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

3 Running Title: Exoprotein function in *Leptospira* life cycle

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19 **ABSTRACT**

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

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

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

41

42 INTRODUCTION

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

57 *Leptospira* resemble Gram negative bacteria in that they contain an inner membrane, a
58 periplasmic space with peptidoglycan and an outer membrane where the lipopolysaccharide
59 (LPS) is anchored. The bacteria are highly motile and previous studies have demonstrated that
60 both full motility (11, 12) and an intact LPS (13) are required for successful colonization of the
61 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

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

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

95

96 MATERIALS AND METHODS

97 *Bacterial Strains and Culturing*

98 *Leptospira interrogans* serovar Manilae strain L495 was culture maintained in
99 Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (25, 26) at 30°C. *L. interrogans*
100 transposon mutagenesis has previously been described (27-29) and L495 transposon mutants
101 used in this study were obtained from an in house maintained library of mutants. The mutant
102 strains were culture maintained as described above for the parent strain. To perform proteomic
103 analysis on culture supernatants EMJH was constituted with the following modifications.
104 Albumin was omitted from the recipe and Tween 80 and glycerol were added to 0.01% (v/v). A
105 separate batch of modified EMJH was made to contain 120 mM NaCl. Prior to shifting to
106 modified EMJH media, *L. interrogans* were cultured in EMJH at 30°C to a density of 10⁹
107 bacteria per ml, and subsequently pelleted via centrifugation at 3, 200 xg for 15 min, using a
108 swinging bucket rotor. Pelleted bacteria were washed 3 times with modified EMJH using 20 ml
109 of media for each washing step. Bacteria were then resuspended in modified EMJH and
110 enumerated via darkfield microscopy. Bacteria were subsequently diluted in modified EMJH to a
111 concentration of 10⁸ bacteria per ml in a total volume of 100 ml for each condition (for
112 incubation at 30°C, 37°C and in modified EMJH with 120 mM NaCl at 30°C), in two biological
113 replicates for each condition. After 18 hours of incubation, bacteria were enumerated via
114 darkfield microscopy to validate bacterial viability. Technical limitations prevented growth of
115 bacteria at 37°C in modified EMJH containing 120 mM NaCl as bacterial viability was affected
116 under these conditions.

117 *Leptospira Culture Supernatants*

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

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

135 ***Recombinant Protein and Antisera***

136 Recombinant LIC12209 (encoding a *Leptospira* beta-propeller protein 52 kDa protein
137 (LBP52)) was produced as previously described (30) with the following modifications. The
138 predicted *lβP52* gene was amplified by PCR from using *L. interrogans* serovar Manilae strain
139 L495 genomic DNA with the primer pairs LIC12209F 5'-
140 GGGGCTAGCATGAGAAAATTTTACATT-3' and LIC12209R 5'-

141 CCCGCGGCCGCTTATCTATATTTTACA-3'. Underlined nucleotides indicate restriction sites
142 (NheI/NotI). The PCR product was cloned into TOPO vector (Invitrogen Life Technologies,
143 Saint Aubin, France) and transformed into *Escherichia coli* DH5a (Invitrogen Life
144 Technologies). The amplicon was released from the vector by digestion with NheI and NotI, and
145 re-cloned into the pET-28(a) expression plasmid. The pET28 vector allows expression of
146 recombinant protein with a N-terminal 6X His-tag (poly-histidine tag). The cloned sequence was
147 confirmed by DNA sequencing (eurofins, Courtaboeuf, France).

148 Protein expression was achieved in *E. coli* BL21 (DE3) strain. *E. coli* BL21 (DE3)
149 containing recombinant plasmid was cultured at 37 °C under kanamycin selection to an optical
150 density (600 nm) of 0.6-0.8. Recombinant protein synthesis was induced by the addition of 1
151 mM (final concentration) Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 3 h, the cells
152 were harvested by centrifugation at 4,000 xg for 30 min and the bacterial pellets were
153 resuspended in 5 ml buffer A (100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 8.0). The bacterial cell
154 pellets were lysed via sonication and recombinant protein, in the form of inclusion bodies, was
155 then separated by centrifugation at 17,000 xg for 20 min, and solubilized in 5 ml of buffer B (100
156 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea pH 8.0). The protein was purified through metal-
157 chelating chromatography in a fast-flow column with 0,4 ml of nickle-nitrilotriacetic acid (Ni-
158 NTA) resin (QIAGEN, Courtaboeuf, France) using the manufacturers protocol. All fractions
159 were analyzed by 12% SDS-PAGE.

160 Rabbit antisera generated against the flagellar protein FlaA-2 (31), chaperone protein
161 GroEL (32) and Ig-like repeat domain protein 1 LigA (32) where generously provided by Dr.
162 David Haake. Rabbit antisera against recombinant LBP52 (rLBP52) protein was generated as
163 follows. The recombinant protein LBP52 was dialyzed against buffer 1 (100 mM NaH₂PO₄, 10

164 mM Tris·Cl, 5M urea pH 8) for 48 h followed by a second dialysis step for 48 h against buffer 2
165 (100 mM NaH₂PO₄, 10 mM Tris·Cl, 2M urea pH 8) and 50 µg of protein was submitted for
166 antiserum production rabbits by Covalab R&D in Biotechnology (France).

167 To obtain *L. interrogans* positive sera, Hartley male guinea pigs (Charles River
168 Laboratories, <http://www.criver.com>) were obtained at 6 weeks of age and weighed between
169 450-500 g. Guinea pigs (N = 6) were anaesthetized via intramuscular injection with 40 mg
170 ketamine and 4 mg xylazine (per kg body weight) and blood (~ 1 ml) was collected into 10 ml
171 Venosafe Plastic Tubes (Terumo, Guyancourt, France) via cardiac heart puncture. Animals were
172 then maintained under normal care conditions for 7 days and then injected intraperitoneal with
173 10⁵ *L. interrogans* strain L495 in 1 ml albumin free EMJH. Blood samples from infected animals
174 were collected by terminal cardiac puncture of anaesthetized animals 14 days post-challenge.
175 Serum was collected by incubation of blood samples in Venosafe Plastic tubes (Terumo) at room
176 temperature for 30 min, followed by centrifugation at 1,500 xg for 10 min. The supernatant was
177 collected for use in immunoblot experiments. Protocols for animal experiments conformed to the
178 guidelines of the Animal Care and Use Committees of the Institut Pasteur (Paris, France).

179 ***Protein Immunoblots***

180 Sodium dodecyl sulfate polyacrylamide gel electrophoresis and protein immunoblots
181 were performed as previously described (33, 34) with the following modifications. The SDS-
182 PAGE and immunoblot experiments were performed using 10 µg of total protein from whole cell
183 lysates and culture supernatants. Antiserum to FlaA-2, GroEL, LigA and LBP52 was used at
184 1:2,000, 1:8,000, 1:750 and 1:1,000, respectively. Guinea pig *Leptospira* positive and pre-
185 immune sera were used at 1:100 and goat polyclonal secondary antibody to guinea pig IgG-Fc

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

188 *Protein Treatment Preceding Mass Spectrometry*

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

200 *Mass Spectrometry*

201 Protein samples were analysed on an Ultimate 3000 RSLCnano high-performance liquid
202 chromatography (HPLC) (Dionex, Camberley, UK) system run in direct injection mode coupled
203 to a QExactive Orbitrap mass spectrometer (Thermo Electron, Hemel Hempstead, UK). Samples
204 were resolved on a 25 cm by 75 µm inner diameter picotip analytical column (New Objective,
205 Woburn, MA, USA) which was packed in-house with ProntoSIL 120-3 C18 Ace-EPS phase, 3
206 µm diameter beads (Bischoff Chromatography, Germany). The system was operated at a flow-
207 rate of 300 nl min⁻¹ and a 120 min gradient was used to separate the peptides. The mass
208 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

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

234 ***Assignment of Proteins to Clusters of Orthologous Groups***

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

244 ***Mutant Strains, Growth Rates and Infection Experiments***

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

255 Exoprotein mutants LIC13006⁻ (*lenC*⁻, encoding an endostatin-like protein), LIC12208⁻
256 (encoding a lipoprotein with Beta-propeller repeats), LIC13060⁻ (*lipL36*⁻, encoding a
257 lipoprotein), LIC10373⁻ (encoding a lipoprotein with Beta-propeller repeats), LIC10898⁻
258 (*lipL48*⁻, encoding an exoprotein with highest absolute abundance in the present study),
259 LIC11977⁻ (encoding a cyclic nucleotide binding protein), LIC11852⁻ (encoding a O-
260 acetylhomoserine (thiol) lyase), LIC10713⁻ (*lruB*⁻, encoding a lipoprotein), LIC10465⁻ (*ligA*⁻),
261 and Manilae L495 were compared for *in vitro* growth rates in EMJH media at 30°C. Bacterial
262 growth was measured on a daily basis by measuring optical densities via spectroscopy at 420 nm.
263 Exoproteins that demonstrated similar growth rates to Manilae L495 were used in subsequent
264 virulence measurement experiments in Mongolian gerbils (Janvier). To measure virulence,
265 groups of 4 gerbils were injected intraperitoneal with 10⁴ bacteria per animal. Animals were
266 administered Manilae L495 or mutants LIC13006⁻ (*lenC*⁻), LIC12208⁻, LIC13060⁻ (*lipL36*⁻),
267 LIC10373⁻, LIC10898⁻ (*lipL48*⁻), LIC10465⁻ (*ligA*⁻), and LIC11977⁻. Animals were
268 monitored on a daily basis for 23 days and sacrificed when moribund. All strains were culture
269 isolated from animals post-mortem and genotypes confirmed via PCR. Protocols for animal
270 experiments conformed to the guidelines of the Animal Care and Use Committees of the Institut
271 Pasteur (Paris, France).

272

273 RESULTS

274 *Validation of Methodology and Overview of Whole Cell and Exoproteins*

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

285 *Leptospira* are flagellated bacteria and the flagellum is anchored into the inner membrane
286 of the bacteria (31). Antiserum generated against the 27.2 kDa periplasmic flagellar protein
287 (FlaA-2) revealed reactivity with proteins migrating between 25-35 kDa in WCP, but no
288 reactivity with CSP in protein immunoblots (Fig. 1B). Antiserum generated against the
289 chaperone protein GroEL was used in immunoblot experiments (Fig. 1C) to validate bacterial
290 response to temperature shift from 30°C to 37°C (44). The WCP from *Leptospira* exposed to
291 37°C displayed a slight increase in GroEL reactivity (Fig. 1C). Detectable levels of GroEL were
292 observed in all CSP samples, albeit at significantly lower levels compared to WCP (Fig. 1C).
293 Using similar reasoning, WCP and CSP were subjected to immunoblot analysis using antiserum
294 cross reactive with *L. interrogans* immunoglobulin protein A (LigA with a MW of 128 kDa)
295 (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 LBP52 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

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

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

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

331 ***Overrepresentation of Exoproteins in COGs Relating to Energy Production and Metabolism***

332 Exoproteins from Table S2 were assigned to clusters of orthologous groups (COGs)
333 based on automatic classification of *Leptospira* genomes in the MicroScope platform (43) (Fig.
334 2A). Compared to genome-wide expected frequencies, exoproteins in the COGs; cell motility
335 (N), signal transduction mechanisms (T), replication recombination and repair (L), general
336 function (R), function unknown (S) and unclassified (-) were underrepresented (Fig. 2B). In
337 contrast, there was a 2 to 4 fold overrepresentation of exoproteins classified in the COGs; energy
338 production and conversion (C), amino acid transport and metabolism (E), nucleotide transport
339 and metabolism (F), carbohydrate transport and metabolism (G) and lipid transport and
340 metabolism (I) (Fig. 2B). Additionally, of the 20 most abundant exoproteins 13 were categorized
341 in at least one of the latter overrepresented categories (Table 2).

342 ***Altered Exoprotein Abundance in Response to Temperature and Osmotic Shifts***

343 The optimal temperature for *in vitro* growth of *Leptospira* is 30°C. Comparison of
344 protein abundance in CSP from *Leptospira* shifted to 37°C or to modified medium containing
345 120 mM NaCl (~300 mosmol/l⁻¹), which mimics physiological conditions in human plasma, to
346 CSP from *Leptospira* at 30°C (37°C vs 30°C S and NaCl vs 30°C S, respectively) suggested
347 altered expression of exoproteins in response to temperature (91 in total) and 120 mM NaCl (140
348 in total) (Table S1). Protein quantities were validated via immunoblot experiments (Fig. 1D and
349 E) for LigA (6 fold higher via relative protein intensity) and LBP52 (20 fold higher via relative
350 protein intensity) proteins, confirming increased expression of these proteins in response to 120
351 mM NaCl.

352 ***Exoproteins with Potential Moonlighting Functions***

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

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

369 ***Exoproteins are Immunogenic and not Essential for Leptospira Virulence***

370 To begin to characterize exoproteins in the context of pathogenesis, WCP and CSP were
371 used in immunoblot experiments with *L. interrogans* positive guinea pig sera (S+) to assess
372 antibody response to exoproteins, which would also be suggestive of exoprotein expression
373 during the infection process (Fig. 3). These analyses revealed IgG and IgM reactivity against *L.*
374 *interrogans* exoproteins in S+ sera (Fig. 3A and B) and no reactivity in the control pre-infection
375 sera (Fig. 3C and D). Exoprotein reactivity with S+ IgG was significantly less prominent when
376 compared to WCP and a similar trend was observed with IgM reactivity (Fig. 3A and B).
377 Comparison of S+ IgG and IgM reactivity with exoproteins was distinguishable in that the
378 proteins displaying reactivity with IgG did not display reactivity with IgM and vice versa (Fig.
379 3A and B). Furthermore, CSP from 120 mM NaCl exposure lead to altered IgG and IgM
380 reactivity of exoproteins when compared to CSP from 30°C and 37°C (Fig. 3A and B).
381 Specifically, reduced IgG and IgM reactivity was observed for protein bands migrating between
382 70-100 kDa (Fig. 3A and B) and increased IgG reactivity was observed for two proteins at 35
383 kDa and 15 kDa (Fig. 3A).

384 To further assess the necessity of exoproteins for *L. interrogans* viability *in vitro* and
385 within the host, select *L. interrogans* mutants which had been inactivated in an exoprotein
386 encoding gene were tested for *in vitro* growth rates and for disease manifestation in gerbils. Nine
387 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

397 **DISCUSSION**

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

408 In addition to their metabolic activities, 32 proteins detected in culture supernatants
409 displayed orthology to moonlighting proteins in other microorganisms. The moonlighting
410 properties of two proteins detected in supernatants have already been demonstrated in *Leptospira*
411 (23, 59). One of these proteins, phosphopyruvate hydratase (Eno/LIC11954) has been
412 characterized as an enolase and displays plasminogen binding activity (23). The other protein,
413 elongation factor Tu (Tuf/ LIC12875) is detected on the surface of *Leptospira* and displays
414 plasminogen and factor H binding (59). A catalase (KatE/LIC12032), another potential
415 moonlighting protein has previously been characterized to be required for *Leptospira* oxidative
416 stress resistance and virulence (34), but the plasminogen binding capacity of this protein (as
417 demonstrated for *Candida albicans* catalase (60)) remains to be elucidated in *L. interrogans*. In
418 accordance with a potential role in host-pathogen interactions, 5 putative *L. interrogans*
419 moonlighting proteins have been shown to be immunoreactive (61); suggestive of their

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

430 While exoprotein function can be associated with disease pathogenesis, the *L.*
431 *interrogans* mutants disrupted in exoprotein encoding genes tested in this study displayed similar
432 disease manifestation in animals to that observed for the parent strain. It should be highlighted
433 that one of the inactivated genes encoded LipL48 (LIC10898) which was the most abundant
434 exoprotein and was detected 11 fold higher than that found within the bacteria. Similarly, two
435 other inactivated genes, LIC11977 encoding a cyclic nucleotide binding protein and *ligA*
436 encoding immunoglobulin-like protein 1, were also detected at high abundance in supernatants.
437 It has previously been demonstrated that LigA confers binding of *Leptospira* spp. to host cells
438 and fibronectin (54), implicative of a role for this protein in the infection process. It should also
439 be noted that immunization of hamsters using LigA antigen provides protection against challenge
440 with *L. interrogans* (64), indicating that LigA is an effective antigen for immune clearance of
441 *Leptospira*. Other notable proteins included the endostatin-like protein LenC which has been
442 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

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

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

505

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737 **FIGURE LEGENDS**

738 **Fig 1. Differential protein localization and expression in leptospiral culture supernatants.**

739 *L. interrogans* were cultured at 30°C, 37°C or in media containing 120 mM NaCl. Proteins from
740 whole cells and culture supernatants were subsequently used in immunoblot experiments with
741 the indicated antisera. Panel A. A representative coomassie stained SDS-PAGE demonstrating
742 disparate protein composition when comparing whole cell lysate proteins to those obtained from
743 culture supernatants. Panel B. Protein immunoblot demonstrating localization of flagellar protein
744 FlaA-2 in whole cells but not in the supernatant. Panel C, Protein immunoblot suggesting
745 increased expression of the chaperone protein GroEL at 37°C. Panel D. Protein immunoblot
746 indicating expression of immunoglobulin protein LigA in CSP and increased expression in the
747 CSP from *L. interrogans* exposed to 120 mM NaCl. Panel E. Protein immunoblot confirming
748 expression of LBP52 protein in culture supernatants and increased expression in CSP from *L.*
749 *interrogans* exposed to 120 mM NaCl.

750 **Fig 2. Classification of *L. interrogans* exoproteins suggests most proteins to be involved in**

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

760 chaperones (3.2%); T: Signal transduction mechanisms (5.9%); U: Intracellular trafficking,
761 secretion, and vesicular transport (1.7%); V: Defense mechanisms (1.6%); Z: Cytoskeleton
762 (.06%); B: Chromatin structure and dynamics (.04%); J: Translation, ribosomal structure and
763 biogenesis (3.5%); K: Transcription (3.1%); L: Replication, recombination and repair (4.6%); C:
764 Energy production and conversion (3.2%); E: Amino acid transport and metabolism (7.0%); F:
765 Nucleotide transport and metabolism (1.6%); G: Carbohydrate transport and metabolism (3.6%);
766 H: Coenzyme transport and metabolism (2.8%); I: Lipid transport and metabolism (2.9%); P:
767 Inorganic ion transport and metabolism (4.5%); Q: Secondary metabolites biosynthesis, transport
768 and catabolism (1.8%); R: General function prediction only (11.0%); S: Function unknown
769 (5.0%); -: Unclassified (39.0%) .

770 **Fig 3. *L. interrogans* positive sera display IgM and IgG reactivity to leptospiral exoproteins.**

771 Guinea pig sera obtained prior to and post *L. interrogans* infection were used in protein
772 immunoblot experiments to test immunoglobulin reactivity with whole *L. interrogans* lysates or
773 culture supernatants. Panel A. Protein immunoblot comparing IgG reactivity with whole cell
774 proteins and exoproteins when using *L. interrogans* positive sera. Panel B. Protein immunoblot
775 comparing IgM reactivity to whole cell proteins and exoproteins when using *L. interrogans*
776 positive sera. Panel C. Protein immunoblot demonstrating a lack of IgG reactivity to whole cell
777 and extracellular proteins when using pre-infection sera. Panel D. Protein immunoblot
778 demonstrating a lack of IgM reactivity to whole cell and extracellular proteins when using pre-
779 infection sera.

780

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
^cUnique 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 ^aWhole cell *Leptospira*, ^bCulture supernatants, ^cCombined experimental replicates, ^dAverage between experiments, ^ePeriplasmic
 784 localization, ^fExtracellular localization

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

EMBL/GenBank/ DDBJ CDS	Locus tag	Protein	^aCOG	^bWCP	^cCSP	^cCSP:^bWCP	^dRQ
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 ^aClusters of orthologous groups (COG). See Fig. 2 legend for description of categories.

787 ^bAverage protein intensity for proteins from whole bacteria.

788 ^cAverage protein intensity for proteins in supernatants.

789 ^dRelative protein quantities in supernatants compared to LipL48

790
791

Table 3. Exoproteins unique to pathogenic *Leptospira*.

^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	<i>ligA</i>	-	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	<i>sphH</i>	-	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	<i>orfC</i>	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	<i>mmsB</i>	I	Yes		Not tested
LIC11996	Uncharacterized protein		-	Yes		Not tested
LIC12032	Catalase (EC 1.11.1.6)	<i>katE</i>	P		Yes	avirulent (ref. 33)
LIC12048	Uncharacterized protein		-	Yes		Not tested
LIC12084	Uncharacterized protein		-			Not tested
LIC12139	UDP-N-acetylglucosamine 2-epimerase	<i>rffE</i>	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 (LBP52)		-	Yes		Not tested
LIC12232	Thymidylate synthase	<i>thyX</i>	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	<i>lipL36</i>	-	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

792

793 ^aPutative orthologous alignment between *L. interrogans* with other pathogenic *Leptospira*

794 species are defined as proteins satisfying a blastP alignment threshold of a minimum of 40%

795 sequence identity on 80% of the length of the protein.

796 ^bClusters of orthologous groups were retrieved from the MicroScope platform.

797 ^cN-terminal signal peptide predictions were predicted using Phobius.

798 ^dNon-classical secretion predictions were performed using SecretomeP 2.0.

799 ^eGerbil survival: Groups of 4 gerbils were inoculated with 10⁴ bacteria. Animals were

800 monitored for 23 days.

801

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 ^aMoonlighting functions were obtained from MoonProt (49).

805 ^bProteins 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

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

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

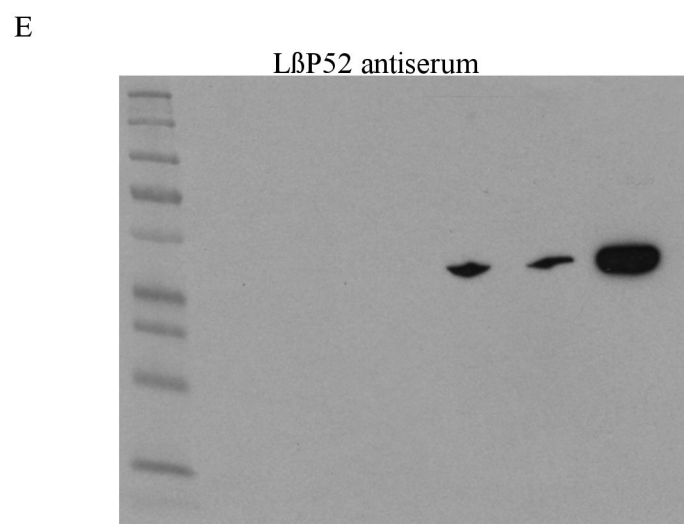
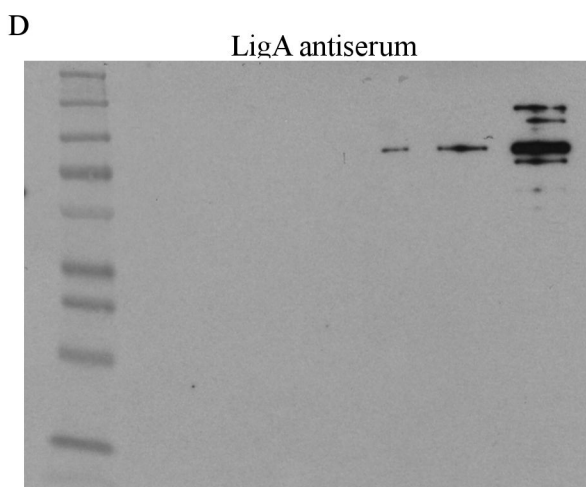
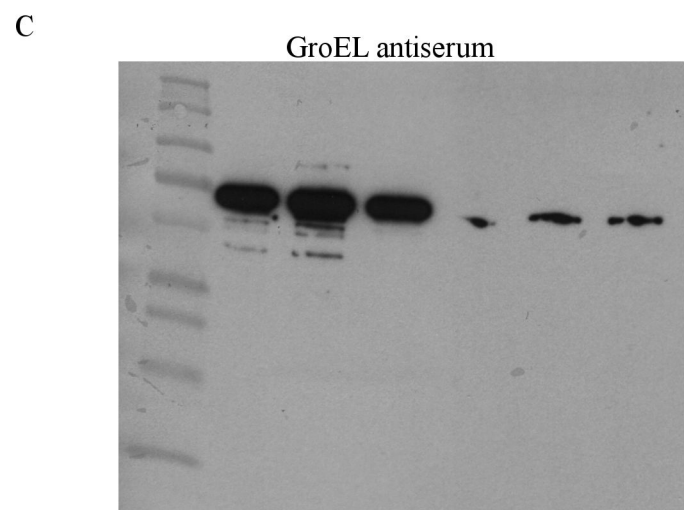
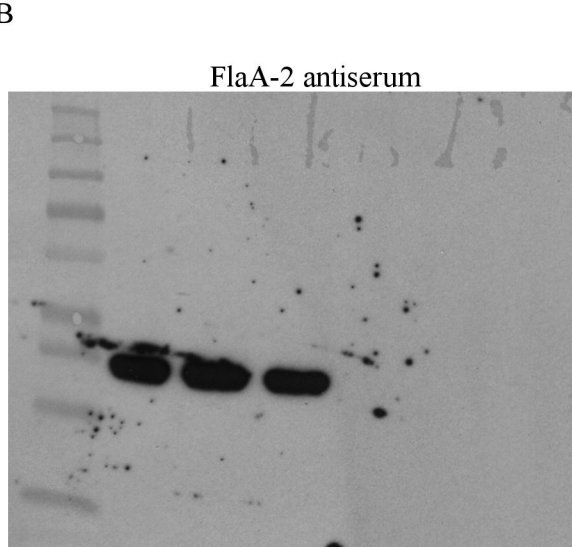
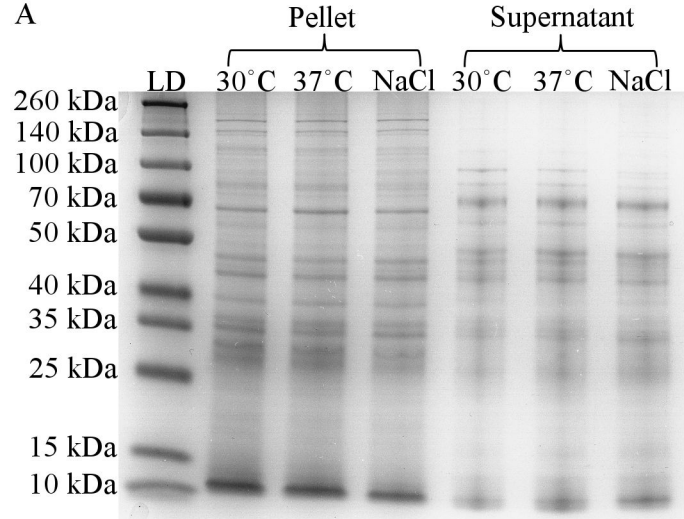
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822 *rrogans* and *L. biflexa* are defined as proteins satisfying a blastP alignment threshold of a
 823 minimum of 40% sequence identity over 80% of the length of the protein.

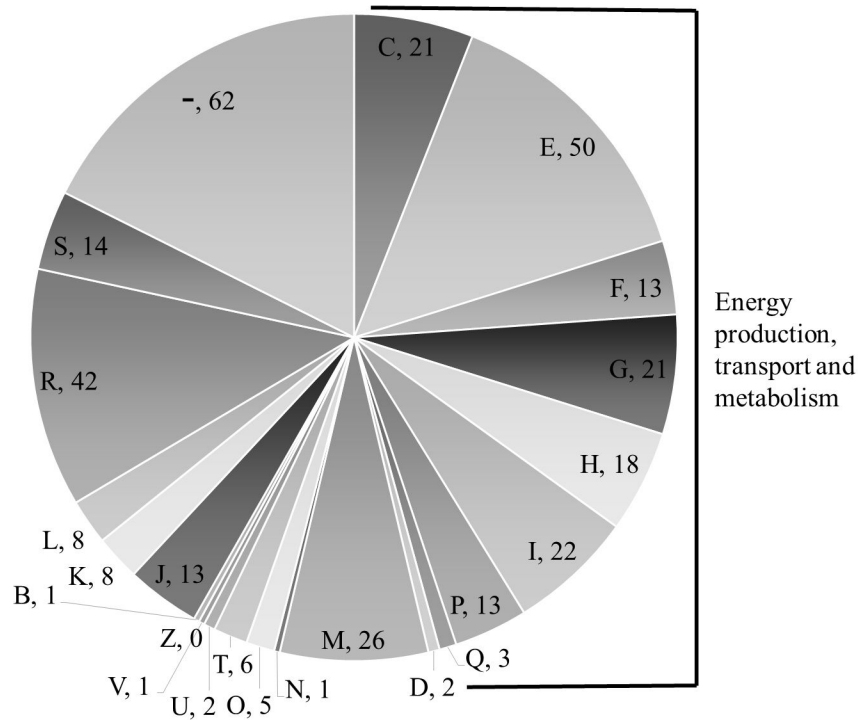
824 ^bGrowth of wild-type and mutant strains in liquid EMJH incubated at 30°C. Growth was
 825 monitored by measuring OD₄₂₀ on a daily basis.

826 ^cGerbil survival: Groups of 4 gerbils were inoculated with 10⁴ bacteria. Animals were
 827 monitored for 23 days.

828



A



B

