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Structure and function of the Leptospira interrogans peroxide stress regulator (PerR), an atypical PerR devoid of a structural metal-binding site

Peroxisome sensing is essential for bacterial survival during aerobiotic metabolism and host infection. Peroxide stress regulators (PerRs) are homodimeric transcriptional repressors with each monomer typically containing both structural and regulatory metal-binding sites. PerR binding to gene promoters is controlled by the presence of iron in the regulatory site, and iron-catalyzed oxidation of PerR by H$_2$O$_2$ leads to the dissociation of PerR from DNA. In addition to a regulatory metal, most PerRs require a structural metal for proper dimeric assembly. We present here a structural and functional characterization of the PerR from the pathogenic spirochete Leptospira interrogans, a rare example of PerR lacking a structural metal-binding site. In vivo studies showed that the leptospiral PerR binds to the peroxide stimulon in pathogenic species and is involved in controlling resistance to peroxide. Moreover, a perR mutant had decreased fitness in other host-related stress conditions, including at 37°C or in the presence of superoxide anion. In vitro, leptospiral PerR could bind to the perR promoter region in a metal-dependent manner. The crystal structure of the leptosomal PerR revealed an asymmetric homodimer, with one monomer displaying complete regulatory metal coordination in the characteristic caliper-like DNA-binding conformation and the second monomer exhibiting disrupted regulatory metal coordination in an open non-DNA-binding conformation. This structure showed that leptospiral PerR assembles into a dimer in which a metal-induced conformational switch can occur independently in the two monomers. Our study demonstrates that structural metal binding is not compulsory for PerR dimeric assembly and for regulating peroxide stress.

Bacteria are unavoidably exposed to reactive oxygen species (ROS) during the course of aerobic metabolism. ROS include superoxide anion radicals (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) produced by the sequential reduction of oxygen catalyzed by membrane-associated respiratory chain enzymes and hydroxyl radicals (•OH) subsequently produced from H$_2$O$_2$ through the Fenton reaction (1). Environmental agents, such as ionizing UV radiation, also contribute to increase the O$_2^-$ cellular content. Pathogenic bacteria are also exposed to a variety of oxidants, such as O$_2^-$, •OH, and H$_2$O$_2$ as well as hypochlorous acid (HOCl) and nitric oxide (NO) produced by the host immune system (2). Defenses against these toxic compounds are therefore crucial for bacterial survival inside and outside the host. Among the multiple enzymes dedicated to ROS detoxification, catalase and other peroxidases catalyze the degradation of H$_2$O$_2$ to H$_2$O. The genes encoding peroxidases in bacteria are under the transcriptional control of H$_2$O$_2$ sensors, OxyR and peroxide stress regulator (PerR). With few exceptions, OxyR and PerR do not co-exist in the same bacterial species but are present in most Gram-negative or Gram-positive bacteria, respectively (3).

PerRs are homodimeric metalloregulators belonging to the ferric uptake regulator (Fur) family, which act mainly as repressors. The paradigm of PerRs is that of Bacillus subtilis, which has been well characterized (4). The individual monomers of PerR consist of an N-terminal DNA binding domain and a C-terminal dimerization domain. Each B. subtilis PerR monomer contains a structural metal (Zn$^{2+}$)-binding site in the dimerization domain and a regulatory metal (Fe$^{2+}$ or Mn$^{2+}$)-binding site in the interdomain region. The structural metal is required for proper folding and dimeric assembly, and the regulatory metal allows PerR to interact with DNA motifs (perR box) present in the promoter regions of regulated genes (5–8). Upon exposure of B. subtilis PerR to H$_2$O$_2$ or O$_2^-$, two histidine residues that participate in regulatory metal coordination are oxidized, and a global conformational change is triggered (9–11). As a result, PerR dissociates from DNA, and target genes are transcribed by RNA polymerase.

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5 The abbreviations used are: ROS, reactive oxygen species; PerR, peroxide stress regulator; BiPerR, B. subtilis PerR; LiPerR, L. interrogans PerR; Fur, ferric uptake regulator; ICP-M5, inductively coupled plasma mass spectrometry; EMUH, Ellingham–McCullough–Johnson–Harris; r.m.s., root mean square; PDB, Protein Data Bank; qRT-PCR, quantitative RT-PCR.
Characterization of the Leptospira peroxide stress regulator

Leptospira are among the few examples of Gram-negative bacteria that possess a PerR-like regulator involved in controlling defenses against peroxide stress (12). These aerobic bacteria of the spirochetal phylum have pathogenic representatives that are the causal agents of leptospirosis, a widespread zoonotic disease (13, 14). Leptospirosis is transmitted to animals and humans by exposure to soils and water contaminated with the urine of reservoir animals carrying pathogenic Leptospira. Once having penetrated an organism, Leptospira enter the bloodstream and rapidly disseminate to multiple tissues and organs, including kidney, liver, and lungs (15). Clinical manifestations range from a mild flu-like febrile state to more severe and fatal cases leading to hemorrhages and multiple-organ failure (16). Although recognized as an emerging disease and a significant health threat among impoverished populations in developing countries and tropical areas (17), leptospirosis is a neglected and underdiagnosed disease. In addition, the lack of efficient tools and techniques for genetic manipulation of Leptospira spp. has greatly hampered our understanding of the mechanism of pathogenicity and virulence as well as the basic biology of this pathogen (13, 14).

Several lines of evidence have indicated the importance of ROS production during infection by Leptospira. The internalization of pathogenic Leptospira by macrophages and concomitant production of ROS has been demonstrated in vitro (18), and leptospirosis-associated oxidative stress has been observed in patients (19) and infected animals (20). Several studies have reported the production of peroxide and other ROS in lung, kidney, and freshly voided urine (21–23), which are colonized by pathogenic Leptospira. In addition, catalase is required not only for survival in the presence of H₂O₂ but also for virulence (24), and perR is up-regulated when cells are grown in rats (25). These findings strongly suggest a role of PerR in the adaptation of pathogenic Leptospira to a mammalian host.

An intriguing particularity of leptospiral PerR, in contrast to most Fur-like regulators, is the absence of a structural metal-binding site. To gain insight into this class of PerR and the molecular mechanism of PerR function in Leptospira, we have carried out a functional, biochemical, and structural characterization of this transcriptional regulator. We show that the binding of Leptospira interrogans PerR with its promoter region is metal-dependent, and we report the crystal structure of the homodimeric PerR from L. interrogans, which provides a snapshot of the metal-induced conformational switch required for DNA binding and dissociation. We have also investigated the effect of perR inactivation on the growth of Leptospira in conditions similar to those encountered in a mammalian host.

Results

Crystal structure of L. interrogans PerR

Leptospira spp. encode a PerR ORF that shares about 30% amino acid identity with PerRs encoded by other bacteria (Campylobacter jejuni, B. subtilis, Staphylococcus aureus, and Streptococcus pyogenes) (Fig. S1A). Alignment of the ORF sequence encoded by the pathogen L. interrogans serovar Copenhagenii (LIC12034, LrPerR) with that of the well-characterized B. subtilis (BsPerR) shows a conservation of the five residues (His37–Asp95–His91–His93–Asp104, BsPerR primary sequence numbering) that coordinate the regulatory metal (Fig. S1A). Surprisingly, the canonical Cys₄ consensus motif that coordinates the structural metal (Cys96, Cys98, Cys136, and Cys139, BsPerR primary sequence numbering) required for PerR dimeric assembly is absent in the primary sequence of LrPerR. PerR orthologues in other Leptospira strains, including L. interrogans serovar Manilae (LMAnV2_280031), Leptospira borgpetersenii serovar Hardjo-Bovis (LBj_1600), and Leptospira biflexa serovar Patoc (LEPBla2461), also lack the Cys₄ consensus motif (Fig. S1B). In contrast, this 4-cysteinate motif is conserved in all PerR regulators whose structures have previously been solved and in most PerR and Fur regulators from sequenced genomes.

To characterize the conformation and assembly of a PerR devoid of a structural metal-binding site, we solved the crystal structure of the LrPerR. The protein crystallized as an asymmetric homodimer with two independent monomers (chains A and B) in the asymmetric unit. The electron density could be traced from residue 1 to 145 in both monomers. Each monomer adopts the characteristic two-domain organization of Fur-like regulators with an N-terminal DNA-binding domain and a C-terminal dimerization domain. The N-terminal domain (residues 1–84) contains four α-helices (H1–H4), followed by a two-stranded antiparallel β-sheet (β1, β2) (Fig. 1). Helices H2, H3, and H4 form a trihelical bundle arranged in a helix-turn-helix DNA-binding motif. The putative DNA-binding helix (H4, residues 54–68) is well conserved in other PerRs (Fig. S1). The C-terminal domain (residues 91–145) consists of a long helix (H5, residues 107–127) and three β strands (β3, β4, and β5) which are involved in dimerization of the regulator. The N- and C-terminal domains of each monomer are connected by a flexible hinge region (residues 85–90), which adopts significantly different conformations in monomers A and B (see below). The overall structure of monomer A is similar to that of the BsPerR-Zn-Mn structure (PDB entry 3F8Ni; Fig. 1) with the
Figure 2. Metal binding in L. interrogans PerR. A, detailed view of the regulatory binding site of LiPerR (left) and LiPerR superimposed with BsPerR (LiPerR in green and BsPerR (PDB entry 3F8N) in white) (right). The coordination residues are labeled in green and gray for LiPerR and BsPerR, respectively, and the metals in the regulatory metal-binding sites are represented by orange (zinc for LiPerR) or blue (manganese for BsPerR) spheres. Ligand coordination is symbolized by black and blue dashed lines for LiPerR and BsPerR, respectively. The electron density map (gray contours, left) was calculated with coefficients $2F_{\text{obs}} - F_{\text{calc}}$, and is contoured at 2σ. An anomalous difference map (red contours, left) is shown contoured at 2σ. B, detailed view of the structural metal-binding site of BsPerR (PDB entry 2FE3; blue) superimposed with the corresponding region in LiPerR (chain A; green). The cysteine residues coordinating the structural metal in BsPerR (zinc, symbolized by a red sphere) are indicated in blue, and the superimposed residues in LiPerR are shown in green. C, overall view of the BsPerR dimerization domain (PDB entry 2FE3; blue) superimposed with the corresponding region in LiPerR (chains A and B in green and yellow, respectively). The structural zinc coordinated by BsPerR is represented by a red sphere.

As expected from the amino acid sequence, the crystal structure of LiPerR confirmed the absence of the canonical Cys$_4$ consensus motif involved in the structural metal coordination (Fig. 2B). Three of the four canonical Cys$_4$ residues of BsPerR (Cys$^{96}$, Cys$^{99}$, and Cys$^{136}$) were structurally aligned with Asp$^{95}$, Thr$^{98}$, and Lys$^{143}$, respectively, in LiPerR (Fig. 2B). This structural alignment differs from the amino acid sequence alignment (Fig. S1). In BsPerR, the structural zinc locks the three β strands (β3, β4, and β5) in close proximity to form a six-stranded β sheet that stabilizes the dimeric assembly (8, 26, 27). LiPerR crystallized as a dimer stabilized by helix H5 and a six-stranded β sheet formed by strands β3, β4, and β5 in the C-terminal domains of each monomer (Fig. 2C). The total buried surface area in the dimer interface is 3570 Å$^2$. The β strands involved in dimerization of LiPerR and BsPerR adopt similar topology and can be closely superimposed (Fig. 2C), indicating that the β sheet could be formed, and dimerization occurred in the absence of a structural metal in LiPerR. Moreover, analysis of purified PerR by dynamic light scattering indicated that the protein is dimeric in solution (Fig. S2). Therefore, structural
analysis of LiPerR shows that the absence of a structural metal-binding site does not affect the overall topology or oligomerization of the protein.

**The asymmetric PerR dimer reveals a conformational switch upon regulatory metal coordination**

In the crystallized LiPerR dimer, monomers A and B share similar overall folding within the individual N-terminal and C-terminal domains, with r.m.s. deviations for equivalent Cα positions of 0.47 and 0.70 Å, respectively (Fig. 3A), but the relative orientation of the two domains differs significantly, resulting in a globally asymmetric overall structure (Fig. 3, B and C). Superposition of the C-terminal domains of the two monomers shows that the centroid of the N-terminal DNA binding domain of monomer B was shifted by 22 Å from the position in monomer A (Fig. 3D). Zinc coordination in the regulatory metal-binding site of monomer A involves five amino acid residues (His36, Asp84, His90, His92, and Asp103) (Figs. 2A and 3B), whereas zinc coordination in monomer B involves only three residues (His90, His92, and Asp103), which are fully exposed in the C-terminal domain (Fig. 3C). His36 and Asp84, which participate in the regulatory metal coordination in monomer A, are fully exposed in monomer B with main-chain Cα positions displaced by 22 and 16 Å, respectively (Fig. 3C). Consequently, His36 and Asp84...
in monomer B do not participate in zinc coordination, leaving the partial metal-binding site exposed to solvent.

Models of symmetrical dimers of LiPerR were generated by superimposing the C-terminal domains of monomers A and B, respectively. The modeled symmetrical dimer of monomer A, with complete regulatory metal coordination in both monomers, exhibits the typical caliper-like structure of Fur family regulators with the regulatory metal bound as in the Mn\(^{2+}\)-bound conformation of BsPerR (Fig. 4A and Fig. S3). The symmetrical dimer of monomer B, in which the regulatory metal-binding sites are disrupted, has an open planar conformation (Fig. 4B) comparable with the apo-conformation of BsPerR (Fig. S3). The orientations of the two DNA-binding helices H4 in symmetrical dimers A and B dramatically differ, and the spacing is significantly greater in symmetrical dimer B. The two helices H4 are 32.6 Å apart in symmetrical dimer A (distance between the C/H9251 atoms of the Lys63 in helix H4). The equivalent distance in the Mn\(^{2+}\)-bound BsPerR (distance between the C atoms of the Lys63) is 36 Å, suggesting that the symmetrical dimer A adopts a conformation suitable for DNA binding, whereas the conformation of symmetrical dimer B is incompatible with DNA binding.

**LiPerR binds to the perR upstream promoter region in a metal-dependent manner**

To investigate the ability of LiPerR to bind DNA, recombinant PerR was purified and incubated with a DNA fragment containing the *perR* promoter region. As shown in Fig. 5A, a 7.5-fold molar excess of PerR led to a total shift of DNA. However, this shift was not observed when an equivalent DNA fragment containing the *dnaK* promoter region was mixed with an excess of purified LiPerR (Fig. 5A) or when the assay was performed in the presence of 20 mM EDTA (Fig. 5B). These results indicate that LiPerR was able to bind its own promoter region in a metal-dependent manner. Preincubation of the purified proteins with Fe\(^{2+}\) in aerobic conditions inhibited the interaction of LiPerR with DNA, whereas preincubation with other metals (Mn\(^{2+}\), Cu\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), or Zn\(^{2+}\)) did not significantly affect the interaction with DNA (Fig. S4).
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Analysis of the metal content of the purified recombinant \( \text{LiPerR} \) by inductively coupled plasma mass spectrometry (ICP-MS) (Table S1) showed that the protein contained 0.18 and 0.39 molar eq of \( \text{Zn}^{2+} \) and \( \text{Ni}^{2+} \), respectively, per monomer. \( \text{Fe}^{2+} \), \( \text{Mn}^{2+} \), and \( \text{Co}^{2+} \) were present at negligible amounts. These ratios indicate that only a fraction of the PerR subunits contained bound metals. By expressing the recombinant protein in minimal medium and chelating metals after purification (extensive dialysis in the presence of EDTA and use of chelated buffer), it was possible to lower the \( \text{Ni}^{2+} \) concentration to undetectable levels and the \( \text{Zn}^{2+} \) concentration to about 0.06 molar eq (Table S1). In this condition, \( \text{LiPerR} \) was still able to bind its promoter region, and this interaction was still inhibited by EDTA, indicating the involvement of a metal-bound promoter region, and this interaction was still inhibited by EDTA, indicating the involvement of a metal.

To test whether metal coordination was important for DNA binding, we substituted with alanine each of the residues of the regulatory metal-binding site. Single alanine substitutions of His\(^{36} \), Asp\(^{84} \), His\(^{90} \), or Asp\(^{103} \) did not affect DNA binding, but when His\(^{92} \) was replaced, \( \text{LiPerR} \) did not interact with DNA (Fig. 5B), suggesting that this residue plays a central role in maintaining the topology of the regulatory metal-binding site. A double alanine substitution of His\(^{36} \) and Asp\(^{84} \) also abolished the ability of \( \text{LiPerR} \) to bind DNA (Fig. 5B). It is noteworthy that these residues do not participate in regulatory metal binding in monomer B. These findings indicate that the impaired regulatory metal coordination in \( \text{LiPerR} \) is associated with a conformational switch to a non-DNA-binding state.

In \( \text{B. subtilis} \), derepression of PerR-regulated genes occurs through the oxidation of His\(^{37} \) and His\(^{91} \) by \( \text{H}_2\text{O}_2 \) or \( \text{O}_2 \) (9, 11). These residues are highly conserved in PerRs, including \( \text{LiPerR} \) (Fig. S1). When \( \text{LiPerR} \) was incubated with \( \text{H}_2\text{O}_2 \) and subsequently mixed with the promoter region DNA fragment, we did not observe inhibition of DNA binding, but more distinctly resolved shifted bands were observed by EMSA when high \( \text{H}_2\text{O}_2 \) concentrations were used (Fig. S6), indicating that \( \text{LiPerR} \) was still able to bind DNA. Because neither \( \text{Zn}^{2+} \) nor \( \text{Ni}^{2+} \) are recognized as regulatory metals that catalyze the \( \text{H}_2\text{O}_2 \)-mediated oxidation in previously characterized PerRs, the effects observed when \( \text{LiPerR} \) is pretreated with \( \text{H}_2\text{O}_2 \) are probably not physiologically meaningful.

The association of PerR with DNA was also examined by DNase I footprinting experiments. In the presence of recombinant \( \text{LiPerR} \) purified without metal chelation (i.e. containing substoichiometric concentrations of \( \text{Zn}^{2+} \) and \( \text{Ni}^{2+} \)), a sequence of 122 bp was protected (Fig. 6A). This sequence exhibits three inverted repeat sequences resembling a \( \text{perR} \) box (underlined sequences in Fig. 6B) as well as four 6-bp motifs (in boldface type in Fig. 6B). These motifs are located between 38 and 152 nucleotides upstream of the transcription start site of the \( \text{perR} \) ORF, which appears to be identical to the first nucleotide of the translation start codon (28).

**Effect of perR inactivation on L. interrogans fitness in host-related conditions**

During infection, virulent leptospires are able to survive host conditions, including a temperature of 37 °C, the presence of serum components, and a variety of ROS. To investigate the role of the PerR protein in the pathogen \( \text{L. interrogans} \), WT and \( \text{perR} \) mutant cells were cultivated in Ellinghamhausen–McCullough–Johnson–Harris (EMJH) medium in a variety of conditions mimicking those encountered in a mammalian host. It is noteworthy that EMJH medium contains 180 \( \mu \text{M} \) \( \text{Fe}^{2+} \), the putative physiological regulatory metal of \( \text{LiPerR} \) (29). As shown in Fig. 7A, the growth rates of WT and \( \text{perR} \) mutant cells were comparable when cultivated in EMJH medium at 30 °C, the optimal laboratory growth conditions. In the presence of 1 \( \text{mM} \) \( \text{H}_2\text{O}_2 \), which prevented growth of WT cells, \( \text{perR} \) mutant cells were still able to grow. The increased resistance of the \( \text{perR} \) mutant to \( \text{H}_2\text{O}_2 \) was also assessed by measuring the survival of \( \text{Leptospira} \) cells after a 30-min exposure to 10 \( \text{mM} \) \( \text{H}_2\text{O}_2 \). In these conditions, WT and \( \text{perR} \) mutant cells had about 0.03 and 100% survival, respectively (Fig. 7B). \( \text{PerR} \) was expressed under its native promoter in mutant cells using the pMaORI replicative vector (30), which restored production of PerR, although to lower amounts than observed in WT cells (Fig. 7C). Complemented \( \text{perR} \) mutant cells lost their ability to grow and survive in the presence of \( \text{H}_2\text{O}_2 \) (Fig. 7, C and D). It is interesting to note that \( \text{perR} \) expression increased when \( \text{L. interrogans} \) cells were exposed to sublethal concentrations of \( \text{H}_2\text{O}_2 \) (Fig. 7E), indicating that the \( \text{perR} \) promoter had a higher activity in the presence of H2O2. The association of PerR with DNA was also examined by DNase I footprinting experiments. In the presence of recombinant \( \text{LiPerR} \) purified without metal chelation (i.e. containing substoichiometric concentrations of \( \text{Zn}^{2+} \) and \( \text{Ni}^{2+} \)), a sequence of 122 bp was protected (Fig. 6A). This sequence exhibits three inverted repeat sequences resembling a \( \text{perR} \) box (underlined sequences in Fig. 6B) as well as four 6-bp motifs (in boldface type in Fig. 6B). These motifs are located between 38 and 152 nucleotides upstream of the transcription start site of the \( \text{perR} \) ORF, which appears to be identical to the first nucleotide of the translation start codon (28).
of low concentrations of peroxide. However, PerR cellular content did not increase accordingly, as seen by immunoblot (Fig. 7E).

We also examined the tolerance of pathogenic *Leptospira* to superoxide stress. WT cells were able to grow in the presence of 2.5 μM paraquat, a superoxide anion-generating reagent, although growth started after an adaptation phase (Fig. 8A). In similar conditions, perR mutant cells grew more slowly than WT cells. The growth of perR mutant cells was also greatly reduced compared with that of WT cells at 37 °C (the host temperature) (Fig. 8B) or in the presence of 0.5% human serum (Fig. 8C). No effects of perR inactivation were observed when cells were cultivated in the presence of 120 mM NaCl (host osmolarity) or in the presence of toxic concentrations of metals.

Figure 7. perR expression is stimulated by H$_2$O$_2$ and involved in controlling tolerance of *L. interrogans* to peroxide. A, WT (solid lines) or perR mutant (dashed lines) cells were cultivated in EMJH medium in the absence (squares) or presence (circles) of 1 mM H$_2$O$_2$ at 30 °C. Cell growth was followed by measuring the absorbance at 420 nm. Data are representative of three independent experiments. B, exponentially growing WT or perR mutant cells were incubated with 10 mM H$_2$O$_2$ for 30 min, and percentage survival was determined as described under “Experimental procedures.” Results are shown as the mean and S.D. (error bars) of three independent experiments. C, 15 μg of total protein extracts from WT and perR mutant cells containing the empty pMaORI-expressing vector, WT(pMa) and perR(pMa), respectively, and perR mutant cells containing the pMaORI vector bearing the perR ORF, perR(pMaPerR), were loaded on a 15% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. FlaA2 (equal loading control) and PerR production (top and middle panels, respectively) were assessed by immunoblot as described under “Experimental procedures.” The corresponding cells were incubated for 30 min with 0 or 10 mM H$_2$O$_2$, and cell viability was assessed by their capacity to reduce blue resazurin into pink resorufin (lower panel). The data are representative of three independent experiments. D, WT and perR mutant cells containing the empty pMaORI vector, WT(pMa) and perR(pMa), respectively, and perR mutant cells containing the pMaORI vector bearing the perR open reading frame, perR(pMaPerR), were cultivated in EMJH medium in the presence of 1 mM H$_2$O$_2$ at 30 °C, and cell growth was followed by measuring absorbance at 420 nm. The data are representative of three independent experiments. E, *L. interrogans* cells were incubated for 30 min in the presence of 0 or 10 μM H$_2$O$_2$ at 30 °C. PerR expression was measured by qRT-PCR as described under “Experimental procedures,” and PerR cellular content was assessed by immunoblot using 10 μg of total protein extracts as described above. The data are the mean and S.D. of three independent biological repeats (qRT-PCR) or representative of two independent experiments (immunoblot).
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(up to 1 mM Cu2+, Co2+, or Zn2+), in the presence of HOCl or with a nitrosative stress–reproducing reagent (diethylamine NONOate) (data not shown). Altogether, these results indicate that the primary role of PerR is to control cell fitness under peroxide stress, although inactivation of perR in pathogenic L. interrogans also affects growth under other host-related conditions.

Discussion

Extensive biochemical and structural characterizations have been conducted for BsPerR, which has become the paradigm for the PerR family of transcriptional regulators. Until now, crystal structures have been reported for PerRs from only two bacteria, B. subtilis and S. pyogenes (6, 8, 10, 26, 27). To our knowledge, the crystal structure of the LiPerR determined in this study is the first structure of a PerR from a Gram-negative bacterium. The structure shows that the regulatory metal-binding site in LiPerR is occupied by zinc, which may have been acquired during protein purification procedures. This zinc ion is coordinated in a manner similar to the regulatory metal (Mn2+) in BsPerR, although zinc is probably not the physiological regulatory metal for LiPerR. Indeed, zinc coordination in the regulatory metal-binding site is not unusual and has been observed in other crystal structures of Fur-like regulators (26, 31–33). The wide conservation of the amino acid residues and architecture of the regulatory metal coordination site and the importance of iron in hydroxyl radical production from peroxide argue in favor of iron as the regulatory metal for LiPerR. Whether Mn2+ could act in vivo as a surrogate metal for Fe2+ in LiPerR remains to be demonstrated. In this study, we show that a LiPerR protein preparation containing substoichiometric concentrations of Zn2+ interacts with DNA in a metal-dependent manner, but we were not able to determine the exact nature of the metal that supported DNA binding. One hypothesis is that the substoichiometric concentrations of Zn2+, even if not the physiological regulatory metal, are sufficient to promote the DNA binding-prone conformation in a small fraction of the LiPerR dimer population. This could explain the presence of smears in the EMSAs indicating a transient or low-affinity interaction with DNA. Alternatively, an additional unidentified metal could be acquired during DNA-binding assays. Our attempts to obtain crystals of LiPerR with bound Fe2+ in aerobic conditions were unsuccessful, and incubating the purified protein with Mn2+ did not increase its ability to bind DNA.

Interestingly, although we could not demonstrate that H2O2 inhibits PerR-DNA interaction in vitro, we provide experiem-
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dential evidence that perR expression in Leptospira cells is influenced by the cellular H$_2$O$_2$ concentration. Because PerR represses its own expression in other bacteria, it is likely that LiPerR dissociates from its promoter in the presence of H$_2$O$_2$, thereby increasing its expression. Our findings, however, demonstrate that PerR cellular content was not increased accordingly. This suggests another level of regulation by inhibition of translation and/or by protein degradation, as seen for BsPerR (34).

A remarkable feature of the LiPerR crystal structure is the globally asymmetric conformation of the homodimer. Monomer A, with complete regulatory metal coordination, has a conformation favorable for DNA binding, whereas monomer B, with disrupted regulatory metal coordination, has an open conformation unfavorable for DNA binding. The two conformations of the LiPerR monomers are similar to those observed in the crystal structures of BsPerR in its apo-form (or after oxidation) and upon regulatory metal binding (6, 8, 10). To our knowledge, this study is the first to provide a snapshot of the conformational switch of PerR, showing that this structural change occurs independently in each protomer without disrupting the dimer interface.

It is generally accepted that a structural metal is required to maintain the dimeric assembly of the Fur-like regulators. However, a striking feature of LiPerR is the absence of a structural metal-binding site. The wide conservation of the Cys$_4$ structural metal-binding site in PerRs led to the hypothesis that this site is a distinctive and obligatory feature of PerR regulators (8). Our study demonstrates that LiPerR, which is devoid of a structural metal-binding site, is fully functional in regulating peroxide stress in vitro and in metal-dependent DNA binding in vitro and is susceptible to a metal-driven conformational switch. This study therefore challenges the generality of the role of a structural metal to stabilize PerR dimers. It is interesting to note that the structural metal-binding site is absent in all leptospiral PerRs (pathogens as well as saprophytes) (Fig. S1), although other putative Fur-like regulators in leptospiral species do contain the Cys$_4$ motif. The Fur regulators from Pseudomonas aeruginosa and Magnetospirillum gryphiswaldense are two other examples of Fur-like regulators devoid of a structural metal-binding site (32, 35). Phylogenetic studies will be required to understand the conservation and the evolution of this trait.

Increased resistance to H$_2$O$_2$ has been associated with perR inactivation in L. interrogans (12) and in many other bacterial species (36–41). In L. interrogans, catalase and cytochrome c peroxidase-encoding genes are up-regulated in the presence of PerR, indicating that the protein represses these genes either directly or indirectly (12). We have shown here that a perR mutant has a several-log greater percentage survival than WT cells in the presence of H$_2$O$_2$ and that perR transcript cellular content is enhanced in the presence of H$_2$O$_2$. These results confirm the critical role of L. interrogans PerR in repressing the defense against hydrogen peroxide and also very likely in sensing H$_2$O$_2$.

PerR regulators have been shown to be crucial for the virulence of many bacteria (40, 42–46) partly because regulation of oxidative and peroxide stress is important for the adaptation of pathogenic bacteria to the host environment. However, if the role of PerR regulators were only restricted to repression of peroxide resistance genes, perR mutants with greater peroxide activities would have a survival advantage in the host environment. The PerR regulators may therefore promote the expression of bacterial factors that favor virulence, as observed previously in S. pyogenes (47). By studying the fitness of the perR mutant in different conditions, we have found that this regulator may help to promote cell growth at the host temperature and in the presence of superoxide anion radicals and human serum. As a pathogen that disseminates through the bloodstream, Leptospira will encounter these conditions in the host. Our findings are consistent with a recent study reporting an increase in perR expression levels when Leptospira were cultivated in the peritoneal cavity of rats (25). Interestingly, about 30 ORFs are deregulated in the perR mutant (12), and one-third of these genes are of unknown function. Progress in understanding of the role of LiPerR in stress and host adaptation will require improvements of the efficiency of allelic exchange in pathogenic Leptospira.

In conclusion, this study shows that Leptospira PerR exists in two conformations: an open conformation that does not bind DNA and a caliper-like DNA-binding conformation (Fig. 9). Regulatory metal coordination shifts the equilibrium toward the DNA-binding conformation, and exposure to peroxide very likely leads to DNA dissociation. LiPerR probably represses its own expression as it represses expression of peroxidases or of other factors necessary to withstand stress. The absence of PerR would therefore be beneficial for survival under conditions such as peroxide stress, and Leptospira cells should maintain the PerR to an appropriate steady-state level by regulated proteolysis to avoid repression of key factors. On the other hand, transcriptional repression or activation of other cellular factors that occurs when active PerR binds to their promoters might be necessary for Leptospira fitness under other stress conditions.

By characterizing the function and structure of LiPerR, our study sheds light on the peculiarity of this regulator. Further studies of leptospiral PerR should provide new insights into the function of the large family of Fur-like transcriptional regulators.

Experimental procedures

Bacterial strains and growth condition

L. interrogans serovar Manilae WT L495 and the perR mutant (M776) strains (12) were grown aerobically at 30 °C in EMJH medium (29) with shaking at 100 rpm. EMJH solid medium was prepared by adding 1% (w/v) noble agar to EMJH liquid medium, and plated cells were incubated at 30 °C for 30 days. When necessary, spectinomycin was added at 50 μg/ml. The perR mutant M776 strain carries a transposon insertion at 62 bp into the 438-bp LA1857/LIC12037/L.ManV2_283013 ORF (12).

Escherichia coli strain BL21(DE3) was cultivated at 30 °C in LB or in minimal medium (M9 salts, 0.4% glucose, 1 mM MgSO$_4$) with shaking at 200 rpm. Kanamycin was added at 30 μg/ml when necessary.

Determination of cell viability

Cell survival was determined by incubating exponentially growing L. interrogans cells (~10$^8$/ml) in EMJH medium in the presence or absence of 10 mM H$_2$O$_2$ for 30 min at 30 °C. Cells were then diluted in EMJH medium without H$_2$O$_2$ and plated
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Figure 9. PerR metal-induced allosteric conformational change in Leptospira adaptation to stress. Regulatory metal coordination triggers a conformational switch allowing DNA binding (1). H₂O₂ oxidation of leptospiral PerR would lead to DNA dissociation (2). This would account for the increase in perR expression as seen in our study. However, our findings indicate that protein production is not increased accordingly, suggesting another level of regulation, probably by proteolysis (3). This additional control would allow Leptospira to lower PerR cellular content and prevent repression of peroxidase-encoding genes (or genes encoding other defense activities) during oxidative stress.

Production and purification of recombinant L. interrogans PerR and its variants

The LIC12034 ORF was amplified from L. interrogans serovar Copenhageni strain Fiocruz L1-130 by PCR using the Lic12034-Nhe5 and Lic12034-Hind3 primers (Table S2) and cloned into the pET28b vector (Novagen) between the NheI and HindIII sites. The plasmid obtained (pNB123) allowed the production of an N-terminal His-tagged PerR. PerR variants were obtained by replacing the indicated amino acid residues with alanine using the QuikChange multisite-directed mutagenesis kit (Agilent Technologies) according to the manufacturer’s recommendations and with the primers listed in Table S2. All amino acid substitutions were confirmed by DNA sequencing.

E. coli strain BL21(DE3) was transformed with the pNB123 plasmid and grown to exponential phase at 30 °C in LB medium supplemented with kanamycin. Protein expression was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h. Cells were harvested by centrifugation at 4 °C and stored at -80 °C until use.

Preparations of purified L. interrogans PerR were incubated with 10% HNO₃ for 15 min at 95 °C and subsequently overnight at room temperature. The reaction was diluted to 3.5% HNO₃, and metal concentrations were determined by ICP-MS. All measurements were recorded in duplicate.
cate using 3.5% (v/v) HNO₃ and expressed in μM metal/μM PerR monomer.

**DNA-binding assays**

For EMSA, 429-bp DNA fragments containing the perR (LIC12034) and dnaK (LIC10524) promoter regions, respectively (−290 to +120, from translation start), were PCR-amplified from L. interrogans serovar Copenhageni strain Fiocruz L1-130 using the primers 12034F/12034R and 10524F/10524R, respectively (Table S2). One pmol of these DNA fragments was incubated for 1 h at 37 °C in the presence or absence of the indicated amount of purified recombinant PerR protein in a 20-μl reaction volume containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, and 10% glycerol. Samples were then analyzed under non-denaturing 6% polyacrylamide gel electrophoresis in 50 mM Tris, pH 7.5, and 50 mM borate. DNA was detected by ethidium bromide staining.

For DNase I footprinting assay, a DNA fragment corresponding to the promoter region of perR (−200 to −1, from translation start; 200 bp) was generated by PCR using the primers Lic12034F2 and Lic12034R2 and Pwo polymerase (Roche Applied Sciences) (Table S2). Labeling of the template strand with [γ³²-P]ATP (PerkinElmer Life Sciences) was performed as described previously (48). Purified recombinant PerR was used for binding assays to radiolabeled DNA (5 × 10⁴ cpm/reaction) at room temperature in a buffer containing 25 mM NaH₂PO₄, pH 8.0, 50 mM NaCl, 1 mM MgCl₂, 0.1 mM DTT, and 10% glycerol. 0.1 mg/ml poly(dI-dC) (Sigma-Aldrich) was added as competitor DNA to avoid nonspecific binding. DNase I treatment and electrophoresis were performed as described previously (49).

**Immunoblotting**

*L. interrogans* serovar Manilae strain L495 cells were cultivated until logarithmic phase and lysed by sonication as described previously (30). Total protein extracts were loaded on a 15% SDS-PAGE and transferred on nitrocellulose membrane. PerR and FlaA2 cellular contents were detected as described (30).

**RNA purification and qRT-PCR experiments**

Exponentially grown *L. interrogans* serovar Manilae strain L495 cells were incubated in the presence or absence of 10 μM H₂O₂ for 30 min at 30 °C. Harvested cells were resuspended in 1 ml of TRIzol (Ambion) and stored at −80 °C. Nucleic acids were extracted with chloroform and precipitated with isopropanol alcohol. Contaminating genomic DNA was removed by DNase treatment using the RNase-free TURBO DNA-free kit (Ambion) as described by the manufacturer. cDNA synthesis was performed with the cDNA synthesis kit (Bio-Rad) according to the manufacturer’s recommendation. Quantitative PCR was conducted with the SsoFast EvaGreen Supermix (Bio-Rad) as described previously (24). *perR* ORF expression was measured with the Lic12034qRTF1 and Lic12034qRTR1 primers using *flab* (LA2017/Lic11890/LManV2_290016) as a reference gene (Table S2).

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**Table 1**

**Crystallographic parameters and statistics**

| Parameter | Value |
|-----------|-------|
| Space group | p21 |
| Unit cell dimensions | | |
| a, b, c (Å) | 48.68, 56.10, 68.79 |
| β (degrees) | 105.82 |
| V₀ (Å³/Da) | 2.66 |
| Resolution (Å) | 46.8–1.90 (2.00–1.90) |
| Unique reflections | 27475 (3919) |
| Multiplicity | 6.3 (6.2) |
| Rmerge | 0.066 (0.852) |
| Rfree | 0.043 (0.566) |
| Completeness (%) | 97.3 (95.3) |
| (I/σ(I)) | 13.9 (2.6) |
| CC | 0.997 (0.789) |

| Refinement | |
| Resolution (Å) | 46.8–1.90 (1.98–1.90) |
| R value, working set | 0.203 (0.327) |
| Rfree | 0.234 (0.344) |
| No. of reflections | 26716 (2613) |
| Non-hydrogen atoms | 2362 |
| Protein residues | 292 |
| Water molecules | 173 |
| Zn²⁺ ions | Monomer A | Occupancy 1.0, B-factor 33.1 |
| | Monomer B | Occupancy 1.0, B-factor 40.7 |
| K⁺ ions | 1 |
| r.m.s. deviations from ideal geometry | Bond length (Å) | 0.010 |
| | Bond angles (degrees) | 1.14 |
| Ramachandran plot (%) | Preferred regions | 98.2 |
| | Allowed regions | 1.40 |
| | Outliers (%) | 0.4 |

**Crystallization, data collection, structure determination, and refinement**

Crystallization screening trials for *Leptospira* PerR were carried out by the sitting drop vapor-diffusion method with a Mosquito® (TTP Labtech) automated nanoliter dispensing system. Sitting drops of 400 nl were set up in Greiner plates for 672 commercially available screening solutions with a 1:1 mixture of protein at 22.7 mg/ml equilibrated against 150 μl of reservoir solution. The plates were stored at 18 °C in a Rockmager (Formulatrix) automated imaging system to monitor crystal growth. Best crystals were obtained with a solution of 0.2 M potassium sodium tartrate and 20% (w/v) PEG 3350 (condition D1 from commercial kit PEG/Ion, Hampton Research). Crystals with dimensions of up to 0.1 × 0.1 × 0.2 mm appeared within 2 weeks. For data collection, the crystals were flash-cooled in liquid nitrogen using a paratone-paraffin oil mixture (50%/50%) as cryoprotectant.

X-ray diffraction data were collected on beamline PROxima 1 at synchrotron SOLEIL (St. Aubin, France). X-ray fluorescence emission scans revealed the presence of zinc and the absence of iron or other metals in the crystals. The structure was solved by the single-wavelength anomalous dispersion method with data collected just above the zinc K-edge as determined by the single-wavelength anomalous dispersion method. Heavy-atom sites were located with Phaser (51). Phasing, density modification, and preliminary chain tracing were performed with Phaser (52), Parrot, and Buccaneer from the CCP4 software suite (53). The models were improved by alternate cycles of manual adjustment and model building with COOT (54) and refinement with BUSTER (55, 56). Crystallographic data and refinement statistics are shown in Table 1. All structural figures were generated with PyMOL (57).
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Author contributions—M. K., R. T., A. L., B. B., and P. W. performed experiments. F. S., A. H., S. D., M. P., and N. B. conceived, performed, and analyzed experiments. N. B. conceived and coordinated the study and wrote the paper. All authors approved the final version of the manuscript.

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