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Role of PerR regulators in oxidative stress response and virulence of pathogenic *Leptospira*

Par Jose Crispin ZAVALA ALVARADO

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Dirigée par Dr. Nadia BENAROUDJ

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Président du jury : Prof Isabelle MARTIN-VERSTRAETE, Institut Pasteur, Université Paris

Rapporteurs : Prof Olga SOUTOURINA, Université Paris Sud

Rapporteurs : Dr Soufian OUCHANE, CNRS

Examineurs : Dr Nienke BUDELMEIJER, Institut Pasteur

Examineurs : Dr Ambroise LAMBERT, Université de Cergy-Pontoise

Directeur de thèse : Dr Nadia BENAROUDJ, Institut Pasteur

Résumé

La leptospirose est une zoonose de répartition mondiale qui touche tous les mammifères, dont l'homme, et qui est causée par les bactéries leptospires. La leptospirose se présente sous une forme modérée pseudo-grippale qui peut évoluer vers une forme sévère caractérisée par des atteintes et des hémorragies multiviscérales. On compte dans le monde un million de cas de leptospirose chaque année avec 10% de mortalité. Lors de l'infection, les leptospires sont soumis aux oxydants produits par l'hôte et l'habilité des leptospires à résister à la présence de ces oxydants est primordiale pour coloniser un hôte. Chez les leptospires, les gènes codant pour les peroxydases sont réprimés par le régulateur transcriptionnel PerR1.

Un des objectifs de cette thèse a été d'identifier les mécanismes utilisés par les leptospires pathogènes pour s'adapter à la présence d'oxydants. Nous avons déterminé le transcriptome de *L. interrogans* en présence de peroxyde d'hydrogène et avons montré que trois peroxydases (catalase, cytochrome C peroxydase et la peroxyredoxine) sont les facteurs cellulaires sollicités par les leptospires pour éliminer peroxyde d'hydrogène. De plus, les chaperones moléculaires et des protéines du système de réparation de l'ADN sont impliqués dans la prévention et la réparation des dommages engendrés par l'oxydation.

Nous avons identifié les gènes régulés par PerR1, ce qui a révélé que les gènes régulés par le peroxyde d'hydrogène ne sont pas tous sous le contrôle de PerR1. Parmi les gènes du régulon de PerR1, nous avons identifié des gènes codants respectivement pour un récepteur de type TonB, FecA, une lipoprotéine LipL48, ainsi que pour le système à deux composants VicKR. Nous avons montré que ces facteurs sont impliqués dans la survie des leptospires en présence de superoxyde. Ces facteurs pourraient participer aux mécanismes de défense contre le superoxyde chez les leptospires pathogènes, des bactéries qui ne possèdent pas de superoxyde dismutase.

Nous avons identifié un deuxième régulateur PerR putatif, spécifique des souches de leptospires pathogènes, PerR2. Un autre objectif de cette thèse a été de déterminer la fonction de PerR2 et de déterminer si ces deux régulateurs coopèrent dans la virulence et l'adaptation des leptospires pathogènes au stress oxydatif. L'étude du régulon de PerR2 et le phénotype d'un mutant *perR2* en présence d'oxydant indique que PerR1 et PerR2 ont des fonctions distinctes et non redondantes dans la survie des leptospires en présence d'oxydants. L'inactivation de *perR2* augmente la capacité des leptospires à tolérer des doses létales de superoxyde alors que l'inactivation de *perR1* entraîne une meilleure survie des leptospires en présence de peroxyde d'hydrogène. L'inactivation simultanée de *perR1* et *perR2* entraîne une meilleure tolérance des leptospires au peroxyde d'hydrogène et au superoxyde et une diminution de la virulence des leptospires et de leur capacité à infecter des macrophages. L'étude transcriptomique du double mutant *perR1perR2* a révélé que l'inactivation simultanée de *perR1* et *perR2* entraîne la dérégulation de plusieurs gènes associée à la virulence des leptospires.

L'ensemble de ces résultats a dévoilé pour la première fois chez les leptospires le réseau de régulation permettant l'adaptation de ces bactéries pathogènes aux oxydants auxquelles elles sont exposées lors de l'infection d'un hôte. La coopération de deux régulateurs PerRs semble primordiale à la virulence des leptospires.

Mots clefs : *Leptospira*, ROS, stress, oxydant, PerR, régulation, virulence, transcriptome, non-coding RNA.

Abstract

Pathogen leptospires are responsible for the zoonotic disease leptospirosis. This neglected but emerging infectious disease has a worldwide distribution and affects people from developing countries. More than one million cases of leptospirosis are currently reported annually in the world, with 10% of mortality. Clinical manifestations of this infection range from a febrile state to a severe life-threatening form characterized by multiple organ hemorrhages. However, these symptoms are not specific of leptospirosis, and they render this disease often underdiagnosed. When infecting host, *Leptospira* are confronted with dramatic adverse environmental changes such as deadly reactive oxygen species (ROS). Withstanding ROS produced by the host cells is a vital strategy evolved by pathogenic *Leptospira* for persisting in and colonizing hosts. In *Leptospira*, genes encoding defenses against ROS are under the control of a Peroxide stress Regulator (PerR1), a metalloprotein from the Fur (Ferric uptake regulator) family.

One aim of this PhD was to identify the cellular factors solicited by pathogenic *Leptospira* to adapt to hydrogen peroxide and to determine the contribution of PerR1 in this adaptive response. We have obtained the transcriptome of *L. interrogans* cells exposed to H₂O₂. shown that three main peroxidase machineries (catalase, cytochrome C peroxidase and peroxiredoxin) constitute the first line of defense against H₂O₂. In addition, canonical chaperones and DNA repair proteins are solicited to prevent and recover from oxidative damage. We have determined the PerR1 regulon and have demonstrated that not all members of the peroxide stimulon are under the control of PerR1. In fact, our study has revealed a regulatory network involving other transcriptional regulators, two-component systems and sigma factors as well as non-coding RNAs that could orchestrate, in concert with PerR1, this adaptive response.

Interestingly, our study has allowed the identification of PerR1-regulated genes encoding a TonB-dependent transport system, a lipoprotein (Lip148) and a two-component system (VicKR) involved in *Leptospira* tolerance to superoxide. These factors could represent the first ever identified defense mechanisms against superoxide in *L. interrogans*, a bacterium lacking canonical superoxide dismutase. By examining the genome of *L. interrogans*, we identified a second putative PerR (PerR2) specific to the *Leptospira* pathogenic clade. Another aim of this thesis was to delineate the function of PerR2 and explore its interplay with PerR1 in the *Leptospira* oxidative stress response and virulence. Comparing the PerR1 and PerR2 regulons suggested that these two regulators do not have a redundant function during oxidative stress response in *L. interrogans*. Inactivating *perR1* in *L. interrogans* leads to an increased tolerance to hydrogen peroxide whereas inactivating *perR2* leads to a higher resistance to superoxide; this difference in fitness is consistent with a distinct function in oxidative stress adaptation. Concomitant inactivation of *perR1* and *perR2* leads to a higher ability to resist both peroxide and superoxide but, surprisingly, this double *perR1perR2* mutant has an attenuated virulence and its ability to infect macrophages was impaired. Interestingly, the transcriptome of the double *perR1perR2* mutant exhibited deregulation in several genes associated with *Leptospira* virulence.

Altogether, our study has uncovered the complex regulatory network of the adaptive response to ROS in *Leptospira* and revealed the interplay between the PerR1 and PerR2, necessary for the defense against ROS and virulence in pathogenic *Leptospira*.

Keywords: *Leptospira*, ROS, stress, oxidant, PerR, regulation, virulence, transcriptome, non-coding RNA.

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Introduction

Chapter I

I Leptospirosis

Leptospirosis is a widespread zoonotic disease that was first described by Dr. Adolph Weil in 1886 as jaundice with acute kidney injury, skin rash, and conjunctivitis. Leptospirosis was then called “Weil disease” (in his honor). Then Inada in 1916 identified for the first time that the causative agent of leptospirosis was an unusual spirochete bacteria. Interestingly, the name of the pathogenic strain “*Leptospira interrogans*” was coined by Noguchi soon after, inspired by the question mark shape of the bacteria (Noguchi 1918).

I.1 Leptospirosis and cycle of infection

Leptospirosis, is the most widespread zoonotic disease due to the vast host diversity that can be infected. This disease can be detected in all types of mammals ranging from small ones, like rodents, bigger animals (dogs, cats, horses, cows, etc.) to even aquatic mammals. More recently, several reports showed that even amphibians could develop the disease (Pratt and Rajeev 2018).

Leptospirosis is a misdiagnosed disease because the first symptoms are flu-like symptoms such as fever, myalgia, and headaches, that resemble the symptoms of malaria and dengue. Later stages of leptospirosis can lead to life-threatening complications such as multiorgan (kidney, lung, and liver) failures, eventually leading to the Weil disease (Ko *et al.*, 2009; Haake *et al.*, 2015). Through the World Health Organization (WHO) surveillance reports, it has been shown that around 10% of leptospirosis cases led to the death of the host.

Humans, animals, and the environment are the key factors of the cycle of a zoonotic disease like leptospirosis. The main host of this disease are rodents, which are asymptomatic carriers of the bacteria. Pathogenic leptospires colonize their kidneys; thus, the rodents serve mostly as ecological niche of *Leptospira*. Once infected, rodents excrete pathogenic leptospires through their urine and contaminate soil and water. Leptospires can survive in the environment until, by

accident, livestock and domestic and wild animals get infected. The bacteria are easily sustained in natural and domestic environments. Humans are accidental hosts that get contaminated through either direct contact with an infected animal or most commonly, through indirect contact via soil or water contaminated with urine from an infected animal (Figure 1) (Costa et al. 2015). Pathogenic leptospires enter the host through abraded skin or mucous membranes such as conjunctival, oral or genital surfaces (Hookey 1991).

Leptospirosis is a neglected, zoonotic disease, considered as a significant public health problem, only, in impoverished population living in slum area in tropical countries. However, due to climate change and globalization problems, there is an increase in outbreaks in urban areas and leptospirosis is now considered as a re-emerging zoonotic disease (Mcbride *et al.*, 2005; Torgerson *et al.*, 2015; Asante *et al.*., 2019).

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Figure 1: Cycle of Leptospirosis. Interspecies contamination between wild animals and the asymptomatic rodent carriers occur and the pathogen is maintained (vertical line). Once the different host are infected, through urine excretion, soil and water can get contaminated with the pathogen, highlighting the role of the environment (bidirectional lines). Although, direct contamination can also occur interacting with the infected animals (thin lines). Maintenance of the pathogen is due continued cycles of reinfection between the animals (curved lines) (Ko et al., 2009).

Usually, the outbreaks occur after a sporting event, adventure tourism, and natural disasters. After the exposure of an individual with pathogenic leptospires, the incubation time is around 7-10 days before leptospiraemia takes place (Figure 2A). During the first stage of infection, pathogenic leptospires penetrate the organisms and migrate to the tissues of several organs. In this early phase of infection, the symptoms of the disease are nonspecific and patients exhibit fever, myalgia and headaches, which makes it difficult to differentiate from other febrile-like diseases such as influenza, dengue, or malaria (Figure 2A) (Ko *et al.*, 2009; Adler, 2014; De Brito *et al.*, 2018).

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Figure 2 Leptospirosis phases of infection. (A) Kinetics of leptospirosis after contamination, the first symptoms start after 7–10 days of infection, and at later stay, some patients develop severe multiple organ dysfunction (Ko *et al.*, 2009). (B) Patient with subconjunctival hemorrhages and icterus, characteristic symptoms of leptospirosis in the late phase of infection (Jansen *et al.*, 2011).

The second stage of infection occurs 14-21 days after exposure to pathogenic leptospires, the bacteria are cleared from the bloodstream and antibodies production increases. At this later stay of infection, the symptoms described by Weil in 1886 are observed (jaundice, multiple organ failure, bleeding). In several cases, hemorrhagic pulmonary syndrome are associated with high fatality rates (Figure 2A).

It is known that leptospirosis in humans depends on several other factors like host susceptibility, the inoculum of the pathogen, and the virulence factors of the strain (Al Hariri *et al.* 2019).

Indeed, the susceptibility of the hosts varies with the age and genetic factors to even skin integrity, and whether the hosts are wearing protective clothes. Pathogenic leptospires differ in their ability to cause the disease, depending on their virulence mechanisms, and ability to survive in the host. It has been shown that there is a serovar specificity towards a particular host. In fact, the types of hosts determine the types of pathogens present in a particular epidemiologic setting (Ben Adler 2014; Divers et al. 2019; Fouts et al. 2016; C. Zhang et al. 2019).

In theory, any animal species can be infected with pathogenic leptospires but it does not mean that they will all develop the disease. The dispersion of the disease depends on the type of host, the most common and the principal reservoir of pathogenic leptospires are the rats. Domestic (cats and dogs) and wildlife animals could also be hosts of pathogenic leptospires. Livestock animals (cattle, cow, pork) are also affected by this disease, resulting in a substantial economic loss due to a decreased production of milk, reproductive failure, abortions, premature birth or stillbirth (Verma *et al.*.,2013; Adler, 2014)

Giving the vast range of animal hosts that can be infected, the symptoms vary a lot from one animal species to another one. In the case of domestic and so far reported livestock animals, the clinical signs of leptospirosis are more similar to that of humans, and infected animals present acute or chronic infections. Acute leptospirosis is observed in the early phase of infection. Clinical signs related to chronic infections in livestock (cattle, cow, pork) are usually associated with decreased milk production, premature birth, stillbirth, reproductive failure and abortion. However, there are cases where animals can recover from the disease, and in those cases, infected animals serve more as carriers, in which leptospires can remain in the renal tubules and be shed in the environment by the urine, contaminating water and soil (Verma *et al.*, 2013; Petrakovsky *et al.*, 2014; Zhang *et al.*, 2019)

The main reservoir of the disease are the rodents, such as rats and mouse, mainly because those hosts present an asymptomatic form of leptospirosis. Pathogenic leptospires are able to evade the immune response to colonize renal tubules from which they are shed in urine, thus serving as carriers (Guernier *et al.*, 2018; Pratt *et al.*, 2018). The reason why these hosts are resistant to leptospirosis is not very well understood, but the innate immune system of the host plays an important role. In particular, the activation of the toll-like receptors (TLR), present in immune cells such as macrophages. This activation of the TLRs is a determinant factor in the difference

between acute and chronic leptospirosis and this will be described in the part of Host responses of the thesis.

I.2 Epidemiology, Diagnosis and Treatment

The fact that leptospirosis symptoms resemble those of other tropical diseases (malaria and dengue) makes it very difficult to give an exact diagnosis; as consequence, there is a massive gap in the incidence of leptospirosis. It has been estimated that the number of people infected is at least 10 per 100 000 people living in tropical climates and the numbers are increasing due to climate change, increased urbanization and sanitation problems (Figure 3) (Costa et al. 2015; Garba et al. 2018). Indeed, it has been reported an increase of leptospirosis outbreaks frequently after periods of seasonal rainfall and flooding in urban slum settings (Casanovas-Massana et al. 2018; Garba et al. 2018). In the case of developing countries, leptospirosis is considered as a professional disease having more incidence in population that are potentially in close contact with infected animals, such as veterinarians, farmworkers, hunters and fieldworkers. Indirect contact with contaminated water or soil with pathogenic leptospires is more common in aquatic recreational activities such as caving, canoeing, kayaking, triathlons (Ricaldi *et al.*, 2013; Al Hariri *et al.*, 2019).

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Figure 3 Global estimation of leptospirosis cases by country. The gradient color from white (0-3), yellow (7-10), orange (20-25) to red (over 100), represents the number of cases per 100,000 persons.

Circles and triangles indicate the countries of origin for published and unpublished data, respectively (Costa et al., 2015)

The disease is underreported for many reasons. Beside the difficulty in distinguishing clinical leptospirosis signs from those of other endemic diseases, there is a massive lack of appropriate diagnostic laboratory services.

The detection of leptospirosis by PCR in the blood can only be carried out during the first days after the symptoms of leptospirosis appeared (Figure 2A), when pathogenic leptospires are still found in the blood and, could potentially be isolated (Bourhy et al. 2011). At a later stages of the disease, leptospirosis is diagnosed by serology, and the gold standard method is the MAT (Microscopic Agglutination Test) (Courdurie et al. 2017; Signorini et al. 2013). The principle of this technique is to mix patient sera with different *Leptospira* strains. If the patient serum has antibodies against any of the *Leptospira* strains, this will induce the agglutination of the leptospires. This technique could be very sensitive at the serogroup level of *Leptospira*. However, this makes MAT to be restricted to a few reference laboratories that have an extensive collection of strains with most of the infective serogroups; also it requires specific equipment and skill technicians and, sometimes, the analysis can be very subjective (Turner, 1968; Ricaldi et al., 2013; Schreier et al., 2013).

There have been many efforts to increase the number of techniques available to detect *Leptospira* at early stage of infection to avoid misdiagnosis and severe cases with high rate of fatality. Another available technique is the ELISA (Enzyme-Linked ImmunoSorbent Assay) to detect antibodies against *Leptospira* (Bourhy et al. 2011), the other one is a rapid diagnostic test (RDT) with strips (Courdurie et al. 2017; Goarant et al. 2013).

The treatment used against leptospirosis in humans will depend on the stage of infection where it has been detected. Antibiotics are the most common treatment against leptospirosis. Doxycycline and azithromycin are mostly used at early stages of the disease and in more severe cases of leptospirosis intravenously injection of penicillin, ampicillin, ceftriaxone, and cefotaxime is preferable (Adler et al., 1976; McClain et al., 1984; Levett, 2001).

In the case of animals, streptomycin is the most used antibiotic. A leptospirosis vaccine using the strain *Leptospira interrogans* serovar *Icterohaemorrhagiae* is available (Guernier et al. 2018; Ido et al. 1917). However, the active component of killed, whole-cell vaccines is leptospiral

LPS, a serovar-specific antigen (Chapman et al. 1991). Thus, the vaccine gives limited protection and is not effective against other serovars. Ongoing researches are performed to obtain a vaccine with a low side-effect profile that can induce long-lasting and cross-protective immunity.

I.3 Approaches to study *Leptospira* pathogenicity

The most accurate way to reproduce the extent of leptospirosis in laboratory conditions is the utilization of animal models. There are different animal models used to study the forms of lethal and chronic leptospirosis. Rats and mice are naturally resistant to the disease, and they will not develop any symptoms of leptospirosis when infected. In the contrary, sensitive animal models such as hamsters, gerbils, and guinea pigs will develop symptoms similar to those detected in humans.

Hamsters are the most used animals in the laboratory to study the total outcome of leptospirosis, because they develop the symptoms, mimicking the severe form of humans leptospirosis leading to fatality. This model has been used to identify and characterize virulence factors of pathogenic leptospires, vaccines, and treatments against leptospirosis (Athanzio et al. 2008; Setubal et al. 2006; Truccolo et al. 2002).

The guinea pig has been used mostly to study severe pulmonary hemorrhage and respiratory failure in the outcome of leptospirosis as it replicates the same failures seen in humans (Bharti et al., 2003; Gomes-Solecki et al., 2017). Interestingly, it has been shown that depending on the age of the guinea pigs, the resistance to leptospirosis will be different. Young guinea pigs will develop acute leptospirosis more comparable to severe leptospirosis in humans than working older guinea pigs (Ben Adler et al. 2011; Nally et al. 2018).

Inoculation through skin, eyes, and peritonea have showed that gerbils are good sensitive models for leptospirosis caused by different serovars of *Leptospira* (Faine et al., 1964; Coutinho et al., 2014). In addition, this model allows working independently of the gender or age of the animals (Branger et al. 2001).

As rats and mouse are the reservoirs of pathogenic leptospires, their infection in laboratories is asymptomatic. Usually, these animal models are used to study renal colonization, mainly because around 1-2 weeks after infection, leptospires are only detected in renal tubes (Athanzio *et al.*, 2008; Ko *et al.*, 2009; Adler *et al.*, 2011; Gomes-Solecki *et al.*, 2017). Also, these models are used to understand mechanisms involved in the resistance to infection and in to study mechanisms of immune evasion.

There is no consensus in terms of which infection doses to use in the animal model to study leptospirosis. Also, it has been shown that the lethal dose (LD50) can differ from the different strains of *Leptospira* you are working. In fact, for the hamster, Ristow and collaborators in 2007, showed that with the strain *L. interrogans* serovars Lai the LD50 is 10^7 for the contrary Silva and collaborators in 2008 showed that for hamster and working with Icterohaemorrhagiae and Canicola the LD50 is between 2 to 100 bacteria.

So, the LD50 depends on the strain but also of the animal model. In the case of guinea pig and working with *L. interrogans* serovars Lai the LD50 is 10^8 compared to the hamster that the LD50 is 10^7 (Ristow et al. 2008).

II *Leptospira*

Leptospira have been identified as causative agents of the severe human syndrome Weil's disease around 100 years ago. Since then, numerous *Leptospira* species have been isolated from almost all mammalian species. At the present times, leptospirosis is recognized as one of the most widespread zoonotic diseases worldwide and also one of the major causes of disease in many domestic animal species.

II.1 Spirochetes phylum

The Spirochete phylum is composed of unique, fascinating, and diverse bacteria. This phylum is composed of 15 genera with around 200 bacterial species with diverse lifestyles (Parte 2018). They can live in a variety of environments going from marine sediments, deep within the soil, to host-associated environments, aerobic or anaerobic (Schwan et al. 2005). Most of the members of this phylum share a distinguishing morphological spiral-like feature, and a particular class of flagella that remains within the periplasm called the endoflagella (C. Li et al. 2008).

Based on the sequence alignment of 22 conserved housekeeping and ribosomal proteins, it has been established that there are three families in this phylum: *Spirochaetaceae*, *Brachyspiraceae*, and *Leptospiraceae* (Figure 4). The most studied genera in the spirochetes phylum are *Treponema*, *Borrelia*, and *Leptospira* which have species that are pathogenic for humans. These species are *Treponema pallidum* (causative agent of Syphilis), *Borrelia burgdorferi* (causative agent of Lyme disease), and *Leptospira interrogans* (causative agent of Leptospirosis) (Chan et al., 2000).

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Figure 4 Phylogenetic tree of the Spirochetes phylum. This tree is based on the amino acid sequences of 22 conserved proteins (Gupta *et al.*, 2013)

The leptospires belong to the *Leptospiraceae* family, and the *Leptospira* genus, is composed of at least 22 species with 300 serovars approximately (Mathieu Picardeau 2017).

The *Leptospira* genus was previously divided into pathogenic and non-pathogenic based on the heterogeneity in the structure of the carbohydrate component of their lipopolysaccharide (LPS)

(Bharti et al. 2003; L. I. O. Croda et al. 2001). However, with different phylogenetic analyses the genus *Leptospira* has been divided into three distinct clades comprising 22 species (Figure 5). All the pathogenic species are grouped in a clade, and those species are responsible for infecting and causing disease in human and animals. This clade consists of ten pathogen species that can be further divided into four subgroups (subgroups 1-IV) (Fouts et al. 2016). Another clade comprises the intermediate species that have been isolated from humans and animals and that cause mild clinical manifestations of leptospirosis. The third clade is the saprophytes, which is composed of seven species, and those are unable to cause disease and are found in the environment (Brenner et al. 2009; Xu et al. 2016).

Recently base on average nucleotide identity (ANI) values of 90 isolates and representative genomes of well known species revealed 30 new *Leptospira* species and proposed to classify *Leptospira* genus into S2, S1, P2 and P1 subclades. P1 been the formerly described as the pathogen group, P2 formely described as the intermediate group, S1 known as the saprophyte groups and S2, new subclade including new saprophyte species (Vincent et al. 2019).

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Figure 5 Phylogenetic tree of the clades of the *Leptospira* genus. Maximum-likelihood phylogenetic tree of the *Leptospira* genus, based on the concatenation of the selection of 491 core genes (Mathieu Picardeau 2017).

II.2 *Leptospira* morphology and physiology

Compare to the other clinical-relevant spirochetes (*Borrelia burgdorferi* and *Treponema pallidum*), *Leptospira* have a unique helicoidal morphological shape. They are thin bacteria with a length of 10-20 μm and a diameter of 0.15 μm . They have periplasmatic endoflagella and a hook-shaped ends, thus resembling a question mark (Figure 6) (Wunder et al. 2016).

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Figure 6 Characteristics of *Leptospira* morphology. *Leptospira* are helical bacteria with a clockwise rotation. They have a cap-like structure, a chemoreceptor that contains methyl-accepting chemotaxis proteins (MCPs), close to the flagellar motor at each polar end. The cell wall is composed of an inner membrane, the peptidoglycan and an outer membrane that has surface-exposed lipopolysaccharide (LPS) (Mathieu Picardeau 2017).

Leptospira cell envelope is like any other diderm Gram-negative bacteria, where the inner membrane and peptidoglycan are close and overlaid by an outer membrane that has membrane lipoproteins and the lipopolysaccharide (LPS) (Figure 6). Interestingly, *Leptospira* is the only genus of the Spirochetes phylum that has an LPS, although the composition of the LPS may be different from other Gram-negative bacteria (Haake *et al.*, 2010).

Usually, the LPS is an endotoxin highly immunogenic present in Gram-negative bacteria. However, the LPS from *Leptospira* is different due to the unusual structure of its lipid A, which is explained by the presence of a unique methylated phosphate not found in other lipids A from any other bacteria. This difference induces less immunity, probably explaining immune evasion response in the different infected hosts (Werts 2010) (See Host responses upon leptospiral infection chapter).

Leptospira spp. are chemoorganotrophic, aerobic or microaerophilic bacteria. Most of their carbon source comes from the oxidation of long-chain fatty acids. Comparing the genomes of several leptospires, complete set of genes for a system of long-chain fatty-acid utilization, a tricarboxylic acid cycle, and a respiratory electron transport chain it has been identified (Ren *et al.* 2003). However, this analysis was done without using all *Leptospira* species genomes. Thus, some exceptions about the nature of carbon source in the *Leptospira* genus.

Contrary to other spirochetes like *Treponema pallidum*, it is possible to cultivate leptospires *in vitro* at 30°C (optimal temperature in laboratory conditions). The medium used is rich and complex and is called EMJH, for the initials of Ellinghausen McCullough Johnson and Harris, who described and modified the medium in the mid-sixties (Ellinghausen *et al.*, 1965; Johnson *et al.*, 1967). This medium is the most used to cultivate *Leptospira* that will be further described in *in vitro* limitations part of the thesis.

The leptospires are also cultivable in solid media, on EMJH complemented with 1.2% of agar (Slamti *et al.* 2011). The colonies of *Leptospira* grow inside the agar (subsurface) but nevertheless can be isolated by taking the colony with a pipette tip (Cinco *et al.* 1996).

II.3 Virulence mechanisms

Virulence mechanisms of pathogenic *Leptospira* spp. are not fully understood mainly because leptospires lack classical virulence factors present in other bacteria such as the recognized systems for translocation of effectors type III, IV and V secretion systems (Nascimento et al. 2004). *Leptospira* probably have novel unidentified virulence mechanisms. In addition, the over-representation of genes that encode proteins with no known orthologs in other bacteria, makes it difficult to assign a function to many ORFs in *Leptospira* genomes (Ren *et al.*, 2003; Ko *et al.*, 2009; Adler *et al.*, 2011). I will review here factors that have been associated with or involved in *Leptospira* virulence and pathogenicity.

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Figure 7 Virulence-associated genes in pathogenic, intermediate, and saprophytic species of *Leptospira*. Many genes that encode for virulence-associated factors, such as catalase, collagenase, sphingomyelinases, ligB, and thermolysin, are only present in pathogenic species. Interestingly, haem oxygenase and loa22 are present in all species (Mathieu Picardeau 2017).

The first approach to identify any putative virulence factors is the comparison of the genomes between saprophytic and pathogenic species of *Leptospira*. The genes that are present only in the genome of pathogenic species are considered as genes encoding putative virulence factors and this warrants their study (Figure 7). By this approach, it has been possible to identify several virulence factors such as sphingomyelinases, phospholipases, hemolysin, catalase, heme oxygenase and collagenase (Eshghi et al. 2012; Kassegne et al. 2013a; Marcsisin et al. 2013; Murray et al. 2009).

In addition, the number of leucine rich repeat-containing proteins (LRR) encoding genes are higher in the genomes of pathogenic species compared to intermediates and saprophytic species, which is consistent with the fact that LRR proteins contain a motif involved in the interaction with host cells important for host-pathogen interactions (Bell et al. 2003; Eshghi et al. 2015; Miras et al. 2015).

Leptospire lack the typical secretion systems found in other pathogenic bacteria such as type III, IV, and VI. However, it has been shown that leptospire genomes encode the T1SS (Type 1 Secretion System), which secretes a wide variety of proteins into the extracellular milieu. Also, some components of the T2SS are encoded in leptospiral genomes, but the system seems to be incomplete and has never been characterized (Abby *et al.*, 2017). Noteworthy, it has been reported that around 325 proteins are secreted by *Leptospira*, where a minority of them share homology with other virulence factors known in other bacteria (Eshghi et al. 2015). Most of the proteins that were secreted were grouped in proteins involved in nutrient uptake and metabolism. This is attributed that most of the secreted proteins could show moonlight function, class of proteins where a single polypeptide chain could perform more than one biochemical function. This moonlight activity has been previously reported in *Leptospira* with an enzyme in the glycolytic pathways (Nogueira et al. 2013).

One attractive candidate for a virulence factor is the lipoprotein LipL32 because it is one of the most abundant proteins in *Leptospira* and is only present in intermediate and pathogenic species (Figure 7) (Malmström et al. 2009). LipL32 has been shown to bind to host-related factors (Murray et al. 2009; Mathieu Picardeau 2017). However, when LipL32-encoding gene was inactivated, no attenuation in virulence was demonstrated either in hamster or rat models (Murray et al. 2009). This observation could be explained by a functional redundancy, which

is a phenomenon widespread in *Leptospira*. Proteins with redundant function could operate in different stages of the disease, in different niches of *Leptospira* or even work synergistically.

Another interesting example is the Lig protein family (LigA, LigB, and LigC). These surface-exposed proteins are members of the bacterial immunoglobulin-like protein superfamily (Koizumi *et al.*, 2004). LigA is present only in some serovars of pathogenic leptospires whereas LigB that is widely distributed in all pathogenic serovars (Figure 7). LigC is found as a pseudogene in some pathogenic serovars (Cerqueira *et al.* 2009). Several findings suggest that these proteins are virulence factors. They are only present in pathogenic serovars, are highly expressed under host-like conditions, and it has been shown that LigA and LigB bind many host proteins, including the complement regulatory proteins (Choy *et al.* 2007, 2011; Matsunaga *et al.* 2003). However, this is another example of proteins with functional redundancy in *Leptospira* because single *ligA* and *ligB* mutants are still virulent (J. Croda *et al.* 2008). In fact, it has been shown that concomitant decreased *ligA* and *ligB* expression by the TALEs (Transcription Activator-Like Effectors, see Limitations in studying *Leptospira* chapter below) technique attenuates *Leptospira* virulence (Pappas *et al.*, 2015).

Loa22 is a surface-exposed putative lipoprotein that is also among the most abundant proteins and it has been shown that it is highly expressed during acute infection (Nally *et al.*, 2018; Malmström *et al.*, 2009). Furthermore, Barbosa and collaborators (2006) showed that Loa22 binds to host-related proteins such as collagen and fibronectin. Interestingly, Loa22 was the first reported virulence factor identified by transposon mutagenesis of *Leptospira interrogans* (Ristow *et al.* 2007).

During infection, the production of reactive oxygen species (ROS) is an essential weapon for phagocytes and also during infection *Leptospira* is confronted against ROS that are produced in different organs such as liver and kidney. Many pathogenic bacteria have genes that encode for catalases which are essential for detoxification of H₂O₂, survival in macrophages and virulence (Elkins *et al.*, 1999; Das *et al.*, 2009; Steele *et al.*, 2010). Interestingly, a catalase-encoding gene (*katE*) is only found in pathogenic species of *Leptospira* (Figure 7), which makes it an interesting putative virulence factor. In fact, it has been shown that catalase of pathogenic species of *Leptospira* is required for resistance to hydrogen peroxide and hamsters infected with a *katE* mutant survived without signs of diseases, indicating that oxidative stress resistance is pivotal for *Leptospira* virulence (Eshghi *et al.* 2012).

Motility is determinant for pathogenesis although it is not by definition a proper virulence factor. It has been shown that the endoflagellar motility of *Leptospira* is a crucial factor for rapidly cross tissues and barriers, and to disseminate in the host in the host, thus crucial for virulence (Lambert et al. 2012; Liao et al. 2009) and also to escape from the immune system (Werts 2010).

Progress has been made in the identification of virulence factors of *Leptospira* that accomplish with Koch's postulates such as Loa22. However, the lack of genetic tools, the functional redundancy in many factors and the moonlighting activity hinders the comprehension of *Leptospira* and how this pathogen elicits its pathogenicity during infection.

II.4 Limitations in studying *Leptospira*

Working with *Leptospira in vitro* in the laboratory conditions is not as easy or straightforward as with other bacteria. The main problems relate to laborious cultivation and difficulty in genetic manipulation.

Saprophytic and pathogenic species of *Leptospira* both grow under aerobic conditions but at a different rate. The optimal growth temperature of *Leptospira* species is between 28-30 °C, but saprophytes can grow at low temperatures (11-13 °C) whereas pathogens are unable to multiply at these low temperatures. The doubling time of saprophytes is of 6-8 h in liquid media and colonies appear on solid media after one week. In contrast, pathogenic species have a doubling time of about 18-24 h in liquid media and colonies are visible on solid media after one month.

The medium used to cultivate *Leptospira* is the EMJH medium (Ellinghausen *et al.*, 1965; Johnson *et al.*, 1967). This medium has a complex composition and its preparation is tedious which results sometimes in reproducibility inconsistencies. *Leptospira* do not grow at the surface of the solid medium; instead the colonies are embedded just below the agar surface and are therefore difficult to be visualized.

The genetic tools to manipulate *Leptospira* are limited. However, progresses have been made primarily to work on saprophytic species of *Leptospira* because of their faster growth and ease to manipulate. Currently, molecular tools enabling targeted mutagenesis, complementation of mutations, heterologous expression are available (Figure 8).

The main breakthrough for the improvement of genetic studies of *Leptospira* was the identification of leptospiral phages or chromosomal prophage regions that were useful for the generation of replicative plasmids. In early nineties, Saint Giron and collaborators isolated three bacteriophages from sewage water, replication of which was limited to the saprophyte species of *Leptospira*, giving birth to the first replicative vectors (Girons et al. 1990). A replicative plasmid vector for pathogenic *Leptospira* species was developed only very recently. In 2015, two groups reported the ability of plasmid replication in pathogenic *Leptospira* species by cloning the replication origin of extrachromosomal replicons from prophages into different pathogenic leptospires (Pappas *et al.*, 2015; Zhu *et al.*, 2015). All these replicative plasmids have been useful to complement mutants and test Koch's molecular postulates and also for the heterologous expression of genes that are pathogen-specific in saprophyte leptospires (Figueira et al. 2011; Toma et al. 2014) (Figure 8). Until now, three replicative vectors are available to work with saprophytes and only one to work with pathogenic species, all of them being low-copy number plasmids.

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Figure 8 Genetic tools to work with species of *Leptospira*. Genetic tools to study and manipulate *Leptospira* spp. Different tools have been developed to manipulate leptospires in laboratory conditions like tools to generate mutants such as transposon mutagenesis, to track *Leptospira* during infection in animals models, and vector for heterologous expression and/or complementation of mutants (Adler *et al.*, 2018).

Two types of mutagenesis approaches are used with *Leptospira* species. The first one consists of targeted mutagenesis using a suicide vector allowing incorporating the inactivated allele of the target gene by a resistance cassette, and this is achieved by inducing an event of homologous recombination (Figure 8 and 9A) (Picardeau *et al.*, 2001). In saprophytic species many chromosomal genes have been inactivated with this technique (Louvel *et al.*, 2007). In contrast, only few genes, including *ligB* (J. Croda *et al.* 2008), *mce* (L. Zhang *et al.* 2012), *colA* (Kassegne *et al.* 2013b), *fliY* (Liao *et al.* 2009), *fcpA* (Wunder *et al.* 2016), and *fliM* (Fontana *et al.* 2016) have been successfully inactivated by targeted mutagenesis in pathogenic species.

The second type of mutagenesis is a system for random mutagenesis using the *HimarI* mariner transposon (Figure 8 and 9B). This system has been successfully used in saprophytic and pathogenic species of *Leptospira* (Bourhy, 2005; Louvel *et al.*, 2005). However, the efficiency is much higher by 2-3 order magnitude in saprophytes than in pathogens (Bourhy 2005). In fact,

this approach allowed the identification of Loa22 and Catalase as virulent factors (Eshghi et al. 2012; Ristow et al. 2007). Interestingly, transposon sequencing (Tn-Seq), an approach using the combination of random mutagenesis and NGS (Next Genome Sequencing) techniques has been recently developed in *Leptospira* (Figure 7) (Lourdault *et al.*, 2016). Tn-Seq could be an excellent tool for screening a large number of mutants in animal models at the same time and identifying putative virulence factors of pathogenic *Leptospira*.

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Figure 9 Scheme representing targeted and random mutagenesis. In targeted mutagenesis (left scheme), a kanamycin-resistance cassette, encoded in the suicide vector, is flanked by the sequence of the target gene. An event of an allelic exchange is induced, interrupting the expression of the target gene. In random mutagenesis (right scheme), the Himar1 mariner transposon carries a kanamycin-resistance cassette and the C9 hyperactive transposase flanks this transposon. This allows the random insertion of the transposon into the chromosome (Adler *et al.*, 2015)

Mutagenesis techniques described so far remain challenging for *Leptospira* pathogenic species. Thus, alternative strategies of targeted gene knockouts has been recently developed such as Transcription Activator-Like Effector (TALE) (Pappas *et al.*, 2015). The TALEs are a group of repressors that bind directly to DNA promoter region and modify transcriptional activity by inhibiting the binding of the RNA polymerase or by abrogating transcription initiation (Figure 7) (Garg *et al.*, 2012; Politz *et al.*, 2013). Recently, it has been described a new method for gene silencing using a modify version of the Cas9 protein with the guide of single-guide RNA (sgRNA) called CRISPR interference (CRISPRi) (L. G. V. Fernandes et al. 2019).

Monitoring *Leptospira* infection have been attempted by several approaches. Different groups successfully transferred the *gfp*, mRFP1 alleles into saprophytic and pathogenic species of *Leptospira* (Aviat et al. 2010). However, these systems were not entirely successful, mainly because the fluorescent signal was not strong enough to be detected and did not allow the monitoring of infection with *L. interrogans*. Luciferase from the *luxCDABE* cassette from *Photobacterium luminescens* was expressed in *L. interrogans*, enabling imaging of *Leptospira* infection in hamster (Ozuru et al. 2017). In addition, Ratet and collaborators (2014) constructed a bioluminescent leptospire expressing the firefly luciferase-derived *luc* gene under the control of the *flgB* promoter from *Leptospira interrogans* and were able to monitor bioluminescent *Leptospira* during infection in live mice (Figure 8).

In order to evaluate gene expression, β -galactosidase and GFP transcriptional fusions have been constructed in saprophyte (Cerqueira et al. 2011) and pathogenic *Leptospira* (Matsunaga et al., 2018). Translational fusions have not been reported in *Leptospira* spp.

II.5 Host responses upon leptospiral infection.

Leptospirosis progression depends on the host and the nature of the leptospiral serovar. The host response to *Leptospira* can be divided into three parts: host detection, innate immune response, and the humoral response.

Leptospira detection by the host mainly relies on a variety of receptors in the mammalian immune cells called pattern recognition receptors (PRRs). PRRs such as toll-like receptors (TLRs) and C-type lectin detect and interact with signatures of the pathogen called pathogen-associated molecular patterns (PAMPs) such as LPS, lipoproteins, peptidoglycan, and motor proteins like flagellins (Iwasaki et al., 2010). Interaction between PRRs and PAMPs trigger the immune response of the host.

Most of the research performed on the host response to *Leptospira* infection have been focused on how the TLR2 and TLR4 of the immune cells, such as macrophages, neutrophils, and dendritic cells, recognize and are activated by *Leptospira* PAMPs and induce subsequent cellular responses.

TLR4 and TLR2 are the central toll-like receptors involved in detecting LPS from most bacteria, especially Gram-negative. Interestingly, the LPS from *Leptospira* is atypical and it activates human immune cells through TLR2 but not through the conventional TLR4 pathway, inducing early inflammatory response (Monahan *et al.*, 2009; Werts, 2010). This recognition by TLR2 could be explained by the unique structure of the lipid A from *Leptospira*, which is the active toxic component of the LPS (Que-Gewirth *et al.* 2004). On the contrary, in mice infection, intact TLR2 and TLR4 pathways are necessary to control infection of pathogenic leptospires (Nahori *et al.* 2014). These differences in the detection of pathogenic *Leptospira* between humans and mice could explain the different leptospirosis susceptibility between acute (human and other animal species) and chronic (rodents) host (see I.1 Leptospirosis and Cycle of Infection). In fact, it has been shown that the presence of TLR4 is pivotal for the production of Immunoglobulin M (IgM) by B humoral cells from mice and thus for the clearance of leptospires from the blood (Chassin *et al.* 2009).

Innate immune response of the host against pathogenic *Leptospira* after macrophage PRR activation occurs during the acute phase of leptospirosis (Isogai *et al.* 1986). PRR activation of the macrophages induces phagocytosis of the pathogen, through cytoskeleton rearrangements, and production of ROS and antimicrobial peptides, which are bactericidal. Interestingly, it has been shown that pathogenic species of *Leptospira* can enter both murine and human macrophages and induce ROS production. However, the fate of intracellular *Leptospira* is different depending on the host. In mouse macrophages, pathogenic leptospires were observed in late phagosomes and did not survive in contrast to human macrophages where leptospires were able to survive, replicate and escape to the cytosol (S. Li *et al.* 2010; Toma *et al.* 2014). Moreover, leptospire death in murine macrophages depends on the presence of ROS whereas leptospire fate in human macrophages is ROS-independent (S. Li *et al.* 2017).

The role of Polymorphonuclear leukocytes (PMNs) including granulocytes, eosinophils and neutrophils, in protection against *Leptospira* is unclear. Neutrophils are the most abundant leukocytes in the blood, they are highly motile cells, and they phagocyte pathogens and destroy them by the generation of ROS. Also, in the presence of a pathogen neutrophils synthesize a mixture of chromatin and protease-forming nets called neutrophils extracellular traps (NETs) in order to kill the pathogen (Segal 2005; De Silva *et al.* 2014). It has been demonstrated that neutrophils could phagocyte leptospires and furthermore it was shown that leptospiral infection

in mice and human PMNs triggered the formation of NETs (Raffray et al. 2016; Scharrig et al. 2015).

Adler and collaborators (1977) showed that antibodies against *Leptospira* are pivotal for protective immunity. They discovered this by demonstrating that infected mice having inhibited B cells with pathogenic *Leptospira* were sensitive (Adler *et al.*, 1976, 1977). In contrast, T cells do not appear to have an essential role in giving protection against *Leptospira* (Chassin et al. 2009). Antibodies production against *Leptospira* has an essential role in providing immune protection against lethal infection in many host species. In fact, it has been shown that leptospiral infection induces a durable and protective antibody response against the LPS. This antibody response is generally short, requiring immunization almost every year and exhibits a limited cross-protection against different serovars (Guerreiro et al. 2001).

Chapter II

I ROS Damage in bacteria

As mentioned earlier, *Leptospira* are aerobic bacteria and as any bacteria exposed to dioxygen, they are exposed to ROS produced during the respiration chain (Figure 10). Pathogenic species are further exposed to the overproduction of ROS by the host innate immune response during infection. Thus, whether leptospires are in the outside environment or inside a host, they are confronted to different ROS damaging coming either from endogenous production or from an exogenous source such as phagocytic cells (Figure 10) (Imlay, 2013; Flannagan *et al.*, 2015). These oxidant species can affect all cellular components (lipids, DNA and proteins) and affect several cellular processes.

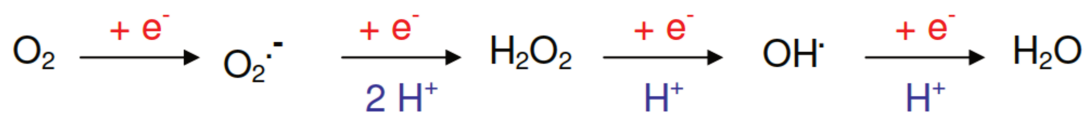


Figure 10 Generation of Reactive oxygen species by the reduction of dioxygen. Once dioxygen is reduced, it will result in superoxide anion ($\text{O}_2^{\cdot-}$). Superoxide may be reduced to form hydrogen peroxide (H_2O_2) that eventually can be reduced and form hydroxyl radicals (HO^{\cdot}) during the Fenton reactions. Following the gain of an electron, hydroxyl radicals can be converted to water (H_2O).

Oxidative damages harm particularly proteins that have metal as a prosthetic group, i. e. metalloenzymes, but they also result in irreversible covalent modifications to amino acid, including carbonylation, histidine oxidation to oxo-histidine or tyrosine oxidation to nitrotyrosine (Traoré *et al.*, 2009; Feeney *et al.*, 2012).

Proteins having cysteine and methionine residues are perhaps the most affected proteins by oxidative stress. Their sensitivity is due to the presence of electron-rich sulfur atoms in their side chain (Figure 11) (Ezraty *et al.* 2017). Enzymes that use iron as a prosthetic group such as epimerases, dehydrogenases, and deaminases are prone to be inactivated by superoxide anion ($\text{O}_2^{\cdot-}$) or hydrogen peroxide (H_2O_2). Inactivation by H_2O_2 usually involves the Fenton reaction, where H_2O_2 oxidizes the ferrous iron (Fe^{2+}) into ferric (Fe^{3+}) producing hydroxyl radical (HO^{\cdot}) (Winterbourn 1995). Some enzymes that are inactivated by this ROS can be reactivated just by the addition of Fe^{2+} , but most of the cases this is not possible due to damage to polypeptides by HO^{\cdot} produced during the Fenton reaction (Figure 11).

$\text{O}_2^{\cdot-}$ also oxidizes and releases the iron atoms of proteins that employ ferrous iron atoms as a catalytic cofactor (Figure 10) (Gu *et al.*, 2013). However, this oxidation does not lead to severe damage to the polypeptide because there is no generation of HO^{\cdot} radicals. However, this oxidation leads to the generation of H_2O_2 , which could lead to the Fenton reaction (Figure 10). Repeated cycles of this process lead to mismetallation of enzymes with zinc, another abundant metal *in vivo*. This mismetallation with zinc leads to a progressive decline of protein functions because zinc is not efficient catalytically as iron (Sobota *et al.*, 2011; Gu *et al.*, 2013). Giving the high reactivity of iron with peroxide to eventually form hydroxyl radical by the Fenton reaction, manganese can be used as a cofactor instead of iron in many enzymes, thus protecting

against oxidative stress. This protection is mainly because manganese is not oxidized by any ROS as iron (Cheton *et al.*, 1988; Aguirre *et al.*, 2012).

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Figure 11 Oxidation of cysteinyl iron-binding proteins by hydrogen peroxide (H_2O_2) and superoxide (O_2^\bullet) . The Oxidation of iron atom used as a cofactor in a protein by H_2O_2 (left pathway) generates hydroxyl radicals (HO^\bullet) and a transient ferryl species ($\text{Fe}^{4+}=\text{O}$) that eventually will be released forming sulfenic species ($-\text{SOH}$) as ultimate product. Reactivation of the proteins damaged by H_2O_2 requires sulfenic reduction before re-metalation (dashed grey arrows) (Imlay, 2013). The oxidation by O_2^\bullet (right pathway) generates Fe^{3+} which dissociates and H_2O_2 is produced. The oxidation by superoxide can be circumvented by re-metalation (dashed black arrows). However, this constant re-metalation can progressively diminish protein activity.

The damage also affects the integrity of the DNA and lipids (Imlay 2013). Interaction of H_2O_2 with DNA produces HO^\bullet , which can eventually oxidize the base and ribose moieties leading to several irreversible lesions and mutagenesis (Dizdaroglu et al. 1991; Henle et al. 1999). One product of H_2O_2 damaging in the DNA is the highly mutagenic 8-hydroxyguanine that can base pair with adenine in a way that inhibits the detection system of the DNA polymerases (Hogg *et al.*, 2005). In the case of lipids, there is peroxidation where many peroxy groups are added in the unsaturated bonds, thus damaging the lipid packing in the cell membrane (Arenas et al. 2011; Semchyshyn et al. 2005).

II Bacterial response against ROS

ROS are ubiquitous and, as described before, they can have several irreversible effects in the bacteria fitness. As a consequence, bacteria have evolved mechanism to detect ROS, prevent and repair their damage.

In this part, I will describe the most known and studied molecular mechanisms to defend and sense ROS and the expression regulation of gene that encode these systems.

II.1 Defenses mechanisms

Scavengers of ROS are the prominent and most studied systems against superoxide and hydrogen peroxide. There essential scavenger systems for superoxide in bacteria such as the superoxide dismutase (SOD) and a superoxide reductase (SOR). For hydrogen peroxide, the most known and studied scavengers are catalase and peroxidases (Imlay 2008; Scandalios 2005).

II.1.a. Scavengers of superoxide

The superoxide dismutase systems are mostly present in Gram-negative bacteria. This metalloenzyme accelerates the reduction of two molecules of O_2^{\bullet} into H_2O_2 and O_2 (Figure 12) through a dismutation metal-dependent (Blanchard et al. 2007).

Gram-negative bacteria usually synthesize both cytoplasmic and periplasmic SOD and they utilize different metal as a cofactor. In *E.coli* the cytoplasmic SOD utilizes iron and the periplasmic zinc as a cofactor (Benov *et al.*, 1996). O_2^{\bullet} is not diffusible in the membranes, so SODs must be localized within the cellular compartments that are intended to protect (Korshunov *et al.*, 2006).

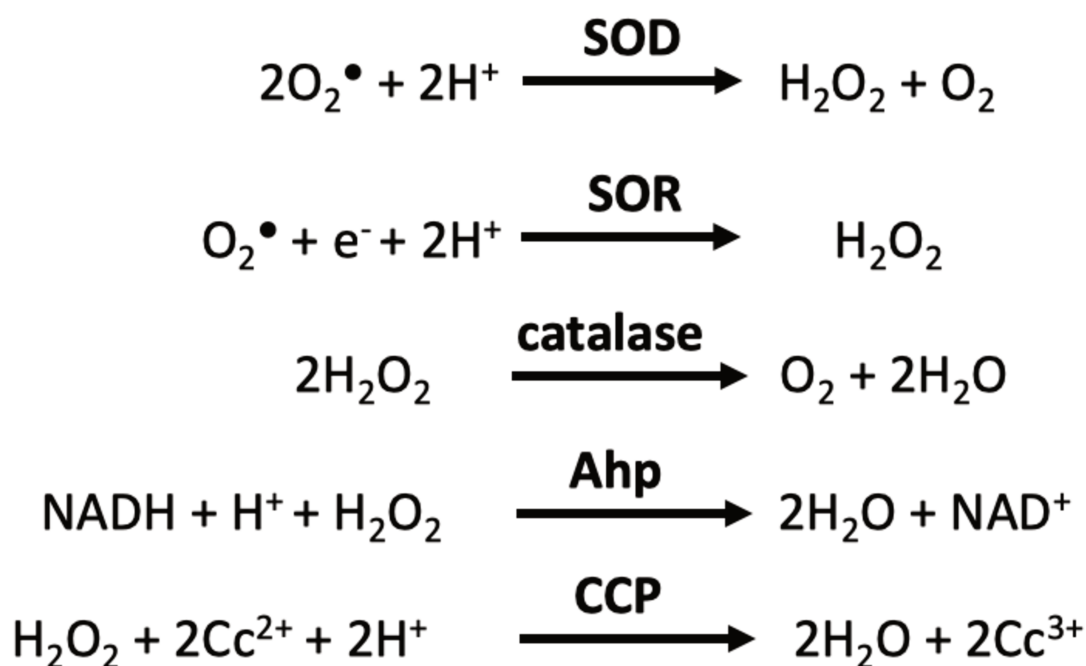


Figure 12 Enzymes involved in ROS scavenging . O_2^{\bullet} is dismuted by superoxide dismutase (SOD) can be reduced by superoxide reductase (SOR). H_2O_2 is degraded by catalase and by different peroxidases (alkyl hydroperoxide reductase (Ahp), cytochrome C peroxidase (CCP)).

SOD was recognized as the main enzyme involved in the elimination of superoxide. However, it has been shown recently that some bacteria, such as *Desulfovibrio baarsi*, *Archeoglobus fulgidus*, and *Treponema pallidum*, lack a SOD system. Instead, they use a superoxide reductase (SOR) that eliminates superoxide by a reduction and produces H_2O_2 . On the contrary to the dismutation by SOD, this reaction does not produce dioxygen and the efficiency in reacting with peroxide its done with a velocity much faster (Adam *et al.*, 2004; Nivière *et al.*, 2004).

II.1.b. Scavengers of hydrogen peroxide

In most organisms, hydrogen peroxide is scavenged by catalases and peroxidases such as alkyl hydroperoxide (AhpC) and cytochrome C peroxidase (CCP) (Figure 12).

Catalase has been one of the first enzymes involved in the oxidative stress response in *E.coli* that was described. This metalloenzyme catalyzes the dismutation of H_2O_2 into water and

oxygen (Figure 12). Interestingly, in the active site of this enzyme it has a heme group. The heme is oxidized (usually by H_2O_2) for the catalase to be activated (Schellhorn *et al.*, 1988).

In *E. coli*, Ahp is a two-component system (AhpC–AhpF) that catalyzes electron transfers from NADH to H_2O_2 , thereby forming water (Figure 12) (Seaver *et al.*, 2001).

It has been shown that the peroxidase Ahp is the primary H_2O_2 scavenging enzyme because of is kinetically more efficient than catalase, mostly reducing the H_2O_2 that is formed endogenously. However, when concentrations of H_2O_2 reach a level that saturates Ahp (approx. 1 μM), catalase is highly induced and becomes the primary scavenging enzyme (Christman *et al.*, 1989; Aslund *et al.*, 1999). Interestingly, this is consistent with the fact that Ahp genes are expressed during the exponential phase of growth and catalase genes during the stationary phase of growth (Schellhorn *et al.*, 1988).

Besides these principal hydrogen peroxide scavenging enzymes, it is essential to highlight that many aerobic bacteria have additional proteins that showed a peroxidase activity. In *E. coli*, thiol peroxidases, bacterioferritin (Bcp) and a homolog of glutathione peroxidase, all scavenge hydrogen peroxide (Cha *et al.*, 1996; Jeong *et al.*, 2000; Arenas *et al.*, 2011). However, their functions have been studied *in vitro* conditions, and whether these functions also occur *in vivo* remains to be demonstrated.

II.2 Repair mechanisms

If the scavenging machinery fails to eliminate or are overwhelmed by the excess of ROS, repair mechanisms are solicited. Different bacteria have evolved several mechanisms in order to repair oxidative damages to cellular constituents.

II.2.a DNA repair

DNA repair after oxidative damage is essential in aerobic organisms. The first step of the repair is the excision of the oxidized bases involving MutM, the glycosylase Fpg, exonucleases, endonuclease IV, and VIII (Jiang *et al.* 1997; Saito *et al.* 1997). These enzymes scan the helical distortions in the DNA, thus enabling the removal of many damaged bases produced by the oxidation. They excise the fracture ribose group of the DNA and restore a 3' primer for the DNA polymerase I repair that fragment (Demple *et al.*, 1986).

When these excision systems fail to recognize and repair lesions, post-replication recombination systems are the next strategy. These recombination systems mainly relied on the RecA protein, which catalyzes the reaction for homologous recombination, mostly homology search and DNA strand invasion (Li *et al.*, 2008).

In fact, bacterial strains without the recombination genes (*rec*) are susceptible to exogenous H₂O₂, and *recA* mutants are lethal for the bacteria in aerobic conditions (Park *et al.*, 2005). Strains that are deficient in both recombination and excision repair systems, such as *recA* and exonuclease II mutants or *recB* and DNA polymerase I mutants are only viable in anaerobic media. This shows that aerobic environments create oxidative DNA lesions, and repair mechanisms become essential for the bacteria (Touati *et al.*, 1995; Keyer *et al.*, 1996). This lethality observed in the absence of RecA might be explained by the fact that RecA controls the expression of the global response to DNA damage in bacteria, the SOS system.

Another critical option to cope with DNA damage is the by-pass of an error-prone lesion, allowing replication to proceed even with a lesion that has not been repaired. It has been shown that this process is facilitated by two polymerases, Pol IV and V, that are also part of the SOS system (Napolitano *et al.* 2012).

II.2.b Cytoplasmic Protein repair

As mention before, proteins containing an iron-sulfur cluster are susceptible to oxidative damage. One very important protein repair mechanism relies on the repairment of oxidized cysteines and methionines.

Oxidoreductases catalyze the transfer of an electron from a donor to an acceptor. Thioredoxins (Trxs) and glutaredoxins (Grxs) are oxidoreductases involved in the repair of oxidized cysteine. Thioredoxins have a CXXC catalytic motif. In this motif, the most exposed cysteine residue (at the amino-terminal of the protein), is in its reduced form and has a thiol group, initiating the reduction reaction of the oxidized cysteine in the oxidatively damaged-substrate (Figure 13) (Collet *et al.*, 2010; Arts *et al.*, 2016). This reaction will lead to the release of an oxidized Trx and reduced cysteines grouping the substrate. The oxidized Trx is then reduced by a thioredoxin reductase regenerating the reduced CXXC motif. The thioredoxin reductase is a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent flavoenzyme (Figure 13) (Collet *et al.*, 2010; Paulsen *et al.*, 2013).

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Figure 13. Repair of oxidized cysteinyl-containing proteins by Trx and Grx in E.coli. (A) Trx activity begins with the attack by the CXXC motif and the formation of a mixed-disulfide intermediate complex. This reaction produces a reduced substrate and an oxidized Trx, that will be subsequently recycled by a thioredoxin reductase (TrxR). (B) Grx activity begins as the same manner of that of Trx with the formation of a mixed-disulfide intermediate complex and the production of a reduced substrate and an oxidized Grx. Grx is recycled by glutathione (GSH), , generating an oxidized glutathione molecule (GSSG). The glutathione reductase (Gor) will reduce GSSG to GSH (Ezraty *et al.*, 2017).

In *E.coli*, if Trx is inactivated, bacteria can survive because the activity of Grx compensates for the loss of Trx (Ritz *et al.*, 2001). Almost all Grx share the same CXXC motif and activity as Trxs. A reduced cysteine of the CXXC motif in Grxs reacts with an oxidized cysteine residue in an oxidatively damaged-substrate protein and becomes oxidized when the target disulfide is reduced (Fernandes *et al.*, 2004) (Figure 13). The main difference with the Trx is the regeneration mechanisms. Grxs are usually reduced by glutathione, a low molecular weight thiol molecule that serves as an electron donor, which reacts with the first cysteine in the CXXC motif of the Grx (Isakov *et al.*, 2000). Eventually, this reaction will lead to a reduced Grx and an oxidized glutathione molecule. The oxidized glutathione is subsequently reduced by the glutathione reductase, an NADPH-dependent enzyme (Vlami-Gardikas 2008) (Figure 13).

For the reduction of oxidized methionine, bacteria use methionine sulfoxide reductases (Msrs). Most bacteria have two Msrs, MsrA, and MsrB both can reduce oxidized methionine (Delaye et al. 2007). Their activity has stereospecificity; MsrA and MsrB will reduce Met-S-O and Met-R-O form, respectively (Moskovitz et al. 2000). Thus, for full repair of protein-containing oxidized methionine, both MsrA and MsrB are required. MsrA and MsrB catalytic activities are quite similar for the reduction of the oxidized methionine (Boschi-Muller 2018). Oxidized methionine is a three-step reaction as shown in Figure 14. A cysteine residue of MsrA or MsrB attacks the oxidized methionine in the oxidatively damaged-protein substrate, leading to the formation and release of a reduced Met residue, the formation of an intramolecular disulfide intermediate in MsrA or MsrB, and the production of water. Interestingly, the disulfide intermediate in MsrA or MsrB is then reduced by a Trx protein generating a catalytically active Msr (Ezraty *et al.*, 2005; Kim *et al.*, 2005). In fact, in *E.coli* mutation of Trx leads to a dramatic reduction in the catalytic activity of Msr (Lee *et al.*, 2008).

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Figure 14. Reduction of oxidized methionine by the methionine sulfoxide reductases MsrA and MsrB. Both MsrA and MsrB have two cysteines (Cys) residues, a catalytic Cys (CysA) and a recycling Cys (CysB). CysA will first attack the oxidized methionine (in the oxidized protein), which will result in the reductive repair of the substrate. In a second step, an intramolecular disulfide intermediate is formed and a molecule of water is produced. The third step consists of the reduction of the intramolecular disulfide intermediate by thioredoxin (Ezraty et al., 2017, Sharov et al., 1999).

II.2.c Cell envelop Protein repair

Leptospira, as typical Gram-negative bacteria, is a dyderm organism with an inner and outer membrane, defining a periplasmic space. The periplasm contains the peptidoglycan cell wall and has a more oxidizing redox potential than the cytoplasm (Sharov et al. 1999).

In the oxidizing environment of the periplasm, most of the Cys residues form disulfide bonds that are essential for the envelope protein folding. Disulfide bond formation is catalyzed by the Disulfide bond family enzymes (Dsb). One member of this family, DsbA, is a soluble

periplasmic oxidoreductase, which has a Trx-like domain with a CXXC catalytic motif (Bardwell *et al.*, 1991). This catalytic motif forms a disulfide bond that will serve as an electron acceptor in the oxide reduction reaction leading to formation of a disulfide bond in proteins translocated to the periplasm (Figure 15). Once DsbA disulfide bond is reduced, it will require DsbB, an inner membrane protein, to be oxidized again and catalytically active (Kadokura *et al.*, 2009). It has been reported that DsbA often introduces inappropriate disulfide bonds into proteins, which are corrected by the periplasmic disulfide isomerase DsbC (Figure 15). DsbC as the other Dsb has a CXXC motif for catalytic activity (Shevchik *et al.*, 1994; Kadokura *et al.*, 2009).

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Figure 15 Mechanisms for the repair of periplasmatic proteins. DsbA will catalyzed the formation of a disulfide bond in unfolded proteins containing Cys, then DsbC will catalyzed the isomerization if there is any incorrect disulfide bond leading in the realse of a correctly folded protein. DsbB accepts electrons from DsbA and they will be transferred to quinones (Q), recycling DsbA to its disulfide bond-containing form. DsbD has three domains (α -domain, β -domain and γ -domain) will transfer electrons from a cytoplasmatic Trx to recycle DsbC and DsbG, which are involved into repair folded reduced proteins that were damaged by ROS (Ezraty *et al.*, 2017, Imlay *et al.*, 2008).

Because of the oxidizing environment in the periplasm, the probabilities that ROS also damage several cysteinyl-containing proteins is very high. DsbG, a periplasmatic oxidoreductase, plays a role in protecting these proteins with exposed Cys residues (Figure 15). DsbG also has a Trx-like domain with a CXXC catalytic motif that acts in the same manner as that of DsbC. DsbC and DsbG work in concert to reduced oxidized cysteine residues and are both recycled by DsbD (Figure 14) (Depuydt *et al.* 2009; Mainardi *et al.* 2007). DsbD has three different domains with a pair of essential redox-active Cys that will receive an electron from the cytoplasmic Trx1 (Figure 14) (Rietsch *et al.*, 1997; Katzen *et al.*, 2000).

Periplasmic Msrs have been identified in a limited number of bacteria. However, an Msr system has been recently discovered in Gram-negative bacteria, the MsrPQ (Gennaris et al. 2015). That is widely conserved MsrP is a soluble periplasmic protein and MsrQ is bound to the membrane, acting as a membrane anchor and a redox partner for MsrP. This Msr system works in the same manner as the other ones with the exception that it has no stereospecificity. Interestingly, this MsrPQ system uses electrons from the respiratory chain, and is therefore independent of Trx; This feature distinguishes the MsrPQ system from the Msr system found in the cytoplasm (Juillan-Binard et al. 2017).

II.2.d Molecular chaperones

As described previously, during oxidative stress, protein modification can affect the proper protein folding, thus eventually forming protein aggregates that can alter the bacterial survival. Most of the molecular chaperones reported so far, use hydrophobic substrate interaction sites for binding and sequester the intermediates of misfolding protein, thus inhibit the number of protein aggregates and promoting the survival of the cell (Bukau *et al.*, 2006; Kumsta *et al.*, 2009).

In terms of mechanisms, there are two groups of molecular chaperones: chaperone holdases and foldases. The foldases are chaperones that use ATP hydrolysis to regulate their affinity for unfolding proteins. They usually work under nonstress conditions, preventing protein aggregation during stress conditions and, after the stress, promote the refolding of the protein. In this group, we found chaperones such as Hsp70 and Hsp60 (Deuerling *et al.*, 2004).

On the contrary, the holdases such as Hsp33 and HdeA, are chaperones that are not dependent on ATP (Winter et al. 2005). Specifically, they prevent protein aggregation formation during any stress condition. With the fact that they do not need ATP to work, it is very common to find them in cellular compartments where lack ATP (e.g., in the periplasm) or during stress conditions that decrease cellular ATP (Graf *et al.*, 2002).

Here, I have described different ways oxidants can damage DNA and proteins and the various defense mechanisms against oxidative damage. Understanding the most relevant repair mechanisms against oxidants in bacteria helps to understand the nature of the bacterial adaptive response to oxidative stress, but one fundamental question remains, how all of this response is orchestrated?

II.3 Transcriptional regulators involved in the oxidative stress response

When bacteria are exposed to ROS, they induced several mechanisms, including ion homeostasis to alleviate the damage caused by reactive ferrous iron (Faulkner *et al.*, 2011). Thus, all these mechanisms have to be tightly regulated, and there are many transcriptional regulators involved in the regulation of the adaptive response to oxidative stress, including as the positive regulator OxyR, the repressor OhrR, SoxR and PerR, a member of the FUR family transcriptional regulators.

II.3.a OxyR

OxyR is a transcriptional regulator from the LysR family and a sensor of H₂O₂. This regulator is a tetramer that contains a sensory cysteine residue. When these residues are oxidized by H₂O₂ into a disulfide bond, the conformation of OxyR is changed, leading to a higher affinity for DNA and favoring, thereby the interaction of the RNA polymerase with DNA. Thus, when OxyR binds DNA, transcription of the target genes is induced (Figure 16).

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Figure 16 Mechanism of transcriptional activation by OxyR in the presence of H₂O₂. Activation by OxyR begins with the oxidation of the sensing cysteine (SH) to sulfenic acid (-SOH). This leads to a conformational change in OxyR that increases its affinity for DNA binding. This will result in a promoter conformation favoring binding of RNA polymerase and induction of gene expression. Oxidized OxyR is eventually reduced by reduced glutathione (GSH). Subsequent reduction of glutathione glutaredoxin (*grxA*)/glutathione reductase (*gor*) system. Red and green boxes indicate the -35 and -10 promoter elements, respectively. Blue boxes are the OxyR binding sites (Dubbs *et al.*, 2012).

OxyR oxidation is reversible, and the reduction of the disulfide bond is performed by reduced glutathione. Oxidized glutathione will be subsequently reduced by the glutathione reductase, with NAD(P)H serving as an electron donor (Figure 16).

OxyR is mostly present in Gram-negative bacteria and, like any other LysR transcriptional regulator, it auto-represses its expression. OxyR is primarily described as a transcriptional activator under oxidizing conditions because of the direct contact with the RNA polymerase. However, it has been reported that it can also act as a repressor under both oxidizing and reducing conditions (Storz *et al.*, 1990; Zeller *et al.*, 2007).

In *E.coli*, the OxyR regulon comprises around 20 genes. Among them, there are the genes that encode for H₂O₂ scavengers such as catalase and AhpCF, factors involved in the heme biosynthesis pathway, proteins involved in the Fe-S cluster assembly (Suf), a ferritin (Dps) that sequesters iron, the ferric uptake regulator (Fur), and proteins involved in disulphide bond oxide reduction reactions (TrxC, GrxA and DsbG) (Jacobson *et al.*, 1989; Chiancone *et al.*, 2010; Mancini *et al.*, 2015).

II.3.b OhrR

OhrR is a transcriptional regulator from the MarR family and senses organics hydroperoxides and sodium hypochlorite (NaOCl) (Chi et al. 2011). When in its reduced form, OhrR dimer binds to DNA, resulting in gene repression (Figure 17). The sensing cysteine is oxidized by organic peroxides or NaOCl into cysteine sulfenic acid, and this OhrR derivative remains bind to promoters until it undergoes further modifications. The sulfenic cysteine acid (Cys-SOH) can either form a disulfide bond (Cys-S-S-R) through the reaction with a reduced cellular thiol or form a cycle amide (Cys-SN) through the interaction with the amino group of an amino acid located in the vicinity. This results in the dissociation of OhrR from DNA and repression alleviation. Derepression can also be induced with the further oxidation of the sulfenic cysteine acid into cysteine sulfinic acid (Cys-SOOH). The formation of Cys-S-S-R and Cys-SN is reversible, and these cysteine derivatives can be reduced into a thiol group. On this opposite, overoxidation of OhrR is irreversible, and cysteine sulfinic acid OhrR derivatives are thought to be degraded (Figure 17) (Antelmann *et al.*, 2011; Dubbs *et al.*, 2016).

OhrR can be present in both Gram-negative and Gram-positive bacteria. Interestingly, it can also co-exists with OxyR or PerR (Panmanee *et al.*, 2006; Antelmann *et al.*, 2011). It has been shown that in *E.coli* OhrR regulates the expression of AhpCF. In fact, AhpCF not only degrades H₂O₂, but it also eliminates organics peroxides (ROOH) (Seaver *et al.*, 2001). In *P. aeruginosa* and other bacteria from where the AhpCF system is absent, the peroxiredoxin Ohr (organic hydroperoxide resistance) functions as a scavenger for organic hydroperoxides and its expression is under the control of OhrR (Fuangthong *et al.*, 2001; Ochsner *et al.*, 2001). Ohr has a thiol peroxidase activity which catalyzes the reduction of ROOH into their corresponding less harmful alcohols (Cussiol et al. 2010).

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Figure 17. *B. subtilis* OhrR binding to DNA and oxidation-controlled dissociation from DNA. Reduced OhrR dimer crystal structure (in green) binds to DNA promoter (in brown), resulting in gene repression. Alkyl peroxide-induced DNA dissociation is summarized below the OhrR/DNA complex. Organic hydroperoxides (ROOH) promote oxidation of OhrR cysteine into cysteine sulfinic acid (Cys-SOH). This protein derivative will be further modified into a mixed thiol (Cys-S-S-R) or a cyclic amide (Cys-SN) or oxidized into cysteine sulfonic acid (Cys-SOOH). These modifications will lead to OhrR dissociation from DNA (Dubbs *et al.*, 2012).

II.3.c SoxR

E. coli and other bacterial species encode the SoxR transcriptional regulator from the MerR family. This regulator contains iron-sulfur [2Fe-2S] clusters that sense stress through the oxidation. Oxidized SoxR binds to DNA, and this will lead to the expression of a second transcription factor SoxS (Figure 18) (Tsaneva *et al.*, 1990). Then together with SoxR or independently, as in some bacteria, SoxS will induce the expression of genes involved in the defenses against O_2^{\bullet} , including the superoxide dismutase and aconitase A-encoding genes (*sodA* and *acrAB*) (Figure 18) (Pomposiello *et al.*, 2001).

It would be logical to think that O_2^{\bullet} is the direct oxidant that activates SoxR, however, different reports have shown that redox-cycling compounds such as quinones and phenazines directly oxidize the SoxR iron-sulfur clusters (Figure 18) (Gu *et al.*, 2011).

Oxidized SoxR is reduced, and SoxS is also activated, hence the SoxR regulon will be expressed. The proteolysis of SoxS is a crucial determinant for the full SoxR regulon stop to be expressed (Griffith *et al.*, 2004).

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Figure 18. SoxRS system in *E. coli*. SoxR dimer is activated when redox-cycling compounds oxidize the iron-sulfur cluster. Once SoxR is oxidized, it will bind to DNA and activate the transcription of *soxS*, coding for a secondary transcription factor SoxS. Thus, the SoxRS regulon, including the *sodA* gene, will be activated (Imlay *et al.*, 2013).

II.3.d FUR family

Transcriptional regulators from the FUR (Ferric uptake regulator) family are present in most bacteria. These regulators act as a metal sensor and regulate the expression of genes involved in metal homeostasis and response to oxidative stress (Lee *et al.*, 2007).

Fur family members are small (17 kDa) proteins that associate into dimers. Each protomer has an amino-terminal DNA binding domain and a carboxy-terminal dimerization domain. The DNA binding domain has a winged Helix-Turn-Helix (HTH) architecture. Proper folding and dimerization required the coordination of a structural metal, which is generally zinc. The binding site for the structural domain is located in the carboxy-terminal domain.

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Figure 19. Characteristic feature of FUR transcriptional regulators family. The FUR regulators act as a dimer, and once the regulatory metal binds in the regulatory site, they interact with a target DNA promoter, repressing the expression of the gene (Left panel). The FUR regulators can also activate the expression of their target gene, either with the regulatory metal (Metal-bound) or without it (Apo-FUR conformation) (Center). These apo-FUR dimers can also bind to the DNA blocking the binding of the RNA polymerase; hence, transcription is repressed (Right panel). Abbreviated lists of organisms where it has been reported these different activities for the FUR regulators. Here we used the Fur regulator model where iron is the regulatory metal. However, it is the same mechanism for the transcriptional regulators of the FUR family (Carpenter *et al.*, 2009).

DNA binding is controlled by the coordination of a regulatory metal (Figure 19). The nature of this regulatory metal depends on the function of the Fur regulator. Indeed, the regulator involved in iron uptake (Fur) will be regulated by iron, the regulator involved in zinc uptake (Zur) will be regulated by zinc, the regulator involved in nickel uptake (Nur) will be regulated by nickel (Figure 19). The member of the Fur family involved in the regulation of the adaptation to oxidative stress (PerR) is controlled by iron (Lee *et al.*, 2007; Fillat, 2014). The binding site for the regulatory metal is located at the hinge of the two domains.

In the presence of the regulatory metal, Fur-like regulators are in conformation that can bind DNA, leading in gene repression (Figure 19). When the availability of the regulatory is scarce, Fur-like regulators switch to a conformation that cannot bind DNA (Figure 19). DNA dissociation leads to the alleviation of gene repression and genes involved in respective metal uptake are transcribed.

There is a vast diversity inside this family in terms of metal sensing and biological function, but the most well-known and representative member of this regulator family is the iron-responsive regulator Fur. The ferric uptake regulator (Fur) as the name says it, the regulatory metal is iron (Fe^{2+}). When cellular levels of iron increase, this metal will bind to Fur, inducing for this regulator to repress further uptake preventing iron uptake (Lee *et al.*, 2007). Whereas, when iron level decreases the Fur regulator will not be able to bind to the DNA, thus allowing the expression of its target genes (Carpenter *et al.*, 2009). Fur regulon will depend on the bacteria. However, it has the same tendency to regulate genes that are involved in iron uptake systems such as the ferric citrate transport system (*fecABCDE*), the ferrichrome-iron receptor and the regulator of *fecABCDE* system (Hantke, 1981, 1984; Angerer *et al.*, 1998). Despite being essential for iron homeostasis, Fur also regulates virulence factors such as in the case for *H. pylori* where a *fur* mutant is less efficient in colonization (Bury-Moné *et al.* 2004), and the case of *S. aureus* infection with a *fur* mutant strain showed attenuation in virulence and the same case with *L. monocytogenes* and *C. jejuni* (Horsburg *et al.*, 2001; Palyada *et al.*, 2004; Rea *et al.*, 2004).

The zinc-dependent Zur protein regulates the expression of genes that are involved in zinc homeostasis, such as an ABC transporter that transports zinc intracellularly (*znuACB*) in *E. coli* (Patzner *et al.*, 2000). Also it has been showed that Zur can regulates the expression of metallophores that are secreted to capture zinc and then these complexes are taken up by a TonB-dependent system that actively transports metallophores through the outer membrane of Gram-negative bacteria (Neumann *et al.*, 2017; Mikhaylina *et al.*, 2018). In fact, identification of genes belonging to the Zur regulon in several bacteria allowed to conclude that Zur functions as a global regulator to control zinc uptake, storage, and mobilization (Lee *et al.*, 2007).

The manganese-sensing Mur transcriptional regulator is also a Fur-like regulator that controls the expression of genes involved in manganese uptake such as an ABC transporter that is activated under high concentration of manganese (Díaz-Mireles *et al.* 2004). Interestingly, this

regulator lacks the structural binding site for zinc and can be regulated either by manganese or iron (Bellini *et al.*, 2006). This suggests that the regulator metal-binding site has plasticity allowing binding of surrogate regulatory metals, but the *in vivo* response is dictated by the available level of metals in the cytosol.

Nickel homeostasis is sensed by the nickel-uptake regulator (Nur) binding directly in the regulatory metal-binding site when nickel levels are higher. Nur negatively regulates the expression of a putative nickel-transporter gene cluster in the presence of nickel in *Streptomyces coelicolor* and also the expression of the superoxide dismutases (SOD) (B. E. Ahn *et al.* 2006; H. M. Kim *et al.* 2015).

In *Bradyrhizobium japonicum*, a protein required for the expression of the heme biosynthesis genes and belonging to the Fur family was discovered. This Fur-like regulator, the iron response regulator, Irr regulates iron uptake and storage but with a different mechanism than FUR (Hamza *et al.* 1998). Irr is active in the absence of the regulatory metal and degraded when heme binds to it, which correlates when there is high iron concentration. Once heme is bound to Irr it will be oxidized by H_2O_2 , thus realizing the heme inducing Irr degradation, hence expression of its target genes (Yang *et al.*, 2006). Interaction of heme to Irr is mediated by the ferrochelatase, the enzyme responsible for the insertion of iron into protoporphyrin (Qi *et al.*, 2002). When iron concentration is low, protoporphyrin binds to ferrochelatase and Irr and ferrochelatase will not form a complex; thus, Irr is free and active. It has been shown that Irr coordinates iron homeostasis and heme biosynthesis in response not only to iron availability but interestingly also to oxidative stress (Yang *et al.*, 2006).

Since the transcriptional regulators from the FUR family are metal-regulated and are involved in the corresponding metal homeostasis, it is not surprising that some of these regulators have evolved to sense oxidants, such as Irr. Besides Irr, PerR is another essential transcriptional regulator of the Fur family that regulates the oxidative stress response by sensing H_2O_2 .

Chapter III

I Peroxide stress regulator (PerR)

PerR was first identified and described in *Bacillus subtilis* by Bsai and collaborators in 1998. Furthermore, the characterization of PerR activity in *B. subtilis* started by analyzing the expression of a gene that its expression is controlled by metals, *mrgA* (Bsai *et al.*, 1998; Huffman *et al.*, 2001). *mrgA* encodes for a protein that protects the DNA during stress, a homolog of Dps (Chen *et al.*, 1995). When iron was abundant *mrgA* expression was down-regulated; however, the expression was induced in the presence of H₂O₂. Moreover, they observed that this regulation exerted in *mrgA* expression was under the control of a PerR regulator in *B. subtilis* (Chen *et al.*, 1993; Chen *et al.*, 1995; Bsai *et al.*, 1998).

PerR is mostly present in Gram-positive bacteria, and it is functionally analogous of OxyR. Generally, PerR and OxyR do not co-exist within the same bacterial species, except for rare examples such as *Deinococcus radiodurans* and *Neisseria gonorrhoeae* (Tseng *et al.* 2003).

PerR it is a transcriptional repressor that is a member of the FUR family. It is an iron-binding protein that senses H₂O₂ and controls the expression of genes involved in the adaption to oxidative stress (Mongkolsuk *et al.*, 2002).

PerR regulates its own expression and expression of genes encoding for catalase, AhpCF, and the DNA-binding protein, MrgA and other genes involved in metal homeostasis such as Fur, the heme biosynthesis machinery (*hemAXCDBL*) among others. (Chen *et al.*, 1995; Bsai *et al.*, 1998; Gaballa *et al.*, 2002).

As any other regulator from the FUR family, PerR contains a binding site for the structural zinc metal and a binding site for the regulatory metal. In *B. subtilis* and *S. pyogenes*, regulatory metal is iron but manganese functions as a surrogate regulatory metal (Duarte *et al.*, 2010). Both iron and manganese allow the interaction between PerR and DNA, leading to gene repression. Thus, DNA binding by PerR is achieved in the reduced state of the protein, with the regulatory metal bound.

The key amino acids that coordinate ferrous iron (Fe^{2+}) binding at the regulatory metal-binding site are three histidines (H37, H91, H93) and two aspartates (D104, D85) (Figure 19) (Jacquamet et al. 2009; Traoré et al. 2006). In the presence of H_2O_2 , H37, and H91 in the iron-bound PerR are oxidized into oxo-histidine (Lee *et al.*, 2006). This oxidation is catalyzed by the Fe^{2+} coordinated in the regulatory metal-binding site through the production of hydroxyl radical (HO^\bullet) by the Fenton reaction (Figure 20) (Lee *et al.*, 2006). This disrupts the regulatory iron coordination and induces a conformational switch leading to PerR dissociation from DNA (Traoré et al. 2009). PerR oxidation is irreversible and, in *B. subtilis*, oxidized PerR is degraded by Lon (Ahn *et al.*, 2016).

PerR has been shown to activate the expression of genes directly. In *B. subtilis* it has been reported that the expression of the gene involved in the synthesis of surfactin (*surfA*) is positively regulated by PerR with direct binding (Brenot *et al.*, 2005; HayashiTaku, 2014). Nevertheless, in some bacteria such as *N. meningitidis* and *S. pyogenes*, it has been shown that in the absence of PerR there is attenuation of virulence, supporting the notion of direct and positive regulation exerted by PerR (Delany *et al.*, 2004; Gryllos *et al.*, 2008).

The structural metal-binding site where zinc binds does not appear to have a role in sensing oxidants. Even though the structural metal-binding site is coordinated by four cysteines (C96, C99, C136, and C139), they are highly resistant to the oxidation exerted by H_2O_2 *in vivo* (Traoré et al. 2006).

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Figure 20. PerR inactivation by H_2O_2 in *Bacillus subtilis*. The structure of the reduced and iron-bound form of PerR (PerR-Zn-Fe) is shown on the left. The amino acid side chains that coordinate the regulatory metal are shown in green and the DNA binding helices are in dark blue. The structural metal (Zn^{2+}) and the regulatory metal (Fe^{2+}) are shown in brown and in red spheres, respectively. In this conformation PerR binds to DNA and represses transcription. The oxidized form of PerR is shown on the right. The Fenton reaction producing hydroxyl radicals and leading to the PerR H37 and H91 oxidation is shown below. This oxidation results in the dissociation of oxidized PerR from the DNA (Dubbs *et al.*, 2012).

**What do we know about oxidative stress response in pathogenic
Leptospira?**

I Peroxide stress regulator (PerR) in *Leptospira interrogans*

The essentiality of defense against hydrogen peroxide in *Leptospira* virulence was established by demonstrating that a catalase mutant was completely avirulent.

As described earlier, generally, OxyR controls the defenses against ROS in Gram-negative bacteria, and in Gram-positive bacteria, these defenses are mainly under the control of the PerR transcriptional regulator. Interestingly, *Leptospira* are among the few examples of Gram-negative bacteria where the oxidative stress response relies on a PerR.

Pathogenic *Leptospira* have 4 ORFs that encode a Fur-like regulator, and it is difficult to assign a precise function of a Fur-like regulator on the sole basis of the protein sequence. The presence of a PerR among these Fur-like regulators was demonstrated by Lo and collaborators in 2010 where they analyzed by microarrays the response of the *L. interrogans* serovar Lai to different iron levels. They did the same with a transposon mutant in one of the ORFs that encoded a Fur-like regulator (LA1857), and they compared the results with the ones of the WT strain. Interestingly, they observed that there was an overlap in their different regulons, which indicated that LA1857 was not a global regulator for iron homeostasis. Instead, they observed an increase of expression of the genes that encode for catalase and the machinery for heme biosynthesis in the *la1857* mutant (Lo et al. 2010). Furthermore, they showed that the *la1857* mutant strain was able to cope against the hydrogen peroxide stress compared to the WT cells. All these results indicated that LA157 is a PerR transcriptional regulator.

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Figure 21. PerR plays a role in the oxidative stress response in *L. interrogans*. (A) WT and *perR* mutant cells (solid and dashed lines, respectively) were cultivated in EMJH medium in the absence (squares) or presence (circles) of 1 mM of H₂O₂ at 30 °C. (B) WT and *perR* mutant cells were incubated with 10 mM of H₂O₂ for 30 min, and the survival was determined. (C) *perR* expression level after exposure with 10 µM of H₂O₂ were quantified by qRT-PCR, and the PerR cellular content was determined by Western-blot using 10 µg of total protein (Kebouchi *et al.*, 2018).

Our group have conducted a thorough structural and functional characterization of PerR in *L. interrogans*. In the optimal laboratory grow conditions (30°C), the growth rate of the *L. interrogans perR* mutant is comparable to that of the WT strain. The growth of the *perR* mutant was also compared to that of WT cells when *Leptospira* are cultivated in different conditions mimicking those encountered in a host. In the presence of 1mM of H₂O₂, the *perR* mutant cells were able to grow, whereas WT cells were not. (Figure 21A). The high resistance of the *perR* mutant to hydrogen peroxide was corroborated by survival test after a 30 min exposure to 10 mM of H₂O₂ (Figure 21B). It is noteworthy to mention that the expression of *perR* increased when *L. interrogans* cells were exposed to sublethal doses of H₂O₂ (Figure

21C). However, this increase in gene expression was not correlated with an increase in the PerR protein level (Figure 21C). This suggests that *Leptospira* PerR is degraded as in *B. subtilis*.

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Figure 22. PerR has a role in other host-related conditions. Growth of *L. interrogans* WT (solid lines) and *perR* mutant (dashed lines) cells in the presence of 2.5 μ M of Paraquat (A), at 37 °C (B), or at 30 °C with 0.5% human serum (C) (Kebouchi2018).

PerR is, therefore, an H₂O₂-responsive gene in *Leptospira*, and the PerR protein represses defenses against peroxide.

Our group also observed that PerR of *Leptospira* has a role under other host-related conditions, such as with the superoxide-generating compound paraquat, at the host temperature of 37 °C and in the presence of 0.5% of human serum (Figure 22). In all these host-related conditions,

the *perR* mutant growth was highly reduced compared to that of WT cells (Figure 22). Altogether, these results demonstrated that PerR of *Leptospira interrogans* has a role in cell fitness under oxidative stress and also under other host-related conditions.

An in vitro DNA binding assay was developed and allowed to show that PerR binds to its own promoter region, indicating that it controls its own expression as previously reported in *B. subtilis* (Jacquamet et al. 2009).

Our group have also determined the crystal structure of PerR in *L. interrogans* and showed that its overall folding was comparable to that of PerR in *B. subtilis*. However, the *Leptospira* PerR exhibited a particular feature, so as the absence of the structural metal-binding site that was considered as a distinctive and obligatory feature of PerR regulators (Traoré et al. 2006). Even in the absence of this structural metal-binding site, PerR of *L. interrogans* is correctly folded, assembled into a dimer, and fully functional in DNA binding and repression of genes coding for defenses against peroxide (Kebouchi et al. 2018a). Another exciting feature of the PerR structure of *L. interrogans* was the obtention of an asymmetric homodimer composed of protomers having a different conformation and regulatory metal coordination. One protomer has the full regulatory metal site and a conformation for the proper DNA binding, and the other protomer had disrupted regulatory metal coordination and a conformation unfavorable for DNA binding. This provided a snapshot of the metal-induced conformational switch controlling DNA binding and dissociation (Kebouchi et al. 2018a).

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Figure 23. Model of the regulation exerted by PerR in *L. interrogans*. The regulatory metal coordination induces a conformational change allowing DNA binding (1). The H₂O₂ sensing of PerR would lead to dissociation from the DNA. PerR protein (oxidized and reduced) would be degraded to keep appropriate and optimal level of the repressor in the cell (3). PerR represses genes that encode peroxidases but it also control the expression of genes involved in survival in other host-related conditions (Kebouchi *et al.*, 2018).

Altogether, these findings allowed to propose a model of the function of PerR in pathogenic leptospires. PerR exists in two conformations, an open one that does not bind DNA and a caliper-like conformation prone to DNA binding (Figure 23). The equilibrium between both conformations is controlled by the regulatory metal binding and, by analogy with the PerR in *B. subtilis*, also by the sensing of peroxide. The binding of regulatory metal will favor the conformation that binds DNA, and H₂O₂ might oxidize PerR and lead to dissociation from DNA and expression of its target genes. Logically, one might think that the absence of PerR is beneficial for survival under oxidative stress. However, *Leptospira* should maintain the levels of PerR by proteolysis in an appropriate steady-state. However, PerR might be involved in regulating other bacterial factors necessary for *Leptospira* fitness under other stress conditions (Figure 23) (Kebouchi et al. 2018b).

Main objectives of this thesis

I To identify PerR1-controlled genes involved in the adaptation to peroxide stress.

We have characterized the structural and functional properties of the first PerR (PerR1) in *L. interrogans* and shown that this regulator is involved not only in repressing defenses against peroxide stress but also in regulating adaptation to other host-related conditions such as the presence of superoxide anions (Kebouchi2018). However, there is no knowledge about the nature of all the cellular factors that are solicited for the adaptation to oxidative stress, nor the exact contribution of PerR1 in orchestrating this adaptive response. We have determined the transcriptome of WT and *perR1* mutant strains in the presence of hydrogen peroxide, allowing the identification of scavenging and repairing machineries. We have also uncovered a complex regulatory network involving not only PerR1 but also additional regulators and non-coding RNAs to control the adaptive response to hydrogen peroxide.

II Investigating the function of PerR2 in *Leptospira*.

By examining the genome of *L. interrogans*, we identified another putative PerR present only in pathogenic *Leptospira* strains and whose function has never been studied. Another goal of this thesis was to delineate the role of this second PerR (PerR2) and investigate whether its function is distinct or redundant with that of PerR1 in *Leptospira* adaptation to oxidative stress. The phenotype of a *perR2* mutant was studied in the presence of different oxidants, and the transcriptome of this mutant was determined.

III To evaluate whether there is an interplay between PerR1 and PerR2 in the oxidative stress response and virulence in *Leptospira*.

Studies in other pathogens have shown that PerR regulators are essential for bacterial virulence. We aimed at investigating whether these two PerR regulators collaborate in *Leptospira* virulence. We have succeeded in concomitantly inactivating PerR1 and PerR2 and obtained, for the first time in a *Leptospira* strain, a double mutant. We have compared the ability of this double *perR1perR2* mutant to grow in the presence of oxidants. In addition, the virulence of single and double *perRs* mutants was tested in the acute animal model (hamsters) and in macrophages.

Experimental results
(Article 1. In preparation)

Article1. Role of the peroxide stress regulator (PerR) in the adaptation of pathogenic *Leptospira* to peroxide stress

Crispin Zavala-Alvarado^{1*}, Odile Sismeiro ², Rachel Legendre², Hugo Varet², Giovanni Bussoti³, Jan Bayram¹, Samuel Garcia Huete¹, Guillaume Rey¹, Jean-Yves Coppé², Mathieu Picardeau¹, and Nadia Benaroudj¹

¹ From the Unité de Biologie des Spirochètes, ² Plate-forme Biomix, ³ C3BI, Institut Pasteur, Paris 75015, France

* Université de Paris, Sorbonne Paris Cité, COMUE BioSPC, Paris 75013, France

Running title: Adaptation of pathogenic *Leptospira* to peroxide stress

To whom correspondence should be addressed: Nadia Benaroudj, Tel: (33) 1-40-61-37-46; Fax: (33) 1-40-61-30-01; E-mail: nadia.benaroudj@pasteur.fr

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Abstract

Pathogenic *Leptospira* spp. are the causative agents of the zoonotic disease leptospirosis. During infection, *Leptospira* are confronted with dramatic adverse environmental changes such as deadly reactive oxygen species (ROS). Withstanding ROS produced by the host innate immunity is an important strategy evolved by pathogenic *Leptospira* for persisting in and colonizing hosts. The peroxide stress regulator, PerR, represses genes involved in ROS defenses in *L. interrogans*. In this study, transcriptomic studies were performed to characterize the *L. interrogans* adaptive response to hydrogen peroxide. We showed that *Leptospira* solicit three main peroxidase machineries (catalase, cytochrome C peroxidase and peroxiredoxin) and heme to adapt to peroxide stress as well as canonical chaperones of the heat shock response, and DNA repair. Determining the PerR regulon allowed to identify the PerR-dependent mechanisms of the peroxide adaptive response and has revealed a regulatory network involving other transcriptional regulators, two-component systems and sigma factors as well as non-coding RNAs that putatively orchestrate, in concert with PerR, this adaptive response. In addition, we have identified other PerR-regulated genes encoding a TonB-dependent transport system, a lipoprotein (LipL48) and a two-component system (VikR). involved in *Leptospira* tolerance to superoxide anion and could represent the first defense mechanism against superoxide anion in *L. interrogans*, a bacterium lacking canonical superoxide dismutase.

Introduction

In order to invade and establish persistent host colonization, pathogens have evolved a variety of strategies to resist, circumvent, or counteract host defenses. Synthesis detoxification enzymes or molecules to eliminate host-produced bactericidal compounds, effective flagella-based motility enabling mucosal barrier crossing and rapid dissemination to host tissues and organs, secretion of effectors inhibiting or subverting the host innate immunity or inflammation allowing intracellular survival, biofilm formation enabling resistance to host defenses, are all examples of mechanisms used by pathogens depending of their lifestyle and niche.

The whole strategies used by pathogenic *Leptospira* for successful host colonization and virulence are not fully unraveled. These aerobic gram-negative bacteria of the spirochetal phylum are the causative agents of leptospirosis, a widespread zoonosis (Haake and Levett, 2015). Although recognized as a health threat among impoverished populations in developing countries and tropical areas (Costa et al., 2015), reported cases of leptospirosis are also on the rise in developed countries under temperate climates (Pijnacker et al., 2016). Rodents are the main reservoir for leptospires as the bacteria asymptotically colonize the proximal renal tubules. They shed the bacteria in the environment by their urine and leptospirosis is transmitted to other animals and humans mostly by exposure to contaminated soils and water. Once having penetrated an organism, *Leptospira* enter the bloodstream and rapidly disseminate to multiple tissues and organs including kidney, liver and lungs. Clinical manifestations range from a mild flu-like febrile state to more severe and fatal cases leading to hemorrhages and multiple organ failure. Lack of efficient tools and techniques for genetic manipulation of *Leptospira* spp. has greatly hampered and limited our understanding of the mechanism of pathogenicity and virulence as well as the basic biology of this pathogen (Ko et al., 2009; Picardeau, 2017).

As part of the host innate immunity response, reactive oxygen species (ROS), *i.e.* superoxide anion (O_2^-), hydrogen peroxide, (H_2O_2), hydroxyl radicals (OH^\bullet), hypochlorous acid (HOCl),

and nitric oxide anion ($\cdot\text{NO}$) are produced upon infection by *Leptospira*. Indeed, the internalization of pathogenic *Leptospira* by macrophages and concomitant production of these oxidants have been demonstrated *in vitro* (Marangoni et al., 2006), and leptospirosis-associated oxidative stress has been observed in human (Araujo 2014) and infected animals (Erdogan et al., 2008). Consistent with these findings was the demonstration that catalase, that catalyzes the degradation of H_2O_2 , is required for *Leptospira interrogans* virulence (Eshghi et al., 2012).

In *L. interrogans*, defenses against peroxide stress such as catalase are controlled by the peroxide stress regulator (PerR), a peroxide-sensing transcriptional regulator (Lo et al., 2010). We have conducted a functional characterization of PerR in *L. interrogans* and showed that not only it represses defenses against H_2O_2 , but also that a *perR* mutant had a decreased fitness in other host-related stress conditions including in the presence of $\cdot\text{O}_2^-$ (Kebouchi et al., 2018). Interestingly, it was shown that *perR* is up-regulated when *Leptospira* are exposed *in vitro* to hydrogen peroxide (Kebouchi et al., 2018) as well as when *Leptospira* are cultivated *in vivo* in rats (Caimano et al., 2014), which strongly suggests a role of PerR in the adaptation of pathogenic *Leptospira* to a mammalian host.

In order to identify the mechanisms solicited by pathogenic *Leptospira* to adapt to an oxidative stress, we have determined the global transcriptional response of *L. interrogans* to H_2O_2 and assessed the role of PerR in the H_2O_2 -mediated regulation. We have also identified novel PerR-regulated factors involved in *Leptospira* survival in the presence of $\cdot\text{O}_2^-$ and assessed their role in *Leptospira* virulence.

Material and Methods

Bacterial strains and growth condition

L. interrogans serovar Manilae WT L495 and transposon mutant strains (see Table S1 for a complete description of the transposon mutant used in this study) were grown aerobically at

30°C in Ellinghausen-McCullough-Johnson-Harris medium (EMJH) (Ellinghausen and McCullough, 1965) with shaking at 100 rpm. Cell growth was followed by measuring the absorbance at 420 nm.

RNA purification

Virulent *L. interrogans* serovar Manilae WT L495 and *perR* mutant M776 with less than three *in vitro* passages were used in this study. Four independent biological replicates of exponentially grown *L. interrogans* cells were incubated in the presence or absence of 10 μ M H₂O₂ for 30 min at 30°C. WT L495 strain was also incubated in the presence of 1 mM H₂O₂ for 60 min at 30°C. Harvested cells were resuspended in 1 ml TRIzol™ (ThermoFisher Scientific) and stored at -80°C. Nucleic Acids were extracted with chloroform and precipitated with isopropanol. Contaminating genomic DNA was removed by DNase treatment using the RNase-free Turbo DNA-free™ turbo kit (ThermoFisher Scientific) as described by the manufacturer. The integrity of RNAs (RIN > 7.6) was verified by the Agilent Bioanalyzer RNA NanoChips (Agilent technologies, Wilmington, DE).

RNA Sequencing

rRNA were depleted using the Ribo Zero kit for bacteria (Illumina) and cDNA libraries were built using the Truseq mRNAseq Library Preparation kit (Illumina), according to the manufacturer's recommendations. Quality controls were performed on Agilent Bioanalyzer DNA NanoChips (Agilent technologies, Wilmington, DE). RNA sequencing was performed on an Illumina HiSeq 2500 instrument (Illumina).

Quantitative RT-PCR experiments

cDNA synthesis was performed with the cDNA synthesis kit (Biorad) according to the

manufacturer's recommendation. Quantitative PCR was conducted with the SsoFast EvaGreen Supermix (Biorad) as previously described (Eshghi et al., 2012; Kebouchi et al., 2018). Gene expression was measured with primers described in Table S2 using *flab* (LA2017/Lic11890/LManV2_290016/LIMLP_09410) as a reference gene.

Non-coding RNA identification

Sequencing data from the *Leptospira* WT and *perR* mutant strains exposed in the absence or presence of 10 μ M H₂O₂ for 30 min at 30°C were processed with Trimmomatic (Bolger et al., 2014) to remove low-quality bases and adapter contaminations. Reads that were shorter than 36 bases after filtering were discarded.

BWA mem (version 0.7.12) was used to discard the reads matching *Leptospira* rRNA, tRNA or polyA sequences and to align the resulting reads against *Leptospira interrogans* serovar Manilae genome assembly available from the NCBI genome resource (accessions GCF_001047635.1 - ASM104763v1, (Satou et al., 2015)). Sequencing and mapping statistics were estimated utilizing Picard CollectAlignmentSummaryMetrics. The Rockhopper software (McClure et al., 2013) was used in combination with available GenBank annotations to re-align reads corresponding to separate replicons and to assemble transcripts models.

The output was filtered to retain all novel transcripts longer than 50nt. The remaining transcripts overlapping within 10nt with NCBI annotated genes on the same orientation were discarded. Eventually, poorly supported transcripts were filtered retaining only those with a Rockhopper raw counts value of 50 in at least two isolates. This high-quality set of 778 new sRNA was subjected to differential expression analysis across different strains and conditions with *Rockhopper*. Genes were considered differentially expressed if they had a Benjamini-Hochberg adjusted P-value < 0.01.

For each non-coding RNAs, putative function was identified by BLAST using the Rfam database (Kalvari et al., 2017).

Determination of cell viability

Cell survival was determined by incubating exponentially growing *L. interrogans* cells ($\approx 10^8$ /ml) in EMJH in the presence or absence of H_2O_2 . Cells were then incubated with resazurin (Alamar Blue® Assay, ThermoFisher Scientific) for 24h. Viability is assessed by the reduction of blue resazurin into pink resorufin. Plating experiments were performed by diluting in EMJH in the absence of H_2O_2 and plating the samples on EMJH agar medium. Colonies were counted after one month incubation at 30°C.

Infection experiments

WT and mutant *L. interrogans* strains were cultivated in EMJH medium until the exponential phase and counted under a dark-field microscope using a Petroff-Hauser cell. 10^6 bacteria (in 1 ml) were injected intraperitoneally in groups of 6-8 male 4 weeks-old Syrian Golden hamsters (RjHan:AURA, Janvier Labs). Animals were monitored daily and sacrificed when endpoint criteria were met (sign of distress, morbidity). The protocol for animal experimentation is conformed to the guidelines of the Animal Care and Use Committees of the Institut Pasteur.

Results

***Leptospira* transcriptional response to sublethal concentration of hydrogen peroxide.**

In order to characterize the transcriptional response of pathogenic *Leptospira* to hydrogen peroxide, we have exposed exponentially growing *L. interrogans* cells to sublethal concentrations of this oxidant. A 30 min. treatment with 10 μ M H₂O₂ (in the presence of iron) was chosen during pilot experiments as having no significant effect on *Leptospira* viability and growth during logarithmic phase while increasing expression of H₂O₂-responsive genes such as *perR* (Kebouchi et al., 2018). RNA sequencing was performed to assess RNA abundance and comparison with untreated cells identified a total of 21 genes with differential transcript abundance (see Table S3 for complete data set). Among those, only 12 and 1 genes were respectively up- and down-regulated by a ≥ 2.0 fold with P-values ≤ 0.005 (See Table 1). Under a low concentration of H₂O₂, *katE*, encoding a catalase, and *mauG* and *AhpC*, coding respectively for a cytochrome C peroxidase and for a peroxiredoxin, were up-regulated with a Log₂FC of 1.79, 4.76 and 3.14 respectively.

The catalase encoded by *katE* (LIMLP_10145) is a monofunctional heme-containing hydroperoxidase, the catalase activity of which and periplasmic localization were experimentally demonstrated (Eshghi et al., 2012; Faine, 1960; RAO et al., 1964). The immediate upstream ORF (LIMLP_10150), encoding an Ankyrin repeat-containing protein, was also up-regulated with a comparable fold. In bacteria such as *Pseudomonas aeruginosa* and *Campylobacter jejuni* (Flint and Stintzi, 2015; Howell et al., 2000), protein with ankyrin repeats were found to be required for the catalase activity, probably by allowing heme binding. A downstream ORF (LIMLP_10140) that encodes a His kinase was also up-regulated by H₂O₂ although with a lower fold change (Log₂FC of 0.82; p-value 5.98e-06). This 85.4 kDa kinase is predicted to be located at the cytoplasmic membrane (PsortB score 7.88). *katE* and *ank* were

organized as an operon that does not contain the kinase-encoding LIMLP_10140 ORF (data not shown). Significant up-regulation of the *ank-katE* operon was confirmed by qRT-PCR (Table 1), however it did not confirm that the expression of the downstream LIMLP_10140 was not significantly increased upon exposure to 10 μ M H₂O₂

The significantly up-regulated *ahpC* gene (LIMLP_05955) encodes a peroxiredoxin that reduces H₂O₂ and ter-butyl peroxide (Arias et al., 2014). The SufB-encoding LIMLP_05960 located in the vicinity of *AhpC* was also up-regulated with a 2-fold (p value 1.60e-08). *SufB* encodes a polypeptide involved in Fe-S cluster assembly proteins. In bacteria such as *E. coli*, SufB is part of a pseudo ABC (ATP-binding cassette)-transporter composed of SufB, SufD and the SufC ATPase. *sufB* is normally found in an operon with *sufC* and *sufD* as well as with the other factors of the Suf machinery, i. e. *sufE* and the *SufS* cysteine desulfurases. Interestingly, none of *suf* genes were present in the vicinity of the SufB-encoding LIMLP_05960. In fact, *L. interrogans* genome contains a putative *suf* cluster (LIMLP_14560-14580 ORFs) as well as a putative SufE (encoded by LIMLP_05090). This cluster is devoid of any SufB-encoding ORF, but it does contain a *subD*, a *sufB* homolog. None of these ORFs were regulated by sublethal dose of H₂O₂. The SufB-encoding LIMLP_05960 shares 40% and 47% identity with SufB from *E. coli* and *B. subtilis* respectively and most importantly it does contain critical residues involved in the Fe-S assembly, including the Cysteine residue (C405 according to the *E. coli* *sufB* numbering) proposed as one of the Fe-S ligands. Predicted secondary structures indicated a central domain mainly composed of β -strands surrounded by N- and C-terminal extremities composed of α -helices (data not shown), similarly to the *E. coli* SufB structure (Hirabayashi et al., 2015). This suggests that the isolated LIMLP_05960-encoded SufB functions as a genuine scaffold in Fe-S biogenesis.

LIMLP_02795 was another peroxidase-encoding ORF that was greatly up-regulated in the presence of H₂O₂ (with a Log₂FC of 4.76, p value of 5.31e-43). LIMLP_02795 encodes a

putative Cytochrome C Peroxidase (CCP) family that catalyzes the reduction of H_2O_2 into H_2O using the ferrocyclochrome as an electron donor. This ORF is annotated as *mauG* in many *L. interrogans* genomes. MauG is a class of Cytochrome C Peroxidase that catalyzes the oxidation of methylamine dehydrogenase (MADH) into tryptophan tryptophylquinone (TTQ) in the methylamine metabolism pathway. LIMLP_02795 exhibits two heme domains with the conventional heme binding motif CXXCH that exist in both CCP and MauG proteins but LIMLP_02795 lacks the Tyrosine axial ligand for heme (Tyr294 in *Paracoccus denitrificans*, (Jensen et al., 2010) that is conserved in all MauGs and replaced by a Methionine or Histidine residue in CCPs. Therefore, it is very likely that LIMLP_02795 encodes a CCP with a peroxidase activity.

In addition to these three peroxidases, several ORFs encoding components of heme biosynthesis (LIMLP_17840-17865) were up-regulated with a 2 to 3.4-fold ($p\text{-value} \leq 1.00e\text{-}03$). *Leptospira*, unlike other spirochetes, possess a complete heme biosynthesis functional pathway (Guégan et al., 2003). The ORFs encoding the glutamyl-tRNA reductase (*hemA*), the porphobilinogen deaminase (*hemC/D*), the delta-aminolevulinic acid dehydratase (*hemB*), the Glutamate-1 semialdehyde aminotransferase (*hemL*), the uroporphyrinogen-III decarboxylase (*hemE*), the coproporphyrinogen-III oxidase (*hemN/F*), as well a two-component system (TCS) (LIMLP_17860 and LIMLP_17865) were organized as an operon (data not shown). qRT-PCR confirms the significance of the up-regulation of *hemA*, *hemC/D* and of the LIMLP_17860-encoded histidine kinase of the TCS (Table 1). The expression of the last ORF of this operon (*hemN/F*) and of ORFs encoding the last enzymes of this pathway, the coproporphyrinogen, the protoporphyrinogen oxidase and the ferrochelatase (LIMLP_17875, LIMLP_17885 and LIMLP_17890, respectively), was only slightly affected by the presence of low concentration of H_2O_2 (Table S3).

When pathogenic *Leptospira* cells are exposed to 10 μ M H₂O₂, the only ORF that was down-regulated was that encoding a permease (LIMLP_18600; with a Log₂FC of -1 and a p-value of 1.18e-04). This permease is a putative Major Facilitator Superfamily (MFS) transporter and is predicted to contain 12 transmembrane helices. This permease-encoding ORF is the second gene of a bicistronic operon where a heme oxygenase-encoding ORF (LIMLP_18595) is the first (data not shown). Expression of heme oxygenase ORF was not significantly changed by the exposure to 10 μ M H₂O₂ (Table S3).

Plotting statistical significance (-log₁₀ of p values) in function of fold change (Log₂FC) indicate that *katE*, *ccp*, *ahpC*, *perR*, and several heme genes of the biosynthesis pathway were among the genes the expression of which was significantly up-regulated (Figure 1).

Noteworthy, after a 2-hour exposure of *L. interrogans* to 10 μ M H₂O₂, the expression of the peroxidases and heme biosynthesis genes returns to a level closer to the level observed in the absence of H₂O₂ (data not shown). Altogether, these data indicate that pathogenic *Leptospira* respond to a low sublethal dose of H₂O₂ by soliciting three peroxidases and heme, and that the peroxidase and catalase activities up-regulated are sufficient to allow survival of *Leptospira*.

***Leptospira* transcriptional response to 1 mM of hydrogen peroxide.**

In order to better reproduce physiological oxidative stress encountered during infection, we performed similar RNASeq experiments upon 1-hour exposure to 1 mM H₂O₂. In this condition, *Leptospira* survival was of 60% \pm 2.735. Comparison with untreated cells identified a total of 2145 genes with differential transcript abundance (see Table S4 for complete data set). Among those, 223 and 268 genes were respectively up- and down-regulated by a ≥ 2.0 fold with P-values ≤ 0.0005 . The volcano representation exhibited more scattered data and a higher number of genes with significantly and statistically changed expression than when *Leptospira* are exposed to sublethal dose of H₂O₂ (Figure 2).

Among the up-regulated genes, the most represented functional categories were the post-translational modification, protein turnover, and chaperones, the carbohydrate and inorganic ion transport and metabolism, and the secondary metabolites biosynthesis, transport and catabolism (Figure 2).

As in the presence of low dose of H₂O₂, the *Ank-katE* operon (LIMLP_10150-10145), *ccp* (LIMLP_02790) and *ahpC* (LIMLP_05955) were up-regulated in the presence of 1 mM H₂O₂ but with higher fold changes (with Log₂FC values of 2.7, 5.8 and 4, respectively, see Table 2). Expression of *PerR* was also greater in the presence of 1 mM H₂O₂ (with Log₂FC value of 3.5, with a pvalue of 1.17e-83). Noteworthy, the ORF upstream *ahpC* that encodes a SufB (LIMLP_05960) was also up-regulated (with a with Log₂FC value of 2.2, p-value of 4.81e-45). All these up-regulations were confirmed by RT-qPCR experiments (Table 2).

Additional ORFs encoding cellular factors related to oxidative stress and redox maintenance were also up-regulated. An ORF encoding a thiol oxidoreductase (LIMLP_07145) exhibiting two cytochrome C-like (heme binding) domains was up-regulated with a Log₂FC value of 2.2 (p-value 1.15e-17). LIMLP_07145 was immediately downstream an ORF (LIMLP_07150) encoding a protein with five chromosome condensation regulator (RCC1) domains that was up-regulated Log₂FC value of about 5 (p-value 9.64e-47). LIMLP_07145-07150 are probably a bicistronic operon as predicted in Zhukova *et al.* (Zhukova et al., 2017). A second thiol peroxidase-encoding ORF (LIMLP_14175) exhibiting a single cytochrome C-like domain was also up-regulated (Log₂FC value of 1.8, p-value 1.02e-940). This ORF might be part of the operon LIMLP_14170-14180 where LIMLP_14170 and LIMP_14180, two ORFs annotated as Imelysins (iron-regulated proteins) were also up-regulated (Log₂FC value of 2.8 and 1.4, p-value 4.04e-144 and 1.15e-13, respectively). Noteworthy, the Imelysin encoded by LIMLP_14170 is the LruB protein that was shown to be associated with *Leptospira*-induced uveitis (Verma et al., 2010).

A thioredoxine disulfide reductase (encoded by LIMLP_07165) was up-regulated (Log₂FC value of 1.9, p-value 9.98e-18). This protein has been shown to catalyze *in vitro* the NADPH-dependent reduction of a thioredoxin encoded by LIMLP_09870 (Sasoni et al., 2016). The LIMLP_09870 was only slightly up-regulated in the presence of 1 mM H₂O₂ (Log₂FC value of 0.8, p-value 3.81e-10).

Other thiol peroxidase-encoding ORFs were up-regulated, including LIMLP_08980 and LIMLP_08985 that encode two glutaredoxins, LIMLP_11965 that codes for the thiol disulfide interchange protein DsbD that might participate in the oxidative folding of periplasmic proteins, and LIMLP_18310 that encodes a bacterioferritin comigratory protein (Bcp, an Ahpc-like). An ORF encoding a putative Glutathione S transferase (LIMLP_13670) had an increased expression in the presence of 1 mM H₂O₂ (Log₂FC value of 1.76, p-value 1.43e-28). Also, an ORF annotated as DNA binding stress protein (Dps) was up-regulated.

Two major cellular pathways involved in reparation of damaged cell components were dramatically up-regulated. Indeed, several genes encoding molecular chaperones had an increased expression in the presence of 1 mM H₂O₂. Two ORFs encoding small heat shock proteins (sHSP), probably organized as a bicistronic operon (LIMLP_10970-10975) exhibited a significant increase in expression (Log₂FC values of about 6, p-value 1.34e-184 and 1.45e-238, respectively). The LIMLP_15105-15120 cluster encoding DnaK/DnaJ/GrpE the molecular chaperone machinery and its putative repressor HrcA, was significantly up-regulated Log₂FC values of 2.6-3.6 (p-value<4.8e-24). Similarly, the GroES-GroEL operon (encoded by LIMLP_06545-06540) was up-regulated Log₂FC values of 3.3 (p-value<3.2e-33). The *clpB* gene (LIMLP_10060) also had an increased expression (Log₂FC value of 2.1, p-value 1.2e-15). Genes encoding several components of the SOS response, a regulatory network stimulated by DNA damage-inducing stress, had a higher expression in the presence of 1 mM H₂O₂. Indeed, ORFs encoding the recombinase A (*recA*, LIMLP_08665), the DNA repair protein RecN

(LIMLP_07915), the DNA polymerase IV (*dinP*, LIMLP_02170) as well as the repressor of the SOS response LexA1 (LIMLP_11440) were significantly up-regulated. Other factors putatively involved in DNA repair but not under the control of LexA1 (Fonseca et al., 2013; Schons-Fonseca et al., 2016) has also an increased expression, including the DNA mismatch repair protein MutS (LIMLP_07780, Log₂FC value of 1, p-value 1.18 e-7), the DNA repair protein RadD (LIMLP_11400, Log₂FC value of 3.4, p-value 1.134e-167).

One remarkably up-regulated ORF was located into a genomic region previously identified as an island enriched in prophage genes ranging from LIMLP_00855 to LIMLP_01005 and referred as prophage 1 (Qin et al., 2008; Schons-Fonseca et al., 2016). LIMLP_00895, encoding a hypothetical protein, had an increased expression Log₂FC value of 3.6 (p-value 4.81e-45). Noteworthy, another cluster enriched in prophage genes ranging from LIMLP_13010 to LIMLP_13095, prophage 2, (Schons-Fonseca et al., 2016) contains 4 ORFs (LIMLP_13010, LIMLP_13015, LIMLP_13020, and LIMLP_13025) that were up-regulated in the presence of 1 mM H₂O₂.

Down-regulated genes were mainly involved in metabolism, translation and ribosomal biogenesis, cell wall and membrane biogenesis (Figure 2). 14 ORFs encoding ribosomal proteins, a translation initiation factor (LIMLP_03190), a ribosome maturation factor (LIMLP_07600), a RNA polymerase RpoA (LIMLP_03215), a transcription termination factor RhoA (LIMLP_13190) were among them (Table 3).

A cluster of gene encoding the ATP synthase complex (LIMLP_06050-06080) was down regulated with a Log₂FC ≤ -1.2 (p-values ≤ 2.88e-05), indicating that *Leptospira* decrease ATP synthesis upon exposure to high dose of H₂O₂. Another metabolic pathway that was down-regulated in this condition was the cobalamin (vitamine B12) biosynthesis pathway. Indeed, 15 out 17 genes of the cobI/III cluster (LIMLP_18460-18530) were significantly down-regulated (with a Log₂FC ≤ -1.5, p-values ≤ 6.28e-08).

Gene encoding proteins involved putatively in secretion, including SecY (LIMLP_03180), SecF (LIMLP_12685) and SecD (LIMLP_12690), and in correct membrane insertion (LIMLP_00815-00830) were down-regulated. Coding sequences annotated as Penicillin Binding Protein and murein transglycosylases (LIMLP_01410, LIMLP_06170, and LIMLP_01540) involved in cell wall biogenesis were also down-regulated. Genes encoding putative MreD (LIMLP_06165), RodA (LIMLP_06175), and FstW (LIMLP_09265) had also a decreased expression in the presence of 1 mM H₂O₂. A least 10 genes annotated as lipoproteins, the genes coding for LipL41 (LIMLP_02605) and LipL46 (LIMLP_09360), as well as 8 genes annotated as membrane proteins were down-regulated.

A cluster of four genes encoding proteins of the CRISPR-CAS machinery (*csh2*, LIMLP_2870; *cas8*, LIMLP_2875; *cas5*, LIMLP_2880; *cas3*, LIMLP_2880) involved in phage defense were down-regulated (with a Log₂FC<1, p-values≤3.90e-05), which is consistent with the increased expression of prophage-related ORFs.

Several genes related to motility/chemotaxis were down-regulated when *Leptospira* are exposed to a high dose of H₂O₂. Several of these genes encode constituent of the endoflagellum basal body (*flgGAHIJ*, LIMLP_06485-06505), of the flagellar export apparatus (*fliOPQR-FlhBA*, LIMLP_06690-06715; *fliL*, LIMLP_14615 and LIMLP_14620), and of the flagellar motor stator (*motAB*, LIMLP14625-14630). A cluster of four down-regulated genes encode chemotaxis-related proteins (*cheBDW-mcp*, LIMLP_07420-07435).

Identification of small RNAs up-regulated in the presence of hydrogen peroxide.

In order to identify non-coding (NC) RNA whose expression is changed in the presence of hydrogen peroxide, non coding genome regions of RNASeq data were also analyzed. When *Leptospira* were exposed to 10 µM H₂O₂ for 30 min, the most highly up-regulated ncRNA was a 322 bp ncRNA (rh859) located 21 bp downstream LIMLP_02795, the ORF encoding the

cytochrome C peroxidase (see Table 4 and Table S5 for the complete set of data). Noteworthy, this ORF was up-regulated in the same condition (Table 1).

Other significantly up-regulated ncRNAs were a 127 bp ncRNA (rh3130) located downstream the two small hsps-encoding ORF (LIMLP_10970-10975) and a 225 bp ncRNA (rh3999) overlapping with the LIMLP_14135 ORF. The expression of these ORFs was not significantly changed by the presence of sublethal concentration of hydrogen peroxide.

When *Leptospira* were exposed to a lethal dose of hydrogen peroxide (1 mM for 1h), a higher number of ncRNAs with a higher transcriptional activity was detected. Indeed, 416 and 102 ncRNAs were up- and down-regulated, respectively.

28 ncRNAs were up-regulated with a Log₂FC above 1.5. Rh3130 and rh859 were the two most highly up-regulated with a Log₂FC of 7.19 and 4.25, respectively. An up-regulated 70 bp ncRNA overlapped LIMLP_00895 (rh288), an ORF located in a prophage locus, was also up-regulated in the presence of 1 mM H₂O₂ (Table 4).

13 ncRNAs were down-regulated with a Log₂ FC below -1.5. Among the most highly down-regulated ncRNAs were a 193 bp RNA (rh967) located downstream a large cluster encoding ribosomal proteins (of LIMLP_03075-3220).

Several of the ncRNA whose expression was up- or down-regulated in the presence of hydrogen peroxide were located in the vicinity or overlapped ORFs that were also up- or down-regulated in the same conditions. For instance, as mentioned earlier, the rh3130 and rh859, two of the most highly up-regulated ncRNAs, were in the vicinity of Hsp20 and CCP-encoding ORFs (LIMLP_10970-10975 and LIMLP_02795, respectively), two genes whose expression was greatly increased in the presence of hydrogen peroxide. LIMLP_05620, LIMLP_13670, and LIMLP_13765 were three up-regulated ORFs that have a downstream ncRNA (rh1641, rh3871, and rh3894, respectively).

This trend was also observed with down-regulated ncRNAs. Rh411, rh967, rh1102, rh1880, rh3186, and rh4281 ncRNAs were also located downstream or upstream, or overlapped ORFs whose expression was decreased in the presence of hydrogen peroxide.

Most of the ncRNAs whose transcriptional activity is modified when *Leptospira* are exposed to hydrogen peroxide did not belong to any identified RNA families. However, this study has allowed the identification of a TPP riboswitch downstream LIMLP_04085, a colabamin riboswitch downstream LIMLP_06575, LIMLP_17100, and upstream LIMLP_11800, an AsrC (Antisense RNA of rseC) downstream LIMLP_10015, and a ligA thermometer downstream LIMLP_05075.

These findings indicate that exposure of *Leptospira* to 1 mM H₂O₂ triggers a drastic regulation of expression of ncRNAs that correlates with dramatic changes in coding sequence expression.

Contribution of PerR in the adaptation of pathogenic *Leptospira* to oxidative stress.

Comparison of the transcriptome of a *perR* mutant with that of wild-type *Leptospira* allowed determination of PerR regulon. In the *perR* mutant, 5 and 13 ORFs were up- and down-regulated, respectively, with a log₂FC cutoff of 1 and a p-value below 0.05 (Table 5). The LIMLP_10150-10145 operon, encoding the Ankyrin-containing protein and catalase, LIMLP_05955, encoding AhpC and LIMLP_02795, encoding the Cytochrome C peroxydase, were up-regulated upon *perR* inactivation. Chromatin immunoprecipitation experiments showed that when *Leptospira* were cultivated in EMJH medium, PerR was bound to DNA fragment comprising the 200 bp upstream region to the LIMLP_10150-10145 operon as well as to the 200 bp upstream region to the LIMLP_10155 ORF encoding PerR (Figure S1). This was consistent with the binding of PerR to its own promoter region as demonstrated previously by *in vitro* DNA binding assay (Kebouchi 2018). A binding upstream 300 bp upstream the LIMLP_02790 was also observed. These findings indicate that PerR represses the expression

of the Ankyrin-containing protein and of catalase, and of the Cytochrome C peroxidase upon directly binding the upstream region of these ORFs (Figure S1).

A cluster composed of genes encoding a TonB-dependent receptor (LIMLP_04240-04255, TonB, two ExbDs and ExbB) were dramatically down-regulated in the *perR* mutant (with a Log₂FC of -5.47 to -4.60), as well as downstream *fecA*- and *lipL48*-encoding ORFs (with Log₂FC of -3.26 and -5.50, respectively). LIMLP_04240, LIMLP_04245, LIMLP_04250 and were organized as an operon and *fecA* (LIMLP_04270), LIMLP_04275 and *lipL48* (LIMLP_04280), and LIMLP_04285 were also organized as an operon (data not shown). *In vivo* binding assay indicated a direct interaction of PerR upstream the LIMLP_04285-04270 operon (Figure S1), suggesting that PerR directly activates the expression of the tonB-dependent receptor-encoding operon.

A bicistronic operon composed of the response regulator VicR (LIMLP_16720) and the histidine kinase VicK (LIMLP_16725) of a two-component system was also down-regulated. The ncRNAs rh859, located upstream the ORF LIMLP_02795 encoding the cytochrome C peroxidase was up-regulated in the *perR* mutant (with a Log₂FC of 2.50 and p-value of 8.77 e-56). A 77 bp ncRNA (rh1263) located upstream the TonB-dependent receptor-encoding operon (LIMLP_04255-04240) and upstream the *fecA* gene (LIMLP_04270) was significantly down-regulated in the *perR* mutant (with a Log₂FC of -3.129 and p-value of 1.96 e-95).

Interestingly, among the PerR regulon, only genes whose expression is repressed by PerR were up-regulated when *Leptospira* were exposed to H₂O₂. Indeed, the expression of the Ankyrin-containing protein, of catalase, of AhpC and of the Cytochrome C peroxidase were both up-regulated in the *perR* mutant and in the presence of H₂O₂ whereas the expression of the ORFs encoding the TonB-dependent receptor, FecA, LipL48, VicR and VicR was not dramatically altered by the presence of H₂O₂.

In order to determine the exact contribution of PerR in the increase in gene expression upon exposure to H₂O₂ in *Leptospira*, the transcriptome of the *perR* mutant exposed to a sublethal dose of H₂O₂ was also obtained (see Table S6 for a complete set of data). The LIMLP_10150-10145 operon, encoding the Ankyrin-containing protein and catalase, whose expression is directly repressed by PerR and increased in the presence of H₂O₂ in WT *Leptospira*, was not up-regulated in the presence of H₂O₂ when *perR* was inactivated (Table S3 and S7). The amount of LIMLP_10150-10145 operon RNA in the *perR* mutant is in fact higher than that in WT *Leptospira* exposed to H₂O₂. This indicates that the derepression of the operon occurring by the absence of PerR is more important than its derepression induced by the presence of H₂O₂. The LIMLP_02795 and LIMLP_05955 ORFs encoding CCP and AhpC, respectively, were still significantly up-regulated in the presence of H₂O₂ in the *perR* mutant (with Log₂FC values of 2.298 and 1.874, respectively). Therefore, an H₂O₂-induced derepression of these two genes still occurs in the absence of PerR even though their expression is repressed by this regulator. Interestingly, the ncRNAs rh859, located upstream the ORF LIMLP_02795, was further up-regulated in the *perR* mutant upon exposure to H₂O₂ (with a Log₂FC of 1.71 and p-value of 5.46 e-07). In that condition, 11 ncRNAs were significantly down-regulated with a log₂FC below -2 whereas only one ncRNAs was significantly down-regulated when WT cells were exposed to 10 µM of H₂O₂ (Table S5). Rh753 was also down-regulated upon inactivation of *perR* and Rh3164 and rh1880 were also down-regulated upon exposure of WT cells to 10 µM and 1 mM of H₂O₂, respectively (Table S5). Rh38, rh96, rh367, rh928, rh2114, rh2850, rh4234, and rh4918 were all specifically down-regulated when the *perR* mutant was exposed to H₂O₂ (Table S5).

Altogether, these findings indicated that not all H₂O₂-regulated genes belong to the PerR regulon in pathogenic *Leptospira* and several PerR-regulated genes were not regulated by H₂O₂.

Role of the PerR-regulated genes in defenses against ROS and in virulence in *Leptospira*

The transcriptomic experiments have allowed the identification of cellular factors putatively required for the adaptation to peroxidic stress. One important question is to experimentally establish and understand the role of this factors in the adaptation to ROS.

Genetic manipulation of pathogenic *Leptospira* is still a challenge and functional studies in these bacteria mainly relies on random insertion transposon. Our laboratory has constructed a transposon mutant library (Bourhy et al., 2005) and several mutants inactivated in ORFs with change in expression upon exposure to H₂O₂ or upon *perR* inactivation were available.

Catalase, the peroxiredoxin AhpC, and the cytochrome C peroxidase (CCP) were the peroxidase machineries up-regulated in the presence of H₂O₂ and repressed by PerR. Only *katE* and *ahpC* mutants were available in the transposon mutant library and we have studied the ability of these mutants to grow in the presence of H₂O₂ and paraquat, a superoxide-generating compound. As seen in Figure 3, these two mutants had a comparable growth rate in EMJH medium but when the medium was complemented with 0.5 mM H₂O₂, the ability of the *katE* mutant to divide was dramatically impaired. The growth rate of the *ahpC* mutant in the presence of H₂O₂ was comparable to that of WT strain, regardless of the H₂O₂ concentration used in the assay (data not shown). However, when the EMJH medium was complemented with 2 μ M paraquat, the growth of the *ahpC* mutant was considerably reduced, indicating a high sensitivity to superoxide.

In other bacteria including *E. coli* and *B. subtilis*, *katE* is produced during stationary phase, and in order to further characterized the role of *katE* in *Leptospira* survival under oxidative stress, we investigated the survival of stationary phase-adapted *Leptospira* in the presence of H₂O₂. *L. interrogans* WT cells were cultivated in EMJH medium and samples were harvested in the logarithmic phase (at OD_{420 nm} \approx 0.3, Figure 4A), at the entry in stationary phase (OD_{420 nm} \approx 0.7, Figure 4A) and in advanced stationary phase (at OD_{420 nm} \approx 0.7, 5 days after the entry in

stationary phase, Figure 4A). Each sample was used to inoculate a new batch of EMJH medium in the absence or presence of 2 mM H₂O₂. As seen in Figure 4A, when EMJH was inoculated with *Leptospira* cells at logarithmic phase, *Leptospira* were not able to divide in the presence of 2 mM H₂O₂. When the culture medium was inoculated with *Leptospira* cells at the beginning of the stationary phase, *Leptospira* acquired a greater resistance to 2 mM H₂O₂ as cells were able to grow (Figure 4A). An even higher ability to grow in the presence of this deadly dose of H₂O₂ was observed when the EMJH medium was inoculated with *Leptospira* at advanced stationary phase (Figure 4A). This indicates that *Leptospira* cell at stationary phase acquire a tolerance to hydrogen peroxide. Interestingly, this acquired tolerance to H₂O₂ was independent of PerR since a *perR* mutant cells also acquire a higher ability to grow in the presence of 2 mM H₂O₂ when at stationary phase (Figure 4A). In order to determine which peroxidase was responsible for this acquired tolerance to H₂O₂, the survival of WT, *ahpC* and *katE* mutant strains in was tested in logarithmic phase was compared with that in stationary phase. As seen in Figure 4B, a 30 min. exposure in the presence of 10 mM H₂O₂ led to dramatic loss of survival of all strains at logarithmic phase. WT and *ahpC* mutant strains were able to acquire a higher resistance to H₂O₂ when placed at stationary phase whereas the *katE* mutant did not. Therefore, *katE* is essential for the stationary phase-acquired resistance to H₂O₂ and this probably involves another regulation mechanism than that exerted by PerR.

Among the genes repressed by PerR, only mutants inactivated in LIMLP_04245 (*exbD*), LIMLP_04270 (*fecA*), and LIMLP_04280 (*lipl48*), LIMLP_16720 (*vicR*), LIMLP_16725 (*vicK*), were available in the transposon mutant library. All these mutants but *vicK* had a growth comparable to that of the WT strain in EMJH medium (Figure 5 and 6). Despite the fact that *vicK* had a reduced ability to divide in EMJH medium, this mutant strain has a greater resistance to 2 μ M paraquat than the WT and *vicR* mutant strains (Figure 5). Among the mutants inactivated in ORFs encoding the TonB-dependent transport system, *exbD*, *fecA* and *lipl48*

mutant strains had a lower ability to grow in the presence of paraquat than the WT strain (Figure 6). Noteworthy, all these mutants exhibited a comparable ability to grow in the presence of H₂O₂ than the WT strain. Altogether, these findings suggest that some of the PerR-repressed ORF are involved in *Leptospira* defense against superoxide.

Catalase has been shown to be essential for *Leptospira* virulence as inactivation of *katE* led to a drastic attenuation in *L. interrogans* virulence (Eshghi et al., 2012). We investigated whether other PerR-controlled genes were also required for *Leptospira* virulence. The different mutants were used in infection experiments in the acute model for leptospirosis. Despite an altered growth of *ahpC*, *vicK*, *exbD*, and *lipI48* mutant strains in the presence of a superoxide-generating compound, none of these mutants had an altered virulence in the hamsters (Data not shown and Figure 7). The *vicR* and *fecA* mutant strains exhibited a slight delay in triggering sign of leptospirosis when injected in hamsters (Figures 7). Therefore, AhpC, the tonB-dependent transport system, and the two-component system VicKR do not have a pivotal role in *Leptospira* virulence.

Discussion

Reactive oxidative species are powerful and efficient weapons used by the host innate immunity response to eliminate infecting microorganisms. The ability of pathogenic *Leptospira* to detoxify hydrogen peroxide, one of the ROS produced upon *Leptospira* infection and pathogenicity, is essential for these pathogenic bacteria virulence. The present study has used RNASeq technology to determine the adaptive response of pathogenic *Leptospira* to hydrogen peroxide. Because *Leptospira* are also environmental aerobic bacteria, they will also face low concentrations of ROS endogenously produced through the respiratory chain or present in the outside environment. In our study, *L. interrogans* were subjected to two different treatments. A short exposure in the presence of a sublethal dose of hydrogen peroxide (30 min. with 10 μ M H₂O₂) and a longer exposure with a lethal concentration of hydrogen peroxide (60 min. with 1 mM H₂O₂) could mimic the hydrogen peroxide concentrations encountered in the environment and inside a host, respectively.

Our study allowed a global identification of all the cellular factors solicited by pathogenic *Leptospira* to adapt to the presence of H₂O₂. Our findings indicate that the peroxidic stress response is timely-orchestrated and dose-dependent. *L. interrogans* can sense and rapidly respond to H₂O₂ concentrations as low as 10 μ M by up-regulating the catalase (encoded by *katE*) and two peroxidases, an AhpC and a CCP. Heme biosynthesis-encoding genes were also up-regulated probably because catalase and CCP have heme-dependent peroxidase activities. These three peroxidases are the first-line of defense allowing detoxification of H₂O₂, and among these three enzymes, *katE*-encoded catalase has a major role in protecting *L. interrogans* from the deadly effect of hydrogen peroxide, during logarithmic phase but also during stationary phase. In our study, an *ahpC* mutant did not exhibit an altered tolerance toward H₂O₂; instead, this mutant had a lower ability to grow in the presence of superoxide. Although we cannot rule

out that the inactivation of *ahpC* triggers an increase in catalase activity to compensate the absence of AhpC, our findings might indicate a role of this peroxidase in detoxification of superoxide or of H₂O₂ produced from the catabolism of superoxide. Determining whether CCP acts for degrading H₂O₂ or as an electron acceptor for the respiratory chain (Khademian and Imlay, 2017) during the hydrogen peroxide stress response in *L. interrogans* will require obtaining a deletion mutant by allelic exchange as a transposon *ccp* mutant was not available in the transposon mutant library.

When H₂O₂ reach a level that overwhelms the H₂O₂ detoxification machinery, not only *L. interrogans* solicited the aforementioned peroxidases but additional enzymes with a putative role as antioxidants and/or in repair of oxidized cysteines in proteins were also up-regulated, including several thiol oxidoreductases, thioredoxin, glutaredoxin, and DsbD and Bcp-like proteins. The induction of several genes of the LexA regulon (*recA*, *recN*, *dinP*) and other genes with putative role in DNA repair (*mutS*, *radC*) suggests that these concentration of H₂O₂ induced oxidative damage to DNA and a need for the SOS response. Surprisingly, the classical repair mechanism for oxidized methionine residues (such as methionine sulfoxide reductases) or damages to iron-sulfur clusters in proteins (the Suf machinery) were not more expressed in the presence of H₂O₂ as if this repair mechanisms were not required under such oxidative damage-inducing condition. Also, the redox-regulated chaperone Hsp33 involved in protecting from aggregation and promoting the refolding of oxidatively-damaged proteins, was not up-regulated. Instead, canonical ATP-dependent (DnaK/J/GrpE, GroEL/ES, ClpB) and -independent (small Hsps) molecular chaperones were dramatically more expressed, suggesting that 1 mM H₂O₂ results in protein aggregation and unfolding.

The up-regulation of all these detoxification and repair mechanisms correlated with a down-regulation of genes encoding transcription and translation factors, protein secretion, *Leptospira*

motility and chemotaxis, as well metabolism pathways including ATP and cobalamin (vitamin B12), that might explain the slowdown in growth induced by the presence of H₂O₂.

Comparing the H₂O₂-induced change in gene expression in the *perR* mutant with that in WT cells, indicated that PerR contributes only partially to the H₂O₂-induced gene regulation. Among the genes whose expression is markedly changed upon exposure to H₂O₂, only *katE*, *ahpC* and *ccp* are under the controlled of PerR. Surprisingly, even in the absence of PerR, as in the *perR* mutant, *ahpC* and *ccp* expression are still increased upon exposure to H₂O₂, suggesting that additional regulatory mechanisms are involved in the H₂O₂-induced gene regulation. In fact, several genes encoding transcriptional regulators, two component systems, and sigma factors had their expression altered by the presence of H₂O₂, corroborating the involvement of other regulators. Moreover, our RNASeq experiments have allowed the identification of several non-coding RNAs that might also influence the expression of the H₂O₂-regulated genes. For instance, many non-coding RNAs with increased or reduced expression upon *Leptospira* exposure to H₂O₂ are located in the vicinity of ORFs with increased or reduced expression in the same condition. Noticeably, rh859 located downstream *ccp* might participate in the increased expression of this gene together with the derepression induced by PerR dissociation from DNA in the presence of H₂O₂. According to the transcriptome of the *perR* mutant, heme biosynthesis genes were not under the control of PerR. Consistent with this, the expression of some of the heme genes was still increased by H₂O₂ even when *perR* was inactivated.

Of note, comparison of the transcriptome of the *perR* mutant determined in this study with that determined previously by Lo et al. (Lo et al., 2010) pinpoints several discrepancies. For instance, our study did not demonstrate that heme biosynthesis genes are under the the control of PerR and the expression of *ahpC* was not affected in the *perR* mutant in the study of Lo et al. These contradictions can be explained by the experimental conditions used to determine the transcriptome of the *perR* mutant in this previous study which, in fact, has compared WT cells

cultivated in EMJH medium with *perR* mutant cells cultivated in EMJH medium the presence of kanamycin. Due to the relation between antibiotic and oxidative stresses, the presence of an antibiotic might have influenced the expression of ROS-related genes, such as heme genes or *ahpC*.

Among the ORF that are significantly up-regulated in the presence of H₂O₂, the catalase and ClpB have been shown to be required for *Leptospira* survival under oxidative stress and virulence. In the present study, we have identified new ORFs that participate in *Leptospira* survival in the presence of ROS. Indeed, our findings indicate that a peroxidase, encoded by *ahpC*, and a TonB-dependent transporter (encoded by a cluster containing *fecA*, *exbD*, and *lipL48*) are required in *Leptospira* survival in the presence of superoxide. Interestingly, pathogenic *Leptospira* genomes do not contain any genes homologs to a superoxide dismutase or superoxide reductase, nor they exhibit a SOD activity (Austin et al., 1981). This is quite intriguing as it is generally believed that all aerobic bacteria do have a SOD. One fundamental question is to understand the mechanism these pathogenic bacteria use to detoxify superoxide produced endogenously during the respiratory chain or exogenously by phagocytic cells during infection. Our study is the first to identify cellular factor in pathogenic *Leptospira* involved in survival in the presence of superoxide. AhpC could detoxify H₂O₂ produced upon the reduction of superoxide and the TonB-dependent transporter could act as an efflux pump. It will be interesting to understand and decipher the exact contribution of AhpC and this TonB-dependent transport system in this defense mechanism. None of the mutants inactivated in these ORF exhibited a dramatic reduction in virulence, suggesting that these mechanisms do not have a pivotal role in *Leptospira* during infection.

Many ORFs of the H₂O₂ adaptive response identified in this study have been shown to be also up-regulated upon other host-related conditions such as at the host temperature 37°C or osmolarity, under iron-limited concentration or in dialysis membrane chamber (DMC)

implanted inside rats. Indeed, molecular chaperones (GroEL/ES, DnaK/J/GrpE, small HSPs, ClpB), DNA repair proteins (RadC) were more expressed at 37°C (Lo et al., 2006, 2009; Lourdault et al., 2011) and in DMC (Caimano et al., 2014). Among the up-regulated genes at 37°C in *L. interrogans* were LIMLP_02520 and LIMLP_02525 (encoding a copper resistance and exporting ATPase proteins, respectively) and *katE* (Lo 2006). In addition, catalase and AhpC are significantly more expressed in DMC (Caimano et al., 2014). ORFs encoding TonB-dependent receptors (LIMLP_14160 and LIMLP_08410), Imelysin (LIMLP_14180), the lipoprotein LruB (LIMLP_14170) have been shown to be up-regulated when *Leptospira* are cultivated under iron-limited concentration, as encountered inside a host (Lo et al., 2010). RadC, the LIMLP_16520-encoded DNA repair exonuclease, DsbD and the LIMLP_00770-encoded dithiol disulfide isomerase were more expressed under host osmolarity (Matsunaga et al., 2007). Therefore, the H₂O₂ adaptive response overlaps to some extent with other stress responses. The accumulation of oxidatively-damaged proteins and DNA could trigger a general stress response. The change in expression of other stress-related regulators such HrcA, the repressor of heat shock proteins, and LexA, the repressor of the SOS response, suggest that the presence of ROS elicits heat shock and SOS responses. In fact, and perhaps most importantly, the overlap between the H₂O₂ adaptive response (determined in this study) with the host adaptive response in a mammalian host (assessed by DMC) imply that the H₂O₂ treatment used in this study mimics the oxidant conditions pathogenic *Leptospira* encountered inside a host during infection.

In conclusion, the present study has revealed, for the first time, the genome-wide general adaptive response to peroxide in pathogenic *Leptospira*, unfolding putative biological pathways *Leptospira* have evolved to overcome the deadly effect of ROS. Peroxide adaptive response involves detoxifying enzymes, molecular chaperones, DNA repair machinery, transporters that could act as efflux pumps. This adaptive response also engages a large number of non-annotated

and sometimes *Leptospira* specific ORFs reflecting the submerged part of the iceberg in these bacteria physiology. We have also uncovered a regulatory network of transcriptional regulators, sigma factors, two component systems and non-coding RNA that orchestrate together with PerR the peroxide adaptive response.

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ORF ID ^a	Gene	Function	Log ₂ Fc	p value adj	RT-qPCR ^b
Up-regulated genes					
LIMLP_02795 (LIC12927/LA0666)	<i>ccp</i>	Cytochrome C peroxidase	4.764*	5.31e-43	38.900
LIMLP_05955 (LIC11219/LA2809)	<i>ahpC</i>	Peroxiredoxin/alkylperoxiredoxin reductase	3.145*	3.63e-20	11.742
LIMLP_05960 (LIC11220/LA2808)	<i>sufB</i>	ABC transporter permease	1.056*	1.60e-08	1.880
LIMLP_10145 (LIC12032/LA1859)	<i>katE</i>	Catalase	1.786	2.11e-08	3.477
LIMLP_10150 (LIC12033/LA1858)		Ankyrin repeat-containing protein	2.051*	2.30e-11	4.183
LIMLP_10155 (LIC12034/LA1857)	<i>perR</i>	Regulator Fur family	2.319*	1.02e-39	6.827
LIMLP_17840 (LIC20008/LB010)	<i>hemA</i>	Glutamyl-tRNA reductase	1.771*	1.16e-10	3.389
LIMLP_17845 (LIC20009/ LB011)	<i>hemC/D</i>	Porphobilinogen deaminase	1.617*	6.57e-13	2.328
LIMLP_17850 (LIC20010/ LB012)	<i>hemB</i>	Delta-aminolevulinic acid dehydratase	1.455*	2.65e-14	2.064
LIMLP_17855 (LIC20011/ LB013)	<i>hemL</i>	Glutamate-1 semialdehyde aminotransferase	1.262*	1.19e-07	2.193
LIMLP_17860 (LIC20012/ LB014)		Signal transduction histidine kinase	1.035*	2.67e-03	2.470
LIMLP_17865 (LIC20013/ LB015)		Response regulator cheY	1.166*	1.01e-03	2.012
Down-regulated genes					
LIMLP_18600 (LIC20149/ LB187)		Permease of the Major facilitator superfamily	-1.001	1.17e-04	0.894

Table 1: Regulated genes upon exposure to sublethal dose of H₂O₂.

Significantly up-and down-regulated genes upon exposure to sublethal dose of H₂O₂ (30 min exposure to 10 μ M H₂O₂) with a Log₂FC cutoff of 1 and a p value cutoff of 0.005.

^a Gene numeration is according to Satou et al.(Satou et al., 2015)

^b Fold change (WT vs WT exposed 30 min to 10 μ M H₂O₂) obtained by RT-qPCR experiments

* Genes significantly up-and down-regulated by Volcano analysis (Log₂FC cutoff of 1 and p value cutoff of 0.005).

ORF Id ^a	Gene	Fonction	Log ₂ FC	p-value adj	RT-qPCR ^b
Miscellaneous					
LIMLP_00430 (Lic10079/LA0093)		Cupin fold metalloprotein	3.062*	3.01e-20	
LIMLP_00770 (LIC10149/LA0169)	<i>frnE</i>	Polyketide dithiol disulfide isomerase	3.786*	1.24e-2	1
LIMLP_01545 (LIC13183/LA3982)		Methyl-accepting chemotaxis protein	2.440*	1.12e-80	
LIMLP_02520 (LIC12983/LA0593)	<i>copZ</i>	Copper resistance protein	2.055	5.77e-08	
LIMLP_02525 (LIC12982/LA0594)	<i>copA/zntA</i>	Copper exporting P-type ATPase	1.941	9.86e-12	
LIMLP_04555 (LIC10592/LA3166-68)		Phosphohydrolase	2.395*	3.79e-56	
LIMLP_05110 (LIC11058/LA3017)		Lipoprotein LemA	3.449*	2.87e-44	
LIMLP_07150 (LIC11467/LA2498)	<i>atsI</i>	Chromosome condensation regulator RCC1	4.959*	9.64e-47	21.851
LIMLP_10925 (LIC12202/LA1580)		NAD-dependent dehydratase	2.615*	2.21e-65	
LIMLP_10935 (LIC12204/LA1578)		FAD-dependent oxidoreductase	2.536*	1.31e-73	
LIMLP_15540 (LIC10440/LA3807)	<i>glnK</i>	Nitrogen assimilation regulatory protein	3.529*	8.00e-53	
LIMLP_16870 (LIC13298/LA4137)		NADPH-dependent FMN reductase	2.050	6.62e-75	
LIMLP_12510 (LIC12503/LA1188)		Tetratricopeptide repeat protein	4.522*	1.40e-60	
LIMLP_14170 (LIC10713/LA3469)	<i>irpA/lruB</i>	Peptidase M75/Imelysin/LruB	2.822*	4.04e-144	3.759
LIMLP_14180 (LIC10711/LA3471)		Peptidase M75/Imelysin	1.411	1.11e-16	1.663
LIMLP_14465 (LIC10657/LA3540)	<i>sph</i>	Sphingomyelinase C	2.044	4.62e-13	
LIMLP_18620 (LIC20152/LB192)		HmuY protein	2.120*	2.98e-38	3.153
LIMLP_18625 (LIC20153/LB194)		Lipoprotein	2.151*	3.04e-84	2.705
Hypothetical					
LIMLP_05115 (LIC11059/LA3016)		Hypothetical	6.384*	2.21e-108	
LIMLP_05120 (LEPIC1091/LA3015)		Hypothetical	6.191*	2.35e-134	
LIMLP_05555 (LIC11145/LA2909)		Hypothetical	3.904*	2.19e-26	
LIMLP_05560 (LIC11145/LA2908)		Hypothetical	4.092*	1.84e-21	
LIMLP_08415 (LIC11695/LA2241)		Hypothetical	2.244*	1.27e-61	
LIMLP_09650 (LIC11935/LA1968)		Hypothetical	5.145*	1.91e-180	
LIMLP_10275 (LIC12077/LA1725)		Hypothetical	5.409*	2.33e-71	
LIMLP_11405 (LIC12298/LA1455)		Hypothetical	3.02*	9.47e-169	
LIMLP_12785 (LIC12555/LA1125)		Hypothetical	2.612*	3.49e-16	
LIMLP_13145 (LIC12628/LA1033)		Hypothetical	3.874*	8.60e-21	
LIMLP_13765 (LIC10790/LA3377)		Hypothetical	3.890*	8.95e-38	
LIMLP_17835 (LIC20007/LB009)		Hypothetical	2.924*	1.43e-97	

Regulators/signaling

LIMLP_00700 (LIC10132/LA0146)	<i>fhlA</i>	Transcriptional regulator Sigma activator 54	1.689	1.47e-12	
LIMLP_02515 (LIC12984/LA0592)	<i>csoR</i>	Copper sensing transcriptional repressor	2.622*	1.96e-15	
LIMLP_04775 (LIC10996/LA3104)	<i>rtn</i>	Cyclic diguanylate phosphodiesterase/histidine kinase	1.898	4.51e-37	
LIMLP_05055 (LIC11048/LA3033)		MolR transcriptional regulator	1.119	3.55e-06	
LIMLP_05565 (LIC11146/LA2907)		DeoR transcriptional regulator	2.389	7.31e-11	
LIMLP_05620 (LIC11158/LA2887)		Fur transcriptional regulator	2.207*	4.96e-16	
LIMLP_10155 (LIC12034/LA1857)	<i>perR</i>	Fur transcriptional regulator	3.566*#	1.17e-83	6.105
LIMLP_10945 (LIC12206/LA1576)		MarR EPS-associated transcriptional regulator	2.301*	4.16e-35	
LIMLP_11440 (LIC12305/LA1447)		LexA repressor	2.402*	1.64e-51	
LIMLP_12430 (LIC12490/LA1205)	<i>rpoE</i>	ECF sigma factor	1.112	1.58e-09	
LIMLP_12515 (LIC12504/LA1186)		TCS response regulator CheY	1.582	2.84e-18	
LIMLP_12520 (LIC12505/LA1185)		TCS response regulator	1.282	4.47e-12	
LIMLP_14415 (LIC10666/LA3531)		ArsR transcriptional regulator	1.65	2.04e-11	
LIMLP_15105 (LIC10525/LA3703)	<i>hrcA</i>	Heat-inducible repressor HrcA	3.591*#	4.85e-24	6.768
LIMLP_16265 (LIC10300/LA0348)		Antagonist anti sigma factor	1.134	4.85e-05	
LIMLP_16805 (LIC13285/LA4122)	<i>rpoE</i>	ECF sigma factor	1.285	4.85e-05	

Oxidative stress and redox-related

LIMLP_02795 (Lic12927/LA0666)	<i>ccp</i>	Cytochrome C peroxidase	5.824*#	3.42e-218	41.68
LIMLP_05955 (Lic11219/LA2809)	<i>ahpC</i>	Peroxiredoxin/alkylperoxiredoxin reductase	4.007*#	1.41e-213	8.57
LIMLP_05960 (Lic11220/LA2808)	<i>sufB</i>	ABC transporter permease	2.234*#	4.81e-453.55	
LIMLP_07145 (LIC11466/LA2499)		Thiol oxidoreductase	2.236#	1.50e-17	1.256
LIMLP_07165 (LIC11470/LA2494)	<i>trxB</i>	Thioredoxin-disulfide reductase TrxB	1.91#	9.98e-18	2.289
LIMLP_08985 (LIC11810/LA2108)		Glutathione S-transferase	1.23	3.66e-08	
LIMLP_10145 (LIC12032/LA1859)	<i>katE</i>	Catalase	2.763*#	2.80e-90	6.086
LIMLP_10150 (LIC12033/LA1858)		Ankyrin repeat-containing protein	2.701*#	1.06e-89	2.424
LIMLP_11965 (LIC12404/LA1321)	<i>dsbD</i>	Disulfide interchange protein	1.474#	4.18e-18	
LIMLP_13670 (LIC10807-LEPIC0823/LA3356)	<i>yfcG/gst</i>	Glutathione S-transferase	1.764	1.43e-28	2.305
LIMLP_14175 (LIC10712/LA3470)		Thiol oxidoreductase	1.802*	1.02e-40	2.132
LIMLP_14715 (LIC10606/LA3598)	<i>dps</i>	Ferritin/DNA-binding stress protein Dps	1.095	8.91e-10	1.497
LIMLP_17840 (LIC20008/LB010)	<i>hemA</i>	Glutamyl-tRNA reductase	1.197	8.07e-06	
LIMLP_18310 (LIC20093/LB117)	<i>ygaF/bcp</i>	Bacterioferritin comigratory protein/peroxiredoxin	1.177	7.73e-11	1.353
LIMLP_18595 (LIC20148/ LB186)	<i>pbsa</i>	Heme oxygenase	1.179	5.57e-05	1.360
LIMLP_18600 (LIC20149/ LB187)		Permease of the Major Facilitator Superfamily	1.070	2.84e-04	

Chaperones

LIMLP_06540 (LIC11335/LA2655)	<i>groEL</i>	Molecular chaperone GroEL	3.355*#	6.72e-35	6.936
LIMLP_06545 (LIC11336/LA2654)	<i>groES</i>	Molecular chaperone GroES	3.328*#	3.21e-33	4.862
LIMLP_10060 (LIC12017/LA1879)	<i>clpB</i>	Disaggregating chaperone ClpB	2.111*#	1.23e-15	2.487

LIMLP_10970 (LIC12210/LA1564)	<i>ibpA</i>	Small heat shock protein Hsp20	6.788 ^{##}	1.33e-184	69.605
LIMLP_10975 (LIC12211/LA1563)	<i>hsp15</i>	Small heat shock protein Hsp15	6.589 ^{##}	1.45e-238	56.431
LIMLP_15110 (LIC10525/LA3704)	<i>grpE</i>	GrpE	3.610 ^{##}	4.47e-27	8.929
LIMLP_15115 (LIC10524/LA3705)	<i>dnaK</i>	Molecular chaperone DnaK	3.353 ^{##}	3.46e-31	6.667
LIMLP_15120 (LIC10523/LA3706)	<i>dnaJ</i>	Molecular chaperone DnaJ	2.611 ^{##}	4.81e-45	2.619
DNA repair/SOS response					
LIMLP_02170 (LIC13052/LA0503)	<i>dinP</i>	DNA polymerase IV/DNA damage inducible protein	2.325*	2.03e-36	
LIMLP_07780 (LIC11596/LA2351)		DNA mismatch repair protein MutS	1.019 [#]	1.19e-07	1.134
LIMLP_07915 (LIC11620/LA2321)	<i>recN</i>	DNA repair protein RecN	5.028 ^{##}	0.00	17.490
LIMLP_08665 (LIC11745/LA2179)	<i>recA</i>	Recombinase RecA	2.652*	1.59e-58	
LIMLP_10880 (LIC12191/LA1589)		Mutator protein MutT/nudix hydrolase	1.255	4.42e-04	
LIMLP_11400 (LIC12297/LA1456)		DNA repair protein RadC	3.459*	1.13e-167	
LIMLP_16520 (LIC10252/LA0294)		DNA repair exonuclease	3.830 ^{##}	6.93e-63	6.113
LIMLP_16525 (LIC10251/LA0293)		DNA repair Rad50 ATPase	2.960 ^{##}	1.32e-86	2.899
Transporter					
LIMLP_04310 (LIC10902/LA3233)	<i>fecR</i>	Iron dicitrate transport regulator FecR	1.594	3.70e-20	
LIMLP_07920 (LIC11621/LA2320)		Biopolymer transporter ExbB/TolQ	2.080*	1.47e-85	
LIMLP_07925 (LIC11622/LA2319)		Biopolymer transporter ExbD/Tol	1.167	9.61e-21	
LIMLP_08410 (LIC11694/LA2242)		TonB-dependent receptor	2.616*	2.59e-20	
LIMLP_11395 (LIC12296/LA1457)		ABC transporter permease	1.617*	1.29e-31	
LIMLP_14160 (LIC10714/LA3468)	<i>fecA</i>	TonB-dependent receptor	2.178 ^{##}	2.17e-83	2.743
LIMLP_15535 (LIC10441/LA3806)	<i>amtB</i>	Ammonium transporter	4.100*	3.88e-50	
Prophage-related					
LIMLP_00895 (LEPIC0178/LA0196)		Hypothetical	3.661*	4.81e-45	
LIMLP_04475 (LIC10401)		Hypothetical/bacteriophage related fragment	1.278	2.91e-04	
LIMLP_04480 (no ortholog)		Hypothetical	1.882	4.37e-09	
LIMLP_13010 (LIC12600/LA1067)		Hypothetical	1.501	1.70e-07	
LIMLP_13015 (LIC12601/LA1066)		Hypothetical	1.232	5.76e-05	
LIMLP_13020 (LIC12602/LA1065)		Hypothetical	1.32	5.07e-06	
LIMLP_19610 (LA1831-33)		Phage replication protein	1.176	2.26e-06	

Table 2: Selected up-regulated genes upon exposure to lethal doses of H₂O₂.

Genes up-regulated upon exposure to 1 mM of H₂O₂ for 1 hour.

^a Gene numeration is according to Satou et al. (Satou et al., 2015).

^b Fold change (WT vs WT exposed 30 min to 10 μ M H₂O₂) obtained by RT-qPCR experiment

* Genes significantly up-and down-regulated by Volcano analysis (Log₂FC cutoff of 2 and p-value cutoff of 0.005) (Figure 2).

ORF Id ^a	Gene	Fonction	log2FC	p-value ^b
<i>Hypothetical</i>				
LIMLP_00510 (LIC10095/LA0107)		Hypothetical	-2.312*	3.42e-14
LIMLP_04180 (LIC12661/LA1000)		Hypothetical	-1.408	8.66e-13
LIMLP_04220 (no ortholog)		Hypothetical	-1.414	3.49e-14
LIMLP_04610 (LIC10963/LA3150)		Hypothetical	-1.37	6.59e-07
LIMLP_05020 (LEPIC1072/LA3048)		Hypothetical	-2.452	3.70e-03
LIMLP_05250 (LIC11086/LA2976)		Hypothetical	-1.676*	2.18e-10
LIMLP_05255 (LIC11087/LA2975)		Hypothetical	-2.446*	3.95e-29
LIMLP_05265 (LIC11089/LA2973)		Hypothetical	-2.100*	3.03e-46
LIMLP_07105 (LIC11458/LA2510)		Hypothetical	-1.328	4.56e-08
LIMLP_07970 (LIC11631/LA2308)		Hypothetical	-1.327	1.79e-24
LIMLP_11180 (LIC12253/LA1508)		Hypothetical	-1.574	1.16e-35
LIMLP_11230 (LIC11262/LA1496)		Hypothetical	-1.612	4.88e-18
LIMLP_11675 (LIC12343/LA1396)		Hypothetical	-1.359	9.78e-06
LIMLP_11685 (LIC12345/LA1393)		Hypothetical	-1.971	4.11e-12
LIMLP_11780 (LIC12365/LA1366)		Hypothetical	-1.719	2.12e-11
LIMLP_12590 (LIC12518/LA1168)		Hypothetical	-1.345	2.60e-09
LIMLP_12910 (LIC12578/LA1097)		Hypothetical	-1.788	4.42e-07
LIMLP_13720 (LIC10797/LA3368)		Hypothetical	-1.437	2.31e-08
LIMLP_13725 (LIC10796/LA3369)		Hypothetical	-1.389	3.29e-09
LIMLP_14190 (LIC10709/LEPIN3051)		Hypothetical	-2.128	5.58e-10
LIMLP_14195 (LIC10708/LA3473)		Hypothetical	-1.702	9.80e-08
LIMLP_14450 (LIC10660/LA3537)		Hypothetical	-1.701	2.14e-09
LIMLP_15090 (LIC10529/LA3697)		Hypothetical	-1.407	1.94e-12
LIMLP_15315 (LIC10980/LA3752)		Hypothetical	-1.444	5.39e-08
LIMLP_15335 (LIC10476/LA3756)		Hypothetical	-1.545	2.84e-04
LIMLP_15620 (no ortholog)		Hypothetical	-1.736	1.98e-04
LIMLP_15715 (LIC10140/LA0470)		Hypothetical	-1.834	2.64e-12
LIMLP_16170 (LEPIC0338/LA0371)		Hypothetical	-1.613	1.08e-11
LIMLP_17425 (LIC12518/LA1168)		Hypothetical	-1.402	7.59e-06
LIMLP_17465 (LA4271)		Hypothetical	-1.814	3.66e-04
LIMLP_17470 (LIC13426/LA4280)		Hypothetical	-1.693	6.02e-04
LIMLP_18675 (LIC20162/LB205)		Hypothetical	-1.372	1.28e-07
LIMLP_18680 (LIC20163/LB207)		Hypothetical	-1.910	2.69e-12
LIMLP_19115 (LIC20244/LB320)		Hypothetical	-1.324	2.41e-05

Protein synthesis/secretion

LIMLP_00815 (LIC10156/LA0177)	<i>yidD</i>	Membrane protein insertion effector	-1.49	2.52e-11
LIMLP_00820 (LIC10157/LA0178)	<i>yidC</i>	Insertase	-1.357*	1.98e-09
LIMLP_03095 (LIC12870/LA0742)	<i>rplB</i>	50S ribosomal protein L2	-1.026	3.40e-03
LIMLP_03100 (LIC12869/LA0743)	<i>rpsS</i>	30S ribosomal protein L19	-1.073	1.71e-03
LIMLP_03105 (LIC12868/LA0744)	<i>rplV</i>	50S ribosomal protein L22	-1.263	9.62e-04
LIMLP_03110 (LIC12867/LA0745)	<i>rpsC</i>	30S ribosomal protein S3	-1.176	1.45e-03
LIMLP_03115 (LIC12866/LA0746)	<i>rplP</i>	50S ribosomal protein L16	-1.197	1.34e-03
LIMLP_03120 (LIC12865/LA0747)	<i>rpmC</i>	50S ribosomal protein L29	-1.313	6.53e-04
LIMLP_03125 (LIC12864/LA0748)	<i>rpsQ</i>	30S ribosomal protein S17	-1.388	6.41e-04
LIMLP_03130 (LIC12863/LA0749)	<i>rplN</i>	50S ribosomal protein L14	-1.403	3.60e-04
LIMLP_03135 (LIC12862/LA0750)	<i>rplX</i>	50S ribosomal protein L24	-1.323	4.32e-04
LIMLP_03140 (LIC12861/LA0751)	<i>rplE</i>	50S ribosomal protein L5	-1.351	3.66e-04
LIMLP_03150 (LIC12859/LA0753)	<i>rpsH</i>	30S ribosomal protein S8	-1.466	2.62e-04
LIMLP_03155 (LIC12858/LA0754)	<i>rplF</i>	50S ribosomal protein L6	-1.496	1.95e-04
LIMLP_03160 (LIC12857/LA0755)	<i>rplR</i>	50S ribosomal protein L18	-1.531	1.95e-04
LIMLP_03165 (LIC12856/LA0756)	<i>rpsE</i>	50S ribosomal protein L5	-1.556	1.11e-04
LIMLP_03170 (LIC12855/LA0757)	<i>rpmD</i>	50S ribosomal protein L30	-1.420	1.54e-04
LIMLP_03175 (LIC12854/LA0758)	<i>rplO</i>	50S ribosomal protein L15	-1.322	1.54e-04
LIMLP_03180 (LIC12853/LA0759)	<i>secY</i>	Translocon SecY subunit	-1.292	9.39e-05
LIMLP_03185 (LIC12852/LA0760)	<i>cdk</i>	Adenylate cyclase	-1.004	1.18e-03
LIMLP_03190 (LIC12851/LA0761)	<i>infA</i>	translation initiation factor IF1	-1.121	5.99e-04
LIMLP_03195 (LIC12850)		50S ribosomal L35	-1.194	1.26e-03
LIMLP_03200 (LIC12849/LA0762)	<i>rpsM</i>	30S ribosomal L13	-1.362	1.29e-04
LIMLP_03205 (LIC12848/LA0763)	<i>rpsK</i>	30S ribosomal S11	-1.198	4.05e-04
LIMLP_03210 (LIC12847/LA0764)	<i>rpsD</i>	30S ribosomal S4	-1.203	1.63e-04
LIMLP_03215 (LIC12846/LA0765)	<i>rpoA</i>	RNA polymerase subunit alpha	-1.742	1.35e-06
LIMLP_03220 (LIC12845/LA0766)	<i>yidC</i>	50S ribosomal L17	-1.693	2.64e-06
LIMLP_07600 (LIC11557/LA2389)	<i>rimM</i>	Ribosome maturation/16S RNA processing	-1.260	4.98e-06
LIMLP_12685 (LIC12537/LA1143)	<i>secF</i>	preprotein translocase SecF	-1.389	5.26e-07

CRISPR

LIMLP_02870 (LIC12914/LA0686)		CRISPR-associated protein Csh2	-1.107	4.62e-13
LIMLP_02875 (LIC12913/LA0687)		CRISPR-associated protein Cas8	-1.285	3.45e-11
LIMLP_02880 (LIC12912/LA0688)		CRISPR-associated protein Cas5	-1.575	5.20e-06
LIMLP_02885 (LIC12911-10/LA0689-90)		CRISPR-associated protein Cas3	-1.212	8.70e-06

Regulators/Cell signaling

LIMLP_00130 (LIC10024/LA0024)		Adenylate/guanylate cyclase	-1.795	9.30e-14
LIMLP_02930 (LIC12901/LA0701-03)		Molybdate metabolism transcriptional regulator MolR	-1.296	2.59e-27

LIMLP_04670 (LIC10975/LA3133)		Transcriptional regulator AraC family	-1.052	1.27e-08
LIMLP_04735 (LIC10989/LA3113)		Ser/Thr kinase	-1.096	8.92e-07
LIMLP_05450 (LIC11125/LA2933)		Diguanylate cyclase	-2.157*	1.40e-12
LIMLP_05455 (LIC11126/LA2932)		Diguanylate cyclase	-1.419	1.31e-07
LIMLP_16420 (LIC10275/LA0316)		Transcriptional regulator XRE family	-1.371	2.72e-03
LIMLP_17475 (LIC13427/LA4281)		Response regulator CheY	-1.649	9.26e-04
LIMLP_18250 (LIC20081/LB104)		Transcriptional regulator TetR family	-1.470	7.21e-06
LIMLP_19320 (LA1770)		Transcriptional regulator AraC family	-1.366	8.71e-08

Cell respiration

LIMLP_03705 (LIC12752/LA0884)	<i>nuoN</i>	NADH quinone oxidoreductase subunit N	-1.390	2.08e-08
LIMLP_03710 (LIC12751/LA0885)	<i>nuoN</i>	NADH quinone oxidoreductase subunit M	-1.478	2.90e-08
LIMLP_03720 (LIC12749/LA0887)	<i>nuoL/nqo</i>	NADH quinone oxidoreductase subunit L12	-1.161	2.30e-07
LIMLP_03725 (LIC12748/LA0888)	<i>nuok</i>	NADH quinone oxidoreductase subunit K	-0.998	5.12e-06
LIMLP_07965 (LIC11630/LA2309)	<i>fadD</i>	Long chain fatty acid CoA ligase/AMP binding protein	-1.891	7.93e-66
LIMLP_10990 (LIC12214/LA1556)		Cytochrome C oxidase assembly factor SenC/SOC1	-1.468	1.55e-20

Metabolism

LIMLP_03290 (LIC12833/LA0789)		Glycosyl transferase/sugar kinase	-1.428	2.54e-07
LIMLP_05260 (LIC11088/LA2974)	<i>maug</i>	Methylamine utilization protein	-2.296*	4.11e-39
LIMLP_06060 (LIC11240/LA2780)		ATP F0F1 synthase subunit δ	-1.283	7.23e-06
LIMLP_06065 (LIC11241/LA2779)		ATP F0F1 synthase subunit α	-1.444	1.70e-06
LIMLP_06070 (LIC11242/LA2778)		ATP F0F1 synthase subunit γ	-1.586	4.02e-07
LIMLP_06075 (LIC11243/LA2776)		ATP F0F1 synthase subunit β	-1.67	2.78e-07
LIMLP_06080 (LIC11244/LA2775)		ATP F0F1 synthase subunit ϵ	-1.434	1.14e-07
LIMLP_06830 (LIC11400/LA2581)		N-acetyl neuraminic acid (sialic) synthase	-1.566	7.06e-09
LIMLP_06835 (LIC11401/LA2580)		Phospho glycerol transferase	-1.546	1.89e-06
LIMLP_07110 (LIC11459/LA2509)	<i>wcaJ</i>	Glycosyl transferase	-1.672	5.49e-09
LIMLP_07190 (LIC11477/LA2486)		GDSL-like lipase/acyl hydrolase	-1.266	7.48e-07
LIMLP_08930 (LIC11799/LA2119)	<i>glpk</i>	Glycerol kinase	-1.399	6.47e-07
LIMLP_09240 (LIC11860/LA2054)	<i>mmsB</i>	3 hydroxylisobutyrate dehydrogenase	-1.510	2.44e-10
LIMLP_10020 (LIC12009/LA1889)		1 aminocyclopropane-1-carboxylate deaminase	-1.420	5.68e-05
LIMLP_10235 (LIC12069/LA1735)		Lipase flippase Murj	-1.389	5.91e-06
LIMLP_12210 (LIC12449/LA1258)	<i>aroA</i>	3-phosphoshikimate-1 carboxylvinyltransferase	-2.234	1.57e-07
LIMLP_12215 (LIC12450/LA1257)	<i>tyrA</i>	Chorismate mutase/prefenate dehydrogenase	-1.473	3.03e-05
LIMLP_14455 (LIC10659/LA3538)		Riboflavin biosynthesis protein RibD	-1.763	2.41e-07
LIMLP_14610 (LIC10625/LA3573)	<i>kdsB</i>	3-deoxymanno-octulosonate cytidylyl transferase	-1.402	2.51e-11
LIMLP_15510 (LIC10446/LA3800)	<i>glmM/manB</i>	Phosphomannomutase/phosphoglucosamine mutase	-1.492	1.38e-11
LIMLP_16035 (LIC10346/LA0397)		Lipase/esterase GDSL-like protein	-1.822	2.27e-07
LIMLP_16925 (LIC13309/LA4149)		Thioesterase	-1.546	2.40e-06

LIMLP_17200 (LIC13366/LA4215)		Strictosidine synthase	-1.478	1.35e-18
LIMLP_17445 (LIC13421/LA4275)		Formate dehydrogenase subunit E	-1.443	9.81e-08
LIMLP_18245 (LIC20080/LB103)	<i>ybgC/YbaW</i>	Acyl-CoA thioester hydrolase	-1.419	5.39e-08
LIMLP_18455 (LIC20120/LB150)	<i>cobD</i>	Colabamin (VitB12) biosynthesis	-1.337	4.59e-07
LIMLP_18460 (LIC20121/LB151)	<i>cobDQ</i>	Colabamin (VitB12) biosynthesis	-2.442*	5.85e-21
LIMLP_18465 (LIC20122/LB152)	<i>cobU</i>	Adenosylcobinamide	-2.536*	5.65e-16
LIMLP_18470 (LIC20123/LB153)		Adenosylcobinamide amidohydrolase	-2.180*	1.15e-11
LIMLP_18475 (LIC20124/LB154)	<i>cobB</i>	Cobyrinic acid a,c-diamide synthase	-2.085*	5.60e-14
LIMLP_18480 (LIC20125/LB155)	<i>cobA/btuR</i>	Cobyrinic acid a,c-diamide adenosyl transferase	-2.074*	1.23e-11
LIMLP_18485 (LIC20126/LB156)	<i>cobM/cbiF</i>	Precorrin-4 C11 methyltransferase	-2.099	3.98e-11
LIMLP_18490 (LIC20127/LB157)	<i>cobJ/cbiH</i>	Precorrin-3B C17 methyltransferase	-2.441	1.58e-09
LIMLP_18495 (LIC20128/LB158)	<i>cbiG</i>	Colabamin (VitB12) biosynthesis	-2.074	2.48e-08
LIMLP_18500 (LIC20129/LB159)	<i>cobI/cobF</i>	Precorrin-2 C20 methyltransferase	-1.927	9.59e-09
LIMLP_18505 (LIC20130/LB160)	<i>cobL/cbiET</i>	Precorrin-6Y C5, 15 methyltransferase	-1.935	2.08e-08
LIMLP_18510 (LIC20131/LB161)	<i>cobH/cbiC</i>	Precorrin-8X methylmutase	-1.922	2.55e-07
LIMLP_18515 (LIC20132/LB162)	<i>cbiD</i>	Cobalt precorrin 6A synthase	-1.659	6.93e-08
LIMLP_18520 (LIC20133/LB163)		Oxidoreductase/FAD-binding flavodoxine reductase	-1.496	3.77e-09
LIMLP_19075 (LB310)	<i>pyrF</i>	Orotidine 5'-phosphate decarboxylase	-1.504	5.11e-05

Table 3: Selected down-regulated genes upon exposure to lethal doses of H₂O₂.

Genes up-regulated upon exposure to 1 mM of H₂O₂ for 1 hour.

^a Gene numeration is according to Satou et al. (Satou et al., 2015).

* Genes significantly up-and down-regulated by Volcano analysis (Log₂FC cutoff of 2 and p value cutoff of 0.005) (Figure 2).

ncRNA ^a	chromosome	Log ₂ Fc	p value adj	Start-End	Overlapping	Upstream	Downstream
<i>Up-regulated</i>							
rh57	NZ_CP011933.1	1.629	1.95e-69	23941-24050	LIMLP_19380	LIMLP_19385	LEPIMA_p0025
rh183	NZ_CP011931.1	1.557	7.61e-80	128941-129006	LIMLP_04875	LIMLP_04870	LIMLP_04880
rh288	NZ_CP011931.1	3.812	0	197282-197352	LIMLP_00895*	LIMLP_00890	LEPIMA_CI0185
rh349	NZ_CP011932.1	1.638	1.98e-120	256577-2566392	NA	LEPIMA_CH0243	LIMLP_18855
rh402	NZ_CP011932.1	1.517	1.31e-92	291497-291605	LIMLP_18995	LIMLP_18990	LIMLP_19000
rh449	NZ_CP011933.1	2.512	0	351592-351649	NA	LIMLP_01545*	LIMLP_01550*
rh608	NZ_CP011931.1	2.476	0	479394-479448	LIMLP_02045*	LIMLP_02040	LIMLP_02045*
rh637	NZ_CP011931.1	1.663	2.16e-137	501388-501477	NA	LIMLP_02105*	LIMLP_02110
rh859	NZ_CP011931.1	4.248	0	683752-684074	NA	LIMLP_02795*	LEPIMA_CI0612
rh1192	NZ_CP011931.1	2.197	5.78e-214	975150-975213	LIMLP_04030	LIMLP_04025	LIMLP_04035
rh1269	NZ_CP011931.1	2.164	2.97e-202	1038822-1038876	Hypo_04290	LIMLP_04285	LEPIMA_CI0938
rh1429	NZ_CP011931.1	1.720	3.15e-90	1181397-1181456	Hypo_04840	LIMLP_04830	LIMLP_04845
rh1641	NZ_CP011931.1	2.013	2.50e-145	1386755-1386830	LIMLP_05625	LIMLP_05620*	LIMLP_05630
rh1807	NZ_CP011931.1	2.928	0	1531048-1531289	NA	LIMLP_06235	LIMLP_06240
rh2088	NZ_CP011931.1	2.000	1.66e-149	1780300-1780403	LIMLP_07195	LIMLP_07195	LIMLP_07200
rh2227	NZ_CP011931.1	3.130	0	1892070-1892135	NA	LIMLP_07695	LIMLP_07700
rh2395	NZ_CP011931.1	1.877	2.09e-123	2013277-2013341	LIMLP_08295	LIMLP_08290	LIMLP_08300
rh2961	NZ_CP011931.1	1.974	8.15e-150	2474618-2474668	LIMLP_10350	LIMLP_10345	LIMLP_10355
rh3130	NZ_CP011931.1	7.189	0	2612368-2612495	LEPIMA_CI2416	LIMLP_10975*	LEPIMA_CI2417
rh3352	NZ_CP011931.1	7.653	0	2787780-2787953	LIMLP_11710*	LIMLP_11705*	LIMLP_11715*
rh3871	NZ_CP011931.1	2.133	2.34e-261	3253035-3253139	LIMLP_13675	LIMLP_13670*	LIMLP_13680
rh3894	NZ_CP011931.1	3.784	0	3271638-3271704	NA	LIMLP_13765*	LIMLP_13770
rh4281	NZ_CP011931.1	1.627	1.38e-71	3584015-3584072	LIMLP_15080**	LIMLP_15075**	LIMLP_15085
rh4345	NZ_CP011931.1	1.765	8.56e-126	3664279-3664343	LIMLP_15310	LIMLP_15305*	LIMLP_15315
rh4413	NZ_CP011931.1	3.507	0	3721204-3721564	NA	LIMLP_15540*	LIMLP_15545
rh4542	NZ_CP011931.1	2.748	0	3822746-3823025	NA	LIMLP_16010	LIMLP_16015*
rh4545	NZ_CP011931.1	1.979	1.54e-233	3825144-3825319	LIMLP_16025*	LIMLP_16015	LEPIMA_CI3523
rh5034	NZ_CP011931.1	1.628	2.24e-72	4229144-4229208	NA	LIMLP_17780	LIMLP_17785
<i>Down-regulated</i>							
rh411	NZ_CP011931.1	-1.854	1.53e-65	310470-310529	NA	LIMLP_01410**	LIMLP_01415**
rh685	NZ_CP011931.1	-1.613	1.73e-40	541558-541624	NA	LEPIMA_CI0489	LIMLP_02275
rh967	NZ_CP011931.1	-2.662	8.54e-202	786700-786893	NA	LIMLP_03220**	LIMLP_03225
rh1101	NZ_CP011931.1	-2.684	4.82e-295	888430-888480	NA	LIMLP_03700	LIMLP_03705
rh1102	NZ_CP011931.1	-2.149	2.11e-80	888546-888608	NA	LIMLP_03700	LIMLP_03705
rh1253	NZ_CP011931.1	-1.608	1.10e-30	1025093-1025156	LEPIMA_CI0924	LEPIMA_CI0923	LEPIMA_CI0925
rh1880	NZ_CP011931.1	-1.896	6.86e-62	1592557-1592621	LEPIMA_CI1441	LIMLP_06480*	LEPIMA_CI1442
rh2578	NZ_CP011931.1	-1.730	2.54e-44	2165614-2165832	LIMLP_08925	LIMLP_08920	LIMLP_08930**

rh3186	NZ_CP011931.1	-1.963	5.74e-64	2658407-2658646	NA	LIMLP_11175**	LIMLP_11180**
rh3190	NZ_CP011931.1	-1.874	6.42e-47	2656130-2656312	NA	LIMLP_11170**	LIMLP_11175**
rh3711	NZ_CP011931.1	-2.030	5.35e-87	3116206-3116269	NA	LIMLP_13120	LEPIMA_CI2881
rh4178	NZ_CP011931.1	-1.509	2.89e-18	3496010-3496183	LEPIMA_CI3239	LIMLP_14745	LIMLP_14750
rh4549	NZ_CP011931.1	-1.821	7.63e-51	3827129-3827377	LEPIMA_CI3525	LIMLP_16030	LIMLP_16035**

Table 4: Regulated ncRNAs upon exposure to lethal dose of H₂O₂.

Significantly up-and down-regulated ncRNAs upon exposure to lethal dose of H₂O₂ (1h exposure to 1 mM H₂O₂) with a Log₂FC cutoff of 1.5.

^a Gene numeration is according to Satou et al. (Satou et al., 2015).

* ORFs significantly up-regulated by RNASeq analysis (Log₂FC cutoff of 1).

** ORFs significantly down-regulated by RNASeq analysis (Log₂FC cutoff of 1).

NA, non-applicable

ORF Id ^a	Gene	Fonction	Log ₂ FC	p value adj	RT-qPCR ^b
Down-regulated genes					
LIMLP_04090 (LIC12679/LA0980)	<i>thic</i>	Thiamine biosynthesis protein	-2.073	3.72e-02	
LIMLP_04240 (LIC10889/LA3247)	<i>tonb</i>	Energy transducer TonB	-4.601	2.03e-13	0.00722
LIMLP_04245 (LIC10890/LA3246)	<i>exbD</i>	Biopolymer transport protein ExbD/TolR	-4.606	7.90e-13	0.00737
LIMLP_04250 (LIC10891/LA3245)	<i>exbD</i>	Biopolymer transport protein ExbD/TolR	-5.355	4.93e-15	0.00128
LIMLP_04255 (LIC10892/LA3244)	<i>exbB</i>	Biopolymer transport protein ExbB/TolQ	-5.478	3.00e-22	0.00193
LIMLP_04260 (LIC10893/LA3243)		Hypothetical	-1.519	4.27e-02	1.261
LIMLP_04270 (LIC10895-96/LA3242)	<i>fecA</i>	TonB-dependent receptor	-3.262	2.32e-05	0.0355
LIMLP_04275 (LIC10897/LA3241)		Hypothetical	-3.888	4.42e-05	0.00918
LIMLP_04280 (LIC10898/LA3240)	<i>lipI48</i>	Hypothetical	-5.506	2.03e-13	0.00372
LIMLP_08590 (LEPIC1767/LA2195)		Hypothetical	-0.859	5.08e-08	
LIMLP_09650 (LIC11935/LA1968)		Hypothetical	-1.787	3.26e-02	
LIMLP_14190 (LIC10709/LEPIN3051)		Hypothetical lipoprotein	-0.679	1.88e-05	
LIMLP_14195 (LIC10708/LA3473)		Hypothetical	-0.813	1.32e-12	
LIMLP_14200 (LIC10707/LA3474)		Hypothetical GDSL-like lipase	-0.853	1.23e-05	
LIMLP_14205 (LIC10706/LA3475)		Hypothetical lipoprotein	-0.847	4.75e-02	
LIMLP_14210 (LIC10705/LA3477)		Hypothetical lipoprotein	-0.915	3.05e-03	
LIMLP_14220 (LIC10703/LA3479)		Hypothetical	-0.793	1.13e-02	
LIMLP_14225 (LIC10702/LA3480)		Hypothetical	-0.775	2.14e-03	
LIMLP_15470 (LIC10454/LA3793)		Putative hemolysin	-2.154	3.32e-12	0.3041
LIMLP_16720 (LIC13269/LA4102)	<i>vicR</i>	Response regulator	-1.611	5.80e-07	0.0752
LIMLP_16725 (LIC13270/LA4104)	<i>vicK</i>	Signal transduction histidine kinase	-0.919	4.02e-03	0.4975
LIMLP_18235 (LIC20078/LB099)		Hypothetical	-0.658	7.87e-03	
Up-regulated genes					
LIMLP_02010 (LIC13086/LA3867)		Hypothetical lipoprotein	1.029	4.08e-02	
LIMLP_02795 (LIC12927/LA0666)	<i>ccp</i>	Cytochrome C peroxidase	2.773	8.69e-18	7.943
LIMLP_05955 (LIC11219/LA2809)	<i>ahpC</i>	Peroxiredoxin/alkylperoxiredoxin reductase	1.539	1.23e-05	2.01
LIMLP_10145 (LIC12032/LA1859)	<i>katE</i>	Catalase	2.637	2.59e-24	4.897
LIMLP_10150 (LIC12033/LA1858)		Ankyrin repeat-containing protein	2.867	4.65e-29	5.783

Table 5 : Regulated genes in the *perR* mutant compare with WT cells

Significantly up-and down-regulated genes *perR* inactivation with a Log₂FC above 1 and below -0.5, and a p-value cutoff of 0.005.

^a Gene numeration is according to Satou et al. (Satou et al., 2015).

^b Fold change (WT vs *perR* mutant) obtained by RT-qPCR experiments.

Figure legends

Figure 1. Volcano representation of transcriptome upon exposure to sublethal doses of hydrogen peroxide. Up- and down-regulated genes upon a 30 min exposure to 10 μ M H₂O₂ were graphically represented by a Volcano analysis. Red and blue dots indicate up- and down-regulated genes, respectively, with significant change in expression (with a Log₂FC cut off of 1, p-value<0.05). Representative genes are labeled.

Figure 2. Genes with change in expression upon exposure to lethal doses of hydrogen peroxide. *L. interrogans* were exposed to 1 mM H₂O₂ for 1h and genes with significantly changed expression (Log₂FC cut off of 1, p-value<0.005) were classified according to the COG functional categories (upper panel). The functional categories are as followed with C, energy production and conversion; D, cell cycle control and division and chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination and repair; M, cell wall structure and biogenesis and outer membrane; N, secretion, motility and chemotaxis; O, molecular chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; T, signal transduction; U, secretion; V, defense mechanisms. Genes with significant changes in expression were also graphically represented by a Volcano analysis (lower panel). Red and blue dots indicate up- and down-regulated genes, respectively, with significant change in expression (with a Log₂FC cut off of 2, p-value<0.05). Representative genes are labeled.

Figure 3. Effect of the of *katE* or *ahpC* inactivation on *Leptospira* growth in the presence

of ROS. *L. interrogans* WT, *katE* (Man69) and *ahpC* (Man1368) mutant strains were cultivated in EMJH medium (upper panel) or in the presence of 2 mM H₂O₂ (medium panel) or of 2μM paraquat (lower panel). Growth was assessed by measure of absorbance at 420 nm.

Figure 4. Role of catalase and AhpC in the stationary phase-adapted *Leptospira* tolerance of hydrogen peroxide. (A) *L. interrogans* WT (black line) and *perR* (M776) mutant (red line) strains were cultivated in EMJH medium and samples were taken at the exponential phase (left upper panel, blue arrow 1), at the entry of stationary phase (left upper panel, blue arrow 2), and at advanced stationary phase (left upper panel, blue arrow 3) and used to inoculate a new EMJH medium in the absence (plain line) or presence of 2 mM H₂O₂ (dashed line). The growth curve with samples taken in the exponential phase (samples 1), in the entry of stationary phase (samples 2) and at advanced stationary phase (samples 3) are represented in the right upper, the left lower, and the right lower panels, respectively. (B) *L. interrogans* WT, *katE* (Man69) and *ahpC* (Man1368) mutant strains were cultivated in EMJH medium until the exponential or stationary phases and incubated for 30 min in the absence or presence to 10 mM H₂O₂. Cell viability was assessed by the ability of the cells to reduce the blue rezasurin into a pink resorufin using the Alamar Blue assay as described in the Material and Methods section.

Figure 5. Effect of the of *vicK* or *vicR* inactivation on *Leptospira* growth in the presence of ROS. *L. interrogans* WT, *vicK* (Man1448) and *vicR* (Man899) mutant strains were cultivated in EMJH medium in the absence (left panel) or presence of 2μM paraquat (right panel). Growth was assessed by measure of absorbance at 420 nm.

Figure 6. Effect of the of *fecA*, *exbD*, or *lipI48* inactivation on *Leptospira* growth in the presence of ROS. *L. interrogans* WT, *fecA* (Man1022) and *exbD* (Man782), *lipI48* (Man1089)

mutant strains were cultivated in EMJH medium in the absence (upper panel) or presence of 2μM paraquat (lower panel). Growth was assessed by measure of absorbance at 420 nm.

Figure 7. Role of PerR-controlled ORF in *Leptospira vitulence*. 10⁶ WT, *vicK* (Man1448) and *vicR* (Man899) mutant strains (**A**), or *fecA* (Man1022), *exbD* (Man782), *lipL48* (Man1089) mutant strains (**B**) were injected intraperitoneally in hamster as described in Material and Methods section.

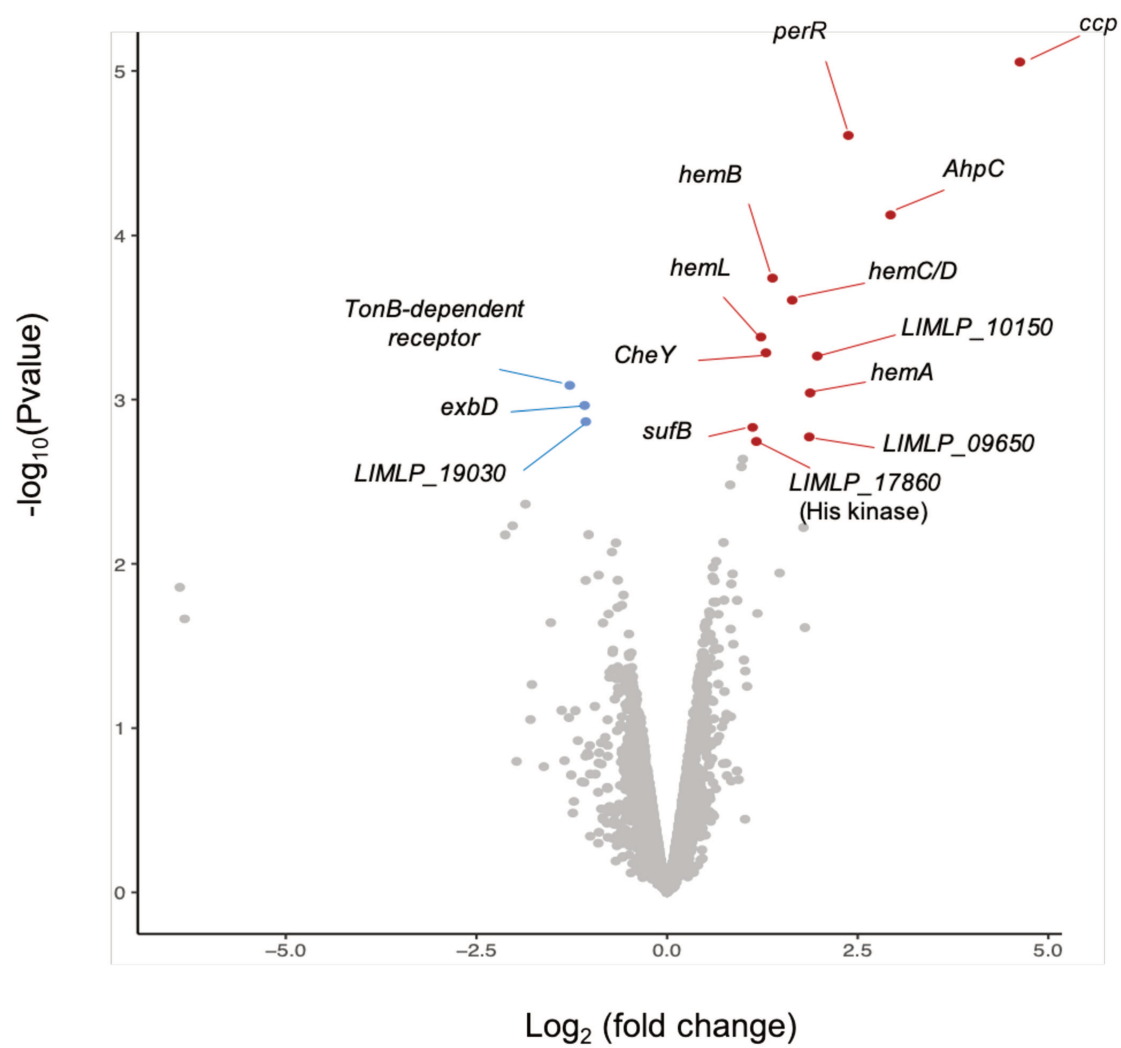


Figure 1

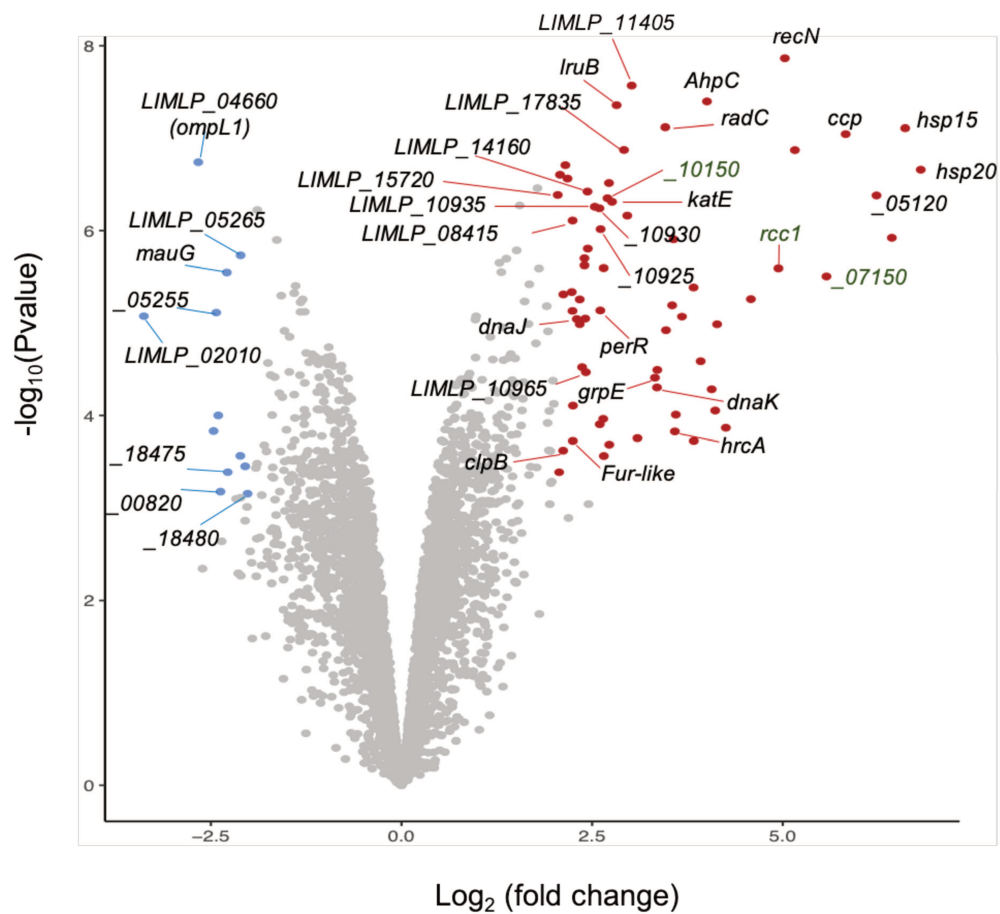
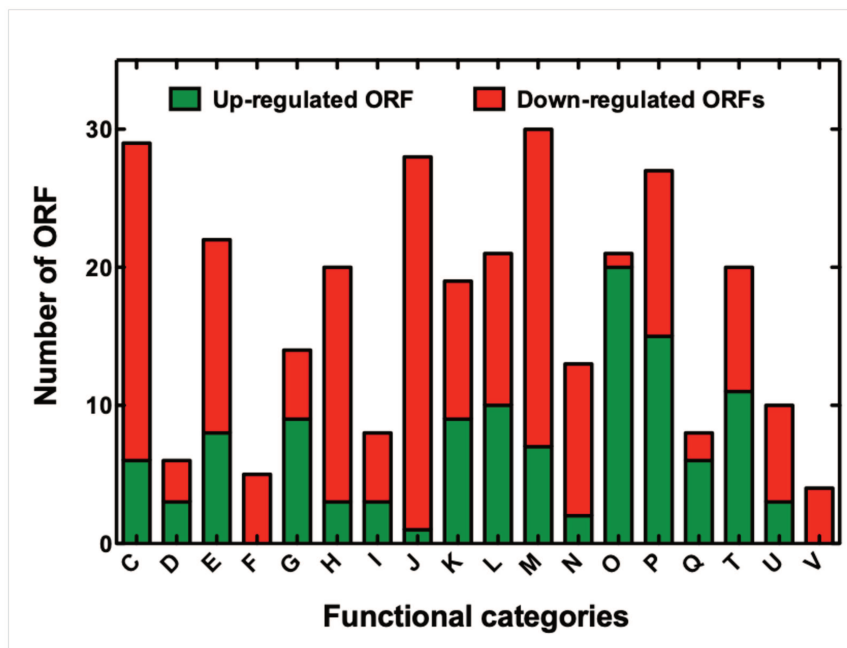


Figure 2

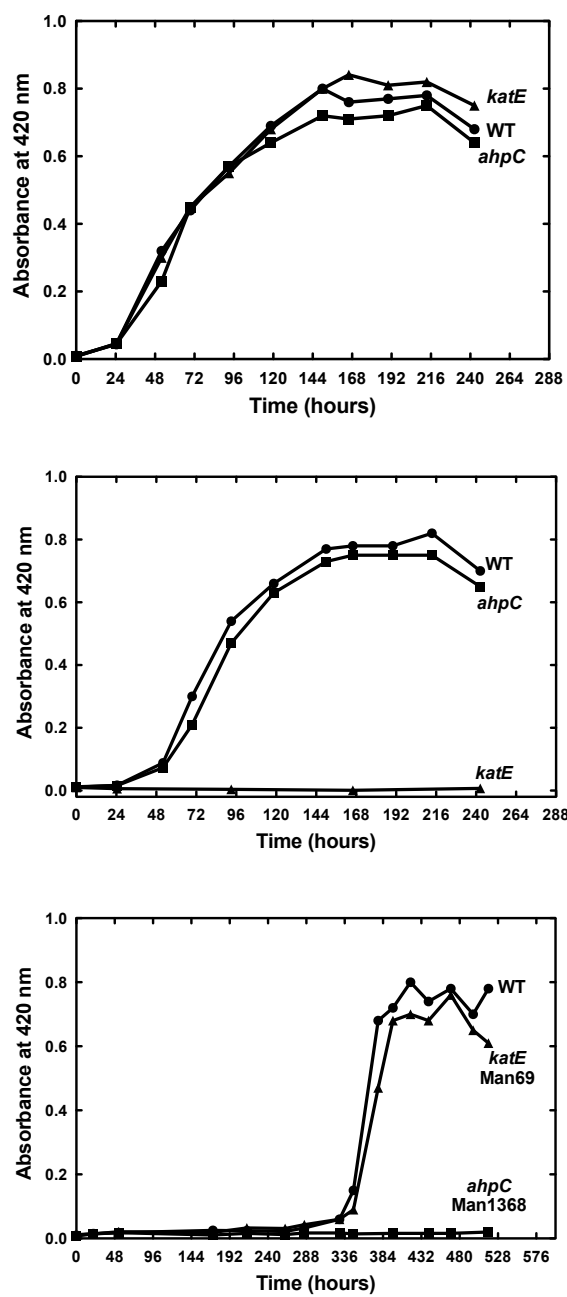
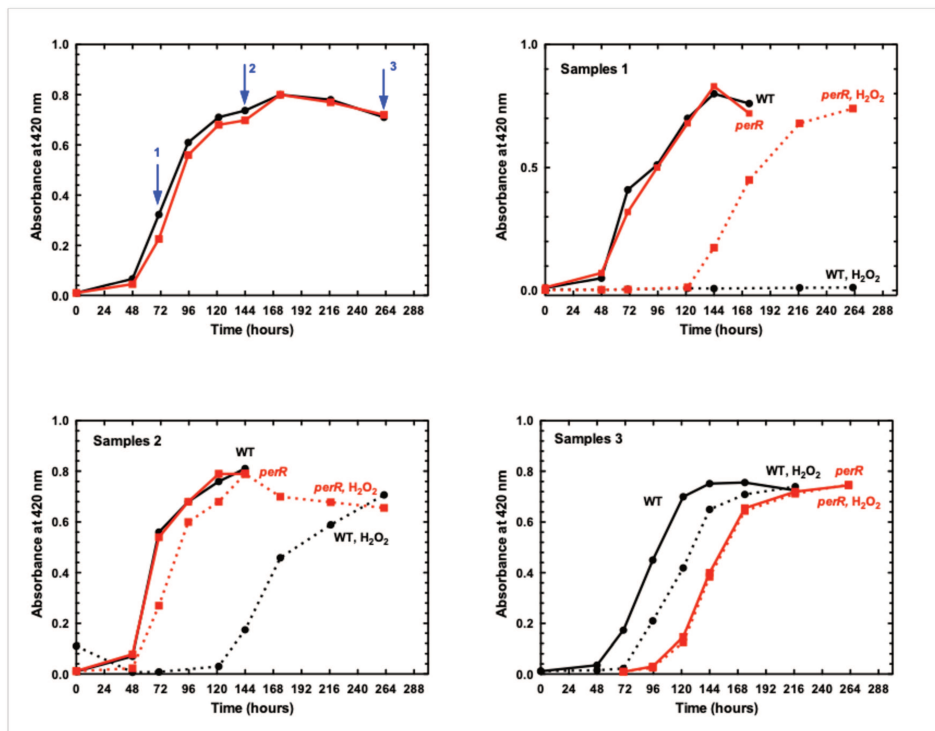


Figure 3

A



B

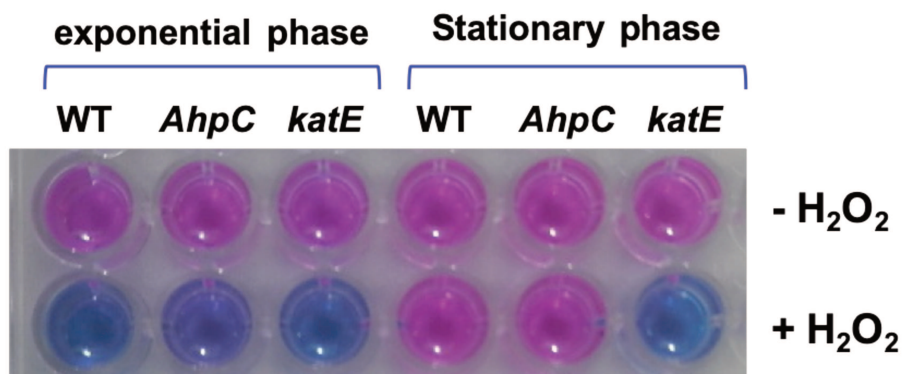


Figure 4

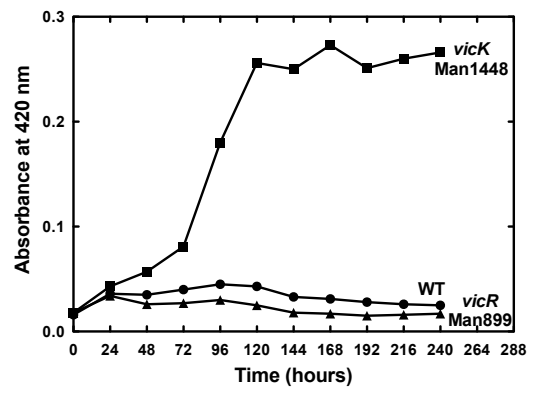
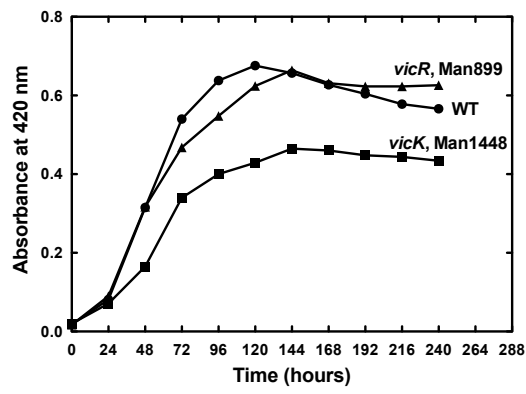


Figure 5

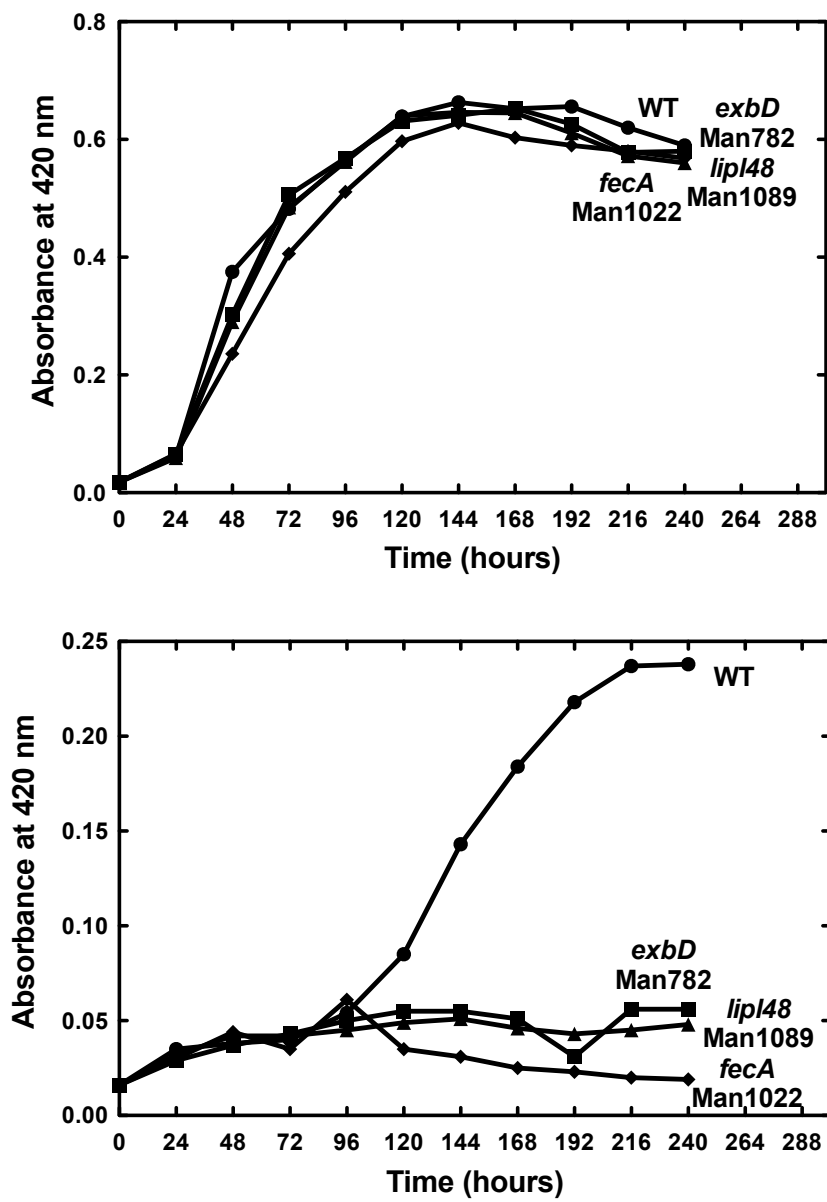
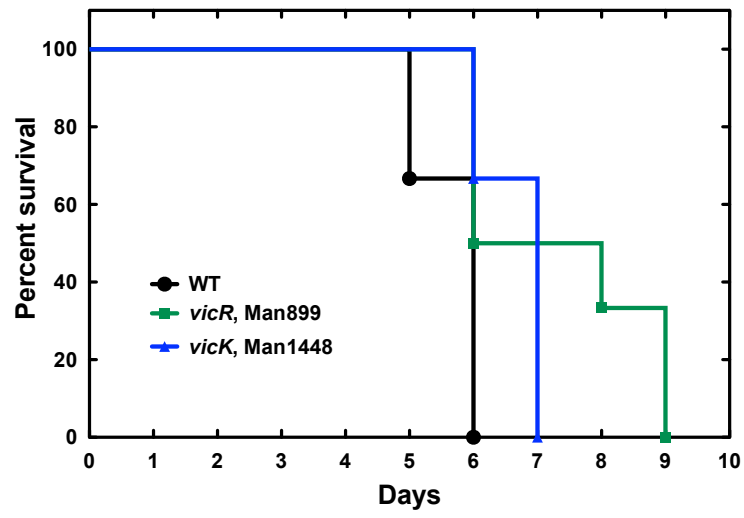


Figure 6

A



B

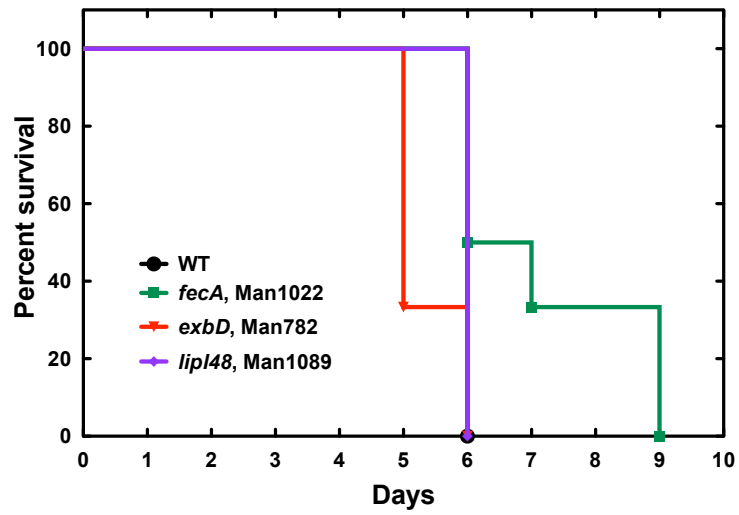


Figure 7

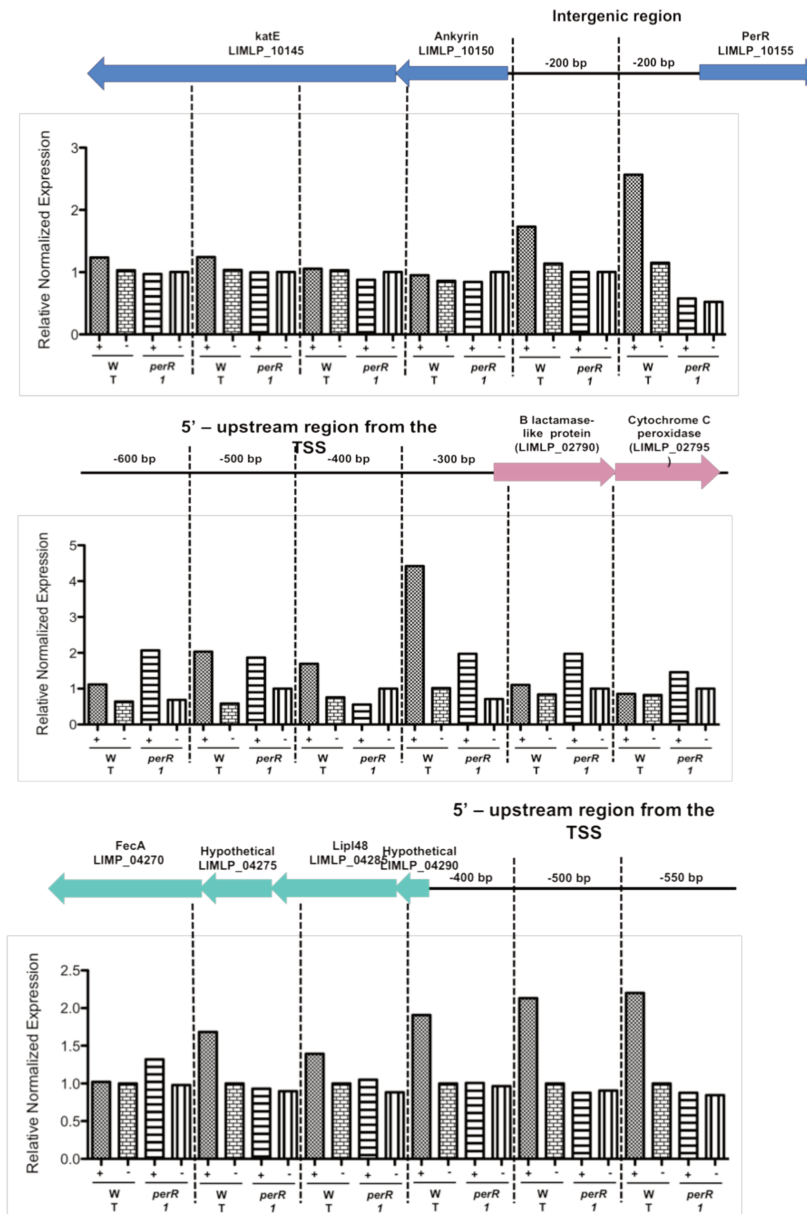


Figure S1. In vivo binding of PerR with *L. interrogans* genomic regions. Chromatine immunoprecipitation was performed by incubating exponentially growing leptospira cells 40 min. with 1% formaldehyde at 30°C. The reaction was stopped by the addition of 400 mM glycine. Cells were then washed with TBS buffer and resuspended in buffer A (50 mM HEPES-KOH pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing protease inhibitor cocktail. Cells were sonicated 7 cycles of 15 min. and centrifuged. The supernatant was incubated 3 hours at 4°C with 50 µl of washed Dynabead Pan rabbit IgG for 3 hrs at 4°C. The samples were incubated in the absence or in the presence of anti-PerR serum (at a dilution of 1:750) for 2 hours at 4°C. The samples were successively washed with buffer A containing 500 mM NaCl, with buffer B (10 mM Tris-HCl pH8, 1 mM EDTA, 0.1% Nonidet-P40, 0.5% sodium deoxycholate) and with buffer C (10 mM Tris-HCl pH7.5, 1 mM EDTA). The elution was performed with 100 µl of elution buffer (50 mM Tris-HCl pH7.5, 10 mM EDTA, 1% SDS, 150 mM NaCl, 0.5% Triton X-100) and an ON incubation at 37°C. An incubation with protease K (2 hours at 65°C) allowed elimination of proteins and DNA fragments were purified. The indicated DNA fragments were amplified by qPCR.

Experimental results
(Article 2. In preparation)

I Identification of an additional putative PerR in pathogenic *Leptospira*

In the previous story, we were able to determine the cellular factors that are involved in the oxidative stress response when *Leptospira* cells are exposed to sublethal doses of hydrogen peroxide, and we showed that some of them are under the control of PerR. Interestingly, at the moment *Leptospira interrogans* cells are exposed to lethal doses of H₂O₂, we can observe that PerR (LIC12034) only partially contributes to the regulation of these cellular factors. We were able to identify additional regulators and non-coding RNAs that were also regulated by H₂O₂. Among these regulators was a member of the FUR family transcriptional regulators that its expression was increased in the presence of lethal doses of H₂O₂.

The *Leptospira interrogans* genome encodes 4 ORFs that share homology with FUR family regulators. As seen in Figure 24, sequence alignment with the Fur transcriptional regulator from *E.coli* shows that these 4 ORFs share the DNA-binding and metal-binding domain of a FUR transcriptional regulator. However, in addition to the PerR1 ORF (LIC12034), one ORF (LIC11158) shares the key amino acids that distinguish a PerR regulator.

For a long time, it was believed that the member of the FUR family could not be distinguished on the sole basis of the primary amino acid sequence. The laboratory of Dr. Victor Duarte have shown that two amino acids could be used to distinguish a Fur from a PerR regulator.

Comparing the respective sequence of the *B. subtilis* Fur and PerR, they identified an asparagine (Asn) in the DNA binding helix, which is crucial for the recognition of DNA sequence (PerR box) (Caux-Thang et al. 2015). This Asn in a Fur transcriptional regulator is arginine amino acid.

An aspartate (Asp) residue located downstream of the regulatory metal binding in PerR is essential for its H₂O₂-sensing (Parent *et al.* 2013). Instead of an Asp in a Fur transcriptional regulator, there is a glutamate residue.

These Asn and Asp residues were both present not only in the PerR1 (LIC12034) ORF (position 60 and 103, respectively) but also in the LIC11158 ORF (position 68 and 112, respectively).

We, therefore, proposed that LIC11158 is a second PerR in *L. interrogans*, and we named this ORF PerR2.

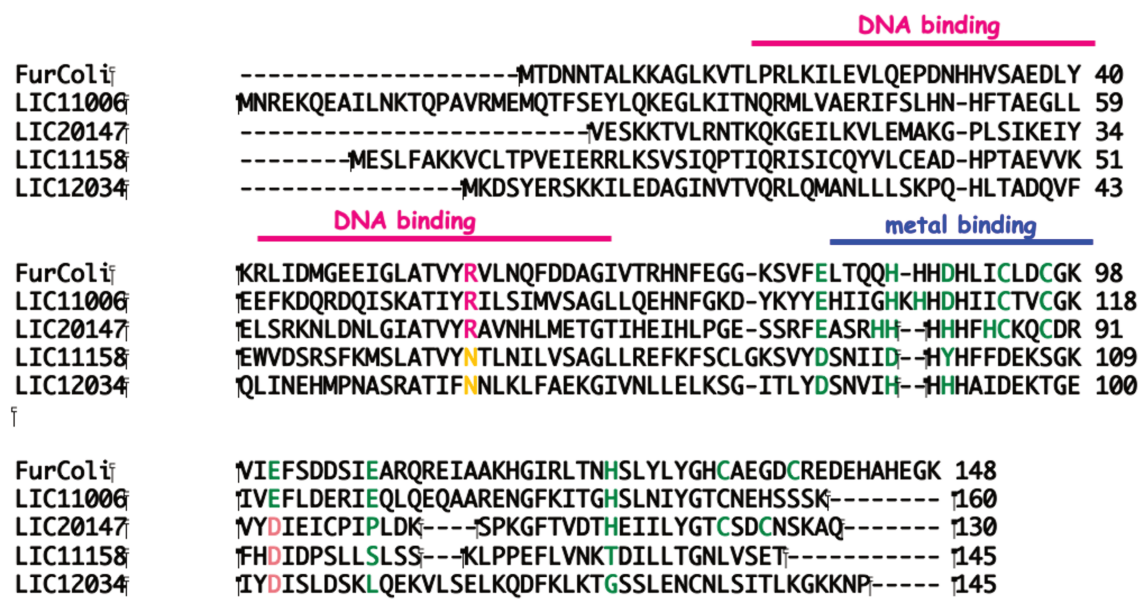


Figure 24. *Leptospira interrogans* has four ORFs that share homology with FUR family. The sequence alignment of four ORFs from *L. interrogans* with the Fur regulator from *E. coli* shows that they share the core DNA-binding (pink bar) and metal-binding motif (blue bar) of a typical FUR family. The key amino acids of a PerR, asparagine (N) and aspartate (D), are shown in orange and light pink, respectively. Amino acids involved in metal coordination are shown in green.

In order to determine the distribution of both PerR regulators among all species of *Leptospira* genus, phylogenetic analysis was performed, in collaboration with Dr. Frederic Veyrier (Institut Armand Frappier, Laval, Quebec). The sequence of *perR1* (LIMLP_10155) and *perR2* (LIMLP_05620) ORFs from the strain *Leptospira interrogans* serovar Manilae were searched and compared in all genome from the *Leptospira* genus.

Interestingly, as seen in Figure 25, the putative second PerR2 regulator is only present in pathogenic species (highly virulent P1 and intermediate P2 species). PerR1 is absent in intermediate species but present in all highly pathogenic P1 and saprophyte species. However, there are two exceptions in the intermediate P2 species since PerR1 is present, in *L. dzoumognesis* and *L. wolffii* Khorat-H2 (Figure 25). This analysis indicates that both PerR regulators only coexist in highly pathogenic species and that PerR2 is specific to pathogenic species.

These analyses are fascinating because this could imply that this second PerR2, which is only present in pathogenic species, is necessary for *Leptospira* adaptation to *in vivo* host-related conditions, whereas PerR1 could be necessary for more several adaptations to H₂O₂ in the outside in the environment and during infection.

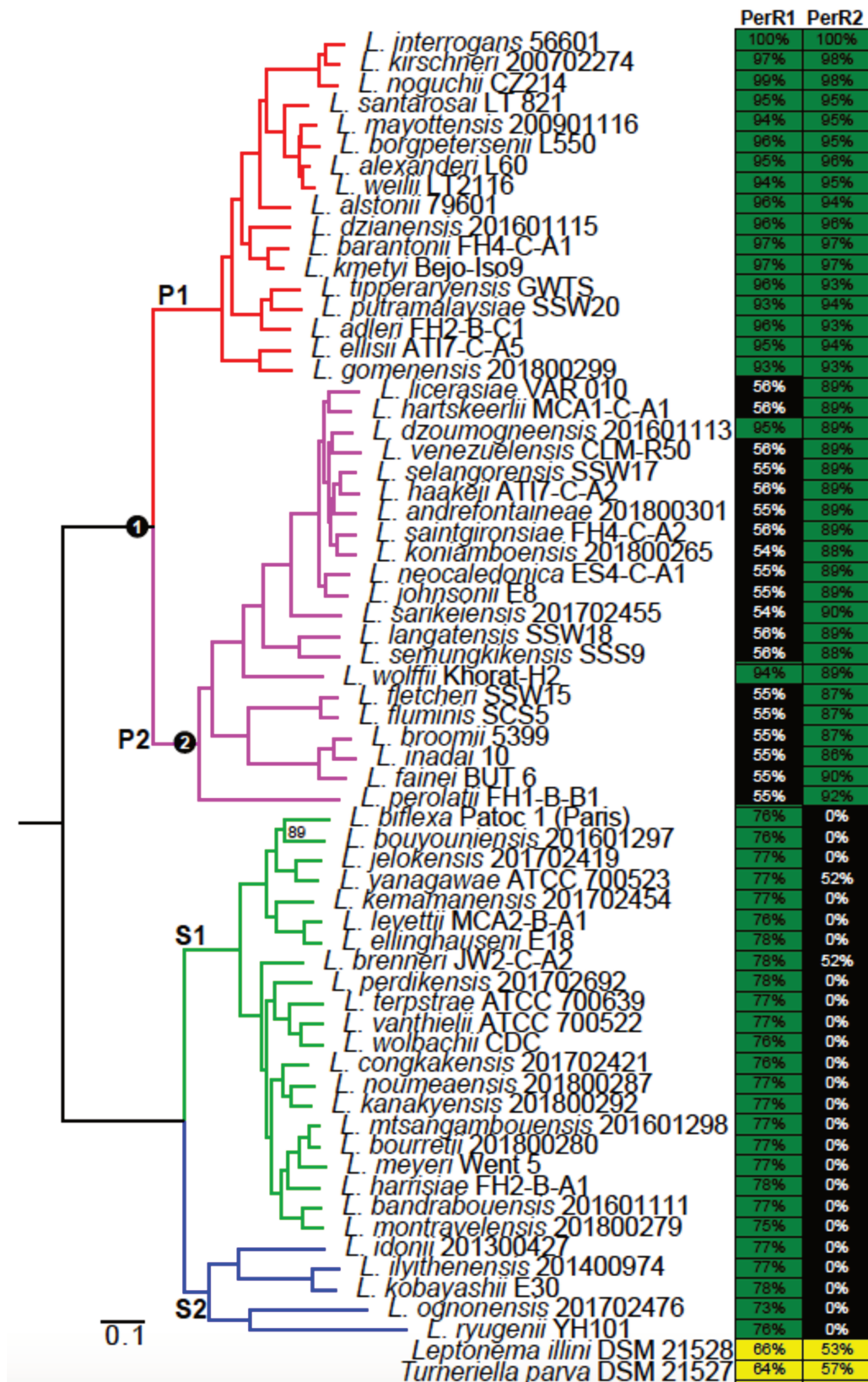


Figure 25. PerR2 is only present in pathogenic and intermediate species of *Leptospira*. Phylogenetic tree of the *Leptospira* genus based on the sequence of *perR1* and *perR2* genes. Blue and green branches correspond to saprophytic species (S1 and S2 clades, respectively), pink branches correspond to intermediate species (pathogenic clade P2) and red branches to pathogenic species (Clade P1). Percentage of similitude of PerR1 and PerR2 from UPLP genome with its homolog sequences its shown in the right. *Leptonema illini* and *Turneriella parva* strains were used as external groups.

Based on the previous results and the results that were published, we believe that the ORF that is up-regulated in lethal doses of H₂O₂ is a PerR2 (see Experimental Results. Article 1.). In order to further understand the proper function of this new PerR regulator we analyzed the expression by RT-qPCR of *perR1* and *perR2* with different doses of H₂O₂

As seen in Figure 26, we were able to observe the expression of *perR1* with sublethal and lethal doses of H₂O₂. On the contrary, *perR2* expression was only detected in the presence of lethal doses of H₂O₂. This result confirms our hypothesis that this new putative PerR2 only is active during in host-related conditions

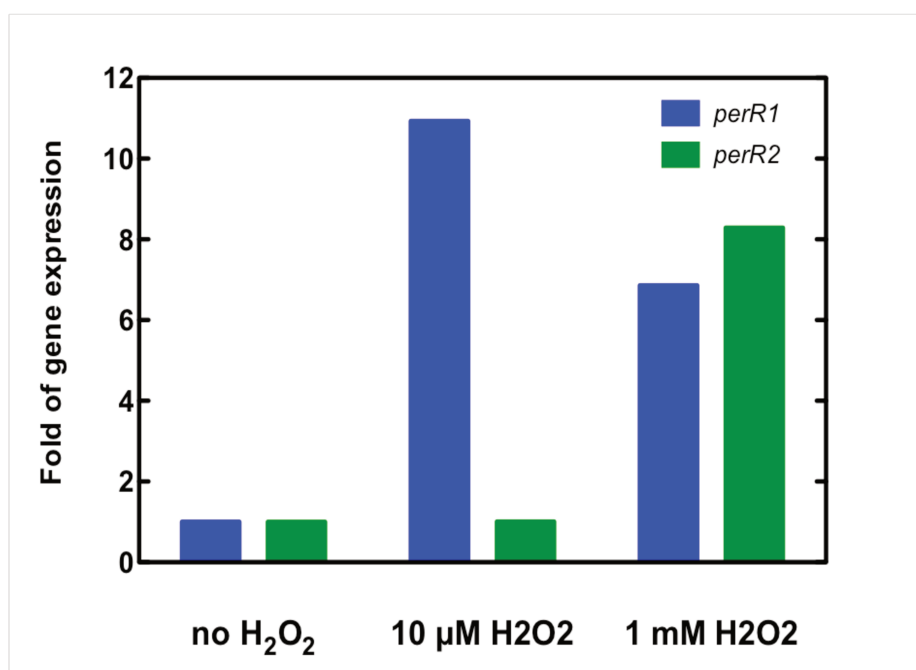


Figure 26. *perR2* is expressed in the presence of lethal doses of H₂O₂. Expression of *perR1* (blue) and *perR2* (green) was determined by RT-qPCR. RNA was purified from log-phase cultures grown *in vitro* and exposed to 10 μM (30 min) or 1 mM (1 hr) of H₂O₂. RT-qPCR experiments were normalized to expression in the absence of H₂O₂ and *flaB* gene was used as a reference gene.

II PerR2 has a role in the oxidative stress response in pathogenic *Leptospira*

We have identified a putative second PerR regulator in *Leptospira* (PerR2) and which is only present in pathogenic and intermediate species, and whose expression is increased in the presence of lethal doses of H₂O₂ (Figure 25 and 26).

We, therefore, studied the role of this second putative PerR in the adaptation of pathogenic leptospires to oxidative stress.

A *perR2* mutant strain was available in our mutant transposon library, and we could investigate the growth of this *perR2* mutant in the presence of different oxidants. WT and *perR2* mutant cells were cultivated in EMJH medium with different *in vitro* conditions mimicking the oxidative stress encountered in the host. Also, the *perR1* mutant cells were included in this study to compare the survival of *perR1* and *perR2* mutant cells in these conditions.

As observed in Figure 27, while the WT and *perR2* mutant cells were not able to grow in the presence of 1 mM of H₂O₂, the *perR1* mutant cells were able to divide, as reported before (Kebouchi et al. 2018a). Interestingly, in the presence of 2 microM of paraquat, a superoxide generating reagent, *perR1* mutant cells growth was impaired compared to that of the WT, whereas *perR2* mutant growth was slightly faster than that of the WT cells.

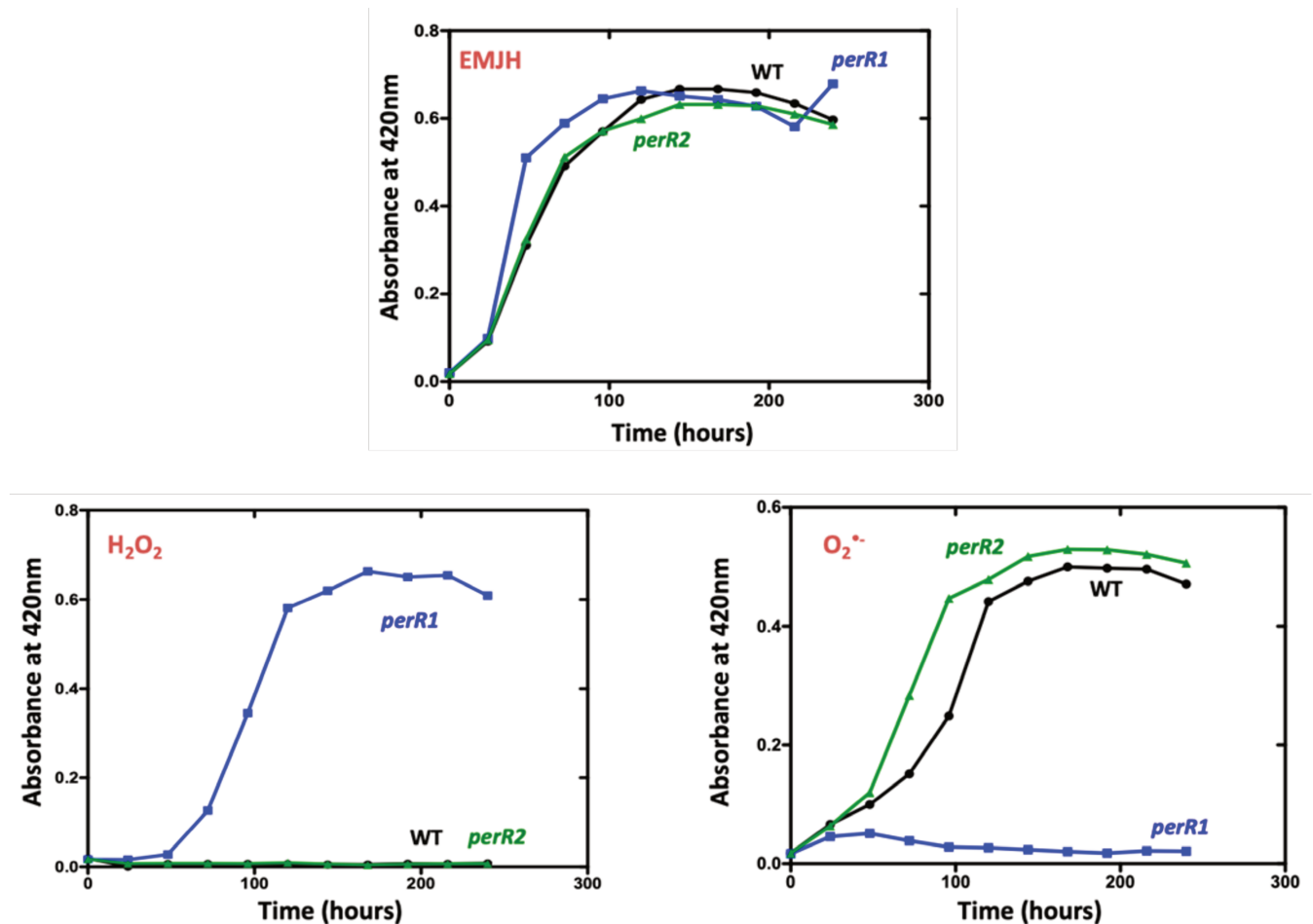


Figure 27. PerR regulators have a role in the oxidative stress response. Growth of WT (black line), single *perR1* (blue line) and *perR2* (green line) mutant strains of *L. interrogans* in EMJH in the presence of 2 mM of hydrogen peroxide (left panel) and 2 μ M of paraquat (a superoxide generating compound) (right panel). All the strains have comparable growth in standard *in vitro* conditions in EMJH (upper panel). *Leptospira* growth was evaluated by measuring absorbance at 420 nm.

Thus, based on the phenotype and transcriptomic results of the *perR1* mutant, as seen in Experimental Results. Article 1., PerR1 represses the expression of genes encoding defenses against peroxide, but it might activate the expression of genes coding for defenses against superoxide. Based on the phenotype of the *perR2* mutant cells, this regulator would repress the expression of genes coding for defenses against superoxide (Figure 28). These results indicate that the two regulators have a different role in the oxidative stress response.

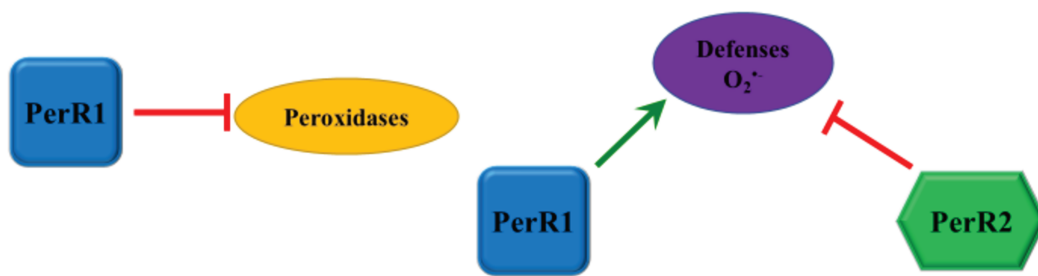


Figure 28. Proposed model of PerR1 and PerR2 regulation during oxidative stress response. Based on the phenotype, we proposed the regulatory model where the main function of PerR1 is repressing the expression of genes that code for peroxidases and inducing the expression of genes that are involved in the superoxide anion stress. On the contrary, PerR2 seems to be repressing the expression of genes involved in the superoxide anion stress.

We did not observe any effect of *perR2* inactivation when cells were cultivated in the presence of other host-related conditions such as the host osmolarity (120 mM NaCl) and with a nitrosative stress-producing agent (15 μ M of sodium nitroprusside dihydrate) (Figure 29).

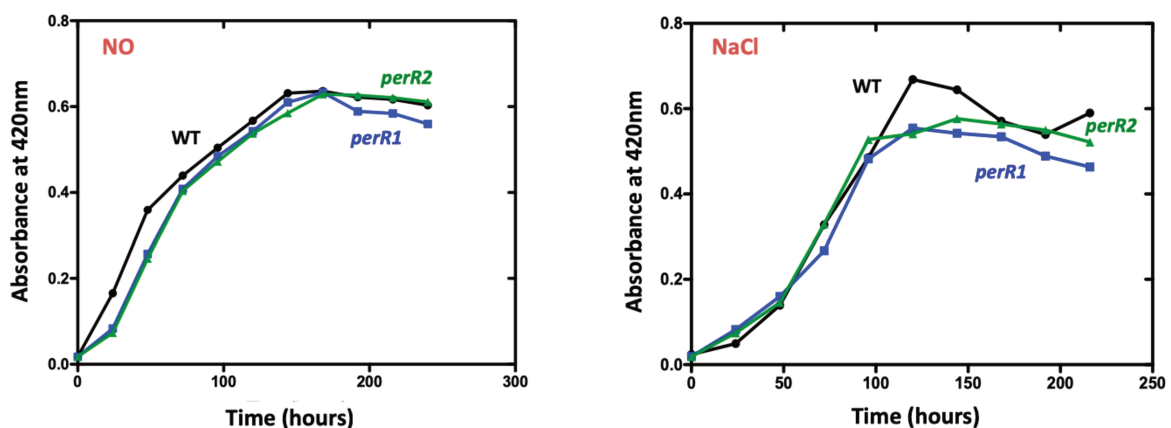


Figure 29. PerR regulators have no role in other host-related condition. Growth of WT (black line), *perR1* (blue line) and *perR2* (green line) mutant strains of *L. interrogans* in the presence of 15 μ M sodium nitroprusside dihydrate (NO stress) (left panel) and 120 mM of NaCl (osmotic stress) (right panel) was evaluated by measuring absorbance at 420 nm.

To further uncover the role of PerR2 in *L. interrogans*, we analyzed the global expression pattern of the *perR2* mutant strain by comparing its transcriptome with that of the WT strain under standard *in vitro* conditions at 30 °C in EMJH medium. Differential gene expression

analyses revealed changes in the transcription of 124 genes, with 59 and 65 up and down-regulated, respectively (Figure 30).

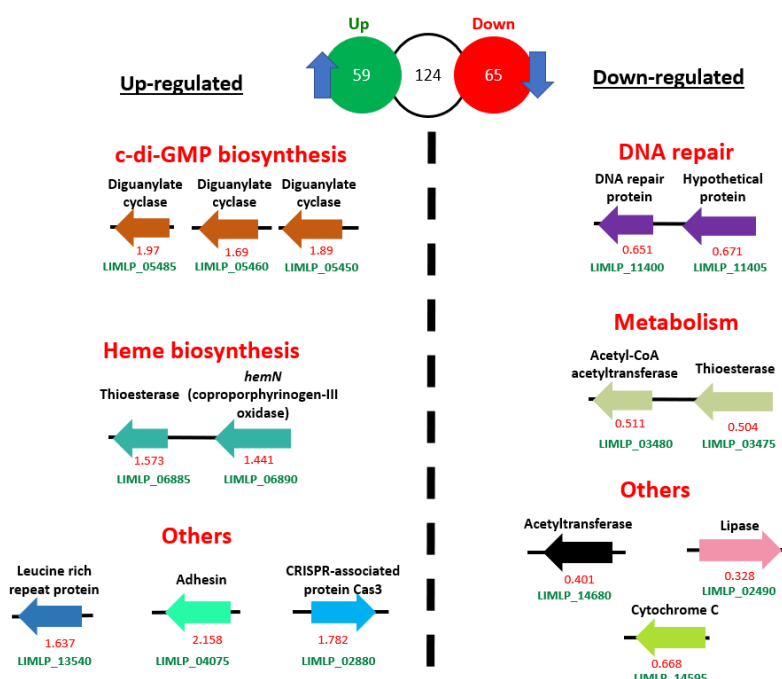


Figure 30. Putative PerR2 regulon. Representative genes that are up-regulated (left panel) and down-regulated (right panel) upon PerR2 inactivation. RNAs were extracted from mid-log phase culture of *L. interrogans* serovars Manilae cultivated in EMJH at 30°C. Numbers in red represent the fold change (FC) value of each ORF.

However, the change in expression observed in these conditions was not significant enough ($-1 < \log_2FC < 1$) to conclusively assign a function to PerR2.

We observed an increase in expression in genes that encode for diguanylate cyclase. These enzymes are involved in the synthesis of c-di-GMP, an essential signaling messenger for the control of many bacterial cellular functions such as virulence, motility, adhesion, biofilm formation, and stress adaptation (Whiteley *et al.*, 2015).

Genes encoding heme biosynthesis, and CRISPR Cas pathways were also up-regulated in the *perR2* mutant as well as genes encoding putative virulence-associated factors such as adhesin and leucine-rich repeat (LRR) proteins. Genes that encode DNA repair factors, metabolism, and lipase were down-regulated. Many genes with unknown annotations and specific for *Leptospira* genomes had their expression affected by PerR2 inactivation.

Importantly, the PerR2 regulon is distinct from that of PerR1. Indeed, the ORFs whose expression is changed upon *perR2* inactivation were not deregulated upon *perR1* inactivation. The transcriptomic results could indicate that PerR2 does not exert a function when *Leptospira* are cultivated in standard *in vitro* conditions. This leads to the hypothesis that PerR2 has a function when cells are confronted with infection-related conditions.

III The concomitant inactivation of *perR1* and *perR2* has a pleiotropic effect in *Leptospira*

PerR1 and PerR2 have no redundant function in the oxidative stress response. In order to determine if there is an interplay between both regulators, it was necessary to obtain a double *perR1perR2* mutant strain. As mention before, manipulating *Leptospira in vitro* remains very challenging, a double mutant had never been obtained in pathogenic *Leptospira*.

We succeeded in concomitantly inactivating PerR1 and PerR2 by allelic exchange technic and were able to obtain a double *perR1perR2* mutant strain. The double *perR1perR2* mutant had a growth rate comparable to that of the single *perR1* and *perR2* mutant and WT strains (Figure 31 upper panel). WT cells, together with single *perR1* and *perR2* mutants and double *perR1perR2* mutant, were also cultivated in EMJH medium in the presence of H₂O₂ or paraquat.

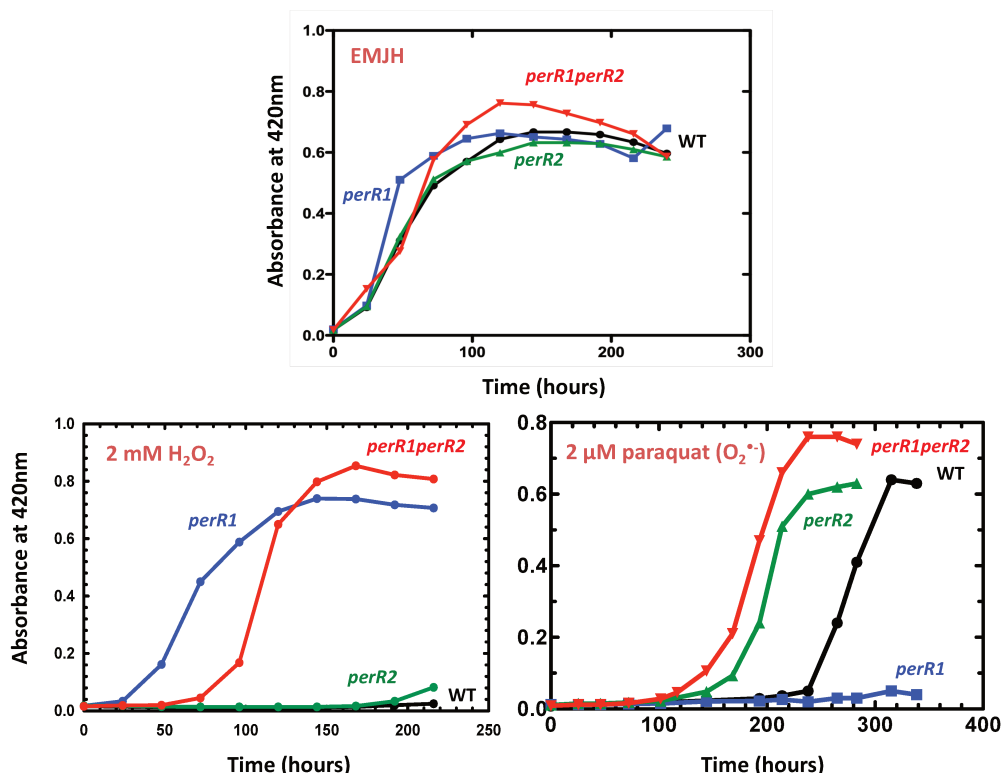


Figure 31. PerRs have a distinct but complementary role in the oxidative stress response. WT (black line), single *perR1* (blue line) and *perR2* (green line) mutant strains, and the double *perR1perR2* (red line) mutant strain of *L. interrogans* were cultivated in the presence of 2 mM of peroxide (left panel) and 2 μM of paraquat (right panel). *Leptospira* growth was evaluated by measuring absorbance at 420 nm.

As seen in Figure 31 and as observed before, the *perR1* mutant strain can grow in the presence of H₂O₂ but not in the presence of Paraquat. On the contrary, the *perR2* mutant strain is not able to grow in the presence of H₂O₂, but it does in the presence of paraquat. Interestingly, the concomitant inactivation of both regulators resulted in a *Leptospira* strain with higher resistance to both H₂O₂ and superoxide stress. This confirms our hypothesis that PerR1 and PerR2 have a distinct role in the oxidative stress response. The double *perR1perR2* mutant exhibits the phenotype of both *perR1* and *perR2* single mutants.

To further investigate whether there is an interplay between PerR1 and PerR2, we analyzed and compared the transcriptome of WT and double *perR1perR2* mutant cells by RNA-Seq in standard *in vitro* conditions. Compared to the single *perR1* and *perR2* mutants, the absence of both transcriptional regulators resulted in a change in expression of 2080 genes, with 1100 and 980 were up and down-regulated, respectively (Figure 32).

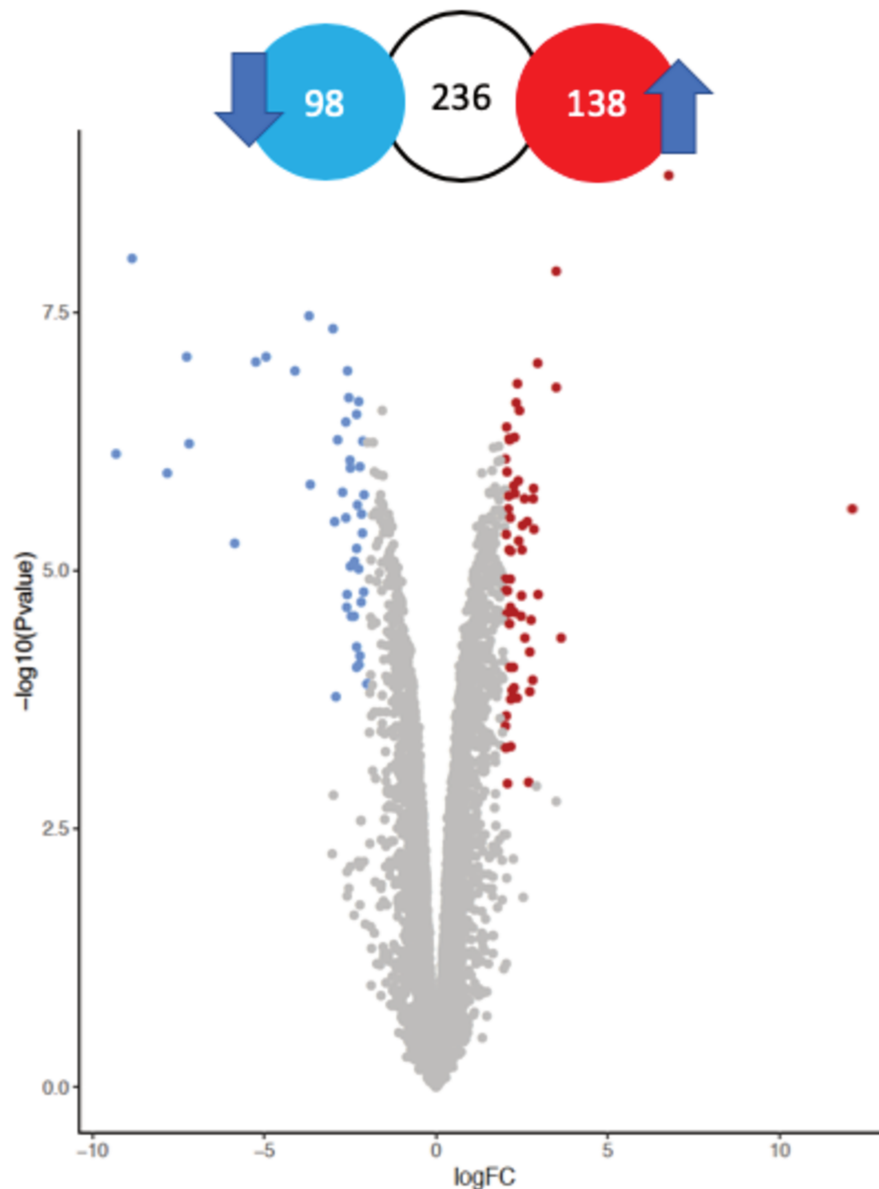


Figure 32. High number of genes deregulated in the absence of PerR1 and PerR2. Volcano plot of the transcriptomic results comparing the double *perR1perR2* mutant with WT strain. Colored circles represent the genes whose expression is significantly changed in the double mutant $\log_2FC > 2$ and a $p\text{-value} < 0.005$. Red and blue dots are genes that are up and down regulated, respectively.

138 and 98 genes were significantly ($p\text{-value} < 0.005$) up and down-regulated respectively ($\log_2FC > 2$) (Figure 32 red and blue dots, respectively). Analyzing the transcriptomic results of the double *perR1perR2* mutant cells, we could observe that most of the PerR1 regulon is also deregulated in the absence of both PerR regulators. The FC of some genes is not the same such as in the case for catalase where its expression is higher in the PerR1 regulon (FC of 2.2 and 6.22, in the double *perR1perR2* and single *perR1* mutant transcriptome, respectively). Interestingly, in the case of the peroxidase AhpC and the Cytochrome C peroxidase, their

expression is higher than that of the PerR1 regulon, meaning that their expression is more enhanced in the double *perR1perR2* mutant strain.

Strikingly, in the case for a cluster that encodes for a TonB transporter system that we showed is down-regulated in the absence of PerR1, the FC in the double *perR1perR2* mutant is not dramatically changed and the same case for the hemolysin. However, for the two-component VicKR, in the double mutant does not appear down-regulated, the histidine kinase VicK. On the other hand, the response regulator VicR, appear at the same level of down-regulation as in the PerR1 regulon (FC of 0.215 and 0.327, in the double *perR1perR2* and single *perR1* mutant transcriptome, respectively). Overall, the PerR1 regulon is still deregulated in the *perR1perR2* mutant transcriptome, although we observed a difference in some factors where we could hypothesize that there might be another factor independent of PerR1 for their regulation in expression.

We did not observe any correlation between the double *perR1perR2* mutant transcriptome and the PerR2 regulon obtained in standard *in vitro* conditions, despite that we did not observe significant deregulation in the PerR2 regulon. Nevertheless, in the double mutant transcriptome, *perR2* expression is down-regulated with an FC of 0.461. These results could mean some genes that appear in the double *perR1perR2* mutant transcriptome are regulated by PerR2.

Remarkably, we also observed genes that their expression is up or down-regulated only in the absence of both PerR regulators. Among these genes are some were up-regulated and encode for putative virulence factors such as the lipoproteins LruA and LruB that have been detected highly expressed in *Leptospira* during infection in humans (Verma et al. 2008). Simultaneously, several Leucine-rich repeat proteins that have been shown to participate in host-pathogen interactions (Eshghi et al. 2015), together with the outer membrane protein LipL32 (Figure 33).

Interestingly, several previously reported virulence factors in *Leptospira* were down-regulated in the double mutant. Among these genes are both surface-exposed proteins LigA and LigB. LigA and LigB were previously shown, by the TALES technique, to be required for *Leptospira* virulence (Pappas et al., 2015a). In the same way, the expression of the signaling system Lvr that has been reported previously as an essential factor for *Leptospira* virulence is down-regulated (Adhikarla et al. 2018). In addition, also the chaperon ClpB that has been reported to

be necessary for *Leptospira* survival under stress and virulence in *Leptospira* (Lourdault et al. 2011). The small heat shock proteins Hsp15 and Hsp20 were also down-regulated in the double *perR1perR2* mutant transcriptome (Figure 33).

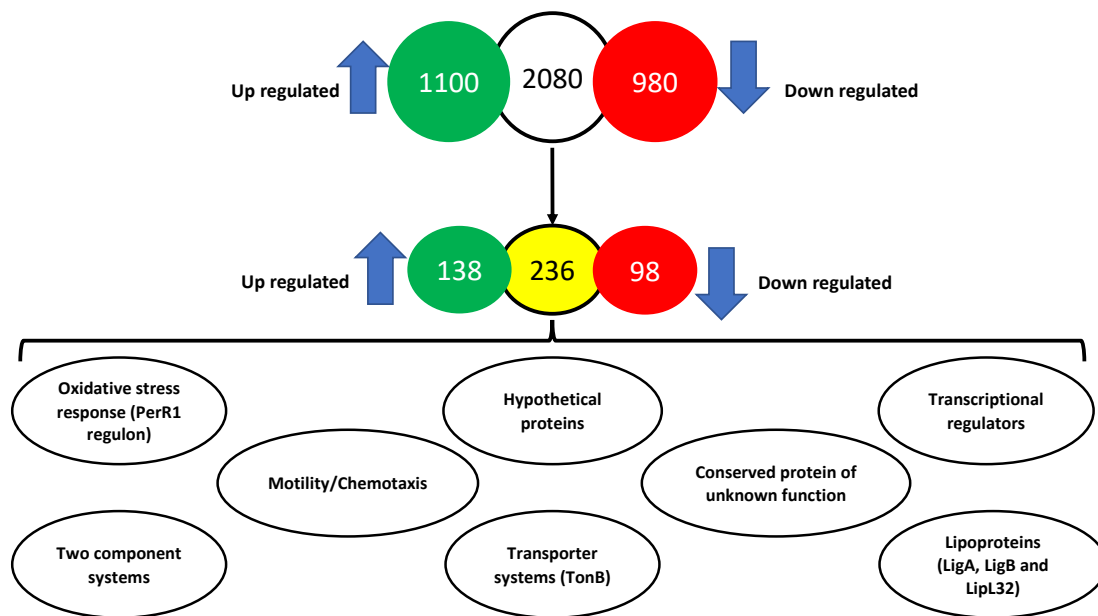


Figure 33. Representative ORFs whose expression is changed in the double mutant. RNA was extracted from mid-log phase culture of *L. interrogans* serovars Manilae incubated in EMJH at 30°C. Among 2080 genes, 236 are statistically significantly deregulated, with 138 and 98 up and down-regulated genes, respectively ($\log_2FC > 2$, $p\text{-value} < 0.005$).

Based on the phenotypes of the mutants in the presence of ROS, PerR1 and PerR2 do not have a redundant role in the oxidative stress response since they are required for *Leptospira* growth under different oxidative stress. However, the transcriptomic studies indicate that they might act together for *Leptospira* virulence, mainly because the inactivation of both regulators leads to genes changes of expression of several genes coding for virulence factors. Also, it seems that many genes that encode for hypothetical proteins are under the control of both PerR regulators. These suggest that PerR1 and PerR2 have an interplay in *L. interrogans* virulence (Figure 33 and 34).

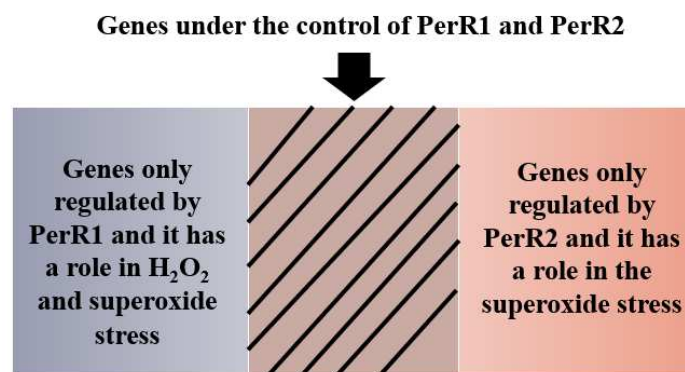


Figure 34. Scheme representing the interplay of both `PerR regulators. Right panel shows the PerR2 regulon that has a role in the superoxide stress according to the phenotype. Left panel shows the PerR1 regulon and, according to the transcriptome and phenotypic results, regulate the expression of genes involved in the H₂O₂ and superoxide stress. Overlapping both regulons we observed genes that are only under the control of both regulators, meaning that PerR1 and PerR2 compensate for each other in the single mutants.

IV Interplay between PerR1 and PerR2 in *Leptospira* virulence.

Since we observed that many relevant virulence factors were deregulated in the double *perR1perR2* mutant strain (Figure 32, 33 and 34), we tested whether the PerR regulators were essential for *L. interrogans* virulence. The virulence of the single *perR1*, and *perR2* mutants and double *perR1perR2* mutant strains were tested in the acute model for leptospirosis (hamster).

As seen in Figure 35, either of the single *perR1* or *perR2* mutant strains did not show attenuation in virulence. Intriguingly, the double *perR1perR2* mutant strain showed attenuation in virulence. These surprising findings indicate that several factors for infecting or surviving inside a host are down-regulated in the double *perR1perR2* mutant.

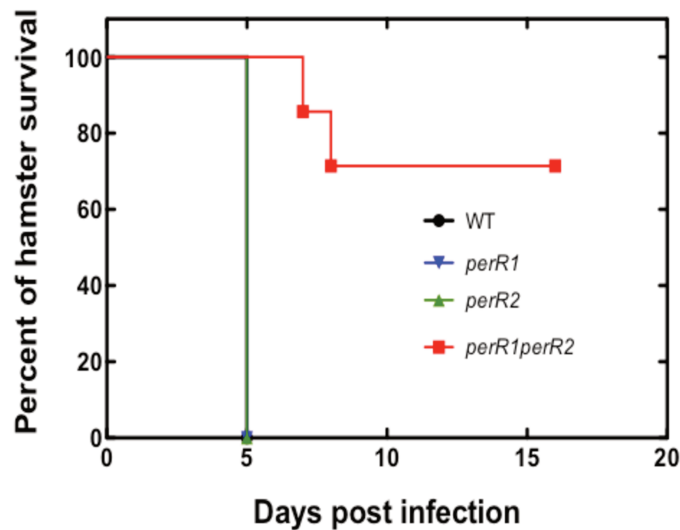


Figure 35. The double *perR1perR2* mutant has an attenuated virulence. Infection of hamsters with WT (black circle), single *perR1* (blue triangle) and *perR2* (green triangle), or double *perR1perR2* (red square) mutant strains were performed by injecting intraperitoneally 10^6 bacteria. Please note that WT, *perR1* and *perR2* strains behave the same

Different groups have shown that *Leptospira* can transiently persist inside macrophages for up to 48 hours (S. Li et al. 2017; Y. Li et al. 2019; Toma et al. 2014). Encouraged by results of the virulence tests, we investigated whether the attenuation in virulence observed in the double *perR1perR2* mutant strain is due to the impairment of surviving inside macrophages. To test this hypothesis, human macrophages were infected with the single *perR1* and *perR2* mutant and the double *perR1perR2* mutant strains.

As seen in Figure 36, we were able to recover *L. interrogans* cells of the WT and the single *perR1* and *perR2* mutant strains after 6, 24 and even 48 h of infection in macrophages. This result indicates that there is no impairment of these strains to enter and survive inside human macrophages. However, we could not recover the double *perR1perR2* mutant, indicating that when both PerR regulators were inactivated, the capacity of *Leptospira* to enter or survive inside macrophages is impaired. Interestingly, we were able to recover the double *perR1perR2* mutant bacteria after six hours of infection, which suggests that even if the double *perR1perR2* mutant had the ability to enter macrophages, this strain is cleared within the first five hours during infection by the innate immune response exerted by macrophages (Figure 36).

Altogether, these results suggest that PerRs are essential for *Leptospira* virulence. PerR1 and PerR2 might regulate genes that are pivotal for survival inside macrophages and also for persist inside host tissue.

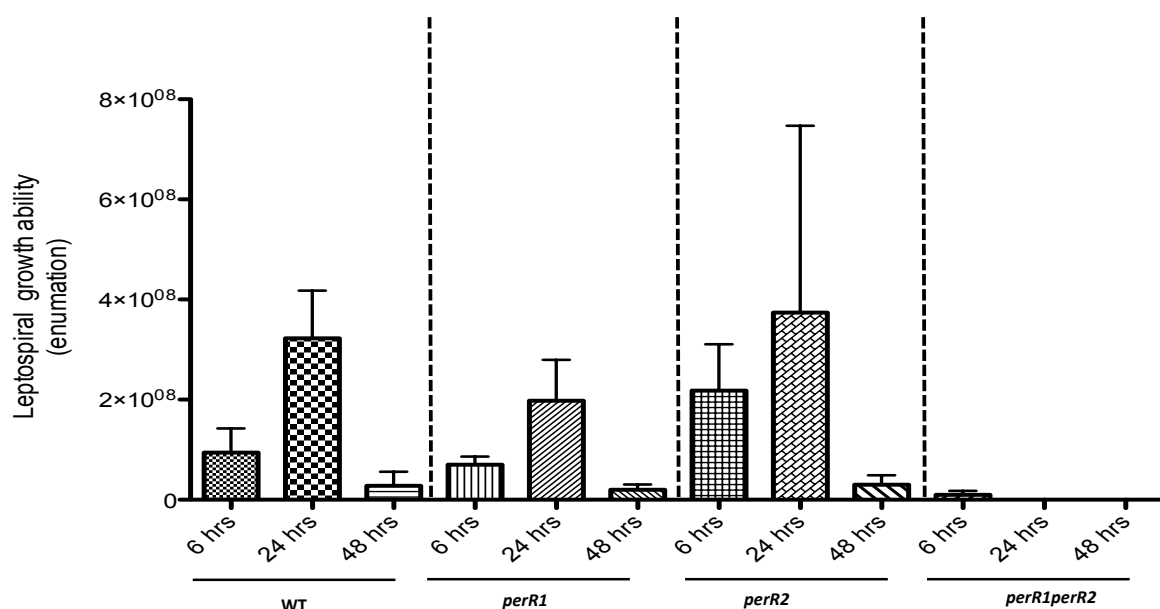


Figure 36. *Leptospira* recovering after macrophages infection. Infection was performed with WT, single *perR1* and *perR2* mutants and the double *perR1perR2* mutant strain at a MOI of 100 in human macrophages (THP-1). Infected cells were lysed and inoculated in EMJH. After 7 days, *Leptospira* were enumerated by counting under a dark field microscope using a PetroA-Houser cell

V Role of PerR1 and PerR2 in *Leptospira* survival inside a host

Transcriptomics results allowed us to raise hypothesis to explain the interplay between PerR1 and PerR2 in *L. interrogans* virulence. However, all the conditions used to perform transcriptomes were standard *in vitro* conditions at 30°C in EMJH medium.

Caimano and collaborators developed in 2014 a system to study gene expression by leptospires inside a host. They cultivated *Leptospira* cells within dialysis membrane chambers (DMCs) implanted into the peritoneal cavities of rats (Caimano et al. 2014). *Leptospira* can be recovered from the DMC, and RNA-Seq or mass spectrometry can be performed. This approach is very

useful because it can give an exact idea of what is the transcriptomic profile of *Leptospira* during infection.

We engaged in collaboration with the team of Melissa Caimano at the University of Connecticut Health Center (USA). The transcriptome of the WT, single *perR1*, and *perR2* mutants and the double *perR1perR2* mutant strains were determined when bacteria were grown in the DMC. We compared the DMC transcriptome with that obtained during *in vitro* conditions in our study.

In the DMC model, 915 genes are deregulated differently from the *in vitro* conditions transcriptome, 637 been statistically significant (cutoff of $\log_2FC > 3$ and $p\text{-value} < 0,005$). 275 and 326 were up and down-regulated, respectively (Figure 37).

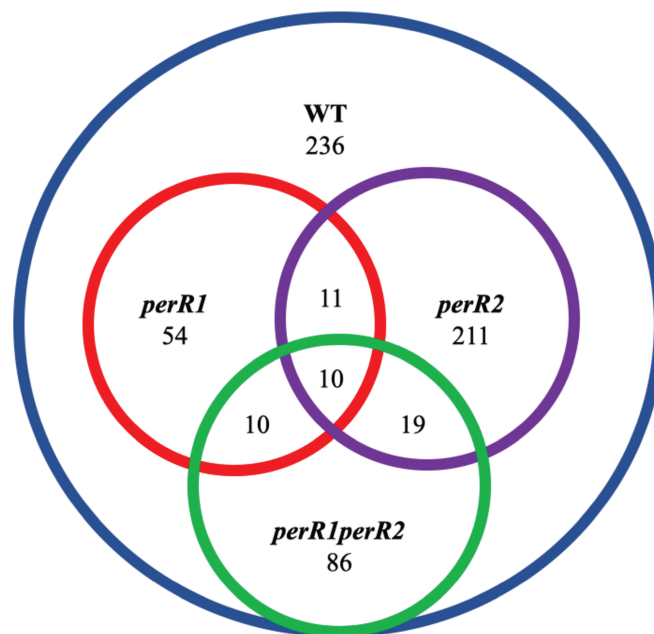


Figure 37. Correlation of the DMC transcriptomic results between the single *perR1* and *perR2* mutants and the double *perR1perR2* mutant cells. 637 genes were statistically significant deregulated in the DMC model from *in vitro* conditions. Among these, 85 under the control of PerR1, 251 under the control of PerR2 and 125 under both regulators. 11 genes seem to be present in PerR1 and PerR2 regulon. 19 genes are present in the single *perR2* mutant and in the double *perR1perR2* mutant transcriptome. 10 with the single *perR1* and the double *perR1perR2* mutant transcriptomes. Comparing all the transcriptomic results only 10 are shared between them.

Interestingly, among these genes that are differentially deregulated in the DMC model, we could identify several genes of the PerR1 regulon that we described in the *in vitro* conditions

(Figure 37). The all gene cluster that code for the TonB transporter system and the hemolysin are also down-regulated, and the all peroxidases that are under the control of PerR1 also appear in the DMC. This comparison allowed us to observe that, in the case of the PerR1 regulon, there is an excellent correlation between our transcriptomic results observed in *in vitro* conditions with the one in DMC.

On the contrary, if we analyzed the PerR2 regulon obtained in standard *in vitro* conditions with that of the DMC, there is no correlation with any gene. This comparison allowed us to confirm our hypothesis that PerR2 has a role in infection-related conditions. In agreement with that, we observed an increased expression of *perR2* in the presence of lethal doses of H₂O₂ (Figure 26).

If we compared the transcriptome obtained in high doses of H₂O₂, where *perR2* expression is induced (see Experimental Results. Article 1.), with that of the PerR2 regulon obtained in the DMC, we could observe some correlation. Repair mechanisms were up-regulated in both transcriptomes such as the DNA repair protein RecN and the same manner oxidoreductases involved in the repairment of oxidized cysteines such as thioredoxins and glutaredoxins. Also, in the *perR2* mutant in DMC we could identify up-regulated genes that code for molecular chaperones such as Hsp20 together with lipoproteins like LipL21 and LipL41.

Interestingly, in the DMC model, we can observe that there is almost no correlation between PerR1 and PerR2 regulon, except the all gene cluster that encodes the TonB transporter system that's is down-regulated in the single mutants transcriptome. Additionally, we observed that some genes that code for hypothetical proteins are shared between PerR1 and PerR2 regulons. Most of those are up-regulated in the absence of PerR1 and down-regulated in the absence of PerR2.

Surprisingly, a comparison of the transcriptome of the double *perR1perR2* mutant obtained *in vitro* with that of the DMC allowed us to see that there is an excellent correlation between them. However, log₂FC seems to be higher in the DMC model, such as the example of the signaling system Lvr (log₂FC in DMC -11.15 and in *in vitro* -2.355). Curiously, in the DMC model but not in *in vitro* conditions, if you remove both PerR regulators, the expression of a gene that codes for the virulence factor collagenase is up-regulated (Kassegne et al. 2013a).

Although the DMC model is an attractive tool to understand the transcriptomic arrangement of *Leptospira* during infection, the results will be only in the asymptomatic colonization model, in the rats. Nevertheless, we were able to compare the transcriptomic profiles with that one *in vitro* and conclude that there is almost a full correlation between them, at least with the PerR1 regulon and the double *perR1perR2* mutant. However, in this host-like model, we were able to determine the PerR2 regulon for the first time.

Discussion

Leptospirosis is one of the most widespread zoonotic diseases, and is classified as being among the neglected and misdiagnosed diseases, affecting livestock and also infecting around 1 million humans, killing 60,000 of those every year (Costa et al. 2015). In recent years, due to climate change, an increasing number of leptospirosis cases have been reported in developed countries such as Germany, France, Croatia, Netherlands, several other European countries, and the USA (Choffnes *et al.*, 2011; Costa *et al.*, 2015). Nevertheless, leptospirosis remains under the radar on the WHO's (World Health Organization) list, and has still not been included among the official top neglected tropical diseases, ironically further highlighting the neglected nature of the disease.

Even though research for leptospirosis is significantly under-resourced, progress has been made. Different approaches have been used and developed for further study of pathogenic species of *Leptospira*, as described in the introduction. One important virulence mechanism in any pathogen is the resistance to oxidative stress, and giving the multiphasic niches *Leptospira* can inhabit it raises the question: how does *Leptospira* cope with these oxidants either in the environment or inside a host during infection?

We aimed to answer this broad question by identifying the different mechanisms that pathogenic *Leptospira* use to adapt to and withstand oxidative stress during infection, and we divided it into three objectives.

I. Identification of all cellular factors involved in the adaptation to peroxide stress.

We have used RNASeq technology to determine the adaptive response of pathogenic *Leptospira* to hydrogen peroxide. *L. interrogans* were subjected to two different treatments, a short exposure in the presence of a sublethal dose of hydrogen peroxide, that might mimic peroxide doses produced during aerobic metabolism and present in the outside environment, and a longer exposure with a lethal concentration of hydrogen peroxide, that could mimic the peroxide concentrations encountered inside a host during infection.

Our findings indicate that H₂O₂ concentrations as low as 10 µM can up-regulate the catalase (encoded by *katE*) and two peroxidases, an AhpC and a CCP, as well as heme biosynthesis-

encoding genes. Heme is also up-regulated probably because it acts a cofactor for catalase and CCP peroxidase activities. These three peroxidases are the first-line of defense allowing detoxification of H_2O_2 , and among these three enzymes, *katE*-encoded catalase has a major role in protecting *L. interrogans* from the deadly effect of hydrogen peroxide, during logarithmic phase but also during stationary phase. In our study, an *ahpC* mutant did not exhibit an altered tolerance toward H_2O_2 ; instead, this mutant had a lower ability to grow in the presence of superoxide. This might indicate a role of this peroxidase in superoxide detoxification or in elimination of H_2O_2 produced from the catabolism of superoxide. Obtaining a deletion mutant by allelic exchange in *ccp* will be required to determine whether CCP acts for degrading H_2O_2 or as an electron acceptor as recently demonstrated in *E. coli* (Khademian *et al.*, 2017).

When H_2O_2 reach a level that overwhelms the H_2O_2 detoxification machinery, not only *L. interrogans* solicited the peroxidase activities of catalase, AhpC and CCP but additional enzymes with a putative role as antioxidants and/or in repair of oxidized cysteines in proteins were also up-regulated, including several thiol oxidoreductases, thioredoxin, glutaredoxin, and DsbD and Bcp-like proteins. The induction of several genes with putative role in DNA repair (*recA*, *recN*, *dinP*, *mutS*, *radC*) suggests that these concentration of H_2O_2 induced oxidative damage to DNA. Surprisingly, the classical repair mechanism for oxidized methionine residues (such as methionine sulfoxide reductases) or damages to iron-sulfur clusters in proteins (the Suf machinery) were not more expressed in the presence of H_2O_2 as if this repair mechanisms were not required under such oxidative damage-inducing condition. Also, the redox-regulated chaperone Hsp33 involved in protecting protein from aggregation and promoting the refolding of oxidatively-damaged proteins, was not up-regulated (Jakob *et al.*, 1999). Instead, canonical molecular chaperones (DnaK/J/GrpE, GroEL/ES, ClpB and small Hsps) were dramatically more expressed, suggesting that 1 mM H_2O_2 results in protein aggregation and unfolding.

The nature of down-regulated genes (encoding factors involved in transcription, translation, protein secretion, motility and chemotaxis, and metabolism pathway) indicates that *Leptospira* decrease their general metabolism, that might explain the slowdown in growth induced by the presence of H_2O_2 .

Comparing the H_2O_2 -induced change in gene expression in the *perR* mutant with that in WT cells indicated that PerR contributes only partially to the H_2O_2 -induced gene regulation. Among the genes whose expression is markedly changed upon exposure to H_2O_2 and is under the

control of PerR, only *katE* had a H₂O₂-induced increase in expression that resulted in the repression alleviation when oxidized PerR dissociates from DNA. Surprisingly, even in the absence of PerR, *ahpC* and *ccp* expression are still increased upon exposure to H₂O₂, suggesting that additional regulatory mechanisms are involved in the H₂O₂-induced gene regulation. In fact, several genes encoding transcriptional regulators (including PerR2, a second putative PerR), two component systems, and sigma factors had their expression altered by the presence of H₂O₂, corroborating the involvement of other regulators. Moreover, our RNASeq experiments have allowed the identification of several non-coding RNAs that might also influence the expression of the H₂O₂-regulated genes. For instance, many non-coding RNAs with increased or reduced expression upon *Leptospira* exposure to H₂O₂ are located in the vicinity of ORFs with increased or reduced expression in the same condition. Noticeably, rh859 located downstream *ccp* might participate in the increased expression of this gene together with the derepression induced by PerR dissociation from DNA in the presence of H₂O₂.

Of note, comparison of the transcriptome of the *perR* mutant determined in this study with that determined previously by Lo and collaborators (Lo et al. 2010) pinpoints several discrepancies. For instance, our study did not demonstrate that heme biosynthesis genes are under the control of PerR and the expression of *ahpC* was not affected in the *perR* mutant in the study of Lo et al. These contradictions can be explained by the experimental conditions used to determine the transcriptome of the *perR* mutant in this previous study which, in fact, has compared WT cells cultivated in EMJH medium with *perR* mutant cells cultivated in EMJH medium the presence of kanamycin. Due to the relation between antibiotic and oxidative stresses, the presence of an antibiotic might have influenced the expression of ROS-related genes, such as heme genes or *ahpC*.

We have identified new ORFs that participate in *Leptospira* survival in the presence of ROS. Indeed, our findings indicate that a peroxidase, encoded by *ahpC*, and a TonB-dependent transporter (encoded by a cluster containing *fecA*, *exbD*, and *lipL48*) are required in *Leptospira* survival in the presence of superoxide. Interestingly, pathogenic *Leptospira* genomes do not contain any genes homologs to a superoxide dismutase or superoxide reductase, nor they exhibit a SOD activity (Nivière *et al.*, 2004). This is quite intriguing as it is generally believed that all aerobic bacteria do have a SOD. One fundamental question is to understand the mechanism these pathogenic bacteria use to detoxify superoxide produced endogenously during the respiratory chain or exogenously by phagocytic cells during infection. AhpC could detoxify

H₂O₂ produced upon the reduction of superoxide and the TonB-dependent transporter could act as an efflux pump. It will be interesting to understand and decipher the exact contribution of AhpC and this TonB-dependent transport system in this defense mechanism. None of the mutants inactivated in these ORFs exhibited a dramatic reduction in virulence, suggesting that these mechanisms do not have a pivotal role in *Leptospira* during infection.

Many ORFs of the H₂O₂ adaptive response identified in this study have been shown to be also up-regulated upon other host-related conditions such as at the host temperature 37°C (GroEL/ES, DnaK/J/GrpE, small HSPs, ClpB, RadC, catalase) (Lo et al. 2006; Lourdault et al. 2011; Murray et al. 2009), host osmolarity (RadC, LIMLP_16520-encoded DNA repair exonuclease, DsbD, the LIMLP_00770-encoded dithiol disulfide isomerase) (Matsunaga *et al.*, 2007), under iron-limited concentration (TonB-dependent receptors LIMLP_14160 and LIMLP_08410, Imelysin LIMLP_14180, the lipoprotein LruB LIMLP_14170) (Lo *et al.*, 2010) or in dialysis membrane chamber (DMC) implanted inside rats (GroEL/ES, DnaK/J/GrpE, small HSPs, ClpB, RadC, catalase, AhpC) (Caimano et al. 2014). Therefore, the H₂O₂ adaptive response overlaps to some extent with other stress responses. The accumulation of oxidatively-damaged proteins and DNA could trigger a general stress response. The change in expression of other stress-related regulators such HrcA, the repressor of heat shock proteins, and LexA, the repressor of the SOS response, suggests that the presence of ROS elicits heat shock and SOS responses. In fact, and perhaps most importantly, the overlap between the H₂O₂ adaptive response (determined in this study) with the host adaptive response in a mammalian host (assessed by DMC) implies that the H₂O₂ treatment used in this study mimics the oxidative conditions pathogenic *Leptospira* encountered inside a host during infection

II Identification of an additional putative PerR in pathogenic *Leptospira* and its function in the oxidative stress response.

In our study, we have identified a second putative PerR regulator that we called PerR2. We have demonstrated that PerR2 has a role different from that of PerR1 in the oxidative stress response.

To the best of our knowledge, this is the first report that has identified the coexistence of two PerR regulators in a Gram-negative bacterium. In most cases, the oxidative stress responses in Gram-negative bacteria are coordinated mainly by the transcriptional regulator OxyR. OxyR can coexist with another regulator involved in the oxidative stress response, such as *P. aeruginosa*, which encodes for an OxyR and OhrR, and they both have a different role, and, thereby, distinct regulons, in the oxidative stress response (Ochsner *et al.*, 2001). In the case of *E. coli*, the oxidative stress responses rely on OxyR, and SoxR transcriptional regulators and their regulons differ entirely from one another, which correlates with the different sensing mechanisms (Imlay 2015).

As previously mentioned, PerR is mostly present in Gram-positive bacteria such as *Bacillus subtilis*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Duarte *et al.*, 2010). *Leptospira* is one of the few examples of Gram-negative bacteria with *Campylobacter jejuni* that has a PerR. PerR also can coexist with other regulators involved in the oxidative stress responses, such as OxyR in *Deinococcus radiodurans* (H. Chen *et al.* 2008) that share some genes of their regulon. In the case of *Bacillus subtilis*, it can coexist with OhrR, with both regulators differing in their regulons and sensing mechanisms, as described before (Dubbs *et al.*, 2016).

Interestingly, the coexistence of a duplicate regulator in the same bacterium is an infrequent event. In fact, the coexistence of three PerR-like regulators has been reported only in *Bacillus licheniformis* (J. H. Kim *et al.* 2016). In this study, they showed that the three PerRs sense hydrogen peroxide by histidine oxidation, although, with different sensitivity. In our results, PerR2 seems to be involved in superoxide sensing, instead of hydrogen peroxide such as PerR1, based on the phenotypic experiments (Figure 27). Also, the transcriptome results did not help

us to identify any gene that might be involved in the PerR2 superoxide stress response, like the different peroxidases controlled by PerR1 (Experimental Results. Article 1.). However, we were able to detect the expression of *perR2* in the presence of high doses of hydrogen peroxide (Figure 26). These results correlate with the previously determined sensing mechanism of any PerR, where PerR self-represses, and the protein dissociates from its own promoter when it senses H₂O₂, thus increasing its own expression in the presence of this oxidant (Giedroc 2009).

One hypothesis is that the overall results of the activity of PerR2 that we are observing are more due to different sensitivity for the detection of oxidants. In our proposed model, PerR1 is more prone to sense sublethal doses of hydrogen peroxide, regulating genes that are required as the first line of defense. Subsequently, when concentrations of hydrogen peroxide are higher and potential lethal, PerR2 is necessary for regulating genes that are involved in more adaptation-repair mechanisms. This hypothesis is supported by the results obtained in the *perR2* transcriptome in the DMC model where we were able to identify genes that encode for DNA repair mechanisms such as the DNA repair protein RecN, and several genes coding for thiol peroxidases such as thioredoxin and glutathione peroxidases that are involved in protein repairment, and a vast number of genes coding for hypothetical proteins (more than the 70% of PerR2 regulon) that could have a function either related to oxidative stress or repair were deregulated in the PerR2 regulon, and the fact that *perR2* expression is only increased in host-like conditions (Figure 26).

The sensing mechanism of PerR2 by protein oxidation could be determined with MALDI-TOF MS after overexpression of the protein in a suitable model bacterium such as *E.coli*. This approach has been used before to detect protein oxidation of PerR from *B. subtilis* and *B. lichenimorfis* (Ji et al. 2015; J. H. Kim et al. 2016; Won et al. 2010). They reported that PerR senses hydrogen peroxide in a concentration-dependent manner by oxidation of the histidine residues characteristically of the PerR from *B. subtilis* (H37 and H91). This same experimental approach could be performed in bacteria expressing leptospiral PerR1. In the work reported by Kebouchi and collaborators in 2018, the authors determined that leptospiral PerR1 has the asparagine and aspartate amino acid residues that are well conserved in the PerR of *B. subtilis*. Furthermore, deletion of PerR1 in *L. interrogans* results in an analogous phenotype to the same mutant in *B. subtilis*. However, they did not demonstrate that H₂O₂ inhibits the interaction of PerR1 with DNA, meaning that the sensing mechanisms for this transcriptional regulator remains unknown but very likely to be similar to that of PerR from *B. subtilis*.

III Interplay between PerR1 and PerR2 in *Leptospira* virulence and their role in *Leptospira* survival inside a host.

Interestingly, looking at the phylogenetic distribution of the PerR regulators in the *Leptospira* genus, the PerR1 protein is present in highly virulent P1 and saprophytic species, and the PerR2 is present in highly virulent P1 and intermediate P2 species. Looking at their different transcriptomic profiles and their phenotypic response allowed us to conclude that they have a non-redundant role in *L. interrogans* in the oxidative stress response (Figure 27 and 31). On the contrary, it seems that they might be redundant for *Leptospira* virulence. This is further supported by the fact that both PerR proteins are necessary for virulence and intracellular survival inside macrophages (Figure 35 and 36). In fact, in the double mutant, we observed new genes that are deregulated compared to the single mutant's transcriptomic results (Figure 32 and 33).

All these results made us wonder about the evolutionary path for the PerR regulators in the *Leptospira* genus. Did *Leptospira* acquire PerR2 in the diversification between saprophytic and intermediate species? Alternatively, was PerR1 subsequently lost in the intermediate species?

Looking at the phylogenetic tree, we could hypothesize that PerR2 was acquired by the common ancestor of intermediate and pathogenic species of *Leptospira* (Figure 25). Then, intermediate species is the clade where PerR1 started to be absent. As seen in Figure 25, in the intermediate species P2, it is still possible to find between 40 – 50 % similitude of PerR1 among those species, which could be explained by the fact that this is the clade where PerR1 started to be lost. Furthermore, there are two intermediate species in which PerR1 is actually present (*L. dzoumognesis* and *L. wolffii* Khorat-H2 (Figure 25). One way to evaluate this hypothesis could be to do the same analysis with genes that are only present in pathogenic species and see if they follow the same evolutionary process as PerR2. Also, once we can determine the PerR2 regulon, we could analyze the evolutionary acquisition of those genes, in order to be able to make further conclusions about the evolutionary process of PerR2.

In some other bacteria, it has been reported that PerR contributes to their virulence, such as *N. meningitidis*, *S. pyogenes*, and *S. aureus* (Horsburgh *et al.*, 2001; Delany *et al.*, 2004; Brenot, King *et al.*, 2005). Interestingly, in our model, there is only attenuation in virulence in the absence of both PerR regulators. Moreover, this is further supported by the massive deregulation observed in the absence of both PerR regulators, where even there is almost no overlap with the individual regulon of PerR1 and PerR2 (Figure 32, 33 and 34). These results highlight the redundancy phenomena that are very common in *Leptospira* (see chapter II.3 Virulence mechanisms).

The fact that *Leptospira's* ability to persist and replicate inside macrophages is decreased in the absence of both PerRs could explain the lack of virulence observed for the double *perR1perR2* mutant strain. However, these results give place to further questions as: is it the impairment of survival observed with the double *perR1perR2* mutant survival due to a problem of internalization in macrophage. If there is high resistance to oxidative stress *in vitro*, what are the mechanisms by which *Leptospira* fails to persist inside macrophages in the absence of both PerRs?

Liu and collaborators in 2014 developed a dye where they were able to differentiate intracellular with extracellular leptospires, called carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), that did not affect motility, viability, or virulence of the bacteria. In order to determine if the lack of survival in macrophages of the double *perR1perR2* mutant strain could be due to a decreased internalization, confocal microscopy using the CFDA-SE-dyed bacteria could be an excellent approach to try to explain the lack of virulence of the double mutant. Nevertheless, when we infected human macrophages after 6hrs, we were able to recover the double *perR1perR2* mutant cells (Figure 36), these results suggest that the lack of survival inside macrophages is not due to a problem of internalization, but instead some other factor that affects the fitness of the cells once internalized into this highly specific environment.

Surprisingly, in the double mutant transcriptomic results, we also observed a considerable number of genes that were deregulated. Among these genes, we can find several that also could explain the lack of virulence observed with the double *perR1perR2* mutant strain (Figure 32 and 33). As seen in Figure 33, among the genes that are down-regulated in the double mutant is the response regulator LvrA of a two components system called LvrA/B that was shown to be essential for *Leptospira interrogans* virulence (Adhikarla *et al.* 2018). These authors also

showed that this system governs a major *Leptospira* virulence pathway through a complex network regulating many genes. Some of these genes we could also observe in our double *perR1perR2* mutant transcriptomic results, such as the putative virulence-related genes encoding for leucine-rich repeat (lrr) proteins (Miras et al. 2015), genes that encode for tetratricopeptide repeats structural motifs (Cervený et al. 2013), and the lipoprotein LigA (Pappas *et al.*, 2015a), among others.

Also, we were able to observe genes deregulated that only appeared in the *perR1perR2* mutant transcriptome. The genes that encode for a TonB-transporter system are down-regulated, as is the gene that encodes for the molecular chaperone ClpB and other molecular chaperones such as Hsp20 and Hsp15 that could have a role in *Leptospira* virulence (Lourdault et al. 2011). We also observed the entire regulon of PerR1 appears to be dysregulated in the double mutant. Together with several hypothetical proteins that are only present in the pathogenic species, around 66 % of the genes do not share homology with the saprophytic species of *Leptospira*.

Even though we observed a huge pleiotropic effect in the absence of both PerR regulators, there remains to be elucidated the exact mechanisms by which PerR1 and 2 regulate leptospiral virulence.

In the *Leptospira* field, there have been relatively few examples in which virulence factors have been able to fulfill Koch's molecular postulates due to different limitations countered *in vitro*. As described in chapter II.3 Virulence mechanisms, Loa22 is among them (Ristow et al. 2007). In this study, we are reporting the activity of transcriptional regulators which were initially considered to be uniquely involved in the oxidative stress response like reported previously for other PerR regulators in other bacteria (Carpenter *et al.*, 2009; Faulkner *et al.*, 2011; Dubbs *et al.*, 2016b). Nonetheless, as seen in this study, PerR has other roles in bacteria such as virulence, which in the case of *Leptospira* could be explained by the lack of survival inside macrophages. Although, we cannot exclude the possibility that the lack of survival in macrophages is due to an impairment in the oxidative stress response ability. However, given the impressive deregulation observed in the transcriptomic results, we are not able to conclude which specific mechanism under the control of both PerR regulators is responsible in this exact phenotype, although we have some hypotheses. We are reasonably convinced that, instead of one specific mechanism, there are several, that, regulated by both PerR regulators, affect the physiology, fitness, motility, and oxidative stress response, and thus, affect virulence.

Further studies will be necessary in order to be able to determine the exact mechanism or mechanisms by which, in the absence of PerR1 and PerR2, we have, as an outcome, attenuation in virulence and lack of survival inside macrophages in *Leptospira interrogans*.

Dual RNA-Seq is the holy grail for the host-pathogen interactions field because it allows you to observe the *in vivo* response of both the pathogen and the host during infection (Westermann *et al.*, 2012; Westermann *et al.*, 2016). However, in the *Leptospira* field, this remains quite challenging because of the different limitations that were previously described (see chapter II.4 Limitations in studying *Leptospira*).

Howbeit, progress has been made in the determination of the *in vivo* response during *Leptospira* infection. As described before, Caimano and collaborators in 2014 published a protocol in which dialysis membrane chambers (DMCs) implanted into the peritoneal cavities of rats allowed for transcriptomic analyses to study how leptospires respond to host-derived signals.

We used this approach with our different mutants: the single *perR1* and *perR2* mutant strains and the double *perR1perR2* mutant strain. Interestingly we were able to observe a nice correlation with our *in vitro* results with the *perR1* and the double *perR1perR2* mutant strains.

Curiously, using the DMC model, we were able to determine the *in vivo* PerR2 regulon with statistical significance. In this regulon, we could observe that many genes that are up-regulated in the absence of PerR2 are involved in repair mechanisms, such as glutaredoxins (LIMLP_08980 and LIMLP_13670), the DNA repair protein RecN (LIMLP_07915) among others. Most of the down-regulated genes encode for hypothetical proteins that do not share homology with the saprophytic species of *Leptospira*. In fact, 70% of the PerR2 regulon consists of hypothetical proteins.

Even though the DMC model more closely approximates the environment of the bacterial host and allows us to study the response *in vivo* of *Leptospira* during infection, this approach is just telling one part of the story. As discussed before (see chapter II.5 Host responses upon leptospiral infection), rats are asymptomatic hosts of *Leptospira*, which means that all the transcriptomic results seen with the DMC will be the bacterial response in the asymptomatic

host which could lack necessary factors promoting *Leptospira* virulence, including those which the bacterium may use as signals in order to avoid the immune response of the sensitive hosts, and other nutrients or internal signals.

However, due to the *in vitro* limitations in the *Leptospira* field, this approach has been very helpful in the determination of several potential virulence factors and observed and confirmed our results *in vitro* obtained with the double *perR1perR2* mutant strain. Nevertheless, also in the asymptomatic host of infections is possible to assess the virulence of *Leptospira*. Infected rats shed *Leptospira* through the urine even been asymptomatic (Nally et al. 2018).

The virulence of the single *perR1* and *perR2* mutants and the double *perR1perR2* mutant cells were assessed in rats to that of the WT. Interestingly all the strains were shed through the urine in rats with the exception of the double *perR1perR2* mutant cells. These results are in agreement with the attenuation in virulence observed in the sensitive model such as hamster and the lack of survival in macrophages. Furthermore, the results observed in both host models for pathogen leptospires are highlighting the cooperative role of both PerR regulators in *Leptospira* virulence.

Pathogenic leptospires have different niches, either outside in the environment or inside a host during infection. During all these conditions, *Leptospira* is constantly confronted to oxidative stress for which *Leptospira* has defense mechanisms. In this study, we have shown that the expression of these defenses are coordinated by two peroxide stress regulators, PerR1 and PerR2. Although they seem to have a non-redundant role in the oxidative stress response, they cooperate for *Leptospira* virulence. PerR1 mainly regulates the expression of peroxidases, the first line of defense against oxidative stress, but when concentrations of hydrogen peroxide elevate to lethal doses, PerR2 is highly expressed and regulates the expression of repair machinery, together with many genes that encode for hypothetical proteins. We also showed that, for some of the peroxidases, PerR1 regulates their expression directly. Additionally, we were able to determine a putative binding sequence for PerR1, and this is among the few examples reported in the literature of a PerR having a determined binding motif (Chen *et al.*, 1995; Brenot *et al.*, 2005; Gryllos *et al.*, 2008).

This study has resulted in the identification of novel factors essential for *Leptospira* virulence, and may also provide the basis for hypothesis-driven research to characterize new virulence

factors that could constitute novel molecular therapeutic targets which may be used in the fight against leptospirosis.

Perspectives

As discussed before, one crucial point that remains is to verify that both PerR regulators are sensing H₂O₂ through protein oxidation. This would be pivotal in order to conclude that our PerR regulators control the expression of genes using the same mechanism as previous PerRs reported in another bacteria.

We were able to propose a putative binding sequence of PerR1 in some of the peroxidases. Further experimental corroboration would be necessary, such as direct mutagenesis of some nucleotides and see if the binding of PerR1 is affected with the CHIP-Seq experiments.

Now that we were able to determine the PerR2 regulon in the DMC model, it would be possible to perform the same evolutionary studies done between PerR1 and PerR2. Choosing some genes that are under the control of PerR2 and observed if they followed the same evolutionary path as PerR2. This analysis could help to precisely elucidate the evolution process of both PerRs through *Leptospira* evolution.

We observed *in vitro* high expression of *perR2* in the presence of lethal doses of H₂O₂. Now that we know the conditions under which *perR2* is highly expressed it would be interesting to analyze the transcriptome of a *perR2* mutant in *L. interrogans* exposed to 1 mM H₂O₂. Also to analyze if *perR2* is up-regulated when *Leptospira* are exposed to superoxide, as we have observed an increased tolerance to this ROS when *perR2* is inactivated, suggesting a role of PerR2 in repressing defenses against superoxide. If there is an up-regulation of *perR2* when *Leptospira* are exposed to superoxide, analyzing the transcriptome of the *perR2* mutant upon exposure to superoxide would be pivotal to understand the full function of this regulator in the oxidative stress response in *Leptospira*.

We can also analyze the potential virulence factors that are under the control of both PerR regulators and observed if the mutant of those ORFs are already available in the mutant transposon libraries reported before (Bourhy2005, Louvel2005). This would help to elucidate the mechanisms that are involved in the lack of virulence observed in the absence of both PerR regulators.

As mentioned in the discussion the fact that we were not able to recover *Leptospira* double mutant cells after infection in macrophages raises the question as to whether there is a problem related to bacterial internalization. The CFDA-SE dye, together with confocal microscopy

experiments, would be an excellent approach in order to determine if there is a problem of macrophage internalization of the double *perR1perR2* mutant strain.

Implementation of Dual-RNA Seq would be revolutionary in the *Leptospira* field, and we would not depend on a model such as DMC that only helped us to elucidate one part of the virulence mechanisms governing *Leptospira* infection. The main problem might rely on the amount of biomass that we will have at the end of the RNA purification because concentrations of eukaryotic RNA will be superior to prokaryotic RNA. Another way to eliminate this limitation could be to find the proper prokaryotic enrichment method for *Leptospira* RNA.

It's very important to determine whether PerR2 is a real PerR or a Fur-like regulator. PerR has a DNA binding activity that is favored by the coordination of a regulatory metal. In *B. subtilis*, the regulatory metal is iron, but manganese can also work as a surrogate metal to allow DNA binding. In the presence of H₂O₂, PerR is oxidized and releases the regulatory iron, which results in a change of conformation and dissociation from DNA. Working in vitro on purified protein with iron requires to be in anaerobic condition to avoid oxidation of ferrous iron into ferric iron. Also, PerR is sensitive to oxidation by oxygen and purification of recombinant PerR might result in a population containing oxidized PerR. To test whether interaction of PerR2 is favored by iron and prevented by the presence of H₂O₂, and also to test whether manganese can work as a surrogate regulatory metal, promoter fusion experiments could be a nice approach. The idea would be to express *perR2* promoter under the control of a reporter gene (GFP or beta-galactosidase) in one plasmid. A second plasmid will be used to express PerR2 under the control of a constitutive promoter (promoter *groES*). Co-transformation of both plasmids in bacteria will allow to see whether PerR2 interacts with its own promoter when the bacteria will be cultivated in the presence of iron, manganese, with H₂O₂ and superoxide.

Annexes

Additional submitted and published articles

The single-step method of RNA purification applied to *Leptospira*

Authors: Crispin Zavala-Alvarado^{1*} & Nadia Benaroudj¹

Affiliations: ¹Unité de Biologie des Spirochètes, Institut Pasteur, Paris 75015, France

^{*} Université de Paris, Sorbonne Paris Cité, COMUE BioSPC, Paris 75013,
France

Corresponding authors: Nadia Benaroudj, nadia.benaroudj@pasteur.fr

Running head: Total RNA extraction from *Leptospira*

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i. Abstract

Establishing a rapid method to obtain pure and intact RNA molecules has revolutionized the field of RNA biology, enabling laboratories to routinely perform RNA analysis such as Northern blot, reverse transcriptase quantitative PCR and RNA sequencing. Here, we describe an application of the effective single-step method of RNA extraction (or guanidinium thiocyanate-phenol-chloroform extraction) applied to *Leptospira* species. This method is based on the powerful ability of guanidinium thiocyanate to inactivate RNases and on the different solubility of RNA and DNA in acidic phenol. This method allows one to reproducibly obtain total RNAs with high yield and integrity, as determined by capillary electrophoresis, suitable for the RNA sequencing technology.

ii. Key words: Spirochetes, *Leptospira*, RNA, Guanidinium Thiocyanate, Phenol-Chloroform Extraction, RIN, RNA-Seq, RT-PCR.

1. Introduction

Efficient acquisition of pure and intact RNA molecule is a prerequisite for numerous analytical techniques such as reverse transcriptase quantitative PCR (RT-qPCR), Northern blotting, microarray analysis, and RNA sequencing (RNA-Seq). Particularly powerful are RNA-Seq technologies that allow for profiling and quantification of RNA. Knowing which gene is expressed and how genes are regulated in a particular condition provides scientists with a comprehensive knowledge of the physiological state of cells. The pioneering transcriptomic studies performed in the 1990's have used hybridization-based microarrays technology (1). Since the development of affordable, high throughput sequencing technologies, transcriptomes are determined by RNA-Seq (2).

Leptospira spp. are microorganisms with remarkable adaptation capacities allowing survival in different ecological niches. Pathogenic strains disseminate in the blood of infected hosts, can persist intracellularly in macrophages, colonize different animal tissues (including kidney, liver, and brain), and are shed in the environment (soil and water) through the urine of infected hosts (3). Knowledge of the molecular basis of *Leptospira* pathogenicity is very limited compared to other bacteria, mainly due to the lack of genetic tools available for manipulation of leptospiral genome. Inactivating a gene by allelic exchange in pathogenic *Leptospira* strain is feasible but very inefficient. To study the function of a given leptospiral gene, scientists usually rely on random transposon insertion mutants (4, 5). The transcriptomic approach is therefore instrumental not only in identifying cellular pathways involved in one particular physiological condition, but also to speculate gene function when mutants are not available. Effective RNA extraction has allowed several laboratories to perform transcriptomic studies in *Leptospira*, thereby leading to a better knowledge of bacterial adaptation to host osmotic stress (6), in the presence of serum (7), upon temperature changes (8, 9), and to the host environment (10, 11).

Different methods can be used to extract RNA from a biological sample. One method relies on the different solubility of cellular components in organic solvents and RNA precipitation by alcohol. Another method is based on the ability of RNA to bind to specific adsorbing material, such as silica and cellulose matrixes, and is used in most commercial RNA purification kits. In a third method, RNA is separated on density gradient centrifugation, but this method is laborious and does not allow for simultaneous processing of multiple samples.

Here, we describe the method based on RNA extraction with an organic solvent and precipitation with alcohol currently applied to *Leptospira* strains and allowing for high yields of pure and intact RNA, compatible with the use of RNA-sequencing technology. In this protocol, harvested *Leptospira* are first lysed in TRIzol™. This reagent contains guanidinium

isothiocyanate, a chaotropic agent which is very effective at inactivating endogenous RNases. It also contains low-pH phenol for separating DNA from RNA (12). After adding chloroform to the samples and subsequent centrifugation, RNAs remain in the upper clear aqueous phase while precipitated proteins and DNA remain in the interphase and lower organic phase, respectively. The RNA contained in the upper phase is transferred to a new tube and undergoes alcohol precipitation. The RNA pellet is then diluted in a suitable buffer. Traces of contaminating DNA are eliminated by DNase treatment. This guanidinium thiocyanate-phenol-chloroform extraction also known as the “single-step method” greatly improved and expedited RNA purification, and has become the gold standard widely used for any type of biological samples (13).

RNA quantification and purity can be determined by absorbance measurement at 260 and 280 nm. A ratio A_{260}/A_{280} of at least 1.80 indicates an acceptable purity with low protein contamination, suitable for RT-PCR. For performing RNA-Seq, RNA preparation should be of the highest quality. The integrity of RNA (i.e. absence of RNA degradation) can be assessed by analyzing the RNA preparation by capillary electrophoresis using, for instance, the chip-based device of the Agilent BioAnalyzer. This analysis will provide with a RIN (RNA Integrity Number) value that represents an objective measurement of RNA integrity ranging from 10 (highly intact RNA) to 1 (completely degraded RNA) (14). For RNA-Seq, a RIN value above 8 should be aimed.

The total RNAs obtained via this method are mostly ribosomal RNAs. Depending of the analysis method used downstream, depletion of ribosomal RNAs allowing enrichment of messenger RNAs might be necessary.

2. Materials

We have applied this protocol to pathogenic *Leptospira* (*L. interrogans* serovar Manilae strain L495) and saprophyte (*L. biflexa* serovar Patoc strain Patoc) strains cultivated *in vitro* in EMJH medium (see **Note 1**).

1. Albumin supplement: 10% (w/v) Bovine Serum Albumin, 0.004% (w/v) zinc sulfate, 0.015% (w/v) magnesium chloride, 0.015% (w/v) calcium chloride, 0.1% (w/v) sodium pyruvate, 0.4% (w/v) Glycerol, 1.25% (v/v) Tween 80, 0.0002% (w/v) Vitamin B12, 0.05% (w/v) ferrous sulfate (added at the last moment) in sterile water for injection (WFI).
2. EMJH base: dissolve 2.3 g of Difco *Leptospira* medium base EMJH (Becton Dickenson) in 900 ml sterile WFI. Autoclave the solution.
3. EMJH medium: add 100 ml albumin supplement to 900 ml EMJH base. Adjust the pH to 7.5 and filter sterilize the solution.
4. Refrigerated centrifuge and rotor reaching 12000×g.
5. Water bath at 55°C and 4°C.
6. Vortexer.
7. Fume hood.
8. P1000, P200, P20, P10/2 micropipettes.
9. RNase-free barrier tips for pipettes.
10. 1-2 ml disposable serological plastic pipettes.
11. 1.5 ml RNase-free polypropylene microcentrifuge tubes (see **Note 2**).
12. 50 and 15 ml RNase-free polypropylene conical tubes (see **Note 2**).
13. Surface RNase decontaminant solution.
14. TRIzol™ reagent or other commercially available guanidinium thiocyanate-acidic phenol solution (see **Note 3**).
15. Chloroform.

16. Isopropanol.
17. 75% Ethanol in RNase-free water (*see Note 4*).
18. RNase-free H₂O.
19. DNase treatment kit (*see Note 5*).
20. UV Spectrophotometer.
21. Tris Acetate EDTA (TAE) running buffer (50×): 242g of Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA, pH 8.0. Adjust the volume to 1 liter with distilled water (the pH should be around 8.5). Dilute the solution with ultrapure water to 1× for use.
22. Nucleic acid staining such as ethidium bromide (supplied in a dropper bottle at 625 µg/ml).
23. 1% Agarose: 1 g of agarose in 100 ml of TAE running buffer. Add 1 drop (about 25 µg/40 µl) of ethidium bromide in 50 ml of the solution before agarose polymerization.
24. 6× gel loading buffer for nucleic acid: 10 mM Tris-HCl, pH 7.6, 60% glycerol, 60 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol FF. Mix 1 volume of the 6× gel loading buffer with 5 volume of RNA solution (containing 0.5-1 µg of RNA).
25. Gel equipment for nucleic acid electrophoresis.
26. Electrophoresis power supply.
27. UV transilluminator to visualize nucleic acids.

3. Methods

Great care should be taken to prevent RNA degradation by exogenous RNases. Gloves should be worn at all times and changed frequently. People with long hair should secure it. If possible, a designated laboratory space should be reserved exclusively for RNA extraction and manipulation (*see Note 6*). All the consumable materials (tips, tubes) and solutions should be RNase-free and protected from the dust. All the non-disposable materials that will be in contact with the RNA (pipettes, benches, centrifuge, gel equipment) should be washed with a surface

RNase decontaminant solution (*see Note 7*). All the steps are performed at room temperature unless otherwise noted.

3.1 Cell lysis

Optimally, the starting material should be *in vitro*-cultured *Leptospira* consisting of at least 10^9 cells. This corresponds to a 30 ml *Leptospira* culture at exponential phase (*see Note 8*).

1. Centrifuge the *Leptospira* cells in a 50 mL conical tube for 15 min at $3000\times g$ at 4°C (*see Note 9*).
2. Resuspend the cell pellet in 1 ml of TRIzolTM reagent and transfer the suspension in a 1.5 ml polypropylene tube (*see Note 10*).
3. Vortex well to fully resuspend the pellet.
4. Flash freeze samples in liquid nitrogen and store them at -80°C until further use (*see Note 11*).

3.2 RNA extraction

1. Thaw the sample(s) at room temperature (*see Note 12*).
2. Add 260 μl chloroform, mix thoroughly by inversion for 15 sec and incubate for 10 min (*see Note 13*).
3. Centrifuge for 15 min at $12000\times g$ at 4°C . After the centrifugation, three phases are observed in the tube. The top clear aqueous phase contains RNA, the white ring at the interphase contains denatured precipitated proteins and the bottom pink organic phase contains DNA.
4. Carefully, transfer the aqueous top layer containing RNA to a new clean 1.5 ml polypropylene tube (*see Note 14*).

5. Add 600 μ l isopropanol to precipitate RNA. Mix thoroughly by gently inverting the tube. Incubate for 5-10 min at room temperature (*see Note 15*).
6. Centrifuge for 10 min at 12000 \times g at 4°C and discard the supernatant (*see Note 16*).
7. Wash the RNA pellet by adding 1 ml of 75% ethanol (*see Note 17*).
8. Centrifuge for 5 min at 12000 \times g at 4°C. Discard the supernatant (*see Note 18*).
9. Air-dry the RNA (*see Note 19*).
10. Resuspend the pellet in 40 μ l RNase-free H₂O by pipetting up and down several times.
11. Incubate for 10 min in a water bath at 55°C in order to enhance the resuspension of the pellet (*see Note 20*).

3.3 DNase treatment

Here, we describe the DNase treatment using the Turbo DNA-free™ kit, but any other commercially available kit might work as well.

1. Add 5 μ l of the 10 \times Turbo DNA-free™ buffer and 4 μ l of RNase-free H₂O (provided in the kit) to 40 μ l of the RNA suspension obtained in step 11 in section 3.2.
2. Add 1 μ l of Turbo DNA-free™ DNase (at 2U/ μ l). Mix by pipetting and incubate 30 min. in a water bath at 37°C (*see Notes 20 and 21*).
3. Add 5 μ l of DNase inactivation reagent (provided in the kit), mix well by flicking the tube to disperse the inactivating reagent, and incubate for 5 min at room temperature. During this incubation, flick the tube to disperse the inactivating reagent each minute in order to increase the binding of the DNase to the reagent (*see Note 22*).
4. Centrifuge for 2 min at 10000 \times g and transfer the supernatant to a new clean polypropylene tube (*see Note 23*).

3.4 Assessing quantity and quality of RNA

The absorbance measurement at 260 nm allows the calculation of RNA concentration. An absorbance value of 1 corresponds to 40 µg/ml of RNA (for a spectrophotometer with 1 cm light path).

For RT-PCR, the quality of the RNA preparation can be assessed by electrophoresis on an agarose gel. When 0.5-1 µg of RNA are loaded on a 1% agarose gel, three main bands can be observed, the 23S, the 16S and the 5S ribosomal RNAs (Figure 1) as the total RNA preparation contains mainly ribosomal RNAs. Messenger RNAs can be sometimes visible as faint smear.

If the RNA is to be used in RNA-Seq, the integrity of RNA should be assessed by capillary electrophoresis (*see Note 24*). A typical electrophoresis pattern of high-quality RNA is shown in Figure 2. In this analysis, the abundant 23S and 16S rRNAs are well resolved and the smaller peak corresponds to the 5S rRNA. Here, a RIN value of 9.5 was obtained, which indicates pure and non-degraded RNAs.

The yield of the purification method presented here can be up to 75 µg of RNA per 10⁹ *Leptospira* and RIN values of at least 8.5 are routinely obtained, which makes RNA obtained suitable for RNA-Seq analysis.

3.5 Storage

RNA can be stored at -20°C for a short-term storage but -80°C is preferential for a long-term storage. RNA samples could be aliquoted into several tubes to minimize freeze-thaw and reduce RNase degradation occurring upon accidental RNase contamination.

4. Notes

1. Only use autoclaved glassware dedicated for EMJH medium preparation. In order to avoid contaminating the glassware with components that could prevent growth of

Leptospira, we rinse beforehand the glassware with sterile WFI and all the chemical stock solutions are prepared with sterile WFI.

2. You do not need to use autoclaved tubes, but tubes exclusively reserved for RNA purification and do not manipulate the tubes without wearing gloves.
3. We recommend using TRIzol™ from ThermoFisher Scientific as it has proven to work optimally with this protocol. Another equivalent commercially available reagent might work with a comparable efficiency. Alternatively, home-made solutions can be prepared (see references 12, 13 and 15) but the process is laborious.
4. Prepare the 75% Ethanol solution in a RNase-free conical tube and discard any left over.
5. We recommend using the Turbo DNA-free™ turbo kit from Invitrogen (ThermoFisher Scientific).
6. If you do not have at your disposal an exclusive designated laboratory space for RNA extraction, it might be wise to perform RNA purification when there are not too much people in the laboratory, lowering air perturbation and the risk of dust movement and contamination. Regardless, the surface and any equipment used should be cleaned with a surface decontaminant RNA/RNase removing solution. We recommend using the RNase Away from Merck.
7. If possible, we recommend having a designated pipette set and gel equipment exclusively used for RNA purification.
8. We cultivate *Leptospira* in EMJH medium. It is possible to extract RNA from lower amounts of cells; however, the yield will be lower and, in our experience, working with low amounts of cells leads to a RNA preparation with a lower integrity. It is not necessary to wash the cells before adding the guanidinium thiocyanate-acidic phenol solution.

9. The cells should be rapidly processed in the guanidinium thiocyanate-acidic phenol solution (TRIzol™) after harvesting them as rapid inactivation of endogenous RNases is essential for obtaining high quality RNA.
10. Manipulation of the guanidinium thiocyanate-acidic phenol solution (TRIzol™) should be done under a fume hood, as the solution is highly volatile and toxic.
11. Even if RNA extraction is conducted right after cell resuspension in the guanidinium thiocyanate-acidic phenol solution (TRIzol™), samples should be frozen at -80°C as freezing promotes cell lysis. Samples can be stored in the guanidinium thiocyanate-acidic phenol solution (TRIzol™) at -80°C for at least several weeks. If you plan to analyze RNAs extracted from different biological samples, it is better to perform the RNA extraction of all samples at the same time.
12. In order to increase efficiency of cell lysis, up to three cycles of freezing/thawing can be applied to the samples. However, as promptness is key to obtain high quality RNAs, we avoid this especially when extracting RNAs for RNA-Seq.
13. This step should be performed under a fume hood as chloroform is highly volatile and toxic.
14. In order to prevent contamination with DNA and precipitated proteins, great care should be taken to avoid perturbing the three phases. It is better not to try to retrieve the totality of the upper phase to prevent carry over.
15. You can pause at this step and store the samples in isopropanol at -20°C until you are ready to proceed with the procedure, although we avoid this especially when extracting RNAs for RNA-Seq.
16. You should be able to see a white gel-like pellet containing RNA at the bottom of the tube. Great care should be taken when removing the supernatant as sometimes the RNA pellet does not tightly stick to the tube and tends to move on the tube wall.

17. You can pause at this step and store the samples in 75% ethanol at -20°C until you are ready to proceed with the procedure, although we avoid this especially when extracting RNAs for RNA-Seq.
18. As washing the RNA pellet with 75% ethanol will dissolve salts contained in the pellet, the aspect of the pellet will change. Very often, the pellet becomes smaller and less visible. Again, great care should be taken when removing the supernatant. You can use a micropipette or a syringe with a 22G needle to remove most of the supernatant without disturbing the pellet.
19. The time for air-drying the RNA pellet will depend of the amount of ethanol left in the tube after removing the supernatant. You can put your samples under a fume hood or laminar flow cabinet to enhance the drying step. If the ethanol is properly removed, this step should take 5-10 min. You should avoid excessively drying the RNA pellet as it will decrease its solubility.
20. You should use a clean water bath. A heat block might work as well.
21. To enhance the DNase reaction, you can perform the reaction with 2 µl (4U) of Turbo DNA-free™ DNase. You can also perform a two-step incubation with the enzyme. In a first step, 1 µl of Turbo DNA-free™ DNase are added and the sample is incubated for 30 min at 37°C. Then, an additional 1 µl of Turbo DNA-free™ DNase is added to the sample and the second incubation at 37°C is conducted for 30 min.
22. The amount of DNase Inactivating Reagent to be added should be adjusted depending on the number of DNase units used for the reaction. The manufacturer recommends using 5 µl of DNase Inactivating Reagent for 1 µl (2 unit) of DNase.
23. It might be more comfortable to perform the DNase treatment in a 0.5 ml polypropylene tube as it will ease removal of the supernatant.

24. We recommend using the chip-based device of the Agilent BioAnalyzer as its software is the only one allowing for a RIN determination. This analysis is performed in transcriptomic facilities.

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Figure captions

Figure 1: Analysis of RNA preparation on agarose gel. 0.5 µg of total RNAs were loaded on a 1% agarose gel in 1×TAE. Nucleic acid was stained with ethidium bromide. The bands corresponding to 23S, 16S and 5S ribosomal RNA are indicated.

Figure 2: Analysis of RNA preparation by capillary electrophoresis. 0.5 µg of total RNAs were analyzed on an Agilent RNA 6000 Nano chip with the 2100 Bioanalyzer. (A) Capillary electrophoresis gel-like image of the Agilent RNA 6000 ladder (lane M) and RNAs (lane RNAs). The migration position of 23S, 16S and 5S rRNA is indicated at the right of the image. (B) Electropherogram trace of the RNA preparation. The peaks corresponding to different rRNAs are indicated. The RNA preparation displayed here has a RIN of 9.5. A degraded RNA preparation would display a decrease in the 23S and 16S rRNA signal and a concomitant increased baseline in the fast-migrating zone (before the position of the 16S rRNA peak).

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