The FUR-like regulators PerRA and PerRB control a complex signaling network required for mammalian host-adaptation and virulence of *Leptospira interrogans*

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Abstract

Leptospira interrogans, the causative agent of most cases of human leptospirosis, must respond to myriad environmental signals during its free-living and pathogenic lifestyles. Previously, we compared *L. interrogans* cultivated *in vitro* and *in vivo* using a dialysis membrane chamber (DMC) peritoneal implant model. From these studies emerged 166 genes that were differentially regulated in response to host signals, including *perRA*, one of two Peroxide stress response (PerR)-like regulators encoded by *L. interrogans*. Zavala-Alvarado *et al*. recently demonstrated that leptospires lacking both PerRA and PerRB are avirulent in hamsters. Herein, we establish that PerRA and PerRB also are required for renal colonization in C3H/HeJ mice. The finding that loss of virulence was observed only with the *perRA/B* double mutant suggests that these regulators serve redundant or overlapping functions *in vivo*. Our finding that the *perRA*/*B* double mutant survives at wild-type levels in DMCs is noteworthy as it demonstrates that the loss of virulence is not due to a metabolic lesion (*i.e.*, metal starvation) but instead reflects dysregulation of virulence-related gene products. Comparative RNA-Seq analyses of *perRA*, *perRB* and *perRA/B* mutants cultivated within DMCs identified 106 genes that are dysregulated in the double mutant, including *ligA, ligB* and *lvrA/B* sensory histidine kinases. Decreased expression of LigA and LigB in the *perRA*/*B* mutant was not due to loss of LvrAB signal transduction. The majority of genes in the *perRA* and *perRB* single and double mutant DMC regulons were differentially expressed only *in vivo*, highlighting the importance of host-specific signals for regulating gene expression in *L. interrogans*. Importantly, the PerRA, PerRB and PerRA/B DMC regulons each contain multiple genes related to environmental sensing and/or transcriptional regulation. Collectively, our data suggest that PerRA and PerRB are part of a complex signaling network required by *L. interrogans* for adaptation to and survival within the host.

Author Summary

Leptospirosis is a neglected tropical disease with a worldwide distribution. Globally, ~1 million cases and ~60,000 deaths are reported each year. The majority of cases of human leptospirosis are associated with *Leptospira interrogans*. Infection begins when a naïve reservoir (or incidental) host comes into direct or indirect contact with urine from an infected reservoir host. While infection in reservoir hosts, including rats and mice, is generally asymptomatic, incidental hosts, including humans, may develop clinical symptoms ranging from mild flu-like illness to fulminant disease. The gene products required by leptospires for infection remain poorly understood. Herein, we establish that the FUR family regulators PerRA and PerRB function either cooperatively or in parallel to promote survival and renal colonization in mice. By comparative transcriptomics, we identified >100 genes that were dysregulated in the *perRA/B* double mutant *in vivo*, including four virulence-related genes. Importantly, the PerRA, PerRB and PerRA/B DMC regulons contain multiple genes related to environmental sensing and/or transcriptional regulation. Our data suggest that PerRA and PerRB are part of a complex signaling network required by *L. interrogans* for adaptation to and survival within the host.

67 **Introduction**

68

69 Leptospirosis is a neglected tropical disease with a worldwide distribution [1, 2]. Globally, ~1
70 million cases and ~60,000 deaths are reported each year [3]. When adjusted for potential under-
71 reporting, the number of cases globally likely exceeds 1.7 million [4]. Leptospirosis is now well
72 recognized as a significant public health problem in developing countries and tropical regions [5-
73 7]. In poor, urban communities in underdeveloped countries, major outbreaks of leptospirosis often
74 are associated with seasonal flooding [5]. Leptospirosis also is of considerable veterinary
75 importance; leptospirosis in cattle and other ruminants can lead to reduced reproductive fitness
76 and diminished milk production, with substantial economic consequences [8, 9].

77 Leptospirosis is caused by infection with pathogenic spirochetes belonging to the genus
78 *Leptospira* [10]. The majority of severe cases of human leptospirosis are associated with *L.*
79 *interrogans* [10]. Infection begins when a naïve reservoir (or incidental) host comes into contact
80 with urine from an infected host, most often *via* contaminated water or soil [11]. Leptospires gain
81 entry to the host through bruises or abrasions in the skin and/or mucous membranes. Following
82 inoculation, leptospires transition from a saprophytic (free-living) to a parasitic lifestyle by a
83 complex and poorly understood process referred to as 'host adaptation'. Once in the bloodstream,
84 leptospires rapidly disseminate to distal tissues but, in reservoir host, are cleared within several
85 days from all sites except the kidney, where they set up long-term residence in the proximal tubules
86 [12-14]. Infected reservoir hosts shed large numbers of leptospires (up to 10^7/mL) in their urine
87 for weeks to months [10, 13, 15, 16]. While infection in reservoir hosts is generally asymptomatic,
88 incidental hosts, including humans, may develop clinical symptoms ranging from mild flu-like
89 illness to fulminant disease (*e.g.*, Weil's disease and pulmonary hemorrhage syndrome). Even with

90 treatment, mortality for severe leptospirosis ranges between 10-70% [5]. The factors driving
91 disease severity in humans are poorly understood but are thought to include the bacterial serovar
92 and strain, inoculum size, and the host's innate and adaptive immune responses [7, 17-19].

93 The ability of *Leptospira* spp. to sense and respond to environmental signals encountered
94 within mammals is generally believed to be critical to sustain the bacterium within its zoonotic
95 lifestyle. The majority of studies investigating gene regulation by *L. interrogans* have done so by
96 manipulating *in vitro* growth conditions [20-28]. However, numerous studies using another
97 enzootic spirochetal pathogen, *Borrelia burgdorferi*, have shown that cultivation *in vitro* under
98 "mammalian host-like" conditions (*i.e.*, increased temperature, increased pH, high osmolality)
99 does not replicate the full range of environmental signals and physiological cues that spirochetes
100 respond to *in vivo* [29-41]. Thus, to gain better insight into the transcriptomic and antigenic
101 changes that *L. interrogans* undergoes within mammals, we developed an *in vivo* model in which
102 leptospires are cultivated within dialysis membrane chambers (DMCs) implanted into the
103 peritoneal cavities of rats, a natural reservoir host [42, 43]. Leptospires within DMCs (6-8 kDa
104 MWCO) are exposed to host-derived nutrients and environmental signals but are protected from
105 the host's cellular and humoral immune responses. Importantly, the DMC model provides
106 sufficient numbers of host-adapted organisms (~10^8 per ml) for genome-wide transcriptomics [42]
107 and proteomics [44]. Using this model, we identified 166 genes (110 upregulated and 56
108 downregulated) differentially-expressed by *L. interrogans* serovar (sv.) Copenhageni strain
109 Fiocruz L1-130 in response to host-specific signals [42]. Almost all of the genes upregulated by
110 the Fiocruz L1-130 strain within DMCs were unique to pathogenic leptospires (*i.e.*, not found in
111 the genomes of saprophytic *Leptospira* species).

112 Not surprisingly, many of the genes upregulated by *L. interrogans* in DMCs encode
113 functions related to environmental signaling and gene regulation [42], including LIMLP10155
114 (LIC12034), which encodes a member of the Ferric Uptake Regulator (FUR) superfamily [45].
115 The namesake of this highly diverse superfamily, Fur, functions as a global regulator of iron
116 homeostasis in Gram-negative and -positive bacteria, controlling both the induction of iron uptake
117 systems under iron limitation and the expression of iron storage proteins and iron-utilizing
118 enzymes under iron sufficiency [46]. Beyond balancing metal uptake and toxicity, FURs also
119 modulate intermediary metabolism, host colonization and virulence [47-53]. Members of the FUR
120 superfamily are diverse in their metal specificities; in addition to the iron-sensing Fur, this family
121 includes regulatory sensors for zinc (Zur), manganese (Mur) and nickel (Nur) [50, 54-59]. Other
122 FUR superfamily members, , PerR uses metal-catalyzed oxidation to sense peroxide stress [46, 60-
123 64] and Irr controls iron uptake and heme homeostasis in a heme/iron-dependent oxidation [64,
124 65]. While Fur and Zur regulators are widely distributed across both Gram positive and Gram-
125 negative bacteria, other FUR family regulators have more limited distribution. PerRs are found
126 mainly in Gram-positive bacteria, and Irrs are limited to α-proteobacteria. [66]. So far, Mur and
127 Nur have been characterized in α-proteobacteria and actinomycetes, respectively, but their
128 distribution within other taxonomic groups is still unclear. Although most often characterized as
129 "repressors", FUR family regulators also may activate transcription in either a metal-bound or -
130 unbound (apo) state [58, 67, 68].
131 In diverse bacteria, iron serves as an essential co-factor for many cellular processes,
132 including energy generation *via* electron transport, intermediary metabolism and DNA biogenesis
133 [51-53, 69, 70]. For many pathogens, the shift from a high- to low-iron environment is a key
134 environmental signal for induction of expression of virulence genes [52]. Unlike other spirochetes,

135 such as *B. burgdorferi* and *Treponema pallidum,* which require iron in trace amounts, if at all [71-
136 75], *Leptospira* spp. require this metal for growth *in vitro* and, presumably, in the host [76].
137 Consequently, leptospires have evolved elaborate mechanisms for iron sensing, scavenging and
138 utilization [77-79]. At the same time, leptospires must balance their physiological need for
139 transition metals with the potential damage caused by highly toxic hydroxyl free radicals generated
140 by Fenton chemistry from H_2O_2 in the presence of ferrous ions [80-82]. Many bacteria, including
141 *Leptospira* spp., encode systems to ameliorate the toxicity of H_2O_2 and repair damage due to
142 oxidative stress [83, 84]. Expression of gene products involved in oxidative stress responses
143 typically are controlled by one of two master regulators – OxyR and PerR. While OxyR acts as a
144 transcriptional activator for gene products (*i.e.*, catalase and superoxide dismutase) related to
145 detoxification of reactive oxygen species (ROS), PerR acts as a repressor and is released from
146 DNA following exposure to peroxide [56, 60, 85, 86]. Both master regulators respond to similar
147 amounts of H_2O_2 [87, 88]; thus, it is unclear why some bacteria have evolved to use PerR while
148 others use OxyR.

149 A genome-wide survey of *L. interrogans* identified four putative FUR family regulators
150 (*LIMLP04825*, *LIMLP05620*/*perRB*, *LIMLP10155*/*perRA* and *LIMLP18690*). Prior studies by Lo
151 *et al*. [89] and Zavala-Alvarado *et al*. [90], suggest that PerRA functions as a metal-dependent
152 peroxide stress regulator. Consistent with its repressor function in other bacteria, a *L. interrogans*
153 *perRA* transposon mutant expresses increased levels of catalase, AhpC and cytochrome c-
154 peroxidase and enhanced survival following exposure to peroxide *in vitro* [89-91]. Recently,
155 Zavala-Alvarado *et al*. [91] demonstrated that expression of *perRB* also was increased by H_2O_2 *in*
156 *vitro*. Inactivation of *perRB* increased survival to superoxide but not H_2O_2 [91]. These data suggest
157 that PerRA and PerRB likely are functionally distinct. Consistent with this notion, Zavala-

158 Alvarado *et al*. [91, 92] saw little overlap between the PerRA and PerRB regulons by RNA-Seq
159 analysis of *in vitro*-cultivated organisms. Interestingly, while *perRA* and *perRB* single mutants are
160 virulent in hamsters [89, 92], a *perRA/B* double mutant was avirulent [91]. Collectively, these data
161 argue that *L. interrogans* requires at least one functional PerR-like regulator for infection in
162 mammals.

163 To investigate the molecular basis for the phenotypic differences between PerRA and
164 PerRB single and double mutants and identify putative virulence-related genes dysregulated by
165 the loss of both regulators *in vivo*, we performed comparative RNA-Seq on all three mutant strains
166 cultivated within DMCs. Similar to RNA-seq data for *in vitro*-cultivated organisms [91, 92], we
167 saw very little overlap between the PerRA and PerRB regulons within mammals. Interestingly, the
168 PerRB DMC regulon was substantially larger than its *in vitro* counterpart [91]. Importantly, by
169 RNA-Seq, we identified 90 genes that are dysregulated only in the double mutant cultivated in
170 DMCs. Of particular note, the "double-only" regulon includes at least four virulence-associated
171 genes, *ligA* and *ligB*, encoding Leptospiral Immunoglobulin-like proteins LigA and LigB, and
172 *lvrAB*, encoding tandem sensory histidine kinases (HKs). Decreased expression of LigA and LigB
173 in the *perRA/B* double mutant was not due to loss of LvrAB signaling. The *perRA/B* double mutant
174 DMC regulon also contains 15 additional genes related to environmental sensing and/or gene
175 regulation, including nine putative hybrid HKs and six putative DNA binding proteins; all but two
176 of the 15 were dysregulated only *in vivo*. Taken together, our data suggest that PerRA and PerRB
177 are part of a complex signaling network that uses mammalian host-specific signals to coordinate
178 the expression of genes required by *L. interrogans* for adaptation to and survival within reservoir
179 and incidental (*i.e.*, human) hosts.
180

181 **Results**

182 **Pathogenic and saprophytic *Leptospira* spp. encode different FUR-like metalloregulator**
183 **repertoires.** To gain insight into the functions of the four FUR-like regulators encoded by *L.*
184 *interrogans*, we performed a phylogenetic comparison of these proteins against well characterized
185 representative FUR family metalloregulators from gram-negative and -positive bacteria (Fig 1A).
186 PerRA and PerRB clustered most closely with PerRs and iron-response regulators (Irrs), while
187 LIMLP18590 and LIMLP04825 clustered with Zur/Nur and Fur/Mur regulators, respectively.

188 We next surveyed the amino acid sequences of the leptospiral FUR-like proteins for
189 conserved regulatory and structural metal binding sites (MBS), which promote DNA binding and
190 folding/dimerization, respectively, in other Fur family regulators [57, 58, 93]. As noted recently
191 by Zavala *et al*. [91], PerRA and PerRB contain two PerR canonical amino acid residues (Asn60
192 and Asn68 in PerRA and PerRB, respectively) involved in peroxide sensitivity and DNA
193 recognition (Asp103 and Asp112 in PerRA and PerRB, respectively) [94, 95]. Based on these
194 features and increased expression of *perRB* upon exposure of *L. interrogans* to peroxide,
195 LIMLP05620 was named *perRB* [91]. Interestingly, as shown in Fig 1B and S1 Fig, the aspartate
196 of the PerR regulatory MBSs and the asparagine in the PerR DNA-binding helices (DBH) also are
197 conserved in Irr proteins. As noted previously by Kebouchi *et al.* [90] and Zavala *et al.* [91], both
198 PerRA and PerRB lack the C-terminal conserved CxxC motif(s) used for structural metal-
199 dependent dimerization by many, but not all, FUR family regulators; this cysteinate motif also is
200 absent in Irrs. Overall, the PerRA and PerRB DBHs are not highly conserved, raising the
201 possibility that they recognize different upstream sequences. LIMLP04825, on the other hand,
202 contains features conserved across Fur, Mur, Zur and Nur regulators, including a glutamic acid at
203 position 103, one or possibly two CxxC motifs (residues 95-98 and 133-136), and an arginine

204 (Arg60) within its DBH (Fig 1B). Interestingly, LIMLP18590 contains features of both PerR (Asp
205 at position 103) and Fur/Mur/Zur/Nur (Arg residue within its putative DBH). The regulatory metal
206 binding site(s) for LIMLP18590 most closely resembles that of a Zur (Fig 1B), which includes two
207 putative tetra-coordinated zinc binding sites rather than the single penta-coordinated site used by
208 PerR. However, without additional data regarding the peroxide responsiveness and/or regulatory
209 metal-binding properties of LIMLP04825 or LIMLP18590, it is not possible to discern their
210 function(s). For this reason, we propose designating them as Fur family regulators 1 (Ffr1) and 2
211 (Ffr2), respectively.

212 We next assessed the conservation of FUR family regulators across pathogenic (P1 and P2)
213 and saprophytic (S1 and S2) leptospiral subclades [96]. Orthologs for PerRA and Ffr1 were
214 identified in all highly pathogenic (P1), some intermediate (P2) and all saprophytic strains (S1 and
215 S2), whereas orthologs for PerRB and Ffr2 were found exclusively in pathogenic strains (Fig 2A
216 and B and S2 Fig). Our analyses also identified two additional FUR family regulators, both of
217 which were found only in saprophytic leptospires (Fig 2A and B and S2 Fig). The first, designated
218 PerRC, contains features of a canonical PerR (two CxxC motifs, an aspartic acid residue within its
219 regulatory MBS and an asparagine within its putative DBH). The second saprophyte-specific FUR
220 family regulator, designated Ffr3, resembles a Fur/Mur/Nur-like regulator (two CxxC motifs, a
221 glutamic acid residue within its regulatory MBS and an arginine within its putative DBH).

222

223 ***L. interrogans* FUR family regulators are expressed at higher levels *in vivo* than *in vitro*.**
224 Previously, we reported that expression of *perRA* in *L. interrogans* sv. Copenhageni strain Fiocruz
225 L1-130 was induced 3.83-fold in response to mammalian host signals compared to *in vitro* [42].
226 Using qRT-PCR, we compared transcript levels for all four FUR-like regulators in *L. interrogans*

227 sv. Manilae strain L495 [97, 98] grown *in vitro* (EMJH at 30°C) and following cultivation within
228 DMCs. As shown in Fig 3, *perRA* (7.59-fold), *ffr1* (3.20-fold), and *ffr2* (5.70-fold) were
229 upregulated significantly ($p<0.05$) *in vivo*. *perRB* was upregulated 1.63-fold in DMCs compared
230 to *in vitro*, but the difference was not statistically significant (Fig 3).
231
232 **Inactivation of both *perRA* and *perRB* in *L. interrogans* results in loss of virulence in mice.**
233 Previously, Murray *et al*. [97] and Zavala-Alvarado *et al*. [92] independently reported that a *L.*
234 *interrogans* Manilae *perRA* Tn mutant is virulent in hamsters. More recently, Zavala-Alvarado *et*
235 *al.* [91] established that *L. interrogans* lacking PerRB also retain virulence in hamsters. Zavala-
236 Alvarado and colleagues also generated a double mutant by insertional inactivation *perRA* in the
237 *perRB* Tn mutant; the resulting double mutant (*perRA/B*) was avirulent in hamsters [91]. Golden
238 Syrian hamsters are exquisitely sensitive to *L. interrogans* and develop acute, fulminant,
239 disseminated disease at doses as low as 10^1 [18, 97, 99, 100]. Mice, on the other hand, are a natural
240 reservoir for *L. interrogans* and relatively resistant to infection; at sublethal doses, susceptible
241 mouse strains develop a self-resolving hematogenous dissemination phase (~1 week) followed by
242 chronic, asymptomatic renal colonization marked by shedding large numbers of leptospires in
243 urine [18, 101-104].
244 Given the differences in leptospiral disease progression and severity between hamsters and
245 mice, we asked whether PerRA, PerRB, or both are required to establish infection and persistence
246 within a reservoir host model. At the outset, we first established that our wild-type serovar Manilae
247 parent (WT) is virulent in C3H/HeJ mice. Female 10-week old mice (n=5 per group) were infected
248 intraperitoneally with 5×10^6, 1×10^6, 1×10^5 and 1×10^4 leptospires. Mice were monitored daily
249 for signs of disease (*i.e.*, weight loss). Within 6 days, all mice in the 5×10^6 group and 3 of 5 mice

250 in the 10^6 group succumbed to infection, while all others survived the entire 42-day experimental
251 time course (Fig 4A). Based on these virulence studies, the LD_{50} for the wild-type (WT) parent
252 was ≅ 7 x 10^5. Beginning 14 days post-infection (p.i.), surviving mice were monitored weekly for
253 the presence of leptospires in their urine by darkfield microscopy. Urine from all but one (10^4
254 group) mouse contained large numbers of leptospires at all three time points (14, 21, and 35 days
255 p.i.) (Fig 4B). At 42 days p.i., kidneys harvested from all surviving mice infected with the WT
256 parent, including the single urine-negative mouse from the 10^4 group, were culture-positive.

257 Prior to using the *perRA* and *perRB* single and double mutants for murine virulence studies,
258 we first confirmed their genotypes by amplicon sequencing using primers listed in S6 Table and
259 immunoblot and established that loss of one regulator had no obvious effect on expression of the
260 other in the corresponding single mutants (S3 Fig). We also confirmed that expression of PerRB
261 in the double mutant was restored by *trans*-complementation using a WT copy of *perRB* plus its
262 native promoter cloned into the pMaORI-genta shuttle vector (S3 Fig). We next compared
263 infectivity of the WT, *perRA, perRB* or *perRA/B* strains in C3H/HeJ mice using a sublethal
264 intraperitoneal inoculum (1 x 10^5). All of the mice inoculated with the WT parent and single
265 mutants were infected, shedding comparable numbers of leptospires in their urine at 14- and 21-
266 days p.i. (Fig 4C). In contrast, no leptospires were detected in urine from mice inoculated with the
267 *perRA/B* double mutant. Consistent with data from urine, at day 28 p.i., all *perRA/B*-infected mice
268 were negative for leptospires by both culture and qPCR (Fig 4D). Lastly, in contrast to mice
269 infected with the WT or single mutant strains, all of which generated robust serological responses
270 against *L. interrogans*, none of the mice infected with the double mutant seroconverted (Fig 4E).
271 The ability of the double mutant to establish infection was restored by *trans*-complementation with
272 *perRB* alone (Fig 4C-E).

273

274 ***perRA* and *perRB* single and double mutants grow normally in rat peritoneal cavities.** PerR
275 regulators have been linked to a wide range of physiological functions outside of oxidative stress,
276 including metal homeostasis, metabolism and virulence [105-107]. In *Bacillus subtilis*,
277 inactivation of *perR* leads to increased expression of *fur* and iron starvation [80]. To examine
278 whether the avirulent phenotype of the *perRA/B* double mutant could be due to an inability to grow
279 in mammals, we took advantage of our DMC model, whereby leptospires are cultivated for 9-10
280 days within dialysis membrane chambers implanted in the peritoneal cavity of a rat [42, 108].
281 Originally developed for *B. burgdorferi*, this model is able to separate genes related to
282 physiological adaption (*i.e.*, nutrient acquisition and metabolism) from those encoding virulence
283 determinants, such as adhesins, motility and immune evasion. In at least four independent
284 experiments (2 rats per strain, per experiment), we saw no obvious difference in the average
285 numbers of wild-type and *perRA/B* double mutant leptospires (2.8×10^8 for WT vs. 4.6×10^8 per
286 ml for *perA/B* strains) recovered from DMCs 9 days post-implantation. These data demonstrate
287 that the virulence-defect observed with the double mutant is not due to a metabolic lesion (*i.e.*,
288 metal starvation).

289

290 **Defining the PerRA and PerRB regulons *in vivo* by comparative RNA-Seq.** Prototypical FUR
291 family regulators, including PerR, modulate transcription by binding to DNA *via* one or more ~19-
292 bp inverted repeats ('boxes') located upstream of their target genes [109]. Kebouchi *et al*. [90]
293 previously identified three potential PerR binding sites upstream of *perRA* in *L. interrogans*.
294 However, searches of the Manilae genome using these sequences, as well as canonical Fur and
295 PerR boxes [109, 110], did not identify additional hits [42, 45, 89]. Therefore, to identify genes

296 controlled by PerRA, PerRB, or both, in response to host signals, we performed comparative RNA-
297 Seq using WT, *perRA*, *perRB* and *perRA/B* strains cultivated in DMCs (3 biological replicates per
298 strain); a summary of the raw Illumina read data is presented in Table S1. Reads were mapped
299 using EDGE-pro [111] and analyzed for differentially-expressed genes using DESeq2 [112].
300 Genes expressed at ≥3-fold higher/lower levels in the WT versus mutant with a False-discovery
301 rate (FDR)-adjusted-*p* value (*q*) <0.05 were considered differentially expressed. Complete RNA-
302 Seq datasets for all comparisons are presented in Tables S2-S4. Raw read files have been deposited
303 in the NCBI Sequence Read Archive (SRA) database (BioProject accession PRJNA659512).

304 *Overview of the PerRA DMC regulon.* The PerRA DMC regulon contained a total of 81
305 differentially expressed genes; 43 were expressed at higher levels (*i.e.*, upregulated directly or
306 indirectly by PerRA) in the WT parent compared to the *perRA* mutant, while 38 were expressed at
307 lower levels (*i.e.*, downregulated/repressed directly or indirectly by PerRA) (Table S2). Notably,
308 the PerRA DMC regulon is substantially larger than its *in vitro* counterpart (17 genes total but only
309 14 dysregulated >3-fold), recently reported by Zavala-Alvarado *et al.* [92]. Overlap between the
310 PerRA DMC and *in vitro* regulons consists primarily of seven genes located in a single
311 chromosomal locus (S4 Fig) containing LipL48 (*LIMLP04280*), a putative outer-membrane
312 embedded TonB-dependent receptor (TBDR, *LIMLP04270*) and one of the 2-3 putative
313 TonB/ExbD/ExbB transporters systems (*LIMLP04245-04230*) encoded by *L. interrogans* [45, 89].
314 TonB-dependent transporters (TBDT) for iron typically are repressed by Fur [113]; thus, it was
315 surprising that this system was upregulated by PerRA both *in vitro* and in DMCs. Interestingly,
316 none of the prototypical oxidative stress-related genes identified by Zavala-Alvarado *et al.* [92] as
317 being under PerRA control *in vitro* were dysregulated in DMCs.

318 More than half (55%) of genes in the PerRA DMC regulon encode proteins of unknown
319 function (S5A Fig). The remaining genes are distributed over a wide range of functional categories
320 (COGs) related to cellular homeostasis and metabolism. Most notably, the PerRA regulon includes
321 five genes (two upregulated, three downregulated) involved in signaling and/or gene regulation
322 (Fig 5A, S5A Fig and Table S2). The two upregulated genes (*LIMLP05780* and *LIMLP01845*)
323 encode putative DNA binding proteins, while the three downregulated genes encode a two-
324 component system (TCS) histidine kinase with four Per-Arnt-Sim (PAS)-type sensor domains
325 (*LIMLP10140*), a putative DNA binding protein (*LIMLP00900*) and a putative serine/threonine
326 kinase with GAF domain (*LIMLP11575*). PAS domains are ubiquitous in bacteria and sense a wide
327 range of ligands, including heme, FAD, fatty acids and divalent metals [114, 115]. GAF domains
328 share a similar fold to PAS domains and often regulate the catalytic activity of cyclic nucleotide
329 phosphodiesterases [116]. Of note, none of these putative regulatory factors were dysregulated ≥3
330 fold by loss of PerRA *in vitro* [92].

331 *Overview of the PerRB DMC regulon.* Inactivation of *perRB* resulted in dysregulation of
332 200 genes (131 upregulated and 69 downregulated) within DMCs (Table S3). In contrast, only 30
333 genes were dysregulated in the *perRB* mutant *in vitro*, with only one affected >3-fold [91].
334 Remarkably, we saw no overlap between the *in vitro* and DMC PerRB regulons. Overlap between
335 the PerRA and PerRB DMC regulons was limited to genes within the TonB-dependent transporter
336 locus described above (S4 Fig). Notably, none of the TonB-related genes were dysregulated ≥3
337 fold in the *perRB* mutant *in vitro* (S4 Fig). The implications of these data are two-fold; differences
338 between the *in vitro* and DMC regulons for the *perRB* mutant imply that PerRB it is not fully
339 activated under normal growth conditions *in vitro*, while the minimal overlap between the PerRA
340 and PerRB DMC regulons suggests that they recognize different upstream binding sites.

341 The majority (66%) of genes in the PerRB DMC regulon were upregulated, most encoding
342 proteins of unknown or poorly function (S5B Fig and Table S3). Notably, however, the PerRB
343 DMC regulon includes 17 genes (11 upregulated, 6 downregulated) related to signaling and/or
344 gene regulation (Fig 5B). The 11 upregulated genes include six related to signal transduction, three
345 putative DNA binding proteins (*LIMLP16420*, *LIMLP07225* and *LIMLP15900*), an ECF-type
346 sigma factor (*LIMLP14515*) and a putative serine/threonine phosphatase with a PAS-type sensor
347 domain (*LIMLP06960*) (Fig 5B). The six downregulated signaling genes include two additional
348 putative DNA binding proteins (LIMLP07895 and LIML10055), a second ECF-type sigma factor
349 (*LIMLP14940*), a putative anti-sigma factor antagonist (*LIMLP04815*), and an EAL-type
350 phosphodiesterase (*LIMLP04775*) (Fig 5B). None of these putative regulators were affected *in*
351 *vitro* by loss of PerRB [91].
352
353 **Inactivation of both PerRA and PerRB results in a DMC regulon that differs dramatically**
354 **from its single mutant counterparts.** The PerRA/B DMC regulon contains 106 differentially
355 expressed genes, 74 upregulated and 32 repressed (Tables 1-2 and S4 Table). Surprisingly, we saw
356 limited overlap between the DMC regulons for the double and single mutants (Fig 6A); all of the
357 overlapping genes were located in the TonB-related chromosomal locus dysregulated in the *perRA*
358 and *perRA/B* mutants *in vitro* [91, 92] (S4 Fig). Ninety genes (62 upregulated and 28 repressed)
359 were dysregulated <u>only</u> in the *perRA/B* double mutant (Fig 6A, Tables 1-2 and S4 Table).
360 <u>*Cellular homeostasis and metabolism*</u>. A handful of genes upregulated by PerRA/B in
361 DMCs encode proteins involved in cellular homeostasis and metabolism (Fig 6B and Table 1).
362 *LIMLP14200* and *LIMLP12135* contain domains found in lipases/esterases (IPR0002489) [117]
363 and alkaline phosphatases and sulfatases (IPR000917), respectively. *LIMLP18070* contains an

364 ATP-NAD kinase domain (IPR022504), suggesting a role in maintaining NADP homeostasis and,
365 by extension, NADPH-dependent reductive biosynthetic pathways. *LIMLP02545*, encoding one
366 of the few gene products in the PerRA/B regulon related to oxidative stress, is a putative
367 methionine sulfoxide reductase, which catalyzes the reversible thioredoxin-dependent oxidation-
368 reduction (repair) of Met-SO to Met [118, 119]. Lastly, *LIMLP15435* contains a domain found in
369 glyoxalase/bleomycin resistance proteins; in bacteria, glyoxalases are used to detoxify
370 methylglyoxal, a reduced derivative of pyruvate, as part of the glutathione-dependent glyoxalase
371 system [120].

372 Eleven genes downregulated by PerRA/B in DMCs are involved in cellular homeostasis
373 and metabolism (Fig 6B, Table 2 and S4 Table). Only three (*LIMLP02795*, *LIMLP14175* and
374 *LIMLP08980*), encoding a cytochrome c peroxidase, a thiol oxidoreductase and a Grx4 family
375 monothiol glutaredoxin, respectively, are involved in oxidative stress adaptation.

376 *Sensing and responding to the mammalian host environment*. The PerRA/B regulon
377 includes at least 17 genes related to environmental sensing, signaling and, potentially, host
378 adaptation. Two (*LIMLP02835* and *LIMLP02840*) encode methyl-accepting chemotaxis proteins
379 co-regulated with *LIMLP02845*, encoding a small (62 aa) hypothetical protein of unknown
380 function (Table 1). Nine, including *lvrAB* (discussed below), encode sensory histidine kinases,
381 most of which contain PAS-type sensor domains (Fig 5C). One of the nine (*LIMLP05830*) encodes
382 a regulator that contains both PAS and GAF domains (Fig 5C). The PerRA/B DMC regulon
383 includes six putative DNA binding proteins, four upregulated and two downregulated (Fig 5C).
384 Three upregulated genes belong to the TetR (*LIMLP02080*), Cro/C1-λ (*LIMLP04315*) and CRP-
385 like (*LIMLP07225*) repressor families, while the fourth (*LIMLP00755*) encodes a hypothetical
386 protein containing a homeobox winged helix-like domain of unknown function (DUF433). A

387 second TetR-like repressor (*LIMLP19135*) and a BolA-like regulator (*LIMLP08975*) were
388 repressed by PerRA/PerRB in DMCs (Fig 5C). In *E. coli*, BolA has been linked to a range of
389 adaptive responses, including biofilm formation and entry into stationary phase [121]. All but two
390 of the regulatory proteins in the PerRA/B DMC regulon were dysregulated only in the double
391 mutant; *LIMLP06340*, encoding a histidine kinase, and *LIMLP07225*, encoding a CRP-like DNA
392 binding protein, also were upregulated by PerRB alone in DMCs (Fig 5B).

393 Although the vast majority (70%) of genes upregulated by PerRA/B encode proteins of
394 unknown function (Fig 6B and Table 1), seven contain conserved domains potentially related to
395 mammalian host adaptation and/or virulence. *LIMLP08585* contains a PPM-type phosphatase
396 domain (IPR001932); PPM domains are found in diverse regulatory proteins, including SpoIIE in
397 *B. subtilis* [122]. *LIMLP15425* contains a putative Lambda_Bor-like domain (PF06291), which in
398 *E. coli* has been associated with increased serum survival [123, 124]. *LIMLP14585*, annotated as
399 a host attachment protein, contains a domain of unknown function (IPR019291) found in
400 virulence-associated proteins from the plant pathogens *Agrobacterium tumefaciens* and
401 *Xanthomonas* spp. [125, 126]. *LIMLP02040* contains a SRPBCC-like domain (cd07812), which
402 forms a deep, hydrophobic ligand binding pocket capable of binding diverse ligands [127, 128].
403 Three hypothetical proteins (*LIMLP04635*, *LIMLP10965* and *LIMLP16555*) upregulated by
404 PerRA/B are predicted to form β-propeller structures, which are associated with a wide range of
405 functions, including ligand-binding, enzymatic activity, cell signaling, and protein-protein
406 interactions [129]. Interestingly, Thibeaux et al. [130] previously noted that proteins with β-
407 propeller repeats are enriched in highly virulent *Leptospira* spp. Six upregulated genes encode
408 uncharacterized lipoproteins of unknown function (Table 1), which could be involved in a variety

409 of host-pathogen interactions, including nutrient acquisition, adherence and/or immune evasion [7,
410 131].

411 Eighteen (56%) genes downregulated by PerRA/B in DMCs encode proteins of unknown
412 function (Table 2). *LIMP04970* and *LIMLP11660*, both predicted to encode lipoproteins, contain
413 domains (pectin lyase-fold/IPR011050 and Ricin B lectin/IPR000772, respectively) potentially
414 involved in binding to and/or cleavage of host-derived carbohydrates. *LIMLP04765* contains an
415 alpha/beta hydrolase domain shared by a wide range of hydrolytic enzymes. Lastly, *LIMLP01455*,
416 encoding an inner membrane protein, contains a DoxX-like domain; in *Mycobacterium*
417 *tuberculosis*, DoxX complexes with a thiosulfate sulfurtransferase (SseA) to promote resistance to
418 agents that disrupt thiol homeostasis [132].

419 *Known or putative virulence determinants*. The upregulated portion of the PerRA/B
420 regulon contains at least four virulence-associated genes (Table 1). Two, *LIMLP15405/ligA* and
421 *LIMLP15415/ligB,* encode the pathogen-specific, multifunctional, Leptospiral Immunoglobulin-
422 like repeat proteins LigA and LigB, respectively [133, 134], while *LIMLP08490* and *LIMLP08485*
423 encode the hybrid histidine kinases LvrA and LvrB, respectively [20]. Although tandemly located
424 on the chromosome, *ligA* and *ligB* are not co-transcribed (Fig 7A). They do, however, have
425 identical upstream regions and respond similarly *in vitro* to conditions used to mimic the
426 mammalian host milieu (*e.g.,* high osmolality and increased temperature) [22-25, 135]. Three
427 genes located downstream of *ligB*, all encoding hypothetical proteins, also were upregulated (Fig
428 7A). Using antisera against the shared N-terminal repeats (Fig 7A), we compared expression of
429 LigA and LigB in WT and mutant strains. As shown in Fig 7B, both LigA and LigB were absent
430 in whole cell lysates prepared from the *perRA/B* double mutant cultivated within DMCs;
431 expression of both Ligs was restored by *trans*-complementation with *perRB* alone. Consistent with

432 our transcriptomic data (Fig 7A), LigA and LigB were detected at near wild-type levels in the
433 *perRA* mutant by immunoblot (Fig 7B). While LigB also was expressed at near wild-type levels
434 in the *perRB* mutant cultivated in DMCs, by immunoblot, we saw a clear decrease in LigA (Fig
435 7A and B). These data suggest that LigA levels may be controlled by PerRB at both the
436 transcriptional and post-transcriptional level.

437 The downregulated portion of the PerRA/B DMC regulon contains at least one gene
438 potentially related to virulence. *LIMLP03665*, encoding a collagenase precursor [136], was
439 expressed at ~500-fold lower levels in the WT parent compared to the *perRA/B* mutant (Table 2).
440 While collagenase-mediated degradation of host tissues likely enhances dissemination of
441 leptospires during early infection [137], once in the kidneys, repression of *colA* could help reduce
442 pathogen-mediated damage to renal epithelial cells. Further transcriptional analysis of this gene is
443 needed to establish its expression profile in different tissues over the course of infection.

444

445 **Loss of LvrAB alone is not responsible for avirulence of the *perRA/B* double mutant in mice.**
446 As noted above, expression of *lvrAB* is disrupted only in the *perRA/B* double mutant (Table 1);
447 similar results were obtained using leptospires grown *in vitro* [91]. Using LvrA- and LvrB-specific
448 antisera, we confirmed our transcriptomic data at the protein level by immunoblot using whole cell
449 lysates from WT, *perRA*, *perRB, perRA/B* mutant and complemented strains cultivated in DMCs
450 (Fig 8A). Previously, Adhikarla *et al*. [20] reported that inactivation of *lvrAB* by transposon
451 mutagenesis results in dysregulation of a large number of genes *in vitro,* including *ligB*. However,
452 in our hands, we saw no decrease in LigA or LigB in the *lvrAB* mutant strain following cultivation
453 in DMCs (Fig 7B). Adhikarla *et al*. [20] also reported that loss of either *lvrAB* or *lvrB* alone resulted
454 in a significant loss of virulence in hamsters. To explore whether the avirulence of the *perRA/B*
455 double mutant in mice (Fig 4C-E) is due solely to loss of LvrAB, we assessed the ability of *lvrAB*

456 and *lvrB* transposon mutants to colonize the kidneys of C3H/HeJ mice (5 mice per strain, per
457 experiment). While mice infected with either the *lvrAB* or *lvrB* mutant shed ~2-$\log_{10}$ less
458 leptospires in their urine compared to the WT controls, all of the urine samples collected from
459 mice infected with either mutant were darkfield positive by day 21 (Fig 8B). At day 28 p.i., kidneys
460 harvested from all mice infected with either the *lvrAB* or *lvrB* mutant were positive for leptospires
461 by both culturing in EMJH and qPCR (Fig 8C). Mice infected with the *lvrAB* mutant also
462 seroconverted (Fig 8D). Thus, while LvrAB signal transduction contributes to virulence, loss of
463 *lvrAB* expression alone is not responsible for the complete loss of virulence observed with the
464 *perRA/B* double mutant in mice.
465

466 **Discussion**

467 *L. interrogans* must sense and respond to diverse signals and threats during the free-living
468 and reservoir host phases of its zoonotic cycle. Not surprisingly, *L. interrogans* encodes
469 substantially more sensory and regulatory proteins than *B. burgdorferi* and *T. pallidum* [138], two
470 pathogenic spirochetes with far more restrictive growth niches. However, the regulatory networks
471 and gene products that sustain *L. interrogans* in nature remain poorly understood. To gain insight
472 into the transcriptomic changes that leptospires undergo within the host, we previously compared
473 *L. interrogans* sv. Copenhageni strain Fiocruz L1-130 cultivated *in vitro* and in mammals using
474 our DMC peritoneal implant model [42, 108]. From these studies emerged >100 genes that were
475 differentially-expressed in response to host-specific signals, including *LIC12034*, encoding the
476 peroxide stress response regulator PerRA, which was upregulated 3.83-fold in DMCs. Herein, we
477 confirmed these data using *L. interrogans* sv. Manilae strain L495 and also established that the
478 three remaining FUR family regulators are transcribed at comparable (*perRB*) or higher (*ffr1* and
479 *ffr2*) levels in DMCs compared to *in vitro*. The importance of FUR family regulators for host
480 adaptation was confirmed recently by Zavala-Alvarado *et al*. [91], who demonstrated that
481 leptospires lacking both PerRA and PerRB are unable to infect hamsters. In our current study, we
482 establish that these regulators also are required for renal colonization of C3H/HeJ mice. In both
483 animal models, loss of virulence was observed only when both PerRA and PerRB were inactivated,
484 suggesting that these regulators may serve redundant or overlapping functions *in vivo*. Our finding
485 that the *perRA*/*B* double mutant survives at wild-type levels in DMCs is particularly noteworthy
486 as it demonstrates that the avirulent phenotype observed for this mutant is not due to a metabolic
487 lesion (*i.e*., metal starvation) but instead reflects dysregulation of one or more virulence-related
488 genes. Transcriptomic analyses of *perRA* and *perRB* single and double mutants cultivated in DMCs

489 brought to light a number of novel aspects of FUR-mediated regulation in *L. interrogans*. Most
490 notably, the majority of genes in the PerRA, PerRB and PerRA/B regulons were differentially
491 expressed only in DMCs, highlighting the importance of mammalian host-specific signals for
492 PerR-mediated regulation in *L. interrogans*. Remarkably, inactivation of both PerRA and PerRB
493 resulted in a DMC regulon that differs substantially from those of either single mutant and includes
494 a large cohort of genes involved in environmental sensing, signal transduction and transcriptional
495 regulation.

496 The presence of multiple FUR family regulators in *Leptospira* spp. was noted previously
497 by Louvel *et al*. [45], who identified five distinct orthologs between *L. interrogans* and *L. biflexa*.
498 Phylogenetic analyses presented herein identified a sixth FUR family regulator and established
499 that two (PerRA and Ffr1) are conserved within both pathogenic and saprophytic species and two
500 each are unique to either pathogenic (PerRB and Ffr2) or saprophytic subclades (PerRC and Ffr3).
501 As their designations suggest, three are predicted to function as PerRs, while the functions of the
502 remaining three cannot be predicted based on sequence alone. Given the importance of trace
503 metals, iron in particular, for *Leptospira* spp., the latter likely encode Fur, Zur or Mur orthologs.
504 Consistent with this notion, transcription of *ffr1* in *L. biflexa* was decreased ~10-fold in response
505 to iron starvation *in vitro* [45], presumably to derepress expression of iron uptake systems. Thus
506 far, only *B. licheniformis* has been shown to encode multiple PerRs ($PerR_{BL}$, PerR2 and PerR3),
507 each of which displays a different level of sensitivity to H_2O_2 (PerR2 > $PerR_{BL}$ > PerR3) [139].
508 The extent of regulatory overlap between these three PerRs has yet to be determined. Our finding
509 that almost all saprophytic and pathogenic *Leptospira* spp. encode closely-related PerRA and Ffr1
510 orthologs implies that these regulators promote survival outside of a host (e.g., within soil and/or
511 water). Nonetheless, studies with the *perRA/B* double mutant, presented here and elsewhere [91,

512 92], indicate that PerRA also contributes to virulence in mammals. The presence of a single PerR
513 (PerRB) in all but one species (*L. wolfii*) in the P2 subclade may help explain the 'intermediate'
514 virulence of these *Leptospira* spp. compared to highly virulent P1 subclade [7, 140].

515 *L. interrogans* cultivated in DMCs express increased levels of catalase, AhpC-type
516 peroxiredoxin and cytochrome c peroxidase [42], three enzymes typically associated with
517 detoxification of reactive oxygen species (ROS) in bacteria [83, 85, 141]. These data also provide
518 strong evidence that *L. interrogans* is exposed to ROS *in vivo* [42]. Consistent with this notion,
519 catalase-deficient leptospires are more susceptible to H_2O_2 *in vitro* and show reduced virulence in
520 hamsters [142]. Host phagocytic cells, which generate oxygen radicals *via* a dedicated NADPH
521 oxidase [143, 144], are one likely source of exogenously-derived ROS *in vivo*. Leptospires within
522 renal tubules, a highly oxygenated niche, also would be exposed to elevated levels of oxygen.
523 Incomplete reduction of oxygen by iron-containing cytochromes is another potential source of
524 endogenous ROS [83, 141]. In bacteria, oxidative stress responses often are coordinated by two
525 evolutionarily distinct master regulators -- OxyR and PerR. OxyR, the more common of the two,
526 belongs to the LysR family and functions primarily as an activator [145]. In its oxidized state,
527 OxyR activates transcription of genes involved in the detoxification of H_2O_2 (catalase and AhpC),
528 the prevention or repair of DNA damage (Dps) and/or redox homeostasis (glutathione reductase,
529 thioredoxin) [83]. PerR, first described in *B. subtilis* [146], typically represses rather than activates
530 many of the same genes as OxyR and is released from DNA by peroxidation [57, 60, 88]. Although
531 OxyR and PerR regulate transcription by different mechanisms, they react with H_2O_2 at essentially
532 the same rate constant (10^5 M^{-1} s^{-1}) [87] and orchestrate highly similar responses. *L. interrogans*
533 does not encode an OxyR homolog but, as noted above, encodes at least two PerR orthologs,
534 PerRA and PerRB. Consistent with PerR functions in other bacteria, as shown here and elsewhere

535 [89, 91, 92], *L. interrogans perRA* mutants show enhanced survival following exposure to lethal
536 levels of H_2O_2 *in vitro* and increased expression levels of catalase, AhpC and cytochrome c
537 peroxidase *in vitro*. While inactivation of *perRB*had no effect on the ability of leptospires to
538 withstand killing by H_2O_2, the *perRB* mutant showed increased tolerance to the superoxide-
539 generating compound paraquat [91]. Moreover, no genes associated with ROS defenses were
540 dysregulated in the *perRB* mutant *in vitro* [91]. In DMCs, only cytochrome c peroxidase, AhpC
541 and a glutaredoxin were dysregulated in the double mutant. Interestingly, all three genes were
542 expressed at higher levels in the WT compared the mutant, suggesting that they are activated rather
543 than repressed by PerRB. Moreover, expression of catalase was not significantly different in the
544 WT vs. *perRB* or *perRA/B* DMC comparison. Taken together, these data argue that while PerRA
545 and PerRB may be 'activated' by ROS, the adaptive responses they control likely extend beyond
546 oxidative stress.

547 Consistent with differences in the putative DNA-binding helices, we saw very little overlap
548 between the PerRA and PerRB DMC regulons. Seven of the eight genes common to both regulons
549 are located in a single locus encoding a TonB-dependent transporter (TBDT) system. In Gram-
550 negative bacteria, TBDT systems promote the uptake of substrates, such as iron siderophores,
551 heme, vitamin B12, and carbohydrates, that are either poorly transported by non-specific outer
552 membrane porins or are present in the extracellular milieu at low concentration [113, 147].
553 Substrate binding and uptake is mediated by high affinity, substrate-specific TonB-dependent
554 receptor (TBDR) proteins, which form outer membrane-embedded 22-stranded β-barrels [113].
555 The energy required for substrate transport is provided in the form of proton motive force, which
556 is transduced from the inner to outer membrane by the TonB-ExbB-ExbD complex [113]. *L.*
557 *interrogans* encodes 11 putative TonB-dependent receptors and at least two complete TonB-ExbB-

558 ExbD transporters. None of the leptospiral TBDRs possess a N-terminal extension capable of
559 interacting with anti-sigma factors, similar to that of the iron and heme TBDRs FecA and HasR in
560 *E. coli* and *Serratia marcescens*, respectively [148, 149]. Only one TBDT system, *LIMLP04240-*
561 *04270*, is differentially regulated by PerRA and PerRB in DMCs. While the substrate(s) recognized
562 by the TBDR (LIMLP04270) cannot be predicted based on sequence, mutagenesis studies on its
563 ortholog in *L. biflexa* suggest that it is not essential for uptake of iron or heme *in vitro* [45].
564 Moreover, a *L. interrogans* transposon mutant containing an insertion in *LIMLP04270* is virulent
565 in hamsters [91]. However, given the large number of TBDRs in *L interrogans*, one of these may
566 compensate for loss of LIMLP04270 *in vitro* and/or *in vivo*. Interestingly, *in vitro*, expression of
567 *LIMLP04240-04270* was dysregulated in the *perRA* and *perRA/B* mutants but not the *perRB* single
568 mutant; only *LIMLP04255* was upregulated 1.93-fold in the WT compared to the *perRB* mutant
569 [91]. A second TBDR, encoded by *LIMLP08410*, was repressed by PerRA/B only within DMCs.
570 Together, these data suggest that mammalian host signals play key role in modulating TonB-
571 dependent nutrient uptake in *L. interrogans* and, moreover, that the activity of PerRB is enhanced
572 *in vivo*.

573 Our finding that both PerRA and PerRB upregulate expression of the TBDT in DMCs
574 implies that they recognize the same upstream region(s). However, outside of this locus, how does
575 one explain the lack of overlap between the single and double mutants? In the simplest scenario,
576 PerRA and PerRB regulate a shared group of genes independently (*i.e.*, recognizing different but
577 adjacent PerR boxes within the same region). In this case, loss of both regulators is required for a
578 significant (>3-fold) difference in expression compared to wild-type. Alternatively, PerRA and
579 PerRB bind overlapping regulatory elements but exert opposite regulatory effects (*i.e.*, one
580 activating, the other repressing). Along this line, Pinochet-Barros and Helmann [80] recently

581 reported that *pfeT*, encoding an iron efflux pump, is co-regulated by both Fur and PerR in *B.*
582 *subtilis*, with the former acting as an activator and the latter a repressor. On the other hand, the
583 PerRA and PerRB single mutant regulons both contain multiple gene products related to
584 environmental sensing, signal transduction and/or DNA binding. Thus, differences between the
585 single versus double mutant regulons (and virulence phenotypes) could be due to "short circuiting"
586 of crosstalk between effector proteins that are controlled independently by PerRA and PerRB.
587 Further studies are needed to determine which, if any, of these non-mutually exclusive scenarios
588 are operative in *L. interrogans*.

589 Surprisingly, the majority of genes controlled by PerRA and/or PerRB in DMCs were
590 upregulated (*i.e.*, expressed at lower levels in the single or double mutants compared to WT) rather
591 than repressed *in vivo*. While only one prior study has demonstrated PerR-mediated activation
592 [150], there are multiple examples of FUR family regulators acting as transcriptional activators
593 [50, 59]. In *Vibrio vulnificus*, apo-Fur positively regulates its own expression by binding upstream
594 of the *fur* promoter [151]. In *Helicobacter pylori* and *Salmonella* Typhimurium, Fur activates
595 expression by binding upstream of target gene and helping to recruit RNA polymerase [152, 153].
596 In α-proteobacteria, Irrs (see below) act as positive and negative transcriptional regulators of
597 genes related to heme homeostasis [154-157]. BosR, a FUR family regulator in the Lyme disease
598 spirochete *B. burgdorferi*, activates transcription of the alternative sigma factor *rpoS* as part of a
599 complex that includes the alternative sigma factor RpoN and the response regulator Rrp2 [158-
600 163]. PerRA and/or PerRB also could activate transcription of target genes indirectly *via*
601 repression of a regulatory small RNA (*e.g.*, RyhB in *E. coli*) [164] or by preventing the binding of
602 another repressor (*i.e.*, anti-repression) [165, 166].

603 Designation of PerRA and PerRB as peroxide stress regulators in *L. interrogans* is based
604 largely on *in vitro* studies showing increased survival of *perRA* and *perRB* mutants following
605 exposure to H_2O_2 and paraquat, respectively [89, 91, 92]. Several lines of evidence, however, raise
606 the possibility that these gene products function as iron response regulators (Irrs) rather than PerRs.
607 Based on amino acid sequence alignments, PerRA and PerRB appear to be more closely related to
608 Irrs than PerRs. In α–proteobacteria, Irrs and their regulatory partner, RirA, coordinate the
609 expression of genes involved in heme biosynthesis with iron availability. Thus, it is worth noting
610 that LIMLP06290 (LIC11283), annotated as a hypothetical protein, contains domains consistent
611 with it being a RirA. At the sequence level, Irrs share a number of features with PerRs, including
612 the presence of Asp and Arg residues in their regulatory metal sites and DNA binding helices,
613 respectively. Irrs and PerRs also are responsive to similar levels of ROS, albeit by a different
614 mechanism, and regulate many of the same effector genes (*i.e.,* catalases and peroxidases) [65,
615 157, 167]. In some, but not all cases, *irr* mutants also show increased survival *in vitro* under high
616 H_2O_2 levels [157, 168]. As noted above, only *B. lichenformis* is known to encode multiple PerRs.
617 Numerous bacteria, on the other hand, encode two or more Irrs [167]. Variable affinity of Irrs for
618 their target promoters enables them to modulate gene expression over a wider range of conditions
619 than PerRs [169]. The autoregulatory sequences identified upstream of *perRA* [90] diverge
620 significantly from canonical PerR and Fur boxes but show strong similarity to "Irr-boxes" [170,
621 171]. Moreover, Irrs are known to act as activators as well as repressors [170] [167]. Although
622 typically associated with peroxide-sensitive regulation of iron/heme acquisition and utilization,
623 Irrs have been shown to control diverse cellular processes, including virulence. Moreover, the vast
624 majority of histidine kinases upregulated by PerRA/B in DMCs contain one or more PAS-type
625 sensor domains, which have been shown to function as heme sensors [172]. Given the established

626 importance of heme for survival of *L. interrogans* in mammals [173-175], our findings raise the
627 possibility that heme sensing by PerRA and/or PerRB in mammals could serve as an important
628 initiating event for host adaptation.

629 Studies presented here and elsewhere [91] demonstrate for the first time that both PerRA
630 and PerRB are required for full transcription of the virulence-related genes *ligA* and *ligB*. These
631 pathogen-specific surface lipoproteins have been studied extensively for their contributions to
632 host-pathogen interactions [176, 177], virulence [178] and potential use as vaccinogens [179-181].
633 Using a TALE-based transcriptional knockdown approach, Pappas and Picardeau [178] reported
634 that both Ligs are required for virulence in hamsters. As noted earlier, *ligA* and *ligB* are not co-
635 transcribed but instead share virtually identical upstream regions and, consequently, are co-
636 regulated by the same environmental signals. Matsunaga, Haake and others previously reported
637 that *ligA* and *ligB* are upregulated in response to physiological osmolarity (EMJH supplemented
638 with 120 mM sodium chloride) [25] and increased temperature [182]. However, temperature-
639 dependent regulation is mediated by a *cis*-acting RNA secondary structure that prevents translation
640 at lower temperature and disruption of this *cis* element had minimal effect on osmoregulation
641 [182]. Eshghi *et al*. [28] reported that inactivation of *lb139* (*LIMLP18410*), encoding a putative
642 anti-ECF sigma factor, resulted in ~2.5-fold decreased expression of *ligB in vitro.* However,
643 *LIMLP18410* is not in the PerRA/B regulon either *in vitro* [91] or in DMCs (this study) and,
644 therefore, is not responsible for dysregulation of *ligA* and *ligB* in the *perRA/B* double mutant.
645 Additional studies are needed to establish whether PerRA and/or PerRB regulate expression of
646 *ligA* and *ligB* directly by binding to the *lig* promoter region or indirectly *via* another effector
647 protein. The presence of multiple sensory and regulatory effector proteins in the PerRA and PerRB
648 DMC regulons argues that activation of PerRA and PerRB, presumably by oxidative stress,

649 initiates a complex regulatory network capable of sensing and responding to a wide range of
650 mammalian host-specific signals. Our finding that LvrAB-deficient leptospires express normal
651 levels of LigA and LigB argues that at least two PerRA/B-dependent regulatory pathways (LvrAB-
652 dependent and -independent) are operative in *L. interrogans in vivo*.
653

654 **Material and Methods**

655

656 **Ethics statement.** All experiments involving animals conducted at UConn Health were performed
657 in accordance with The Guide for the Care and Use of Laboratory Animals (8th Edition) (Guide
658 for the Care and Use of Laboratory Animals, 1996) using protocols reviewed and approved by the
659 UConn Health Institutional Animal Care and Use Committee [Animal Welfare Assurance (AWA)
660 number A347-01].

661

662 **Bacterial cultivation *in vitro.*** *L. interrogans* strains are described in Table S5. Leptospires were
663 cultivated routinely *in vitro* in Ellinghausen, McCullough, Johnson and Harris medium (EMJH)
664 [183, 184] supplemented with 1% rabbit serum at 30°C under static conditions. Mutants were
665 maintained in EMJH under appropriate antibiotic selection (spectinomycin, 40 μg/ml; kanamycin,
666 40 μg/ml; and/or gentamycin, 8 μg/ml). Cultures were harvested at late logarithmic phase (1-5 ×
667 10^8 per ml). Culture viability (*i.e.*, motility and cell morphology) was evaluated by darkfield
668 microscopy. Leptospires were enumerated using a Petroff-Hausser counting chamber (Hausser
669 Scientific Co., Horsham, PA). *Escherichia coli* strains were maintained in Lysogeny broth (LB)
670 or LB agar supplemented with the appropriate antibiotics (ampicillin, 100 μg/ml; spectinomycin,
671 100 μg/ml; kanamycin, 100 μg/ml; and/or gentamycin, 50 μg/ml). The genotypes of *L. interrogans*
672 mutants used in these studies were confirmed by PCR and amplicon sequencing using primers
673 listed in Table S6.

674

675 **Routine DNA manipulation and cloning.** Routine cloning was performed using In-Fusion® HD
676 Cloning Plus (Takara Bio USA Inc., Mountain View, CA) according to the manufacturer's

677 instructions. Plasmids were maintained in *E. coli* Top10 (Life Technologies, Grand Island, NY) or
678 Stellar (TaKaRa, Mountain View, CA) cells and purified using QIAprep spin and midi kits
679 (Qiagen, Valencia, CA). Bacterial genomic DNA was extracted from *L. interrogans* using the
680 Gentra Puregene Yeast/Bacteria kit (Qiagen) according to the manufacturer's recommendations.
681 Routine and high-fidelity PCR amplifications were performed using RedTaq (Denville Scientific,
682 Metuchen, NJ, United States) and CloneAmp HiFi (Takara Bio USA Inc., Mountain View, CA),
683 respectively. DNA sequencing was performed by Genewiz, Inc. (Cambridge, MA). Routine
684 sequence analyses were performed using MacVector (version 17.0.1, MacVector, Inc., Cary, NC,
685 United States). Oligonucleotide primers used in these studies were purchased from Sigma-Aldrich
686 (St. Louis, MO); primer sequences are provided in Table S6.
687
688 **Generation of polyclonal antisera using recombinant protein.** Recombinant His-tagged PerRA
689 (LIMLP10155), PerRB (LIMLP05620), LvrA (LIMLP08490) and LvrB (LIMLP08485) cloned
690 into pET28a vector (Novagen) and FlaB1 (LIMLP09410) cloned into pAE [185], were expressed
691 in *E. coli* BL21 Star (DE3) or OverExpress™ C43 (DE3) (Lucigen/VWR, Radnor, PA). Following
692 induction with IPTG, recombinant proteins were purified by nickel affinity chromatography using
693 HisTrap™ Column (GE Healthcare Life Sciences Pittsburgh, PA). Rat polyclonal antisera against
694 *L. interrogans* PerRA, PerRB, LvrA, LvrB and FlaB1 were generated by hyperimmunization of
695 female Sprague-Dawley rats (Envigo, South Easton, MA) with 40-60 μg of recombinant His-
696 tagged proteins co-administered with Freund's Complete Adjuvant. After three weeks, two
697 additional boosts of 40-60 μg of protein mixed 1:1 with Freund's Incomplete Adjuvant were co-
698 administered at two-week intervals. Two weeks after the second boost, animals were euthanized

699 by anesthetic overdose and blood was collected by cardiac puncture. Sera was collected by
700 centrifugation, aliquoted and frozen at -80°C.
701
702 **SDS–PAGE and immunoblot analyses.** To analyze *L. interrogans* whole cell lysates, equivalent
703 amounts of cells (~10^8 leptospires per lane) were re-suspended and boiled in reducing Laemmli
704 sample buffer (BioRad, Hercules, CA), separated through 10-12.5% separating polyacrylamide
705 mini-gels and then visualized by GelCode Blue Stain Reagent (ThermoFisher Scientific, Grand
706 Island, NY). Recombinant proteins expressed in *E. coli* were separated by SDS-PAGE and stained
707 with GelCode Blue Stain Reagent (ThermoFisher). For immunoblotting, proteins were transferred
708 to nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA) using Trans-Blot SD
709 semi-dry transfer cell (BioRad, Hercules, CA). Membranes were blocked using milk block solution
710 (MBS; 5% dry milk, 0.1% Tween 20, 5% fetal calf serum in PBS) for 1 h at room temperature.
711 His-tagged recombinant proteins were detected using an HRP-conjugated anti-His monoclonal
712 antibody (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Antisera
713 against recombinant His-tagged leptospiral proteins were diluted 1:250 (PerRA and PerRB),
714 1:1000 (LvrA, LvrB and FlaB1), 1:10,000 (LigA/B repeat region) in MBS and incubated overnight
715 at 4°C. After washing with PBS containing 0.05% Tween 20 (PBST), bound antibody was detected
716 with horseradish peroxidase-conjugated secondary antibody (Southern Biotechnology Associates,
717 Birmingham, AL) diluted 1:30,000. After 1 hr at room temperature, membranes were washed at
718 least five times with PBST and developed using the SuperSignal West Pico chemiluminescence
719 substrate (Pierce, Rockford, IL).
720

721 **Generation of host-adapted leptospires.** To obtain mammalian host-adapted organisms, *L.*
722 *interrogans* sv. Manilae strain L495 wild-type and mutant strains were cultivated in DMCs as
723 previously described [42, 43, 108]. Briefly, DMCs were prepared with 9-10 mls of EMJH medium
724 (supplemented with an additional 10% bovine serum albumin to maintain osmotic pressure) at a
725 starting inoculum of 10^4 organisms per ml. Using strict aseptic technique, DMCs were implanted
726 into the peritoneal cavity of an anesthetized female Sprague-Dawley rat. After nine days, animals
727 were euthanized by CO2 narcosis and DMCs harvested. The viability and density of leptospires
728 were evaluated by dark field microscopy using a Petroff-Hausser counting chamber (Hausser
729 Scientific Co., Horsham, PA).
730
731 **Murine infection experiments.** To determine the lethal dose to 50% of mice (LD_{50}) for *L.*
732 *interrogans* sv. Manilae strain L495, ten-week-old female C3H/HeJ mice (Jackson Laboratories,
733 Bar Harbour, ME) were inoculated intraperitoneally (IP) with 200 μl of EMJH containing 5×10^6,
734 10^6, 10^5 or 10^4 leptospires (5 mice per group). Animals were monitored twice a day for signs of
735 leptospirosis and, when moribund, were euthanized by anesthetic overdose. LD50 was calculated
736 using the Reed-Muench method [186]. For virulence studies, 10^5 of wild-type parent, mutant or
737 complemented strains were used to infect C3H/HeJ mice (5 animals per group, per experiment).
738 Beginning 14 days post-infection (p.i.), animals were monitored for the presence of leptospires in
739 urine, collected in a metabolic chamber for ~45 min following IP administration of furosemide (2-
740 10 mg/kg, IP). Burdens in urine were assessed by darkfield microcopy using a Petroff-Hausser
741 counting chamber. Twenty-eight or 42 days p.i. (virulence and LD_{50} experiments, respectively),
742 animals were euthanized by CO_2 narcosis and blood and kidneys were collected for serology,
743 culturing in EMJH, and qPCR. Sera from individual mice were used to immunoblot whole cell

744 lysates (~10^8 leptospires per lane) prepared from the wild-type parent grown *in vitro* in EMJH at
745 30°C.
746
747 **qRT-PCR.** Total RNA was isolated from leptospires (four biological replicates per condition)
748 cultivated *in vitro* at 30ºC or following cultivation in DMCs as previously described [42]. cDNAs
749 (+ and – RT) were assayed in quadruplicate in 25 µl reactions performed with SsoAdvanced
750 Universal SYBR or Probe (*lipL32*) Super Mixes (Bio-Rad). Oligonucleotide primers used for qRT-
751 PCR are provided in S6 Table. Copy numbers were calculated using internal standard curves (10^7
752 – 10^1 copies) generated using purified amplicons for *perRA*, *perRB*, *LIMLP18590* and
753 *LIMLP04825* and then normalized against *lipL32* [179]. The standard curve for *lipL32* was
754 generated using a copy of the *lipL32* amplicon cloned into pCR2.1-TOPO plasmid (Invitrogen).
755 Normalized copy numbers were compared using an unpaired *t* test with two-tailed *p* values and
756 95% confidence interval (Prism v. 6, GraphPad Software).
757
758 **Quantitation of burdens by qPCR**. DNA was extracted from infected kidneys using the Qiagen
759 DNeasy Blood & Tissue kit according to the manufacturer's recommendations. DNAs were
760 analyzed by quantitative PCR (qPCR) using a TaqMan-based assay for *lipL32* [179] in 25 µl
761 reactions performed with SsoAdvanced Universal Probes Super Mix (Bio-Rad). Copy numbers for
762 *lipL32* were determined using an internal standard curve for the *lipL32* amplicon cloned into
763 pCR2.1 TOPO (Invitrogen). Average values for each strain were compared using an unpaired *t*
764 test with two-tailed *p* values and 95% confidence interval (Prism v. 6, GraphPad Software).
765

766 **RNA sequencing and comparative transcriptomics.** Total RNA was prepared from leptospires
767 cultivated in DMCs using TRIzol Reagent (ThermoFisher) (3 biological replicates per strain) and
768 then treated twice with TURBO™ DNase (ThermoFisher) followed by purification using RNeasy
769 columns (Qiagen) as previously described [42]. Samples were eluted in RNAse-free water and
770 purified RNA was analyzed using Qubit RNA HS Assay Kit (Thermo) and Agilent TapeStation
771 4200 (Agilent Technologies, Santa Clara, CA, USA) using the RNA High Sensitivity assay. Only
772 samples with Ribosomal Integrity number (RIN*e*) values >7.5 were used for library preparation.
773 Stranded libraries were prepared from ribo-depleted RNA using Zymo-Seq RiboFree Total RNA
774 Library Kit according to manufacturer's instructions. Libraries were validated for length and
775 adapter dimer removal using the Agilent TapeStation 4200 D1000 high-sensitivity assay and then
776 quantified and normalized using the double-stranded DNA (dsDNA) high-sensitivity assay for
777 Qubit 3.0 (Life Technologies, Carlsbad, CA). Libraries were run on an Illumina High Output 75-
778 cycle v2.5 NextSeq 500 flow cell. Raw reads for each sample were trimmed using Sickle (v. 1.3.3;
779 available from https://github.com/najoshi/sickle) and then mapped using EDGE-pro version 1.1.3
780 [111] using fasta, protein translation table (ptt) and ribosomal/transfer RNA table (rnt) files based
781 on the *L. interrogans* sv. Manilae strain UP-MMC-NIID LP genome (NZ_CP011931.1,
782 NZ_CP011932.1 and NZ_CP011933.1). Differential expression was determined using DESeq2
783 [112]. Genes expressed at ≥3-fold higher/lower levels in the mutant compared to the wild-type
784 parent with a False Discovery Rate (FDR)-adjusted *p*-value (*q*-value) ≤ 0.05 were considered
785 differentially expressed. Raw read data have been deposited in the NCBI Sequence Read Archive
786 (SRA) database (BioProject accession PRJNA659512, samples SRR12604412, SRR12604413,
787 SRR12604414, SRR12604415, SRR12604416, SRR12604417, SRR12604418, SRR12604419,
788 SRR12604420, SRR12604421, SRR12604422 and SRR12604423).

789

790 **Bioinformatics**. Routine and comparative sequence analyses were performed using MacVector
791 (version 17.5.4; MacVector, Inc., Apex, NC). Clusters of Orthologous Group (COG)
792 classifications are based on MicroScope, an integrated platform for the annotation of bacterial gene
793 function through genomic, pangenomic and metabolic comparative analysis [187]. Conserved
794 domain searches were performed using Conserved Domain Database (CDD) Search [188], UniProt
795 [189] and InterPro [190]. Candidate lipoproteins were identified based on Setubal *et al*. [191] and
796 LipoP server [192]. Subcellular localization predictions were performed by BUSCA (Bologna
797 Unified Subcellular Component Annotator) [193]. Multiple sequence alignments were generated
798 by Clustal Omega [194] and MAFFT 7 [195]. Phylogenetic trees were generated using PhyML 3.0
799 [196] with LG substitution model chosen after an Akaike Information Criterion (AIC) model
800 selection [197]. Tree improvement was done by subtree pruning and regrafting (SPR) method
801 [198] with ten random starting trees. Robustness of branches was assessed by Approximate
802 Likelihood-Ratio Test (aLRT-SH) [199]. The resulting trees were visualized and annotated using
803 Interactive Tree of Life (iTOL, v 4.3) [200]. FUR domain-containing proteins in 26 *Leptospira*
804 spp. genomes (10, 5, 6 and 5 species from subclade P1, P2, S1 and S2, respectively [96]) were
805 identified using the *Leptospira* species name as a query in the Ferric-uptake regulator domain entry
806 (IPR002481) in EMBL-EBI InterProScan [201]. Orthologs shared between *L. interrogans* sv.
807 Manilae strain L495 and sv. Copenhageni strain Fiocruz L1-130 strains were identified using
808 OrthoVenn 2.0 [202].

809

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Author contributions statement

AG, CZ, NB, MP and MC contributed to the design of experiments. AG, EB and MC performed the experiments. AG and MC analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

833

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1148 interests: MSG and MGS are or were employed in part by a commercial company, Immuno Technologies,
1149 Inc. MGS holds more than 5% financial interest in Immuno Technologies, Inc. NN and CW declare no
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Table 1. Genes significantly upregulated by PerRA/PerRB in *L. interrogans* cultivated within DMCs

Gene[1]	LIC[2]	Description[1]	Localization[3]	Fold-reg.[4]	Single mutants DMC[5]	*in vitro*[6]
Virulence genes						
LIMLP08490	LIC11709	LvrA	CY	11.34		↑AB
LIMLP08485	LIC11708	LvrB	CY	6.96		↑AB
LIMLP15405	LIC10465	LigA	LIPO	5.18		↑AB
LIMLP15415	LIC10464	LigB	LIPO	3.57		↑AB
Unknown or poorly characterized functions						
LIMLP08590	-	Hypothetical protein	OM	111.42		↑A, ↑AB
LIMLP14210	LIC10705	Hypothetical protein	CY	75.96		↑A, ↑AB
LIMLP14215	LIC10704	Putative lipoprotein	LIPO	47.82		↑AB
LIMLP04320	LIC10905	Hypothetical protein	CY	19.52		
LIMLP13810	-	DUF1563 domain-containing protein	CY	19.25		
LIMLP14205	-	Putative lipoprotein	CY	14.88		↑AB
LIMLP08585	LIC11730	PPM-type phosphatase domain-containing protein	IM	14.83	↑B	↑AB
LIMLP14220	LIC10703	Hypothetical protein	IM	12.34		↑A, ↑AB
LIMLP00435	LIC10080	Putative cytoplasmic membrane protein	IM	10.66		
LIMLP14195	LIC10708	Hypothetical protein	IM	8.64		↑A, ↑AB
LIMLP15400	-	Hypothetical protein	CY	8.15		
LIMLP04275	LIC10897	Putative cytoplasmic membrane protein	IM	6.72	↑A, ↑B	↑A, ↑AB
LIMLP16540	LIC10244	Hypothetical protein	CY	6.61		
LIMLP02845	LIC12920	Hypothetical protein	CY	6.54		
LIMLP14225	LIC10702	Hypothetical protein	CY	6.18		↑A, ↑AB
LIMLP00875	-	Hypothetical protein	CY	5.49	↓A	↓AB
LIMLP00885	-	Hypothetical protein	CY	5.43	↓A	↓AB
LIMLP19335	-	Cytoplasmic membrane protein	IM	5.18		↑AB
LIMLP00880	LIC10167	Hypothetical protein	CY	5.00	↓A	↓AB
LIMLP00870	LIC10166	Hypothetical protein	CY	4.97	↓A	↓AB
LIMLP04280	LIC10898	Hypothetical protein/LipL48	LIPO	4.9	↑A, ↑B	↑A, ↑AB
LIMLP16575	LIC10235	Hypothetical protein	CY	4.86		↑AB
LIMLP18725	LIC20172	LruC domain-containing protein	LIPO	4.81		↑AB
LIMLP02550	LIC12977	Hypothetical protein	CY	4.56		
LIMLP01200	-	Putative lipoprotein	CY	4.49	↓B	
LIMLP15410	-	Hypothetical protein	CY	4.37		
LIMLP04635	LIC10968	TolB-like β-propeller repeat protein	LIPO	4.12		
LIMLP16555	LIC10239	Beta-propeller repeat protein	EC	4.11		↑AB
LIMLP05930	LIC11215	Hypothetical protein	OM	3.99		
LIMLP11850	LIC10903	Putative cytoplasmic membrane protein	IM	3.70	↓A	
LIMLP14905	LIC10567	Hypothetical protein	IM	3.70	↓A, ↑B	↑AB
LIMLP15430	LIC10461	Putative lipoprotein	LIPO	3.6		↑AB
LIMLP04325	LIC10906	HK-like N-terminal 7TM region	IM	3.58		
LIMLP15640	LIC10422	Hypothetical protein	CY	3.47		
LIMLP15420	LIC10463	Putative lipoprotein	LIPO	3.37		↑AB
LIMLP06815	LIC11397	Hypothetical protein	CY	3.36		
LIMLP10965	LIC12209	TolB-like β-propeller repeat protein	LIPO	3.34		
LIMLP02040	LIC13081	Hypothetical protein	CY	3.28	↑B	↑AB
LIMLP15920	LIC10372	Putative cytoplasmic membrane protein	IM	3.25		
LIMLP15425	LIC10462	Putative lipoprotein, Bor domain	LIPO	3.24		↑AB

LIMLP00860	LIC10164	Hypothetical protein	CY	3.24	↓A	
LIMLP15645	LIC10421	Hypothetical protein	CY	3.23	↓B	
LIMLP14585	LIC10630	Host attachment protein	CY	3.19		
LIMLP01545	LIC13183	Hypothetical protein	CY	3.16		
Cellular homeostasis and metabolism						
LIMLP14200	LIC10707	SGNH/GDSL hydrolase family protein	EC	8.5		↑A, ↑AB
LIMLP00865	LIC10165	Host-nuclease inhibitor	CY	4.52	↓A	
LIMLP12135	LIC12436	Sulfatase, Alk. phosphatase-like domain-containing protein	IM	4.30		↑AB
LIMLP04245	LIC10890	Biopolymer transporter ExbD	IM	3.83	↑A, ↑B	↑A, ↑AB
LIMLP18070	LIC20049	ATP-NAD kinase	CY	3.76		↑AB
LIMLP02545	LIC12978	Peptide methionine sulfoxide reductase MsrA	CY	3.70		
LIMLP04270	LIC10896	TonB-dependent receptor	OM	3.70	↑A, ↑B	↑A
LIMLP04250	LIC10891	Biopolymer transporter ExbD	IM	3.29	↑A, ↑B	↑A, ↑AB
LIMLP04240	LIC10889	Energy transducer TonB	IM	3.24	↑A, ↑B	↑A, ↑AB
LIMLP15905	LIC10374	PPK2 domain-containing protein	CY	3.15	↑B	
LIMLP15435	LIC10460	Glyoxalase/Bleomycin resistance protein	CY	3.12		
Chemotaxis and motility						
LIMLP02840	LIC12921	Methyl-accepting chemotaxis protein	IM	8.24		↑AB
LIMLP02835	-	Methyl-accepting chemotaxis protein	IM	3.24		
Signaling and regulation						
LIMLP02080	LIC13073	TetR/AcrR family transcriptional regulator	CY	14.94		
LIMLP07025	LIC11439	Sensory Histidine kinase	CY	12.18		
LIMLP07225	LIC11484	Crp/Fnr family transcriptional regulator	CY	10.04	↑B	
LIMLP07030	LIC11440	Response regulator	CY	6.47		
LIMLP04315	LIC10904	Lambda repressor-like, DNA-binding domain protein	CY	4.91		
LIMLP00755	LIC10143	Homeobox-like and winged helix-like DNA-binding domain-containing protein	CY	4.29		
LIMLP11545	LIC12319	Serine/threonine-protein phosphatase	CY	4.19		↑AB
LIMLP06340	LIC11292	Histidine kinase	IM	3.59	↑B	
LIMLP08685	LIC11749	Acyl transferase/hydrolase/lysophospholipase	CY	3.57		
LIMLP00425	LIC10078	ATPase domain of Hsp90/DNA topoisomerase II/HK	CY	3.45		
LIMLP05840	LIC11202	Hybrid sensor HK/RR	CY	3.14		
LIMLP06990	LIC11432	PAS domain-containing sensor HK/RR	CY	3.09		
LIMLP05830	LIC11200	PAS domain S-box HK	CY	3.04		

1 Gene identifications and descriptions are based on *L. interrogans* sv. Manilae strain UP-MMC-NIID LP (accession numbers NZ_CP011931.1, NZ_CP011932.1 and NZ_CP011933.1). In some cases, annotations have been manually curated to conform with prior studies or bioinformatics.

2 *L. interrogans* sv. Copenhageni strain Fiocruz L1-130 (accession numbers NC_005823.1 and NC_005824.1) orthologs were identified using OrthoVenn 2.0 [202]. Dashes (-) indicate genes for which no clear ortholog was identified.

3 Localization is based on cumulative data from BUSCA [193], LipoP [192] and SpLip [191]. Abbreviations: CY, cytoplasmic; EC, extracellular; LIPO, lipoprotein; IM, inner membrane; OM, outer membrane.

4 Fold-regulation and adjusted-*p* values determined using DESeq [112] based on WT vs. *perRA/B* RNA-Seq

comparison. See Table S4.

5 Behavior of the corresponding gene in the WT vs. *perRA* (A) and *perRB* (B) comparisons by RNA-Seq using *L. interrogans* cultivated within DMCs. Arrows are used to indicate significant (≥3-fold, $q<0.05$) upregulation (↑) or repression (↓) in the WT compared to the mutant in DMCs. See Tables S2 and S3.

6 Behavior of the corresponding gene in the WT vs. *perRA* (A), WT vs. *perRB* (B) and WT vs. *perRA/B* (AB) RNA-Seq using *L. interrogans* cultivated *in vitro* [91, 92]. Arrows are used to indicate significant (≥3-fold, $p\leq0.05$) upregulation (↑) or repression (↓) in the WT compared to the corresponding mutant *in vitro*. See Tables S2 and S3.

Table 2. Genes significantly downregulated by PerRA/PerRB in *L. interrogans* cultivated within DMCs

Gene[1]	LIC[2]	Description[1]	Localization[3]	Fold-reg[4]	Single mutant DMC[5]	*in vitro*[6]
Virulence genes						
LIMLP03665	LIC12760	collagenase	EC/LIPO	-513.15		
Unknown or poorly characterized functions						
LIMLP05765	LIC11190	Hypothetical protein	CY	-30.51		
LIMLP04970	LIC11030	Putative lipoprotein (Pectin lyase fold domain)	LIPO	-12.14		↓AB
LIMLP08420	LIC11696	Hypothetical protein	LIPO	-9.52		↓AB
LIMLP05735	LIC11184	Putative lipoprotein with Ig-like domain	LIPO	-8.00		
LIMLP11660	LIC12340	DUF1561 domain-containing protein (Ricin B lectin domains)	EC	-7.51		
LIMLP11655	LIC12339	DUF1561 domain-containing protein	EC	-5.81		
LIMLP08415	LIC11695	Putative lipoprotein	LIPO	-5.48		
LIMLP01455	LIC13200	Hypothetical protein (DoxX domain)	IM	-5.05		
LIMLP04580	LIC10957	Hypothetical protein	CY	-4.06		↓AB
LIMLP03670	LIC12759	LRR domain containing protein	CY	-3.96		
LIMLP01990	LIC13089	Hypothetical protein	EC	-3.41	↓B	
LIMLP01965	LIC13095	TPR protein	CY	-3.39		
LIMLP09385	LIC11707	LRR domain containing protein (Internalin-like)	CY	-3.38		↓AB
LIMLP04765	LIC10995	Alpha/beta hydrolase	CY	-3.26	↓B	
LIMLP05980	LIC11224	Hypothetical protein (TM protein 43 family)	IM	-3.25		
LIMLP15220	-	Hypothetical protein	CY	-3.25		
LIMLP18200	-	Hypothetical protein	CY	-3.21	↓B	
LIMLP02405	LIC13005	Putative cytoplasmic membrane protein	IM	-3.06		↓AB
Cellular homeostasis and metabolism						
LIMLP02795	LIC12927	Cytochrome-c peroxidase	LIPO	-6.89		↓A, ↓AB
LIMLP04590	LIC10958	NAD(P)-dependent alcohol dehydrogenase	CY	-5.79		
LIMLP05325	LIC11101	Dihydroxy-acid dehydratase	CY	-4.82		
LIMLP03780	LIC12737	Site-specific modification, DNA-methyltransferase	CY	-4.63		
LIMLP05610	LIC11156	Putative citrate transporter	IM	-3.94		
LIMLP11840	LIC12378	Class I SAM-dependent methyltransferase	CY	-3.53		↓AB
LIMLP14175	LIC10712	Thiol oxidoreductase	IM	-3.48		↓AB
LIMLP08410	LIC11694	TonB-dependent receptor	OM	-3.46		
LIMLP08980	LIC11809	Grx4 family monothiol glutaredoxin	CY	-3.36	↓B	
LIMLP14170	LIC10713	Peptidase M75/lrub	LIPO	-3.33		↓AB
Signaling and regulation						
LIMLP19135	LIC20248	TetR/ArcR family transcriptional regulator	CY	-4.60		↓AB
LIMLP14755	LIC10598	Serine phosphatase	IM	-3.54		
LIMLP08975	LIC11808	BolA-like transcriptional regulator	CY	-3.02		

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cytoplasmic; EC, extracellular; LIPO, lipoprotein; IM, inner membrane; OM, outer membrane.

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5 Behavior of the corresponding gene in the WT vs. *perRA* (A) and *perRB* (B) comparisons using *L. interrogans* cultivated within DMCs. Arrows are used to indicate significant upregulation (↑) or repression (↓) in the WT compared to the corresponding mutant in DMCs. See Tables S2 and S3.

6 Behavior of the corresponding gene in the WT vs. *perRA* (A), *perRB* (B), and *perRA/B* (AB) RNA-Seq using *L. interrogans* cultivated *in vitro* [91, 92]. Arrows are used to indicate significant ($\geq$3-fold, $p \leq 0.05$) upregulation (↑) or repression (↓) in the WT compared to the corresponding mutant *in vitro*. See Tables S2 and S3.

1484
1485 **Figure Legends**
1486
1487 **Figure 1. Comparative sequence analysis of Ferric uptake regulator (FUR) domain-**
1488 **containing proteins from *L. interrogans* and other bacteria. A.** Phylogenetic analysis of *L.*
1489 *interrogans* FUR-like regulators LIMLP10155 (PDB:5NL9, PerRA), LIMLP05620 (PerRB),
1490 LIMLP04825 (Ffr1) and LIMLP18590 (Ffr2) with well-characterized FUR superfamily members
1491 from diverse bacteria. Phylogenetic analyses were performed as described in Materials and
1492 Methods. A midpoint rooted tree was generated using iTOL [200]. Fur family regulators
1493 represented in the tree: *Bacillus subtilis* PerR (Uniprot: P71086, PDB: 3F8N); *Bradyrhizobium*
1494 *japonicum* Irr (Uniprot: A0A0A3XTB2); *Campylobacter jejuni* PerR (Uniprot: Q0PBI7, PDB:
1495 6DK4); *Escherichia coli* Fur (Uniprot: P0A9A9, PDB: 2FU4); *Francisella tularensis* Fur
1496 (Uniprot: Q5NIN6, PDB: 5NBC); *Magnetospirillum gryphiswaldense* Irr (Uniprot: V6F4I4) and
1497 Fur (Uniprot: V6F4Q0, PDB: 4RB1); *Brucella abortus* Irr (Uniprot: Q2YQQ7); *Mycobacterium*
1498 *tuberculosis* Zur (Uniprot: P9WN85, PDB: 2O03); *Pseudomonas aeruginosa* Fur (Uniprot:
1499 Q03456, PDB: 6H1C); *Rhizobium leguminosarum* Irr (Uniprot: Q8KLU1) and Mur (Uniprot:
1500 O07315, PDB: 5FD6); *Rhodobacter sphaeroides* Irr (Uniprot: Q3IXE0); *Staphylococcus aureus*
1501 PerR (Uniprot: Q2G282); *Streptococcus pyogenes* PerR (Uniprot: A0A0H2UT39, PDB: 4I7H);
1502 *Streptomyces coelicolor* Zur (Uniprot: Q9L2H5, PDB: 3MWM) and Nur (Uniprot: Q9K4F8, PDB:
1503 3EYY); and *Vibrio cholerae* Fur (Uniprot: P0C6C8, PDB: 2W57). **B.** Multiple sequence alignment
1504 of FUR-like regulators in A. Residues confirmed to be involved in regulatory metal coordination
1505 (●) are highlighted in yellow, green or gray; position 103 is used to discriminate between PerR/Irrs
1506 (Asp, green) and Fur/Zur/Mur/Nur regulators (Glu, gray). CxxC-motif residues (○) confirmed to
1507 be involved in structural metal coordination are highlighted in cyan. Residues in red are predicted
1508 but not confirmed by X-ray crystallography to be involved in regulatory or structural metal

1509 coordination. Asparagine (N) or arginine (R) residues (□) in blue, located in DNA binding helix
1510 H4, can be used to distinguish between PerR and Fur, respectively [95]. *, the PDB structure for
1511 *E. coli* Fur includes only the DNA binding domain. Numbers on the top correspond to residues
1512 positions in *L. interrogans* PerRA.
1513
1514 **Figure 2. Distribution of Ferric-uptake regulator (FUR) domain-containing proteins across**
1515 **pathogenic and saprophytic *Leptospira* spp. A.** FUR domain-containing proteins in
1516 representative *Leptospira* spp. from pathogenic (P1 and P2) and saprophytic (S1 and S2)
1517 subclades. Genomic locus tags for each FUR family protein in *Leptospira* spp. are indicated. **B.**
1518 Phylogenetic analysis of *Leptospira* spp. FUR family proteins shown in **A**. Unrooted tree was
1519 generated using iTOL [200].
1520
1521 **Figure 3**. ***L. interrogans* express increased transcript levels for three FUR family regulators**
1522 **in response to mammalian host signals compared to *in vitro***. Transcripts for *LIMLP10155*
1523 (*perRA*), *LIMLP05620* (*perRB*), *LIMLP04825* (*ffr1*) and *LIMLP18590* (*ffr2*) and were accessed by
1524 qRT-PCR using cDNAs from wild-type *L. interrogans* sv. Manilae strain L495 cultivated *in vitro*
1525 in EMJH at 30°C (IV) or within rat peritoneal dialysis membrane chambers (DMC). Transcript
1526 copy numbers for each gene of interest were normalized per 1000 copies of *lipL32*. Bars show the
1527 average of four biological replicates for each condition, assayed in quadruplicate. *p*-values were
1528 determined using a two-tailed *t*-test; *, $p < 0.05$.
1529
1530 **Figure 4. *L. interrogans* requires both PerRA and PerRB for renal colonization of C3H/HeJ**
1531 **mice. A.** Female 10-week old C3H/HeJ mice (5 per group) were inoculated intraperitoneally with

1532 the indicated numbers of leptospires and monitored for 42 days. At 42 days, animals were
1533 euthanized, and kidneys harvested for culturing in EMJH. **B**. Enumeration of leptospires in urine
1534 collected from C3H/HeJ mice shown in A 14-, 21- and 35-days post-infection. Circles represent
1535 data for urine from individual mice. Burdens per ml of urine were assess by darkfield microscopy
1536 using a Petroff-Hauser counting chamber. Bars show the average and standard error of the mean.
1537 *p*-values were determined using a two-tailed *t*-test. **C.** Enumeration of leptospires in urine collected
1538 from C3H/HeJ mice inoculated intraperitoneally with 10^5 of WT, *perRA*, *perRB*, *perRA/B* or
1539 *perA/B+perRB trans*-complemented (Comp) strains. Burdens per ml of urine were assess by
1540 darkfield microscopy using a Petroff-Hauser counting chamber. Circles represent data for urine
1541 from individual mice in three independent experiments (5 mice per group, per strain, per
1542 experiment). One mouse infected with the WT strain died between day 14-21 due to circumstances
1543 unrelated to infection with *L. interrogans*. **D**. Burdens of leptospires in kidneys harvested from
1544 mice in panel C. DNA samples from kidneys harvested 28 days post-inoculation were assessed by
1545 qPCR using a Taqman-based assay for *lipL32* (in quadruplicate). Bars in B-D represent the average
1546 and standard error of the mean. *p*-values in C and D were determined by comparing burdens in
1547 mice infected with wild-type (WT) and mutant strains at the same timepoint using a two-tailed *t*-
1548 test. ***, $p \leq 0.0001$; **, $p = 0.0079$. **E**. Immunoblot analysis of sera collected from mice with
1549 WT, *perRA*, *perRB*, *perRA/B* or *perA/B+perRB trans*-complemented (Comp) strains, collected 28
1550 days post-infection and tested against whole cell lysates of *L. interrogans* sv. Manilae strain L495
1551 grown in EMJH at 30°C.
1552
1553 **Figure 5. The PerRA and PerRB DMC regulons contain genes related to environmental**
1554 **sensing, signaling and/or transcriptional regulation.** Proteins containing conserved domains

1555 related to signal transduction systems, DNA binding or other regulatory functions identified as
1556 being differentially expressed in the wild-type vs. *perRA* (**A**), *perRB* (**B**) or *perRA/B* (**C**) RNA-
1557 Seq comparisons. Values indicate fold-regulation (up or down) in each comparison. Shading
1558 indicates genes differentially expressed ≥3-fold (adjusted-p <0.05). Abbreviations for conserved
1559 domain names and Interpro (IPR) designations: σ PP2C, PPM-type phosphatase domain
1560 superfamily (IPR036457); σ^{70} r2, RNA polymerase sigma factor region 2 (IPR007627); σ^{70} r3/4,
1561 RNA polymerase sigma factor, region 3/4-like (IPR013324); Arc, Arc-type ribbon-helix-helix
1562 (IPR013321); ArsR, ArsR-type helix-turn-helix DNA-binding domain (IPR001845); BolA, BolA
1563 family domain (IPR002634); Cro/C1-λ, Cro/C1-type helix-turn-helix domain (IPR001387) and/or
1564 Lambda repressor-like, DNA-binding superfamily domain (IPR010982); Crp cNBD, Cyclic
1565 nucleotide-binding domain (IPR000595); Crp HTH, Crp-type helix-turn-helix domain
1566 (IPR012318); DBD, Putative DNA-binding domain superfamily (IPR009061); DUF433, domain
1567 of unknown function DUF433 (IPR007367) and Homeobox-like superfamily domain
1568 (IPR009057); EAL, EAL-type phosphodiesterase domain (IPR001633); GAF, GAF-like domain
1569 superfamily domain (IPR029016); HAMP, HAMP domain (IPR003660); HK, Histidine kinase
1570 (IPR005467, IPR003594) and dimerization/phosphoacceptor (IPR003661) domains; HK*,
1571 Histidine kinase (IPR005467, IPR003594) only (no dimerization domain); HTH/TetR, DNA-
1572 binding helix-turn-helix/TetR-type domain (IPR001647); Kinase, Protein kinase domain
1573 (IPR000719); NTPase, P-loop containing nucleoside triphosphate hydrolase (IPR027417); PAS,
1574 PAS domain (IPR000014); PHA DBD, PHA accumulation regulator DNA-binding, N-terminal
1575 (IPR012909); REC, Signal transduction response regulator receiver (IPR001789) and/or CheY-
1576 like superfamily (IPR011006) domain; STAS, Sulphate Transporter and Anti-Sigma factor
1577 antagonist domain (IPR002645); TetR, Tetracyclin repressor-like superfamily C-terminal domain

1578 (IPR036271); TM, transmembrane helix; TMx6, six transmembrane helices; and TPR,
1579 Tetratricopeptide-like helical domain superfamily (IPR011990).
1580
1581 **Figure 6. Overview of comparative RNA-Seq analyses of *L. interrogans* wild-type, *perRA*,**
1582 ***perRB* and *perRA/B* mutant strains. A.** Venn diagram showing overlap of genes differentially
1583 expressed ≥3-fold (q≤0.05) in wild-type versus single and double mutant comparisons. ↑ and ↓
1584 symbols denote genes upregulated (*i.e.*, expressed at higher levels in the wild-type vs. mutant) or
1585 downregulated (*i.e.*, expressed at lower levels in the wild-type vs. mutant), respectively, by PerRA,
1586 PerRB or both (PerRA/B). Complete datasets of all comparisons are presented in Tables S2-S4.
1587 **B.** Cluster of Orthologous Genes (COG) categorization of differentially expressed genes (DEGs)
1588 in the wild-type vs. *perRA/B* double mutant RNA-Seq comparison. COG predictions for individual
1589 genes are presented in Table S4. Number of DEGs in each COG are indicated on the *x*-axis.
1590
1591 **Figure 7. Expression of LigA and LigB *in vivo* is dysregulated only in the *perRA/B* double**
1592 **mutant. A.** Cartoon depiction of *ligA*, *ligB* and surrounding genes in *L. interrogans* sv. Manilae
1593 strain L495. Hatched bars are used to show Immunoglobulin-like repeats. Red and Blue colored
1594 regions indicate LigA- and LigB-specific regions, respectively. Values below each cartoon
1595 indicate the fold-regulation in the wild-type (WT) strain L495 parent compared to *perRA*, *perRB*
1596 and *perRA/B* mutant strains based on the corresponding RNA-Seq comparisons. **B.** Whole cell
1597 lysates of *L. interrogans* sv. Manilae strain L495 isogenic WT, *perRA*, *perRB*, *perRA/B*, *perRA/B*
1598 *trans*-complemented with *perRB* (Comp) and *lvrAB* strains were generated from leptospires
1599 cultivated within DMCs, separated by SDS-PAGE, and immunoblotted with rabbit polyclonal
1600 antiserum against repeats conserved in both LigA and LigB (gray region in panel A). After

1601 detection, membranes were striped and re-blotted using rat polyclonal antiserum against
1602 recombinant FlaB1 as a loading control. Molecular weight markers (kDa) are shown on the left.
1603
1604 **Figure 8. Expression of LvrAB requires both PerRA and PerRB but the absence of LvrAB**
1605 **alone is not solely responsible for avirulence of the *perRA/B* double mutant. A.** Whole cell
1606 lysates of *L. interrogans* sv. Manilae strain L495 wild-type (WT), *perRA*, *perRB*, *perRA/B*,
1607 *perRA/B* mutant complemented in *trans* with *perRB* (Comp) and *lvrAB* mutant strains were
1608 generated from leptospires cultivated within DMCs. Lysates were separated by SDS-PAGE,
1609 transferred to nitrocellulose, and immunoblotted with rabbit polyclonal LvrA- or LvrB-specific
1610 antiserum. Membranes were striped and re-blotted using rat polyclonal antiserum against
1611 recombinant FlaB1 as a loading control. Molecular weight markers (kDa) are shown on the left.
1612 **B.** Enumeration of leptospires in urine collected from C3H/HeJ mice 14- and 21-days post-
1613 infection following intraperitoneal inoculation with 10^5 of wild-type (WT), *lvrAB* or *lvrB* mutant
1614 strains. Circles represent data for urine from individual mice (5 mice per group, per strain, per
1615 experiment). **C.** Burdens of leptospires in kidneys harvested from mice in panel B. DNA samples
1616 from kidneys harvested 28 days post-inoculation were assessed (in quadruplicate) by qPCR using
1617 a Taqman-based assay for *lipL32*. Bars in B and C represent the average and standard error of the
1618 mean. *p*-values were determined by comparing burdens in mice infected with wild-type (WT) and
1619 mutant strains at the same timepoint using a two-tailed *t*-test; ***, $p < 0.0001$; **, $p = 0.0007$; and
1620 *, $p = 0.0079$. **D**. Immunoblot analysis of sera collected from C3H/HeJ mice 28-days following
1621 intraperitoneal inoculation with 10^5 wild-type or *lvrAB* mutant strains and then blotted against
1622 whole cell lysates of *L. interrogans* sv. Manilae strain L495 grown in EMJH at 30°C.
1623

1624 **Supplementary Material**

1625

1626 **S1 Figure. Multiple sequence alignment with full-length sequences of FUR-like regulators in**
1627 **Figure 1**. Residues confirmed to be involved in regulatory metal coordination (●) are highlighted
1628 in yellow, green or gray; position 103 is used to discriminate between PerR/Irrs (Asp, green) and
1629 Fur/Zur/Mur/Nur regulators (Glu, gray). CxxC-motif residues (○) confirmed to be involved in
1630 structural metal coordination are highlighted in cyan. Residues in red are predicted but not
1631 confirmed by X-ray crystallography to be involved in regulatory or structural metal coordination.
1632 The asparagine (N) or arginine (R) residues in blue (□) are located in the DNA binding helix H4
1633 and usually are used to distinguish between PerR and Fur, respectively [95]. Bars at the top of the
1634 alignment indicate DNA binding helices H2, H3 and H3. *, the PDB structure for *E. coli* Fur
1635 includes only the DNA binding domain. Numbers on the top correspond to residues positions in
1636 *L. interrogans* PerRA.

1637

1638 **S2 Figure. Multiple sequence alignment of FUR domain-containing proteins in**
1639 **representative *Leptospira* spp. from Figure 2.** Species from pathogenic subclades P1 and P2 are
1640 colored in black and blue, respectively, while saprophytic species from subclades S1 and S2 are in
1641 red and green, respectively. Genomic locus tags for each FUR family proteins in *Leptospira* spp.
1642 are indicated. Highly conserved residues predicted as regulatory metal binding sites are highlighted
1643 in yellow, green and magenta. Putative structural metal binding sites are highlighted in cyan.
1644 *Leptospira* species are abbreviated as follow: L. int, *L. interrogans*; L. kir, *L. kirschneri*; L. adl, *L.*
1645 *adleri*; L. als, *L. alstonii*; L. san, *L. santarosai*; L. bor, *L. borgpetersoni*; L. ale, *L. alexanderi*; L.
1646 wol, *L. wolfii*; L. lis, *L. liscerasiae*; L. ina, *L. inadai*; L. fai, *L. fainei*; L. bif, *L. biflexa*; L. mey, *L.*

1647 *meyeri*; L. ter, *L. terpstrae*; L. van, *L. vanthielii*; L. ryu, *L. ryugenii*; L. ily, *L. ilyithenensis*; L. ido,
1648 *L. idonii*.
1649
1650 **S3 Figure. Expression of PerRA and PerRB in *L. interrogans* wild-type, mutant and**
1651 **complemented strains**. Whole-cell lysates from *L. interrogans* sv Manilae strain L495 wild-type
1652 (WT), *perRA*, *perRB*, *perRA/B* and the *perRA/B* mutant complemented in *trans* with *perRB* on
1653 pMaORI (Comp) were separated by SDS-PAGE, immobilized on nitrocellulose and then
1654 immunoblotted with rat polyclonal antisera against PerRA, PerRB and FlaB1.
1655
1656 **S4 Figure. PerRA and PerRB regulate the expression of a locus (*LIMLP04285-04240*) that**
1657 **includes a TonB-dependent transport system.** Data from comparative RNA-Seq analysis of
1658 wild-type vs. *perRA*, *perRB* and *perRA/B* strains identified a nine gene chromosomal locus that
1659 includes *lipL48* and genes encoding a TonB-dependent receptor and ExbB/ExbD/TonB
1660 transporter. Fold-of-regulation for each gene are based on RNA-Seq data from wild-type and
1661 mutant leptospires grown in DMCs, presented in Tables S2-S4, and *in vitro* in EMJH at 30°C (IV),
1662 presented in Zavala-Alvarado *et al.* [92]
1663
1664 **S5 Figure. Overview of genes differentially expressed by *L. interrogans perRA* and *perRB***
1665 **single mutants.** Cluster of Orthologous Genes (COG) categorization of differentially expressed
1666 genes (DEGs) in the wild-type vs. *perRA* (**A**) and *perRB* (**B**) RNA-Seq comparisons. COG
1667 predictions for individual genes are presented in Tables S2-S3. Number of DEGs in each COG are
1668 indicated on the *x*-axis.
1669

1670

1671 **Table S1. Summary of RNA-Seq raw read data.**

1672

1673 **Table S2. Comparative RNA-Seq data for *L. interrogans* sv. Manilae L495 wild-type and**
1674 ***perRA* strains cultivated in dialysis membrane chambers (DMCs)**. The genome sequence of *L.*
1675 *interrogans* sv. Manilae strain UP-MMC-NIID LP (accession numbers NZ_CP011931.1,
1676 NZ_CP011932.1 and NZ_CP011933.1) was used for mapping and differential gene expression
1677 analysis. All non-coding RNAs and pseudogenes were removed before DESeq2 analysis. **Column**
1678 **A**: RefSeq locus tag. **Column B**: Locus tag. **Column C**: *L. interrogans* serovar Copenhageni strain
1679 Fiocruz L1-130 (accession numbers NC_005823.1 and NC_005824.1) orthologs identified using
1680 OrthoVenn 2.0 [202]. Dashes (-) indicate genes for which no clear ortholog was identified.
1681 **Column D**: Description of gene product, following genome annotation. **Columns E, F**: Clusters
1682 of Orthologous Group (COG) classifications based on MicroScope [187]. **Column G**: Fold-
1683 regulation of the corresponding gene based on RNA-Seq analysis by Zavala-Alvarado *et al.* [91,
1684 92] performed using the same strains cultivated *in vitro*. Positive and negative numbers indicate
1685 upregulation and repression, respectively, in the WT compared to the mutant strain. Dashes (-)
1686 indicate genes that are not differentially regulated at least 3-fold ($p \leq 0.05$) by PerRA *in vitro*.
1687 **Column H**: Fold-regulation determined using DESeq2 based on WT vs. *perRA* mutant RNA-Seq
1688 analysis using leptospires cultivated within DMCs. **Column I**: Type of regulation by PerRA in
1689 DMCs. Genes expressed at ≥3-fold higher/lower levels in the WT vs. mutant with a False-
1690 discovery rate-adjusted-p value (q) ≤0.05 were considered differentially expressed. "NO" indicates
1691 genes that are not regulated by PerRA in DMCs; "Up" indicates genes upregulated by PerRA in
1692 DMCs (expressed at lower levels in the mutant vs. WT); "Down" indicates genes downregulated

1693 by PerRA in DMCs (expressed at higher levels in the mutant vs WT). **Columns J-O**: Number of
1694 mapped reads per gene for each one of the three biological replicates per strain. **Columns P-AC**:
1695 Output from DESeq2 for WT vs. *perRA* mutant strains cultivated in DMCs (3 biological replicates
1696 per strain). **Column P**: Mean DESeq2 values for each gene. **Column Q**: Log_2-fold change in gene
1697 expression. **Column R**: Power function transformation of log_2-fold change. **Column S**: Fold
1698 regulation. **Column T-W**. Statistical analysis of differential gene expression including standard
1699 error estimate for the log_2-fold change estimate (lfcSE, column T) and adjusted *p*-value (W).
1700 **Columns X-AC**: Normalized copy numbers per gene (3 biological replicates per strain).
1701
1702 **Table S3. Comparative RNA-Seq data for *L. interrogans* sv. Manilae L495 wild-type and**
1703 ***perRB* strains cultivated in dialysis membrane chambers (DMCs)**. The genome sequence of *L.*
1704 *interrogans* sv. Manilae strain UP-MMC-NIID LP (accession numbers NZ_CP011931.1,
1705 NZ_CP011932.1 and NZ_CP011933.1) was used for mapping and differential gene expression
1706 analysis. All non-coding RNAs and pseudogenes were removed before DESeq2 analysis. **Column**
1707 **A**: RefSeq locus tag. **Column B**: Locus tag. **Column C**: *L. interrogans* serovar Copenhageni strain
1708 Fiocruz L1-130 (accession numbers NC_005823.1 and NC_005824.1) orthologs identified using
1709 OrthoVenn 2.0 [202]. Dashes (-) indicate genes for which no clear ortholog was identified.
1710 **Column D**: Description of gene product, following genome annotation. **Columns E, F**: Clusters
1711 of Orthologous Group (COG) classifications based on MicroScope [187]. **Column G**: Regulation
1712 of the corresponding gene based on RNA-Seq analysis by Zavala-Alvarado *et al.* [91, 92]
1713 performed using the same strains cultivated *in vitro*. Positive and negative numbers indicate
1714 upregulation and repression, respectively, in the WT compared to the mutant strain. Dashes (-)
1715 indicate genes that are not differentially regulated at least 3-fold ($p \leq 0.05$) by PerRB *in vitro*.

1716 **Column H**: Fold-regulation determined using DESeq2 based on WT vs. *perRB* mutant RNA-Seq
1717 analysis using leptospires cultivated within DMCs. **Column I**: Type of regulation by PerRB in
1718 DMCs. Genes expressed at ≥3-fold higher/lower levels in the WT vs. mutant with a False-
1719 discovery rate-adjusted-*p* value (*q*) ≤0.05 were considered differentially expressed. "NO" indicates
1720 genes that are not regulated by PerRB in DMCs; "Up" indicates genes upregulated by PerRB in
1721 DMCs (expressed at lower levels in the mutant vs. WT); "Down" indicates genes downregulated
1722 by PerRB in DMCs (expressed at higher levels in the mutant vs. WT). **Columns J-O**: Number of
1723 mapped reads per gene for each one of the three biological replicates per strain. **Columns P-AC**:
1724 Output from DESeq2 for WT vs. *perRB* mutant strains cultivated in DMCs (3 biological replicates
1725 per strain). **Column P**: Mean DESeq2 values for each gene. **Column Q**: Log_2 fold change in gene
1726 expression. **Column R**: Power function transformation of log_2-fold change. **Column S**: Fold
1727 regulation. **Column T-W**. Statistical analysis of differential gene expression including standard
1728 error estimate for the log_2-fold change estimate (lfcSE, column T) and adjusted *p*-value (W).
1729 **Columns X-AC**: Normalized copy numbers per gene (3 biological replicates per strain).
1730
1731 **Table S4. Comparative RNA-Seq data for *L. interrogans* sv. Manilae L495 wild-type and**
1732 ***perRA/B* strains cultivated in dialysis membrane chambers (DMCs)**. The genome sequence of
1733 *L. interrogans* sv. Manilae strain UP-MMC-NIID LP (accession numbers NZ_CP011931.1,
1734 NZ_CP011932.1 and NZ_CP011933.1) was used for mapping and differential gene expression
1735 analysis. All non-coding RNAs and pseudogenes were removed before DESeq2 analysis. **Column**
1736 **A**: RefSeq locus tag. **Column B**: Locus tag. **Column C**: *L. interrogans* sv. Copenhageni strain
1737 Fiocruz L1-130 (accession numbers NC_005823.1 and NC_005824.1) orthologs identified using
1738 OrthoVenn 2.0. [202]. Dashes (-) indicate genes for which no clear ortholog was identified.

1739 **Column D**: Description of gene product, following genome annotation. **Column E**. Identification
1740 of conserved domain(s) within the corresponding gene product based on search of the Interpro
1741 database [190, 201]. The domain identification for each gene is followed by description; [D]
1742 indicates a domain; [F] indicates a protein family; [H] indicates a homologous superfamily.
1743 **Column F**. Uniprot entry for the orthologous gene in *L. interrogans* sv. Copenhageni strain
1744 Fiocruz L1-130 genome. **Columns G, H**: Clusters of Orthologous Group (COG) classifications
1745 based on MicroScope [187]. **Column I**: Regulation of the corresponding gene based on RNA-Seq
1746 analysis by Zavala-Alvarado *et al.* [91, 92] performed using the same strains cultivated *in vitro*.
1747 Positive and negative numbers indicate upregulation and repression, respectively, in the WT
1748 compared to mutant strain. Dashes (-) indicate genes that are not differentially regulated at least 3-
1749 fold ($p \leq 0.05$) by PerRA/B *in vitro*. **Column J**: Fold-regulation determined using DESeq2 based
1750 on WT vs. the *perRA/B* double mutant RNA-Seq analysis using leptospires cultivated within
1751 DMCs. **Column K**: Type of regulation by PerRA/B in DMCs. Genes expressed at ≥3-fold
1752 higher/lower levels in the WT versus mutant with a False-discovery rate-adjusted-p value (q) ≤ 0.05
1753 were considered differentially expressed. "NO" indicates genes that are not regulated by PerRA/B
1754 in DMCs; "Up" indicates genes upregulated by PerRA/B in DMCs (expressed at lower levels in
1755 the mutant vs. WT); "Down" indicates genes downregulated by PerRA/B in DMCs (expressed at
1756 higher levels in the mutant vs. WT). **Column L**: Behavior of the corresponding gene in the WT
1757 vs. *perRA* (A) and *perRB* (B) mutants by RNA-Seq using *L. interrogans* cultivated in DMCs. "Up"
1758 and "Down", respectively, are used to significant (>3-fold, $q<0.05$) upregulation or repression of
1759 the gene in the WT compared to the mutant. **Columns M-R**: Number of mapped reads per gene
1760 for each one of the three biological replicates per strain. **Columns S-AF**: Output from DESeq2 for
1761 WT vs. *perRA/B* mutant strains cultivated in DMCs (3 biological replicates per strain). **Column**

1762 **S**: Mean DESeq2 values for each gene. **Column T**: Log_2 fold change in gene expression. **Column**

1763 **U**: Power function transformation of Log_2 fold change. **Column V**: Fold regulation. **Column W-**

1764 **Z**. Statistical analysis of differential gene expression including standard error estimate for the log2

1765 fold change estimate (lfcSE, column T) and adjusted *p*-value (W). **Columns AA-AF**: Normalized

1766 copy numbers per gene (3 biological replicates per strain).

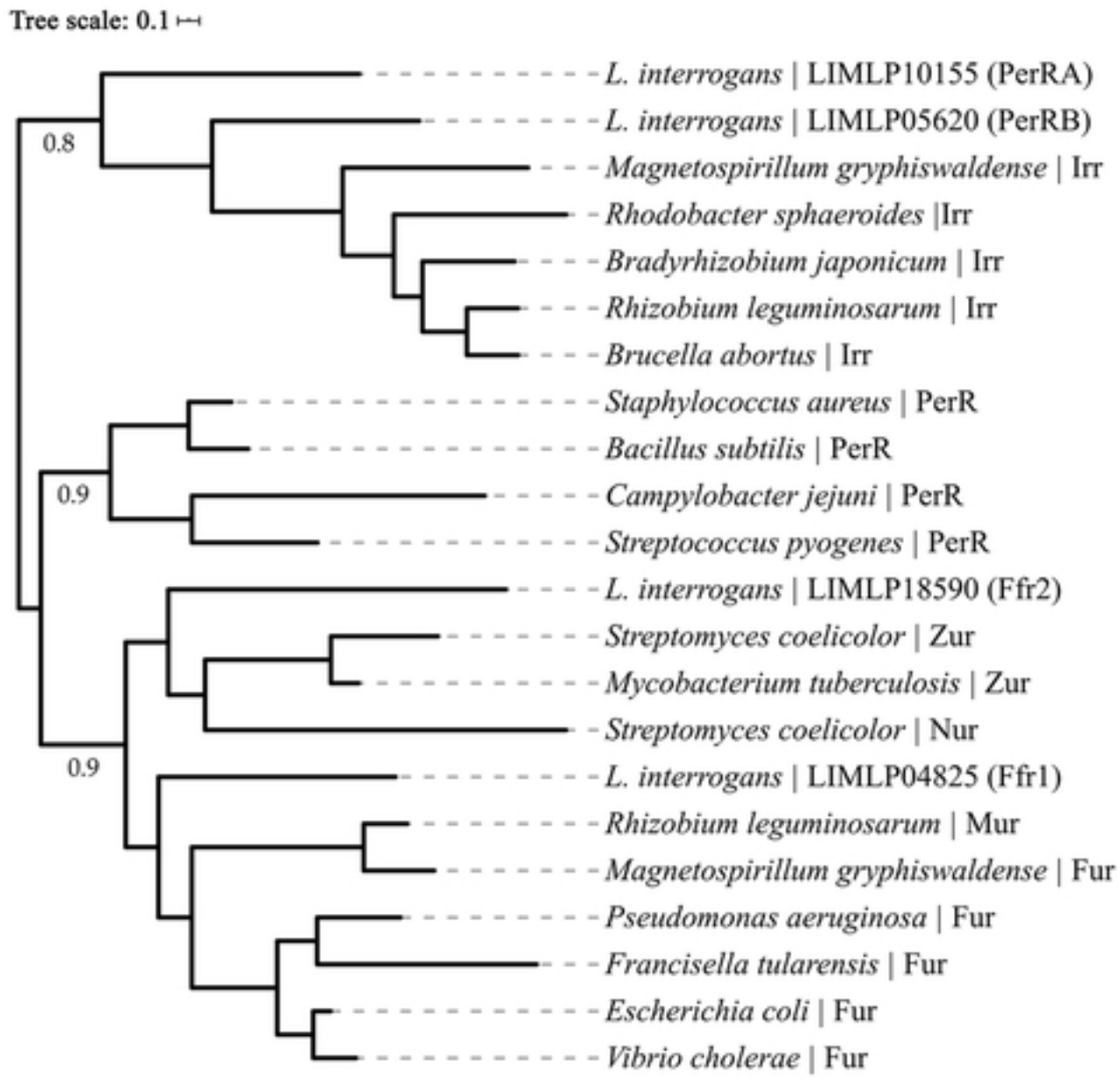
1767

1768 **Table S5. Bacterial strains used in these studies.**

1769

1770 **Table S6. Oligonucleotide primers used in these studies.**

A


Tree scale: 0.1
0.8
0.9
0.9
L. interrogans | LIMLP10155 (PerRA)
L. interrogans | LIMLP05620 (PerRB)
Magnetospirillum gryphiswaldense | Irr
Rhodobacter sphaeroides |Irr
Bradyrhizobium japonicum | Irr
Rhizobium leguminosarum | Irr
Brucella abortus | Irr
Staphylococcus aureus | PerR
Bacillus subtilis | PerR
Campylobacter jejuni | PerR
Streptococcus pyogenes | PerR
L. interrogans | LIMLP18590 (Ffr2)
Streptomyces coelicolor | Zur
Mycobacterium tuberculosis | Zur
Streptomyces coelicolor | Nur
L. interrogans | LIMLP04825 (Ffr1)
Rhizobium leguminosarum | Mur
Magnetospirillum gryphiswaldense | Fur
Pseudomonas aeruginosa | Fur
Francisella tularensis | Fur
Escherichia coli | Fur
Vibrio cholerae | Fur

B

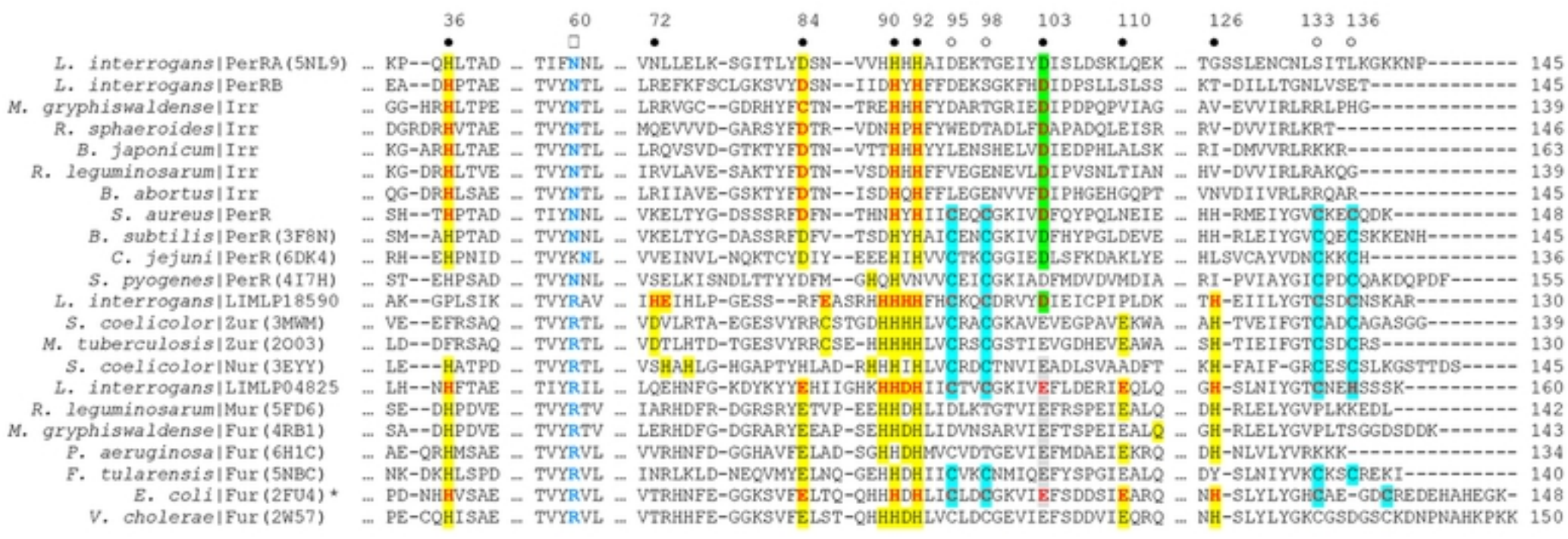

36 60 72 84 90 92 95 98 103 110 126 133 136
L. interrogans|PerRA(5NL9) … KP--QHLTAD … TIFNNL … VNLLELK-SGITLYDSN--VVHHHHAIDEKTGEIYDISLDSKLQEK … TGSSLENCNLSITLKGKKNP-------- 145
L. interrogans|PerRB … EA--DHPTAE … TVYNTL … LREFKFSCLGKSVYDSN--IIDHYHFFDEKSGKFHDIDPSLLSLSS … KT-DILLTGNLVSET---------- 145
M. gryphiswaldense|Irr … GG-HRHLTPE … TVYNTL … LRRVGC--GDRHYFCTN--TREHHHFYDARTGRIEDIPDPQPVIAG … AV-EVVIRLRRLPHG---------- 139
R. sphaeroides|Irr … DGRDRHVTAE … TVYNTL … MQEVVVD-GARSYFDTR--VDNHPHFYWEDTADLFDAPADQLEISR … RV-DVVIRLKRT---------- 146
B. japonicum|Irr … KG-ARHLTAE … TVYNTL … LRQVSVD-GTKTYFDTN--VTTHHHYYLENSHELVDIEDPHLALSK … RI-DMVVRLRKKR---------- 163
R. leguminosarum|Irr … KG-DRHLTVE … TVYNTL … IRVLAVE-SAKTYFDTN--VSDHHHFFVEGENEVLDIPVSNLTIAN … HV-DVVIRLRAKQG---------- 139
B. abortus|Irr … QG-DRHLSAE … TVYNTL … LRIIAVE-GSKTYFDTN--ISDHQHFFLEGENVVFDIPHGEHGQPT … VNVDIIVRLRRQAR---------- 145
S. aureus|PerR … SH--THPTAD … TIYNNL … VKELTYG-DSSSRFDFN--THNHYHIICEQCGKIVDFQYPQLNEIE … HH-RMEIYGVCKECQDK---------- 148
B. subtilis|PerR(3F8N) … SM--AHPTAD … TVYNNL … VKELTYG-DASSRFDFV--TSDHYHAICENCGKIVDFHYPGLDEVE … HH-RLEIYGVCQECSKKENH-------- 145
C. jejuni|PerR(6DK4) … RH--EHPNID … TVYKNL … VVEINVL-NQKTCYDIY--EEEHIHVVCTKCGGIEDLSFKDAKLYE … HLSVCAYVDNCKKCH---------- 136
S. pyogenes|PerR(4I7H) … ST--EHPSAD … TVYNNL … VSELKISNDLTTYYDFM--GHQHVNVVCEICGKIADFMDVDVMDIA … RI-PVIAYGICPDCQAKDQPDF------ 155
L. interrogans|LIMLP18590 … AK--GPLSIK … TVYRAV … IHEIHLP-GESS--RFEASRHHHHHFHCKQCDRVYDIEICPIPLDK … TH-EIILYGTCSDCNSKAR--------- 130
S. coelicolor|Zur(3MWM) … VE--EFRSAQ … TVYRTL … VDVLRTA-EGESVYRRCSTGDHHHHLVCRACGKAVEVEGPAVEKWA … AH-TVEIFGTCADCAGASGG-------- 139
M. tuberculosis|Zur(2O03) … LD--DFRSAQ … TVYRTL … VDTLHTD-TGESVYRRCSE-HHHHHLVCRSCGSTIEVGDHEVEAWA … SH-TIEIFGTCSDCRS---------- 130
S. coelicolor|Nur(3EYY) … LE---HATPD … TVYRTL … VSHAHLG-HGAPTYHLAD-RHHHIHLVCRDCTNVIEADLSVAADFT … KH-FAIF-GRCESCSLKGSTTDS----- 145
L. interrogans|LIMLP04825 … LH--NHFTAE … TIYRIL … LQEHNFG-KDYKYYEHIIGHKHHDHIICTVCGKIVEFLDERIEQLQ … GH-SLNIYGTCNEHSSSK---------- 160
R. leguminosarum|Mur(5FD6) … SE--DHPDVE … TVYRTV … IARHDFR-DGRSRYETVP-EEHHDHLIDLKTGTVIEFRSPEIEALQ … DH-RLELYGVPLKKEDL---------- 142
M. gryphiswaldense|Fur(4RB1) … SA--DHPDVE … TVYRTV … LERHDFG-DGRARYEEAP-SEHHDHLIDVNSARVIEFTSPEIEALQ … GH-RLELYGVPLTSGGDSDDK------- 143
P. aeruginosa|Fur(6H1C) … AE-QRHMSAE … TVYRVL … VVRHNFD-GGHAVFELAD-SGHHDHMVCVDTGEVIEFMDAEIEKRQ … DH-NLVLYVRKKK---------- 134
F. tularensis|Fur(5NBC) … NK-DKHLSPD … TVYRVL … INRLKLD-NEQVMYELNQ-GEHHDHIICVKCNMIQEFYSPGIEALQ … DY-SLNIYVKCKSCREKI---------- 140
E. coli|Fur(2FU4)* … PD-NHHVSAE … TVYRVL … VTRHNFE-GGKSVFELTQ-QHHHDHLICLDCGKVIEFSDDSIEARQ … NH-SLYLYGHCAE-GDCREDEHAHEGK- 148
V. cholerae|Fur(2W57) … PE-CQHISAE … TVYRVL … VTRHHFE-GGKSVFELST-QHHHDHLVCLDCGEVIEFSDDVIEQRQ … NH-SLYLYGKCGSDGSCKDNPNAHKPKK 150

Figure 1

A

Strain	Clade	PerRA	PerRB	PerRC	Ffr1	Ffr2	Ffr3
L. interrogans	P1	LIMLP10155	LIMLP05620	-	LIMLP04825	LIMLP18590	-
L. adleri	P1	CH380_01660	CH380_02860	-	CH380_17490	CH380_14590	-
L. kirschneri	P1	LEP1GSC008_0476	LEP1GSC008_1438	-	LEP1GSC008_0367	LEP1GSC008_3669	-
L. alstonii	P1	LEP1GSC193_0427	LEP1GSC193_0507	-	LEP1GSC193_0044	LEP1GSC193_1728	-
L. santarosai	P1	LSS_20032	LSS_01772	-	LSS_17135	-	-
L. borgpetersoni	P1	LEP1GSC103_3665	LEP1GSC103_3950	-	LEP1GSC103_0991	-	-
L. alexanderi	P1	LEP1GSC062_0896	LEP1GSC062_1140	-	LEP1GSC062_0662	-	-
L. wolfii	P2	LEP1GSC061_1520	LEP1GSC061_1439	-	LEP1GSC061_0663	LEP1GSC061_2348	-
L. liscerasiae	P2	-	LEP1GSC185_0014	-	LEP1GSC185_0212	LEP1GSC185_3622	-
L. inadai	P2	-	LEP1GSC047_4095	-	LEP1GSC047_2424	LEP1GSC047_0399	-
L.fainei	P2	-	LEP1GSC058_1675	-	LEP1GSC058_1243	LEP1GSC058_0965	-
L. biflexa	S1	LEPBI_I2461	-	LEPBI_I2152	LEPBI_I2330	-	LEPBI_I2849
L. meyeri	S1	CLV96_3482	-	CLV96_0017	CLV96_3352	-	CLV96_2858
L. terpstrae	S1	LEP1GSC203_0849	-	LEP1GSC203_1114	LEP1GSC203_0981	-	LEP1GSC203_3918
L. vanthielii	S1	LEP1GSC199_1434	-	LEP1GSC199_2345	LEP1GSC199_0490	-	LEP1GSC199_2661
L. ryugenii	S2	LPTSP4_14140	-	LPTSP4_11630	LPTSP4_12510	-	LPTSP4_33290
L. ilyithenensis	S2	EHS11_13520	-	EHS11_06550	EHS11_06315	-	EHS11_02435
L. idonii	S2	EHS15_06365	-	EHS15_05735	EHS15_16565	-	EHS15_08445

B

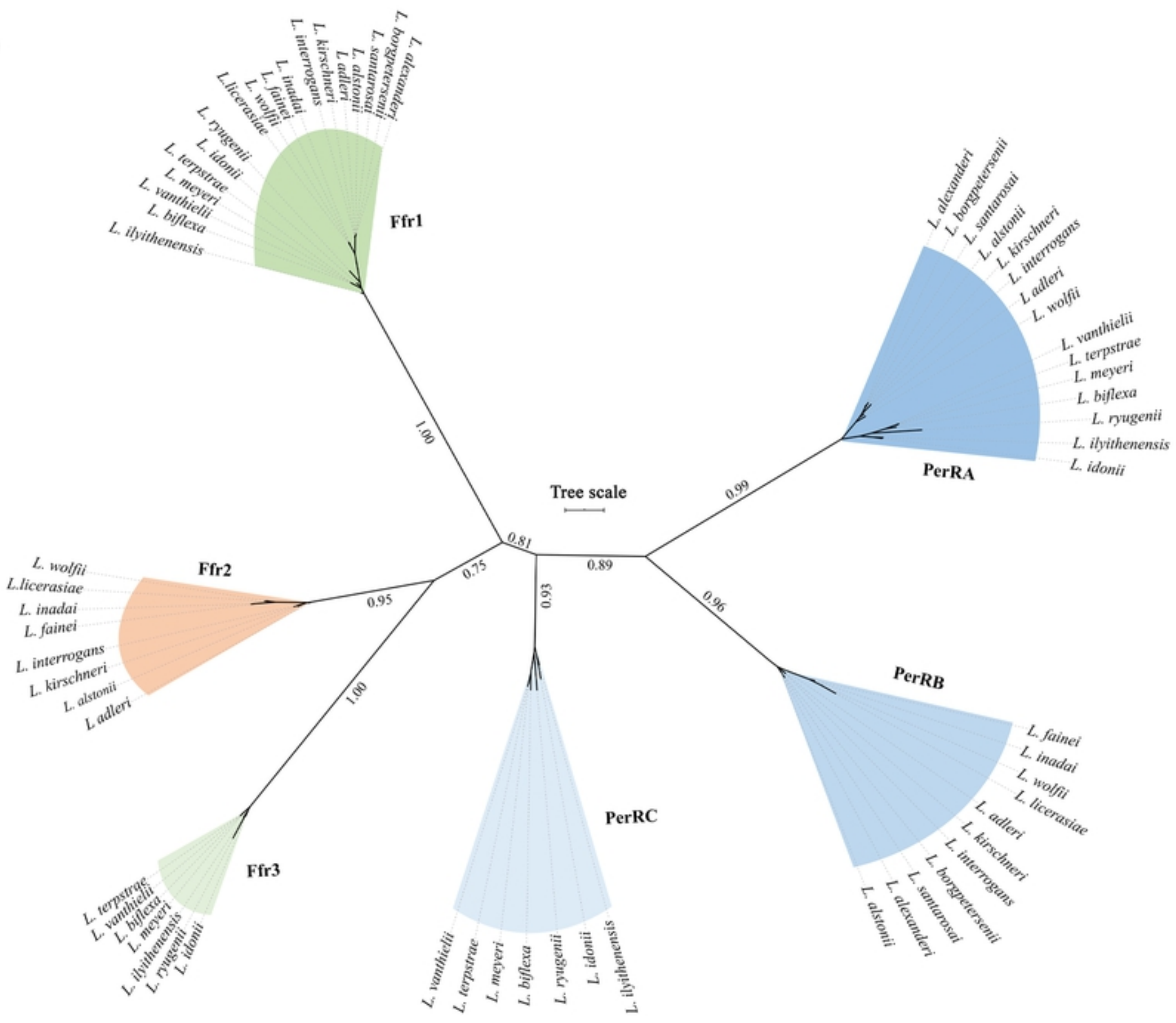
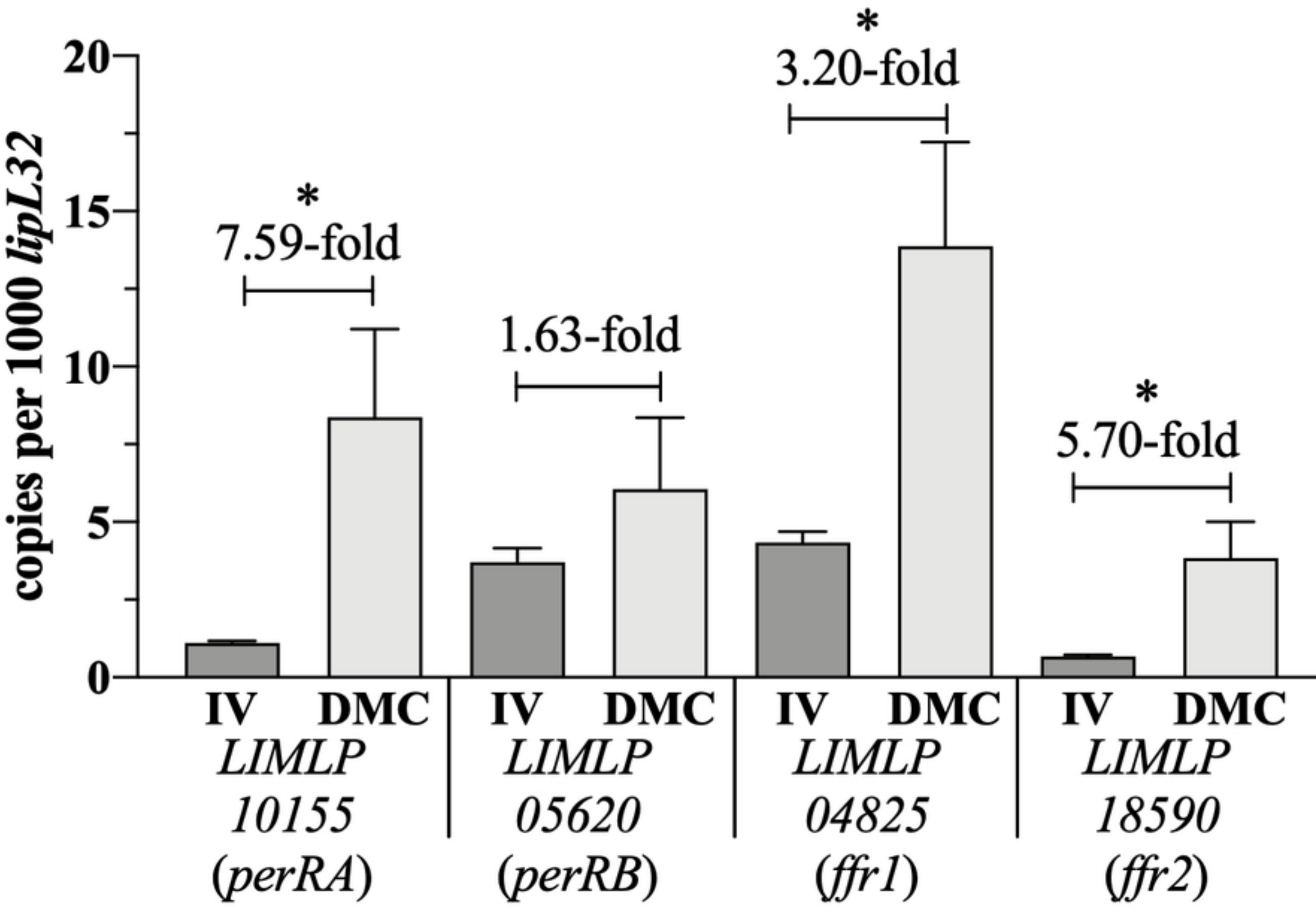


Figure 2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7.59-fold
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IV
DMC
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DMC
IV
DMC
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DMC
LIMLP 10155 (perRA)
LIMLP 05620 (perRB)
LIMLP 04825 (ffr1)
LIMLP 18590 (ffr2)

Figure 3

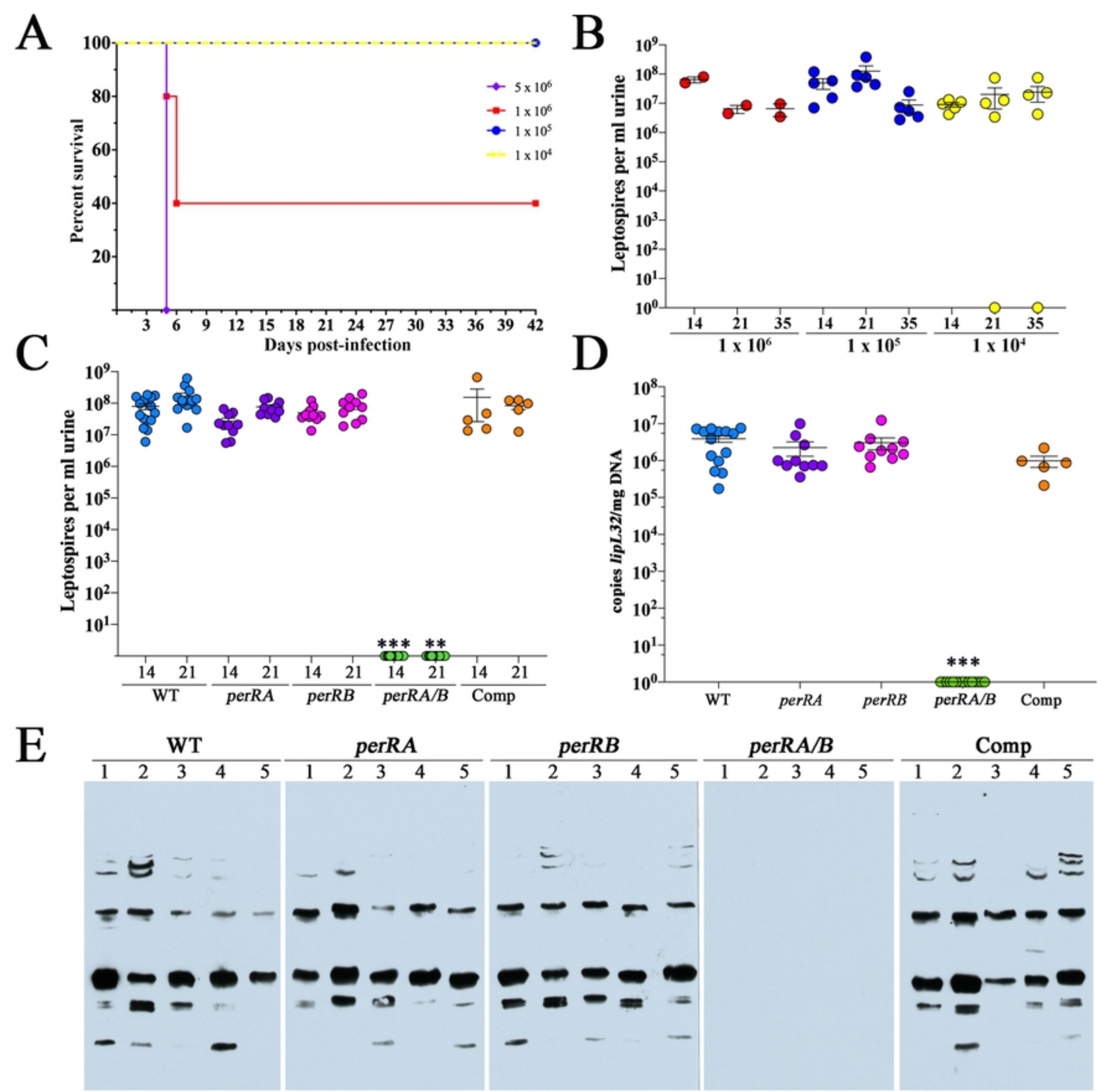

A
Percent survival
Days post-infection
5 x 10^6
1 x 10^6
1 x 10^5
1 x 10^4
B
Leptospires per ml urine
1 x 10^6
1 x 10^5
1 x 10^4
C
Leptospires per ml urine
WT
perRA
perRB
perRA/B
Comp
D
copies lipL32/mg DNA
E
WT
perRA
perRB
perRA/B
Comp

Figure 4

A

Locus tag	Fold-change in WT vs. *perRA*	*perRB*	*perRA/B*	
LIMLP05780	4.23	-1.19	1.05	Arc
LIMLP01845	3.00	1.17	1.29	PHA DBD
LIMLP10140	-3.94	-1.22	-1.40	PAS PAS PAS PAS HK
LIMLP11575	-3.79	-2.42	1.44	Kinase NTPase TPR GAF σ PP2C
LIMLP00900	-3.52	1.20	1.05	DBD

B

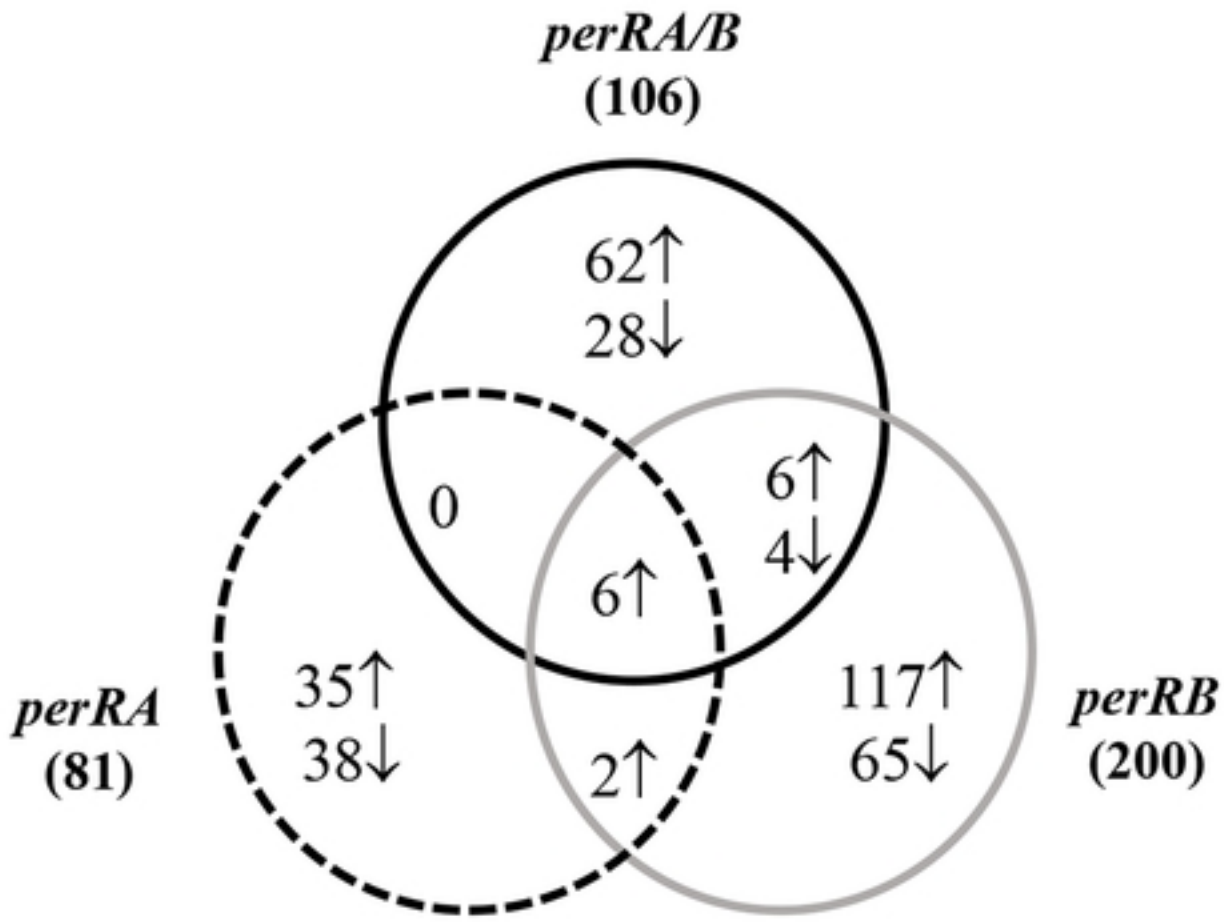
Locus tag	Fold-change in WT vs. *perRA*	*perRB*	*perRA/B*	
LIMLP16420	-2.83	8.25	1.45	Cro/C1-λ
LIMLP17475	-2.14	5.94	2.58	Rec
LIMLP06955	1.48	3.52	-1.65	PAS HK
LIMLP06340	-1.57	3.49	3.59	TM HK
LIMLP11010	-2.05	3.26	1.33	TMH x6 HK
LIMLP07225	-2.06	3.23	10.04	Crp cNBD Crp HTH
LIMLP14515	1.25	3.12	1.16	σ^{70} r2 σ^{70} r3/4
LIMLP06960	1.40	3.10	-1.43	PAS σ PP2C
LIMLP15900	1.07	3.10	2.18	HTH/ TetR TetR
LIMLP16725	2.56	3.07	2.22	TM HAMP HK
LIMLP16825	-1.34	3.06	2.89	TM HK
LIMLP04775	1.59	-4.83	-2.38	EAL
LIMLP10055	1.77	-3.76	1.22	Cro/C1-λ
LIMLP07895	1.53	-3.56	-1.30	ArsR
LIMLP14940	2.87	-3.53	-1.30	σ^{70} r2 σ^{70} r3/4
LIMLP11860	1.27	-3.17	-1.21	Rec
LIMLP04815	1.41	-3.11	-1.35	STAS

C

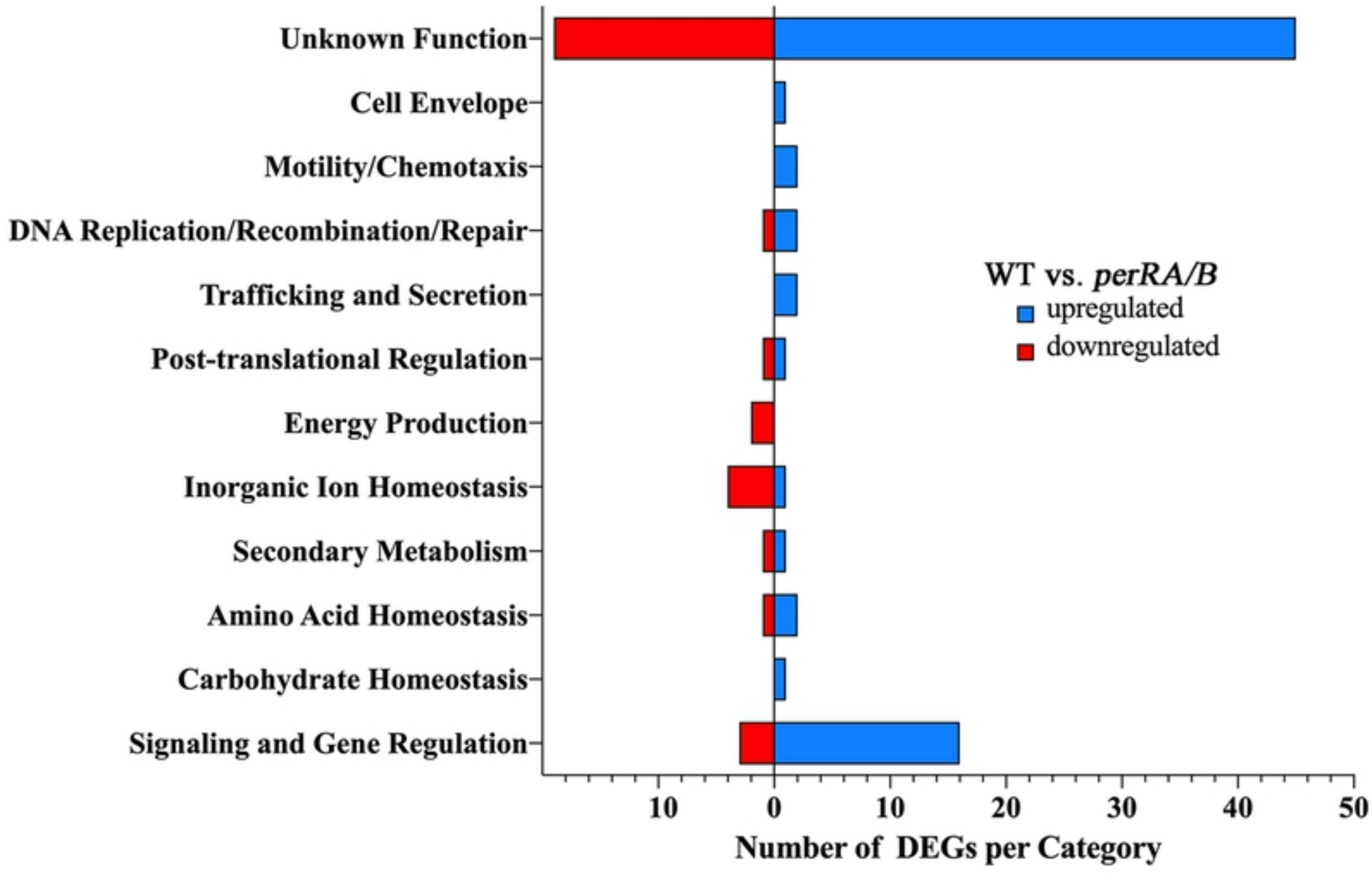
Locus tag	Fold-change in WT vs. *perRA*	*perRB*	*perRA/B*	
LIMLP02080	1.02	1.49	14.94	HTH/ TetR TetR
LIMLP07025	-2.52	2.05	12.18	HK
LIMLP08490	-2.03	-1.19	11.34	PAS HK Rec HK LvrA
LIMLP07225	-2.06	3.23	10.04	Crp cNBD Crp HTH
LIMLP08485	-1.34	-1.58	6.96	Rec HK LvrB
LIMLP07030	-1.91	1.17	6.47	Rec PAS HK
LIMLP04315	1.11	1.19	4.91	Cro/C1-λ
LIMLP00755	-1.74	2.11	4.29	DUF433
LIMLP06340	-1.57	3.49	3.59	TM HK
LIMLP00425	-1.21	2.20	3.45	HK*
LIMLP05840	-2.22	1.29	3.14	HK Rec
LIMLP06990	-1.29	1.01	3.09	PAS PAS PAS HK Rec Rec
LIMLP05830	-1.61	-1.21	3.04	PAS PAS GAF PAS HK
LIMLP19135	-1.28	1.92	-4.60	HTH/ TetR
LIMLP08975	1.25	-1.25	-3.02	BolA

Figure 5

A


perRA/B
(106)
62↑
28↓
0
6↑
4↓
6↑
perRA
(81)
35↑
38↓
2↑
117↑
65↓
perRB
(200)

B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Cell Envelope
Motility/Chemotaxis
DNA Replication/Recombination/Repair
Trafficking and Secretion
Post-translational Regulation
Energy Production
Inorganic Ion Homeostasis
Secondary Metabolism
Amino Acid Homeostasis
Carbohydrate Homeostasis
Signaling and Gene Regulation
WT vs. perRA/B
upregulated
downregulated
10
0
10
20
30
40
50
Number of DEGs per Category

Figure 6

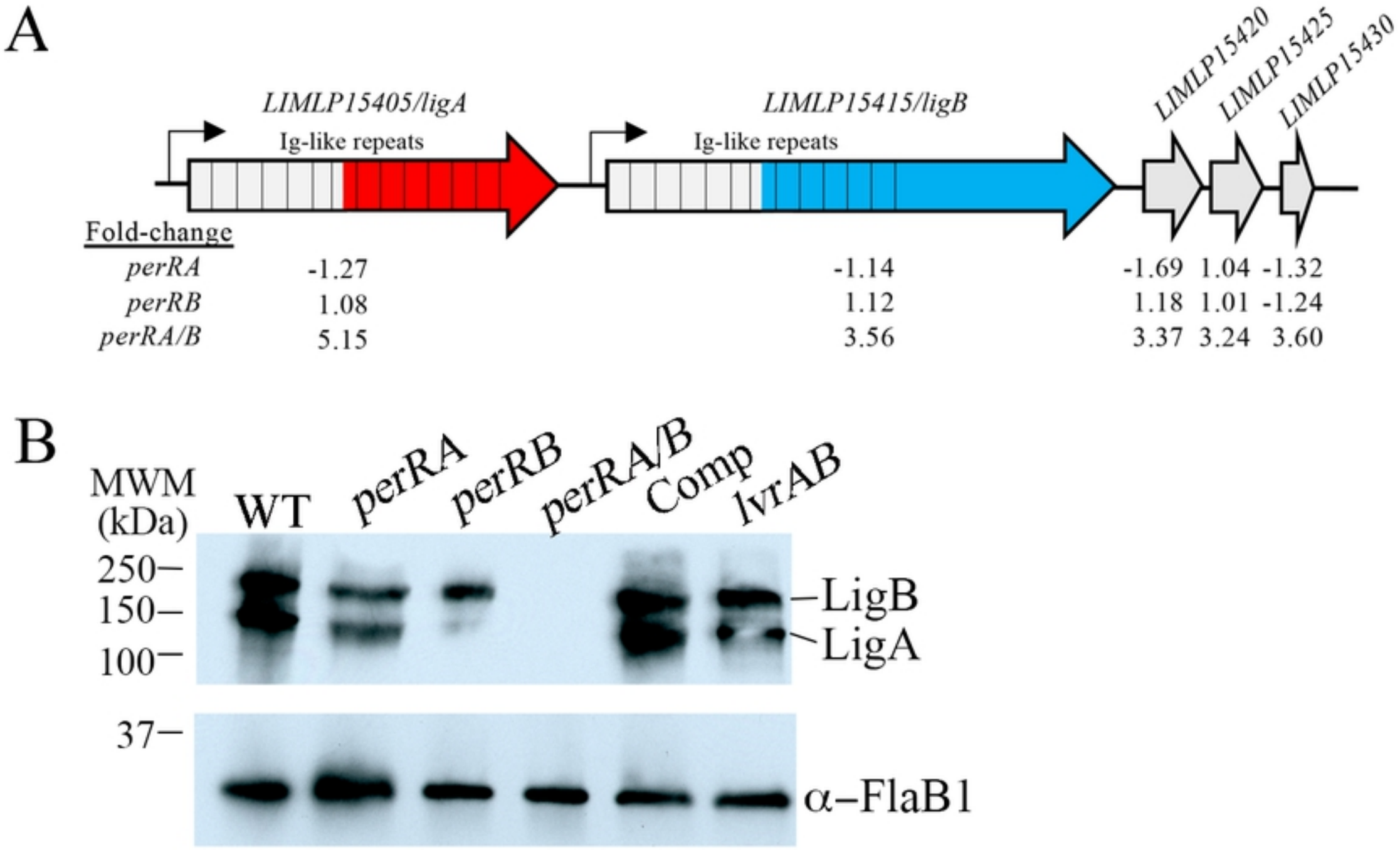
A
LIMLP15405/ligA
Ig-like repeats
LIMLP15415/ligB
Ig-like repeats
LIMLP15420
LIMLP15425
LIMLP15430
Fold-change
perRA -1.27 -1.14 -1.69 1.04 -1.32
perRB 1.08 1.12 1.18 1.01 -1.24
perRA/B 5.15 3.56 3.37 3.24 3.60
B
MWM (kDa)
WT
perRA
perRB
perRA/B
Comp
lvrAB
250
150
100
LigB
LigA
37
α–FlaB1

Figure 7

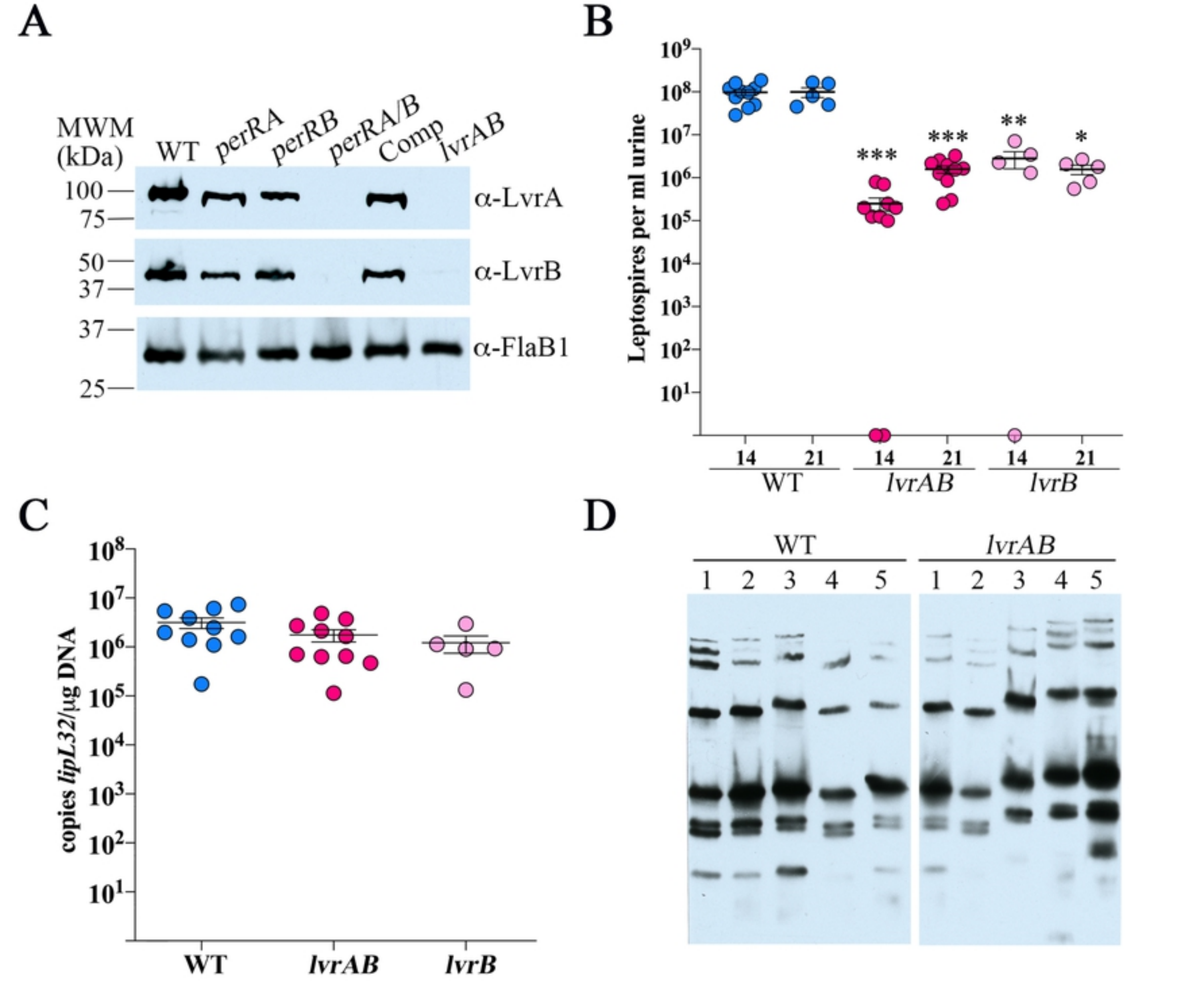
A
MWM (kDa)
WT
perRA
perRB
perRA/B
Comp
lvrAB
100
75
50
37
37
25
α-LvrA
α-LvrB
α-FlaB1
B
Leptospires per ml urine
14
21
14
21
14
21
WT
lvrAB
lvrB
C
copies lipL32/µg DNA
WT
lvrAB
lvrB
D
WT
lvrAB
1 2 3 4 5
1 2 3 4 5

Figure 8