Pathogenic Leptospira interrogans Exoproteins Are Primarily Involved in Heterotrophic Processes

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The Pathogenic *Leptospira interrogans* Exoproteins are Primarily Involved in Heterotrophic Processes

Running Title: Exoprotein function in *Leptospira* life cycle

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ABSTRACT

Leptospirosis is a life-threatening and emerging zoonotic disease with a worldwide annual occurrence of more than one million cases. Leptospirosis is caused by spirochetes belonging to the genus *Leptospira*. Mechanisms of disease manifestation in the host remain elusive and the roles of leptospiral exoproteins in these processes have yet to be determined. Our aim in this study was to assess the composition and quantity of exoproteins of pathogenic *Leptospira interrogans* and to construe how these proteins contribute to disease pathogenesis.

Label-free quantitative mass spectrometry of proteins, obtained from *Leptospira* cultured *in vitro* under conditions mimicking infection, identified 325 exoproteins. The majority of these proteins are conserved in the non-pathogenic *Leptospira biflexa* and proteins involved in metabolism and energy generating functions were overrepresented and displayed the highest relative abundance in culture supernatants. Conversely, proteins of unknown function, which represent the majority of pathogen-specific proteins (presumably involved in virulence mechanisms), were underrepresented. Characterization of various *L. interrogans* exoprotein mutants in the animal infection model revealed similar host mortality rates relative to infection with wild type *L. interrogans*.

Collectively, these results indicate that pathogenic *Leptospira* exoproteins primarily function in heterotrophic processes (the processes by which organisms utilize organic substances as a nutrient sources) to maintain the saprophytic lifestyle of the bacteria, rather than virulence. The underrepresentation of proteins homologous to known virulence factors such as toxins and effectors in the exoproteome also suggests that disease manifesting from *Leptospira* infection is likely caused by a combination of primary and potentially moonlight functioning of exoproteins.

INTRODUCTION

Leptospira are spirochaete bacteria classified into non-pathogenic, intermediate or pathogenic species and inhabit soil and fresh water reservoirs, predominately in tropical regions (1). Infection with pathogenic *Leptospira* spp. can result in a large range of clinical manifestations including fever, renal failure, jaundice, hemorrhage, meningitis and death. Leptospirosis, the severe manifestation of disease, has an annual global incidence of over one million human cases and a case fatality of 5%-20% (2, 3). Pathogenic *Leptospira* spp. can colonize renal tubules of a wide variety of wild and domesticated mammals. Rats are asymptomatic carriers and serve as the main reservoir of pathogenic *Leptospira* spp. The bacteria are shed in the urine of infected animals and persist in fresh water (4, 5), providing an opportunity for the bacteria to infect a new host. *Leptospira* are unable to cross the skin barrier, they can however access the underlying tissues via cuts/wounds in the skin. After gaining access to underlying tissue, *Leptospira* rapidly disseminate to the interstitial space in the kidneys and liver (6, 7). While generally extracellular, studies have demonstrated transient intracellular localization in macrophages (8-10).

Leptospira resemble Gram negative bacteria in that they contain an inner membrane, a periplasmic space with peptidoglycan and an outer membrane where the lipopolysaccharide (LPS) is anchored. The bacteria are highly motile and previous studies have demonstrated that both full motility (11, 12) and an intact LPS (13) are required for successful colonization of the host. Unlike the majority of other pathogenic Gram negative bacteria, *Leptospira* genomes lack

62 the genes encoding delivery systems such as type III and IV secretion systems and "classical"
63 virulence proteins such as toxins and effectors (14).

64 Protein secretion systems have not been experimentally identified in *Leptospira* (15).
65 Orthologous genes to those encoding secretion systems are present in *Leptospira* genomes,
66 including a type I secretion system which consists of an inner membrane ATP binding cassette
67 protein, a periplasmic adaptor and a TolC channel-forming outer membrane protein (16). While
68 relatively simple, the type I secretion system is responsible for the export of a variety of proteins
69 with different functions in bacteria (17). *Leptospira* genomes also contain genes orthologous to
70 those encoding type II secretion proteins. The type II secretion system consists of various
71 proteins that reside in the inner membrane and a pilus-like structure that can polymerize to drive
72 proteins to the extracellular space through an outer membrane pore (for a review see (18)). To
73 date, other bacterial secretion systems have also been characterized (non-flagellar type III, and
74 type IV, V, VI and VII) but *Leptospira* genomes do not contain orthologous genes that encode
75 proteins which would assemble into these systems. Consequently, protein export to the
76 extracellular space is likely mediated by types I and II secretion systems or through other yet to
77 be discovered mechanism(s) in *Leptospira*.

78 The extracellular proteome of cells is more accurately termed as the exoproteome, which
79 is defined as the proteins in extracellular proximity to a biological system arising from secretion,
80 other transport mechanisms and/or cell lysis (19). Proteins delivered to the extracellular space
81 likely serve essential functions for the pathogenic and saprophytic lifestyles of *Leptospira* spp..
82 Previous studies focusing on extracellular proteins identified *Leptospira interrogans* proteins in
83 culture supernatants, (20) while another study used a bioinformatics based approach to identify
84 potential outer membrane and extracellular proteins (21). It has also been demonstrated that

culture supernatants of *L. interrogans* contain proteases which can interfere with host complement defense against *Leptospira* spp. (22), that an extracellular enolase interacts with host plasminogen (23) and that an extracellular collagenase can degrade host collagen (24). Combined, these studies implicated extracellular proteins in the leptospiral infection process.

Here, we used label-free quantitative proteomics to analyze and characterize the *L. interrogans* exoproteome. We identified proteins transported to the extracellular space and categorize these proteins functionally to gain further insight into *L. interrogans* biology, encompassing the pathogenic and saprophytic existence of these bacteria. These experiments guided our subsequent *in vivo* experiments which provided significant insight into the extent of *L. interrogans* pathogenicity.

MATERIALS AND METHODS

Bacterial Strains and Culturing

Leptospira interrogans serovar Manilae strain L495 was culture maintained in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (25, 26) at 30˚C. *L. interrogans* transposon mutagenesis has previously been described (27-29) and L495 transposon mutants used in this study were obtained from an in house maintained library of mutants. The mutant strains were culture maintained as described above for the parent strain. To perform proteomic analysis on culture supernatants EMJH was constituted with the following modifications. Albumin was omitted from the recipe and Tween 80 and glycerol were added to 0.01% (v/v). A separate batch of modified EMJH was made to contain 120 mM NaCl. Prior to shifting to modified EMJH media, *L. interrogans* were cultured in EMJH at 30˚C to a density of 10^9 bacteria per ml, and subsequently pelleted via centrifugation at 3, 200 x*g* for 15 min, using a swinging bucket rotor. Pelleted bacteria were washed 3 times with modified EMJH using 20 ml of media for each washing step. Bacteria were then resuspended in modified EMJH and enumerated via darkfield microscopy. Bacteria were subsequently diluted in modified EMJH to a concentration of 10^8 bacteria per ml in a total volume of 100 ml for each condition (for incubation at 30°C, 37°C and in modified EMJH with 120 mM NaCl at 30°C), in two biological replicates for each condition. After 18 hours of incubation, bacteria were enumerated via darkfield microscopy to validate bacterial viability. Technical limitations prevented growth of bacteria at 37°C in modified EMJH containing 120 mM NaCl as bacterial viability was affected under these conditions.

Leptospira Culture Supernatants

Leptospira were centrifuged at 3,200 x*g* for 10 minutes and the pelleted bacteria were separated from culture supernatants by siphoning supernatants. The pellets were stored at -20°C and the culture supernatants were transferred to Vivaspin 20 1,000,000 MWCO (molecular weight cutoff) PES (polyethersulfone) ultrafiltration devices (Sartorius Stedim Biotech, Goettingen, Germany). The latter step ensured removal of any remaining whole *L. interrogans* cells from the culture supernatants. Subsequent centrifugation was performed according to manufacturer's instructions and all manipulations were carried out at room temperature. The flow-through was then concentrated using Amicon Ultra Centrifugal Filters Ultracel-3K (Merck Millipore Ltd., Cork, Ireland) and subsequently with low volume Amicon Ultra – 0.5 ml 3k (Merck Millipore Ltd.). Each 100 ml culture supernatant was concentrated down to a final volume of 20 µl.

To obtain protein from the pelleted whole *Leptospira* cells, bacterial pellets were resuspended in the respective modified EMJH media to a final volume of 200 µl and sonicated to lyse the bacteria. Protein concentration was measured via UV spectrometry at 280 nm. Samples were diluted 3:1 in Laemmli (4x) sample loading buffer and equal concentrations of protein were used in sodium dodecyl sulfate polyacrylamide gel electrophoresis. Equal protein loading across all gel lanes was confirmed via densitometry.

Recombinant Protein and Antisera

Recombinant LIC12209 (encoding a *Leptospira* beta-propeller protein 52 kDa protein (LßP52)) was produced as previously described (30) with the following modifications. The predicted *lßP52* gene was amplified by PCR from using *L. interrogans* serovar Manilae strain L495 genomic DNA with the primer pairs LIC12209F 5'-GGGGCTAGCATGAGAAAATTTTACATT-3' and LIC12209R 5'-

CCCGCGGCCGCTTATCTATATTTTACA-3'. Underlined nucleotides indicate restriction sites (NheI/NotI). The PCR product was cloned into TOPO vector (Invitrogen Life Technologies, Saint Aubin, France) and transformed into *Escherichia coli* DH5α (Invitrogen Life Technologies). The amplicon was released from the vector by digestion with NheI and NotI, and re-cloned into the pET-28(a) expression plasmid. The pET28 vector allows expression of recombinant protein with a N-terminal 6X His-tag (poly-histidine tag). The cloned sequence was confirmed by DNA sequencing (eurofins, Courtaboeuf, France).

Protein expression was achieved in *E. coli* BL21 (DE3) strain. *E. coli* BL21 (DE3) containing recombinant plasmid was cultured at 37 ºC under kanamycin selection to an optical density (600 nm) of 0.6-0.8. Recombinant protein synthesis was induced by the addition of 1 mM (final concentration) Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 3 h, the cells were harvested by centrifugation at 4,000 x*g* for 30 min and the bacterial pellets were resuspended in 5 ml buffer A (100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0). The bacterial cell pellets were lysed via sonication and recombinant protein, in the form of inclusion bodies, was then separated by centrifugation at 17,000 x*g* for 20 min, and solubilized in 5 ml of buffer B (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea pH 8.0). The protein was purified through metal-chelating chromatography in a fast-flow column with 0,4 ml of nickle-nitrilotriacetic acid (Ni-NTA) resin (QIAGEN, Courtaboeuf, France) using the manufacturers protocol. All fractions were analyzed by 12% SDS-PAGE.

Rabbit antisera generated against the flagellar protein FlaA-2 (31), chaperone protein GroEL (32) and Ig-like repeat domain protein 1 LigA (32) where generously provided by Dr. David Haake. Rabbit antisera against recombinant LßP52 (rLßP52) protein was generated as follows. The recombinant protein LßP52 was dialyzed against buffer 1 (100 mM NaH_2PO_4, 10

mM Tris·Cl, 5M urea pH 8) for 48 h followed by a second dialysis step for 48 h against buffer 2 (100 mM NaH_2PO_4, 10 mM Tris·Cl, 2M urea pH 8) and 50 µg of protein was submitted for antiserum production rabbits by Covalab R&D in Biotechnology (France).

To obtain *L. interrogans* positive sera, Hartley male guinea pigs (Charles River Laboratories, http://www.criver.com) were obtained at 6 weeks of age and weighed between 450-500 g. Guinea pigs (N = 6) were anaesthetized via intramuscular injection with 40 mg ketamine and 4 mg xylazine (per kg body weight) and blood (~ 1 ml) was collected into 10 ml Venosafe Plastic Tubes (Terumo, Guyancourt, France) via cardiac heart puncture. Animals were then maintained under normal care conditions for 7 days and then injected intraperitoneal with 10^5 *L. interrogans* strain L495 in 1 ml albumin free EMJH. Blood samples from infected animals were collected by terminal cardiac puncture of anaesthetized animals 14 days post-challenge. Serum was collected by incubation of blood samples in Venosafe Plastic tubes (Terumo) at room temperature for 30 min, followed by centrifugation at 1,500 x*g* for 10 min. The supernatant was collected for use in immunoblot experiments. Protocols for animal experiments conformed to the guidelines of the Animal Care and Use Committees of the Institut Pasteur (Paris, France).

Protein Immunoblots

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and protein immunoblots were performed as previously described (33, 34) with the following modifications. The SDS-PAGE and immunoblot experiments were performed using 10 µg of total protein from whole cell lysates and culture supernatants. Antiserum to FlaA-2, GroEL, LigA and LßP52 was used at 1:2,000, 1:8,000, 1:750 and 1:1,000, respectively. Guinea pig *Leptospira* positive and pre-immune sera were used at 1:100 and goat polyclonal secondary antibody to guinea pig IgG-Fc

(HRP) (Abcam, Paris, France) and IgM-Fc (HRP) (Acris, Montluçon, France) were used at a dilution of 1:20,000.

Protein Treatment Preceding Mass Spectrometry

For mass spectrometry experiments, 10 µg of protein was allowed to migrate 10 mm into a 4-12% gradient TGX gel (Bio-Rad, Marnes-la-Coquette, France), without the addition of any loading dyes. Samples were excised, diced and digested with trypsin as previously describe (35). Briefly gel pieces were washed multiple times in 25 mM ammonium bicarbonate in 50% acetonitrile, reduced with 10 mM dithiothreitol (DTT) at 37 °C for 60 min and then alkylated using 55 mM iodoacetamide for 60 min. Gel pieces were dried in a SpeedVac (Thermo Savant, Hemel Hempstead, UK) and then 200 ng of trypsin (Promega, Southampton, United Kingdom) in 25mM ammonium bicarbonate was added to each gel piece and digested overnight at 37 °C. Peptides were extracted by sonicating the gel pieces in 50 % acetonitrile containing 0.1% formic acid for 30 min. After vacuum concentration the tryptic peptides were concentrated on an in-house manufactured C18 desalting tip prior to mass spectrometry analysis (36).

Mass Spectrometry

Protein samples were analysed on an Ultimate 3000 RSLCnano high-performance liquid chromatography (HPLC) (Dionex, Camberley, UK) system run in direct injection mode coupled to a QExactive Orbitrap mass spectrometer (Thermo Electron, Hemel Hempstead, UK). Samples were resolved on a 25 cm by 75 µm inner diameter picotip analytical column (New Objective, Woburn, MA, USA) which was packed in-house with ProntoSIL 120-3 C18 Ace-EPS phase, 3 µm diameter beads (Bischoff Chromatography, Germany). The system was operated at a flow-rate of 300 nl min^{-1} and a 120 min gradient was used to separate the peptides. The mass spectrometer was operated in a “Top 10” data dependent acquisition mode. Precursor scans were

209 performed in the orbitrap at a resolving power of 70,000, from which the ten most intense
210 precursor ions were selected by the quadrupole and fragmented by higher-energy collisional
211 dissociation (HCD) at a normalized collision energy of 28%. The quadrupole isolation window
212 was set at 3 *m/z*. Charge state +1 ions and undetermined charge state ions were rejected from
213 selection for fragmentation. Dynamic exclusion was enabled for 40 s. Data were converted from
214 .RAW to .MGF using ProteoWizard (36).

215 ***Mass Spectrometry Data Analysis***

216 Data were searched against the *L. interrogans* serovar Copenhageni strain Fiocruz L1-
217 130 proteome using the Andromeda (37) algorithm, through MaxQuant software (38) (version
218 1.5.2.8). Parameters can be found in supplementary Table S3. Contaminants, reverse decoy
219 proteins, and proteins identified only by a modification site were removed; majority protein ids
220 were used as protein identifiers. Raw protein abundances were used for further analysis. Missing
221 values were replaced with the minimum for each sample. Resulting protein intensities were log2
222 transformed and quantile normalized (39), using the method implemented in the
223 'normalizeBetweenArrays' function of the limma package (40, 41). An empirical Bayes
224 moderated *t*-statistics, as implemented in the R limma package (40), was applied to identify
225 proteins with a significant difference in abundance. Temperature and osmotic variables were
226 used as a blocking factors, to account for any additional variance in the data. The Benjamini and
227 Hochberg multiple testing correction was applied to control the false discovery rate (42).

228 ***Data Analyses Downstream of Identification and Quantification***

229 To be deemed as an exoprotein and/or a protein potentially localized to the extracellular
230 space via active transport, proteins must have displayed an enriched abundance in supernatants
231 compared to the respective abundance in whole cells with an adjusted p value < 0.05. To

compare exoprotein quantities in culture supernatants (37˚C vs 30˚C S and NaCl vs 30˚C S), protein abundances were compared to assess relative abundance (Table S1).

Assignment of Proteins to Clusters of Orthologous Groups

The protein products of *Leptospira* genomes have been automatically classified into clusters of orthologous groups (COG) by the MicroScope platform (43). This data was used to sort the detected exoproteins into COGs and the resulting frequencies were compared to those predicted genome-wide. Statistical analysis was performed assuming a binomial distribution where assignment of exoproteins into a COG would be considered a "success" and absence a "failure". The percentage of coding sequences classified into a COG (as calculated in MicroScope genome-wide) was used as the probability of observing a "success" and the total number of detected exoproteins (325) was used as the sampling size to generate COG specific binomial probability distributions. A p value of $p < 0.01$ was used as a cut-off for significance.

Mutant Strains, Growth Rates and Infection Experiments

The LIC10465^{-} (*ligA*, encoding Ig-like repeat domain protein 1) mutant was obtained in Manilae L495 by cloning an internal fragment of *ligA* lacking the 5′ and 3′ ends of the open reading frame in a conjugative suicide vector carrying a spectinomycin cassette. We obtained integration of the plasmid via a single crossover event, which generated two copies of the targeted gene, one with a deletion at the 5′ end of the gene and the other with a deletion at the 3′ end, thereby rendering it inactive, as confirmed by immunoblotting and immunofluorescence assay (data not shown). Other mutants were obtained by transposon mutagenesis in the parental strain (Manilae L495) and insertion sites initially identified via semi-random PCR (27-29). The insertion sites for exoprotein mutants were validated via PCR and the primers, insertion sites and transposon mutants are listed in Table S4.

Exoprotein mutants LIC13006^{-} (*lenC*$^{-}$, encoding an endostatin-like protein), LIC12208^{-} (encoding a lipoprotein with Beta-propeller repeats), LIC13060^{-} (*lipL36*$^{-}$, encoding a lipoprotein), LIC10373^{-} (encoding a lipoprotein with Beta-propeller repeats), LIC10898^{-} (*lipL48*$^{-}$, encoding an exoprotein with highest absolute abundance in the present study), LIC11977^{-} (encoding a cyclic nucleotide binding protein), LIC11852^{-} (encoding a O-acetylhomoserine (thiol) lyase), LIC10713^{-} (*lruB*$^{-}$, encoding a lipoprotein), LIC10465^{-} (*ligA*$^{-}$), and Manilae L495 were compared for *in vitro* growth rates in EMJH media at 30°C. Bacterial growth was measured on a daily basis by measuring optical densities via spectroscopy at 420 nm. Exoproteins that demonstrated similar growth rates to Manilae L495 were used in subsequent virulence measurement experiments in Mongolian gerbils (Janvier). To measure virulence, groups of 4 gerbils were injected intraperitoneal with 10^4 bacteria per animal. Animals were administered Manilae L495 or mutants LIC13006^{-} (*lenC*$^{-}$), LIC12208^{-}, LIC13060^{-} (*lipL36*$^{-}$), LIC10373^{-}, LIC10898^{-} (*lipL48*$^{-}$), LIC10465^{-} (*ligA*$^{-}$), and LIC11977^{-}. Animals were monitored on a daily basis for 23 days and sacrificed when moribund. All strains were culture isolated from animals post-mortem and genotypes confirmed via PCR. Protocols for animal experiments conformed to the guidelines of the Animal Care and Use Committees of the Institut Pasteur (Paris, France).

RESULTS

Validation of Methodology and Overview of Whole Cell and Exoproteins

The method utilized in the present study for separation of culture supernatants from whole bacteria has not been previously described and required validation that culture supernatants were free of non-extracellular proteins. To survey proteins found in culture supernatants, whole cell proteins (WCP) and culture supernatant proteins (CSP) from each culture condition were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses (Fig. 1). The Coomasie stained SDS-PAGE gel revealed a visual difference in protein banding between WCP and CSP (Fig. 1A). Additionally, the majority and/or the most abundant CSP migrated between 25-90 kDa, as opposed to WCP where protein bands as large as 260 kDa were observed (Fig. 1A). There was no visual difference in protein banding in response to temperature or osmotic shifts in WCP or CSP (Fig. 1A).

Leptospira are flagellated bacteria and the flagellum is anchored into the inner membrane of the bacteria (31). Antiserum generated against the 27.2 kDa periplasmic flagellar protein (FlaA-2) revealed reactivity with proteins migrating between 25-35 kDa in WCP, but no reactivity with CSP in protein immunoblots (Fig. 1B). Antiserum generated against the chaperone protein GroEL was used in immunoblot experiments (Fig. 1C) to validate bacterial response to temperature shift from 30°C to 37°C (44). The WCP from *Leptospira* exposed to 37˚C displayed a slight increase in GroEL reactivity (Fig. 1C). Detectable levels of GroEL were observed in all CSP samples, albeit at significantly lower levels compared to WCP (Fig. 1C). Using similar reasoning, WCP and CSP were subjected to immunoblot analysis using antiserum cross reactive with *L. interrogans* immunoglobulin protein A (LigA with a MW of 128 kDa) (Fig. 1D) to assess bacterial response to osmotic shift (45, 46). The LigA protein was detected in

296 CSP samples, with pronounced reactivity being observed in CSP samples from *L. interrogans*
297 exposed to 120 mM NaCl (Fig. 1D). Immunoblot analysis of WCP and CSP also confirmed
298 detection of LßP52 protein in CSP samples only, with elevated expression being observed in 120
299 mM NaCl samples (Fig. 1E).

300 The immunoblot experiments described above, demonstrated that CSP preparations and
301 temperature and osmotic shift experiments were performed in a manner acceptable for
302 subsequent quantitative global proteome analyses using mass spectrometry. Thus, WCP and CSP
303 (in replicate) were subjected to LC-MS/MS QExactive orbitrap mass spectrometry for
304 identification and relative quantification of proteins, using label-free quantification. These
305 analyses resulted in the detection of 1,073-1,293 proteins in WCP preparations and 540-712
306 proteins in CSP preparations (Table 1, Table S1). The range of proteins detected was culture
307 condition dependent. Other notable differences between the samples included detection of
308 relatively large proteins (300 kDa) in WCP which were absent in CSP, and detection of FlaA-2
309 and LIC10371 proteins in WCP and CSP, respectively, but not *vice versa* (Table 1). The
310 LIC10371 protein consists of multiple repeating beta-propeller domains which we found to also
311 be present in 4 other exoproteins and displayed high abundance in all culture supernatants. Both
312 FlaA-2 and LIC10371 proteins served as controls for demonstrating appropriate separation of
313 CSP from WCP. Label-free protein quantification revealed a dynamic range approaching 5
314 orders of magnitude in protein quantities (Table 1, Table S1).

315 ***Identification of Exported Proteins via Relative Protein Quantities***

316 Relative protein abundances in CSP to WCP enabled assignment of exoproteins and those
317 potentially localized to the extracellular space via active transport and this approach identified
318 325 exoproteins (Table S2). The relative abundance of these exoproteins in supernatants

compared to whole cells ranged from 2 fold higher to only being detected in supernatants (Table S1 and Table 2).

To further evaluate assignment of exoproteins, corresponding primary sequences were used in bioinformatic analyses to predict N-terminal signal peptides using Phobius (47) and non-classical protein export using SecretomeP 2.0 server (48) (Table S2). Of the 325 proteins deemed to be exported, 89 were predicted to contain an N-terminal signal peptide and 23 were predicted to be exported via a non-classical pathway (defined as pathways that export proteins lacking classical signal peptides) (Table S2).

Exoproteins identified in Table S2 were then used in basic local alignment searches to identify the numbers of proteins unique to pathogenic *Leptospira* (Table 3) and those conserved in the non-pathogenic *Leptospira biflexa* (Table S2). These analyses revealed the majority (274 out of 325, 84.3%) of exoproteins to be conserved in *L. biflexa*.

Overrepresentation of Exoproteins in COGs Relating to Energy Production and Metabolism

Exoproteins from Table S2 were assigned to clusters of orthologous groups (COGs) based on automatic classification of *Leptospira* genomes in the MicroScope platform (43) (Fig. 2A). Compared to genome-wide expected frequencies, exoproteins in the COGs; cell motility (N), signal transduction mechanisms (T), replication recombination and repair (L), general function (R), function unknown (S) and unclassified (-) were underrepresented (Fig. 2B). In contrast, there was a 2 to 4 fold overrepresentation of exoproteins classified in the COGs; energy production and conversion (C), amino acid transport and metabolism (E), nucleotide transport and metabolism (F), carbohydrate transport and metabolism (G) and lipid transport and metabolism (I) (Fig. 2B). Additionally, of the 20 most abundant exoproteins 13 were categorized in at least one of the latter overrepresented categories (Table 2).

Altered Exoprotein Abundance in Response to Temperature and Osmotic Shifts

The optimal temperature for *in vitro* growth of *Leptospira* is 30°C. Comparison of protein abundance in CSP from *Leptospira* shifted to 37°C or to modified medium containing 120 mM NaCl (~300 mosmol/l^{-1}), which mimics physiological conditions in human plasma, to CSP from *Leptospira* at 30°C (37˚C vs 30˚C S and NaCl vs 30˚C S, respectively) suggested altered expression of exoproteins in response to temperature (91 in total) and 120 mM NaCl (140 in total) (Table S1). Protein quantities were validated via immunoblot experiments (Fig. 1D and E) for LigA (6 fold higher via relative protein intensity) and LßP52 (20 fold higher via relative protein intensity) proteins, confirming increased expression of these proteins in response to 120 mM NaCl.

Exoproteins with Potential Moonlighting Functions

Moonlighting proteins are a class of proteins in which a single polypeptide chain performs more than 1 one biochemical function (49). Classification of exoproteins into COGs revealed overrepresentation of proteins involved in nutrient uptake and metabolism with the latter comprising of numerous proteins involved in the glycolytic pathway (Fig. 2 and Table S2). Enzymes in the glycolytic pathway have been implicated for moonlighting properties in other bacteria (50-53) and in *Leptospira* (23). To assess potential moonlighting properties for *Leptospira* exoproteins, proteins that have been experimentally characterized to display moonlighting properties in other microorganisms were collected from MoonProt (49). The primary sequence of these proteins were then used in basic local alignment searches against *L. interrogans* proteome. The search results were compared with proteomic data in Table S1 and Table S2 to identify orthologous proteins in the proteomic data. This approach identified 32 proteins detected in the supernatant that could be classified as moonlighting proteins (Table 4). A

total of 45 orthologous proteins to moonlighting proteins were identified in *L. interrogans*, 15 of these were exoproteins and 17 others were detected in supernatants (to be deemed as exoproteins, proteins must have displayed a statistically significant higher abundance in supernatants).

Exoproteins are Immunogenic and not Essential for Leptospira Virulence

To begin to characterize exoproteins in the context of pathogenesis, WCP and CSP were used in immunoblot experiments with *L. interrogans* positive guinea pig sera (S+) to assess antibody response to exoproteins, which would also be suggestive of exoprotein expression during the infection process (Fig. 3). These analyses revealed IgG and IgM reactivity against *L. interrogans* exoproteins in S+ sera (Fig. 3A and B) and no reactivity in the control pre-infection sera (Fig. 3C and D). Exoprotein reactivity with S+ IgG was significantly less prominent when compared to WCP and a similar trend was observed with IgM reactivity (Fig. 3A and B). Comparison of S+ IgG and IgM reactivity with exoproteins was distinguishable in that the proteins displaying reactivity with IgG did not display reactivity with IgM and vice versa (Fig. 3A and B). Furthermore, CSP from 120 mM NaCl exposure lead to altered IgG and IgM reactivity of exoproteins when compared to CSP from 30°C and 37°C (Fig. 3A and B). Specifically, reduced IgG and IgM reactivity was observed for protein bands migrating between 70-100 kDa (Fig. 3A and B) and increased IgG reactivity was observed for two proteins at 35 kDa and 15 kDa (Fig. 3A).

To further assess the necessity of exoproteins for *L. interrogans* viability *in vitro* and within the host, select *L. interrogans* mutants which had been inactivated in an exoprotein encoding gene were tested for *in vitro* growth rates and for disease manifestation in gerbils. Nine mutants which had been inactivated in genes encoding exoproteins, which previous studies

388 suggested to be involved in the infection process (LigA (54) and LruB (55-57)), or were detected
389 in high abundance in supernatants in the present study, were used for further characterization. *In*
390 *vitro* growth rates identified two genes *lruB* (LIC10713) and O-acetylhomoserine (thiol) lyase
391 (LIC11852), that when inactivated, resulted in significantly reduced *in vitro* growth of *L.*
392 *interrogans* (Table 5). The other tested *L. interrogans* mutants (in exoprotein encoding genes)
393 did not display an *in vitro* growth defect (Table 5) and were subsequently used to challenge
394 gerbils via intraperitoneal injection (Table 5). Gerbils challenged with these mutants displayed
395 similar mortality rates compared to those challenged with the wt parent strain (Table 5).
396

DISCUSSION

Global characterization of *L. interrogans* exoproteins has revealed the majority of exoproteins to be involved in metabolic and energy generation functions, which are likely essential for survival in the diverse environments encountered by these bacteria. It has been suggested that pathogenic *L. interrogans* evolved from non-pathogenic strains (58) and the former likely retained the majority of these exoprotein encoding genes from the predecessors. Classification of exoproteins into COGs indicated that proteins with unknown functions were underrepresented suggesting involvement of these proteins in yet to be characterized biological processes occurring within the bacteria. In contrast, COGs amino acid, carbohydrate and lipid uptake and metabolism were overrepresented, indicative of exoprotein involvement in nutrient acquisition and metabolism.

In addition to their metabolic activities, 32 proteins detected in culture supernatants displayed orthology to moonlighting proteins in other microorganisms. The moonlighting properties of two proteins detected in supernatants have already been demonstrated in *Leptospira* (23, 59). One of these proteins, phosphopyruvate hydratase (Eno/LIC11954) has been characterized as an enolase and displays plasminogen binding activity (23). The other protein, elongation factor Tu (Tuf/ LIC12875) is detected on the surface of *Leptospira* and displays plasminogen and factor H binding (59). A catalase (KatE/LIC12032), another potential moonlighting protein has previously been characterized to be required for *Leptospira* oxidative stress resistance and virulence (34), but the plasminogen binding capacity of this protein (as demonstrated for *Candida albicans* catalase (60)) remains to be elucidated in *L. interrogans*. In accordance with a potential role in host-pathogen interactions, 5 putative *L. interrogans* moonlighting proteins have been shown to be immunoreactive (61); suggestive of their

expression during the infection process. An exoprotein (not detected in the present study) directly associated with pathogenesis has been characterized as a collagenase required for tissue invasiveness and virulence in animals (24) while another protein (Lsa32/LIC11089), detected in culture supernatants in the present study, has been characterized and demonstrates laminin and plasminogen binding capacity (62). A known *Leptospira* virulence factor, high-temperature protein G (HtpG) (63), was detected at high abundance in all culture supernatants. Suggesting the extracellular presence of this protein as a contributing factor to disease pathogenesis in animals, either through unidentified moonlighting properties or host inflammatory response to this protein. Taken together, these observations make a compelling case for exoprotein mediated host-pathogen interactions and disease pathogenesis.

While exoprotein function can be associated with disease pathogenesis, the *L. interrogans* mutants disrupted in exoprotein encoding genes tested in this study displayed similar disease manifestation in animals to that observed for the parent strain. It should be highlighted that one of the inactivated genes encoded LipL48 (LIC10898) which was the most abundant exoprotein and was detected 11 fold higher than that found within the bacteria. Similarly, two other inactivated genes, LIC11977 encoding a cyclic nucleotide binding protein and *ligA* encoding immunoglobulin-like protein 1, were also detected at high abundance in supernatants. It has previously been demonstrated that LigA confers binding of *Leptospira* spp. to host cells and fibronectin (54), implicative of a role for this protein in the infection process. It should also be noted that immunization of hamsters using LigA antigen provides protection against challenge with *L. interrogans* (64), indicating that LigA is an effective antigen for immune clearance of *Leptospira*. Other notable proteins included the endostatin-like protein LenC which has been shown to bind fibronectin (65) and was unique to supernatants and an *L. interrogans* antigen,

443 LipL36. Two other *L. interrogans* mutants were inactivated in genes annotated as lipoproteins,
444 our curiosity for these genes (LIC12208 and LIC10373) stemmed from the observation that these
445 exoproteins, amongst 3 others, contained multiple repeating beta-propeller domains and were
446 unique to culture supernatants. In contrast to the lack of impact on disease pathogenesis,
447 inactivation of two exoprotein encoding genes, annotated as a lipoprotein (containing an
448 imelysin peptidase domain) (*lruB*/LIC10713) and an O-acetylhomoserine (thiol) lyase
449 (LIC11852), rendered the bacteria with *in vitro* growth defects. The LruB (imelysine-like)
450 protein is categorized in the COG class P: Inorganic ion transport and metabolism and structural
451 analysis of imelysine-like proteins implicates a role in iron uptake (66). The other vital
452 exoprotein O-acetylhomoserine (thiol) lyase, is in the COG class E: Amino acid transport and
453 metabolism and likely involved in methionine and cysteine regulation (67). It follows that of the
454 325 exoproteins 50 were classified in COG class E, further implication of exoprotein function in
455 heterotrophic processes.

456 The lack of involvement in disease pathogenesis by the majority of exoproteins is in
457 agreement with previous studies on *L. interrogans* virulence genes which have demonstrated that
458 the majority of genes are dispensable for virulence (27, 68). Moreover, with the exception of a
459 catalase gene (*katE*) (34) and a collagenase gene (24) the genes that have been demonstrated to
460 be essential for virulence such as HtpG (63), Loa22 (69) and those involved in motility (11, 12),
461 LPS biosynthesis (13), heme metabolism (70), and adhesion (71, 72), have orthologous in the
462 non-pathogenic *L. biflexa.* In combination with the finding that the majority of *L. interrogans*
463 exoproteins have orthologous in *L. biflexa*, these evidence further support the theory that *L.*
464 *interrogans* evolved from *L. biflexa*. How *L. interrogans* became successful at establishing
465 infection and *L. biflexa* did not remains to be clarified. The present study identified 51

466 exoproteins that were unique to *L. interrogans* and the functioning of these proteins in addition
467 to those conserved in *L. biflexa* may confer the pathogenic traits observed for *L. interrogans*.
468 The methodology used in the present study identified 325 exoproteins (the *L. interrogans*
469 genome encodes ~4,000 putative proteins) , which is nearly 5 fold higher than the 66 proteins
470 reported in a previous study (20). We propose two explanations for the observed difference in the
471 number of proteins being detected; firstly protein precipitation, which can result in protein loss
472 due to inefficient precipitation and resolubilization, performed in the previous study, was omitted
473 in the present study. Secondly, the present study identified proteins under three different culture
474 conditions, which would increase the number of proteins detected. Comparing protein quantities
475 in whole cells versus those in the extracellular space, under different culturing conditions, had
476 the advantage of identifying proteins unique to the extracellular space. It follows that at least a
477 subset of the proteins in culture supernatants would be detected as a result of bacterial lysis or
478 release from the outer membrane due to experimental manipulation and relative protein
479 quantification allows satisfactory identification of putative actively transported proteins.
480 Quantitative proteomics also provided information on the relative abundance of proteins in the
481 extracellular space. In line with a role in heterotrophic processes, of the top 20 most abundant
482 proteins in culture supernatants the majority were involved in metabolic processes.
483 Bioinformatic analyses of the primary sequences of the 325 exoproteins suggested that
484 *Leptospira* predominantly export proteins via a mechanism yet to be characterized. Of the 325
485 identified exoproteins 89 (~27 %) contained an N-terminal signal sequence and 23 (~7 %) were
486 predicted to be exported through a non-classical protein export system. How the other 213 (~66
487 %) proteins are being exported by *L. interrogans* remains to be determined. It should be
488 emphasized that these proteins are likely being exported and were not detected as a result of

experimental manipulation. Evidence supporting this claim was the lack of flagellar proteins and relatively low abundance of highly expressed proteins (in the bacteria) such as LipL32 and GroEL, in supernatants.

The present study provides significant insight into exoprotein function in the context of the lifestyle of *L. interrogans* both inside and outside the host. A survey of the *L. interrogans* exoproteins under laboratory growth conditions has revealed that the majority of exoproteins are dedicated to heterotrophic process but that these same proteins may also contribute to the pathogenic life cycle of the bacteria through plasticity or moonlighting functions. These results lead us to propose a shift in how we view disease manifestation resulting from an infection with the extracellular pathogen *L. interrogans*. The numerous exoproteins functioning in nutrient uptake within the host and their potential moonlighting activities in binding to host components could lead to disruption of normal biological processes in the host. Finally, any resulting deleterious effects on the host would likely be further compounded by the host inflammatory response to these proteins. Thus, future research should focus on how these proteins function within the host to acquire nutrients and how they interact with host components and on the ensuing immune response of the host to these proteins.

ACKNOWLEDGMENTS

The authors would like to thank David A. Haake for rabbit antisera against FlaA2, GroEL and LigA, Kristel Lourdault for the characterization of *ligA* mutant, and Karim Sebastien for his help with guinea pig heart punctures. Gabriela Pretre is a recipient of a Bernado Houssay grant. This work (including a postdoctoral grant) was funded by the FUI 14 (Fonds Unique Interministériel)

511 "COVALEPT" and BPIFrance. Azad Eshghi was funded by a Carnot grant from the "Institut
512 Carnot- Pasteur Maladies Infectieuses".

REFERENCES

1. **Ko AI, Goarant C, Picardeau M.** 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat Rev Micro **7:**736-747.
2. **J.E H, F. C, J. C, M. K, P. T, M.S. M-S, C. S, B. A-R, A. K.** 2013. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. Scientific meeting of the International Leptospirosis Society. Fukuoka, Japan. p.66
3. **Goswami RP, Basu A, Tripathi SK, Chakrabarti S, Chattopadhyay I.** 2014. Predictors of mortality in leptospirosis: an observational study from two hospitals in Kolkata, eastern India. Trans R Soc Trop Med Hyg **108:**791-796.
4. **Trueba G, Zapata S, Madrid K, Cullen P, Haake D.** 2004. Cell aggregation: a mechanism of pathogenic *Leptospira* to survive in fresh water. Int Microbiol **7:**35-40.
5. **Barragan VA, Mejia ME, Trávez A, Zapata S, Hartskeerl RA, Haake DA, Trueba GA.** 2011. Interactions of *Leptospira* with environmental bacteria from surface water. Curr Microbiol **62:**1802-1806.
6. **Eshghi A, Cullen PA, Cowen L, Zuerner RL, Cameron CE.** 2009. Global proteome analysis of *Leptospira interrogans*. J Proteome Res **8:**4564-4578.
7. **Eshghi A, Pinne M, Haake DA, Zuerner RL, Frank A, Cameron CE.** 2012. Methylation and *in vivo* expression of the surface-exposed *Leptospira interrogans* outer-membrane protein OmpL32. Microbiology **158:**622-635.
8. **Toma C, Okura N, Takayama C, Suzuki T.** 2011. Characteristic features of intracellular pathogenic *Leptospira* in infected murine macrophages. Cell Microbiol **13:**1783-1792.
9. **Davis JM, Haake DA, Ramakrishnan L.** 2009. *Leptospira interrogans* stably infects zebrafish embryos, altering phagocyte behavior and homing to specific tissues. PLoS Neglect Trop D **3:**e463.
10. **Toma C, Murray GL, Nohara T, Mizuyama M, Koizumi N, Adler B, Suzuki T.** 2014. Leptospiral outer membrane protein LMB216 is involved in enhancement of phagocytic uptake by macrophages. Cell Microbiol **9:**1366-1377
11. **Lambert A, Picardeau M, Haake DA, Sermswan RW, Srikram A, Adler B, Murray GA.** 2012. FlaA proteins in *Leptospira interrogans* are essential for motility and virulence but bre not required for formation of the flagellum sheath. Infect Immun **80:**2019-2025.
12. **Liao S, Sun A, Ojcius DM, Wu S, Zhao J, Yan J.** 2009. Inactivation of the fliY gene encoding a flagellar motor switch protein attenuates mobility and virulence of *Leptospira interrogans* strain Lai. BMC Microbiol **9:**253-262.
13. **Murray GL, Srikram A, Henry R, Hartskeerl RA, Sermswan RW, Adler B.** 2010. Mutations affecting *Leptospira interrogans* lipopolysaccharide attenuate virulence. Mol Microbiol **78:**701-709.
14. **Murray GL.** 2015. The molecular basis of leptospiral pathogenesis. Curr Top Microbiol Immunol **387:**139-185.
15. **Haake DA, Matsunaga J.** 2010. *Leptospira*: a spirochaete with a hybrid outer membrane. Mol Microbiol. doi.org/10.1111/j.1365-2958.2010.07262.x
16. **Masi M, Wandersman C.** 2010. Multiple signals direct the assembly and function of a type 1 secretion system. J Bacteriol **192:**3861-3869.

17. **Delepelaire P.** 2004. Type I secretion in gram-negative bacteria. Biochim Biophys Acta **1694:**149-161.
18. **Sandkvist M.** 2001. Biology of type II secretion. Mol Microbiol **40:**271-283.
19. **Armengaud J, Christie-Oleza JA, Clair G, Malard V, Duport C.** 2012. Exoproteomics: exploring the world around biological systems. Expert Rev Proteomic **9:**561-575.
20. **Zeng L, Zhang Y, Zhu Y, Yin H, Zhuang X, Zhu W, Guo X, Qin J.** 2013. Extracellular proteome analysis of *Leptospira interrogans* serovar Lai. OMICS **17:**527-535.
21. **Viratyosin W, Ingsriswang S, Pacharawongsakda E, Palittapongarnpim P.** 2008. Genome-wide subcellular localization of putative outer membrane and extracellular proteins in *Leptospira interrogans* serovar Lai genome using bioinformatics approaches. BMC Genomics **9:**181.
22. **Fraga TR, Courrol DoS, Castiblanco-Valencia MM, Hirata IY, Vasconcellos SA, Juliano L, Barbosa AS, Isaac L.** 2014. Immune evasion by pathogenic *Leptospira* strains: the secretion of proteases that directly cleave complement proteins. J Infect Dis **209:**876-886.
23. **Nogueira SV, Backstedt BT, Smith AA, Qin JH, Wunder EA, Ko A, Pal U.** 2013. *Leptospira interrogans* enolase is secreted extracellularly and interacts with plasminogen. PLoS One **8:**e78150.
24. **Kassegne K, Hu W, Ojcius DM, Sun D, Ge Y, Zhao J, Yang XF, Li L, Yan J.** 2014. Identification of collagenase as a critical virulence factor for invasiveness and transmission of pathogenic *Leptospira* species. J Infect Dis **209:**1105-1115.
25. **Ellinghausen HC, Jr., McCullough WG.** 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: Fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Am J Vet Res **26:**45-51.
26. **Johnson RC, Harris VG.** 1967. Differentiation of pathogenic and saprophytic leptospires. Growth at low temperatures. J Bacteriol **94:**27-31.
27. **Murray GL, Morel V, Cerqueira GM.** 2009. Genome-wide transposon mutagenesis in pathogenic *Leptospira* spp. Infect Immun **77:**810 - 816.
28. **Picardeau M.** 2008. Conjugative transfer between *Escherichia coli* and *Leptospira* spp. as a new genetic tool. Appl Environ Micro **74:**319-322.
29. **Bourhy P, Louvel H, Saint Girons I, Picardeau M.** 2005. Random insertional mutagenesis of *Leptospira interrogans*, the agent of leptospirosis, using a mariner transposon. J Bacteriol **187:**3255-3258.
30. **Pretre G, Lapponi MJ, Atzingen MV, Schattner M, Nascimento AL, Gómez RM.** 2013. Characterization of LIC11207, a novel leptospiral protein that is recognized by human convalescent sera and prevents apoptosis of polymorphonuclear leukocytes. Microb Pathogenesis **56:**21-28.
31. **Trueba GA, Bolin CA, Zuerner RL.** 1992. Characterization of the periplasmic flagellum proteins of *Leptospira interrogans*. J Bacteriol **174:**4761-4768.
32. **Matsunaga J, Barocchi MA, Croda J, Young TA, Sanchez Y, Siqueira I, Bolin CA, Reis MG, Riley LW, Haake DA, Ko AI.** 2003. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol **49:**929-946.

33. **King AM, Bartpho T, Sermswan RW, Bulach DM, Eshghi A, Picardeau M, Adler B, Murray GL.** 2013. Leptospiral outer membrane protein LipL41 is not essential for acute leptospirosis but requires a small chaperone protein, lep, for stable expression. Infect Immun **81:**2768-2776.
34. **Eshghi A, Lourdault K, Murray GL, Bartpho T, Sermswan RW, Picardeau M, Adler B, Snarr B, Zuerner RL, Cameron CE.** 2012. *Leptospira interrogans* catalase is required for resistance to H_2O_2 and for virulence. Infect Immun **80:**3892-3899.
35. **Raposo RA, Thomas B, Ridlova G, James W.** 2011. Proteomic-based identification of CD4-interacting proteins in human primary macrophages. PLoS One **6:**e18690.
36. **Chambers MC, Maclean B, Burke R, Amodei D, Ruderman DL, Neumann S, Gatto L, Fischer B, Pratt B, Egertson J, Hoff K, Kessner D, Tasman N, Shulman N, Frewen B, Baker TA, Brusniak MY, Paulse C, Creasy D, Flashner L, Kani K, Moulding C, Seymour SL, Nuwaysir LM, Lefebvre B, Kuhlmann F, Roark J, Rainer P, Detlev S, Hemenway T, Huhmer A, Langridge J, Connolly B, Chadick T, Holly K, Eckels J, Deutsch EW, Moritz RL, Katz JE, Agus DB, MacCoss M, Tabb DL, Mallick P.** 2012. A cross-platform toolkit for mass spectrometry and proteomics. Nat Biotechnol **30:**918-920.
37. **Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M.** 2011. Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res **10:**1794-1805.
38. **Cox J, Mann M.** 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol **26:**1367-1372.
39. **Goldstein DR, Yang YH, Thorne NP.** 2003. Normalization for two-color cDNA microarray data, p 403-418, Statistics and science: a Festschrift for Terry Speed, vol Volume 40. Institute of Mathematical Statistics, Beachwood, OH.
40. **Smyth Gordon K.** 2004. Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments, vol 3, p 1.
41. **Smyth GK, Speed T.** 2003. Normalization of cDNA microarray data. Methods **31:**265-273.
42. **Benjamini Y, Y.** 1995. Controlling the false discovery rate : A practical and powerful approach to multiple testing. J R Statist Soc **57:**289-300.
43. **Vallenet D, Engelen S, Mornico D, Cruveiller S, Fleury L, Lajus A, Rouy Z, Roche D, Salvignol G, Scarpelli C, Médigue C.** 2009. MicroScope: a platform for microbial genome annotation and comparative genomics. Database (Oxford) **2009:**bap021.
44. **Stamm LV, Gherardini FC, Parrish EA, Moomaw CR.** 1991. Heat shock response of spirochetes. Infect Immun **59:**1572-1575.
45. **Matsunaga J, Medeiros MA, Sanchez Y, Werneid KF, Ko AI.** 2007. Osmotic regulation of expression of two extracellular matrix-binding proteins and a haemolysin of *Leptospira interrogans*: differential effects on LigA and Sph2 extracellular release. Microbiology **153:**3390-3398.
46. **Matsunaga J, Sanchez Y, Xu X, Haake DA.** 2005. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. Infect Immun **73:**70-78.
47. **Käll L, Krogh A, Sonnhammer EL.** 2004. A combined transmembrane topology and signal peptide prediction method. J Mol Biol **338:**1027-1036.

48. **Bendtsen JD, Kiemer L, Fausbøll A, Brunak S.** 2005. Non-classical protein secretion in bacteria. BMC Microbiol **5:**58.
49. **Mani M, Chen C, Amblee V, Liu H, Mathur T, Zwicke G, Zabad S, Patel B, Thakkar J, Jeffery CJ.** 2015. MoonProt: a database for proteins that are known to moonlight. Nucleic Acids Res **43:**D277-282.
50. **Sukumaran SK, Fu NY, Tin CB, Wan KF, Lee SS, Yu VC.** 2010. A soluble form of the pilus protein FimA targets the VDAC-hexokinase complex at mitochondria to suppress host cell apoptosis. Mol Cell **37:**768-783.
51. **Blau K, Portnoi M, Shagan M, Kaganovich A, Rom S, Kafka D, Chalifa Caspi V, Porgador A, Givon-Lavi N, Gershoni JM, Dagan R, Mizrachi Nebenzahl Y.** 2007. Flamingo cadherin: a putative host receptor for *Streptococcus pneumoniae*. J Infect Dis **195:**1828-1837.
52. **Ling E, Feldman G, Portnoi M, Dagan R, Overweg K, Mulholland F, Chalifa-Caspi V, Wells J, Mizrachi-Nebenzahl Y.** 2004. Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse. Clin Exp Immunol **138:**290-298.
53. **Kinnby B, Booth NA, Svensäter G.** 2008. Plasminogen binding by oral streptococci from dental plaque and inflammatory lesions. Microbiology **154:**924-931.
54. **Figueira C, Croda J, Choy H, Haake D, Reis M, Ko A, Picardeau M.** 2011. Heterologous expression of pathogen-specific genes ligA and ligB in the saprophyte *Leptospira biflexa* confers enhanced adhesion to cultured cells and fibronectin. BMC Microbiology **11:**129-137.
55. **Verma A, Artiushin S, Matsunaga J, Haake DA, Timoney JF.** 2005. LruA and LruB, novel lipoproteins of pathogenic *Leptospira interrogans* associated with equine recurrent uveitis. Infect Immun **73:**7259-7266.
56. **Verma A, Rathinam SR, Priya CG, Muthukkaruppan VR, Stevenson B, Timoney JF.** 2008. LruA and LruB antibodies in sera of humans with leptospiral uveitis. Clin Vaccine Immunol **15:**1019-1023.
57. **Verma A, Kumar P, Babb K, Timoney JF, Stevenson B.** 2010. Cross-reactivity of antibodies against leptospiral recurrent uveitis-associated proteins A and B (LruA and LruB) with eye proteins. PLoS Neglect Trop D **4:**e778.
58. **Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, Wilson PJ, Creno S, Kuczek ES, Bommezzadri S, Davis JC, McGrath A, Johnson MJ, Boursaux-Eude C, Seemann T, Rouy Z, Coppel RL, Rood JI, Lajus A, Davies JK, Médigue C, Adler B.** 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLoS One **3:**e1607.
59. **Wolff DG, Castiblanco-Valencia MM, Abe CM, Monaris D, Morais ZM, Souza GO, Vasconcellos SA, Isaac L, Abreu PA, Barbosa AS.** 2013. Interaction of *Leptospira* elongation factor Tu with plasminogen and complement factor H: a metabolic leptospiral protein with moonlighting activities. PLoS One **8:**e81818.
60. **Crowe JD, Sievwright IK, Auld GC, Moore NR, Gow NA, Booth NA.** 2003. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. Mol Microbiol**47:**1637-1651.
61. **Sakolvaree Y, Maneewatch S, Jiemsup S, Klaysing B, Tongtawe P, Srimanote P, Saengjaruk P, Banyen S, Tapchaisri P, Chonsa-nguan M, Chaicumpa W.** 2007.

Proteome and immunome of pathogenic *Leptospira spp.* revealed by 2DE and 2DE-immunoblotting with immune serum. Asian Pac J Allergy Immunol **25:**53-73.

62. **Domingos R, Fernandes L, Romero E, de Morais Z, Vasconcellos S, Nascimento AL.** 2015. The novel *Leptospira interrogans* protein Lsa32 is expressed during infection and binds laminin and plasminogen. Microbiology **161:**851-864
63. **King AM, Pretre G, Bartpho T, Sermswan RW, Toma C, Suzuki T, Eshghi A, Picardeau M, Adler B, Murray GL.** 2014. High-temperature protein G is an essential virulence factor of *Leptospira interrogans*. Infect Immun **82:**1123-1131.
64. **Lourdault K, Wang LC, Vieira A, Matsunaga J, Melo R, Lewis MS, Haake DA, Gomes-Solecki M.** 2014. Oral immunization with *Escherichia coli* expressing a lipidated form of LigA protects hamsters against challenge with *Leptospira interrogans* serovar Copenhageni. Infect Immun **82:**893-902.
65. **Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, DeMoll E, Kraiczy P, Cooley AE, Creamer TP, Suchard MA, Brissette CA, Verma A, Haake DA.** 2007. *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. PLoS ONE **2:**e1188.
66. **Xu Q, Rawlings ND, Farr CL, Chiu HJ, Grant JC, Jaroszewski L, Klock HE, Knuth MW, Miller MD, Weekes D, Elsliger MA, Deacon AM, Godzik A, Lesley SA, Wilson IA.** 2011. Structural and sequence analysis of imelysin-like proteins implicated in bacterial iron uptake. PLoS One **6:**e21875.
67. **Hullo MF, Auger S, Soutourina O, Barzu O, Yvon M, Danchin A, Martin-Verstraete I.** 2007. Conversion of methionine to cysteine in *Bacillus subtilis* and its regulation. J Bacteriol **189:**187-197.
68. **Marcsisin RA, Bartpho T, Bulach DM, Srikram A, Sermswan RW, Adler B, Murray GL.** 2013. Use of a high-throughput screen to identify *Leptospira* mutants unable to colonize the carrier host or cause disease in the acute model of infection. J Med Microbiol **62:**1601-1608.
69. **Ristow P, Bourhy P, McBride FW, Figueira CP, Huerre M, Ave P, Girons IS, Ko AI, Picardeau M.** 2007. The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog **3:**e97.
70. **Murray GL, Srikram A, Henry R, Puapairoj A, Sermswan RW, Adler B.** 2009. *Leptospira interrogans* requires heme oxygenase for disease pathogenesis. Microbes Infect **11:**311-314.
71. **Zhang K, Murray GL, Seemann T, Srikram A, Bartpho T, Sermswan RW, Adler B, Hoke DE.** 2013. Leptospiral LruA is required for virulence and modulates an interaction with mammalian apolipoprotein AI. Infect Immun **81:**3872-3879.
72. **Zhang L, Zhang C, Ojcius DM, Sun D, Zhao J, Lin X, Li L, Yan J.** 2012. The mammalian cell entry (Mce) protein of pathogenic *Leptospira* species is responsible for RGD motif-dependent infection of cells and animals. Mol Microbiol **83:**1006-1023.

FIGURE LEGENDS

Fig 1. **Differential protein localization and expression in leptospiral culture supernatants**. *L. interrogans* were cultured at 30˚C, 37˚C or in media containing 120 mM NaCl. Proteins from whole cells and culture supernatants were subsequently used in immunoblot experiments with the indicated antisera. Panel A. A representative coomassie stained SDS-PAGE demonstrating disparate protein composition when comparing whole cell lysate proteins to those ubtained from culture supernatants. Panel B. Protein immunoblot demonstrating localization of flagellar protein FlaA-2 in whole cells but not in the supernatant. Panel C, Protein immunoblot suggesting increased expression of the chaperone protein GroEL at 37˚C. Panel D. Protein immunoblot indicating expression of immunoglobulin protein LigA in CSP and increased expression in the CSP from *L. interrogans* exposed to 120 mM NaCl. Panel E. Protein immunoblot confirming expression of LßP52 protein in culture supernatants and increased expression in CSP from *L. interrogans* exposed to 120 mM NaCl.

Fig 2. Classification of *L. interrogans* exoproteins suggests most proteins to be involved in metabolic processes. Panel A. Exoproteins were assigend to clusters of orthologous groups (COG) using GenoScope (43) guidelines. Panel B. The frequencie of exoproteins in each COG was compared to those expected genome-wide. Statistical analyses was performed by assuming a binomial distribution using a p value cut off of $p < 0.01$. The * symbol represents a significant difference between the observed number of exoproteins and the genome-wide expected probabilities for the given COG, in a sample size of 325 proteins. The COG categories and the predicted precentages of *L. interrogans* genes in the respective COG are as follows: D: Cell cycle control, cell division, chromosome partitioning (0.9%); M: Cell wall/membrane/envelope biogenesis (5.3%); N: Cell motility (2.4%); O: Posttranslational modification, protein turnover,

chaperones (3.2%); T: Signal transduction mechanisms (5.9%); U: Intracellular trafficking, secretion, and vesicular transport (1.7%); V: Defense mechanisms (1.6%); Z: Cytoskeleton (.06%); B: Chromatin structure and dynamics (.04%); J: Translation, ribosomal structure and biogenesis (3.5%); K: Transcription (3.1%); L: Replication, recombination and repair (4.6%); C: Energy production and conversion (3.2%); E: Amino acid transport and metabolism (7.0%); F: Nucleotide transport and metabolism (1.6%); G: Carbohydrate transport and metabolism (3.6%); H: Coenzyme transport and metabolism (2.8%); I: Lipid transport and metabolism (2.9%); P: Inorganic ion transport and metabolism (4.5%); Q: Secondary metabolites biosynthesis, transport and catabolism (1.8%); R: General function prediction only (11.0%); S: Function unknown (5.0%); -: Unclassified (39.0%) .

Fig 3. ***L. interrogans*** **positive sera display IgM and IgG reactivity to leptospiral exoproteins**. Guinea pig sera obtained prior to and post *L. interrogans* infection were used in protein immunoblot experiments to test immunoglobulin reactivity with whole *L. interrogans* lysates or culture supernatants. Panel A. Protein immunoblot comparing IgG reactivity with whole cell proteins and exoproteins when using *L. interrogans* positive sera. Panel B. Protein immunoblot comparing IgM reactivity to whole cell proteins and exoproteins when using *L. interrogans* positive sera. Panel C. Protein immunoblot demonstrating a lack of IgG reactivity to whole cell and extracellular proteins when using pre-infection sera. Panel D. Protein immunoblot demonstrating a lack of IgM reactivity to whole cell and extracellular proteins when using pre-infection sera.

781 **Table 1. Overview of proteomic results. The protein intensity is displayed for the periplasmic flagellar sheath protein FlaA-2**

782 **and for an exoprotein (LIC10371) which was detected in high abundance in culture supernatants.**

	P30°C[a]	**P37°C[a]**	**PNaCl[a]**	**S30°C[b]**	**S37°C[b]**	**SNaCl[b]**
[c]Unique proteins detected	1073	1141	1293	712	553	540
Smallest protein (Da)	~11000	~11000	~9000	~9000	~10000	~10000
Largest protein (Da)	~300000	~300000	~300000	~270000	~224000	~219000
Protein intensity (highest/lowest)	1.5E10/1.0E6	1.9E10/3.1E5	3.1E10/3.1E5	1.2E10/2.3E5	2.2E10/1.6E5	1.7E10/1.0E5
[d,e]FlaA-2	5.5E8	2.8E8	1.1E8	Not detected	Not detected	Not detected
[d,f]LIC10371	Not detected	Not detected	Not detected	3.2E8	2.5E8	4.2E8

783 [a]Whole cell *Leptospira*, [b]Culture supernatants, [c]Combined experimental replicates, [d]Average between experiments, [e]Periplasmic

784 localization, [f]Extracellular localization

785 **Table 2. The 20 most abundant exoproteins in the culture supernatant of *L. interrogans*.**

EMBL/GenBank/ DDBJ CDS	Locus tag	Protein	[a]COG	[b]WCP	[c]CSP	[c]CSP:[b]WCP	[d]RQ
AAS69512.1	LIC10898	LipL48	U	1.2E+09	1.3E+10	10.8	1.0
AAS70653.1	LIC12082	Cysteine synthase	E	2.5E+09	1.1E+10	4.6	0.9
AAS71860.1	LIC13318	Fatty acid synthase subunit beta	I	6.5E+08	3.8E+09	5.8	0.3
AAS72009.1	LIC13470	Ferredoxin--NADP reductase (EC 1.18.1.2)	P	3.6E+08	2.3E+09	6.4	0.2
AAS72270.1	LIC20249	Aconitate hydratase	C	8.8E+08	2.2E+09	2.5	0.2
AAS70370.1	LIC11781	Malate dehydrogenase	C	6.0E+08	2.1E+09	3.4	0.2
AAS69960.1	LIC11359	MaoC	I	3.2E+08	2.0E+09	6.2	0.2
AAS71788.1	LIC13244	Isocitrate dehydrogenase	C	8.3E+08	1.9E+09	2.4	0.1
AAS68881.1	LIC10253	Alcohol dehydrogenase	C	3.0E+08	1.9E+09	6.3	0.1
AAS71429.1	LIC12876	Elongation factor G	J	1.2E+09	1.8E+09	1.5	0.1
AAS68844.1	LIC10216	Phosphoenolpyruvate carboxykinase	C	4.2E+08	1.8E+09	4.3	0.1
AAS70553.1	LIC11977	Cyclic nucleotide binding protein	R	5.3E+08	1.8E+09	3.3	0.1
AAS71933.1	LIC13393	Ketol-acid reductoisomerase	E/H	8.2E+08	1.6E+09	1.9	0.1
AAS69801.1	LIC11194	Putative citrate lyase	-	9.8E+08	1.5E+09	1.5	0.1
AAS68639.1	LIC10002	DNA polymerase III beta subunit	L	3.6E+08	1.4E+09	4.0	0.1
AAS70661.1	LIC12090	Glyceraldehyde-3-phosphate dehydrogenase	G	8.2E+08	1.3E+09	1.6	0.1
AAS69456.1	LIC10842	4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7)	M/E	1.6E+08	1.1E+09	6.8	0.1
AAS69086.1	LIC10465	LigA	-	1.7E+08	1.1E+09	6.4	0.1
Q72NJ3.1	LIC12841	LL-diaminopimelate aminotransferase (EC 2.6.1.83)	E	1.4E+08	1.0E+09	7.0	0.1
AAS70438.1	LIC11852	O-acetylhomoserine (Thiol) lyase	E	2.4E+08	9.8E+08	4.1	0.1

786 [a]Clusters of orthologous groups (COG). See Fig. 2 legend for description of categories.

787 [b]Average protein intensity for proteins from whole bacteria.

788 [c]Average protein intensity for proteins in supernatants.

789 [d]Relative protein quantities in supernatants compared to LipL48

790 **Table 3. Exoproteins unique to pathogenic *Leptospira*.**
791

[a]Locus tag	[a]Protein name	Gene	[b]COG	[c]N-terminal signal peptide	[d]Non-classical secretion	[e]Virulence
LIC10061	Uncharacterized protein		-	Yes		Not tested
LIC10241	Uncharacterized protein		D			Not tested
LIC10371	Putative lipoprotein		-	Yes		Not tested
LIC10373	Putative lipoprotein		-	Yes		0/4
LIC10465	Ig-like repeat domain protein 1	*ligA*	-	Yes		0/4
LIC10520	Uncharacterized protein		-	Yes		Not tested
LIC10548	Uracil-DNA glycosylase		L			Not tested
LIC10637	Ribosomal protein serine acetyltransferase		J			Not tested
LIC10657	Sphingomyelinase C	*sphH*	-	Yes		Not tested
LIC10711	Cytoplasmic membrane protein		-	Yes		Not tested
LIC10713	Putative lipoprotein		P	Yes		Not tested
LIC10774	Putative lipoprotein		-	Yes		Not tested
LIC10793	Surface antigen	*orfC*	S	Yes		Not tested
LIC10927	Putative lipoprotein		-	Yes		Not tested
LIC10987	Uncharacterized protein		-			Not tested
LIC10988	Cytoplasmic membrane protein		E	Yes		Not tested
LIC11009	Uncharacterized protein		-	Yes		Not tested
LIC11046	Uncharacterized protein		-			Not tested
LIC11096	Uncharacterized protein		S			Not tested
LIC11207	Putative lipoprotein		-	Yes		Not tested
LIC11334	Uncharacterized protein		S	Yes		Not tested
LIC11687	Endonuclease		-	Yes		Not tested
LIC11860	3-hydroxyisobutyrate dehydrogenase	*mmsB*	I	Yes		Not tested
LIC11996	Uncharacterized protein		-	Yes		Not tested
LIC12032	Catalase (EC 1.11.1.6)	*katE*	P		Yes	avirulent (ref. 33)
LIC12048	Uncharacterized protein		-	Yes		Not tested
LIC12084	Uncharacterized protein		-			Not tested
LIC12139	UDP-N-acetylglucosamine 2-epimerase	*rffE*	M			Not tested
LIC12158	Putative hydroxyacid aldolase protein		G			Not tested
LIC12166	Alcohol dehydrogenase		C			Not tested

LIC12193	Uncharacterized protein		-			Not tested
LIC12208	Putative lipoprotein		-	Yes		0/4
LIC12209	Putative lipoprotein (LßP52)		-	Yes		Not tested
LIC12232	Thymidylate synthase	*thyX*	F			Not tested
LIC12323	Type III beta-ketoacyl synthase-like protein		I		Yes	Not tested
LIC12331	Uncharacterized protein		G	Yes		Not tested
LIC12341	Uncharacterized protein		-	Yes		Not tested
LIC12353	Uncharacterized protein		-	Yes		Not tested
LIC12410	Uncharacterized protein		-	Yes		Not tested
LIC12581	Uncharacterized protein		S		Yes	Not tested
LIC12736	Putative lipoprotein		-	Yes		Not tested
LIC12739	MaoC family protein		I	Yes		Not tested
LIC13060	LipL36	*lipL36*	-	Yes		0/4
LIC13066	Putative lipoprotein		-	Yes		Not tested
LIC13261	Uncharacterized protein		R			Not tested
LIC13354	Uncharacterized protein		-		Yes	Not tested
LIC13361	Uncharacterized protein		L	Yes		Not tested
LIC13428	Uncharacterized protein		-			Not tested
LIC20053	Uncharacterized protein		-			Not tested
LIC20077	Polysaccharide deacetylase		G		Yes	Not tested
LIC20196	Uncharacterized protein		-	Yes		Not tested

[a]Putative orthologous alignment between *L. interrogans* with other pathogenic *Leptospira* species are defined as proteins satisfying a blastP alignment threshold of a minimum of 40% sequence identity on 80% of the length of the protein.

[b]Clusters of orthologous groups were retrieved from the MicroScope platform.

[c]N-terminal signal peptide predictions were predicted using Phobius.

[d]Non-classical secretion predictions were performed using SecretomeP 2.0.

[e]Gerbil survival: Groups of 4 gerbils were inoculated with 10^4 bacteria. Animals were monitored for 23 days.

802 **Table 4. Potential and confirmed moonlighting proteins in *Leptospira* culture supernatants.**
803

EMBL/GenBank/ DDBJ CDS	Locus tag	Gene/annotation	[a]Moonlighting function in other organisms
[b]AAS71252.1	LIC12694	Glutamate synthase (NADPH) alpha chain precursor	Binds plasminogen, fibronectin, laminin and collagen I
[b]AAS69825.1	LIC11219	Peroxiredoxin	Molecular chaperones
AAS71651.1	LIC13105	Glucose-6-phosphate isomerase	Binds laminin and collagen I
AAS72117.1	LIC20088	Pyrophosphate-fructose-6-phosphate 1-phosphotransferase	Binds, invertase and plasminogen
AAS69569.1	LIC10958	Alcohol dehydrogenase	Binds plasminogen, fibronectin, laminin and collagen II
AAS70661.1	LIC12090	Glyceraldehyde-3-phosphate dehydrogenase	NAD ribosylating activity, binds mucin, Caco-2 cells, invertase, fibronectin, laminin, type I collagen, plasminogen, uPAR/CD87 receptor, transferrin-binding protein
AAS69802.1	LIC11195	Ornithine carbamoyltransferase	Binds fibronectin
AAS70607.1	LIC12032	Catalase	Binds plasminogen
AAS70662.1	LIC12091	Phosphoglycerate kinase	Binds plasminogen
AAS72270.1	LIC20249	Aconitate hydratase	Iron-responsive protein, binds iron-responsive elements
[b]AAS68899.1	LIC10272	Translation elongation factor G	Binds mucin
AAS69466.1	LIC10852	Uridylate kinase	Transcriptional regulator
AAS70653.1	LIC12082	Cysteine synthase	Transcriptional regulator
AAS71909.1	LIC13367	Sulfite reductase	Transcriptional regulator
[b]AAS69936.1	LIC11335	GroEL	Binds glycosphingolipids, mucins, epithelial cells, DNA. Toxin.
[b]AAS69145.1	LIC10524	Heat shock protein 70 (DnaK)	Binds plasminogen and invertase
[b]AAS71428.1	LIC12875	Elongation factor Tu (Tuf)	Binds human cells, mucins, fibronectin, factor H, plasminogen
[b]AAS70536.1	LIC11954	Enolase	Binds plasminogen, laminin, fibronectin and mucin

[b]AAS70976.1	LIC12407	Glutamine synthetase protein (GlnA)	Binds plasminogen, fibronectin, laminin, collagen I, transcription factor TnrA
[b]AAS68791.1	LIC10162	Fumarate hydratase (Aspartate ammonia-lyase)	Binds plasminogen
[b]AAS71913.1	LIC13371	Sulfate adenylyltransferase subunit 2 (Transcription elongation factor)	Binds plasminogen
[b]AAS70665.1	LIC12094	Triosephosphate isomerase	Binds plasminogen
AAS72270.1	LIC20249	Aconitate hydratase	Binds untranslated regions mRNA
AAS71933.1	LIC13393	Ketol-acid reductoisomerase (EC 1.1.1.86)	Maintain mitochondrial DNA stability
[b]AAS69525.1	LIC10913	Transketolase alpha subunit protein	Transcriptional regulator
[b]AAS70287.1	LIC11698	Tyrosyl tRNA synthetase	Promotes folding of group 1 introns
AAS70990.1	LIC12422	Aspartate aminotransferase A	Transcription regulation
AAS69466.1	LIC10852	Uridylate kinase (UK) (EC 2.7.4.22)	Required for microautophagy
[b]AAS69380.1	LIC10763	Alanyl-tRNA synthetase	Transcription regulator
[b]AAS69367.1	LIC10750	50S ribosomal protein L1	Translational repressor
[b]AAS71870.1	LIC13328	Isocitrate dehydrogenase	Binds mRNA
[b]AAS68702.1	LIC10065	Deoxycytidine triphosphate deaminase	Hydrolysis of the triphosphate moiety

804 [a]Moonlighting functions were obtained from MoonProt (49).

805 [b]Proteins were detected in supernatants but displayed an adjusted *p* value >0.05 when comparing

806 protein abundances between supernatants and whole cell pellets. A *p* value >0.05 is indicative of

807 equal or lower protein abundance in culture supernatants relative to that in whole c

Table 5. Inactivation of select exoprotein encoding genes has no effect on *L. interrogans* virulence in the animal infection model.

Inactivated locus	**Gene**	**CSP vs WCP**	**[a]Orthologue in *L. biflexa***	**[b]*in vitro* growth**	**[c]Virulence**
LIC10713	*lruB*	S only	No	Limited	Not tested
LIC11852	O-acetylhomoserine (thiol) lyase	4.7	Yes	Limited	Not tested
LIC13006	*lenC*	S only	Yes	Same as wt	0/4
LIC12208	putative lipoprotein (beta-propeller repeats)	S only	No	Same as wt	0/4
LIC13060	*lipL36*	4.3	No	Same as wt	0/4
LIC10373	putative lipoprotein (beta-propeller)	490	No	Same as wt	1/4
LIC10898	*lipL48*	10.8	Yes	Same as wt	0/4
LIC11977	cyclic nucleotide binding protein	3.4	Yes	Same as wt	0/4
LIC10465	*ligA*	6.4	No	Same as wt	0/4

[a]Orthologous alignment between *L. interrogans* and *L. biflexa* are defined as proteins satisfying a blastP alignment threshold of a minimum of 40% sequence identity over 80% of the length of the protein.

[b]Growth of wild-type and mutant strains in liquid EMJH incubated at 30°C. Growth was monitored by measuring OD_{420} on a daily basis.

[c]Gerbil survival: Groups of 4 gerbils were inoculated with 10^4 bacteria. Animals were monitored for 23 days.

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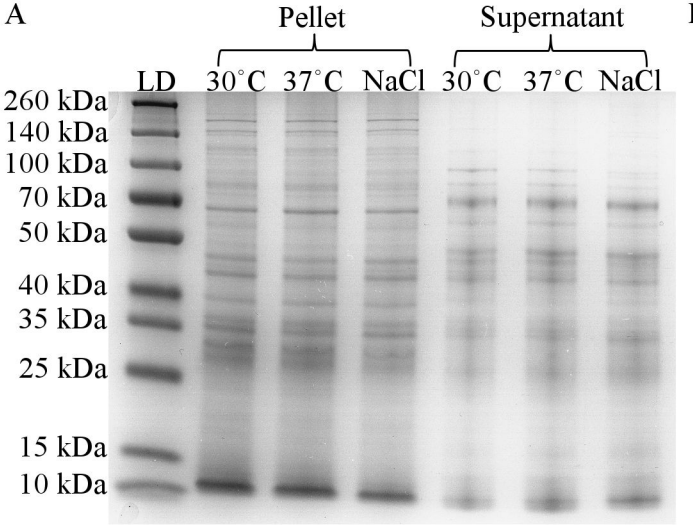
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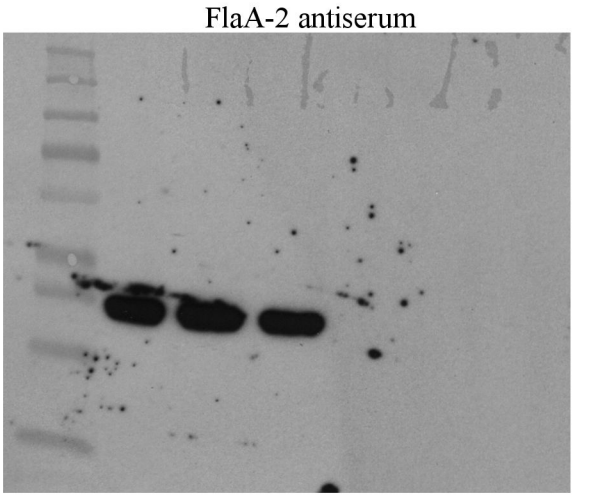
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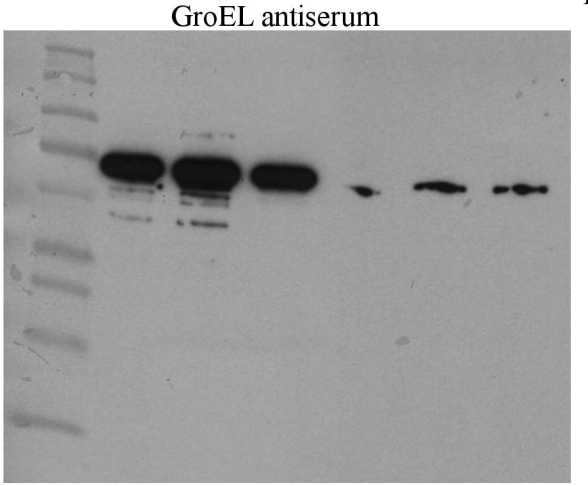
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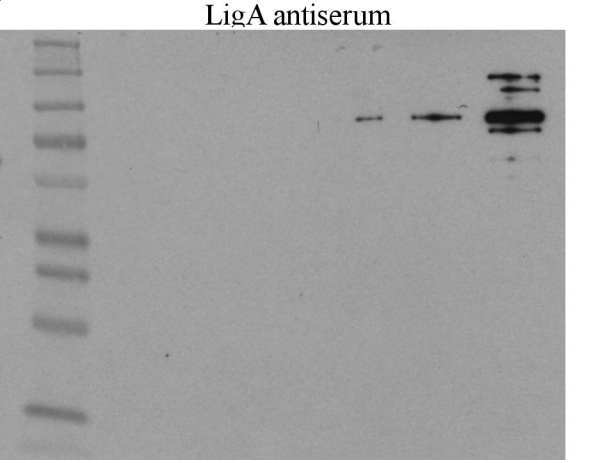
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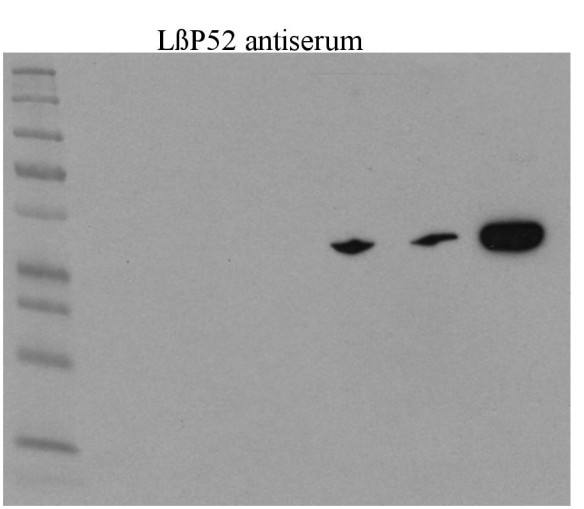
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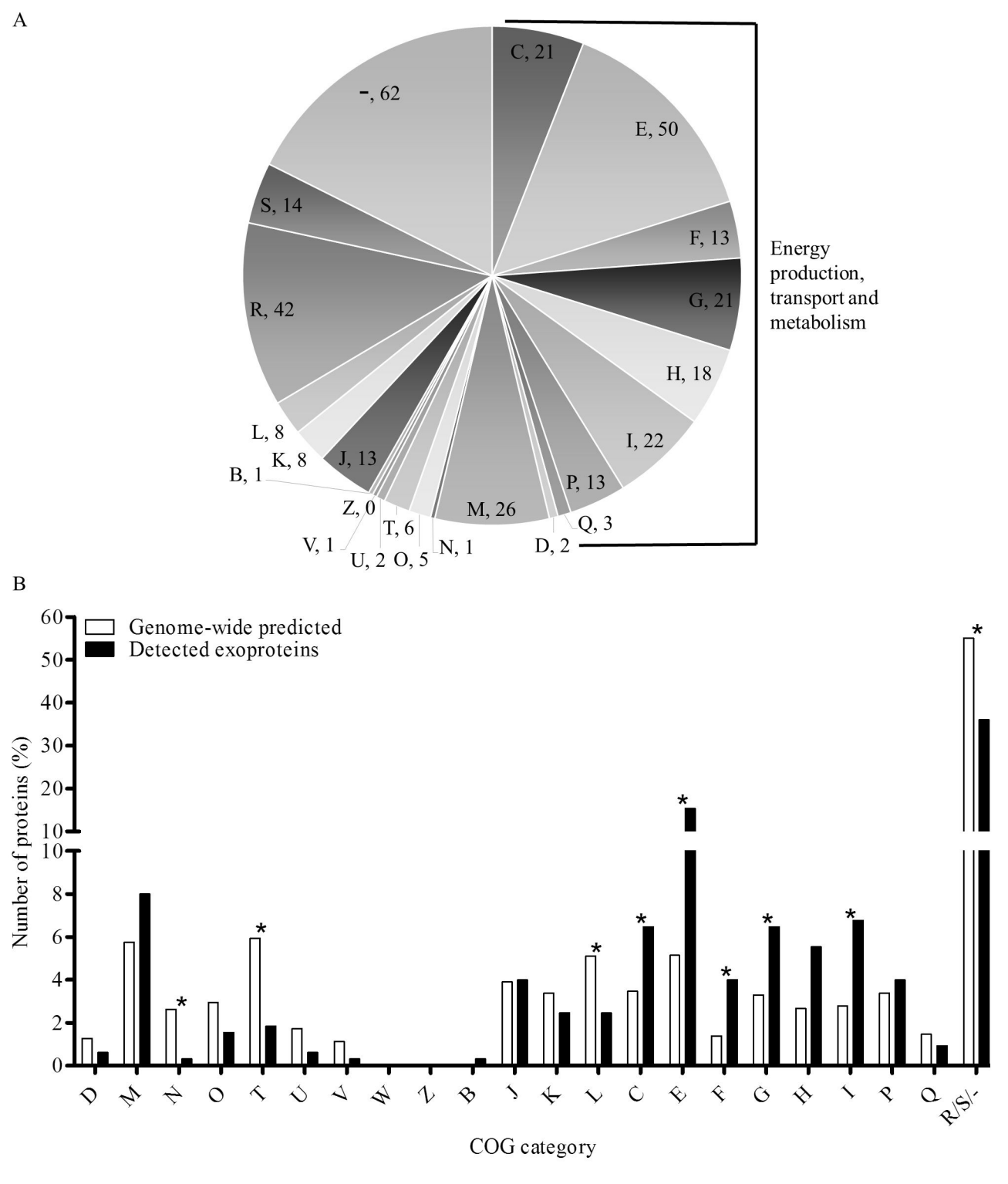
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Energy production, transport and metabolism
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Genome-wide predicted
Detected exoproteins
Number of proteins (%)
COG category
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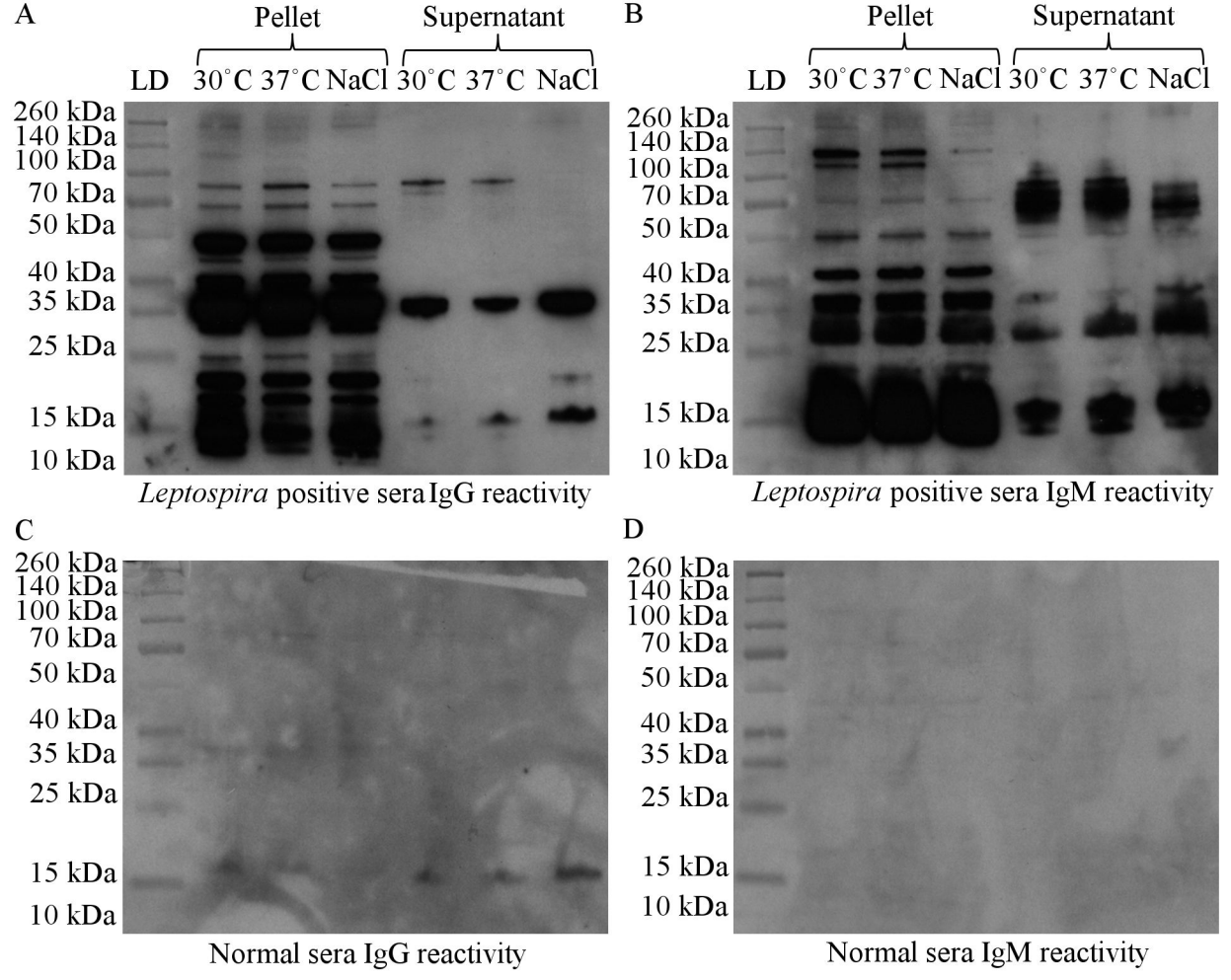
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Leptospira positive sera IgG reactivity
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