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

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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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23

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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 ( $\text{H}_2\text{O}_2$ ), 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 ( $\text{O}_2$ ). Dismutation of superoxide  
95 will give rise to  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  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 H<sub>2</sub>O<sub>2</sub>  
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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub>. 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 H<sub>2</sub>O<sub>2</sub> 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  $\alpha/\beta$  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 H<sub>2</sub>O<sub>2</sub>. The corresponding residue in Fur is a glutamate and  
179 mutating this residue in aspartate leads to H<sub>2</sub>O<sub>2</sub> 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 (C<sub>113</sub>xxC<sub>116</sub> and C<sub>86</sub>xxC<sub>89</sub>-  
213 C<sub>122</sub>xxC<sub>125</sub>, 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 H<sub>2</sub>O<sub>2</sub> (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 H<sub>2</sub>O<sub>2</sub> (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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> [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 H<sub>2</sub>O<sub>2</sub> (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 Log<sub>2</sub>FC  
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 <sup>a</sup>	Gene	<i>L. biflexa</i> <sup>b</sup>	Function	Log2FC	Adjusted p-value
<b>Down-regulated genes</b>					
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
<b>LIMLP_04255 (LIC10892/LA3244)</b>	<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
<b>LIMLP_15470** (LIC10454/LA3793)</b>		<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
<b>LIMLP_18235** (LIC20078/LB099)</b>			Hypothetical	-1.098	1.69e-02
<b>Up-regulated genes</b>					
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 <sup>a</sup> 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 <sup>b</sup> 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> (S3 Fig) [27]. Thus, 66% of  
344 the PerRB regulon is also regulated by the presence of H<sub>2</sub>O<sub>2</sub>. 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 H<sub>2</sub>O<sub>2</sub> (with Log<sub>2</sub>FCs 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 H<sub>2</sub>O<sub>2</sub>. 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 H<sub>2</sub>O<sub>2</sub> with Log<sub>2</sub>FCs 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> (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 10<sup>4</sup> 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 10<sup>4</sup> 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 10<sup>6</sup> 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 Log<sub>2</sub>FC 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 (Log<sub>2</sub>FC 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 (Log<sub>2</sub>FC 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 H<sub>2</sub>O<sub>2</sub> 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 (Log<sub>2</sub>FC 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 H<sub>2</sub>O<sub>2</sub> and it is required for *Leptospira* virulence [27,42]. The ClpB-encoding ORF  
451 (LIMLP\_10060) is dramatically down-regulated in the *perRAperRB* mutant (Log<sub>2</sub>FC 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 (Log<sub>2</sub>FC 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 (Log<sub>2</sub>FC 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 H<sub>2</sub>O<sub>2</sub> [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 Log<sub>2</sub>FC 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 Log<sub>2</sub>FC cutoff of  $\pm 1$ ) 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> sensing histidine and aspartate residues are conserved in *Leptospira*  
529 PerRA and PerRB and, interestingly, both genes are H<sub>2</sub>O<sub>2</sub>-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 H<sub>2</sub>O<sub>2</sub>,  
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 H<sub>2</sub>O<sub>2</sub> [51,52]. In fact, PerR represses *fur*, whose expression is up-regulated in the presence  
545 of H<sub>2</sub>O<sub>2</sub> as a consequence of dissociation of PerR from the *fur* promoter [53,54]. Metal-  
546 catalyzed oxidation of the H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> sensitivity, exhibited  
554 H<sub>2</sub>O<sub>2</sub> sensing by metal-catalyzed histidine oxidation and a higher H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub>. Therefore, even though *perRB* is up-regulated upon exposure to H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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.  $\beta$ 2163 and  $\Pi$ 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  $\mu$ g/ml, 30  $\mu$ g/ml, and 8  $\mu$ 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<sup>R</sup>*) 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 $\Delta$ 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  $\mu$ 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<sup>R</sup>*) 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*  $\beta$ 2163 conjugating strain as previously described  
690 [66]. *Leptospira* conjugants were selected on EMJH plates containing 50  $\mu$ 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 ( $\Delta$ *perRA*, *perRB::Km<sup>R</sup>*) 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*  $\beta$ 2163 conjugating strain as  
706 previously described [66]. *Leptospira* conjugants were selected on EMJH plates containing 8  
707  $\mu$ 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 ( $\text{Log}_2\text{FC}$ ). 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  $\text{Log}_2\text{FC}$  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  
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799

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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 H<sub>2</sub>O<sub>2</sub>.**

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 H<sub>2</sub>O<sub>2</sub> (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 H<sub>2</sub>O<sub>2</sub>. Log<sub>2</sub>FC of differentially-expressed ORFs upon *L. interrogans* exposure to

1064 1 mM H<sub>2</sub>O<sub>2</sub> (as determined previously [27]) was plotted against the Log<sub>2</sub>FC 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 Log<sub>2</sub>FC 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  $\alpha$ -helix and  $\beta$ -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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub>

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  $\mu$ M  
1177 (for 30 min.) or 1 mM H<sub>2</sub>O<sub>2</sub> (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  $\mu$ 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 H<sub>2</sub>O<sub>2</sub> (B) or of 2  $\mu$ 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  $\mu$ 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  $\text{Log}_2\text{FC}$  cutoff of  $\pm 1$  (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 Log<sub>2</sub>FC.

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.

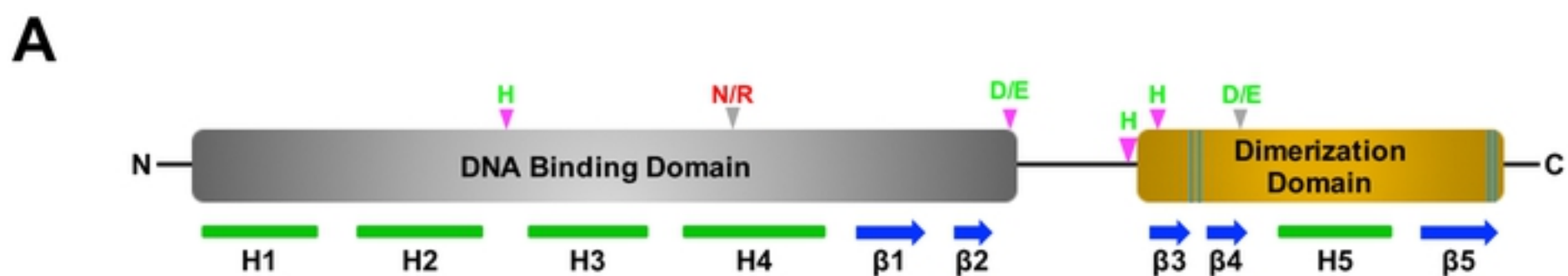
1243

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 H<sub>2</sub>O<sub>2</sub> 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  $\text{Log}_2\text{FC}$  of ncRNAs expression normalized with that in WT and the respective expression  
1252 of these ORF ( $\text{Log}_2\text{FC}$ ) are indicated into parenthesis with the color corresponding to that of  
1253 the ORF in the cartoon. NSC, non-significantly changed.

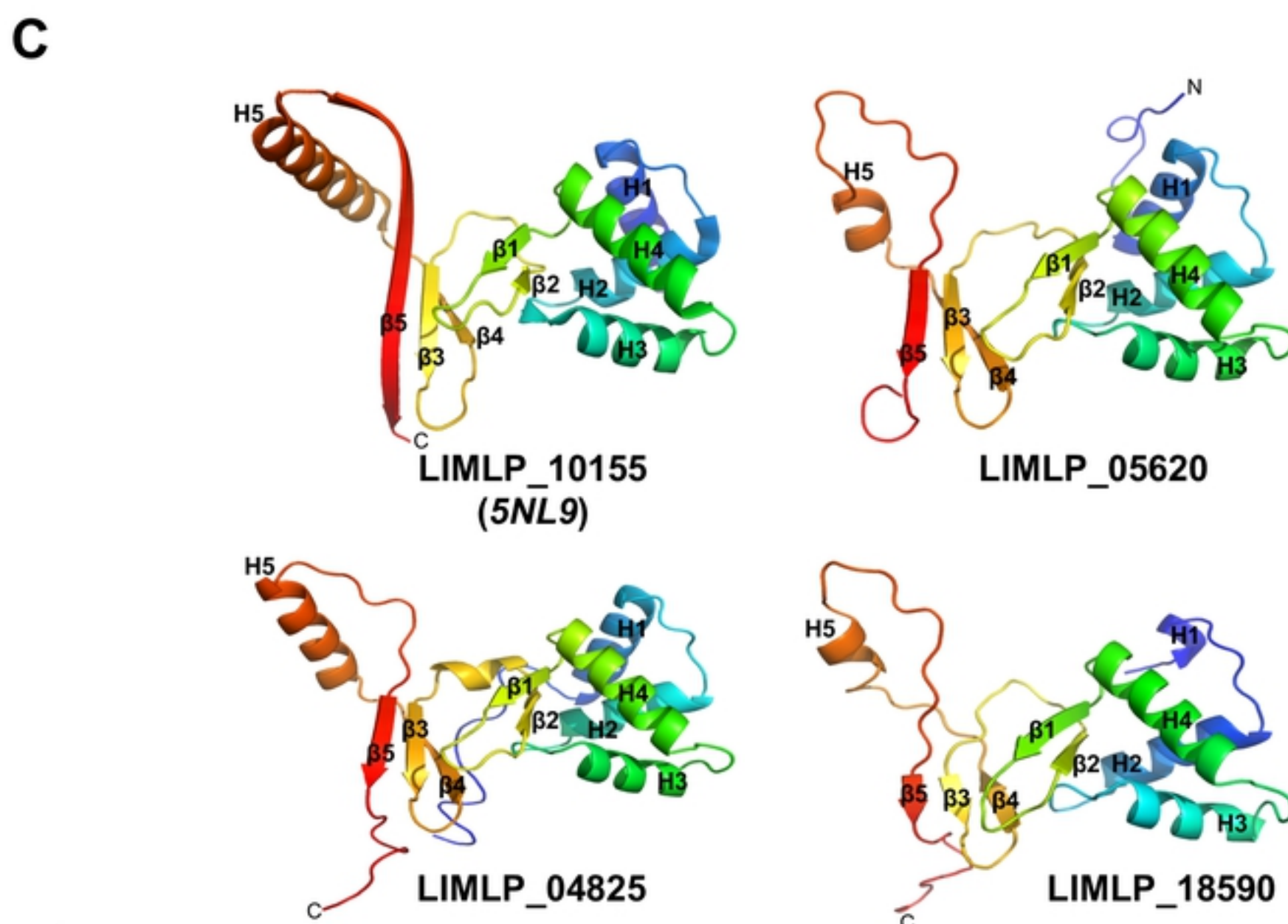




**B**

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BsFur	-----MENRIDRIKKQLHSSSYKLTPOREATV RVLLENEED*HLSAEDVY	44
BsPerR	-----MAAHELKEALET LKETGVRITPQRHAILEY-LVNSMAHPTADDIY	44
10155	-----MKDSYERSKKILE DAGINVTVQRLQMANL-LLSKPQH LTA DQVF	43
05620	-----MESLFAKKVCL---TPVEIERRLKS VSIQPTIQRISICQY-VLCEADHPTAEVVK	51
04825	MNREKQEA I LNKTPAVRMEMQTFSEY LQKEGLKITNQRMLVAER-IFSLHNHFTAEGLL	59
18590	-----VESKRPV LNWFRQAGEILKV-LEMAKGPLSIKEIY	34
BsFur	LLVKEKSPEIGLATVYRTLELLTELKVVDKINFGD-GVSRYDLRKEGAAHF*HHLVCMEC	103
BsPerR	KALEGKFPNMSVATVYNNLRVFRESGLVKELTYGD-ASSRFD FVT----SDHYHAICENC	99
10155	QLINEHMPNASRATIFNNLKLFAEKGI VNLLELKS-GITLYDSNV----VHHHHAIDEKT	98
05620	EWVDSRSFKMSLATVYNTLNILVSAGLLREFKFSCLGKSVYDSNI----IDHYHFFDEKS	107
04825	EEFKDQRDQISKATIYRILS IMVSAGLLQEHNF GK-DYKYYEHIIG--HKHHDHI ICTVC	116
18590	ELSRKNLDNLGIATVYRAVNHLMETGTIHEIHLPG-ESSRFEASR----HHHHHFHCKQC	89
BsFur	GAVDIEEDLLEDVEE I IERDWKFKIKDHRLTFHGI CHRCNGK-----ETE-	149
BsPerR	GKIVDFHYPGLDEVEQLAAHVTGFKVSHHRLEIYGV CQEC SSK-----ENH-	145
10155	GEIYDISLDSKLQEKVLSELKQDFKLKTGSS-----LENCNLSITLKGKKNP-	145
05620	GKFHDIDPSLLSL---SSKLPPEFLVNKTDILLTGNLVSET-----	145
04825	GKIVEFOLDERIEQLQEQA AKENGFKITGHSLNIYGTCNEHSSS-----K---	160
18590	DRVYDIEICPI----PLDKSPKGFTVDTHEI ILYGTCSDCNSK-----AR--	130



**Fig 1**

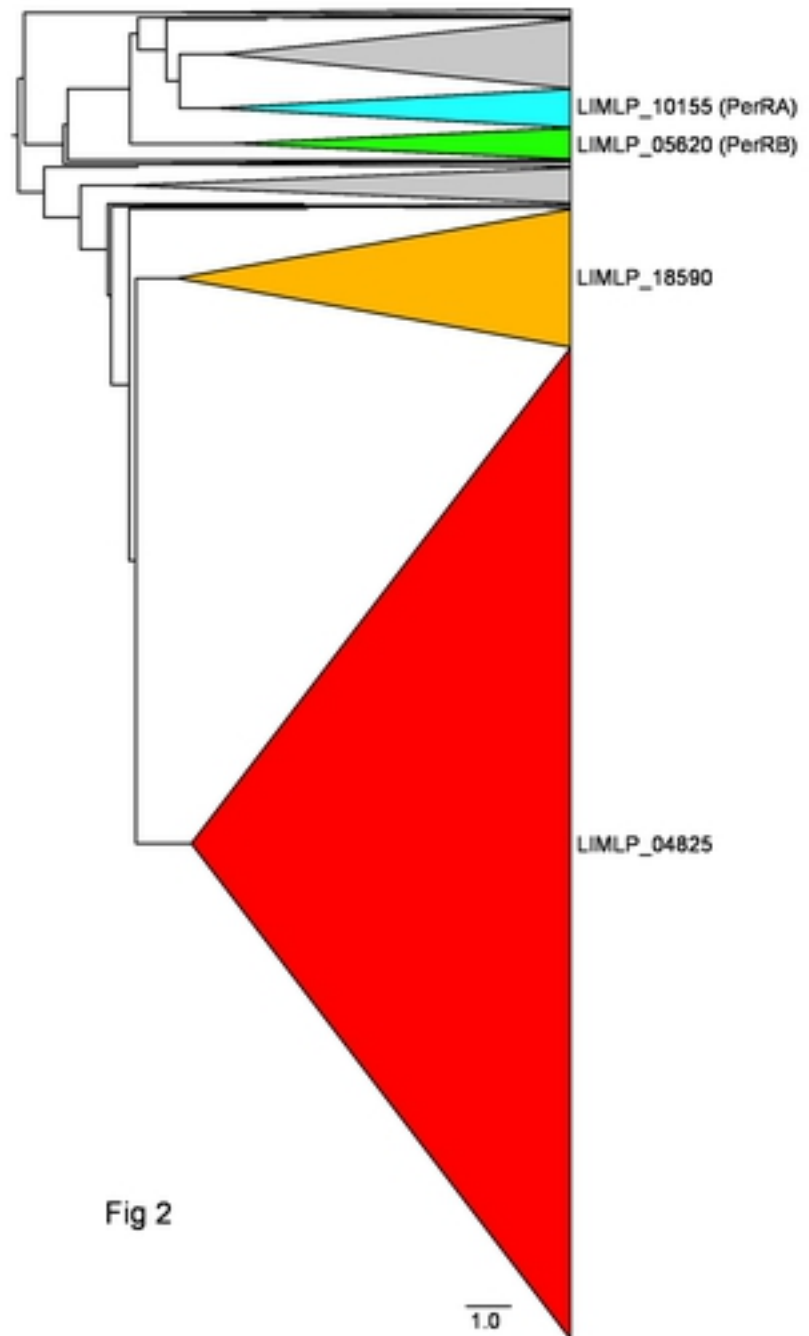
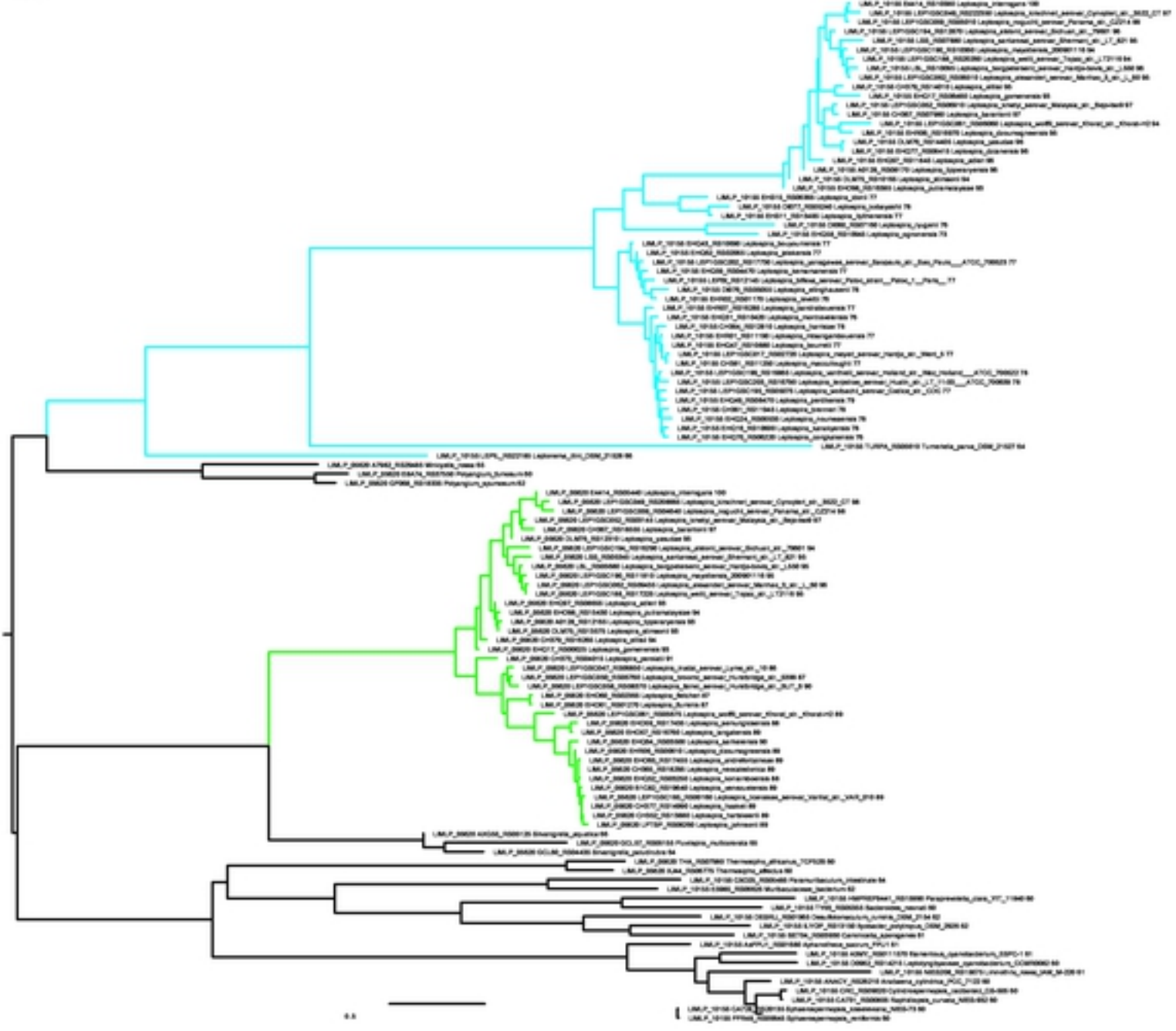
**A****B**

Fig 2





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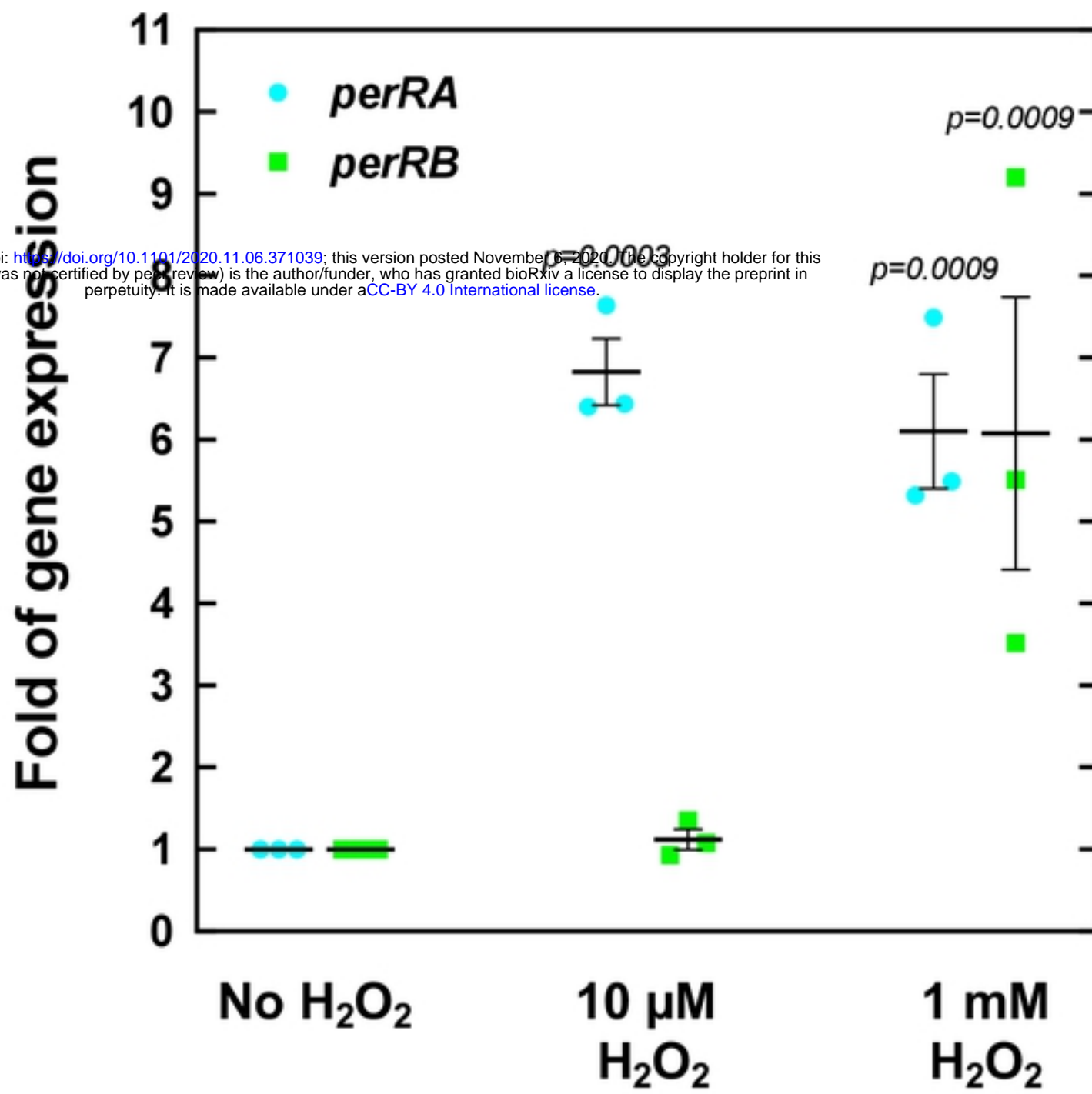
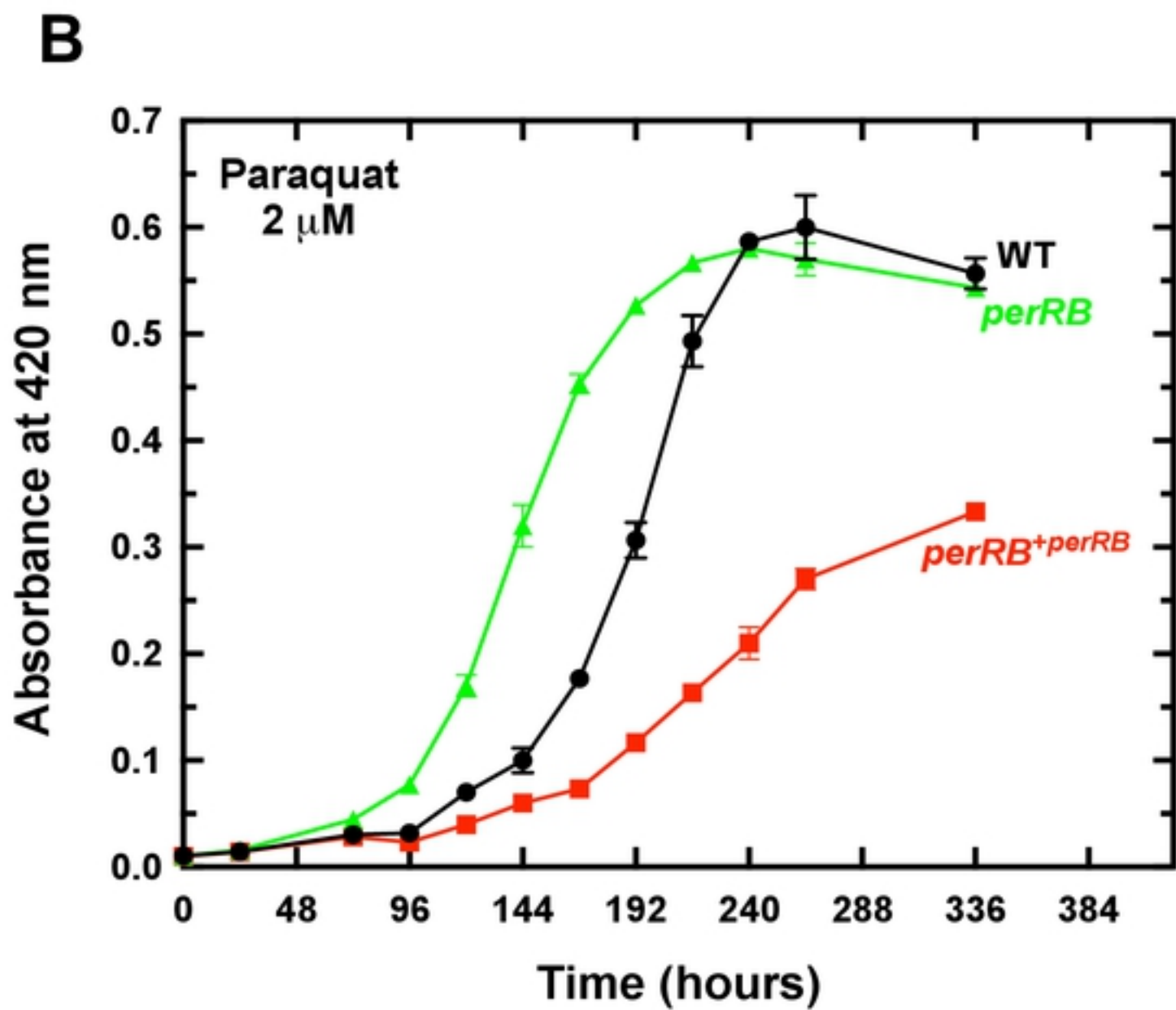
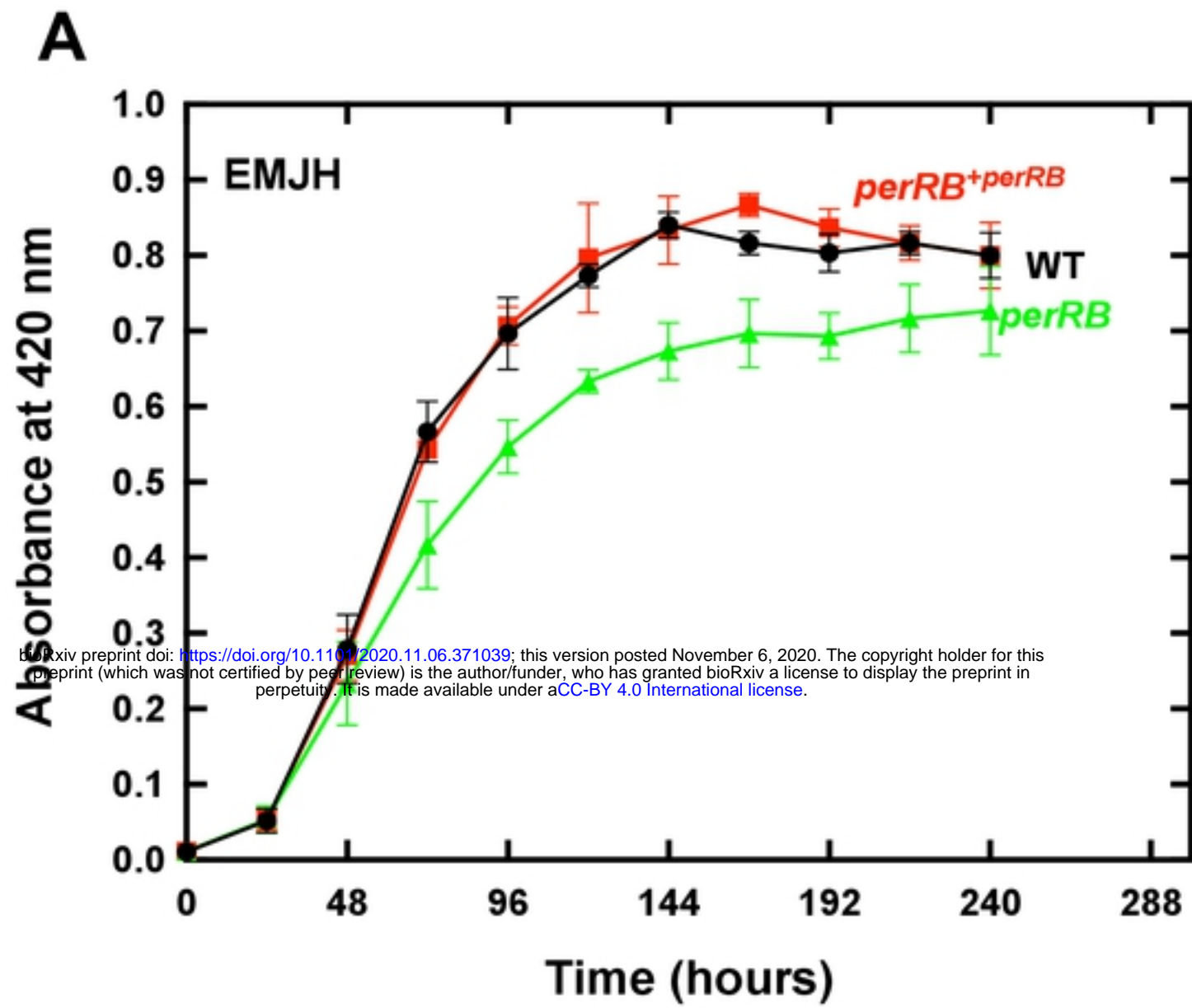


Fig 4



**Fig 5**



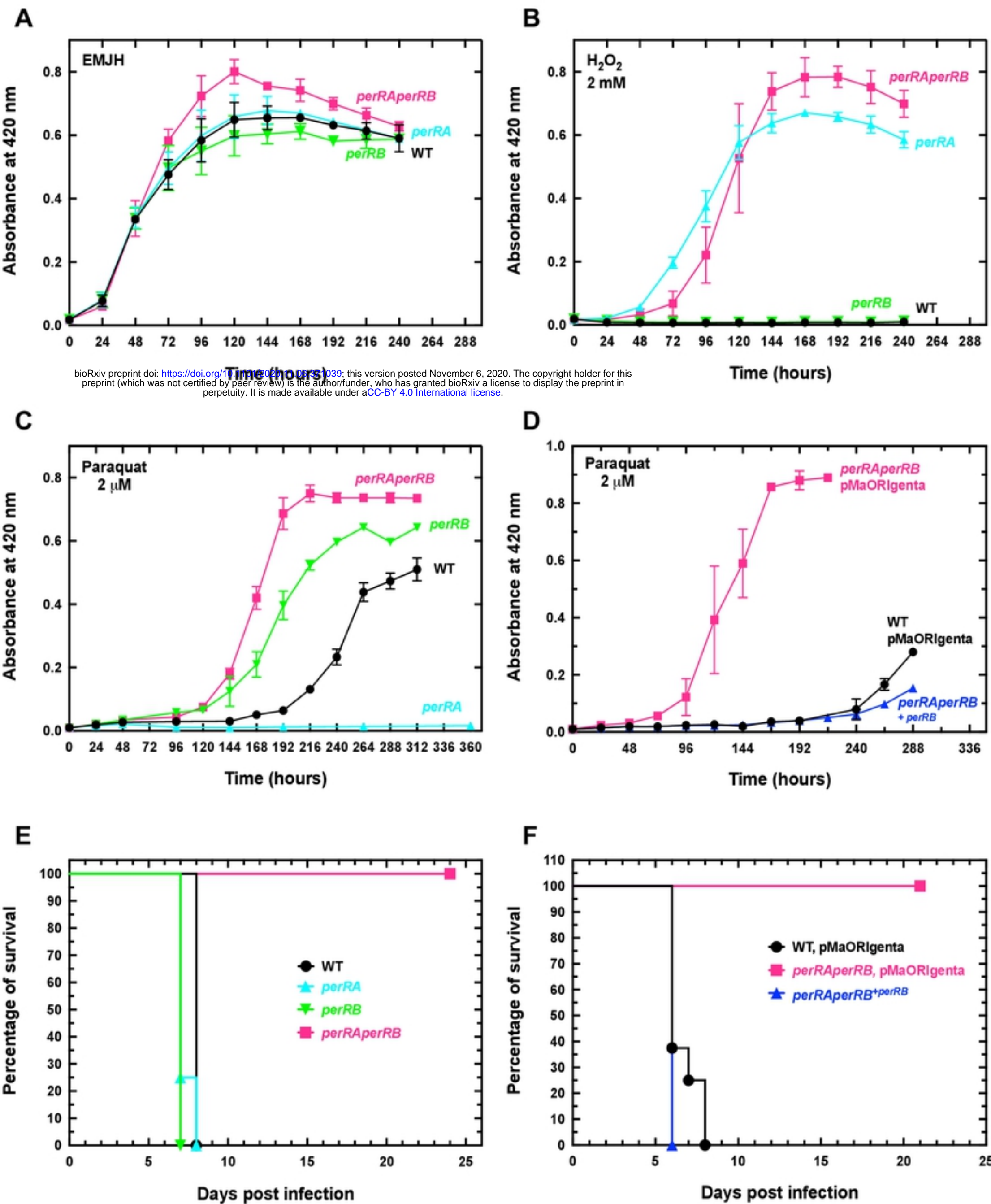
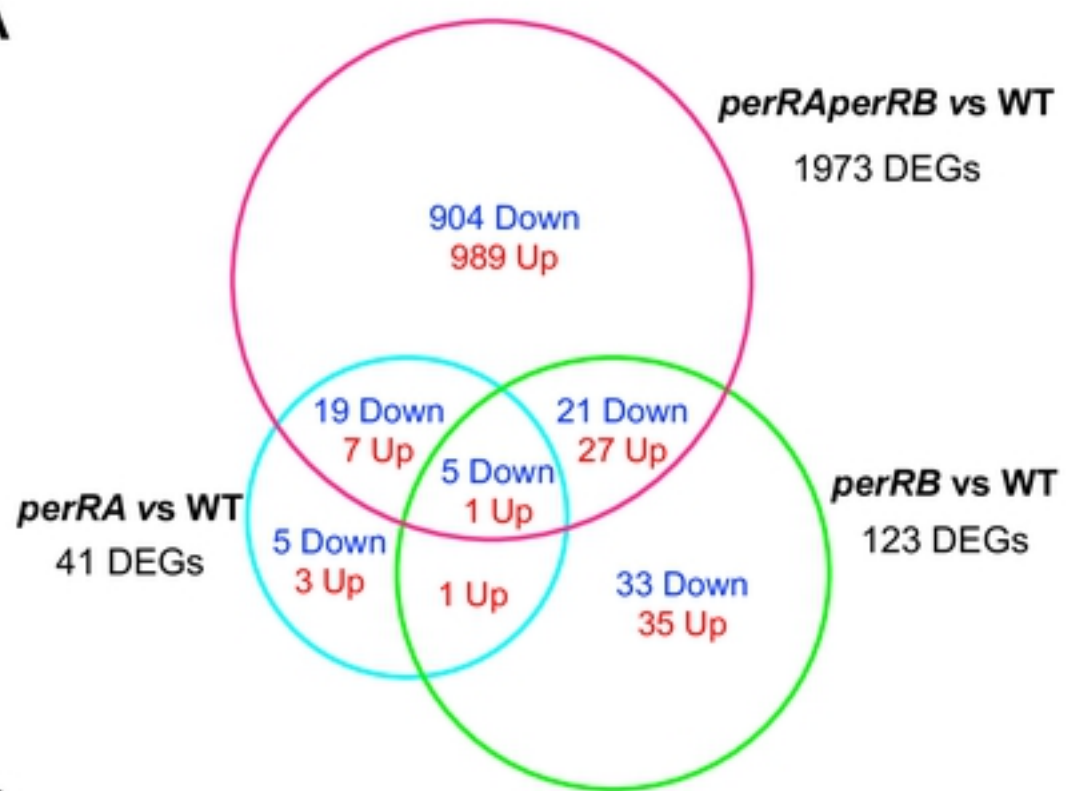
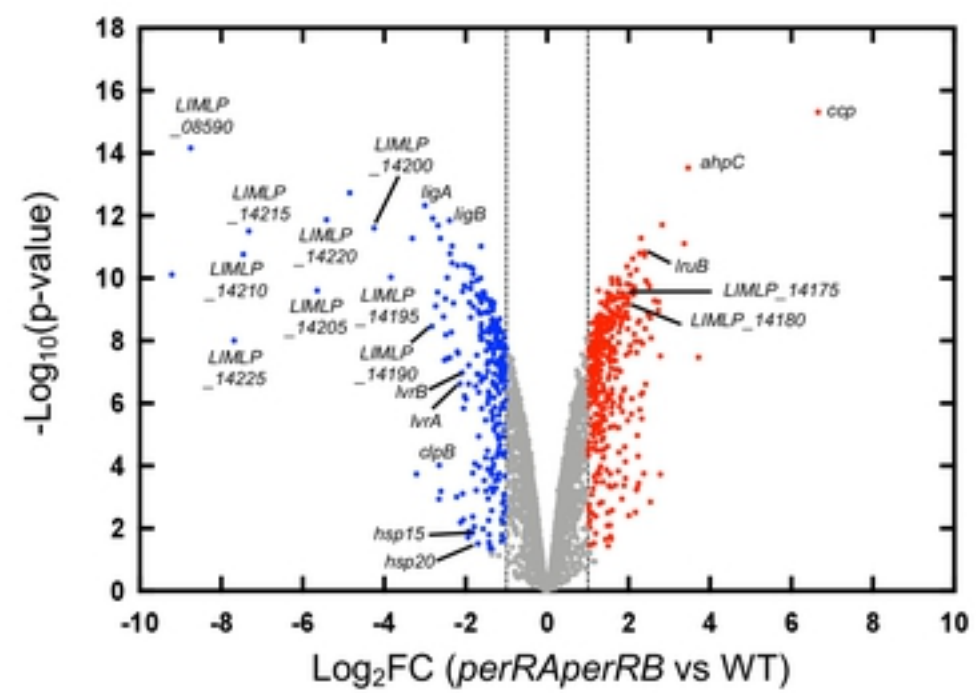
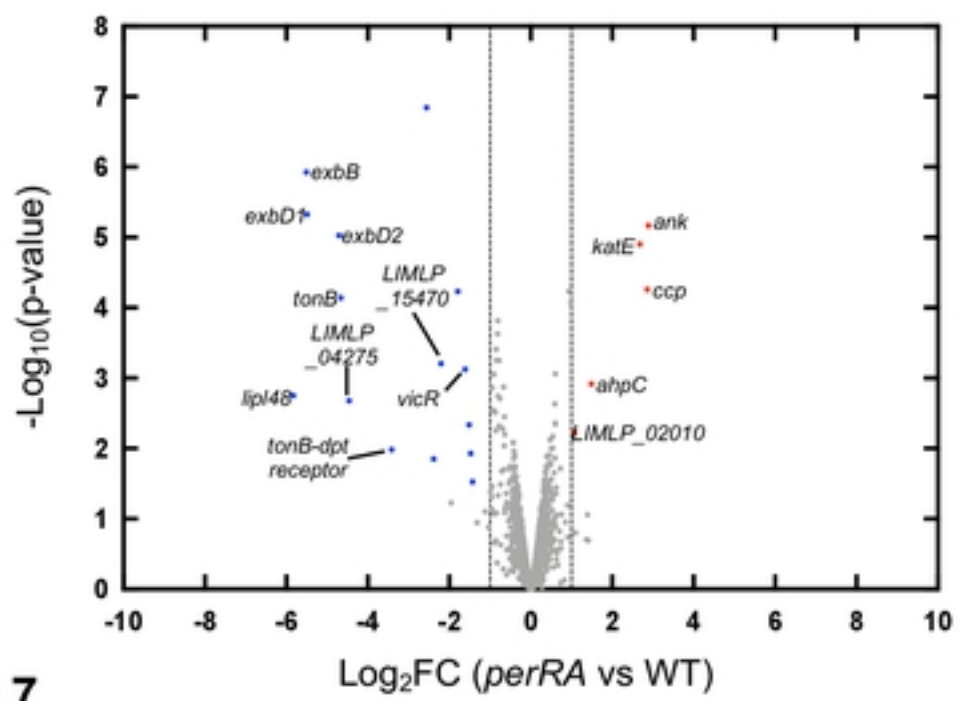
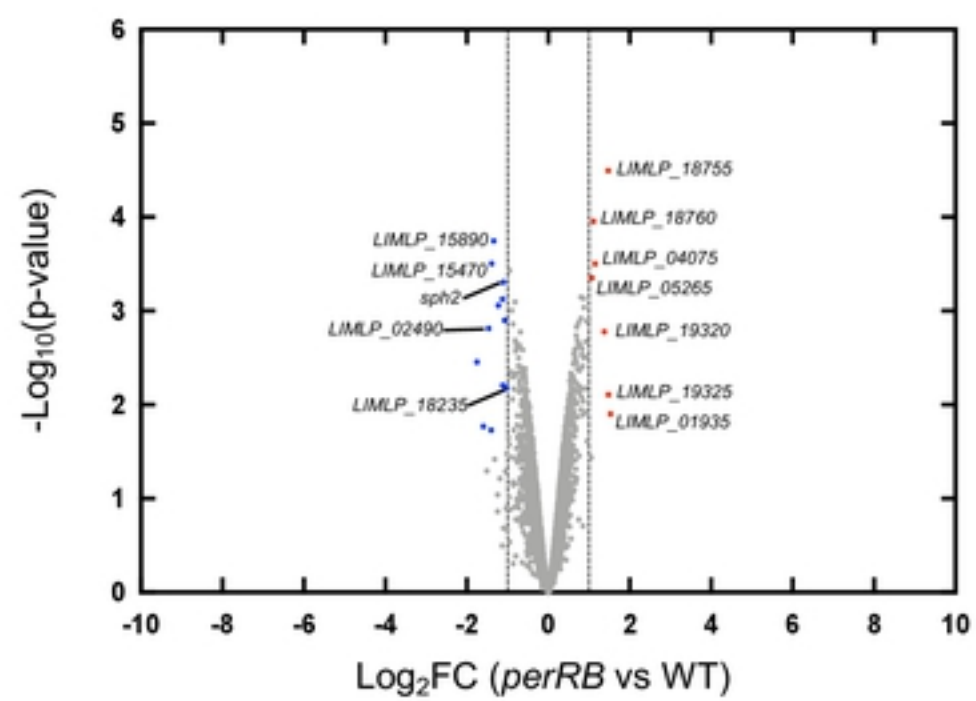


Fig 6

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

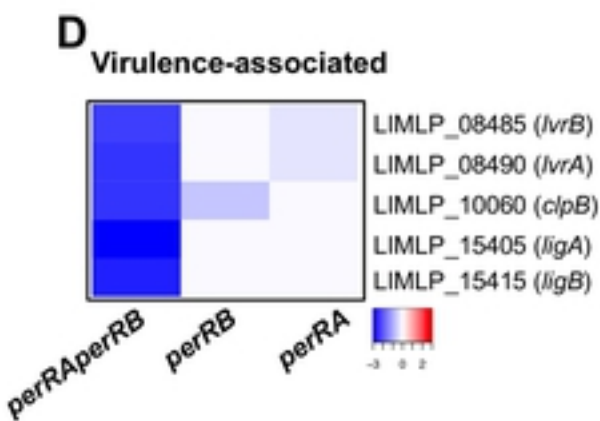
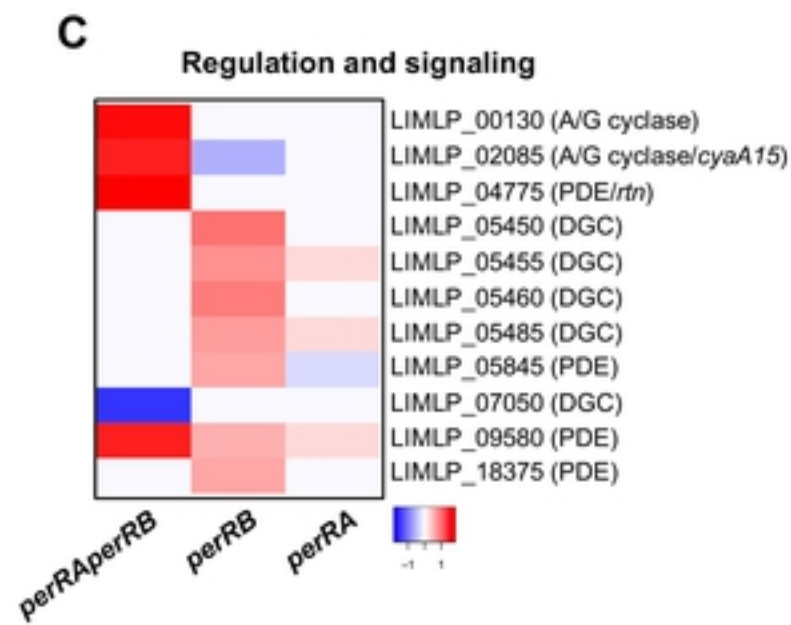
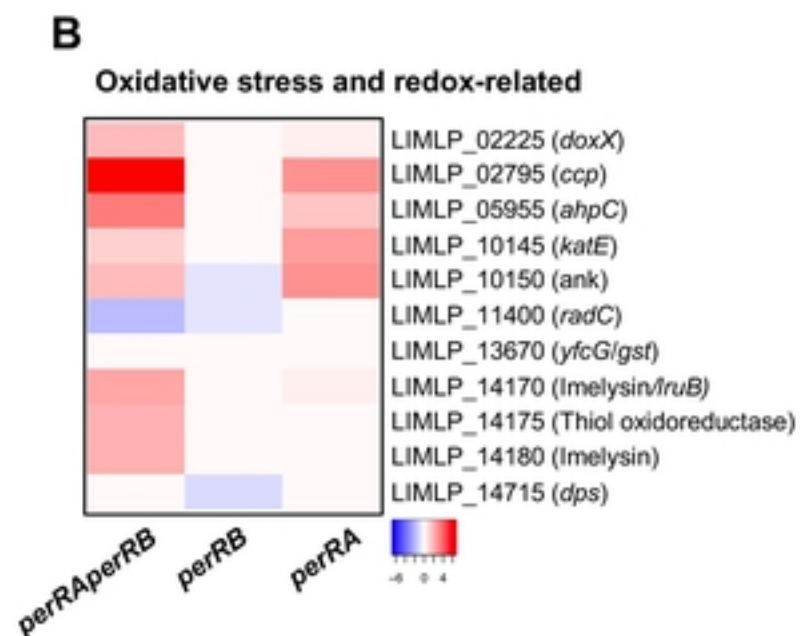
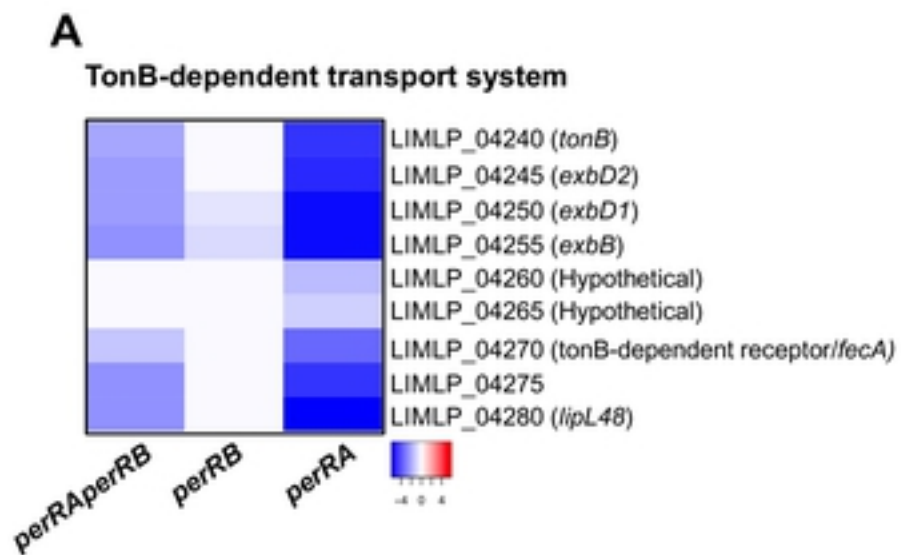


Fig 8

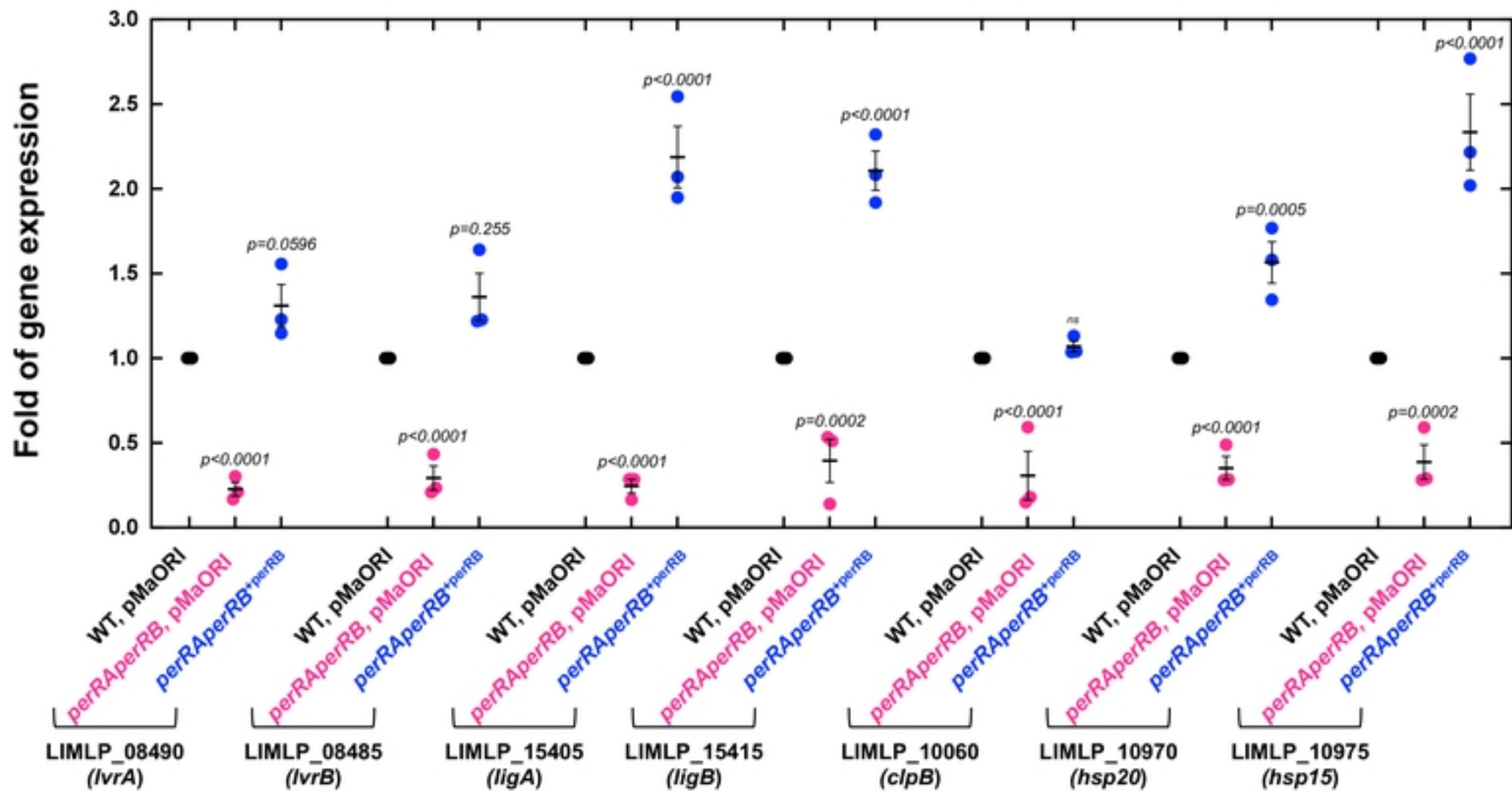


Fig 9

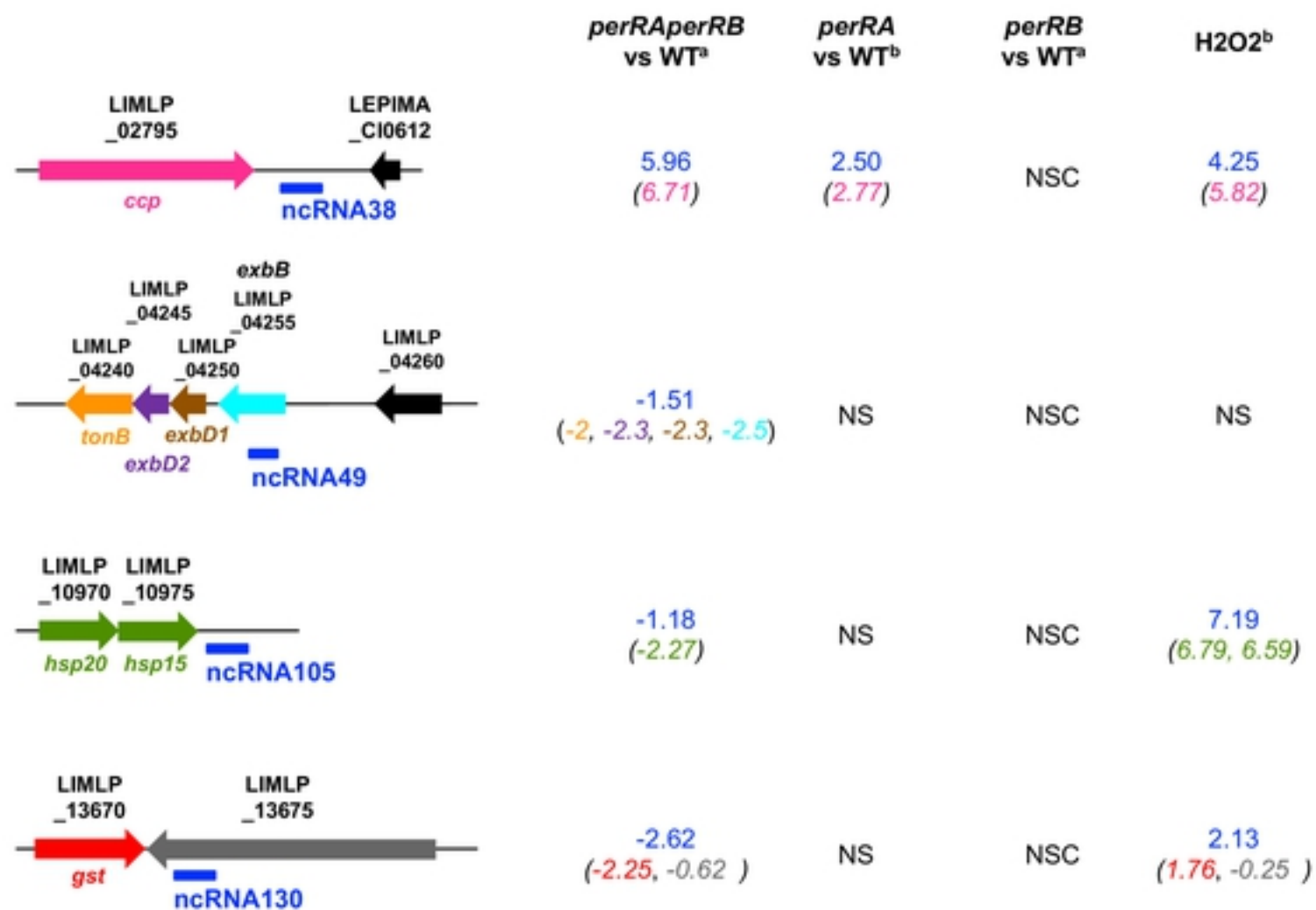


Fig 10