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1 The oxidative stress response and virulence of pathogenic *Leptospira* are controlled by the
2 interplay of two peroxide stress regulators

3

4 Short title: Identification of a second PerR-like regulator in pathogenic *Leptospira*.

5

6

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25 stress; virulence; leptospirosis; RNASeq; non-coding RNAs; *Leptospira*; Spirochetes; PerR;
26 Fur

27

28 **Abstract**

29 Pathogenic *Leptospira* are the causative agents of leptospirosis, the most widespread
30 zoonotic infectious disease. Leptospirosis is a potentially severe and life-threatening emerging
31 disease with highest burden in sub-tropical areas and impoverish populations. Mechanisms
32 allowing pathogenic *Leptospira* to survive inside a host and induce acute leptospirosis are not
33 fully understood. The ability to resist deadly oxidants produced by the host during infection is
34 pivotal for *Leptospira* virulence. We have previously shown that genes encoding defenses
35 against oxidants in *L. interrogans* are repressed by PerRA (encoded by LIMLP_10155), a
36 peroxide stress regulator of the Fur family. In this study, we describe the identification and
37 characterization of another putative PerR-like regulator (LIMLP_05620) in *L. interrogans*.
38 Protein sequence and phylogenetic analyses indicated that LIMLP_05620 displayed all the
39 canonical PerR amino acid residues and is restricted to pathogenic *Leptospira* clades. We
40 therefore named this PerR-like regulator PerRB. In *L. interrogans*, the PerRB regulon is
41 distinct from that of PerRA. While a *perRA* mutant had a greater tolerance to peroxide,
42 inactivating *perRB* led to a higher tolerance to superoxide, suggesting that these two
43 regulators have a distinct function in the adaptation of *L. interrogans* to oxidative stress. The
44 concomitant inactivation of *perRA* and *perRB* resulted in a higher tolerance to both peroxide
45 and superoxide and, unlike the single mutants, to the loss of *Leptospira* virulence.
46 Interestingly, this correlated with major changes in gene and non-coding RNA expression,
47 only observed in the double *perRAperRB* mutant. Notably, several virulence-associated genes
48 (*clpB*, *ligA/B*, and *lvrAB*) were repressed. By obtaining the first double mutant in a pathogenic

49 *Leptospira* strain, our study has uncovered for the first time the interplay of two PerRs, not
50 only in the adaptation of *Leptospira* to oxidative stress, but also in their virulence and
51 pathogenicity, most likely through the transcriptional control of a complex regulatory
52 network.

53

54 **Author summary**

55 Leptospirosis is a widespread infectious disease responsible for over one million of
56 severe cases and 60 000 fatalities annually worldwide. This neglected and emerging disease
57 has a worldwide distribution, but it mostly affects populations from developing countries in
58 sub-tropical areas. The causative agents of leptospirosis are pathogenic bacterial *Leptospira*
59 spp. There is a considerable deficit in our knowledge of these atypical bacteria, including their
60 virulence mechanisms. In addition to the *Leptospira* PerRA regulator that represses defenses
61 against peroxide, we have identified and characterized a second PerR regulator in pathogenic
62 *Leptospira* species (PerRB) that participates in *Leptospira* tolerance to superoxide.
63 Phenotypic and transcriptomic analyses of single PerRA and PerRB mutants suggest that the
64 two PerRs fulfill distinct functions in the adaptation to oxidative stress. However,
65 concomitant inactivation of PerRA and PerRB resulted in a higher tolerance to both peroxide
66 and superoxide, but to a loss virulence.

67 The absence of the two PerR regulators resulted in global and major changes in the
68 transcriptional profile, including a dramatic decrease of several virulence factor expression.
69 Our study has demonstrated that PerRA and PerRB cooperate to orchestrate a complex
70 regulatory network involved in *Leptospira* virulence.

72 **Introduction**

73

74 Pathogenic *Leptospira* spp. are aerobic Gram-negative bacteria of the spirochetal
75 phylum that are the causative agents of leptospirosis, the most widespread zoonosis [1]. More
76 than one million cases of leptospirosis are currently reported annually in the world, with 10%
77 of mortality [2]. This disease is considered as a health threat among impoverished populations
78 in developing countries under tropical areas [2], but the number of reported cases of
79 leptospirosis are also on the rise in developed countries under temperate climates [3]. Rodents
80 are asymptomatic reservoir for leptospires as the bacteria colonize the proximal renal tubules
81 of these animals. Leptospires are shed in the environment through the urine of infected
82 animals and leptospirosis is transmitted to other animal species and humans mostly by
83 exposure to contaminated soils and water. *Leptospira* penetrate an organism through abraded
84 skins and mucous membranes, subsequently disseminate within the bloodstream and rapidly
85 spread to multiple tissues and organs (including kidney, liver, lungs). Clinical manifestations
86 range from a mild flu-like febrile state to more severe and fatal cases leading to hemorrhages
87 and multiple organ failure. Because of the lack of efficient tools for genetic manipulation of
88 *Leptospira* spp. and their fastidious growth in the laboratory conditions, our understanding of
89 the mechanism of pathogenicity and virulence as well as the basic biology of these pathogens
90 have been greatly hampered [4,5].

91 During their life cycle, most bacteria will be exposed to reactive oxidative species (ROS),
92 such as superoxide anion ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2), that are produced
93 endogenously or encountered in the environment [6]. The superoxide is formed during the
94 aerobic respiratory chain upon the reduction of the dioxygen (O_2). Dismutation of superoxide
95 will give rise to H_2O_2 which, in turn, can react with ferrous iron to generate the highly
96 reactive hydroxyl radicals ($\cdot\text{OH}$) through the Fenton reaction. These ROS are also produced

97 together with hypochlorous acid (HOCl), and nitric oxide anion ($\cdot\text{NO}$) as powerful and
98 efficient weapons by eukaryotic innate immune response upon infection by pathogenic
99 bacteria [7]. ROS cause oxidative damage to cellular components (proteins, DNA and lipids)
100 and this would result in bacterial death if bacteria had not developed scavenging enzymes to
101 counteract the deadly effect of ROS, including catalase, peroxidases and superoxide
102 dismutase or reductase (SOD, SOR). Indeed, in response to any increase in ROS
103 concentration, as occurred during environmental oxidative stress or when infecting a host,
104 bacteria trigger an adaptive response allowing the fine-tuning of scavenging enzyme synthesis
105 rate that appropriately adapts the defenses against ROS to the oxidant assault. ROS
106 production is increased upon infection by *Leptospira* [8] and with the development of severe
107 leptospirosis in patients and infected animals [9,10]. In fact, the ability to detoxify H_2O_2 is
108 essential for *Leptospira* virulence as inactivation of the catalase-encoding gene led to
109 virulence attenuation in *L. interrogans* [11].

110 The oxidative stress adaptive response in bacteria is controlled by specialized ROS sensing
111 transcription factors. OxyR and PerR are the main peroxide-sensing regulators found in
112 Gram-negative and Gram-positive bacteria, respectively [12]. OxyR and PerR are functional
113 equivalents but they do control expression of peroxide scavenging enzymes by different
114 mechanisms. OxyR is a transcriptional regulator from the LysR family. Tetrameric OxyR
115 binds promoters and activates the expression of genes coding for catalase and peroxidases
116 through the oxidation of its H_2O_2 sensing cysteine residue [13,14]. PerR belongs to the Fur
117 (Ferric uptake regulator) transcriptional factor family. It is a metalloprotein that binds DNA in
118 the presence of iron or manganese and represses the expression of catalase and peroxidases
119 [15–17]. In the presence of peroxide, PerR is oxidized on the histidine residues participating
120 in iron coordination, PerR dissociates from DNA and peroxide-scavenging enzyme repression
121 is alleviated [18,19]. In Gram-negative bacteria, SOD expression is controlled by the SoxRS

122 system [20,21] by a redox-sensing mechanism and oxidation of a Fe-S cluster [22]. In Gram-
123 positive bacteria, SOD expression regulation probably involves the alternative Sigma Factor
124 SigB among other mechanisms [23]. Pathogenic *Leptospira* spp. are among the few examples
125 of Gram-negative bacteria where the expression of peroxide-scavenging enzymes is regulated
126 by a PerR (encoded by LIMLP_10155/LIC12034/LA1857) and not an OxyR [24]. Moreover,
127 their genomes do not contain any ORF encoding a SOD and they do not exhibit canonical
128 SOD activity [25,26].

129 We have very recently characterized the transcriptional response to hydrogen peroxide in the
130 pathogen *L. interrogans* and shown that these bacteria respond to sublethal H₂O₂
131 concentration by increasing the expression of catalase and of two peroxidases
132 (Alkylperoxiredoxin (AhpC) and Cytochrome C peroxidase (CCP)) [27]. When *Leptospira*
133 were exposed to deadly H₂O₂ concentration, additional enzymes with a putative role as
134 antioxidants and/or in repair of oxidized cysteines in proteins were up-regulated, including
135 several thiol oxidoreductases (thioredoxin, glutaredoxin, DsbD, and Bcp-like proteins) [27].
136 Several genes of the LexA regulon (*recA*, *recN*, *dinP*) and other genes with putative role in
137 DNA repair (*mutS*, *radC*) had a higher expression as well as genes encoding for canonical
138 chaperones (*dnaK/dnaJ/grpE*, *groES/EL*, and *hsp15/20*) [27]. Only genes coding for the
139 catalase and peroxidases were under the control of PerR and our study has revealed a complex
140 regulatory network independent of PerR involving other transcriptional regulators, sigma
141 factors, two component systems and non-coding RNAs [27]. During the course of this study,
142 we noticed that an ORF encoding a Fur-like regulator (LIMLP_05620/LIC11158/LA2887)
143 was up-regulated when *Leptospira* were exposed to a deadly concentration of H₂O₂. Here, we
144 report the functional characterization of this pathogenic *Leptospira*-specific Fur-like regulator
145 in the adaptation of *Leptospira* to oxidative stress. Phenotypic analyses of a mutant
146 inactivated in LIMLP_05620 indicates a role of this Fur-like regulator in *Leptospira* tolerance

147 to superoxide. This, together with the presence of the canonical amino acid residues of the
148 PerR DNA binding helix and H₂O₂ sensitivity, strongly suggest that LIMLP_05620 encodes a
149 second PerR-like regulator in pathogenic *Leptospira* species. By obtaining a double mutant
150 where LIMLP_05620 and *perR* (LIMLP_10155) are concomitantly inactivated, we have also
151 investigated the interplay between these two PerR-like regulators in the adaptation to
152 oxidative stress and virulence of *L. interrogans*. We have demonstrated a cooperation of
153 LIMLP_05620 with PerR in controlling several *Leptospira* virulence-associated genes,
154 including *ligA*, *ligB*, *lvrAB*, and *clpB*, perhaps illustrating the importance of functional
155 redundancy in pathogenic *Leptospira* virulence and pathogenicity.

157 **Results**

158

159 **Identification of an ORF that encodes a novel putative PerR regulator in pathogenic** 160 ***Leptospira* species.**

161 Regulators of the Fur family are homodimeric metalloproteins with a two-domain
162 organization composed of an amino-terminal DNA binding domain and a carboxy-terminal
163 dimerization domain (Fig 1A). The DNA binding domain contains a winged helix-turn-helix
164 (HTH) DNA binding motif (H2-H4, in Fig1A) where DNA binding is mediated by the H4
165 helix. The dimerization domain consists of an α/β domain. The regulatory iron that controls
166 DNA binding and dissociation is coordinated by histidine, aspartate, and glutamate residues
167 located in a loop at the hinge of the amino and carboxy-terminal domains. Most of Fur-like
168 regulators also coordinate a structural metal (zinc) through 2 cysteinate motifs (CxxC, where
169 x designates any AA). This structural metal allows correct folding and dimeric assembly of
170 the regulator.

171 Due to a great conservation in protein folding, metal coordination and similarity in the metal-
172 induced conformational switch controlling DNA binding, it is difficult to distinguish
173 members of the Fur family on the sole basis of their primary sequence. However, in *B.*
174 *subtilis*, a single amino acid residue in the H4 helix of the amino-terminal DNA binding
175 domain (N61 and R61 for *B. subtilis* PerR and Fur, respectively) allows PerR and Fur to
176 discriminate between their respective DNA sequence targets (PerR and Fur boxes,
177 respectively) [28] (Fig 1B). In addition, D104 residue in the carboxy-terminal domain is
178 pivotal in the PerR sensitivity to H₂O₂. The corresponding residue in Fur is a glutamate and
179 mutating this residue in aspartate leads to H₂O₂ sensitivity [29] (Fig 1B). Therefore, N61 and
180 D104 both allow to differentiate a PerR from a Fur in *B. subtilis* and the presence of these

181 canonical amino acid residues in PerR sequences is well-conserved in other bacterial species
182 [28,29].

183 *L. interrogans* genome encodes 4 ORFs that share homology with regulators of the Fur
184 family. Among those *Leptospira* Fur-like regulators, LIMLP_10155 is the only *Leptospira*
185 Fur-like regulator that is functionally and structurally characterized. It encodes a PerR that
186 represses genes encoding defenses against peroxide, including catalase and other peroxidases
187 [24,27,30]. Our previous structural characterization of the PerR from *L. interrogans* indicates
188 that this member of the Fur family lacks a structural metal binding site despite a correct
189 folding and functional dimeric assembly [30].

190 Sequence alignment of these ORFs with the Fur and PerR from *B. subtilis* shows a good
191 conservation of the two-domain organization and residues involved in the regulatory metal
192 coordination (Fig 1B). Interestingly, two of the 4 Fur-like ORFs of *L. interrogans*,
193 LIMLP_10155 (LIC12034/LA1857 encoding a PerR) and LIMLP_05620
194 (LIC11158/LA2887), exhibit the canonical asparagine (N60 and N68, respectively) and
195 aspartate (D103 and D112, respectively) residues of a typical PerR. The third ORF encoding a
196 putative Fur-like regulator, LIMLP_04825 (LIC11006/LA3094), possesses the two Fur
197 typical residues, R76 and E121, respectively. The fourth ORF encoding a putative Fur-like
198 regulator, LIMLP_18590 (LIC20147/LB183) possesses the typical Fur arginine residue in its
199 putative H4 DNA binding helix (R51) but a typical PerR aspartate residue in the carboxy-
200 terminal domain (D94). Of note, LIMLP_18590 has a glutamate residue at the position 96.
201 Fold prediction suggests that the three Fur regulators encoded by LIMLP_05620,
202 LIMLP_04825 and LIMLP_18590 adopt the two-domain organization typical of the Fur
203 family depicted in the crystal structure of LIMLP_10155 (Fig 1C).

204 The closest relative of the PerR-encoding LIMLP_10155 is LIMLP_05620 with about 26% of
205 sequence identity, and LIMLP_04825 and LIMLP_18590 are closest relatives that share 20%

206 of sequence identity. LIMLP_05620 shares about 27% identity with the well-characterized *B.*
207 *subtilis* PerR. The putative H4 helix in LIMLP_05620 (Leu63-Ser75) is relatively well
208 conserved with that of *B. subtilis* (Val56-Ser69) (Fig1B-C) and LIMLP_05620 displays a
209 typical regulatory metal coordination site (His44-Asp93-His99-His101-Asp112). As the
210 LIMLP_10155-encoded PerR, LIMLP_05620 lacks the cysteinate motif involved in structural
211 metal coordination [30] (Fig 1B). On the contrary, both LIMLP_04825 and LIMLP_18590
212 have one or two cysteinate motifs for structural metal coordination (C₁₁₃xxC₁₁₆ and C₈₆xxC₈₉-
213 C₁₂₂xxC₁₂₅, respectively). Therefore, LIMLP_05620 encodes a putative PerR-like regulator
214 closely related to the PerR-encoding LIMLP_10155 whereas LIMLP_04825 and
215 LIMLP_18590 could encode other type of Fur-like regulators (Fur, Zur, or Nur). We therefore
216 annotated the LIMLP_10155 and LIMLP_05620 ORFs as *perRA* and *perRB*, respectively.

217

218 **Phylogenetic analysis of PerRA and PerRB in *Leptospira* species.**

219 To get a better understanding of the evolutionary relationship of the four Fur-like
220 regulators in pathogenic *Leptospira*, we undertook phylogenetic analyses by searching for
221 homologous sequences of the LIMLP_10155, LIMLP_05620, LIMLP_18590 and
222 LIMLP_04825 proteins among the representative genomes present in GenBank. This revealed
223 a large phylogenetic distribution with several branches (Fig 2A). The sequences homologous
224 to the LIMLP_04825 and LIMLP_18590 proteins form two distinct groups (red and orange,
225 respectively) separated by a common ancestor. To get better definition of phylogenetic
226 relationships of PerR-like homologues, we performed analysis with only a subset of sequence
227 (Fig 2B). This phylogenetic analysis shows two separated groups composed of the sequences
228 of LIMLP_10155 (*PerRA*) and LIMLP_05620 (*PerRB*) (see S1Fig for a more complete and
229 detailed tree).

230 The sequences of LIMLP_10155 and LIMLP_05620 ORFs from the strain *L.*
231 *interrogans* serovar Manilae were searched and compared in all available genomes from
232 the *Leptospira* genus (S1 Table). As seen in Fig 3, LIMLP_10155 is present in the
233 saprophytes S1 and S2 clades and in the P1 clade (highly virulent strains). This ORF is absent
234 from most of the P2 clade (intermediate strains). However, there are two exceptions in the P2
235 clade species since homologues of LIMLP_10155, which might have been acquired by a
236 recent horizontal gene transfer, are present in *L. dzoumognesis* and *L. wolffii*. Additionally,
237 this ORF is also present in other bacteria from the order *Leptospirales* such as *Turneriella*
238 *parva* and *Leptonema illini* (Fig 2B). This suggests that LIMLP_10155 (PerRA) was present
239 in *Leptospirales* ancestor before *Leptospira* divergence and lost in the P2 clade. On the other
240 side, LIMLP_05620 ORF is only present in P1 and P2 clades and absent in all species from
241 S1 and S2 clades (Fig 3). LIMLP_05620 is also not found in other bacteria from the
242 *Leptospirales* order (Fig 2B and S1 Fig). This restricted distribution suggests that the ancestor
243 of pathogenic strains (P1 and P2 clades) has likely acquired LIMLP_05620 after their
244 divergence with other *Leptospira*. Overall, both PerR-encoding LIMLP_10155 and
245 LIMLP_05620 ORFs only coexist in P1 clade that comprises the highly virulent *Leptospira*
246 species. Altogether, these findings indicate that pathogenic *Leptospira* strains encode a
247 second putative PerR-like regulator that is absent in saprophytes.

248

249 **Role of PerRB in *L. interrogans* tolerance to ROS.**

250 As demonstrated previously [27], when *L. interrogans* are exposed to a sublethal dose
251 of H₂O₂ (10 μM for 30 min) *perRA* expression is increased by a 7-fold whereas that of *perRB*
252 is unchanged (Fig 4). In the presence of a higher dose of H₂O₂ (1 mM for 1h), expression of
253 both *perRA* and *perRB* was increased significantly by a 6-fold (Fig 4). This suggests that
254 PerRB, like PerRA, responds to deadly H₂O₂ dose.

255 We have previously shown that inactivating *perRA* led to the derepression of *katE*, *ahpC* and
256 *ccp* and to a higher tolerance to H₂O₂ [27,30] (see S2 Fig). The *perRA* mutant exhibited a
257 reduced ability to grow in the presence of the superoxide-generating compound paraquat [30].
258 A mutant with a transposon inserted into the PerRB-encoding LIMLP_05620 ORF was
259 available in our random transposon mutant library and was used to investigate *L. interrogans*
260 tolerance of ROS in the absence of PerRB. When cultivated in EMJH medium, the
261 *perRB* mutant did not reach the same density than the WT strain at stationary phase (Fig 5A
262 and S2 Fig). Inactivating *perRB* did not have any effect on the ability of *L. interrogans* to
263 tolerate deadly concentration of H₂O₂ (S2 Fig); however, it increases the capability of
264 *Leptospira* to grow in the presence of paraquat (Fig 5B). Complementing in trans the *perRB*
265 mutant restored the WT strain phenotype in the absence of paraquat (Fig 5A) and it decreased
266 the growth rate of the cells to a somehow lower level than that of the WT in the presence of
267 paraquat (Fig 5B). This indicates that PerRB is involved in *Leptospira* tolerance to
268 superoxide, very likely by repressing (directly or indirectly) defenses against this ROS.
269 Therefore, PerRA and PerRB have distinct function in pathogenic *Leptospira* survival in the
270 presence of oxidants.

271

272 **Identification of differentially-expressed genes upon *perRB* inactivation.**

273 To understand the role of PerRB in *L. interrogans* tolerance to ROS, we compared the
274 global transcriptional profiles of the *perRB* mutant and WT strains. Differential gene
275 expression analysis revealed changes in the transcription of 123 genes, with 59 and 64 down-
276 and up-regulated, respectively (see S2 Table for a complete set of data). However, *perRB*
277 inactivation did not lead to dramatic changes in gene expression as the majority of Log₂FC
278 (108 out of 123) ranged between -1 and 1 (S2 Table). These findings indicate that the absence
279 of an active PerRB did not lead to substantial significant changes in genes expression when

280 *Leptospira* are cultivated in the laboratory conditions (in EMJH medium at 30°C) and during
281 the exponential phase.
282 Nevertheless, when examining the nature of the highest differentially-expressed genes in the
283 *perRB* mutant, some tendencies could be observed. Many of the differentially-expressed
284 ORFs were annotated as protein with unknown function and did not have homologs in the
285 saprophyte *L. biflexa* strain (S2 Table and Table 1).

ORF ID ^a	Gene	<i>L. biflexa</i> ^b	Function	Log2FC	Adjusted p-value
Down-regulated genes					
LIMLP_02490* (LIC12988/LA0587)		<i>LEPBI_I0886</i>	Lipase putative extracellular lipoprotein	-1.608	6.11e-04
LIMLP_02845 (LIC12920)			Hypothetical	-1.084	3.46e-02
LIMLP_03640** (LIC12763/LA0865)			Hypothetical	-1.102	1.06e-02
LIMLP_03790* (LIC12736/LA0905)			Hypothetical	-1.244	1.42e-05
LIMLP_04255 (LIC10892/LA3244)	<i>exbB</i>	<i>LEPBI_I0149</i>	Biopolymer transport protein ExbB/TolQ	-0.947	1.07e-02
LIMLP_06190** (LIC11265/LA2751)		<i>LEPBI_I3113</i>	Disulfide oxidoreductase	-0.723	6.96e-03
LIMLP_11400** (LIC12297/LA1456)	<i>radC</i>		DNA repair protein RadC	-0.619	1.17e-02
LIMLP_13165** (LIC12631/LA1029)	<i>sph2</i>		Sphingomyelinase C	-1.152	8.10e-03
LIMLP_14595* (LIC10628/LA3571)		<i>LEPBI_I2694</i>	Cytochrome oxidase CcoP subunit	-0.583	3.40e-02
LIMLP_14715** (LIC10606/LA3598)	<i>dps</i>	<i>LEPBI_I2540</i>	DNA-binding stress protein Dps	-0.896	1.61e-03
LIMLP_15470** (LIC10454/LA3793)		<i>LEPBI_I0671</i>	Hemolysin (N-acyltransferase domain)	-1.317	3.40e-03
LIMLP_15890 (LIC10377/LA0430)			Hypothetical putative lipoprotein	-1.353	3.90e-10
LIMLP_16695 (LEPIC3326/LA4096)			Hypothetical	-1.124	8.14e-04
LIMLP_18235** (LIC20078/LB099)			Hypothetical	-1.098	1.69e-02
Up-regulated genes					
LIMLP_02880* (LIC12912/LA0688)	<i>cas5</i>		CRISPR-associated protein Cas5	0.881	5.84e-03
LIMLP_02885* (LIC12911-12910/LA0689-0690)	<i>cas3</i>		CRISPR-associated protein Cas3	0.834	4.51e-03
LIMLP_04075** (LIC12680/LA0974)			Adhesin/FimH-like protein/DuF1566 domain	1.110	4.29e-03
LIMLP_05450* (LIC11125/LA2933)			Diguanylate cyclase	0.983	2.12e-04
LIMLP_05455* (LIC11126/LA2932)			Diguanylate cyclase	0.757	1.81e-02
LIMLP_05460* (LIC11127/LA2930)			Diguanylate cyclase	0.922	1.11e-03
LIMLP_05480 (LA2928)			Hypothetical	0.968	2.67e-02
LIMLP_05485** (LIC11131/LA2926)			Diguanylate cyclase	0.679	8.14e-04
LIMLP_05845 (LIC11203/LA2827)			Diguanylate phosphodiesterase	0.608	3.85e-02
LIMLP_09580 (LIC11921/LA1980)		<i>LEPBI_I1269</i>	Diguanylate phosphodiesterase	0.547	1.61e-02
LIMLP_17875 (LIC20015/LB017)	<i>hemN</i>	<i>LEPBI_I1166</i>	Coproporphyrinogen III oxidase HemN	0.727	9.53e-03
LIMLP_18375** (LIC20106/LB133)			Diguanylate phosphodiesterase	0.593	1.99e-02
LIMLP_18755 (LIC20176/LB225)			Hypothetical putative lipoprotein	1.453	3.77e-04
LIMLP_18760 (LIC20177/LB226)			Adhesin/FimH-like protein/ DUF1566 domain-containing protein	1.095	3.39e-02
LIMLP_19320* (LA1770)			AraC family transcriptional regulator	1.279	4.51e-03
LIMLP_19325* (LA1771)			Hypothetical	1.262	4.05e-02

287

288 **Table 1. Differentially-expressed ORFs upon *perRB* inactivation**

289 Selected up-and down-regulated genes in the LIMLP_05620 (*perRB*) mutant with an adjusted p-value cutoff of 0.05.

290 ^a Gene numbering is according to Satou *et al* [31]. Corresponding genes of *L. interrogans* serovar Lai strain 56601 and serovar Copenhageni
291 Fiocruz strain L1-130 are indicated in parenthesis.

292 ^b Closest ortholog in the saprophytes *L. biflexa* serovar Patoc strain Patoc1. The absence of synteny is indicated in italic.

293 Genes that are down-regulated upon *perRA* inactivation as determined previously [27] are indicated in bold.

294 Down (*) and up (**)-regulated genes upon exposure to lethal H₂O₂ dose as determined previously [27].

295 Among the highest up-regulated genes, two ORFs (LIMLP_04075 and LIMLP_18760)
296 encoded lipoproteins with a putative adhesin function. These proteins contain DUF1566
297 domain repeats which is also share by Lsa25, a *Leptospiral* surface adhesin that binds
298 extracellular matrix (ECM) [32].

299 Several genes involved in the metabolism of c-di GMP were differentially-expressed upon
300 *perRB* inactivation. C-di GMP is a secondary messenger in bacteria that regulates a variety of
301 processes such as biofilm formation, motility, stress adaptation, and virulence. C-di GMP
302 synthesis is catalyzed by diguanylate cyclases (DGCs) whereas its hydrolysis is catalyzed by
303 phosphodiesterases (PDEs). DGCs and PDEs are numerous in pathogenic *Leptospira*,
304 suggesting that c-di GMP fulfills an important role in sensing environmental signals when
305 *Leptospira* infect and colonize a host. C-di GMP has been recently shown to regulate biofilm
306 formation, motility and protection against environmental stress in pathogenic *Leptospira* [33].
307 Four DGCs (LIMLP_05450, LIMLP_05455, LIMLP_05460, LIMLP_05485) were up-
308 regulated upon *perRB* inactivation (Table 1). These DGC-encoding ORFs are located in a
309 gene cluster (LIMLP_05485-05450) that contains 7 ORFs coding for DGCs. LIMLP_05450,
310 LIMLP_05455, LIMLP_05460, and LIMLP_05485 display the typical diguanylate cyclase
311 GGDEF and sensory PAS domains. A DGC activity was demonstrated *in vitro* for
312 LIMLP_05450, LIMLP_05455, LIMLP_05460 [34]. Three PDE-encoding ORFs
313 (LIMLP_05845, LIMLP_9580, and LIMLP_18375) were also up-regulated in the *perRB*
314 mutant.

315 LIMLP_13165 was among the most down-regulated ORFs when *perRB* was inactivated. It
316 encodes a secreted protein with sphingomyelinase C and hemolytic activities [35]. Another
317 significantly down-regulated ORF encoded a protein with an acyl CoA acetyl tranferase
318 domain annotated as a putative hemolysin (LIMLP_15470). This ORF is up-regulated when
319 *L. interrogans* is cultivated in DMC implemented in rats [36].

320 An ORF encoding an AraC transcriptional regulator (LIMLP_19320), and two ORFs of
321 unknown function (LIMLP_18755 and 19325) were among the most up-regulated. The
322 orthologs of LIMLP_19320 and LIMLP_19325 in *L. interrogans* serovar Lai belongs to a
323 genomic island (Lai GI B, LA1747-1851) that can excise from the chromosome and form an
324 independent replicative plasmid [37,38].

325 Among the down-regulated genes, several ORFs encode factors related to oxidative stress.
326 LIMLP_04255 is part of a gene cluster (LIMLP_04240-04285) which code for a putative
327 TonB-dependent transport system repressed by PerRA. We have previously shown that some
328 genes of this cluster (LIMLP_04245, LIMLP_04270 and LIMLP_04280) are involved in *L.*
329 *interrogans* tolerance to superoxide [27]. LIMLP_11400 encodes the DNA repair protein
330 RadC and LIMLP_14715 is a homolog of the *E. coli* Dps, a protein that sequesters iron and
331 protects DNA from oxidative damage. LIMLP_06190 encodes a putative disulfide
332 oxidoreductase with the N-terminal ScdA domain (DUF1858). In *S. aureus*, ScdA is a di-iron
333 protein involved in repair of oxidatively damaged iron-sulfur cluster proteins [39].
334 LIMLP_14595 encodes a putative transmembrane lipoprotein with a cytochrome-like domain
335 that shows homology with the CcoP subunit of the cytochrome C oxidase and could function
336 in the respiratory chain or be an enzyme cofactor.

337 Only 7 out of the 123 differentially-expressed genes in the *perRB* mutant were also
338 differentially-expressed upon *perRA* inactivation with a similar inclination (S3 Fig) [27].
339 LIMLP_02010 and LIMLP_04325 were up-regulated whereas LIMLP_04255,
340 LIMLP_11810, LIMLP_14225, LIMLP_15470 and LIMLP_18235 were down-regulated in
341 the two mutants.

342 Notably, 82 out of the 123 differentially-expressed ORFs in the *perRB* mutant were also
343 differentially-expressed upon exposure of *L. interrogans* to H₂O₂ (S3 Fig) [27]. Thus, 66% of
344 the PerRB regulon is also regulated by the presence of H₂O₂. Interestingly, the majority of

345 ORFs down-regulated in the *perRB* mutant, including the RadC and the Dps-encoding ORFs,
346 were up-regulated in the presence of H₂O₂ (with Log₂FCs of 3.46 and 1.10, respectively) (S3
347 Fig and Table 1). On the contrary, many up-regulated ORFs in the *perRB* mutant had a lower
348 expression in the presence of H₂O₂. For instance, the ORFs that code for Cas5
349 (LIMLP_02880), Cas3 (LIMLP_02885), and two DGCs (LIMLP_05450 and LIMLP_05455)
350 were down-regulated upon exposure to H₂O₂ with Log₂FCs lower than -1.21 (S3 Fig and
351 Table 1).

352

353 **Concomitant inactivation of *perRA* and *perRB* leads to a higher resistance to ROS but to** 354 **a lower virulence**

355 In order to investigate whether PerRA and PerRB cooperate in regulating the adaptive
356 response to ROS, we inactivated *perRA* by allelic exchange in the *perRB* mutant (S4 Fig).
357 This allowed obtaining a double *perRAperRB* mutant in a *L. interrogans* strain, the first
358 double mutant constructed in a pathogenic *Leptospira*.

359 The double *perRAperRB* mutant had a growth rate comparable to that of the single *perRA* and
360 *perRB* mutants and WT strains when *L. interrogans* were cultivated in EMJH medium (Fig
361 6A). However, entry in exponential phase was delayed if the culture medium was inoculated
362 with stationary phase-adapted *perRAperRB* mutant (S5 Fig). We had already shown that a
363 *perRA* mutant had a higher ability to grow and survive in the presence of deadly concentration
364 of H₂O₂ but a slower growth in the presence of the superoxide-generating paraquat ([30], and
365 see in S2 Fig and Figs 6B and 6C). On the contrary, inactivating *perRB* leads to a higher
366 resistance to paraquat (Fig 5 and Fig 6C). The concomitant inactivation of *perRA* and *perRB*
367 led to a greater growth in the presence of both H₂O₂ (Fig 6B) and paraquat (Fig 6C). To
368 complement the double *perRAperRB* mutant strain, the PerRB-encoding ORF LIMLP_05620
369 was expressed in trans using the replicative pMaORIGenta vector (S4 Fig). The growth of the

370 trans-complemented *perRAperRB* mutant resumed to an impaired growth, as that of the WT
371 and single *perRA* mutant strains, in the presence of paraquat (Fig 6D). Therefore, the double
372 *perRAperRB* mutant exhibited cumulative phenotypes of the respective single *perRA* and
373 *perRB* mutants when *L. interrogans* are cultivated in the presence of ROS.
374 We then tested whether *perRA* and *perRB* inactivation had any influence on *L. interrogans*
375 virulence in the animal model of acute leptospirosis. All hamsters infected intraperitoneally
376 with 10⁴ bacteria of the *perRA* or *perRB* single mutant strains exhibited morbidity sign after
377 7-8 days (Fig 6E), similarly to the WT strain. In contrast, all animals infected
378 intraperitoneally with 10⁴ bacteria of the double *perRAperRB* mutant strain did not show any
379 sign of morbidity three weeks post-infection (Fig 6E), even when the animals were infected
380 with 10⁶ bacteria of the double *perRAperRB* mutant strain (Fig 6F). Virulence was restored in
381 the double *perRAperRB* mutant strain complemented in trans only with *perRB* (Fig 6F).
382 Therefore, the concomitant inactivation of *perRA* and *perRB* resulted in a loss of virulence in
383 *L. interrogans* and expressing only *perRB* in the double mutant restored *Leptospira* virulence.
384 Altogether, these results demonstrate that despite a higher resistance to ROS, the double
385 *perRAperRB* mutant exhibited a dramatically reduced virulence.

386

387 **Concomitant inactivation of *perRA* and *perRB* has a pleiotropic effect in *L.***
388 ***interrogans* gene expression.**

389 To further understand the interplay between PerRA and PerRB in controlling the
390 oxidative stress response and virulence in *L. interrogans*, we performed RNA-Seq
391 experiments on the double *perRAperRB* mutant and compared its transcriptomic profile with
392 that of WT and single *perRA* and *perRB* mutant strains.

393 949 and 1024 ORFs were down- and up-regulated, respectively, in the double
394 *perRAperRB* mutant (Fig 7A and see S3 Table for a complete set of data). Therefore,

395 concomitant *perRA* and *perRB* inactivation resulted in differential expression of almost half of
396 the total coding sequences of *L. interrogans*; in comparison, only about 1% and 3% of the
397 total coding sequences of *L. interrogans* were differentially-expressed in the single
398 *perRA* and *perRB* mutants, respectively (S2 Table) [27]. Volcano scatter plot representation
399 indicated not only a higher magnitude of fold changes but also a greater statistical
400 significance in the double *perRAperRB* mutant (Fig 7B-D).

401 Most of the differentially-expressed ORFs in the *perRA* mutant were also differentially-
402 expressed in the double *perRAperRB* mutant (Fig 7A). Many genes of the LIMLP_04240-
403 04280 cluster encoding a putative TonB-dependent transport system, the two-component
404 system VicKR (LIMLP_16720-16725), a putative hemolysin (LIMLP_15470) and several
405 ORFs of unknown function (from LIMLP_14190 to LIMLP_14225) were down-regulated in
406 the *perRA* [24,27] and *perRAperRB* mutants (Fig 7 and 8A, S4 Table). Likewise, the ORFs
407 encoding the catalase, AhpC and CCP (LIMLP_10145, LIMLP_05955 and LIMLP_02795,
408 respectively), that are repressed by PerRA and up-regulated in the single *perRA* mutant
409 [24,27], were also up-regulated in the double *perRAperRB* mutant (Fig 7 and 8B, S5 Table).

410 21 and 27 ORFs that are respectively down- and up-regulated in the *perRB* mutant were also
411 down- and up-regulated in the *perRAperRB* mutant (Fig 7A). LIMLP_11400 (encoding
412 RadC), LIMLP_04255, encoding ExbB of the TonB-dependent transport system, the
413 hemolysin-encoding ORF LIMLP_15470, and LIMLP_15890 were down-regulated in the
414 single *perRB* and double *perRAperRB* mutants (Fig 7 and S2-S4 Tables).

415 Interestingly, the vast majority of the differentially-expressed ORFs in the double
416 *perRAperRB* mutant did not exhibit any change in their expression in the single *perRA* and
417 *perRB* mutants. For instance, of the DGCs and PDEs that were up-regulated in the *perRB*
418 mutant, only LIMLP_09580 was up-regulated in the double *perRAperRB* mutant (S2, S3 and
419 S5 Tables). In fact, the *perRAperRB* double mutant exhibited a distinct expression pattern of

420 genes involved in signaling (Fig 8C). The LIMLP_07050 ORF that codes for a DGC was
421 down-regulated; two ORFs encoding adenylate/guanylate cyclases (LIMLP_00130 and
422 LIMLP_02085) and the PDE-encoding LIMLP_04775 ORF were up-regulated in the
423 *perRAperRB* mutant. Finally, only 6 ORFs were differentially expressed in all mutants (Fig
424 7A), including LIMLP_04255 and LIMLP_15470 (S4 and S5 Tables). Moreover, a
425 substantial number of regulatory factors (transcriptional regulators, two-component systems,
426 sigma factors) were differentially-expressed exclusively in the *perRAperRB* mutant (S4 and
427 S5 Tables).

428 In the double *perRAperRB* mutant, several ORFs encoding factors putatively involved in cell
429 growth (cell division, respiration and cell wall homeostasis), chemotaxis and motility are
430 significantly up-regulated, with a Log₂FC greater than 1.5 (S5 Table). This correlates with a
431 higher ability of the *perRAperRB* mutant to reach a higher cell number when cultivated *in*
432 *vitro* at 30°C (Fig 6 and S4 Fig).

433 In addition to the PerRA-repressed peroxidases (catalase, AhpC, CCP), other oxidative-stress
434 related factors exhibited a higher expression in the *perRAperRB* mutant (Fig 8B). DoxX-
435 encoding ORF, which is up-regulated upon concomitant *perRA* and *perRB* inactivation
436 (Log₂FC 1.65), is an integral membrane protein that interacts with SodA in *M. tuberculosis*
437 and participates in tolerance to oxidative and redox stress [40]. Two imelysins
438 (LIMLP_14170/LruB and LIMLP_14180) and a thiol peroxidase (LIMLP_14175) exhibited also
439 a higher expression in the *perRAperRB* mutant (Log₂FC of 2.36, 1.93, and 2.04 respectively,
440 Fig 8B). All these up-regulated factors (except DoxX) were also up-regulated upon exposure
441 to deadly H₂O₂ dose [27] and they probably participate in the higher tolerance of the double
442 mutant in the presence of oxidants. Despite the up-regulation of several factors involved in
443 the defense against ROS, the DNA repair protein RadC (encoded by LIMLP_11400) and the

444 glutathione S transferase (encoded by LIMLP_13670) were notably down-regulated in the
445 *perRAperRB* mutant (Log₂FC of -1.9 and -2.3, respectively) (Fig 8B and S4 Table).

446 Strikingly, several down-regulated ORFs in the double *perRAperRB* mutant such as *clpB*,
447 *ligA*, *ligB*, and the operon *lvrAB* have been associated with *Leptospira* virulence. As in many
448 bacteria, leptospiral ClpB ATPase is involved in disaggregating protein aggregates arising
449 upon stress-induced protein denaturation [41]. *ClpB* expression is increased upon exposure to
450 H₂O₂ and it is required for *Leptospira* virulence [27,42]. The ClpB-encoding ORF
451 (LIMLP_10060) is dramatically down-regulated in the *perRAperRB* mutant (Log₂FC of -2.99)
452 (Fig 8D and S4 Table).

453 Another virulence factors in *Leptospira* are the immunoglobulin-like LigA (LIMLP_15405)
454 and LigB (LIMLP_15415) proteins. These surface-exposed proteins are involved in adhesion
455 to host cells through ECM binding [43] and participate in the immune evasion through
456 binding to the host complement Factor H and C4b binding protein [44]. Simultaneous down-
457 regulation of *ligA* and *ligB* expression led to attenuation of *Leptospira* virulence [45]. *LigA*
458 and *ligB* were down-regulated in the *perRAperRB* mutant (Log₂FC of -3 and -2.44,
459 respectively) (Fig 8D and S5 Table).

460 *LvrA* (LIMLP_08490) and *lvrB* (LIMLP_08485) encode a hybrid histidine kinase and a
461 hybrid response regulator, respectively. Inactivation of the *lvrAB* operon led to virulence
462 attenuation in *L. interrogans* [46]. *LvrA* and *lvrB* had both a decreased expression in the
463 *perRAperRB* mutant (Log₂FC of -2.3) (Fig 8D and S5 Table).

464 Additional ORFs encoding chaperones (the small heat shock proteins Hsp15 and Hsp20) or
465 enzymes involved in protein folding (the disulfide isomerase DsbD and the peptidyl-prolyl
466 cis-trans isomerase SlyD) and degradation (HtpX) were down-regulated in the *perRAperRB*
467 mutant. The involvement of these factors in *Leptospira* virulence has not been demonstrated
468 but small Hsps participate in *M. tuberculosis* growth in macrophages [47]. DsbD and these

469 two small Hsps were up-regulated in *L. interrogans* upon exposure to H₂O₂ [27]. All these
470 factors might protect *Leptospira* proteostasis under adverse conditions as encountered during
471 infection inside a host. The down-regulation of these virulence-associated genes together with
472 the differential expression of several other genes was confirmed by RT-qPCR (S5 Fig).
473 Interestingly, gene expression of the virulence-associated genes (*lvrA*, *lvrB*, *ligA*, *ligB*, *clpB*,
474 *hsp15*, and *hsp20*) was increased to the WT level (or even to a higher level) in the double
475 *perRAperRB* mutant strain complemented in trans only with *perRB* (Fig 9).
476 Taken together, these findings indicate that the loss of virulence resulting from the
477 concomitant inactivation of *perRA* and *perRB* correlated with a global deregulation of a
478 complex gene network, including genes associated with virulence.

479

480 **Identification of differentially-expressed non-coding RNAs in the *perRB* and**
481 ***perRAperRB* mutants.**

482 Intergenic regions were also analyzed to identify differentially expressed predicted
483 non-coding RNAs (ncRNAs). As observed for coding sequences, inactivation of *perRB* led to
484 the deregulation of only a few putative ncRNAs and most of the changes in expression were
485 below two folds (see S6 Table for a complete set of data). Nonetheless, a few numbers of
486 ncRNAs were significantly down-regulated with a Log₂FC below -1 (S7 Table). Some of the
487 differentially-expressed ncRNAs (*LepncRNA36*, *LepncRNA87*, *LepncRNA89*,
488 *LepncRNA109*, *LepncRNA139*) were located in the proximate vicinity of differentially-
489 expressed ORFs in the *perRB* mutant. Three ncRNAs (*LepncRNA35*, *LepncRNA89* and
490 *LepncRNA109*) were also differentially expressed upon *perRA* inactivation (S7 Table) [27].
491 55 putative ncRNAs were differentially-expressed (with a Log₂FC cutoff of ±1) in the
492 *perRAperRB* mutant and several of them were adjacent or overlapped differentially-expressed

493 ORFs (S8 Table). Only a few of these differentially-expressed ncRNAs had an altered
494 expression in the single *perRA* and *perRB* mutant (S8 Table) [27].

495 Among the most highly differentially-expressed ncRNAs was LepncRNA38 that was located
496 downstream *ccp*, a highly up-regulated ORF in the *perRAperRB* mutant (Fig 10 and S8
497 Table). LepncRNA38 and *ccp* were also up-regulated in the *perRA* mutant [27]. The ncRNA
498 LepncRNA49, which was down-regulated in the *perRAperRB* mutant, overlapped with *exbB*
499 (LIMLP_04255), an ORF that was also down-regulated in the double *perRAperRB* mutant as
500 well as in the single *perRA* and *perRB* mutants (Fig 10). The down-regulated LepncRNA105
501 and LepncRNA130 ncRNAs were located downstream the *hsp20-15* operon and *gst*,
502 respectively, three ORFs whose expression is decreased in the *perRAperRB* mutant (Fig 10
503 and S8 Table). It is worth noting that LepncRNA38, LepncRNA105 and LepncRNA130 are
504 up-regulated by H₂O₂ as were *ccp*, *hsp20-15* and *gst* ([27]; Fig 10).

505 Altogether, these findings indicate that the absence of both PerRA and perRB triggers major
506 changes in the transcriptional activity of many ncRNAs in *L. interrogans*, that could
507 consequently alter the expression of many ORFs.

508

509

510 Discussion

511

512 Virulence mechanisms are poorly characterized in pathogenic *Leptospira*. These
513 bacteria possess a high number of genes encoding proteins of unknown function (40% of the
514 genomes) and many of them are pathogen-specific. Pathogenic *Leptospira* spp. lack many
515 classical virulence factors, such as a type III to type VI secretion systems, and it is unclear
516 which factors are important for its pathogenesis. It is therefore generally agreed that these
517 pathogens possess unique virulence factors. Nonetheless, studying heme oxygenase and
518 catalase mutants have shown that, *in vivo*, iron acquisition and defense against peroxide stress
519 are important virulence-associated mechanisms in *L. interrogans* [11,48]. Catalase is
520 repressed by PerRA [24,27] and genes encoding factors involved in iron uptake are very
521 likely controlled by regulators of the Fur-family.

522 In addition to PerRA, pathogenic *Leptospira* contain three other ORFs annotated as Furs. In
523 the present study, we have characterized the *L. interrogans* Fur-like regulator encoded by
524 LIMLP_05620 and showed that it exhibits characteristic features of a PerR regulator. We
525 consequently named this ORF *perRB*. Sequence alignment and phylogenetic analyses
526 revealed that PerRB is the closest relative to the already characterized PerRA, and perhaps
527 more importantly, they both do exhibit the canonical amino acid residues that are the hallmark
528 of a PerR. The H₂O₂ sensing histidine and aspartate residues are conserved in *Leptospira*
529 PerRA and PerRB and, interestingly, both genes are H₂O₂-responsive, albeit with different
530 apparent sensitivity. This is consistent with a mechanism whereby PerRA and PerRB would
531 repress their own transcription and dissociate from their promoter upon oxidation by H₂O₂,
532 leading to alleviation of repression. Moreover, the higher survival of the *perRB* mutant in the
533 presence of superoxide suggests a derepression of genes encoding defenses against ROS and
534 therefore the participation of PerRB in controlling the adaptation to oxidative stress. Neither

535 *perRA* nor *perRB* expression was up-regulated in iron-limiting condition [24]. Although the
536 putative lipoprotein LIMLP_18755 was significantly up-regulated in the *perRB* mutant and
537 under iron-limiting condition, there was no strong overlap between PerRB regulon and the
538 transcriptional response to iron-limiting condition [24]. Altogether, these findings could argue
539 in favor of LIMLP_05620 encoding a PerR-like regulator rather than a Fur. However, because
540 iron homeostasis and oxidative stress are intertwined, a certain functional relationship has
541 been observed between PerR and Fur. In several bacteria where PerR and Fur coexist,
542 including *B. subtilis* and *C. jejuni*, the PerR regulon overlaps with that of Fur [49,50]. In
543 addition, *fur* and several Fur-regulated genes are also differentially expressed in the presence
544 of H₂O₂ [51,52]. In fact, PerR represses *fur*, whose expression is up-regulated in the presence
545 of H₂O₂ as a consequence of dissociation of PerR from the *fur* promotor [53,54]. Metal-
546 catalyzed oxidation of the H₂O₂ sensing residues will be fundamental in establishing that the
547 Per-like regulator encoded by LIMLP_05620 is a *bona fide* PerR.

548 To the best of our knowledge, this is the first report that has identified the coexistence of two
549 PerR regulators in a pathogenic Gram-negative bacterium. The coexistence of three PerR-like
550 regulators has been reported only in Gram-positive bacteria such as *B. licheniformis* and *M.*
551 *smegmatis*. It was shown that the three *B. licheniformis* PerRs sense hydrogen peroxide by
552 histidine oxidation, although with different sensitivity [55]. In *M. smegmatis*, three Fur-like
553 paralogs displayed the canonical PerR Asp residue involved in H₂O₂ sensitivity, exhibited
554 H₂O₂ sensing by metal-catalyzed histidine oxidation and a higher H₂O₂ resistance when their
555 genes were inactivated [56].

556 One important question was to understand the mechanism that have led to the coexistence of
557 PerRA and PerRB exclusively in highly virulent species (P1 clade). Virulent mammalian-
558 adapted strains in the *Leptospira* genus might have originated from a free-living ancestor
559 inhabiting soils. The phylogenetic analysis presented here indicates that the coexistence of

560 PerRA and PerRB is not due to gene duplication. Indeed, PerRA was already present in the
561 leptospirales ancestor whereas PerRB was probably acquired by pathogenic species by
562 horizontal transfer from a soil/aquatic bacterium of another phylum. In this scenario, PerRA
563 would had been lost by the P2 clade intermediate species but maintained together with PerRB
564 by the P1 clade species to establish full virulence.

565 We had previously shown that when *perRA* was inactivated, *L. interrogans* acquired a higher
566 resistance to H₂O₂ explained by the derepression of *katE*, *ahpC* and *ccp* [24,27]. Here, we
567 have demonstrated that inactivating *perRB* resulted in a higher survival of *L. interrogans* in
568 the presence of the superoxide but it did not affect the survival of *L. interrogans* in the
569 presence of H₂O₂. Therefore, even though *perRB* is up-regulated upon exposure to H₂O₂ as
570 *perRA*, the consequence of *perRB* inactivation is different than that of *perRA*, suggesting that
571 PerRA and PerRB have a distinct and non-redundant function in *Leptospira* adaptation to
572 oxidative stress. The distinct repartition of PerRA and PerRB in the *Leptospira* genus and
573 differences in their respective regulon support the hypothesis of a non-redundant function in
574 the adaptation to oxidative stress. The PerRA and PerRB regulons determined when *L.*
575 *interrogans* are cultivated inside a host using DMC implemented in rats confirmed the limited
576 overlap between PerRA and PerRB regulons [57].

577 Phenotypic studies suggest that PerRB represses (directly or indirectly) genes encoding
578 defenses against superoxide. Highly pathogenic *Leptospira* species (from the P1 clade) do not
579 encode any SOD or SOR that could be responsible for detoxification of superoxide whereas
580 intermediated species (P2 clade) and saprophyte non-pathogenic *Leptospira* do have such
581 enzymes. Understanding how pathogenic *Leptospira* detoxify superoxide encountered during
582 infection is a very important question. Overall, the differentially-expressed genes upon *perRB*
583 inactivation are mostly *Leptospira*-specific and poorly characterized. Examining the PerRB
584 regulon determined when *Leptospira* are cultivated in laboratory conditions did not allow to

585 draw a conclusive hypothesis on the identity of the factors that could participate in superoxide
586 detoxification in *L. interrogans*. The highest differentially-expressed ORFs were mainly
587 involved in regulation and cell signaling (transcription and sigma factors,
588 adenylate/diguanylate cyclase, TCSs) and could be involved in regulating the adaptation to
589 various challenging stress encountered in the environment or within a host. Further studies
590 will be required to determine whether superoxide detoxification in *L. interrogans* is mediated
591 by enzymatic detoxification or metal-dependent scavenging mechanisms and to clarify the
592 exact role of PerRB in controlling those pathways.

593 The low number of significantly differentially-expressed genes in the *perRB* mutant when *L.*
594 *interrogans* are cultivated *in vitro* led us to propose that PerRB exerts its function during
595 oxidative stress or upon host-related conditions. Consistent with this hypothesis is the up-
596 regulation of *perRB* in the presence of lethal H₂O₂ dose. It is worth noting that there is, to
597 some extent, an overlap between the PerRB regulon and the differentially-expressed genes
598 upon exposure to lethal H₂O₂ dose [27]. Moreover, the exclusive presence of PerRB in the
599 pathogenic *Leptospira* clades strongly suggests that PerRB function is more related to
600 regulating adaptation to infection-related conditions rather than to environmental survival.
601 Very interestingly, when the *perRB* mutant was cultivated in the host conditions using the
602 DMC implants, a higher number of genes were differentially-expressed with greater fold
603 changes than when the *perRB* mutant was cultivated at 30°C in the laboratory conditions [57].
604 Notably, in the host condition, the absence of *perRB* also led to the deregulation of several
605 genes involved in signaling and regulation, which is consistent with a role of PerRB in
606 regulating adaptation to the host environment [57].

607 One feature of *Leptospira* genus is the genetic and functional redundancy where multiple
608 genes commonly encode for a similar function. The development of genetic tools has made
609 random and targeted mutagenesis possible, albeit with a low efficiency. Due to this limitation,

610 only a few *Leptospira* virulence factors have been identified and have fulfilled Koch's
611 molecular postulates. The present study is the first to report the concomitant inactivation of
612 two genes and complementation of a double mutant in a pathogenic *Leptospira*. Obtaining a
613 double *perRAperRB* mutant gave us the unique opportunity to investigate the functional
614 relationship between two PerR-like regulators in a pathogenic bacterium.

615 In many pathogens which contain only one PerR paralog, such as *N. gonorrhoeae*, *S.*
616 *pyogenes*, and *S. aureus*, PerR was shown to be involved in virulence [54,58–61]. The single
617 *L. interrogans perRA* and *perRB* mutants still retain full virulence in the model for acute
618 leptospirosis (this study and [24]). Interestingly, virulence attenuation was only observed
619 upon the concomitant inactivation of *perRA* and *perRB*, suggesting an interplay in controlling
620 (directly or indirectly) *L. interrogans* virulence-associated genes. The loss of virulence
621 correlated with a large differential gene and ncRNA expression compared not only with the
622 WT but also with the single mutant strains. In other words, the double *perRAperRB* displayed
623 differential gene expression that were not observed in the single *perRA* and *perRB* mutants.
624 This could indicate that a subset of genes and ncRNAs is controlled by both PerRA and
625 PerRB. The absence of one regulator could be compensated by the other and most of the
626 genes and ncRNAs that can be regulated by the two regulators would not be differentially
627 expressed in the single mutants. The few genes (LIMLP_02010, LIMLP_04255,
628 LIMLP_04235, LIMLP_11810, LIMLP_14225, LILP_15470, and LIMLP_18235) and
629 ncRNAs that display differential expression in the single mutants in our transcriptomic
630 studies (this study and [27]) indicate a certain functional redundancy of the two regulators
631 even if the phenotypic analyses of the mutants suggest distinct functions. The change in
632 expression of a few regulators when PerRA and PerRB are both absent could lead to major
633 changes in expression of many ORFs or ncRNAs in cascade. One can also speculate that
634 among the large differentially-expressed genes only observed in the double *perRAperRB*

635 mutant some deregulation might be due to a compensatory effect to maintain a productive
636 fitness.

637 Despite a higher ability to resist ROS, the double *perRAperRB* mutant has lost its virulence; it
638 could not trigger acute leptospirosis-associated morbidity. This could be obviously explained
639 by a significant lower expression of several virulence-associated factors in the double
640 *perRAperRB* mutant, such as LigA, LigB, LvrA, LvrB, and ClpB. In addition, other
641 dramatically down-regulated genes encode factors such as small Hsps (Hsp15 and Hsp20) for
642 which a role in bacterial virulence is demonstrated in other bacteria including *M. tuberculosis*
643 [62]. Moreover, several differentially-expressed ORFs of unknown function could also be
644 responsible for the loss of virulence of the double *perRAperRB* mutant. It is noteworthy that
645 the double *perRAperRB* mutant was also unable to colonize mice [57] and, consistent with
646 what is observed with *in vitro*-cultivated *perRAperRB* mutant, this correlated with dramatic
647 gene dysregulation including down-regulation of *ligA* and *ligB* [57].

648 In summary, this study has allowed to identify a second PerR-like regulator in pathogenic *L.*
649 *interrogans* strains that cooperates with PerRA to control the adaptation to oxidative stress
650 and virulence. By concomitantly inactivating *perRA* and *perRB* and establishing the molecular
651 Koch' postulates, we have unveiled a complex regulatory network that reveals, for the first
652 time, a functional relationship between PerR regulators and *Leptospira* virulence, most likely
653 through the regulation of virulence- and pathogenicity-associated factors.

654

655 **Materials and Methods**

656 **Bacterial strains and growth condition**

657 *L. interrogans* serovar Manilae strain L495, *perRA* (M776), *perRB* (M1474), and the double
658 *perRAperRB* mutant strains (see S9 Table for a complete description of the strains used in this
659 study) were grown aerobically at 30°C in Ellinghausen-McCullough-Johnson-Harris medium
660 (EMJH) [63] with shaking at 100 rpm. *Leptospira* growth was followed by measuring the
661 absorbance at 420 nm. β 2163 and Π 1 *E. coli* strains were cultivated at 37°C in Luria-Bertani
662 medium with shaking at 37°C in the presence of 0.3 mM thymidine or diaminopimelic acid
663 (Sigma-Aldrich), respectively. When needed, spectinomycin, kanamycin and gentamycin
664 were added at the respective concentration of 50 μ g/ml, 30 μ g/ml, and 8 μ g/ml.

665

666 **Concomitant inactivation of *perRA* (LIMLP_10155) and *perRB* (LIMLP_05620).**

667 *PerRA* gene (LIMLP_10155/LIC12034/LA1857) was inactivated in the *perRB*
668 (LIMLP_05620/LIC11158/LA2887) mutant strain (M1474, *perRB::Km^R*) by introduction of a
669 spectinomycin resistance cassette (S4 Fig). For this, a spectinomycin resistance cassette
670 flanked by 0.8 kb sequences homologous to the sequences flanking *perRA* was created by
671 gene synthesis (GeneArt, Life Technologies) and cloned into a kanamycin-resistant
672 *Escherichia coli* vector unable to replicate in *Leptospira*. The obtained suicide plasmid
673 (pK Δ perRA) (S10 Table) was introduced in the *perRB* mutant strain by electroporation as
674 previously described [64] using a Gene Pulser Xcell (Biorad). Individual spectinomycin-
675 resistant colonies were selected on EMJH plates containing 50 μ g/ml spectinomycin and
676 screened by PCR (using the P1 and P2 primer set, see S11 Table) for proper replacement of
677 the *perRA* coding sequence by the spectinomycin resistance cassette. *PerRA* inactivation in
678 the double *perRAperRB* mutant was verified by western blot using an anti-PerRA serum (S4
679 Fig).

680

681 **Complementation of the single *perRB* and double *perRAperRB* mutants**

682 The *perRB* mutant (*perRB::Km^R*) complementation was performed by expressing the *perRB*
683 ORF in the pMaORI replicative vector [65]. The *perRB* (LIMLP_05620) ORF together with
684 its native promoter region (200 bp upstream region) were amplified from genomic DNA of *L.*
685 *interrogans* serovar Manilae strain L495 (using the ComPerR2_5Not and ComPerR2_3Xba
686 primer set, S11 Table) and cloned between the NotI and XbaI restriction sites in the pMaORI
687 vector. The absence of mutation in the *perRB* ORF in the obtained plasmid (pNB139) was
688 checked by DNA sequencing and the pNB139 plasmid was introduced in the *perRB* mutant
689 (M1474) by conjugation using the *E. coli* β 2163 conjugating strain as previously described
690 [66]. *Leptospira* conjugants were selected on EMJH plates containing 50 μ g/ml
691 spectinomycin and resistant colonies were then inoculated into liquid EMJH medium
692 supplemented with spectinomycin for further analysis. The restoration of *PerRB* expression in
693 the complemented *perRB* mutant was verified by RT-qPCR.

694 The double *perRAperRB* mutant (Δ *perRA*, *perRB::Km^R*) complementation required the
695 construction of a gentamycin resistant pMaORI complementation vector. A gentamycin
696 resistant cassette [67] was cloned into the pMaORI between the NotI and XbaI restriction
697 sites and the integrity of the gentamycin resistance cassette in the pMaORIGenta plasmid (S10
698 Table) was checked by DNA sequencing. Then, the *perRB* ORF (LIMLP_05620) together
699 with its native promoter region (248 bp upstream region) were amplified (using the F-ApaI-
700 PerR2genta and R-ApaI-PerR2genta primer set, S11 Table) from genomic DNA of *L.*
701 *interrogans* serovar Manilae strain L495 and cloned into the PCR-blunt II TOPO vector (Zero
702 Blunt TOPO PCR cloning kit, Invitrogen). The *perRB* locus was subsequently subcloned into
703 the pMaORIGenta plasmid at the ApaI restriction site. The obtained plasmid (pCZ3, S10
704 Table) or the empty pMaORIGenta plasmids were introduced in *perRAperRB* double

705 *Leptospira* mutant strain by conjugation using the *E. coli* β 2163 conjugating strain as
706 previously described [66]. *Leptospira* conjugants were selected on EMJH plates containing 8
707 μ g/ml gentamycin and resistant colonies were then inoculated into liquid EMJH medium
708 supplemented with gentamycin and spectinomycin for further analysis. The restoration of
709 *PerRB* expression in the trans-complemented double *perRAperRB* mutant was verified by RT-
710 qPCR (S4 Fig).

711

712 **Phylogenetic analyses**

713 The sequences homologous to the LIMLP_10155 (*PerRA*), LIMLP_05620 (*PerRB*),
714 LIMLP_18590 and LIMLP_04825 proteins were searched with BLASTP version 2.10.0
715 among the other *Leptospira* species (Fig 3 and S1 Table) or among the protein sequences of
716 11,070 representative genomes (Fig 2), as previously described [68]. In that case, only the
717 sequences with an e-value less than $1e-10$ and a percentage of similarity greater than 60%
718 were retained. Sequences with percent identity equal to 100% were clustered by CD-HIT
719 version 4.8.1 and only one sequence was retained. The resulting 1671 sequences were
720 subsequently aligned by MAFFT version 7.471. A phylogenetic tree was finally built with IQ-
721 TREE version 2.1.1 under the best-fit model LG + R10. A second phylogenetic tree was made
722 with a subset of sequences to improve the resolution of the separation between *PerRA* and
723 *PerRB*. The same procedure was followed, except that the best-fit model used for
724 phylogenetic reconstruction is LG + R5. Both trees were visualized with FigTree version
725 1.4.4 (<https://github.com/rambaut/figtree>).

726

727 **RNA purification**

728 Virulent *L. interrogans* serovar Manilae strain L495 and *perRB* (M1474) mutant strains with
729 less than three *in vitro* passages were used in this study. Four independent biological

730 replicates of exponentially grown *L. interrogans* WT, *perRB* (M1474) and double
731 *perRAperRB* mutant strains were harvested and resuspended in 1 ml TRIzol (ThermoFisher
732 Scientific) and stored at -80°C. Nucleic Acids were extracted with chloroform and
733 precipitated with isopropanol as described earlier [69]. Contaminating genomic DNA was
734 removed by DNase treatment using the RNase-free Turbo DNA-free turbo kit
735 (ThermoFisher Scientific) as described by the manufacturer. The integrity of RNAs (RIN >
736 8.0) was verified by the Agilent Bioanalyzer RNA NanoChips (Agilent technologies,
737 Wilmington, DE).

738

739 **RNA Sequencing**

740 rRNA were depleted from 0.5 µg of total RNA using the Ribo-Zero rRNA Removal Kit
741 (Bacteria) from Illumina. Sequencing libraries were constructed using the TruSeq Stranded
742 mRNA Sample preparation kit (20020595) following the manufacturer's instructions
743 (Illumina). The directional libraries were controlled on Bioanalyzer DNA1000 Chips (Agilent
744 Technologies) and concentrations measured with the Qubit dsDNA HS Assay Kit
745 (ThermoFisher). Sequences of 65 bases were generated on the Illumina Hiseq 2500
746 sequencer.

747 Bioinformatics analyses were performed using the RNA-seq pipeline from Sequana [70].
748 Reads were cleaned of adapter sequences and low-quality sequences using cutadapt version
749 1.11 [71]. Only sequences at least 25 nt in length were considered for further analysis. Bowtie
750 version 1.2.2 [72], with default parameters, was used for alignment on the reference genome
751 (*L. interrogans* serovar Manilae strain UP-MMC-NIID LP, from MicroScope Platform).
752 Genes were counted using featureCounts version 1.4.6-p3 [73] from Subreads package
753 (parameters: -t gene -g locus_tag -s 1).

754 Count data were analyzed using R version 3.5.1 [74] and the Bioconductor package DESeq2
755 version 1.20.0 [75]. The normalization and dispersion estimation were performed with
756 DESeq2 using the default parameters and statistical tests for differential expression were
757 performed applying the independent filtering algorithm. Differential expressions were
758 expressed as logarithm to base 2 of fold change (Log_2FC). A generalized linear model
759 including the replicate effect as blocking factor was set in order to test for the differential
760 expression between *Leptospira* samples. Raw p-values were adjusted for multiple testing
761 according to the Benjamini and Hochberg (BH) procedure [76] and genes with an adjusted p-
762 value lower than 0.05 and a Log_2FC higher than 1 or lower than -1 were considered
763 differentially expressed. Heat maps and Volcano plots were generated using the Galaxy
764 platform (<https://usegalaxy.eu/>).

765

766 **Quantitative RT-PCR experiments**

767 cDNA synthesis was performed with the cDNA synthesis kit (Biorad) according to the
768 manufacturer's recommendation. Quantitative PCR was conducted in triplicate with the
769 SsoFast EvaGreen Supermix (Biorad) as previously described. LIMLP_06735 was used as a
770 reference gene.

771

772 **Non-coding RNA identification**

773 Sequencing data from the *L. interrogans* WT, *perRB* (M1474) and double *perRAperRB*
774 mutant strains were processed with Trimmomatic [77] to remove low-quality bases and
775 adapter contaminations. BWA mem (version 0.7.12) was used to discard the reads matching
776 *Leptospira* rRNA, tRNA or polyA sequences and to assign the resulting reads to *Leptospira*
777 replicons. Then Rockhopper [78] was used to re-align reads corresponding to separate
778 replicons and to assemble transcripts models. The output was filtered to retain all transcripts

779 longer than 50 nucleotides not overlapping within 10 nucleotides with NCBI annotated genes
780 on the same orientation, and showing a minimum Rockhopper raw count value of 50 in at
781 least two isolates. This high-quality set of new sRNA was subjected to differential expression
782 analysis with Rockhopper, adopting a Benjamini-Hochberg adjusted P-value threshold of
783 0.01. For each non-coding RNAs, putative function was identified by BLAST using the Rfam
784 database [79].

785

786 **Infection experiments**

787 WT and mutant *L. interrogans* strains were cultivated in EMJH medium until the exponential
788 phase and counted under a dark-field microscope using a Petroff-Hauser cell. 10^4 or 10^6
789 bacteria (in 0.5 ml) were injected intraperitoneally in groups of 4-8 male 4 weeks-old Syrian
790 Golden hamsters (RjHan:AURA, Janvier Labs). Animals were monitored daily and sacrificed
791 by carbon dioxide inhalation when endpoint criteria were met (sign of distress, morbidity).

792

793 **Ethics Statement**

794 The protocol for animal experimentation was reviewed by the Institut Pasteur (Paris, France),
795 the competent authority, for compliance with the French and European regulations on Animal
796 Welfare and with Public Health Service recommendations. This project has been reviewed
797 and approved (CETEA #2016-0019) by the Institut Pasteur ethic committee for animal
798 experimentation, agreed by the French Ministry of Agriculture.

799

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813 **References**

- 814 1. Haake DA, Levett PN. Leptospirosis in humans. *Curr Top Microbiol Immunol*.
815 2015;387:65-97.
- 816 2. Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al.
817 Global Morbidity and Mortality of Leptospirosis: A Systematic Review. *PLoS Negl Trop Dis*.
818 2015;9(9):e0003898-e0003898.
- 819 3. Pijnacker R, Goris MGA, te Wierik MJM, Broens EM, van der Giessen JWB, de Rosa
820 M, et al. Marked increase in leptospirosis infections in humans and dogs in the Netherlands,
821 2014. *Eurosurveillance* [Internet]. 2016;21(17).
822 <https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2016.21.17.30211>
- 823 4. Ko AI, Goarant C, Picardeau M. *Leptospira*: the dawn of the molecular genetics era
824 for an emerging zoonotic pathogen. *Nat Rev Microbiol*. 2009;7(10):736-47.
- 825 5. Picardeau M. Virulence of the zoonotic agent of leptospirosis: still terra incognita? *Nat*
826 *Rev Microbiol*. 2017;15(5):297-307.
- 827 6. Imlay JA. Where in the world do bacteria experience oxidative stress? *Environmental*
828 *Microbiology*. 2019;21(2):521-30.
- 829 7. Winterbourn CC, Kettle AJ. Redox Reactions and Microbial Killing in the Neutrophil
830 Phagosome. *Antioxidants & Redox Signaling*. 2012;18(6):642-60.
- 831 8. Marangoni A, Accardo S, Aldini R, Guardigli M, Cavrini F, Sambri V, et al.
832 Production of reactive oxygen species and expression of inducible nitric oxide synthase in rat
833 isolated Kupffer cells stimulated by *Leptospira interrogans* and *Borrelia burgdorferi*. *World J*
834 *Gastroenterol*. 2006;12(19):3077-81.
- 835 9. Araújo AM, Reis EAG, Athanazio DA, Ribeiro GS, Hagan JE, Araujo GC, et al.
836 Oxidative stress markers correlate with renal dysfunction and thrombocytopenia in severe
837 leptospirosis. *Am J Trop Med Hyg*. 2014;90(4):719-23.
- 838 10. Erdogan HM, Karapehliyan M, Cital M, Atakisi O, Uzlu E, Unver A. Serum sialic acid
839 and oxidative stress parameters changes in cattle with leptospirosis. *Veterinary Research*
840 *Communications*. 2008;32(4):333-9.
- 841 11. Eshghi A, Lourdault K, Murray GL, Bartpho T, Sermswan RW, Picardeau M, et al.
842 *Leptospira interrogans* Catalase Is Required for Resistance to H₂O₂ and for Virulence.
843 Blanke SR, éditeur. *Infect Immun*. 2012;80(11):3892.
- 844 12. Dubbs JM, Mongkolsuk S. Peroxide-sensing transcriptional regulators in bacteria. *J*
845 *Bacteriol*. 2012;194(20):5495-503.
- 846 13. Choi H-J, Kim S-J, Mukhopadhyay P, Cho S, Woo J-R, Storz G, et al. Structural Basis
847 of the Redox Switch in the OxyR Transcription Factor. *Cell*. 2001;105(1):103-13.
- 848 14. Zheng M, Åslund F, Storz G. Activation of the OxyR Transcription Factor by
849 Reversible Disulfide Bond Formation. *Science*. 1998;279(5357):1718.
- 850 15. Herbig AF, Helmann JD. Roles of metal ions and hydrogen peroxide in modulating
851 the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA.
852 *Mol Microbiol*. 2001;41(4):849-59.
- 853 16. Jacquamet L, Traoré D a. K, Ferrer J-L, Proux O, Testemale D, Hazemann J-L, et al.
854 Structural characterization of the active form of PerR: insights into the metal-induced

- 855 activation of PerR and Fur proteins for DNA binding. *Mol Microbiol.* 2009;73(1):20-31.
- 856 17. Traoré DAK, El Ghazouani A, Ilango S, Dupuy J, Jacquamet L, Ferrer J-L, et al.
857 Crystal structure of the apo-PerR-Zn protein from *Bacillus subtilis*. *Mol Microbiol.*
858 2006;61(5):1211-9.
- 859 18. Lee J-W, Helmann JD. The PerR transcription factor senses H₂O₂ by metal-catalysed
860 histidine oxidation. *Nature.* 2006;440(7082):363-7.
- 861 19. Traoré DAK, El Ghazouani A, Jacquamet L, Borel F, Ferrer J-L, Lascoux D, et al.
862 Structural and functional characterization of 2-oxo-histidine in oxidized PerR protein. *Nat*
863 *Chem Biol.* 2009;5(1):53-9.
- 864 20. Nunoshiba T, Hidalgo E, Amábile Cuevas CF, Demple B. Two-stage control of an
865 oxidative stress regulon: the *Escherichia coli* SoxR protein triggers redox-inducible
866 expression of the soxS regulatory gene. *J Bacteriol.* 1992;174(19):6054-60.
- 867 21. Wu J, Weiss B. Two-stage induction of the soxRS (superoxide response) regulon of
868 *Escherichia coli*. *J Bacteriol.* 1992;174(12):3915-20.
- 869 22. Gu M, Imlay JA. The SoxRS response of *Escherichia coli* is directly activated by
870 redox-cycling drugs rather than by superoxide. *Mol Microbiol.* 2011;79(5):1136-50.
- 871 23. Price CW, Fawcett P, Cérémonie H, Su N, Murphy CK, Youngman P. Genome-wide
872 analysis of the general stress response in *Bacillus subtilis*. *Mol Microbiol.* 2001;41(4):757-74.
- 873 24. Lo M, Murray GL, Khoo CA, Haake DA, Zuerner RL, Adler B. Transcriptional
874 response of *Leptospira interrogans* to iron limitation and characterization of a PerR homolog.
875 *Infect Immun.* 2010;78(11):4850-9.
- 876 25. Austin FE, Barbieri JT, Corin RE, Grigas KE, Cox CD. Distribution of superoxide
877 dismutase, catalase, and peroxidase activities among *Treponema pallidum* and other
878 spirochetes. *Infect Immun.* 1981;33(2):372-9.
- 879 26. Fouts DE, Matthias MA, Adhikarla H, Adler B, Amorim-Santos L, Berg DE, et al.
880 What Makes a Bacterial Species Pathogenic?: Comparative Genomic Analysis of the Genus
881 *Leptospira*. *PLOS Neglected Tropical Diseases.* 2016;10(2):e0004403.
- 882 27. Zavala-Alvarado C, Sismeiro O, Legendre R, Varet H, Bussotti G, Bayram J, et al.
883 The transcriptional response of pathogenic *Leptospira* to peroxide reveals new defenses
884 against infection-related oxidative stress. *PLOS Pathogens.* 2020;16(10):e1008904.
- 885 28. Caux-Thang C, Parent A, Sethu R, Maïga A, Blondin G, Latour J-M, et al. Single
886 asparagine to arginine mutation allows PerR to switch from PerR box to fur box. *ACS Chem*
887 *Biol.* 2015;10(3):682-6.
- 888 29. Parent A, Caux-Thang C, Signor L, Clémancey M, Sethu R, Blondin G, et al. Single
889 glutamate to aspartate mutation makes ferric uptake regulator (Fur) as sensitive to H₂O₂ as
890 peroxide resistance regulator (PerR). *Angew Chem Int Ed Engl.* 2013;52(39):10339-43.
- 891 30. Kebouchi M, Saul F, Taher R, Landier A, Beaudeau B, Dubrac S, et al. Structure and
892 function of the *Leptospira interrogans* peroxide stress regulator (PerR), an atypical PerR
893 devoid of a structural metal-binding site. *J Biol Chem.* 2018;293(2):497-509.
- 894 31. Satou K, Shimoji M, Tamotsu H, Juan A, Ashimine N, Shinzato M, et al. Complete
895 Genome Sequences of Low-Passage Virulent and High-Passage Avirulent Variants of
896 Pathogenic *Leptospira interrogans* Serovar Manilae Strain UP-MMC-NIID, Originally
897 Isolated from a Patient with Severe Leptospirosis, Determined Using PacBio Single-Molecule

- 898 Real-Time Technology. *Genome Announc.* 2015;3(4):e00882-15.
- 899 32. Domingos RF, Vieira ML, Romero EC, Gonçalves AP, de Moraes ZM, Vasconcellos
900 SA, et al. “Features of two proteins of *Leptospira interrogans* with potential role in host-
901 pathogen interactions”. *BMC Microbiology.* 2012;12(1):50.
- 902 33. Thibeaux R, Soupé-Gilbert M-E, Kainiu M, Girault D, Bierque E, Fernandes J, et al.
903 The zoonotic pathogen *Leptospira interrogans* mitigates environmental stress through cyclic-
904 di-GMP-controlled biofilm production. *npj Biofilms and Microbiomes.* 2020;6(1):24.
- 905 34. Xiao G, Kong L, Che R, Yi Y, Zhang Q, Yan J, et al. Identification and
906 Characterization of c-di-GMP Metabolic Enzymes of *Leptospira interrogans* and c-di-GMP
907 Fluctuations After Thermal Shift and Infection. *Frontiers in Microbiology.* 2018;9:764.
- 908 35. Narayanavari SA, Lourdault K, Sritharan M, Haake DA, Matsunaga J. Role of sph2
909 Gene Regulation in Hemolytic and Sphingomyelinase Activities Produced by *Leptospira*
910 *interrogans*. *PLOS Neglected Tropical Diseases.* 2015;9(8):e0003952.
- 911 36. Caimano MJ, Sivasankaran SK, Allard A, Hurley D, Hokamp K, Grassmann AA, et
912 al. A Model System for Studying the Transcriptomic and Physiological Changes Associated
913 with Mammalian Host-Adaptation by *Leptospira interrogans* Serovar Copenhageni. *PLOS*
914 *Pathogens.* 2014;10(3):e1004004.
- 915 37. Bourhy P, Salaün L, Lajus A, Médigue C, Boursaux-Eude C, Picardeau M. A
916 Genomic Island of the Pathogen *Leptospira interrogans* Serovar Lai Can Excise from Its
917 Chromosome. *Infect Immun.* 2007;75(2):677.
- 918 38. He P, Sheng Y-Y, Shi Y-Z, Jiang X-G, Qin J-H, Zhang Z-M, et al. Genetic diversity
919 among major endemic strains of *Leptospira interrogans* in China. *BMC Genomics.*
920 2007;8:204-204.
- 921 39. Overton TW, Justino MC, Li Y, Baptista JM, Melo AMP, Cole JA, et al. Widespread
922 Distribution in Pathogenic Bacteria of Di-Iron Proteins That Repair Oxidative and Nitrosative
923 Damage to Iron-Sulfur Centers. *J Bacteriol.* 2008;190(6):2004.
- 924 40. Nambi S, Long JE, Mishra BB, Baker R, Murphy KC, Olive AJ, et al. The Oxidative
925 Stress Network of *Mycobacterium tuberculosis* Reveals Coordination between Radical
926 Detoxification Systems. *Cell Host & Microbe.* 2015;17(6):829-37.
- 927 41. Krajewska J, Modrak-Wójcik A, Arent ZJ, Więckowski D, Zolkiewski M, Bzowska
928 A, et al. Characterization of the molecular chaperone ClpB from the pathogenic spirochaete
929 *Leptospira interrogans*. *PLOS ONE.* 2017;12(7):e0181118.
- 930 42. Lourdault K, Cerqueira GM, Wunder EA, Picardeau M. Inactivation of clpB in the
931 pathogen *Leptospira interrogans* reduces virulence and resistance to stress conditions. *Infect*
932 *Immun.* 2011;79(9):3711-7.
- 933 43. Choy HA, Kelley MM, Chen TL, Møller AK, Matsunaga J, Haake DA. Physiological
934 osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular
935 matrix proteins and fibrinogen. *Infect Immun.* 2007;75(5):2441-50.
- 936 44. Castiblanco-Valencia MM, Fraga TR, Silva LB da, Monaris D, Abreu PAE, Strobel S,
937 et al. Leptospiral Immunoglobulin-like Proteins Interact With Human Complement
938 Regulators Factor H, FHL-1, FHR-1, and C4BP. *The Journal of Infectious Diseases.*
939 2012;205(6):995-1004.
- 940 45. Pappas CJ, Picardeau M. Control of Gene Expression in *Leptospira* spp. by
941 Transcription Activator-Like Effectors Demonstrates a Potential Role for LigA and LigB in

- 942 *Leptospira interrogans* Virulence. *Appl Environ Microbiol.* 2015;81(22):7888-92.
- 943 46. Adhikarla H, Wunder EA Jr, Mechaly AE, Mehta S, Wang Z, Santos L, et al. Lvr, a
944 Signaling System That Controls Global Gene Regulation and Virulence in Pathogenic
945 *Leptospira*. *Front Cell Infect Microbiol.* 2018;8:45-45.
- 946 47. Yuan Y, Crane DD, Simpson RM, Zhu Y, Hickey MJ, Sherman DR, et al. The 16-kDa
947 α -crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth
948 in macrophages. *Proc Natl Acad Sci USA.* 1998;95(16):9578.
- 949 48. Murray GL, Srikram A, Henry R, Puapairoj A, Sermswan RW, Adler B. *Leptospira*
950 *interrogans* requires heme oxygenase for disease pathogenesis. *Microbes and Infection.*
951 2009;11(2):311-4.
- 952 49. Hayashi K, Ohsawa T, Kobayashi K, Ogasawara N, Ogura M. The H₂O₂ Stress-
953 Responsive Regulator PerR Positively Regulates *srfA* Expression in *Bacillus subtilis*. *Journal*
954 *of Bacteriology.* 2005;187(19):6659–6667.
- 955 50. Palyada K, Sun Y-Q, Flint A, Butcher J, Naikare H, Stintzi A. Characterization of the
956 oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. *BMC Genomics.*
957 2009;10(1):481.
- 958 51. Mostertz J, Scharf C, Hecker M, Homuth G. Transcriptome and proteome analysis of
959 *Bacillus subtilis* gene expression in response to superoxide and peroxide stress. Vol. 150,
960 *Microbiology.* 2004. p. 497-512.
- 961 52. Zhou A, He Z, Redding-Johanson AM, Mukhopadhyay A, Hemme CL, Joachimiak
962 MP, et al. Hydrogen peroxide-induced oxidative stress responses in *Desulfovibrio vulgaris*
963 *Hildenborough*. *Environmental Microbiology.* 2010;12(10):2645-57.
- 964 53. Fuangthong M, Herbig AF, Bsat N, Helmann JD. Regulation of the *Bacillus subtilis*
965 *fur* and *perR* Genes by PerR: Not All Members of the PerR Regulon Are Peroxide Inducible.
966 *Journal of Bacteriology.* 2002;184(12):3276–3286.
- 967 54. Horsburgh MJ, Ingham E, Foster SJ. In *Staphylococcus aureus*, Fur Is an Interactive
968 Regulator with PerR, Contributes to Virulence, and Is Necessary for Oxidative Stress
969 Resistance through Positive Regulation of Catalase and Iron Homeostasis. *Journal of*
970 *Bacteriology.* 2001;183(2):468–475.
- 971 55. Kim J-H, Ji C-J, Ju S-Y, Yang Y-M, Ryu S-H, Kwon Y, et al. *Bacillus licheniformis*
972 Contains Two More PerR-Like Proteins in Addition to PerR, Fur, and Zur Orthologues.
973 *PLOS ONE.* 2016;11(5):e0155539.
- 974 56. Lee H-N, Ji C-J, Lee H-H, Park J, Seo Y-S, Lee J-W, et al. Roles of three FurA
975 paralogs in the regulation of genes pertaining to peroxide defense in *Mycobacterium*
976 *smegmatis mc2155*. *Molecular Microbiology.* 2018;108(6):661-82.
- 977 57. Grassmann AA, Zavala-Alvarado C, Bettin E, Picardeau M, Benaroudj N, Caimano
978 MJ. The Fur-like regulators PerRA and PerRB control a complex signaling network required
979 for mammalian host-adaptation and virulence of *Leptospira interrogans*. submitted. 2020;
- 980 58. Brenot A, King KY, Caparon MG. The PerR regulon in peroxide resistance and
981 virulence of *Streptococcus pyogenes*. *Molecular Microbiology.* 2005;55(1):221-34.
- 982 59. Rea RB, Gahan CGM, Hill C. Disruption of putative regulatory loci in *Listeria*
983 *monocytogenes* demonstrates a significant role for Fur and PerR in virulence. *Infect Immun.*
984 2004;72(2):717-27.
- 985 60. Ricci S, Janulczyk R, Björck L. The Regulator PerR Is Involved in Oxidative Stress

- 986 Response and Iron Homeostasis and Is Necessary for Full Virulence of *Streptococcus*
987 *pyogenes*. *Infect Immun*. 2002;70(9):4968.
- 988 61. Wu H-J, Seib KL, Srikhanta YN, Kidd SP, Edwards JL, Maguire TL, et al. PerR
989 controls Mn-dependent resistance to oxidative stress in *Neisseria gonorrhoeae*. *Molecular*
990 *Microbiology*. 2006;60(2):401-16.
- 991 62. Stewart GR, Newton SM, Wilkinson KA, Humphreys IR, Murphy HN, Robertson BD,
992 et al. The stress-responsive chaperone α -crystallin 2 is required for pathogenesis of
993 *Mycobacterium tuberculosis*. *Molecular Microbiology*. 2005;55(4):1127-37.
- 994 63. Ellinghausen HC, McCullough WG. Nutrition of *Leptospira pomona* and growth of 13
995 other serotypes: a serum-free medium employing oleic albumin complex. *Am J Vet Res*.
996 1965;26:39-44.
- 997 64. Picardeau M, Brenot A, Saint Girons I. First evidence for gene replacement in
998 *Leptospira* spp. Inactivation of *L. biflexa* *flaB* results in non-motile mutants deficient in
999 endoflagella. *Molecular Microbiology*. 2001;40(1):189-99.
- 1000 65. Pappas CJ, Benaroudj N, Picardeau M. A Replicative Plasmid Vector Allows Efficient
1001 Complementation of Pathogenic *Leptospira* Strains. *Parasites*, éditeur. *Appl Environ*
1002 *Microbiol*. 2015;81(9):3176.
- 1003 66. Picardeau M. Conjugative transfer between *Escherichia coli* and *Leptospira* spp. as a
1004 new genetic tool. *Appl Environ Microbiol*. 2008;74(1):319-22.
- 1005 67. Poggi D, Oliveira de Giuseppe P, Picardeau M. Antibiotic resistance markers for
1006 genetic manipulations of *Leptospira* spp. *Appl Environ Microbiol*. 2010;76(14):4882-5.
- 1007 68. Vincent AT, Schiettekatte O, Goarant C, Neela VK, Bernet E, Thibeaux R, et al.
1008 Revisiting the taxonomy and evolution of pathogenicity of the genus *Leptospira* through the
1009 prism of genomics. *PLoS Negl Trop Dis*. 2019;13(5):e0007270-e0007270.
- 1010 69. Zavala-Alvarado C, Benaroudj N. The Single-Step Method of RNA Purification
1011 Applied to *Leptospira*. *Methods Mol Biol*. 2020;2134:41-51.
- 1012 70. Cokelaer, T, Desvillechabrol, D, Legendre, R, Cardon, M. ‘Sequana’: a Set of
1013 Snakemake NGS pipelines. *The Journal of Open Source Software*. 2(16).
- 1014 71. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing
1015 reads. *EMBnet.journal*; Vol 17, No 1: Next Generation Sequencing Data Analysis DO -
1016 1014806/ej171200 [Internet]. 2011;
1017 <http://journal.embnet.org/index.php/embnetjournal/article/view/200>
- 1018 72. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient
1019 alignment of short DNA sequences to the human genome. *Genome Biology*. 2009;10(3):R25.
- 1020 73. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for
1021 assigning sequence reads to genomic features. *Bioinformatics*. 2013;30(7):923-30.
- 1022 74. R core Team. R, a language and environment for statistical computing [Internet].
1023 2016. [https://www.gbif.org/en/tool/81287/r-a-language-and-environment-for-statistical-](https://www.gbif.org/en/tool/81287/r-a-language-and-environment-for-statistical-computing)
1024 [computing](https://www.gbif.org/en/tool/81287/r-a-language-and-environment-for-statistical-computing)
- 1025 75. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for
1026 RNA-seq data with DESeq2. *Genome Biology*. 2014;15(12):550.
- 1027 76. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and
1028 Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B*

- 1029 (Methodological). 1995;57(1):289-300.
- 1030 77. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina
1031 sequence data. *Bioinformatics*. 2014;30(15):2114-20.
- 1032 78. McClure R, Balasubramanian D, Sun Y, Bobrovskyy M, Sumbly P, Genco CA, et al.
1033 Computational analysis of bacterial RNA-Seq data. *Nucleic Acids Res*.
1034 2013;41(14):e140-e140.
- 1035 79. Kalvari I, Argasinska J, Quinones-Olvera N, Nawrocki EP, Rivas E, Eddy SR, et al.
1036 Rfam 13.0: shifting to a genome-centric resource for non-coding RNA families. *Nucleic
1037 Acids Research*. 2017;46(D1):D335-42.
- 1038 80. Madeira F, Park Y mi, Lee J, Buso N, Gur T, Madhusoodanan N, et al. The EMBL-
1039 EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research*.
1040 2019;47(W1):W636-41.
- 1041 81. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. The Phyre2 web portal
1042 for protein modeling, prediction and analysis. *Nature Protocols*. 2015;10(6):845-58.
1043

1044

1045 **Supporting information**

1046

1047 **S1 Fig. Phylogenetic analysis of the four Fur-like regulators of *L. interrogans*.**

1048 Extended phylogenetic tree showing the separation between PerRA (red) and PerRB (blue).

1049

1050 **S2 Fig. Growth of the *L. interrogans* *perRA* and *perRB* mutants in the presence of H₂O₂.**

1051 *L. interrogans* WT (black circles), *perRA* (cyan up-pointing triangles) and *perRB* (green
1052 down-pointing triangles) mutant strains were cultivated in EMJH medium at 30°C in the
1053 absence (A) or presence of 2 mM H₂O₂ (B). *Leptospira* growth was assessed by absorbance at
1054 420 nm. Data are means and standard errors of three independent biological experiments.

1055

1056 **S3 Fig. Analysis of the *L. interrogans* PerRB regulon.**

1057 (A) Venn diagram showing the overlap of differentially-expressed ORFs (with an adjusted p-
1058 value < 0.05) in the *perRA* and *perRB* mutants. Differentially-expressed genes in the *perRB*
1059 mutant (as determined in this study) (in green) were compared with those in the *perRA* mutant
1060 as determined previously [27] (in cyan). The down- and up-regulated ORFs in both mutants
1061 were indicated in blue and red, respectively. (B) Comparison of differentially-expressed
1062 ORFs (with an adjusted p-value < 0.05) in the *perRB* mutant and upon *L. interrogans*
1063 exposure to H₂O₂. Log₂FC of differentially-expressed ORFs upon *L. interrogans* exposure to
1064 1 mM H₂O₂ (as determined previously [27]) was plotted against the Log₂FC of differentially-
1065 expressed ORFs upon *perRB* inactivation. Down- and up-regulated ORFs in the *perRB* mutant
1066 were represented by blue and red symbols, respectively, and the name of selected ORFs was
1067 indicated. The dashed lines indicate a Log₂FC value of zero. Please note that only
1068 differentially-expressed ORFs in both conditions were considered.

1069

1070 **S4 Fig. Characterization and complementation of the double *perRAperRB* mutant strain.**

1071 (A) Schematic representation of the double *perRAperRB* mutant construction. *PerRA*
1072 (LIMLP_10155) was inactivated by allelic exchange in the transposon *perRB* mutant. The
1073 kanamycin (Km) and spectinomycin (Spc) resistance cassettes inactivating *perRB* and *perRA*,
1074 respectively, are indicated. (B) Production of PerRA in the WT, in the single *perRA* and
1075 *perRB* mutants and in the double *perRAperRB* mutant strains. *L. interrogans* strains were
1076 cultivated in EMJH medium at 30°C until the logarithmic phase and lyzed by sonication. 10
1077 µg of total lysates were resolved on a 15% SDS-PAGE and transferred on nitrocellulose
1078 membrane. PerRA was detected by immunoblot using a 1/2000 antibody dilution as described
1079 previously [30]. (C) *PerRB* expression in the WT, in the double *perRAperRB* mutant and in
1080 the trans-complemented *perRAperRB* mutant with the *perRB* ORF. RNAs were extracted
1081 from exponentially-grown *L. interrogans* strains and *perRB* expression was assessed by RT-
1082 qPCR in triplicate using *flaB* gene (LIMLP_09410) as reference gene. *PerRB* expression in
1083 the *perRAperRB* and trans-complemented mutant strains were normalized against that in the
1084 WT strain. (D) Growth of stationary phase-adapted WT, *perRAperRB* and trans-
1085 complemented *perRAperRB* mutant strains. *L. interrogans* WT (black circles), *perRAperRB*
1086 mutant (pink squares) and *perRAperRB* mutant trans-complemented with the *perRB* ORF
1087 (blue triangles) were cultivated in EMJH medium at 30°C until late stationary phase (7 days
1088 after the entry in the stationary phase) and used to inoculate EMJH medium. Bacteria were
1089 then cultivated at 30°C and growth was assessed by absorbance at 420 nm. Data are means
1090 and standard errors of three independent biological experiments.

1091

1092 **S5 Fig. RT-qPCR experiments in the double *perRAperRB* mutant.**

1093 RNAs were extracted from exponentially-grown *L. interrogans* strains WT or double

1094 *perRAperRB* mutant (*m*). Expression of the indicated genes was measured by RT-qPCR using
1095 the LIMLP_06735 as reference gene. Gene expression in the *perRAperRB* mutant was
1096 normalized against that in the WT strain. Fold change values are indicated in blue. Statistical
1097 significance was determined by a Two-way Anova test in comparison with the WT samples.
1098 ****, p-value<0.0001; **, p-value<0.005.

1099

1100 **S1 Table. Distribution of the four Fur-like regulators of *Leptospira interrogans* in the**
1101 **genus *Leptospira*.**

1102

1103 **S2 Table. Complete set of ORF expression in *Leptospira interrogans* WT and M1474**
1104 ***perRB* mutant.**

1105

1106 **S3 Table. Complete set of ORF expression in *Leptospira interrogans* WT and double**
1107 ***perRAperRB* mutant.**

1108

1109 **S4 Table. Selected down-regulated genes in the *perRAperRB* double mutant.**

1110

1111 **S5 Table. Selected up-regulated genes in the *perRAperRB* double mutant.**

1112

1113 **S6 Table. Complete set of differentially-expressed predicted non coding RNAs in the**
1114 ***perRB* (M1474) and in the double *perRAperRB* mutant strains of *Leptospira interrogans*.**

1115

1116 **S7 Table. Selected differentially-expressed non-coding RNAs in the *perRB* mutant**

1117

1118 **S8 Table. Selected differentially-expressed non-coding RNAs in the *perRAperRB* mutant.**

1119

1120 **S9 Table. Strains used in this study**

1121

1122 **S10 Table. Plasmids used in this study**

1123

1124 **S11 Table. Primers used in this study**

1125

1126

1127 **Figure legends**

1128

1129 **Fig 1. Analysis of the four Fur-like regulators of *L. interrogans*.**

1130 (A) Schematic representation of the domain organization of a typical Fur-like regulator. The
1131 N-terminal DNA binding domain and the C-terminal dimerization domain are represented in
1132 grey and golden, respectively. The α -helix and β -strand secondary structures are indicated
1133 below in green and blue, respectively. The His, Asp and Glu residues involved in regulatory
1134 metal coordination are designated in green. The Arg/Asn residue involved in DNA binding
1135 specificity is marked in red. The Arg/Asn (involved in DNA binding specificity) and Asp/Glu
1136 residues (involved in H₂O₂ sensitivity) that allow distinguishing a Fur from a PerR are further
1137 emphasized with a grey arrow head. The two cysteinate motifs in the C-terminal domain
1138 involved in structural metal coordination are represented by the double blue lines in the C-
1139 terminal dimerization domain. (B) Comparison of the four Fur-like regulators of *L.*
1140 *interrogans* (LIMLP_10155, LIMLP_05620, LIMLP_04825, LIMLP_18590) with *B. subtilis*
1141 Fur and PerR. Primary sequence alignment was obtained by Clustal Omega
1142 (<https://www.ebi.ac.uk/Tools/msa/clustalo/>; [80]). The H4 DNA binding helix is underlined
1143 and the Arg/Asn residue involved in DNA binding specificity is designated in red. The
1144 residues of the regulatory metal coordination, including the Asp/Glu residue involved in H₂O₂
1145 sensitivity, are marked in green and indicated with an asterisk. The Arg/Asn and Asp/Glu
1146 residues that allow distinguishing a Fur from a PerR are further emphasized with a grey arrow
1147 head. The cysteine residues of the structural metal coordination are marked in cyan. (C)
1148 Cartoon representation of the crystal structure of LIMLP_10155 (5NL9) and of the modeled
1149 structure of LIMLP_05620, LIMLP_04825 and LIMLP_18590. The modeled structures were
1150 obtained by searching homologies between LIMLP_05620, LIMLP_04825 and

1151 LIMLP_18590 and protein with known crystal structure using PHYRE2
1152 (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>; [81]). Secondary structures are
1153 numbered as in (A).

1154

1155 **Fig 2.** (A) Phylogenetic tree with a cartoon representation showing the distribution of the
1156 1671 sequences putatively homologous to the LIMLP_10155 (PerRA, cyan triangle),
1157 LIMLP_05620 (PerRB, green triangle), LIMLP_18590 (yellow triangle) and LIMLP_04825
1158 (red triangle) proteins. The gray triangles represent groups which are not monophyletic with
1159 the *Leptospira* sequences and which may therefore originate from other types of PerR or have
1160 had a species-specific evolution. (B) Phylogenetic tree showing the separation between
1161 PerRA (cyan) and PerRB (green).

1162

1163 **Fig 3. Distribution of the four Fur-like regulators of *L. interrogans* in the genus**
1164 ***Leptospira*.**

1165 Circular phylogenetic tree with inner circles indicating the homology between each Fur-like
1166 regulator of *L. interrogans* with the closest homolog among representative genomes of
1167 *Leptospira* species. The branches are colored according to their classification into the four
1168 main subclades with P1 (highly pathogenic) in red, P2 (intermediates) in magenta, S1
1169 (saprophytes) in yellow and S2 (new clade saprophytes) in blue [68]. The inner circles are,
1170 from the inside to the outside, LIMLP_10155, LIMLP_05620, LIMLP_04825 and
1171 LIMLP_18590. The green color gradient indicates the degree of homology (See S1 Table),
1172 grey and black indicate the presence of a false positive and the absence of orthologs,
1173 respectively.

1174

1175 **Fig 4. Increased *PerRA* and *perRB* expression upon exposure to hydrogen peroxide.**

1176 Exponentially growing *L. interrogans* were incubated in the absence or presence of 10 μ M
1177 (for 30 min.) or 1 mM H₂O₂ (for 60 min.) and *perRA* (cyan circles) and *perRB* (green squares)
1178 expression was measured by RT-qPCR as described in Material and Methods section. Gene
1179 expression was normalized by that in untreated samples. Data are the means and standard
1180 errors of three independent biological replicates. P-values obtained by a Two-way Anova test
1181 indicates the statistical significance in comparison with untreated samples.

1182

1183 **Fig 5. Effect of *perRB* inactivation on *Leptospira* growth in the presence of superoxide-**
1184 **generating paraquat.**

1185 *L. interrogans* WT containing the empty pMaORI vector (black circles), the *perRB* mutant
1186 containing the empty pMaORI vector (green triangles) or the *perRB* mutant containing the
1187 pMaORI vector expressing LIMLP_05620 (red squares) were cultivated in EMJH medium in
1188 the absence (A) or in the presence of 2 μ M Paraquat (B). Growth was assessed by measure of
1189 absorbance at 420 nm. Data are means and standard errors of three independent biological
1190 replicates.

1191

1192 **Fig 6. Effect of concomitant inactivation of *perRA* and *perRB* on *Leptospira* growth in**
1193 **the presence of ROS and virulence.**

1194 *L. interrogans* WT (black circles), the single *perRA* mutant (cyan up-pointing triangles), the
1195 single *perRB* mutants (green down-pointing triangles) or the double *perRAperRB* mutant
1196 (pink squares) were cultivated in EMJH medium in the absence (A), or in the presence of 2
1197 mM H₂O₂ (B) or of 2 μ M paraquat (C). Complementation of the double *perRAperRB* mutant
1198 with *perRB* ORF (presented in (D)) was performed by cultivating *L. interrogans* WT
1199 containing the empty pMaORIGenta vector (black circles), the double *perRAperRB* mutant
1200 containing the empty pMaORIGenta vector (pink squares) or the double *perRAperRB* mutant

1201 containing the pMaORIGenta vector expressing LIMLP_05620 (blue triangles) in the
1202 presence of 2 μ M paraquat. Growth was assessed by measure of absorbance at 420 nm and
1203 the data are means and standard errors of three independent biological replicates. (E)
1204 Virulence was assessed by infecting hamsters (n=4) by peritoneal route with 10^4 of WT
1205 (black circles), single *perRA* or *perRB* mutants (cyan and green triangles, respectively), or the
1206 double *perRAperRB* mutant (pink squares) as described in Material and Methods section. (F)
1207 Complementation of the double *perRAperRB* mutant with *perRB* was performed by infecting
1208 hamsters (n=8) by peritoneal route with 10^6 of WT containing the empty pMaORIGenta vector
1209 (black circles), of the double *perRAperRB* mutant containing the empty pMaORIGenta vector
1210 (pink squares) or the double *perRAperRB* mutant containing the pMaORIGenta vector
1211 expressing LIMLP_05620 (blue triangles).

1212

1213 **Fig 7. Differential gene expression in the *perRAperRB* mutant.**

1214 (A) Venn diagram showing the overlap of differentially-expressed ORFs (with an adjusted p-
1215 value < 0.05) in the double *perRAperRB* mutant (in pink) with those of the *perRA* mutant (as
1216 determined by Zavala-Alvarado *et al.* [27]) (in cyan) and of the *perRB* mutant (as determined
1217 in this study) (in green). (B)-(D) Volcano scatter representation of differentially-expressed
1218 genes in the *perRAperRB* mutant (B), in the single *perRA* mutant (as determined by Zavala-
1219 Alvarado *et al.* [27]) (C), and in the single *perRB* mutant (as determined in this study) (D).
1220 Red and blue dots indicate significantly up- and down-regulated genes, respectively, with a
1221 Log_2FC cutoff of ± 1 (dashed vertical lines) and p-value<0.05. Selected genes are labelled.

1222

1223 **Fig 8. Comparison of differential gene expression in the double *perRAperRB* mutant**
1224 **with that in the single *perRA* and *perRB* mutants.**

1225 Expression of selected genes of the TonB-dependent transport cluster (A), involved in

1226 oxidative stress and redox homeostasis (B), in regulation and signaling (C), and in virulence
1227 (D) determined by RNASeq in the double *perRAperRB* mutant was compared to that in the
1228 single *perRA* mutant determined by Zavala-Alvarado *et al.* [27] and single *perRB* mutant (as
1229 determined in this study). Differential expression in each mutant strain was normalized with
1230 that in the WT strain. Gene names are indicated on the right. The Heat Map color from blue to
1231 red indicates low to high Log₂FC.

1232

1233 **Fig 9. Complementation of the *perRAperRB* mutant with *perRB* restores expression of**
1234 **virulence associated genes.**

1235 The WT containing the empty pMaORIGenta vector (black circles), the *perRAperRB* mutant
1236 containing the empty pMaORIGenta vector (pink circles) and the complemented *perRAperRB*
1237 mutant (blue circles) strains were cultivated in EMJH medium until the exponential phase.
1238 Cells were harvested and RNAs were purified, and the expression of *lvrA*, *lvrB*, *ligA*, *ligB*,
1239 *clpB*, *hsp20* and *hsp15* was measured by RT-qPCR. Gene expression was normalized by that
1240 in the WT strains. Data are the means and standard errors of three independent biological
1241 replicates. P-values obtained by a Two-way Anova test indicates the statistical significance in
1242 comparison with WT samples.

1243

1244 **Fig 10. Non-coding RNAs expression in the double *perRAperRB* mutant.**

1245 Differential expression of selected ncRNAs (LepncRNA38, 49, 105, and 130) in the
1246 *perRAperRB* mutant (determined in this study) (a) was compared to those in the single *perRA*
1247 mutant, as determined by Zavala-Alvarado *et al.* [27] (b), in the single *perRB* mutant
1248 (determined by this study) (a), and upon exposure to 1 mM H₂O₂ for 1h00 as determined by
1249 Zavala-Alvarado *et al.* [27] (b). The location of the ncRNAs LepncRNA38, 49, 105, and 130
1250 were represented schematically with the adjacent or overlapping ORFs. The values indicate

1251 the Log_2FC of ncRNAs expression normalized with that in WT and the respective expression
1252 of these ORF (Log_2FC) are indicated into parenthesis with the color corresponding to that of
1253 the ORF in the cartoon. NSC, non-significantly changed.

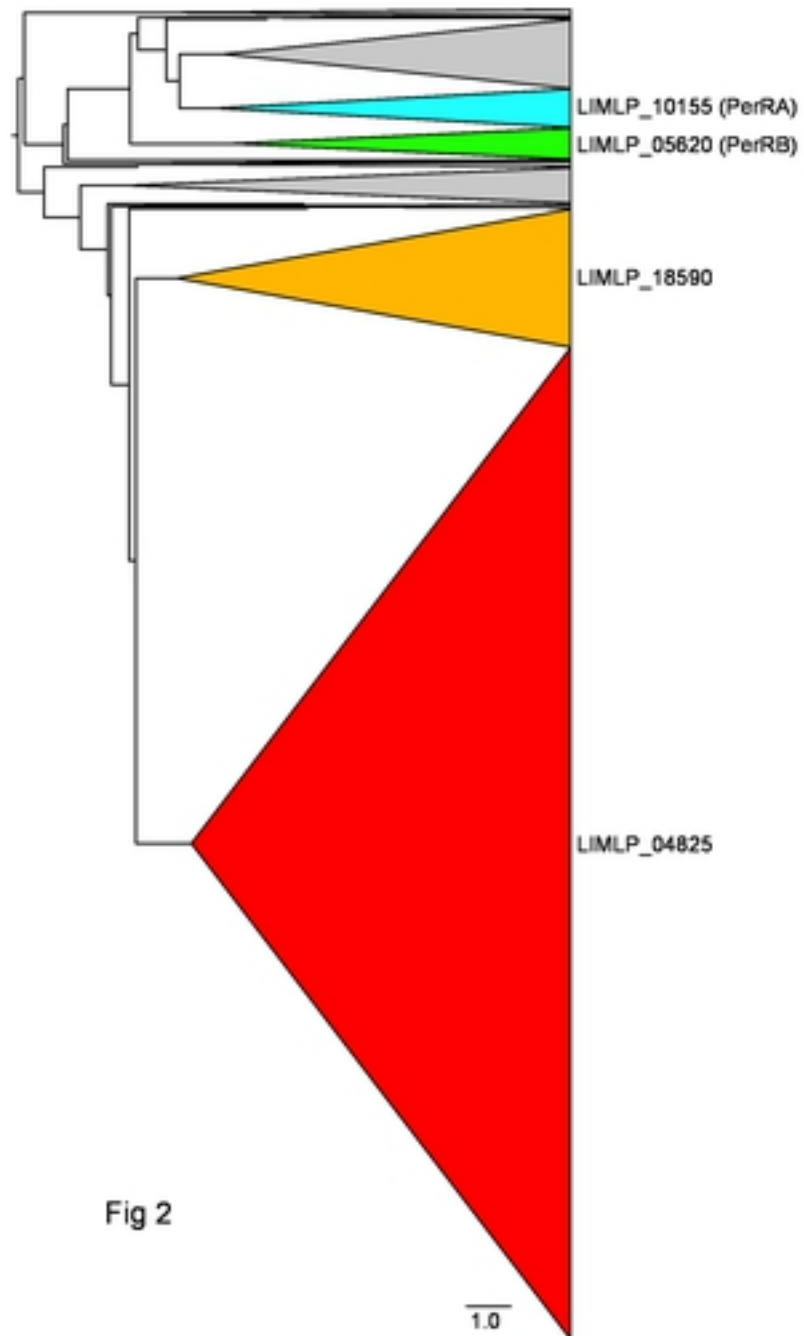
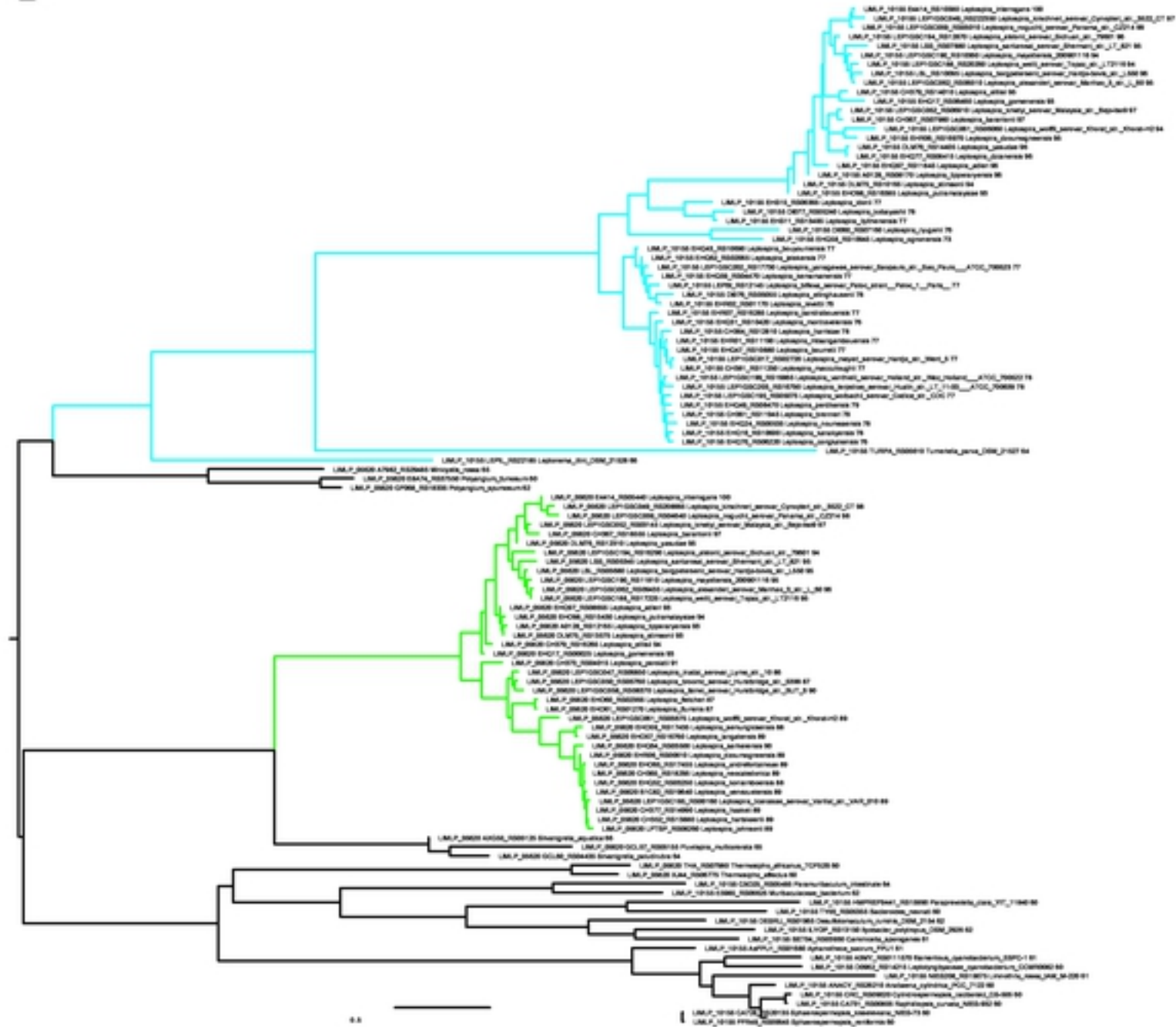
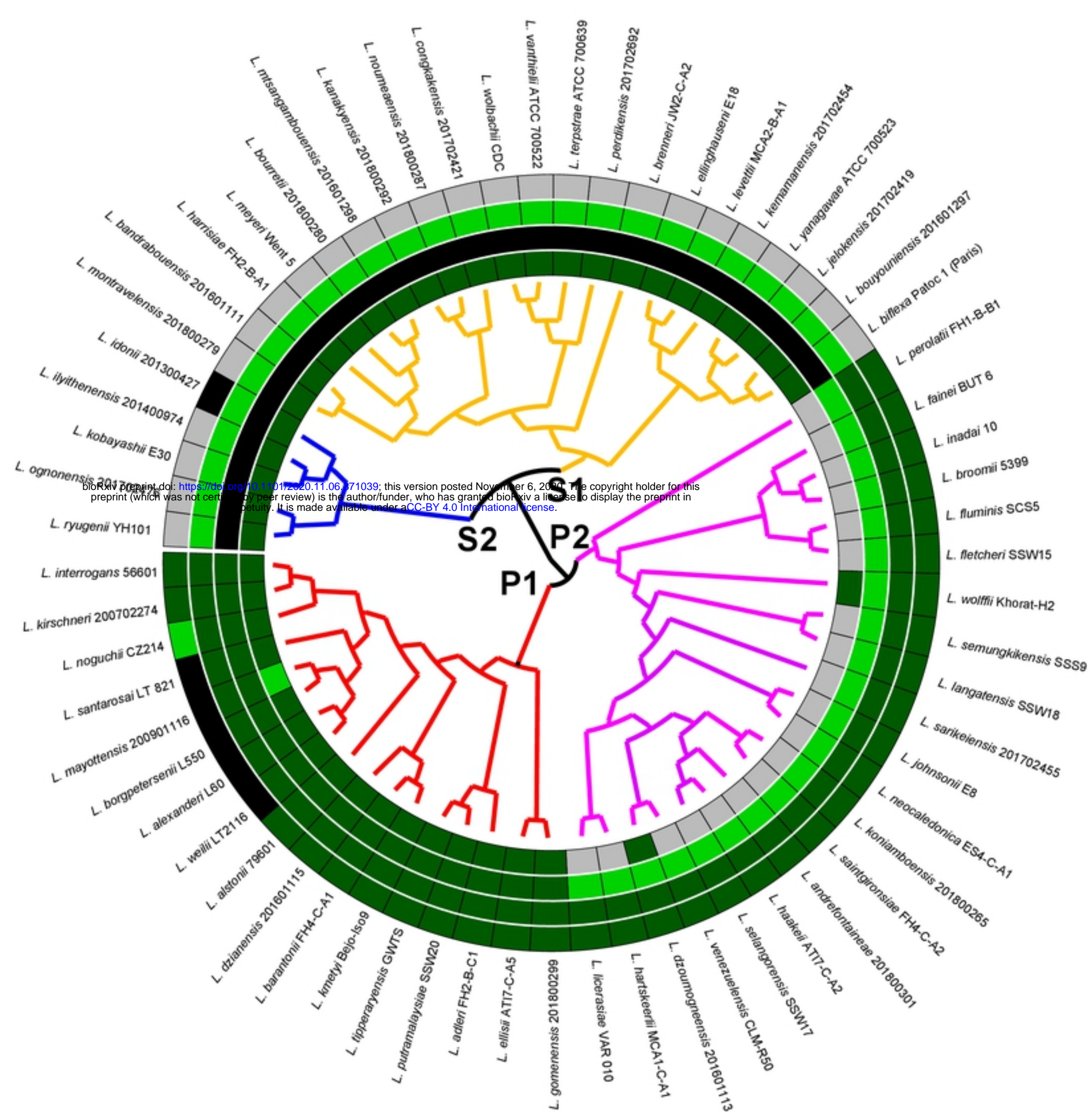
A**B**

Fig 2



- Present
- Present, but may have a structural difference
- False positive
- Absent

Fig 3

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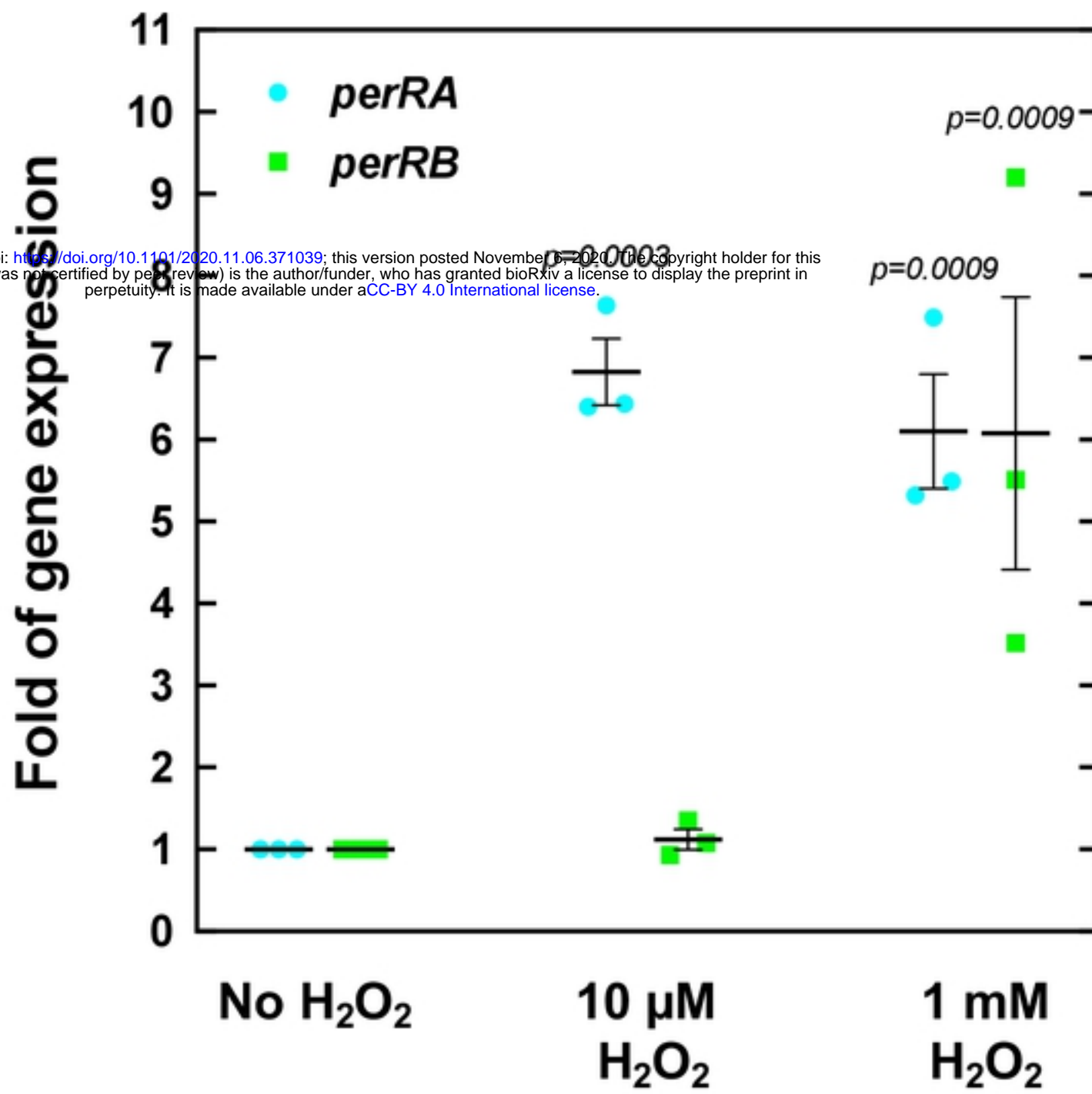


Fig 4

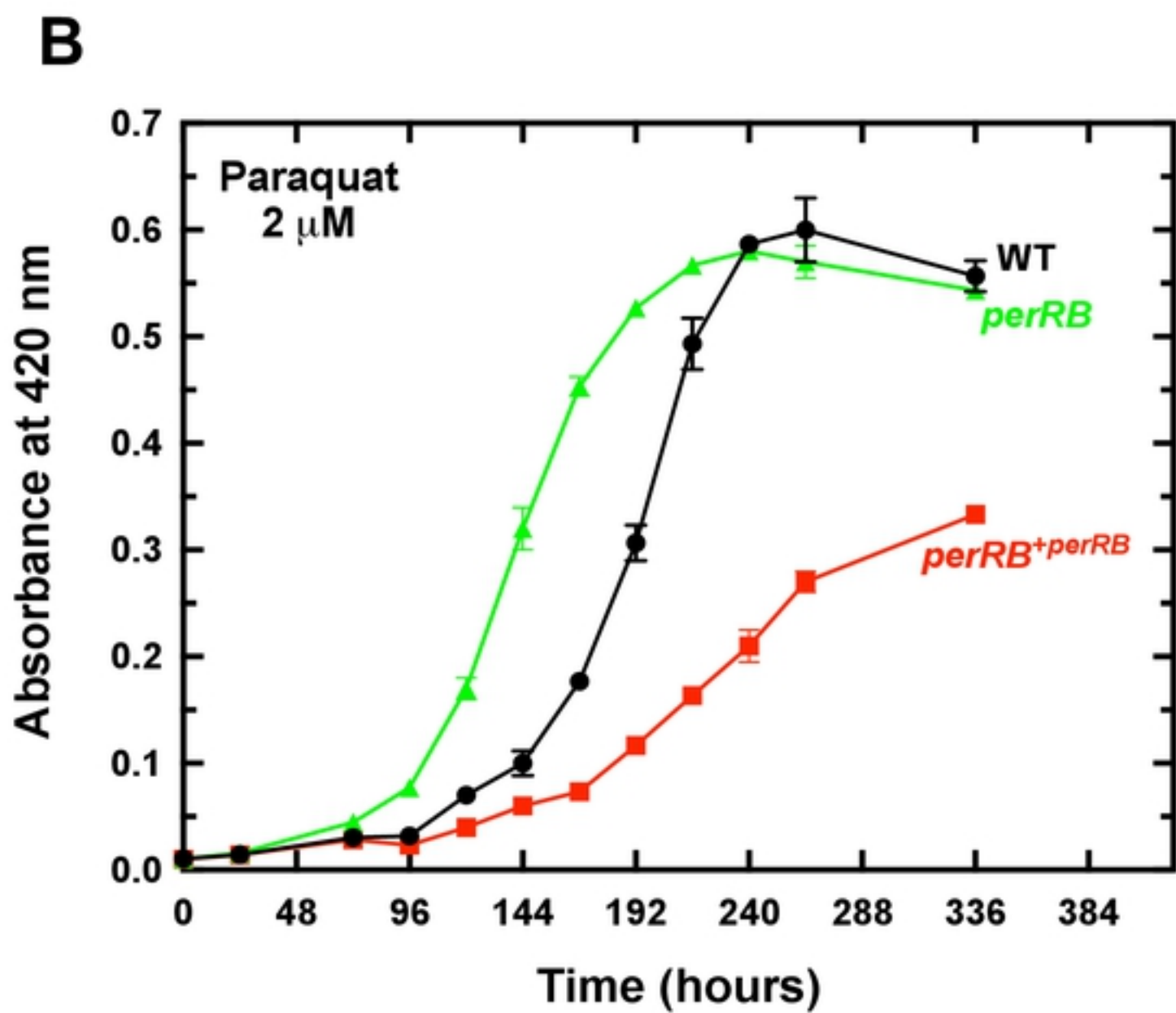
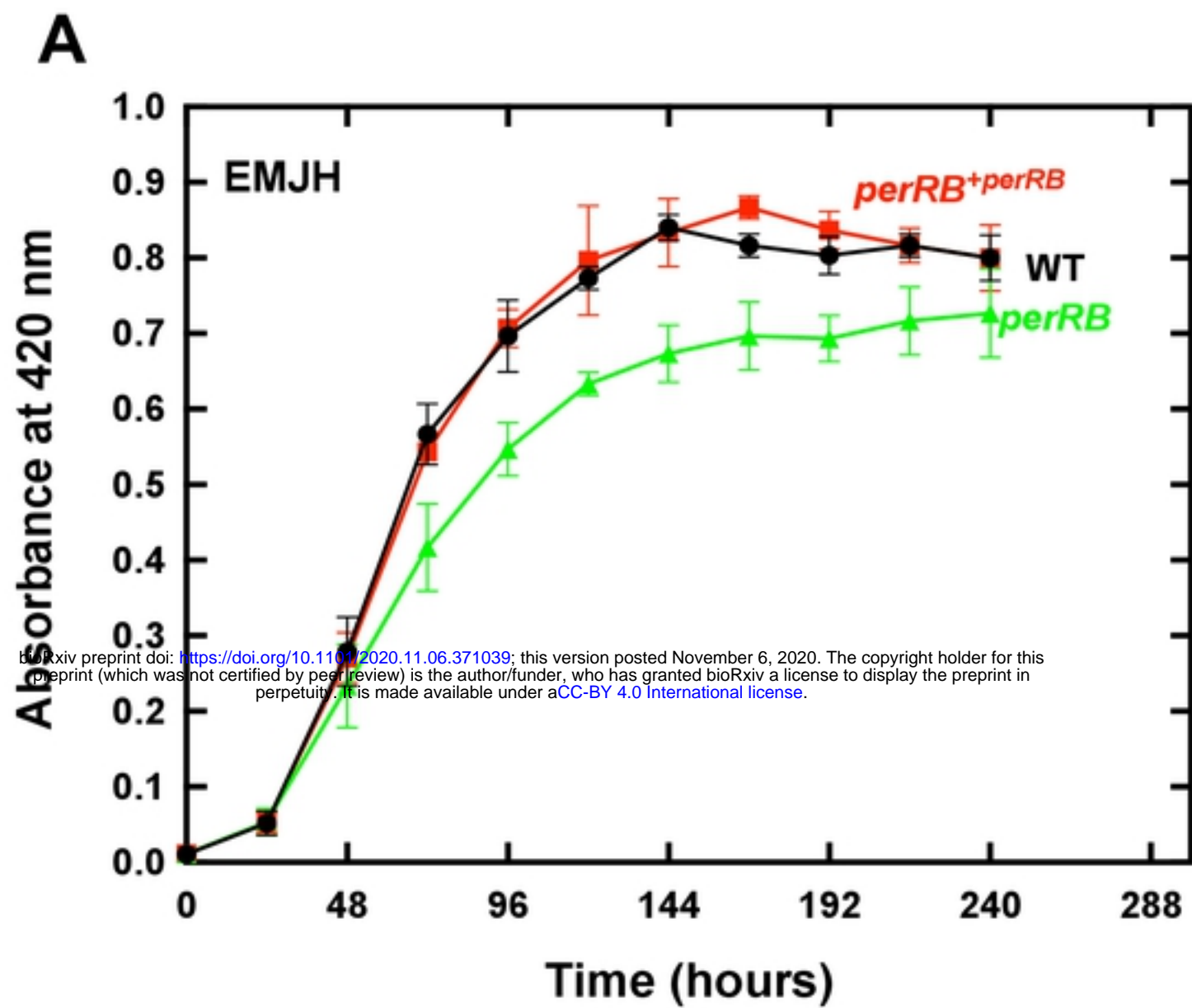


Fig 5

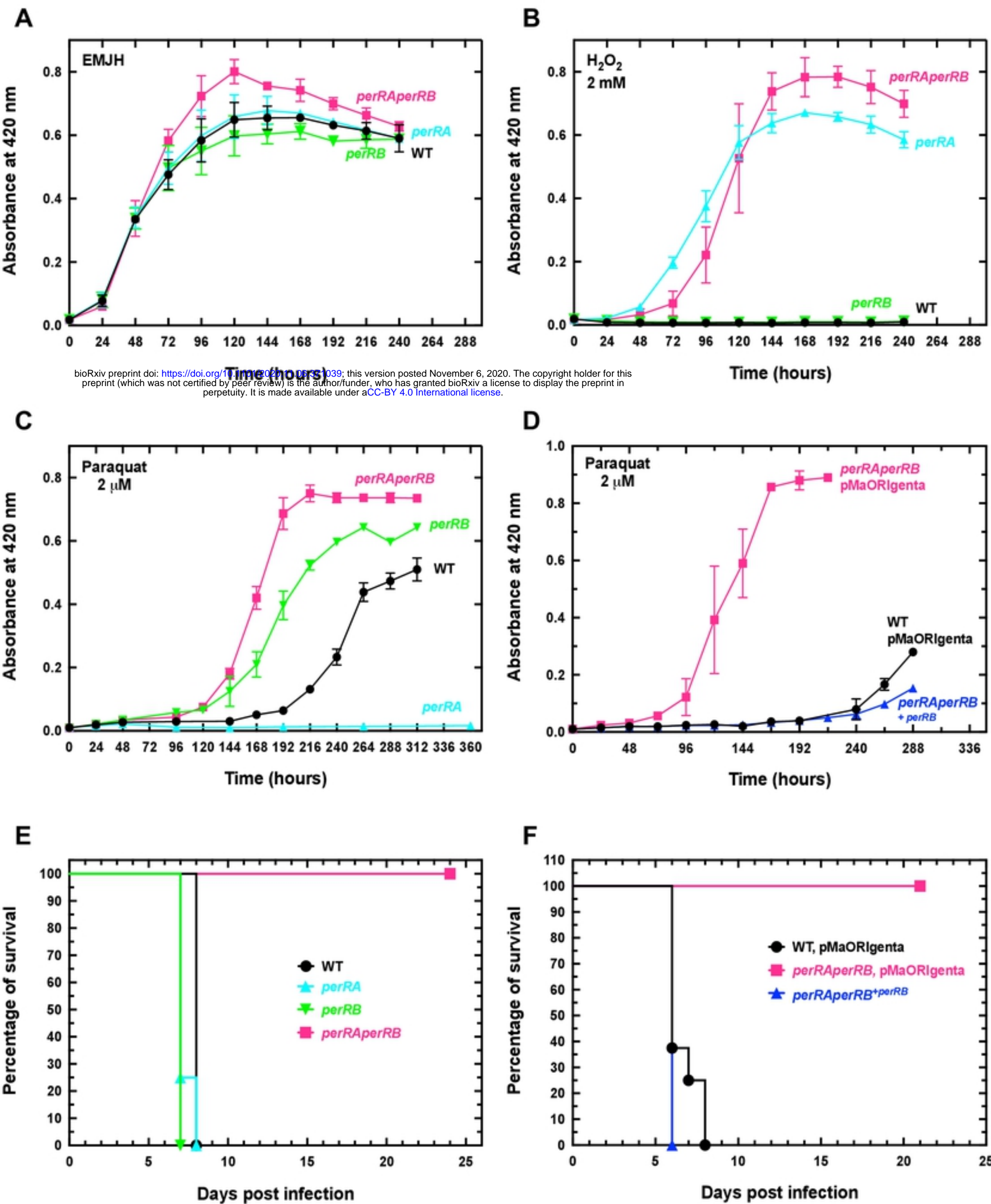
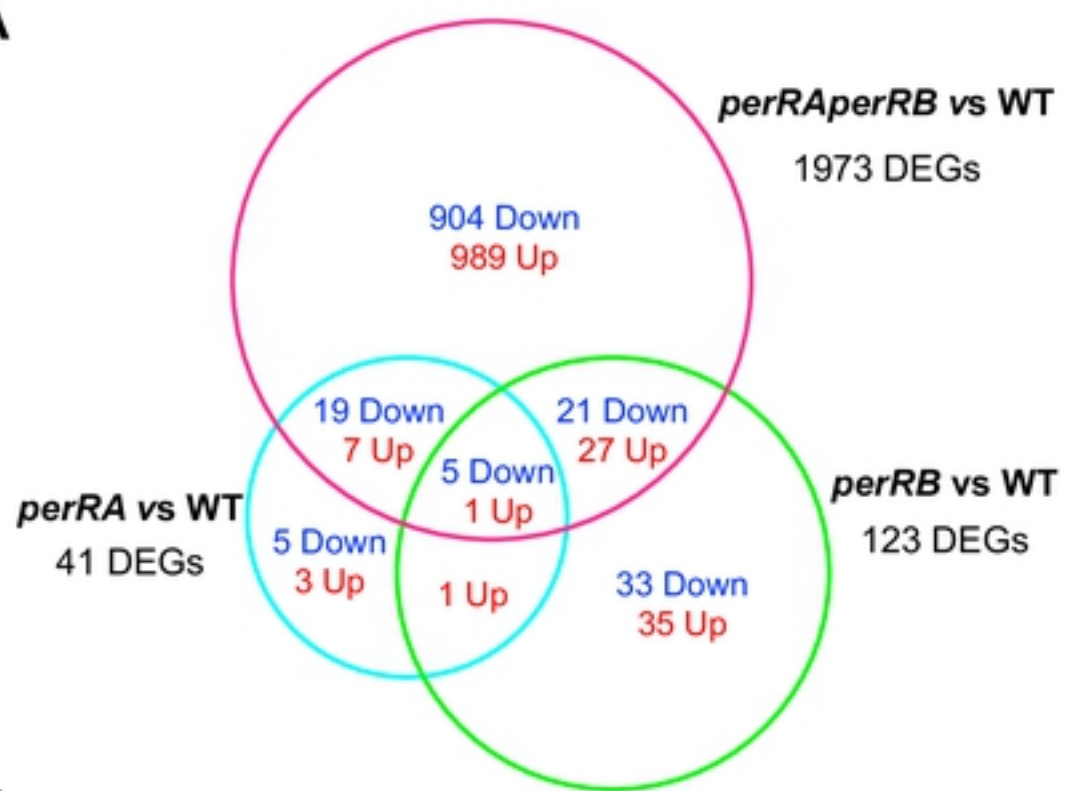
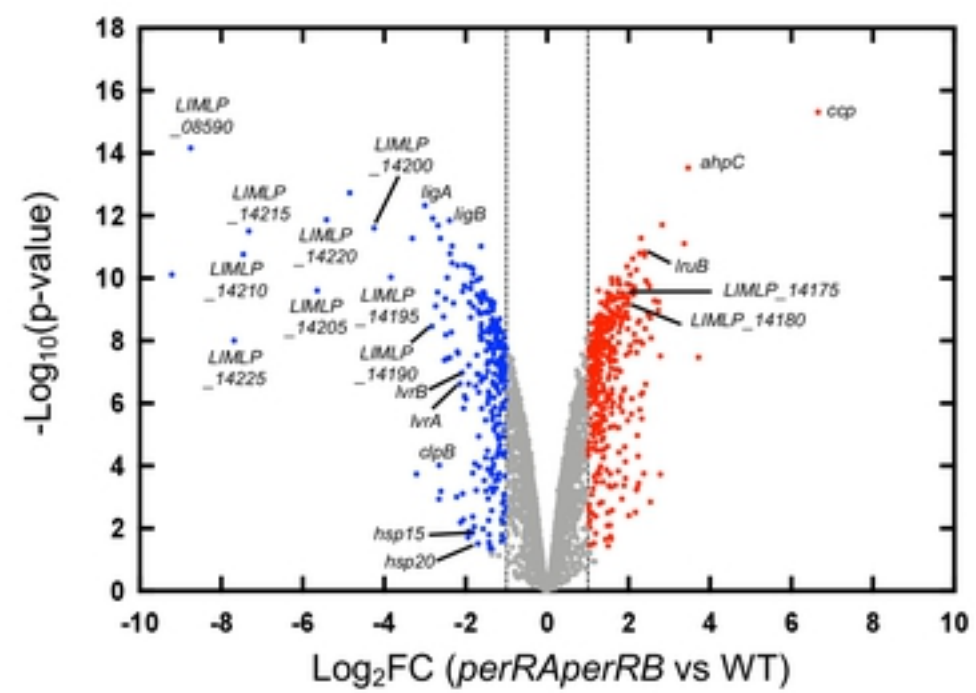
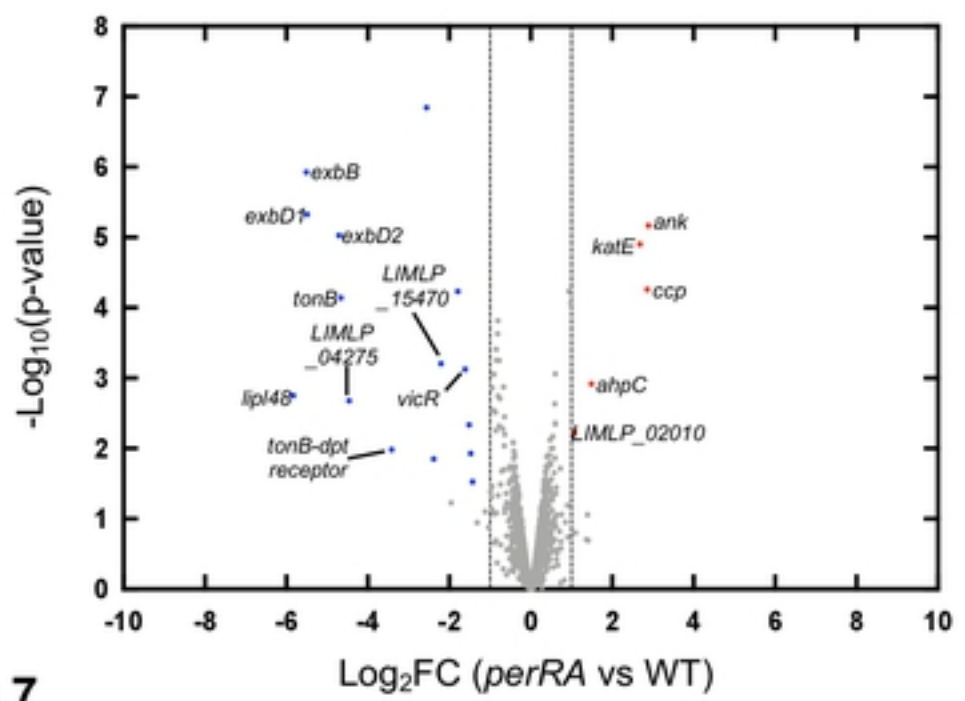
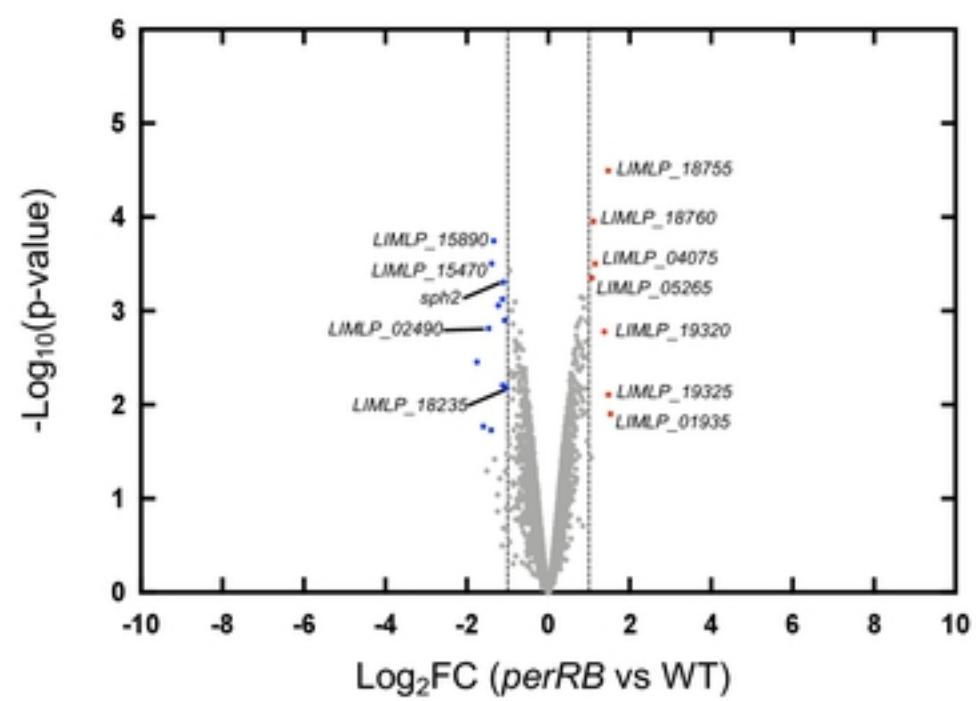


Fig 6

A**B****C****D****Fig 7**

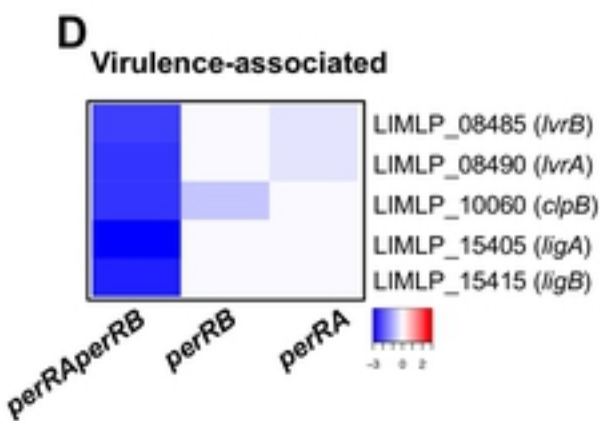
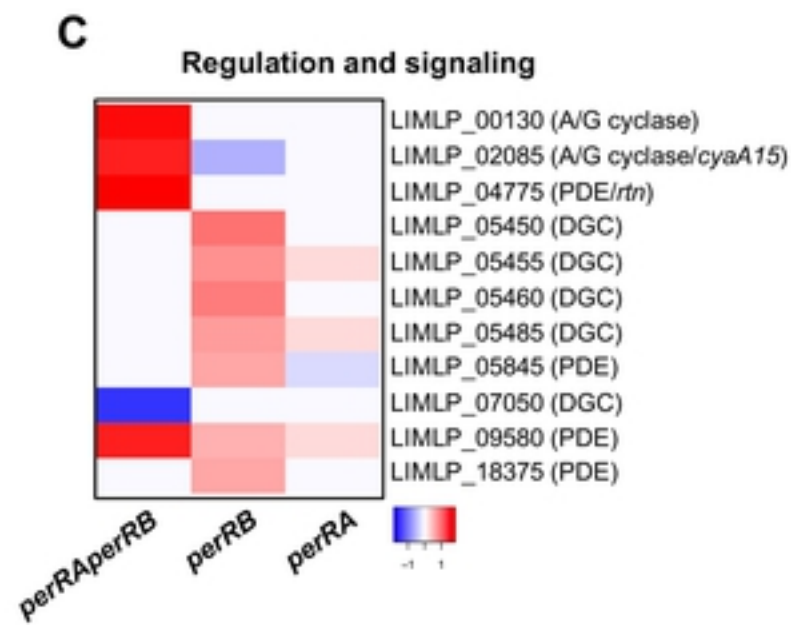
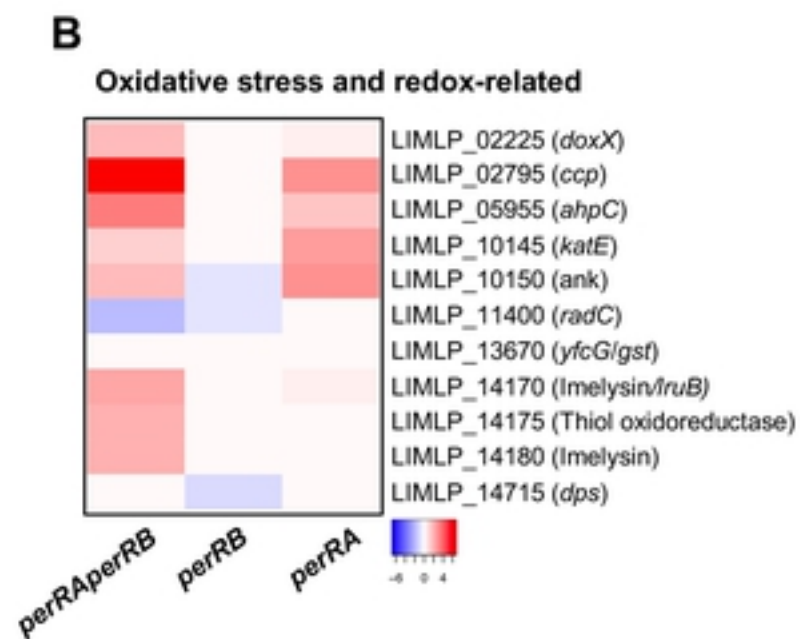
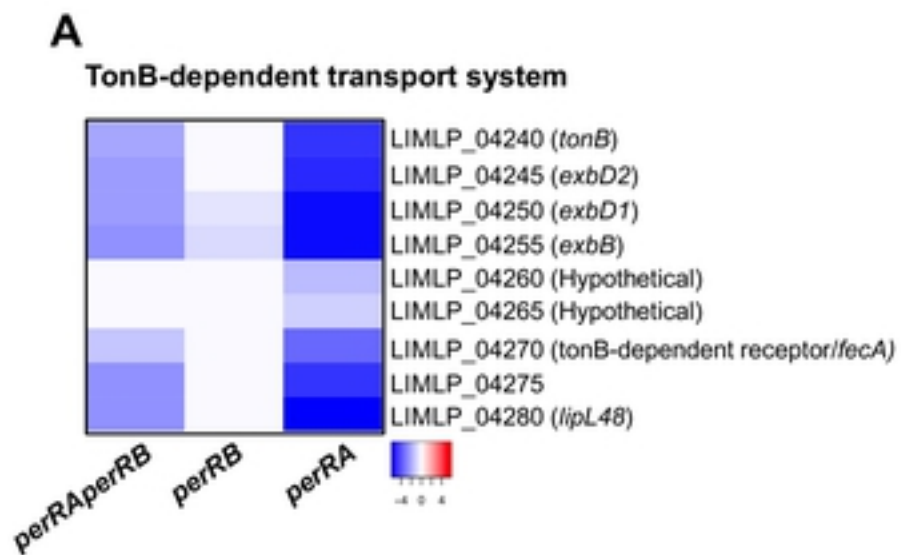


Fig 8

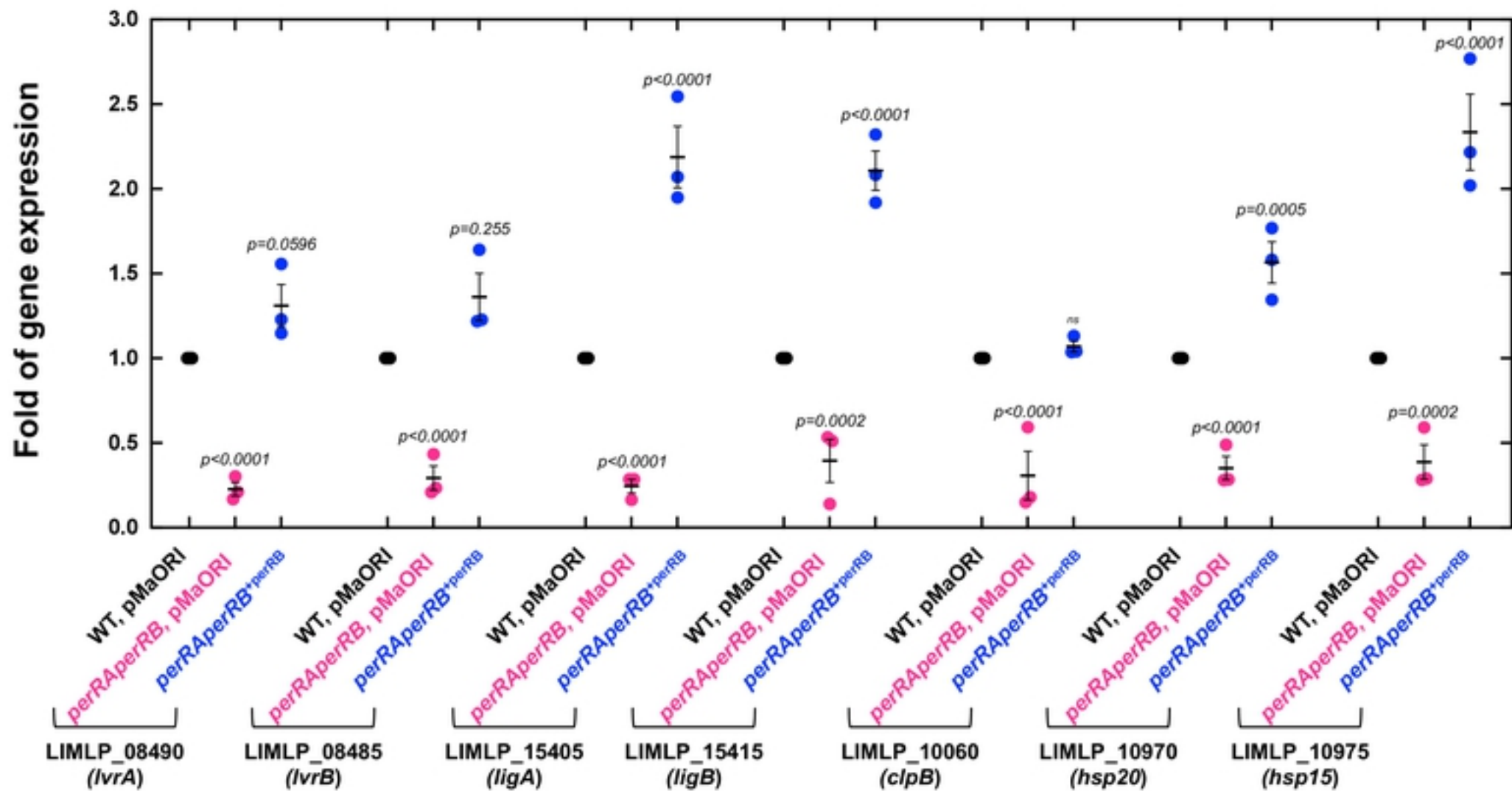


Fig 9

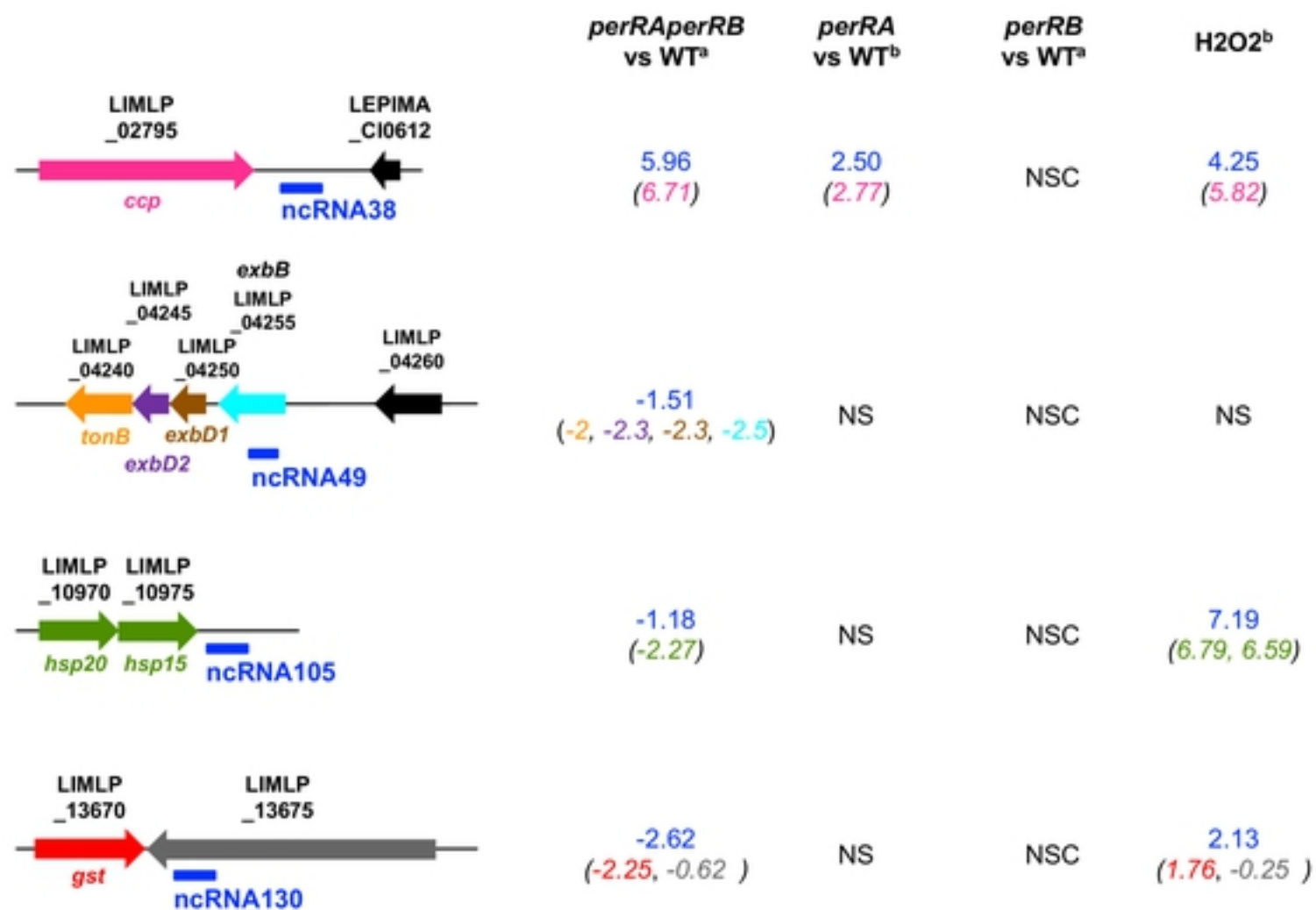


Fig 10