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

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Abstract

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

Author Summary

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

Introduction

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

Leptospirosis is caused by infection with pathogenic spirochetes belonging to the genus *Leptospira* [10]. The majority of severe cases of human leptospirosis are associated with *L. interrogans* [10]. Infection begins when a naïve reservoir (or incidental) host comes into contact with urine from an infected host, most often *via* contaminated water or soil [11]. Leptospire gain entry to the host through bruises or abrasions in the skin and/or mucous membranes. Following inoculation, leptospire transition from a saprophytic (free-living) to a parasitic lifestyle by a complex and poorly understood process referred to as ‘host adaptation’. Once in the bloodstream, leptospire rapidly disseminate to distal tissues but, in reservoir host, are cleared within several days from all sites except the kidney, where they set up long-term residence in the proximal tubules [12-14]. Infected reservoir hosts shed large numbers of leptospire (up to 10⁷/mL) in their urine for weeks to months [10, 13, 15, 16]. While infection in reservoir hosts is generally asymptomatic, incidental hosts, including humans, may develop clinical symptoms ranging from mild flu-like illness to fulminant disease (*e.g.*, Weil’s disease and pulmonary hemorrhage syndrome). Even with

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

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

Not surprisingly, many of the genes upregulated by *L. interrogans* in DMCs encode functions related to environmental signaling and gene regulation [42], including LIMLP10155 (LIC12034), which encodes a member of the Ferric Uptake Regulator (FUR) superfamily [45]. The namesake of this highly diverse superfamily, Fur, functions as a global regulator of iron homeostasis in Gram-negative and -positive bacteria, controlling both the induction of iron uptake systems under iron limitation and the expression of iron storage proteins and iron-utilizing enzymes under iron sufficiency [46]. Beyond balancing metal uptake and toxicity, FURs also modulate intermediary metabolism, host colonization and virulence [47-53]. Members of the FUR superfamily are diverse in their metal specificities; in addition to the iron-sensing Fur, this family includes regulatory sensors for zinc (Zur), manganese (Mur) and nickel (Nur) [50, 54-59]. Other FUR superfamily members, PerR uses metal-catalyzed oxidation to sense peroxide stress [46, 60-64] and Irr controls iron uptake and heme homeostasis in a heme/iron-dependent oxidation [64, 65]. While Fur and Zur regulators are widely distributed across both Gram positive and Gram-negative bacteria, other FUR family regulators have more limited distribution. PerRs are found mainly in Gram-positive bacteria, and Irrs are limited to α -proteobacteria. [66]. So far, Mur and Nur have been characterized in α -proteobacteria and actinomycetes, respectively, but their distribution within other taxonomic groups is still unclear. Although most often characterized as “repressors”, FUR family regulators also may activate transcription in either a metal-bound or -unbound (apo) state [58, 67, 68].

In diverse bacteria, iron serves as an essential co-factor for many cellular processes, including energy generation *via* electron transport, intermediary metabolism and DNA biogenesis [51-53, 69, 70]. For many pathogens, the shift from a high- to low-iron environment is a key environmental signal for induction of expression of virulence genes [52]. Unlike other spirochetes,

such as *B. burgdorferi* and *Treponema pallidum*, which require iron in trace amounts, if at all [71-75], *Leptospira* spp. require this metal for growth *in vitro* and, presumably, in the host [76]. Consequently, leptospires have evolved elaborate mechanisms for iron sensing, scavenging and utilization [77-79]. At the same time, leptospires must balance their physiological need for transition metals with the potential damage caused by highly toxic hydroxyl free radicals generated by Fenton chemistry from H₂O₂ in the presence of ferrous ions [80-82]. Many bacteria, including *Leptospira* spp., encode systems to ameliorate the toxicity of H₂O₂ and repair damage due to oxidative stress [83, 84]. Expression of gene products involved in oxidative stress responses typically are controlled by one of two master regulators – OxyR and PerR. While OxyR acts as a transcriptional activator for gene products (*i.e.*, catalase and superoxide dismutase) related to detoxification of reactive oxygen species (ROS), PerR acts as a repressor and is released from DNA following exposure to peroxide [56, 60, 85, 86]. Both master regulators respond to similar amounts of H₂O₂ [87, 88]; thus, it is unclear why some bacteria have evolved to use PerR while others use OxyR.

A genome-wide survey of *L. interrogans* identified four putative FUR family regulators (*LIMLP04825*, *LIMLP05620/perRB*, *LIMLP10155/perRA* and *LIMLP18690*). Prior studies by Lo *et al.* [89] and Zavala-Alvarado *et al.* [90], suggest that PerRA functions as a metal-dependent peroxide stress regulator. Consistent with its repressor function in other bacteria, a *L. interrogans* *perRA* transposon mutant expresses increased levels of catalase, AhpC and cytochrome c-peroxidase and enhanced survival following exposure to peroxide *in vitro* [89-91]. Recently, Zavala-Alvarado *et al.* [91] demonstrated that expression of *perRB* also was increased by H₂O₂ *in vitro*. Inactivation of *perRB* increased survival to superoxide but not H₂O₂ [91]. These data suggest that PerRA and PerRB likely are functionally distinct. Consistent with this notion, Zavala-

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

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

Results

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

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

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

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

***L. interrogans* FUR family regulators are expressed at higher levels *in vivo* than *in vitro*.**

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

sv. Manilae strain L495 [97, 98] grown *in vitro* (EMJH at 30°C) and following cultivation within DMCs. As shown in Fig 3, *perRA* (7.59-fold), *ffr1* (3.20-fold), and *ffr2* (5.70-fold) were upregulated significantly ($p<0.05$) *in vivo*. *perRB* was upregulated 1.63-fold in DMCs compared to *in vitro*, but the difference was not statistically significant (Fig 3).

Inactivation of both *perRA* and *perRB* in *L. interrogans* results in loss of virulence in mice.

Previously, Murray *et al.* [97] and Zavala-Alvarado *et al.* [92] independently reported that a *L. interrogans* Manilae *perRA* Tn mutant is virulent in hamsters. More recently, Zavala-Alvarado *et al.* [91] established that *L. interrogans* lacking PerRB also retain virulence in hamsters. Zavala-Alvarado and colleagues also generated a double mutant by insertional inactivation *perRA* in the *perRB* Tn mutant; the resulting double mutant (*perRA/B*) was avirulent in hamsters [91]. Golden Syrian hamsters are exquisitely sensitive to *L. interrogans* and develop acute, fulminant, disseminated disease at doses as low as 10^1 [18, 97, 99, 100]. Mice, on the other hand, are a natural reservoir for *L. interrogans* and relatively resistant to infection; at sublethal doses, susceptible mouse strains develop a self-resolving hematogenous dissemination phase (~1 week) followed by chronic, asymptomatic renal colonization marked by shedding large numbers of leptospire in urine [18, 101-104].

Given the differences in leptospiral disease progression and severity between hamsters and mice, we asked whether PerRA, PerRB, or both are required to establish infection and persistence within a reservoir host model. At the outset, we first established that our wild-type serovar Manilae parent (WT) is virulent in C3H/HeJ mice. Female 10-week old mice (n=5 per group) were infected intraperitoneally with 5×10^6 , 1×10^6 , 1×10^5 and 1×10^4 leptospire. Mice were monitored daily for signs of disease (*i.e.*, weight loss). Within 6 days, all mice in the 5×10^6 group and 3 of 5 mice

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

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

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

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

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

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

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

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

The majority (66%) of genes in the PerRB DMC regulon were upregulated, most encoding proteins of unknown or poorly function (S5B Fig and Table S3). Notably, however, the PerRB DMC regulon includes 17 genes (11 upregulated, 6 downregulated) related to signaling and/or gene regulation (Fig 5B). The 11 upregulated genes include six related to signal transduction, three putative DNA binding proteins (*LIMLP16420*, *LIMLP07225* and *LIMLP15900*), an ECF-type sigma factor (*LIMLP14515*) and a putative serine/threonine phosphatase with a PAS-type sensor domain (*LIMLP06960*) (Fig 5B). The six downregulated signaling genes include two additional putative DNA binding proteins (*LIMLP07895* and *LIMLP10055*), a second ECF-type sigma factor (*LIMLP14940*), a putative anti-sigma factor antagonist (*LIMLP04815*), and an EAL-type phosphodiesterase (*LIMLP04775*) (Fig 5B). None of these putative regulators were affected *in vitro* by loss of PerRB [91].

Inactivation of both PerRA and PerRB results in a DMC regulon that differs dramatically from its single mutant counterparts. The PerRA/B DMC regulon contains 106 differentially expressed genes, 74 upregulated and 32 repressed (Tables 1-2 and S4 Table). Surprisingly, we saw limited overlap between the DMC regulons for the double and single mutants (Fig 6A); all of the overlapping genes were located in the TonB-related chromosomal locus dysregulated in the *perRA* and *perRA/B* mutants *in vitro* [91, 92] (S4 Fig). Ninety genes (62 upregulated and 28 repressed) were dysregulated only in the *perRA/B* double mutant (Fig 6A, Tables 1-2 and S4 Table).

Cellular homeostasis and metabolism. A handful of genes upregulated by PerRA/B in DMCs encode proteins involved in cellular homeostasis and metabolism (Fig 6B and Table 1). *LIMLP14200* and *LIMLP12135* contain domains found in lipases/esterases (IPR0002489) [117] and alkaline phosphatases and sulfatases (IPR000917), respectively. *LIMLP18070* contains an

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

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

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

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

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

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

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

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

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

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

Loss of LvrAB alone is not responsible for avirulence of the *perRA/B* double mutant in mice.

As noted above, expression of *lvrAB* is disrupted only in the *perRA/B* double mutant (Table 1); similar results were obtained using leptospires grown *in vitro* [91]. Using LvrA- and LvrB-specific antisera, we confirmed our transcriptomic data at the protein level by immunoblot using whole cell lysates from WT, *perRA*, *perRB*, *perRA/B* mutant and complemented strains cultivated in DMCs (Fig 8A). Previously, Adhikarla *et al.* [20] reported that inactivation of *lvrAB* by transposon mutagenesis results in dysregulation of a large number of genes *in vitro*, including *ligB*. However, in our hands, we saw no decrease in LigA or LigB in the *lvrAB* mutant strain following cultivation in DMCs (Fig 7B). Adhikarla *et al.* [20] also reported that loss of either *lvrAB* or *lvrB* alone resulted in a significant loss of virulence in hamsters. To explore whether the avirulence of the *perRA/B* double mutant in mice (Fig 4C-E) is due solely to loss of LvrAB, we assessed the ability of *lvrAB*

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

Discussion

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

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

The presence of multiple FUR family regulators in *Leptospira* spp. was noted previously by Louvel *et al.* [45], who identified five distinct orthologs between *L. interrogans* and *L. biflexa*. Phylogenetic analyses presented herein identified a sixth FUR family regulator and established that two (PerRA and Ffr1) are conserved within both pathogenic and saprophytic species and two each are unique to either pathogenic (PerRB and Ffr2) or saprophytic subclades (PerRC and Ffr3). As their designations suggest, three are predicted to function as PerRs, while the functions of the remaining three cannot be predicted based on sequence alone. Given the importance of trace metals, iron in particular, for *Leptospira* spp., the latter likely encode Fur, Zur or Mur orthologs. Consistent with this notion, transcription of *ffr1* in *L. biflexa* was decreased ~10-fold in response to iron starvation *in vitro* [45], presumably to derepress expression of iron uptake systems. Thus far, only *B. licheniformis* has been shown to encode multiple PerRs (PerR_{BL}, PerR2 and PerR3), each of which displays a different level of sensitivity to H₂O₂ (PerR2 > PerR_{BL} > PerR3) [139]. The extent of regulatory overlap between these three PerRs has yet to be determined. Our finding that almost all saprophytic and pathogenic *Leptospira* spp. encode closely-related PerRA and Ffr1 orthologs implies that these regulators promote survival outside of a host (e.g., within soil and/or water). Nonetheless, studies with the *perRA/B* double mutant, presented here and elsewhere [91,

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

L. interrogans cultivated in DMCs express increased levels of catalase, AhpC-type peroxiredoxin and cytochrome c peroxidase [42], three enzymes typically associated with detoxification of reactive oxygen species (ROS) in bacteria [83, 85, 141]. These data also provide strong evidence that *L. interrogans* is exposed to ROS *in vivo* [42]. Consistent with this notion, catalase-deficient leptospires are more susceptible to H₂O₂ *in vitro* and show reduced virulence in hamsters [142]. Host phagocytic cells, which generate oxygen radicals *via* a dedicated NADPH oxidase [143, 144], are one likely source of exogenously-derived ROS *in vivo*. Leptospires within renal tubules, a highly oxygenated niche, also would be exposed to elevated levels of oxygen. Incomplete reduction of oxygen by iron-containing cytochromes is another potential source of endogenous ROS [83, 141]. In bacteria, oxidative stress responses often are coordinated by two evolutionarily distinct master regulators -- OxyR and PerR. OxyR, the more common of the two, belongs to the LysR family and functions primarily as an activator [145]. In its oxidized state, OxyR activates transcription of genes involved in the detoxification of H₂O₂ (catalase and AhpC), the prevention or repair of DNA damage (Dps) and/or redox homeostasis (glutathione reductase, thioredoxin) [83]. PerR, first described in *B. subtilis* [146], typically represses rather than activates many of the same genes as OxyR and is released from DNA by peroxidation [57, 60, 88]. Although OxyR and PerR regulate transcription by different mechanisms, they react with H₂O₂ at essentially the same rate constant (10⁵ M⁻¹ s⁻¹) [87] and orchestrate highly similar responses. *L. interrogans* does not encode an OxyR homolog but, as noted above, encodes at least two PerR orthologs, PerRA and PerRB. Consistent with PerR functions in other bacteria, as shown here and elsewhere

[89, 91, 92], *L. interrogans perRA* mutants show enhanced survival following exposure to lethal levels of H₂O₂ *in vitro* and increased expression levels of catalase, AhpC and cytochrome c peroxidase *in vitro*. While inactivation of *perRB* had no effect on the ability of leptospires to withstand killing by H₂O₂, the *perRB* mutant showed increased tolerance to the superoxide-generating compound paraquat [91]. Moreover, no genes associated with ROS defenses were dysregulated in the *perRB* mutant *in vitro* [91]. In DMCs, only cytochrome c peroxidase, AhpC and a glutaredoxin were dysregulated in the double mutant. Interestingly, all three genes were expressed at higher levels in the WT compared the mutant, suggesting that they are activated rather than repressed by PerRB. Moreover, expression of catalase was not significantly different in the WT vs. *perRB* or *perRA/B* DMC comparison. Taken together, these data argue that while PerRA and PerRB may be ‘activated’ by ROS, the adaptive responses they control likely extend beyond oxidative stress.

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

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

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

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

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

Designation of PerRA and PerRB as peroxide stress regulators in *L. interrogans* is based largely on *in vitro* studies showing increased survival of *perRA* and *perRB* mutants following exposure to H₂O₂ and paraquat, respectively [89, 91, 92]. Several lines of evidence, however, raise the possibility that these gene products function as iron response regulators (Irrs) rather than PerRs. Based on amino acid sequence alignments, PerRA and PerRB appear to be more closely related to Irrs than PerRs. In α -proteobacteria, Irrs and their regulatory partner, RirA, coordinate the expression of genes involved in heme biosynthesis with iron availability. Thus, it is worth noting that LIMLP06290 (LIC11283), annotated as a hypothetical protein, contains domains consistent with it being a RirA. At the sequence level, Irrs share a number of features with PerRs, including the presence of Asp and Arg residues in their regulatory metal sites and DNA binding helices, respectively. Irrs and PerRs also are responsive to similar levels of ROS, albeit by a different mechanism, and regulate many of the same effector genes (*i.e.*, catalases and peroxidases) [65, 157, 167]. In some, but not all cases, *irr* mutants also show increased survival *in vitro* under high H₂O₂ levels [157, 168]. As noted above, only *B. lichenformis* is known to encode multiple PerRs. Numerous bacteria, on the other hand, encode two or more Irrs [167]. Variable affinity of Irrs for their target promoters enables them to modulate gene expression over a wider range of conditions than PerRs [169]. The autoregulatory sequences identified upstream of *perRA* [90] diverge significantly from canonical PerR and Fur boxes but show strong similarity to “Irr-boxes” [170, 171]. Moreover, Irrs are known to act as activators as well as repressors [170] [167]. Although typically associated with peroxide-sensitive regulation of iron/heme acquisition and utilization, Irrs have been shown to control diverse cellular processes, including virulence. Moreover, the vast majority of histidine kinases upregulated by PerRA/B in DMCs contain one or more PAS-type sensor domains, which have been shown to function as heme sensors [172]. Given the established

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

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

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

Material and Methods

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

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

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

instructions. Plasmids were maintained in *E. coli* Top10 (Life Technologies, Grand Island, NY) or Stellar (TaKaRa, Mountain View, CA) cells and purified using QIAprep spin and midi kits (Qiagen, Valencia, CA). Bacterial genomic DNA was extracted from *L. interrogans* using the Gentra Puregene Yeast/Bacteria kit (Qiagen) according to the manufacturer's recommendations. Routine and high-fidelity PCR amplifications were performed using RedTaq (Denville Scientific, Metuchen, NJ, United States) and CloneAmp HiFi (Takara Bio USA Inc., Mountain View, CA), respectively. DNA sequencing was performed by Genewiz, Inc. (Cambridge, MA). Routine sequence analyses were performed using MacVector (version 17.0.1, MacVector, Inc., Cary, NC, United States). Oligonucleotide primers used in these studies were purchased from Sigma-Aldrich (St. Louis, MO); primer sequences are provided in Table S6.

Generation of polyclonal antisera using recombinant protein. Recombinant His-tagged PerRA (LIMLP10155), PerRB (LIMLP05620), LvrA (LIMLP08490) and LvrB (LIMLP08485) cloned into pET28a vector (Novagen) and FlaB1 (LIMLP09410) cloned into pAE [185], were expressed in *E. coli* BL21 Star (DE3) or OverExpress™ C43 (DE3) (Lucigen/VWR, Radnor, PA). Following induction with IPTG, recombinant proteins were purified by nickel affinity chromatography using HisTrap™ Column (GE Healthcare Life Sciences Pittsburgh, PA). Rat polyclonal antisera against *L. interrogans* PerRA, PerRB, LvrA, LvrB and FlaB1 were generated by hyperimmunization of female Sprague-Dawley rats (Envigo, South Easton, MA) with 40-60 µg of recombinant His-tagged proteins co-administered with Freund's Complete Adjuvant. After three weeks, two additional boosts of 40-60 µg of protein mixed 1:1 with Freund's Incomplete Adjuvant were co-administered at two-week intervals. Two weeks after the second boost, animals were euthanized

by anesthetic overdose and blood was collected by cardiac puncture. Sera was collected by centrifugation, aliquoted and frozen at -80°C.

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

Generation of host-adapted leptospires. To obtain mammalian host-adapted organisms, *L. interrogans* sv. Manilae strain L495 wild-type and mutant strains were cultivated in DMCs as previously described [42, 43, 108]. Briefly, DMCs were prepared with 9-10 mls of EMJH medium (supplemented with an additional 10% bovine serum albumin to maintain osmotic pressure) at a starting inoculum of 10^4 organisms per ml. Using strict aseptic technique, DMCs were implanted into the peritoneal cavity of an anesthetized female Sprague-Dawley rat. After nine days, animals were euthanized by CO₂ narcosis and DMCs harvested. The viability and density of leptospires were evaluated by dark field microscopy using a Petroff-Hausser counting chamber (Hausser Scientific Co., Horsham, PA).

Murine infection experiments. To determine the lethal dose to 50% of mice (LD₅₀) for *L. interrogans* sv. Manilae strain L495, ten-week-old female C3H/HeJ mice (Jackson Laboratories, Bar Harbour, ME) were inoculated intraperitoneally (IP) with 200 µl of EMJH containing 5×10^6 , 10^6 , 10^5 or 10^4 leptospires (5 mice per group). Animals were monitored twice a day for signs of leptospirosis and, when moribund, were euthanized by anesthetic overdose. LD₅₀ was calculated using the Reed-Muench method [186]. For virulence studies, 10^5 of wild-type parent, mutant or complemented strains were used to infect C3H/HeJ mice (5 animals per group, per experiment). Beginning 14 days post-infection (p.i.), animals were monitored for the presence of leptospires in urine, collected in a metabolic chamber for ~45 min following IP administration of furosemide (2-10 mg/kg, IP). Burdens in urine were assessed by darkfield microscopy using a Petroff-Hausser counting chamber. Twenty-eight or 42 days p.i. (virulence and LD₅₀ experiments, respectively), animals were euthanized by CO₂ narcosis and blood and kidneys were collected for serology, culturing in EMJH, and qPCR. Sera from individual mice were used to immunoblot whole cell

lysates ($\sim 10^8$ leptospire per lane) prepared from the wild-type parent grown *in vitro* in EMJH at 30°C.

qRT-PCR. Total RNA was isolated from leptospire (four biological replicates per condition) cultivated *in vitro* at 30°C or following cultivation in DMCs as previously described [42]. cDNAs (+ and – RT) were assayed in quadruplicate in 25 μ l reactions performed with SsoAdvanced Universal SYBR or Probe (*lipL32*) Super Mixes (Bio-Rad). Oligonucleotide primers used for qRT-PCR are provided in S6 Table. Copy numbers were calculated using internal standard curves (10^7 – 10^1 copies) generated using purified amplicons for *perRA*, *perRB*, *LIMLP18590* and *LIMLP04825* and then normalized against *lipL32* [179]. The standard curve for *lipL32* was generated using a copy of the *lipL32* amplicon cloned into pCR2.1-TOPO plasmid (Invitrogen). Normalized copy numbers were compared using an unpaired *t* test with two-tailed *p* values and 95% confidence interval (Prism v. 6, GraphPad Software).

Quantitation of burdens by qPCR. DNA was extracted from infected kidneys using the Qiagen DNeasy Blood & Tissue kit according to the manufacturer's recommendations. DNAs were analyzed by quantitative PCR (qPCR) using a TaqMan-based assay for *lipL32* [179] in 25 μ l reactions performed with SsoAdvanced Universal Probes Super Mix (Bio-Rad). Copy numbers for *lipL32* were determined using an internal standard curve for the *lipL32* amplicon cloned into pCR2.1 TOPO (Invitrogen). Average values for each strain were compared using an unpaired *t* test with two-tailed *p* values and 95% confidence interval (Prism v. 6, GraphPad Software).

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

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

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Author contributions statement

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

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1147 PubMed PMID: 31905198; PubMed Central PMCID: PMC6964914 the following competing
1148 interests: MSG and MGS are or were employed in part by a commercial company, Immuno Technologies,
1149 Inc. MGS holds more than 5% financial interest in Immuno Technologies, Inc. NN and CW declare no
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Table 1. Genes significantly upregulated by PerRA/PerRB in *L. interrogans* cultivated within DMCs

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

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

- 1 Gene identifications and descriptions are based on *L. interrogans* sv. Manilae strain UP-MMC-NIID LP (accession numbers NZ_CP011931.1, NZ_CP011932.1 and NZ_CP011933.1). In some cases, annotations have been manually curated to conform with prior studies or bioinformatics.
- 2 *L. interrogans* sv. Copenhageni strain Fiocruz L1-130 (accession numbers NC_005823.1 and NC_005824.1) orthologs were identified using OrthoVenn 2.0 [202]. Dashes (-) indicate genes for which no clear ortholog was identified.
- 3 Localization is based on cumulative data from BUSCA [193], Lipop [192] and SpLip [191]. Abbreviations: CY, cytoplasmic; EC, extracellular; LIPO, lipoprotein; IM, inner membrane; OM, outer membrane.
- 4 Fold-regulation and adjusted-*p* values determined using DESeq [112] based on WT vs. *perRA/B* RNA-Seq

comparison. See Table S4.

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

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

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

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

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

3 Localization is based on cumulative data from BUSCA [193], LipoP [192] and SpLip [191]. Abbreviations: CY,

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

- 4 Fold-regulation and adjusted-*p* values determined using DESeq [112] based on WT vs. double mutant RNA-Seq comparison. See Table S4.
- 5 Behavior of the corresponding gene in the WT vs. *perRA* (A) and *perRB* (B) comparisons using *L. interrogans* cultivated within DMCs. Arrows are used to indicate significant upregulation (↑) or repression (↓) in the WT compared to the corresponding mutant in DMCs. See Tables S2 and S3.
- 6 Behavior of the corresponding gene in the WT vs. *perRA* (A), *perRB* (B), and *perRA/B* (AB) RNA-Seq using *L. interrogans* cultivated *in vitro* [91, 92]. Arrows are used to indicate significant (≥ 3 -fold, $p \leq 0.05$) upregulation (↑) or repression (↓) in the WT compared to the corresponding mutant *in vitro*. See Tables S2 and S3.

Figure Legends

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

coordination. Asparagine (N) or arginine (R) residues (□) in blue, located in DNA binding helix H4, can be used to distinguish between PerR and Fur, respectively [95]. *, the PDB structure for *E. coli* Fur includes only the DNA binding domain. Numbers on the top correspond to residues positions in *L. interrogans* PerRA.

Figure 2. Distribution of Ferric-uptake regulator (FUR) domain-containing proteins across pathogenic and saprophytic *Leptospira* spp. **A.** FUR domain-containing proteins in representative *Leptospira* spp. from pathogenic (P1 and P2) and saprophytic (S1 and S2) subclades. Genomic locus tags for each FUR family protein in *Leptospira* spp. are indicated. **B.** Phylogenetic analysis of *Leptospira* spp. FUR family proteins shown in A. Unrooted tree was generated using iTOL [200].

Figure 3. *L. interrogans* express increased transcript levels for three FUR family regulators in response to mammalian host signals compared to *in vitro*. Transcripts for *LIMLP10155* (*perRA*), *LIMLP05620* (*perRB*), *LIMLP04825* (*ffr1*) and *LIMLP18590* (*ffr2*) and were accessed by qRT-PCR using cDNAs from wild-type *L. interrogans* sv. Manilae strain L495 cultivated *in vitro* in EMJH at 30°C (IV) or within rat peritoneal dialysis membrane chambers (DMC). Transcript copy numbers for each gene of interest were normalized per 1000 copies of *lipL32*. Bars show the average of four biological replicates for each condition, assayed in quadruplicate. *p*-values were determined using a two-tailed *t*-test; *, *p* < 0.05.

Figure 4. *L. interrogans* requires both PerRA and PerRB for renal colonization of C3H/HeJ mice. **A.** Female 10-week old C3H/HeJ mice (5 per group) were inoculated intraperitoneally with

the indicated numbers of leptospire and monitored for 42 days. At 42 days, animals were euthanized, and kidneys harvested for culturing in EMJH. **B.** Enumeration of leptospire in urine collected from C3H/HeJ mice shown in A 14-, 21- and 35-days post-infection. Circles represent data for urine from individual mice. Burdens per ml of urine were assessed by darkfield microscopy using a Petroff-Hauser counting chamber. Bars show the average and standard error of the mean. *p*-values were determined using a two-tailed *t*-test. **C.** Enumeration of leptospire in urine collected from C3H/HeJ mice inoculated intraperitoneally with 10^5 of WT, *perRA*, *perRB*, *perRA/B* or *perA/B+perRB* *trans*-complemented (Comp) strains. Burdens per ml of urine were assessed by darkfield microscopy using a Petroff-Hauser counting chamber. Circles represent data for urine from individual mice in three independent experiments (5 mice per group, per strain, per experiment). One mouse infected with the WT strain died between day 14-21 due to circumstances unrelated to infection with *L. interrogans*. **D.** Burdens of leptospire in kidneys harvested from mice in panel C. DNA samples from kidneys harvested 28 days post-inoculation were assessed by qPCR using a Taqman-based assay for *lipL32* (in quadruplicate). Bars in B-D represent the average and standard error of the mean. *p*-values in C and D were determined by comparing burdens in mice infected with wild-type (WT) and mutant strains at the same timepoint using a two-tailed *t*-test. ***, $p \leq 0.0001$; **, $p = 0.0079$. **E.** Immunoblot analysis of sera collected from mice with WT, *perRA*, *perRB*, *perRA/B* or *perA/B+perRB* *trans*-complemented (Comp) strains, collected 28 days post-infection and tested against whole cell lysates of *L. interrogans* sv. Manilae strain L495 grown in EMJH at 30°C.

Figure 5. The PerRA and PerRB DMC regulons contain genes related to environmental sensing, signaling and/or transcriptional regulation. Proteins containing conserved domains

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

(IPR036271); TM, transmembrane helix; TMx6, six transmembrane helices; and TPR, Tetratricopeptide-like helical domain superfamily (IPR011990).

Figure 6. Overview of comparative RNA-Seq analyses of *L. interrogans* wild-type, *perRA*, *perRB* and *perRA/B* mutant strains. **A.** Venn diagram showing overlap of genes differentially expressed ≥ 3 -fold ($q \leq 0.05$) in wild-type versus single and double mutant comparisons. $\uparrow$ and $\downarrow$ symbols denote genes upregulated (*i.e.*, expressed at higher levels in the wild-type vs. mutant) or downregulated (*i.e.*, expressed at lower levels in the wild-type vs. mutant), respectively, by PerRA, PerRB or both (PerRA/B). Complete datasets of all comparisons are presented in Tables S2-S4. **B.** Cluster of Orthologous Genes (COG) categorization of differentially expressed genes (DEGs) in the wild-type vs. *perRA/B* double mutant RNA-Seq comparison. COG predictions for individual genes are presented in Table S4. Number of DEGs in each COG are indicated on the *x*-axis.

Figure 7. Expression of LigA and LigB *in vivo* is dysregulated only in the *perRA/B* double mutant. **A.** Cartoon depiction of *ligA*, *ligB* and surrounding genes in *L. interrogans* sv. Manilae strain L495. Hatched bars are used to show Immunoglobulin-like repeats. Red and Blue colored regions indicate LigA- and LigB-specific regions, respectively. Values below each cartoon indicate the fold-regulation in the wild-type (WT) strain L495 parent compared to *perRA*, *perRB* and *perRA/B* mutant strains based on the corresponding RNA-Seq comparisons. **B.** Whole cell lysates of *L. interrogans* sv. Manilae strain L495 isogenic WT, *perRA*, *perRB*, *perRA/B*, *perRA/B* *trans*-complemented with *perRB* (Comp) and *lvrAB* strains were generated from leptospirees cultivated within DMCs, separated by SDS-PAGE, and immunoblotted with rabbit polyclonal antiserum against repeats conserved in both LigA and LigB (gray region in panel A). After

detection, membranes were striped and re-blotted using rat polyclonal antiserum against recombinant FlaB1 as a loading control. Molecular weight markers (kDa) are shown on the left.

Figure 8. Expression of LvrAB requires both PerRA and PerRB but the absence of LvrAB alone is not solely responsible for avirulence of the *perRA/B* double mutant.

A. Whole cell lysates of *L. interrogans* sv. Manilae strain L495 wild-type (WT), *perRA*, *perRB*, *perRA/B*, *perRA/B* mutant complemented in *trans* with *perRB* (Comp) and *lvrAB* mutant strains were generated from leptospires cultivated within DMCs. Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with rabbit polyclonal LvrA- or LvrB-specific antiserum. Membranes were striped and re-blotted using rat polyclonal antiserum against recombinant FlaB1 as a loading control. Molecular weight markers (kDa) are shown on the left.

B. Enumeration of leptospires in urine collected from C3H/HeJ mice 14- and 21-days post-infection following intraperitoneal inoculation with 10^5 of wild-type (WT), *lvrAB* or *lvrB* mutant strains. Circles represent data for urine from individual mice (5 mice per group, per strain, per experiment). **C.** Burdens of leptospires in kidneys harvested from mice in panel B. DNA samples from kidneys harvested 28 days post-inoculation were assessed (in quadruplicate) by qPCR using a Taqman-based assay for *lipL32*. Bars in B and C represent the average and standard error of the mean. *p*-values were determined by comparing burdens in mice infected with wild-type (WT) and mutant strains at the same timepoint using a two-tailed *t*-test; ***, $p < 0.0001$; **, $p = 0.0007$; and *, $p = 0.0079$.

D. Immunoblot analysis of sera collected from C3H/HeJ mice 28-days following intraperitoneal inoculation with 10^5 wild-type or *lvrAB* mutant strains and then blotted against whole cell lysates of *L. interrogans* sv. Manilae strain L495 grown in EMJH at 30°C.

Supplementary Material

S1 Figure. Multiple sequence alignment with full-length sequences of FUR-like regulators in

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

S2 Figure. Multiple sequence alignment of FUR domain-containing proteins in

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

meyeri; *L. ter*, *L. terpstrae*; *L. van*, *L. vanthielii*; *L. ryu*, *L. ryugenii*; *L. ily*, *L. ilyithenensis*; *L. ido*,
L. idonii.

S3 Figure. Expression of PerRA and PerRB in *L. interrogans* wild-type, mutant and complemented strains. Whole-cell lysates from *L. interrogans* sv Manilae strain L495 wild-type (WT), *perRA*, *perRB*, *perRA/B* and the *perRA/B* mutant complemented in *trans* with *perRB* on pMaORI (Comp) were separated by SDS-PAGE, immobilized on nitrocellulose and then immunoblotted with rat polyclonal antisera against PerRA, PerRB and FlaB1.

S4 Figure. PerRA and PerRB regulate the expression of a locus (*LIMLP04285-04240*) that includes a TonB-dependent transport system. Data from comparative RNA-Seq analysis of wild-type vs. *perRA*, *perRB* and *perRA/B* strains identified a nine gene chromosomal locus that includes *lipL48* and genes encoding a TonB-dependent receptor and ExbB/ExbD/TonB transporter. Fold-of-regulation for each gene are based on RNA-Seq data from wild-type and mutant leptospires grown in DMCs, presented in Tables S2-S4, and *in vitro* in EMJH at 30°C (IV), presented in Zavala-Alvarado *et al.* [92]

S5 Figure. Overview of genes differentially expressed by *L. interrogans perRA* and *perRB* single mutants. Cluster of Orthologous Genes (COG) categorization of differentially expressed genes (DEGs) in the wild-type vs. *perRA* (A) and *perRB* (B) RNA-Seq comparisons. COG predictions for individual genes are presented in Tables S2-S3. Number of DEGs in each COG are indicated on the *x*-axis.

1670

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

1672

1673 **Table S2. Comparative RNA-Seq data for *L. interrogans* sv. Manilae L495 wild-type and**

1674 ***perRA* strains cultivated in dialysis membrane chambers (DMCs).** The genome sequence of *L.*

1675 *interrogans* sv. Manilae strain UP-MMC-NIID LP (accession numbers NZ_CP011931.1,

1676 NZ_CP011932.1 and NZ_CP011933.1) was used for mapping and differential gene expression

1677 analysis. All non-coding RNAs and pseudogenes were removed before DESeq2 analysis. **Column**

1678 **A:** RefSeq locus tag. **Column B:** Locus tag. **Column C:** *L. interrogans* serovar Copenhageni strain

1679 Fiocruz L1-130 (accession numbers NC_005823.1 and NC_005824.1) orthologs identified using

1680 OrthoVenn 2.0 [202]. Dashes (-) indicate genes for which no clear ortholog was identified.

1681 **Column D:** Description of gene product, following genome annotation. **Columns E, F:** Clusters

1682 of Orthologous Group (COG) classifications based on MicroScope [187]. **Column G:** Fold-

1683 regulation of the corresponding gene based on RNA-Seq analysis by Zavala-Alvarado *et al.* [91,

1684 92] performed using the same strains cultivated *in vitro*. Positive and negative numbers indicate

1685 upregulation and repression, respectively, in the WT compared to the mutant strain. Dashes (-)

1686 indicate genes that are not differentially regulated at least 3-fold ($p \leq 0.05$) by PerRA *in vitro*.

1687 **Column H:** Fold-regulation determined using DESeq2 based on WT vs. *perRA* mutant RNA-Seq

1688 analysis using leptospires cultivated within DMCs. **Column I:** Type of regulation by PerRA in

1689 DMCs. Genes expressed at ≥ 3 -fold higher/lower levels in the WT vs. mutant with a False-

1690 discovery rate-adjusted- p value (q) ≤ 0.05 were considered differentially expressed. “NO” indicates

1691 genes that are not regulated by PerRA in DMCs; “Up” indicates genes upregulated by PerRA in

1692 DMCs (expressed at lower levels in the mutant vs. WT); “Down” indicates genes downregulated

1693 by PerRA in DMCs (expressed at higher levels in the mutant vs WT). **Columns J-O**: Number of
 1694 mapped reads per gene for each one of the three biological replicates per strain. **Columns P-AC**:
 1695 Output from DESeq2 for WT vs. *perRA* mutant strains cultivated in DMCs (3 biological replicates
 1696 per strain). **Column P**: Mean DESeq2 values for each gene. **Column Q**: Log₂-fold change in gene
 1697 expression. **Column R**: Power function transformation of log₂-fold change. **Column S**: Fold
 1698 regulation. **Column T-W**. Statistical analysis of differential gene expression including standard
 1699 error estimate for the log₂-fold change estimate (lfcSE, column T) and adjusted *p*-value (W).
 1700 **Columns X-AC**: Normalized copy numbers per gene (3 biological replicates per strain).

1701
 1702 **Table S3. Comparative RNA-Seq data for *L. interrogans* sv. Manilae L495 wild-type and**
 1703 ***perRB* strains cultivated in dialysis membrane chambers (DMCs).** The genome sequence of *L.*
 1704 *interrogans* sv. Manilae strain UP-MMC-NIID LP (accession numbers NZ_CP011931.1,
 1705 NZ_CP011932.1 and NZ_CP011933.1) was used for mapping and differential gene expression
 1706 analysis. All non-coding RNAs and pseudogenes were removed before DESeq2 analysis. **Column**
 1707 **A**: RefSeq locus tag. **Column B**: Locus tag. **Column C**: *L. interrogans* serovar Copenhageni strain
 1708 Fiocruz L1-130 (accession numbers NC_005823.1 and NC_005824.1) orthologs identified using
 1709 OrthoVenn 2.0 [202]. Dashes (-) indicate genes for which no clear ortholog was identified.
 1710 **Column D**: Description of gene product, following genome annotation. **Columns E, F**: Clusters
 1711 of Orthologous Group (COG) classifications based on MicroScope [187]. **Column G**: Regulation
 1712 of the corresponding gene based on RNA-Seq analysis by Zavala-Alvarado *et al.* [91, 92]
 1713 performed using the same strains cultivated *in vitro*. Positive and negative numbers indicate
 1714 upregulation and repression, respectively, in the WT compared to the mutant strain. Dashes (-)
 1715 indicate genes that are not differentially regulated at least 3-fold ($p \leq 0.05$) by PerRB *in vitro*.

1716 **Column H:** Fold-regulation determined using DESeq2 based on WT vs. *perRB* mutant RNA-Seq
 1717 analysis using leptospires cultivated within DMCs. **Column I:** Type of regulation by PerRB in
 1718 DMCs. Genes expressed at ≥ 3 -fold higher/lower levels in the WT vs. mutant with a False-
 1719 discovery rate-adjusted-*p* value (*q*) ≤ 0.05 were considered differentially expressed. “NO” indicates
 1720 genes that are not regulated by PerRB in DMCs; “Up” indicates genes upregulated by PerRB in
 1721 DMCs (expressed at lower levels in the mutant vs. WT); “Down” indicates genes downregulated
 1722 by PerRB in DMCs (expressed at higher levels in the mutant vs. WT). **Columns J-O:** Number of
 1723 mapped reads per gene for each one of the three biological replicates per strain. **Columns P-AC:**
 1724 Output from DESeq2 for WT vs. *perRB* mutant strains cultivated in DMCs (3 biological replicates
 1725 per strain). **Column P:** Mean DESeq2 values for each gene. **Column Q:** Log₂ fold change in gene
 1726 expression. **Column R:** Power function transformation of log₂-fold change. **Column S:** Fold
 1727 regulation. **Column T-W.** Statistical analysis of differential gene expression including standard
 1728 error estimate for the log₂-fold change estimate (lfcSE, column T) and adjusted *p*-value (W).
 1729 **Columns X-AC:** Normalized copy numbers per gene (3 biological replicates per strain).

1730

1731 **Table S4. Comparative RNA-Seq data for *L. interrogans* sv. Manilae L495 wild-type and**
 1732 ***perRA/B* strains cultivated in dialysis membrane chambers (DMCs).** The genome sequence of
 1733 *L. interrogans* sv. Manilae strain UP-MMC-NIID LP (accession numbers NZ_CP011931.1,
 1734 NZ_CP011932.1 and NZ_CP011933.1) was used for mapping and differential gene expression
 1735 analysis. All non-coding RNAs and pseudogenes were removed before DESeq2 analysis. **Column**
 1736 **A:** RefSeq locus tag. **Column B:** Locus tag. **Column C:** *L. interrogans* sv. Copenhageni strain
 1737 Fiocruz L1-130 (accession numbers NC_005823.1 and NC_005824.1) orthologs identified using
 1738 OrthoVenn 2.0. [202]. Dashes (-) indicate genes for which no clear ortholog was identified.

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

1762 **S:** Mean DESeq2 values for each gene. **Column T:** Log₂ fold change in gene expression. **Column**
1763 **U:** Power function transformation of Log₂ fold change. **Column V:** Fold regulation. **Column W-**
1764 **Z.** Statistical analysis of differential gene expression including standard error estimate for the log2
1765 fold change estimate (lfcSE, column T) and adjusted *p*-value (W). **Columns AA-AF:** Normalized
1766 copy numbers per gene (3 biological replicates per strain).

1767

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

1769

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

Figure 1

A

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

B

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Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B

Figure 7

A

B

C

D

Figure 8