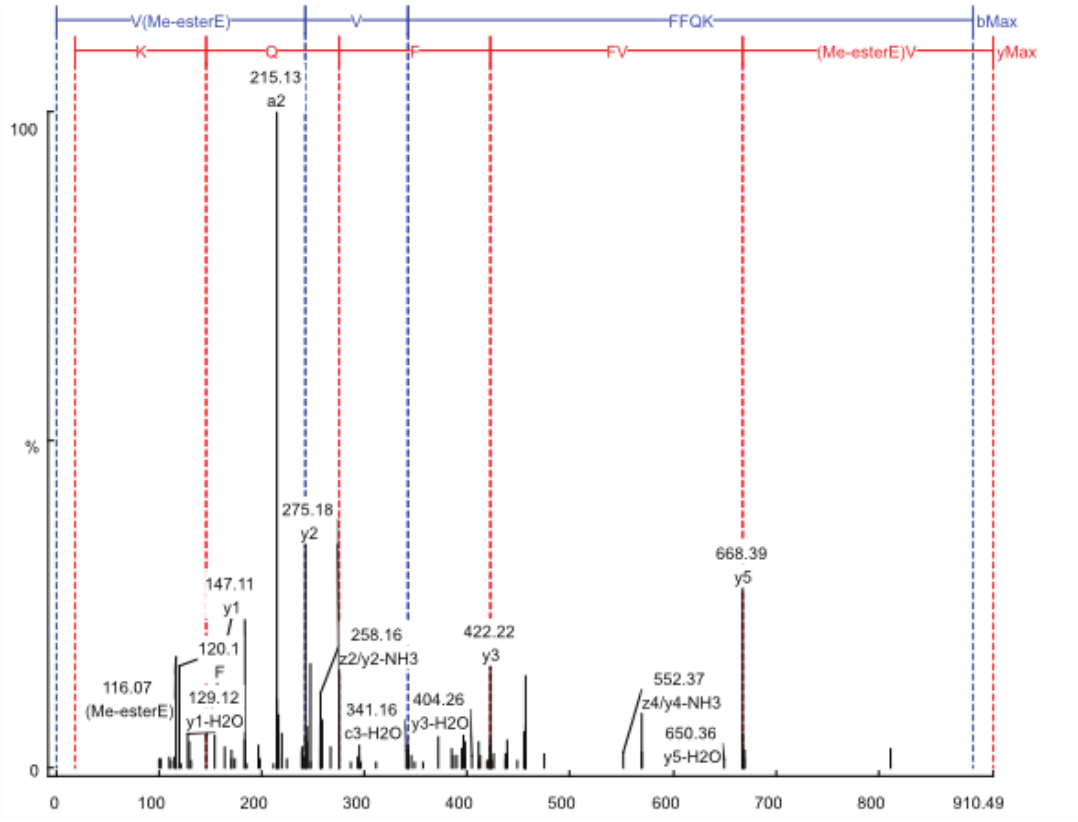
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				105.0686		S						17
2	216.1033	198.0718	188.1155		101.0915	Q						16
3	313.1747		285.1761			P						15
4	400.2280	382.1736	372.2776			S						14
5	487.2984	469.2187	459.2402			S						13
6	558.2917	540.2543		575.3403		A	1358.7...					12
7					101.0915	Q						11
8						L	1159.6...	1141.6...		1143.6...		10
9						G	1046.5...					9
10					101.0915	Q	989.5436					8
11						S						7
12						I						6
13						L	661.3624					5
14					102.0628	E	548.2692			532.2572		4
15						T	419.2196	401.1926	402.1941			3
16					116.0766	(Me-est...	318.1927		301.1563			2
17						R	175.1177	157.1023	158.1162	159.0827		1



Peptide: V(Me-EsterE)VFFQK

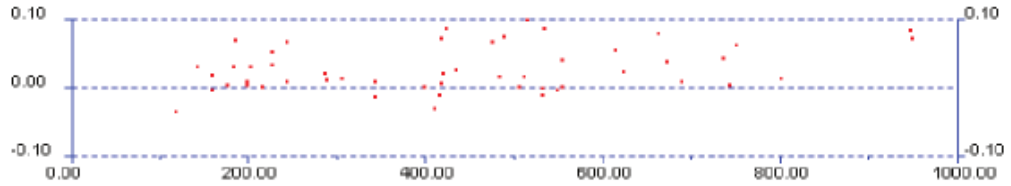
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				117.0786		V						7
2	243.1424	225.1021	215.1336		116.0718	(Me-est...						6
3	342.2035					V	668.3917	650.3588				5
4					120.0959	F			552.3721			4
5					120.0959	F	422.2213	404.2638				3
6					101.1050	Q	275.1838	257.1558	258.1584	259.1631		2
7						K	147.1053	129.1191	130.1148	131.0835		1

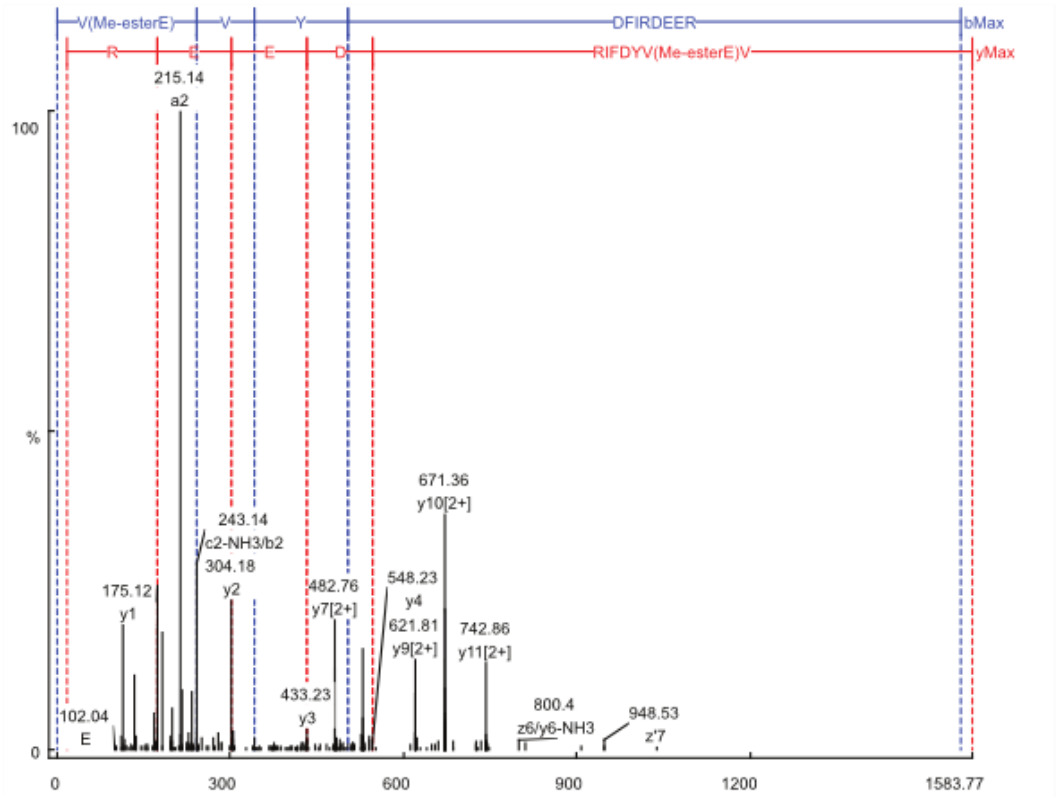




Peptide: V(Me-EsterE)VYDFIRDEER

#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				117.0690		V						12
2	243.1424	225.1557	215.1402		116.0766	(Me-est...					742.8557	11
3	342.2117					V					671.3595	10
4	505.2686				136.0855	Y					621.8121	9
5						D						8
6		749.4125			120.0812	F			947.5421	948.5312	482.7587	7
7						I			800.4032		409.1767	6
8					129.1039	R			687.3145			5
9						D	548.2274	530.2108				4
10					102.0448	E	433.2296	415.1824	416.1830	417.2487		3
11					102.0448	E	304.1758	286.1715	287.1460			2
12					129.1039	R	175.1236		158.0881	159.1108		1

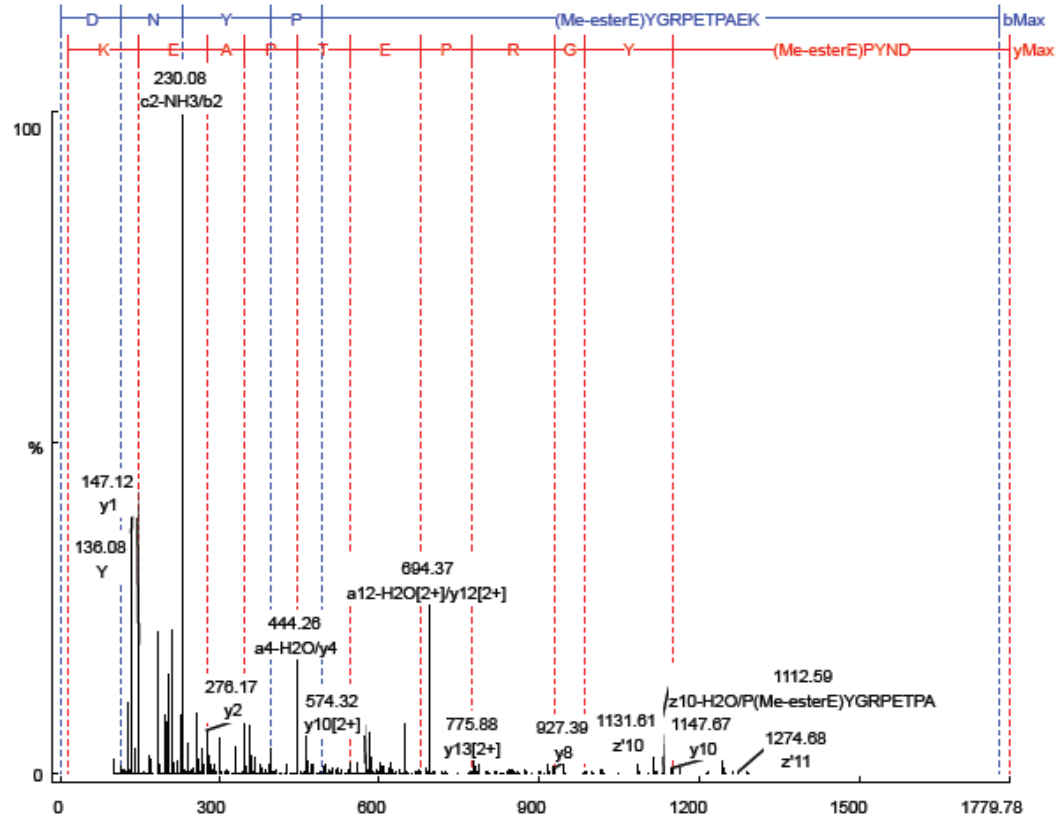




Supplementary Figure 3. Tandem mass spectral evidence of glutamic acid methylation in LIC11848 protein isoform 3. MS/MS analysis identified glutamic acid methylation of five peptides. For each peptide the identified peptide sequence and position of methylesterification is provided followed by ion coverage and the mass spectrum.

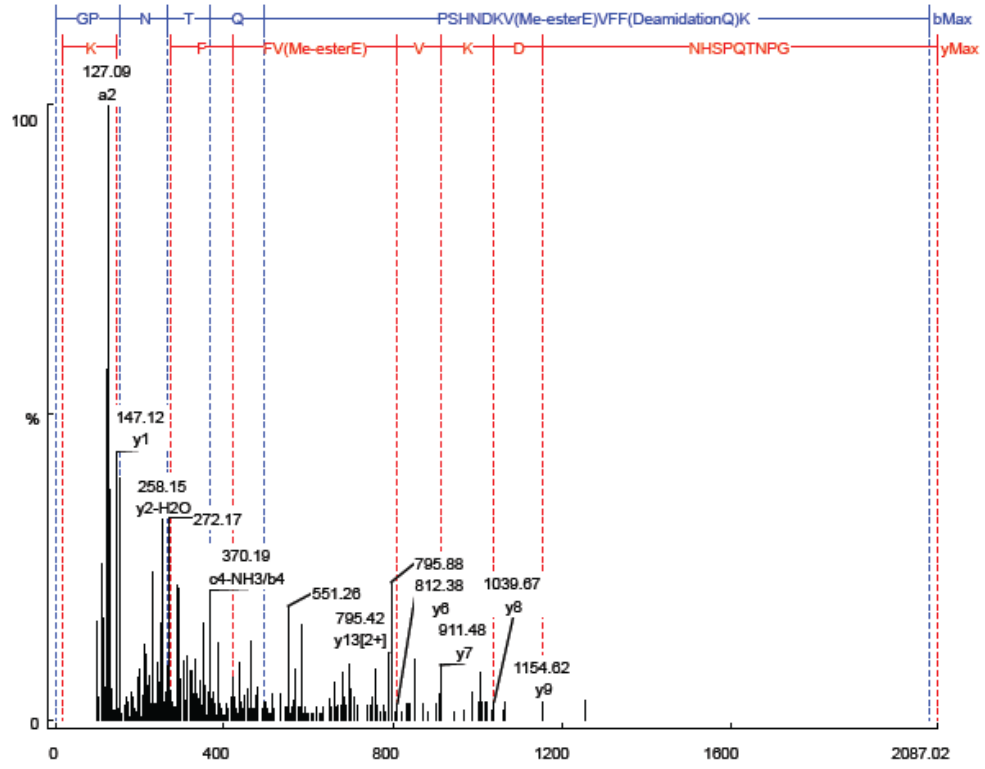
Peptide: DNYP(Me-EsterE)YGRPETPAEK

#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1	116.0766			133.0628		D						15
2	230.0763	212.0688	202.0856	247.1312		N						14
3	393.1456	375.1239	365.1581		136.0802	Y					775.8783	13
4	490.1095			507.2362		P					694.3742	12
5		615.3094			116.0766	(Me-est...				1274.6...	645.8381	11
6					136.0802	Y	1147.6...			1131.6...	574.3241	10
7			825.3610			G	984.4833				492.7702	9
8					129.1090	R	927.3924				464.2353	8
9						P	771.3735				386.1439	7
10					102.0493	E	674.3455					6
11						T	545.3166	527.3175		529.3173		5
12						P	444.2573	426.2557	427.2050	428.1647		4
13						A	347.1819		330.1213	331.1355		3
14					102.0493	E	276.1691	258.1513	259.1559	260.1192		2
15						K	147.1161	129.1090	130.0944	131.0938		1



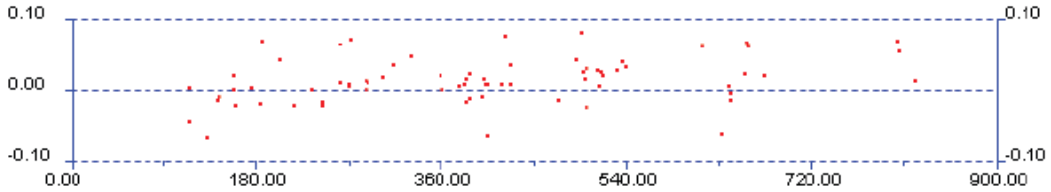
Peptide: GPNTQPSHNDKV(Me-EsterE)VFF(Deamidation Q)K

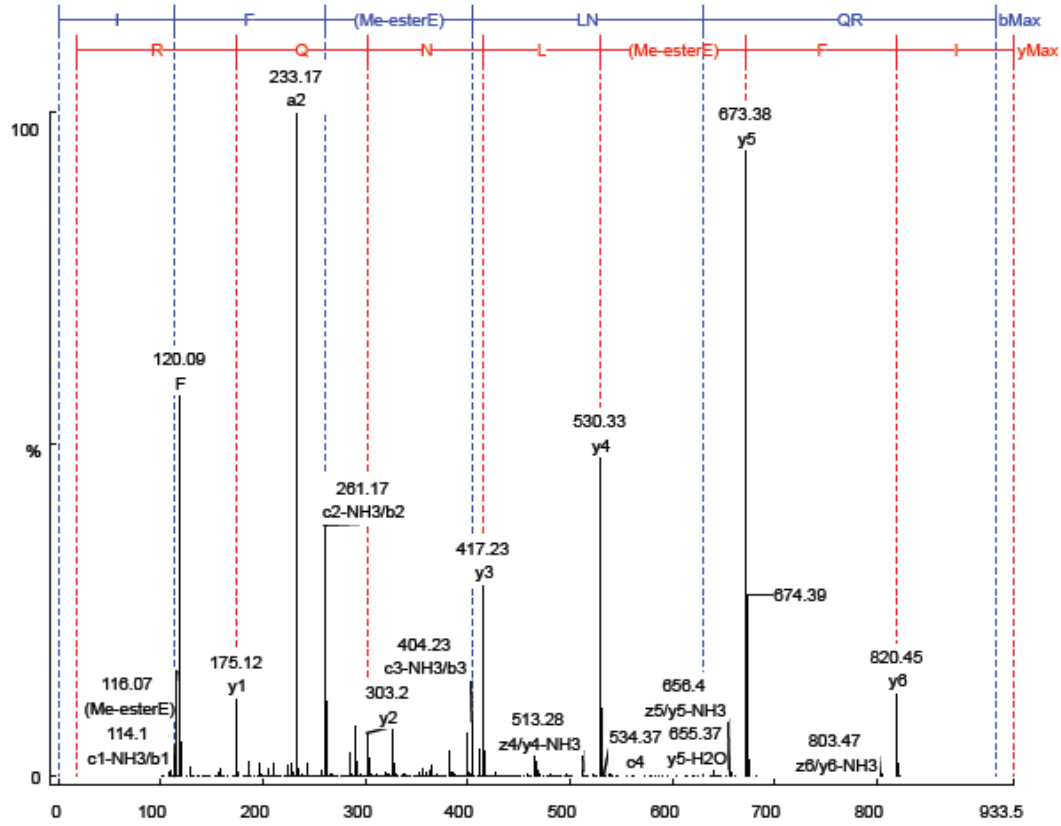
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1						G						18
2	155.0844	137.1023	127.0941	172.0902		P						17
3	269.1408	251.1101	241.1498	286.1338		N						16
4	370.1886	352.1628	342.2447			T						15
5	498.2426	480.2328	470.2921			Q						14
6		577.2790	567.2567			P					795.4249	13
7			654.2903			S					746.8132	12
8				836.4601	110.0707	H					703.3029	11
9						N						10
10						D	1154.6...					9
11					101.0691	K	1039.6...					8
12						V	911.4824					7
13					116.0718	(Me-est...	812.3763	794.4437		796.4057		6
14						V		651.3833				5
15					120.0861	F		552.2882	553.3583	554.2403		4
16					120.0861	F	423.1937	405.1793	406.2038			3
17					102.0628	(Deami...	276.2284	258.1513	259.1702			2
18					101.0691	K	147.1161	129.1090	130.0944	131.0784		1



Peptide: IF(Me-EsterE)LNQR

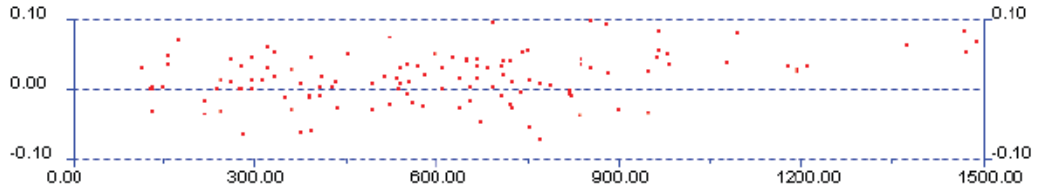
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1	114.0950			131.0529		I						7
2	261.1709	243.1285	233.1659		120.0861	F	820.4461		803.4735	804.4603		6
3	404.2279	386.1965	376.2308	421.3234	116.0718	(Me-est...	673.3842	655.3749	656.4033	657.3982		5
4		499.2690	489.3492	534.3695		L	530.3341	512.3235	513.2834	514.3048		4
5	631.2826	613.3962				N	417.2304	399.2022	400.2102	401.2015		3
6					101.0556	Q	303.1961	285.1685	286.1640	287.1611		2
7						R	175.1236	157.1303	158.0938	159.0714		1

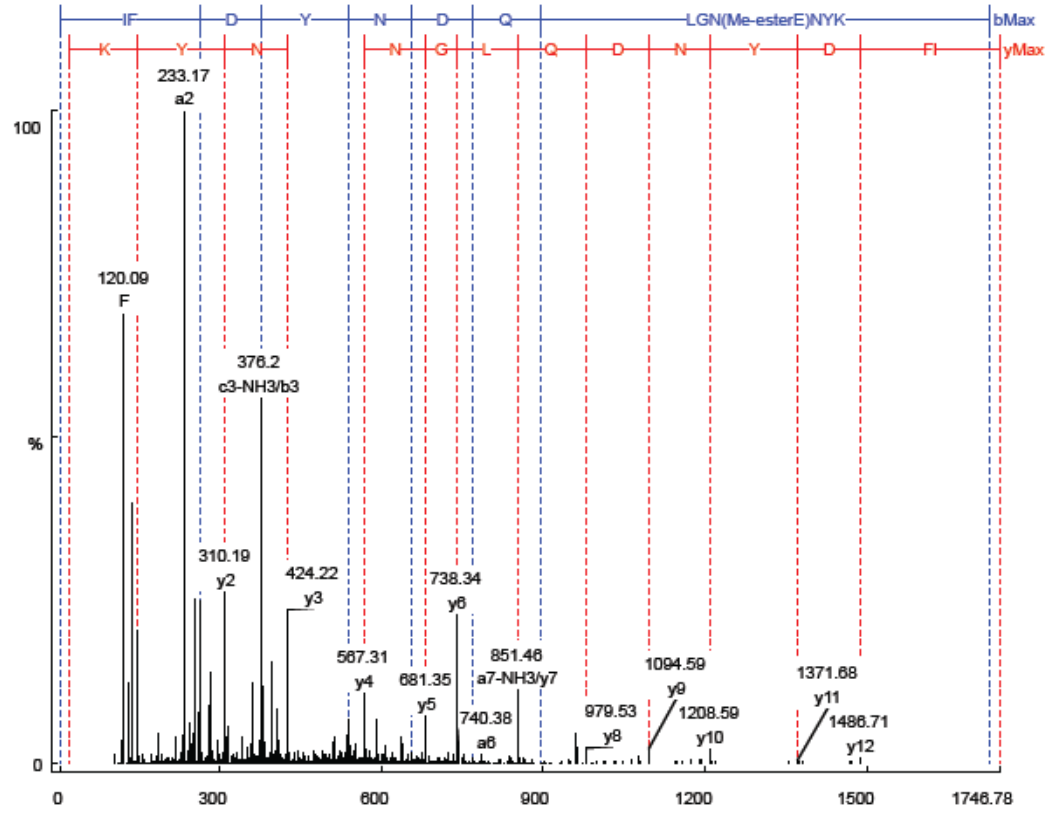




Peptide: IFDYNDQLGN(Me-EsterE)NYK

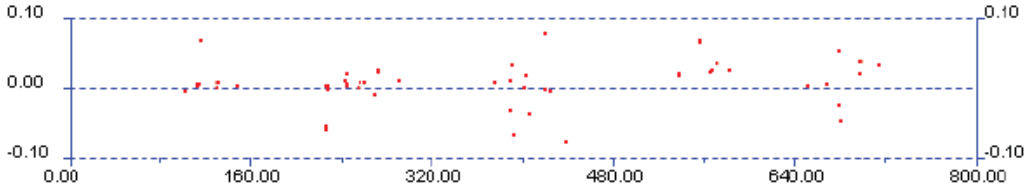
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				131.0886		I						14
2	261.1709	243.1633	233.1659	278.1226	120.0861	F						13
3	376.1962	358.1496	348.1802	393.1544		D	1486.7...	1468.7...		1470.6...		12
4	539.2799	521.2186	511.2737	556.2589	136.0802	Y	1371.6...					11
5	653.2977	635.2573	625.3440	670.2733		N	1208.5...		1191.5...	1192.5...		10
6	768.3281	750.2556	740.3770	785.3524		D	1094.5...		1077.5...			9
7	896.3511	878.4613			101.0691	Q	979.5338	961.5198	962.5024	963.5131		8
8			981.5037			L	851.4568	833.3789	834.4354	835.4410		7
9						G	738.3388	720.3731	721.2955	722.2904		6
10						N	681.3502	663.3259	664.3375	665.3269		5
11					116.0718	(Me-est...	567.3099	549.2621	550.2873	551.2611		4
12						N	424.2223	406.2128	407.1846	408.2116		3
13					136.0802	Y	310.1893	292.1806	293.1958	294.1515		2
14						K	147.1161	129.1039	130.0893	131.0886		1

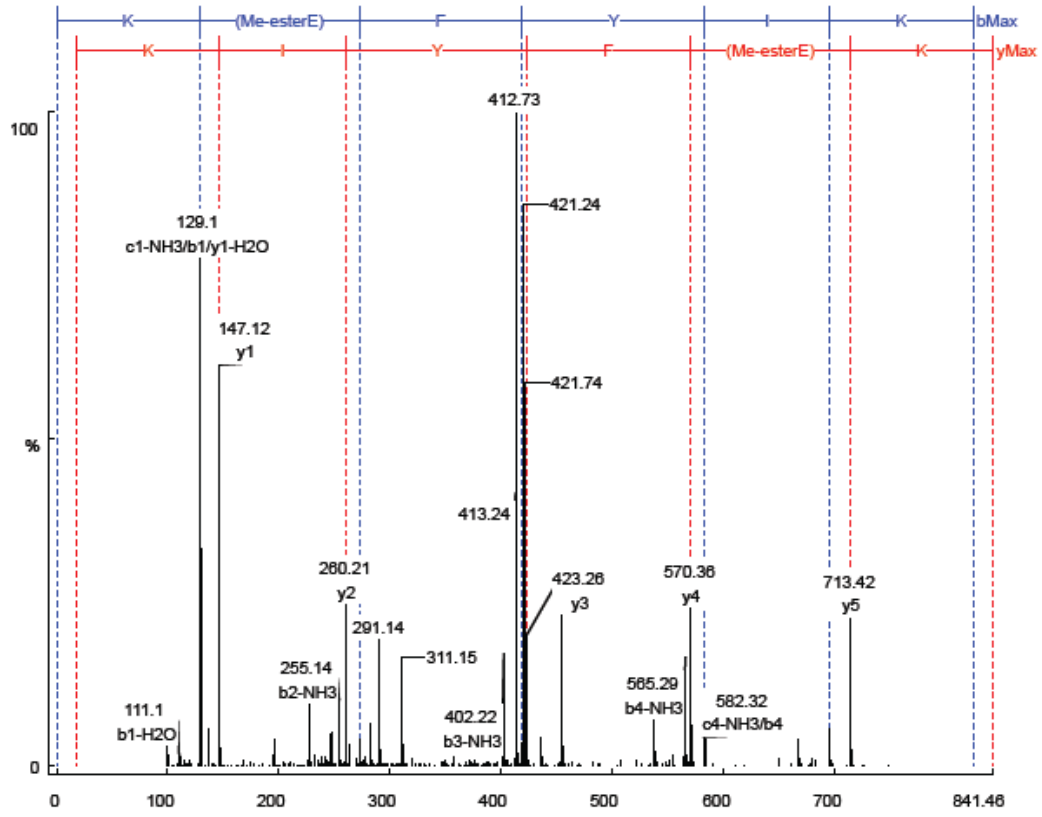




Peptide: K(Me-EsterE)FYIK

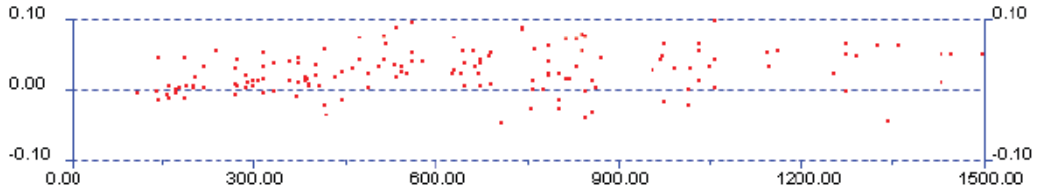
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1	129.1039	111.0982	101.1050			K						6
2	272.1870	254.1524	244.1732	289.1986	116.0766	(Me-est..	713.4203	695.3975	696.3979	697.3991		5
3	419.2287	401.2194	391.1664	436.1785	120.0861	F	570.3639			554.3663		4
4	582.3177	564.3064	554.3663		136.0750	Y	423.2580	405.2153				3
5	695.3975	677.4194	667.3886			I	260.2056	242.1972	243.1911	244.1732		2
6						K	147.1161	129.1039	130.0944	131.0938		1

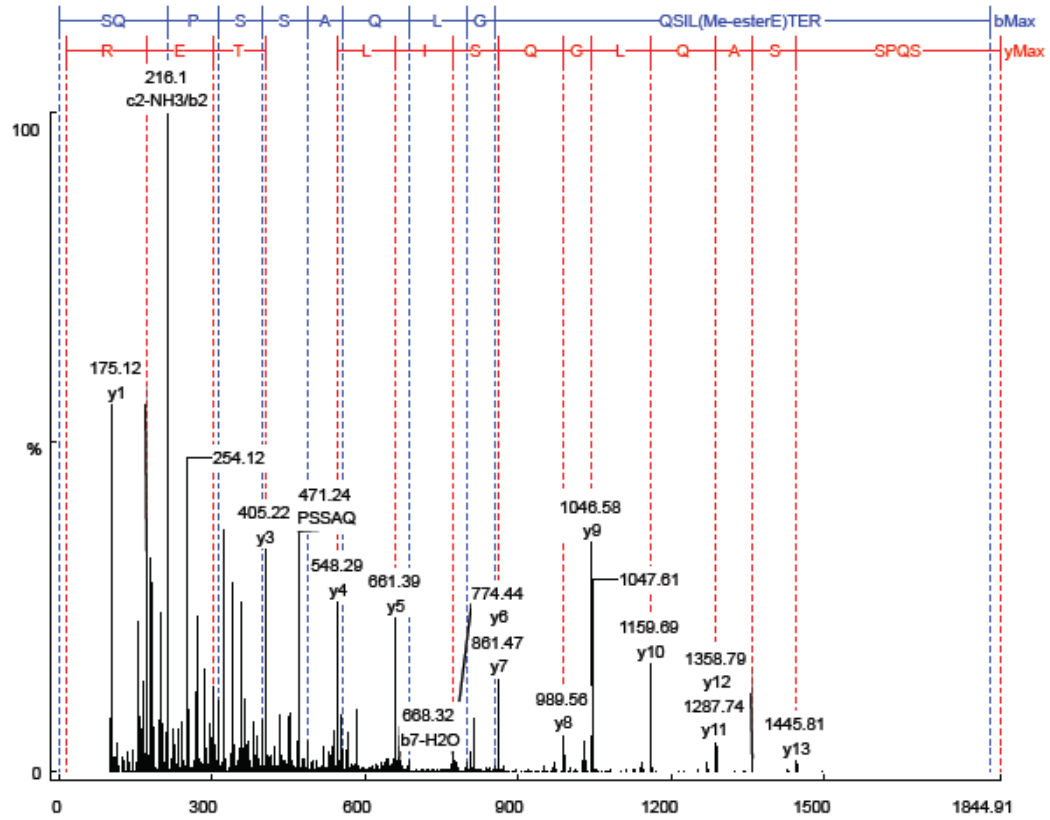




Peptide: SQPSSAQLGQSIL(Me-EsterE)TER

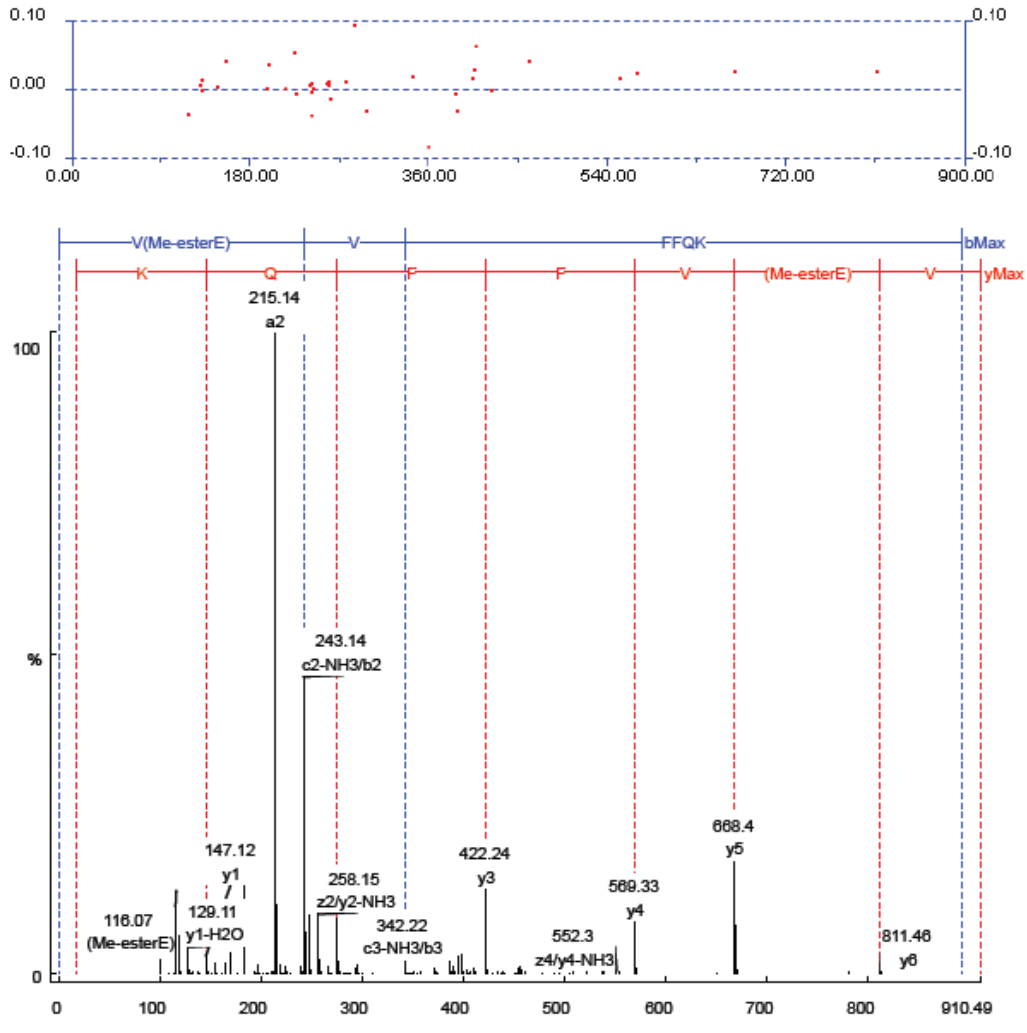
#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				105.0641		S						17
2	216.1033	198.0906	188.1094	233.1795	101.0691	Q						16
3	313.1668	295.1472	285.1610	330.1781		P						15
4	400.2191	382.1911	372.2001	417.1758		S						14
5	487.2393	469.2477	459.2498	504.2762		S	1445.8...	1427.7...	1428.7...			13
6	558.2917	540.2750	530.2930	575.3189		A	1358.7...		1341.6...			12
7	686.3206	668.3225	658.3710	703.2911	101.0691	Q	1287.7...	1269.6...	1270.7...	1271.7...		11
9	799.4189	781.4062		816.4381		L	1159.6...		1142.6...	1143.6...		10
9	856.3852	838.4615				G	1046.5...	1028.6...	1029.5...	1030.5...		9
10		966.5069			101.0691	Q	989.5576	971.5626	972.5641	973.4827		8
11		1053.5...				S	861.4719	843.4558	844.5187	845.4007		7
12						I	774.4373	756.4399	757.4096	758.4660		6
13			1269.6...			L	661.3853	643.3799	644.3309	645.3619		5
14					116.0718	(Me-est...	548.2901	530.2930	531.2592	532.3293		4
15						T	405.2153	387.2143	388.1894	389.1922		3
16					102.0583	E	304.1758	286.1715	287.1460	288.1449		2
17						R	175.1236	157.1023	158.0994	159.0827		1





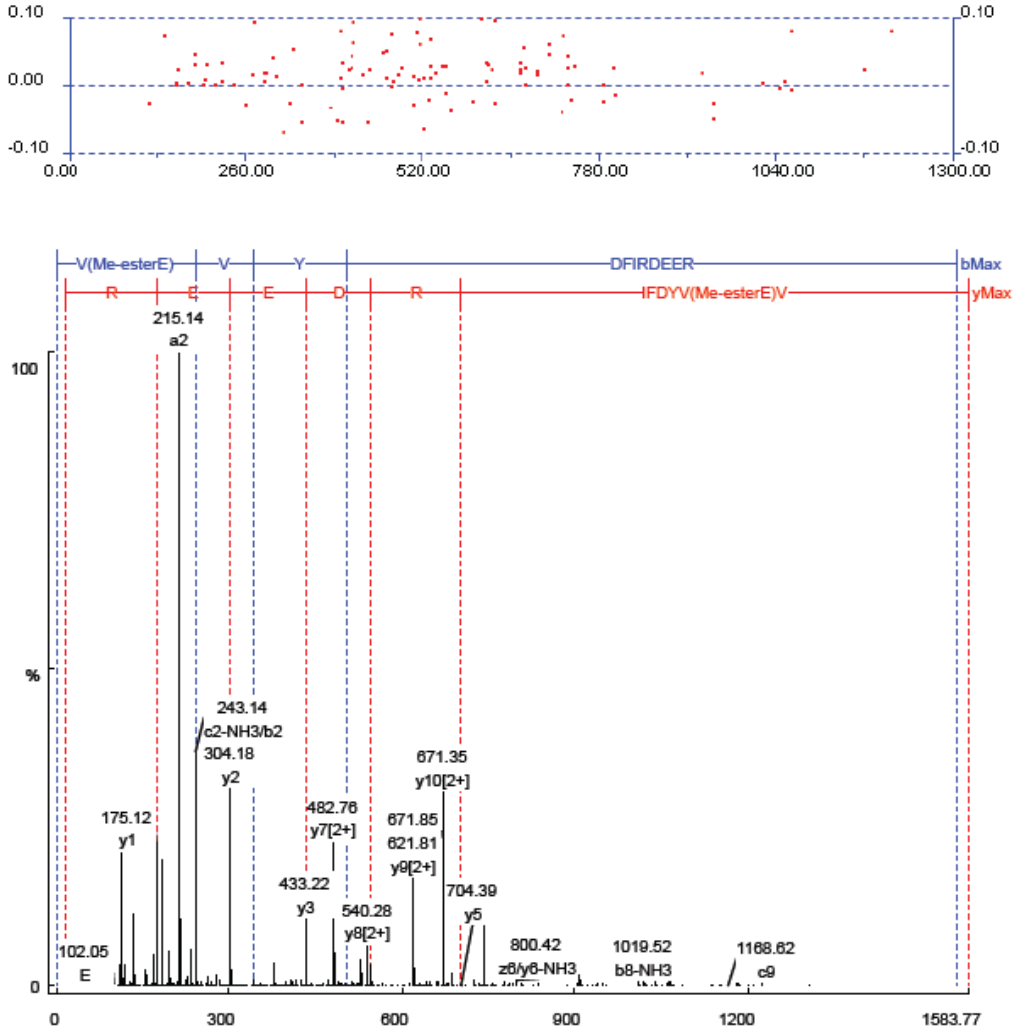
Peptide: V(Me-EsterE)VFFQK

#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				117.0690		V						7
2	243.1355	225.1758	215.1402	260.1480	116.0718	(Me-est...	811.4610					6
3	342.2200			359.1466		V	668.4033					5
4			461.3174		120.0861	F	569.3308		552.2987			4
5					120.0861	F	422.2397	404.2458	405.2422	406.2758		3
6					101.0646	Q	275.1838	257.1701	258.1513	259.1559		2
7						K	147.1161	129.1090	130.0842	131.0989		1

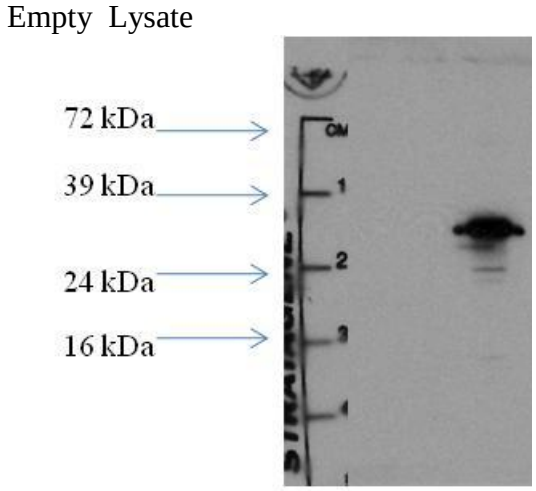


Peptide: V(Me-EsterE)VYDFIRDEER

#	b	b-H2O	a	c	Immonium	Seq	y	y-H2O	z	z'	y (2+)	#
1				117.0786		V						12
2	243.1355	225.1557	215.1402	260.1336	116.0718	(Me-est...					742.8800	11
3	342.2035	324.1657	314.1387			V					671.3480	10
4	505.2385			522.2275	136.0802	Y					621.8121	9
5			592.2761			D			1062.5...	1063.4...	540.2750	8
6			739.3454	784.3649	120.0812	F			947.4322	948.4076	482.7587	7
7						I			800.4158	801.3755	409.2309	6
8				1053.5...	129.1090	R	704.3919		687.3262	688.3208		5
9				1168.6...		D	548.2587	530.2005				4
10					102.0538	E	433.2203	415.2188	416.2194	417.2396		3
11					102.0538	E	304.1758	286.1564	287.1536	288.1524		2
12					129.1090	R	175.1236	157.1135	158.0938	159.1164		1



Supplementary Figure 4. Tandem mass spectral evidence of glutamic acid methylation in LIC11848 protein isoform 4. MS/MS analysis identified glutamic acid methylation of eight peptides. For each peptide the identified peptide sequence and position of methylesterification is provided followed by ion coverage and the mass spectrum.



Supplementary figure 5. Reactivity of rLIC11848 antiserum with leptospiral lysate. Approximately 1 x 10^8 *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 were used to conduct immunoblot analyses with rLIC11848 antiserum. Reactivity was detected with a protein migrating to approximately the molecular weight corresponding to that of rLIC11848 (~32 kDa).