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Characterization of the SOS response in *Leptospira interrogans* serovar Copenhageni

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“The scientist does not study nature because it is useful; he studies it because he delights in it, and he delights in it because it is beautiful. If nature was not beautiful it would not be worth knowing, and life would not be worth living.”

Jules Henri Poincaré

ABSTRACT

Fonseca, L.S. **Characterization of the SOS response in *Leptospira interrogans* serovar Copenhageni**. 2014. 116p. Thesis – Graduate Program in Biological Sciences (Biochemistry). Chemistry Institute, University of São Paulo, São Paulo.

Leptospira is a basal genus in an ancient group of bacteria, the spirochetes. The pathogenic species are responsible for leptospirosis, a disease with worldwide distribution and of public health importance in developed tropical countries. *L. interrogans* serovar Copenhageni is the agent for the majority of human leptospirosis in Brazil. In this work, we used a great variety of experimental approaches to characterize the SOS system in this serovar, to identify its impact in general DNA damage response, as well as to assess the DNA repair toolbox owned by pathogenic and saprophytic leptospires. We identified an additional repressor LexA, acquired by lateral gene transfer, exclusively in serovar Copenhageni. We also observed that UV-C irradiation led to massive death of cells and blockage of cell division in the survivors. Both repressors were active and we identified the sequences responsible for binding to promoters. However, the LexA1 SOS box was redefined after a *de novo* motif search on LexA1 ChIP-seq enriched sequences. This regulator was able to bind to at least 25 loci in the genome. DNA damage also caused a massive rearrangement of metabolism: increase in expression was observed in transposon and prophage genes, in addition to DNA repair pathways and mutagenesis inducers; on the other hand, motility, general metabolism and almost all virulence genes were repressed. Two induced prophages provided several proteins with useful functions. We also assessed the DNA repair-related genes presented by the three species of *Leptospira*: the saprophytic *L. biflexa*, the facultative pathogen *L. interrogans* and the obligatory pathogen *L. borgpetersenii*. There are more diversity and redundancy of repair genes in *L. interrogans* in comparison with the other species. Lateral gene transfer seems to be an important supplier of DNA repair functions. In addition, leptospires share characteristics of both Gram-positives and Gram-negatives bacteria. Representative genes from several different pathways were induced during infection of susceptible mice kidneys, suggesting DNA repair genes are active while causing disease. All these data suggest mobile genetic elements are the major forces in leptospiral evolution. Moreover, during DNA damage response, several SOS-dependent and independent mechanisms are employed to decrease cell growth and virulence in favor of controlled induction of mechanisms involved in genetic variability.

Key-words: leptospira, leptospirosis, DNA repair, SOS system, lateral gene transfer

RESUMO

Fonseca, L.S. **Caracterização da resposta SOS em *Leptospira interrogans* sorovar Copenhageni**. 2014. 116p. Tese – Programa de Pós-Graduação em Ciências Biológicas (Bioquímica). Instituto de Química, Universidade de São Paulo, São Paulo.

Leptospira é um gênero basal em um grupo já considerado um dos mais ancestrais, as espiroquetas. As espécies patogênicas são responsáveis pela leptospirose, uma doença presente em todo o mundo e de principal importância em países tropicais em desenvolvimento. *L. interrogans* sorovar Copenhageni é o agente da maior parte dos casos no Brasil. Nesse trabalho, utilizamos diversas abordagens experimentais para caracterizar o sistema SOS nesse sorovar, identificar seu impacto na resposta geral a danos no DNA, assim como avaliar as funções de reparo de DNA disponíveis em leptospiras patogênicas e saprofíticas. Identificamos um repressor LexA adicional, adquirido por transferência horizontal e exclusivo do sorovar Copenhageni. Observamos também que irradiação por UV-C causou significativa morte celular e bloqueio da divisão celular dos sobreviventes. Ambos os repressores são ativos e identificamos as sequências que utilizam para se ligar aos promotores dos genes regulados. Entretanto, o *SOS box* de LexA1 foi redefinido após uma busca de novo por motivos enriquecidos nas sequências recuperadas por ChIP-seq. Esse regulador ligou-se ao menos a 25 locais do genoma. A maioria desses alvos teve aumento de expressão após UV-C. Danos no DNA também causaram um importante rearranjo metabólico: houve aumento de expressão em transposons e profagos, além de indutores de mutagênese e vias de reparo; por outro lado, mobilidade, crescimento celular e quase todos os fatores de virulência foram reprimidos. Dois profagos induzidos durante essa resposta, possivelmente proporcionam algumas proteínas de funções importantes. Nós também avaliamos a presença de genes envolvidos no reparo de DNA em três espécies de leptospira: *L. biflexa*, *L. interrogans* e *L. borgpetersenii*. *L. interrogans* é a espécie com maior diversidade e redundância de genes de reparo. Além disso, transferência horizontal parece ser um importante fornecedor de funções de reparo nesse gênero. Leptospiras também apresentam genes característicos tanto de bactérias Gram-positivas quanto Gram-negativas. Genes representando diferentes vias de reparo foram induzidos durante infecção em modelo animal, sugerindo que essas vias estão ativas no curso da doença. Todos esses dados, em conjunto, sugerem que elementos genéticos móveis são de extrema importância na evolução do gênero e das vias de reparo. Assim, durante a resposta a danos no DNA, diversos mecanismos dependentes e independentes de SOS são empregados para frear o crescimento celular e virulência em favor da indução controlada de mecanismos para aumentar variabilidade genética.

Palavras-chave: leptospira, leptospirose, reparo de DNA, sistema SOS, transferência horizontal

ABBREVIATIONS

AP	apurinic/apyrimidinic
ATL	alkyltransferase-like
BER	base excision repair
Cas	CRISPR-associated
cDNA	complementary DNA
ChIP-seq	chromosome immunoprecipitation coupled with massive parallel sequencing
CPD	cyclobutane pyrimidine dimer
CRISPR	clustered regularly interspaced short palindromic repeats
dRpases	DNA deoxyribophosphodiesterases
EMJH	Ellinghausen-McCullough-Johnson-Harris medium
EMSA	electrophoretic migration shift assay
gDNA	genomic DNA
HR	homologous recombination
LGT	lateral gene transfer
LPS	lipopolysaccharide
MMC	mitomycin C
MMR	mismatch repair
NER	nucleotide excision repair
NHEJ	non-homologous end joining
ORF	open reading frame
PP1	prophage 1
PP2	prophage 2
SDS-PAGE	polyacrylamide gel electrophoresis with Sodium Dodecil Sulfate
SMC	structural maintenance of the chromosome
SSB	single stranded DNA binding protein
ssDNA	single stranded DNA
TLS	translesion
UDG	uracil-DNA glycosylases

SUMMARY

1. INTRODUCTION

Traditional evolutionary biology would consider mutations as randomly occurring phenomena, happening constantly and gradually, with vertical transmission of genetic information. However, the study of molecular mechanisms of mutagenesis shows that mutations do not occur randomly in space or time. There are hotspots for mutations and strand breaks in the DNA. Moreover, bacteria activate mutagenesis mechanisms in response to environmental stresses, increasing their ability to evolve in specific windows of time (Rosenberg and Queitsch, 2014). Even when there is no direct influence in virulence, stress responses can allow faster adaptation and increased diversity in the pathogen population. In the long term, understanding the processes that generate variation and provide adaptability invites the possibility of new ways to approach the rise of virulent strains and development of antimicrobial therapies.

Some findings are presented here on how the human pathogen *Leptospira interrogans* serovar Copenhageni deals with DNA damage. This characterization goes from general phenotypic and survival traits to genetic expression control dependent of these lesions. There is also a comparison of DNA repair pathways existent in the genus *Leptospira*. Possible consequences for virulence, pathogenicity and evolution are presented. In conjunction, these are the most extensive studies on DNA repair in the genus.

1.1. The pathogen: *L. interrogans*

1.1.1. Biology of spirochetes and leptospires

Spirochetes are mobile helicoidal bacteria, considered one of the oldest phylogenetic eubacterial group (Paster *et al.*, 1991). Apart from the helicoidal shape, they possess a typical Gram-negative structure, with a peptidoglycan cell wall between an internal and an external membrane. The periplasmatic space is defined as the interval between the cell wall and the external membrane (Fig. 1). Motility of spirochetes is result of endoflagella function, a variable number of flagella anchored in the extremities of the cell that lie within the periplasm and extends to the other extremity (Adler and Faine, 2006; Faine et al., 1999).

They are widely spread in nature, as free-living or parasitic bacteria. Some important human diseases caused by spirochetes are periodontitis (*Treponema denticola*), syphilis (*T. pallidum pallidum*), Lyme's disease (*Borrelia bourgdorferi*) and leptospirosis (*Leptospira*).

Leptospires are deeply branched in the Spirochaetales order (Paster *et al.*, 1991). The genus *Leptospira* is composed by pathogenic and saprophytic bacteria, presenting two axial endoflagella and characteristic low guanine-cytosine content, between 33.5 to 43.4%. They are usually 0.1µm thick by 6-20µm long, depending on the subgroup and growth conditions (Faine *et al.*, 1999; Levett, 2001). Growth is slow and it requires a rich medium, containing their preferable source of carbon, long-chained fatty acids (Ellinghausen and McCullough, 1967; Faine et al., 1999). As a result of doubling times from 8-18h, leptospires can take up to four weeks to form colonies in solidified medium and 5-10 days to grow in liquid medium (Faine et al., 1999). As pathogenic leptospires are continuously cultivated *in vitro* (each cycle being named passage), they start growing faster, up to 6h of doubling time. However, virulence drops with increased passages until a point where it cannot cause disease anymore. To keep the virulence of cultivated leptospires, after three or four *in vitro* passages they are again passed in hamsters and reisolated (da Silva et al., 2012).

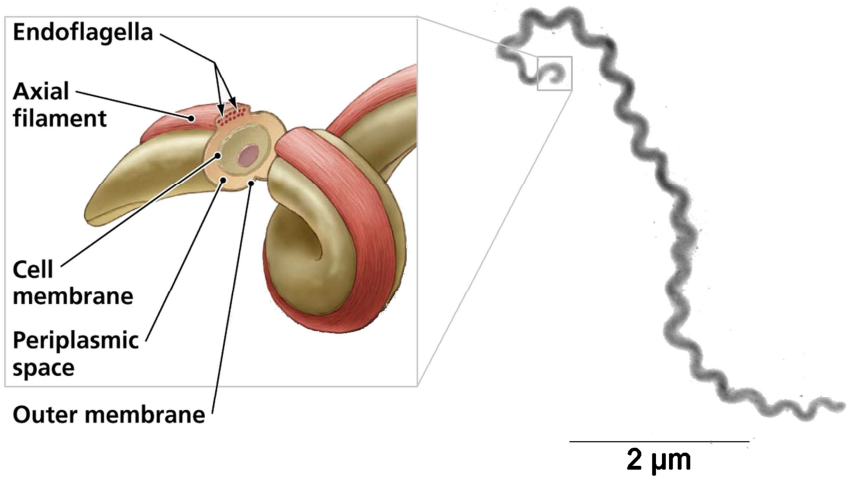

Figure 1. Morphology and cell structure of *L. interrogans*. Electron microscopy of a *L. interrogans* serovar Copenhageni cell in regular culture. Inset: organization of outer and inner membranes, periplasmic space and the localization of axial filaments, where endoflagella are located. Modified from Pearson Education, 2006 and Fraga, 2015 (Fraga et al., 2015).

1.1.2. Classification inside genus Leptospira

The basic systematic unit for *Leptospira* is the serovar, defined by cross agglutination absorption test. Using this parameter, about 250 pathogenic serovars are recognized. They are clustered in 24 serogroups, containing antigenically related serovars (Cerqueira and Picardeau, 2009). All this diversity is caused by the heterogeneity of sugars contained in the lipopolysaccharides (LPS) on the membrane (Bharti et al., 2003). However, the serogroup classification has poor correlation with the molecular one, possibly as a result of LPS determinants being exchanged between species by lateral gene transfer (LGT) (de la Peña-Moctezuma et al., 1999). Molecular classification is based on homology and DNA hybridization, and divides the genus into 20 defined species (Cerqueira and Picardeau, 2009) (Fig. 2).

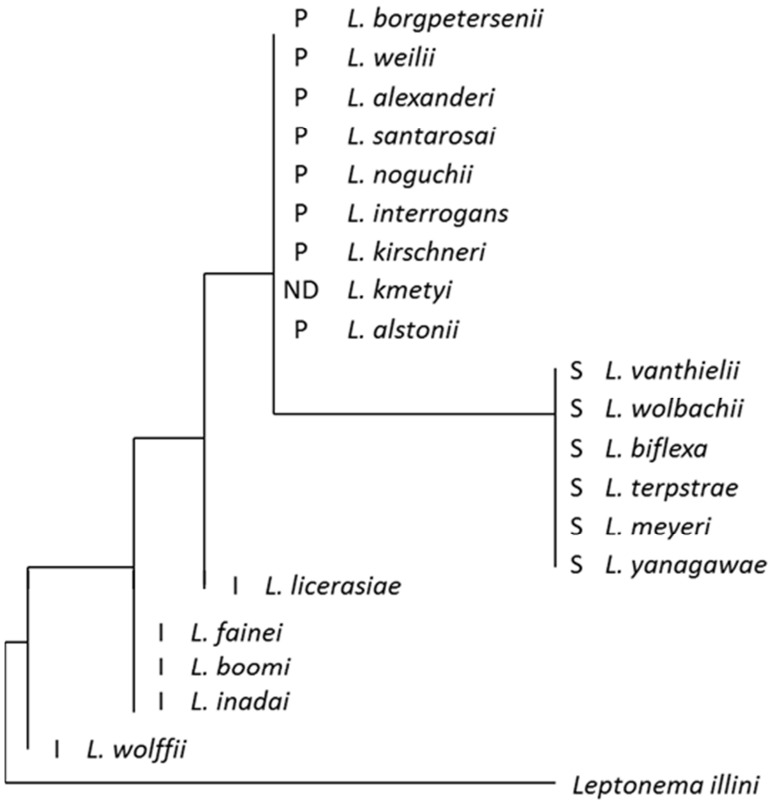

Figure 2. Phylogenetic tree based on the rRNA gene sequences from various Leptospira spp. strains. Species are indicated for the correspondent pathogenicity status as pathogens (P), intermediates (I), saprophytes (S) or not determined (ND). Modified from Cerqueira *et al.*, 2009.

Phylogeny based on the 16S rRNA sequence separates leptospires into three clades reflecting their pathogenicity status: non-pathogenic (as *L. biflexa, L. wolbachii*), pathogenic (as *L. interrogans, L. borgpetersenii*) and intermediate (as *L. licerasiae, L. inadai*). Intermediates are, in general, strains isolated from patients but unable to reproducibly cause disease in animal models (Levett et al., 2006; Matthias et al., 2008). Ancestral leptospires were probably free-living, and the ability to colonize and infect hosts was acquired during evolution of the genus (Faine et al., 1999).

1.2. The disease: leptospirosis

Pathogenic leptospires are causative agents of leptospirosis, a zoonotic disease of worldwide occurrence (Faine et al., 1999). Several mammals serve as reservoirs of bacteria, which colonize the kidneys and chronically infect their renal tubules, usually not causing any important symptoms (Levett, 2001). Reservoirs can shed leptospires in the urine throughout their lives. Even after weeks in mud or water, *L. interrogans* maintains the ability to colonize a new host, invading it by skin abrasions or contact with mucous membranes (Faine et al., 1999). The different species and serovars of leptospires are adapted to a relatively specific group of reservoir, but capable to infect other species, the accidental hosts. In this situation, bacteria colonize kidneys, liver, lungs, eyes and meninges, causing disease (Faine et al., 1999). Humans can get infected by contact with contaminated water (Fig. 3). In developed countries, leptospirosis is considered an occupational and recreational disease, as people can be infected through work with animals, hunting, camping, or swimming (Levett, 2001). However, in tropical developing countries, leptospirosis is considered an important public health concern. Frequent flooding and poor sanitary conditions in urban areas is a powerful combination for spread of the disease, which is mainly maintained in this environment by rats (Bharti et al., 2003; Sarkar et al., 2002).

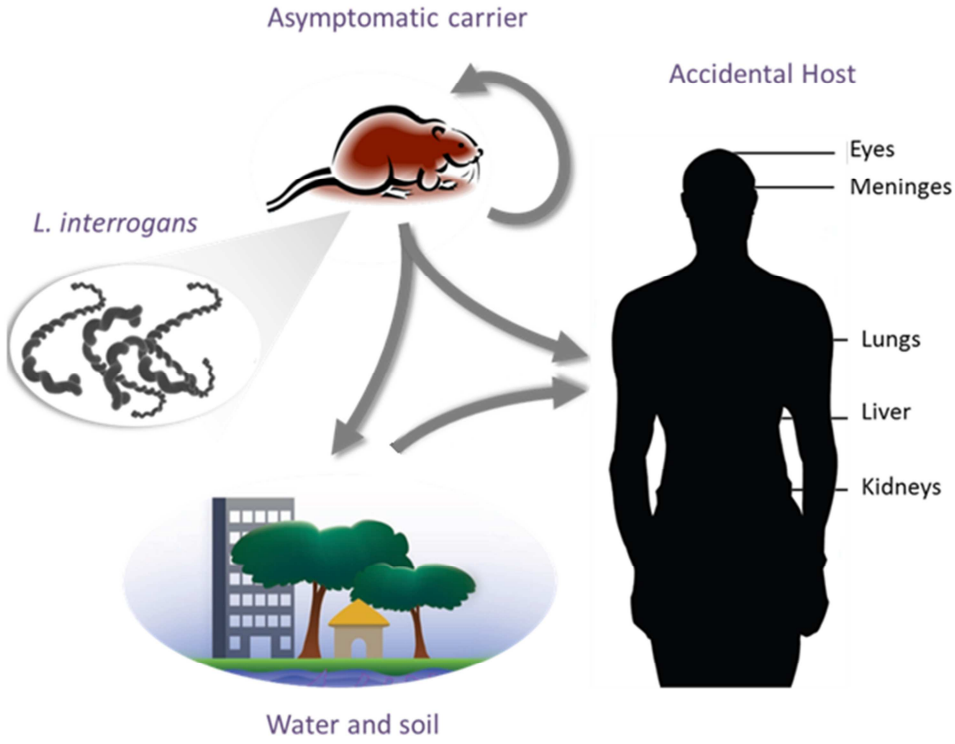

Figure 3. Transmission cycle of leptospirosis in urban environments. Pathogenic bacteria colonize rats chronically, being shed in their urine and contaminating water and soil. Humans can get infected through mucous membranes or abrasions on the skin. Leptospires can infect several organs, but mainly kidneys, livers and lungs.

1.2.1. Symptoms, treatment and prevention

As accidental hosts, humans can present a wide range of symptoms. The majority of cases has mild severity: fever, chills, headache and abdominal pain. These general symptoms, similar to dengue or malaria, make diagnosis difficult (Faine et al., 1999; Ko et al., 1999). However, up to 10% of patients develop the icteric manifestation, with a mortality rate of 15%. The Weil's syndrome, as it is called, presents acute renal failure, thrombocytopenia, uveitis and pulmonary hemorrhage (Levett, 2001). Leptospirosis-associated pulmonary hemorrhagic syndrome can be responsible for more than 50% lethality (Gouveia et al., 2008). These severe manifestations are usually associated with the serovars Icterohaemorrhagiae, Copenhageni or Lai (Adler and de la Peña Moctezuma, 2010). In Brazil, the most prevalent serovar in human leptospirosis is Copenhageni (Gouveia et al., 2008; Ko et al., 1999). In dairy cattle, serovars Hardjo and Pomona are responsible for mastitis and abortion (Levett, 2001).

Treatment depends on the specific symptoms, but use of doxycycline reduces the duration and severity of the illness (McClain et al., 1984). Immunity against leptospires is humoral, predominantly targeting LPS, and after infection there is protection against same and similar serovars only for a limited amount of time (Adler and de la Peña Moctezuma, 2010; Felzemburgh et al., 2014; Levett, 2001). Accordingly, immunization against leptospires achieves limited success, since it is relatively serovar-specific and requires annual boosters. Veterinary vaccines composed of killed or attenuated bacteria are available, although human vaccines are not licensed in Western countries (Levett, 2001; Palaniappan et al., 2007). Until now, the best way to prevent further cases of leptospirosis is to decrease the possibility of infection, mainly by improving sanitary conditions in urban areas (Faine et al., 1999; Felzemburgh et al., 2014).

1.3. Molecular biology of leptospires

1.3.1. Genomics

Genome sequencing of four species, representing the whole range of leptospiral life-styles, provided tools to a whole new chapter in the study of these organisms: the saprophytic *L. biflexa* (strains Patoc and Ames) (Picardeau et al., 2008), the intermediate pathogenic *L. licerasiae* (strains VAR010 and MMD0835, draft sequences) (Ricaldi et al., 2012), the facultative pathogen *L. interrogans* (serovar Copenhageni strain FioCruz L1-130 and serovar Lai strain 56601) (Nascimento *et al.*, 2004; Nascimento *et al.*, 2004; Ren *et al.*, 2003) and the obligate pathogen *L. borgpetersenii* (serovar Hardjo, strains L550 and JB197) (Bulach et al., 2006). The genome size of leptospires varies from approximately 3.6 to 4.7Mb, being *L. borgpetersenii* the smaller and, *L. interrogans*, the largest. They all have at least two circular replicons, containing essential genes, called chromosome I (CI) and chromosome II (CII), approximately 12-fold smaller than CI. However, *L. biflexa* has a third replicon, p74 and a plasmid, LE1 (Bourhy et al., 2007).

The most interesting feature from an evolutive point of view is the role of insertion sequences (ISs) elements in differentiation of species and strains. The saprophytic *L. biflexa*,

possibly closer to the common ancestor, possesses only five ISs (Picardeau et al., 2008), while *L. borgpetersenii* has 167 ISs, with huge impact on coding sequences and the synteny between close strains (Bulach et al., 2006). This IS-mediated genomic deterioration is centered on genes involved in environmental sensing and metabolite transport and utilization. On the other hand, *L. interrogans* presents 36 ISs in serovar Copenhageni and 69 in serovar Lai, with drastic differences in distribution (Nascimento *et al.*, 2004). IS elements were even responsible for a large CI inversion that took place in Lai.

The molecular mechanisms by which leptospires cause disease are still not clear. However, comparison between saprophytic and pathogenic-specific genes can facilitate the search for vaccine candidates, as well as to help understand the biology and pathogenesis of leptospires, through reverse vaccinology (Rappuoli, 2001) and pathogenomic (Lehmann et al., 2014; Lehmann et al., 2013; Pallen and Wren, 2007) approaches. Genomics also provided the knowledge for developing genetic manipulation tools (Girons et al., 2000; Kameni et al., 2002; Picardeau, 2008; Picardeau et al., 2001).

1.3.2. Genetic manipulation

Transformation efficiencies in leptospires are extremely low, being lower for pathogenic bacteria. Identification of LE1 plasmid in *L. biflexa* supplied an important start for genetic manipulation in leptospires, allowing genetic replacement through conjugative transfer (Girons et al., 2000; Louvel and Picardeau, 2007; Picardeau, 2008). However, LE1-based plasmids do not replicate in pathogenic strains. Gene transfers in those strains were only validated by random insertion of *Himar1* transposon, at very low frequencies (Bourhy *et al.*, 2005; Murray *et al.*, 2009). Some serovars, as Copenhageni, are even more difficult to transform (Murray *et al.*, 2009). However, some serovars are able to achieve sufficient efficiency in transposon insertion to create libraries, as Lai and Manilae. The use of such mutant libraries provided tools for the discovery of virulence factors in *L. interrogans* (Bourhy *et al.*, 2005; Murray *et al.*, 2009). Targeted mutagenesis in *L. interrogans* is difficult, with few

examples (Liao et al., 2009; Zhang et al., 2012). Likewise, serovar Copenhageni has only one targeted mutant, *ligB*$^{-}$ (Croda et al., 2008).

1.3.3. Omics to understand virulence

Several advances from the last decade in genomics and mutagenesis are helping to elucidate the key molecular players in as adhesion, colonization, evasion and toxicity. There are currently nine genes considered virulence factors in *L. interrogans*, due to loss of capacity to cause disease in the correspondent mutants, and several putative virulence-associated factors, involved in adhesion and toxicity (Fig. 4).

The first step in the establishment of infection is attachment to host cells, and leptospires have the ability to enter phagocytic and non-phagocytic cells (Merien et al., 1997; Thomas and Higbie, 1990). Consequently, OMPs are obvious targets for testing adhesion and protective potential (Cullen et al., 2004). Based on the concept of reverse vaccinology to look for host-interacting proteins (Rappuoli, 2001), several hypothetical proteins containing signal peptides were tested. These searches revealed a myriad of potential adhesins, mainly lipoproteins, able to bind to extracellular matrix components (Ching et al., 2012; Lima et al., 2013; Lin and Chang, 2007; Longhi et al., 2009; Mendes et al., 2011; Merien et al., 2000; Souza et al., 2012). The redundancy of adhesins can indicate the importance of adhesion to the bacteria. However, the knockout of some important adhesins such as LipL32 and LigB does not affect colonization or virulence (Croda *et al.*, 2008; Murray *et al.*, 2009). On the other hand, the OMP Loa22 was shown to be essential for virulence (Ristow et al., 2007), as well as Mce (mammalian cell entry). The *mce* gene has high expression in leptospires in contact with macrophages, and a *mce*$^{-}$ mutant was less efficient invading them (Zhang et al., 2012). The same is true for LPS: disruption of the LPS-locus abolish colonization of the kidney (Marcsisin et al., 2013) or cause disease in the hamster model (Murray et al., 2010).

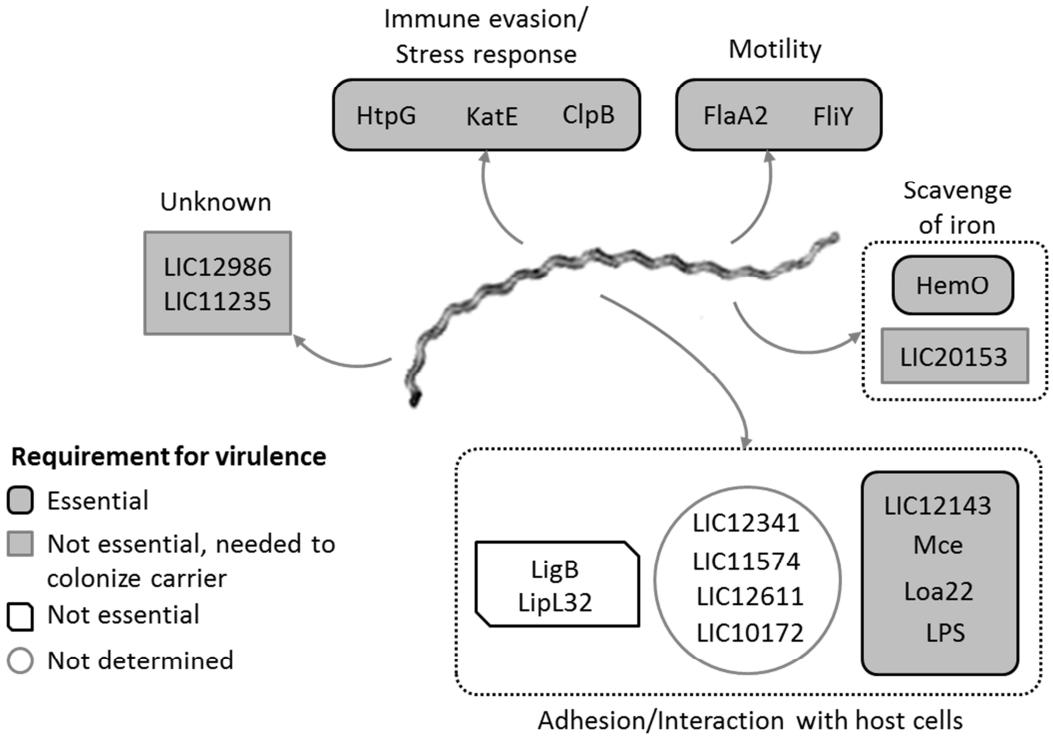

Figure 4. Virulence factors and virulence-associated proteins of *L. interrogans*. The potential mechanisms in which these factors influence pathogenesis are indicated, as well as the level of requirement of the corresponding genes to virulence.

Some interesting factors involved in immune system evasion and host cell binding were also identified applying pathogenomics (Pallen and Wren, 2007). Two prophage structural genes possibly important in pathogenesis were identified after a search for genes carrying mutations in a attenuated strain of serovar Lai in comparison to the ancestral, highly virulent strain (Lehmann et al., 2013): one putatively involved in the onset of leptospirosis-associated thrombocytopenia (LIC12611), and a toxin (LIC10172). In addition, comparison between gene expression profiles of low and high passage strains in contact with macrophages recognized the role of a hypothetical lipoprotein and LigB in enhancing invasion of macrophages (Toma et al., 2014). Finally, Marcsisin *et al*. identified five genes not necessarily essential for virulence in the susceptible host (hamster), but required for colonization of the reservoir host (mouse), like LIC11235, LIC12986 and LIC20153 (Marcsisin et al., 2013).

Motility is also usually involved with virulence. For *L. interrogans*, flagellum protein FliY (Liao et al., 2009), and flagellin FlaA-2 (Lambert et al., 2012), are absolutely required for pathogenesis. A sensor protein involved in regulation of motility was needed as well (Eshghi et al., 2014). Finally, the transition from the environment to the host induces several stress responses, and some of the induced genes are also vital for infection, as catalase *katE* (Eshghi et al., 2012) and chaperones *clpB* (Lourdault et al., 2011) and *htpG* (King et al., 2014). The *hemO* gene, involved in the iron acquisition, is necessary to cause disease, but not for colonization of the kidney (Murray *et al.*, 2008; Murray *et al.*, 2009).

1.4. DNA damage responses

The DNA is constantly subjected to a myriad of damaging agents, from intracellular and external origin. In face of that, all organisms have the challenge of maintaining its integrity, essential for survival and correct transmission of genetic information for the next generation. Some agents used to induced DNA damage and to study its consequences are: (*i*) UV-C, which causes cyclobutane pyrimidine dimers (CPD) and other photoproducts that cause severe distortions in the DNA (van Steeg and Kraemer, 1999); (*ii*) mitomycin C (MMC), an alkylating agent which also forms DNA crosslinks (Dusre et al., 1989); (*iii*) reactive species, responsible for innumerous base alterations modifying their properties of pairing (Cooke et al., 2003). This list is far from exhaustive, but all these lesions have the capacity to generate mutations or block duplication and transcription of DNA (Tornaletti et al., 1997; Tornaletti and Hanawalt, 1999; Tornaletti et al., 2004).

1.4.1. DNA repair mechanisms

Cells apply distinct repair pathways for different kinds of lesions, in several levels and with important superposition between them. Even when these barriers fail, the cells still have mechanisms to tolerate lesions (Friedberg et al., 2006). The main DNA repair systems are direct, excision and recombinational repair, with extensive reviews in the literature (Alberts et al., 2007; Cooper, 2000; Friedberg et al., 2006; Tropp, 2012) and schematized in Fig. 5.

The direct repair is a simple and error-free way to reverse damage caused by UV-C or alkylation (Goosen and Moolenaar, 2008). CPD lesions are repaired by photolyases through photo reactivation, a mechanism that uses energy derived from visible light to break the covalent bond between pyrimidines. Alkylated guanines can be reversed by the transfer of methyl groups from the base to a cysteine region in the methyltransferases active site.

Repair of a great variety of DNA lesions can be achieved by base or nucleotide excision, including deamination, alkylation, CPDs and mismatches. The usually bulky lesions are detected and removed, either as free bases or nucleotides (David et al., 2007; Smith et al., 2001; Van Houten et al., 2005). The formed gap is then filled using the undamaged strand as template. The excision systems are nucleotide excision repair (NER, composed by *uvrABC*), base excision repair (BER, carried out by DNA glycosylases, AP endonucleases and dRpases) and mismatch repair (MMR, composed by *mutS* and *mutL*, with an additional *mutH* in *E. coli* (Radman and Wagner, 1986)). All of them are finished by the actions of UvrD, DNA polymerase II and DNA ligase (Fig 5).

Finally, the recombinational repair can deal with broken chromosomes and single-stranded gaps. Homologous recombination permits the use of an undamaged homologous chromosome as template to repair the gap. The search for this template and the strand invasion are catalyzed by RecA, loaded in the damaged site by one of the classical (RecBCD and RecFOR) or alternative (RecQ, RecS, RecJ) pathways. When a homologous chromosome is not available, double strand breaks can be repaired by the highly mutagenic non-homologous end joining (NHEJ), carried out by homologous of the eukaryotic Ku protein and ATP-dependent DNA ligases (Pitcher et al., 2007; Schwartz et al., 2005).

Several transcriptional regulators can modulate the response of DNA damage and induce these repair systems, through mechanisms not always determined (Hong et al., 2009) and connections to oxidative and general stress responses (Storz and Hengge, 2010). The most extensively studied DNA damage response network is the SOS system, which relies in the sensing of ssDNA by RecA and inactivation of the LexA transcriptional repressor.

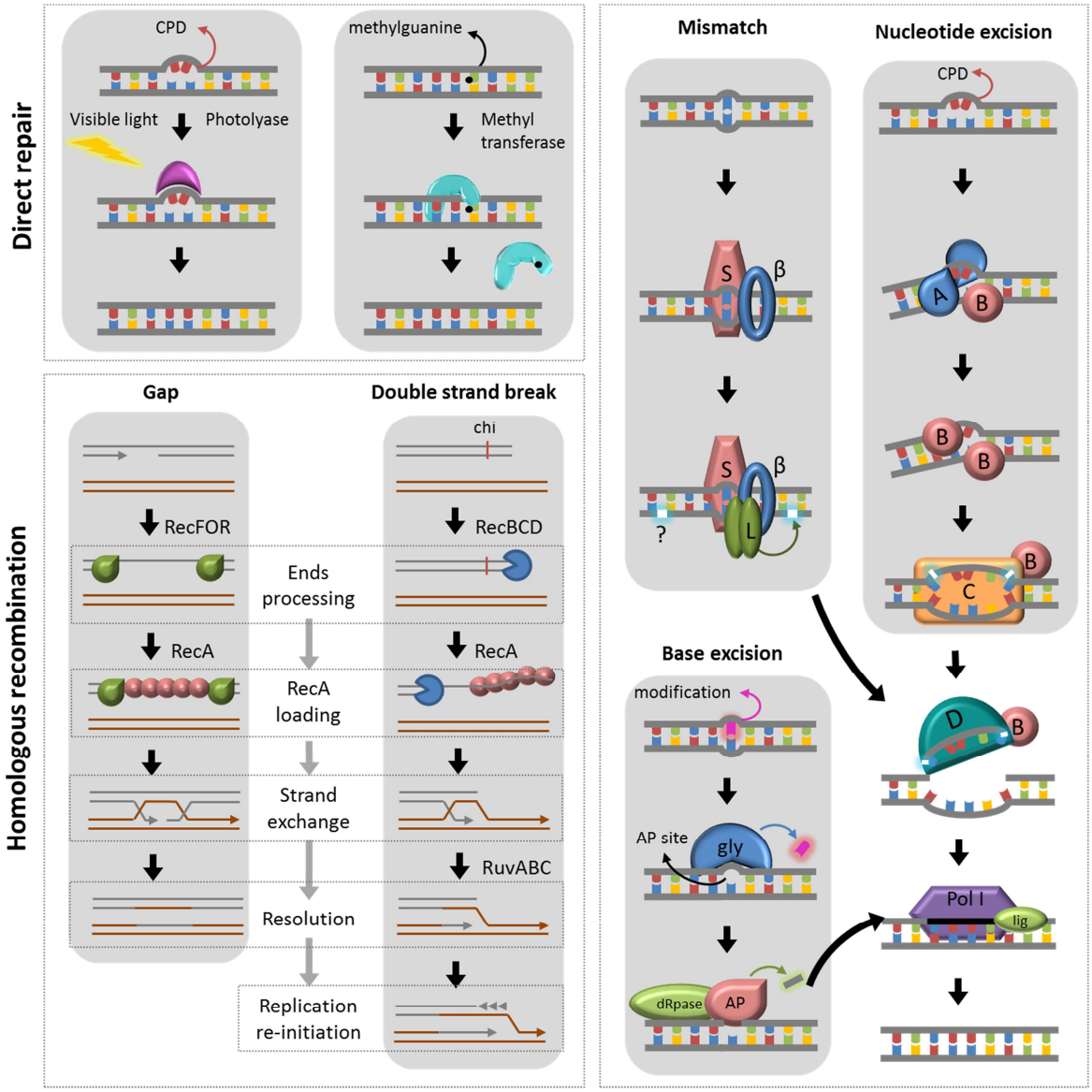

Figure 5. Main DNA repair pathways. Direct repair is carried out with photolyase or methyltransferases; classical pathways of homologous recombination are catalyzed by RecA, loaded onto DNA by RecFOR at ssDNA gaps and by RecBCD at double strand breaks; after strand invasion, RuvABC resolve the Holliday junctions and replication initiation occurs at former double strand break sites. The enzymes responsible for mismatch repair are MutS and MutL, which interacts with the β clamp of the replisome. Nucleotide repair is carried out by UvrABC. Both excision repair pathways use also UvrD. Finally, base excision repair is catalyzed by specific DNA glycosylases (gly), AP endonucleases (AP) and dRpases. The repair is finalized by DNA polymerase I and DNA ligase.

1.4.2. SOS induction cycle

The mechanisms of SOS activation have been extensively studied in *Escherichia coli* and *Bacillus subtilis* (Cheo et al., 1993; Friedberg et al., 2006; Janion, 2001; Little et al., 1981; Winterling et al., 1997). During normal bacterial growth, without any significant level of lesions on the DNA, LexA molecules are bound as dimers to operator sequences usually

located at the promoter of the regulated genes, the SOS box. Binding of LexA represses expression by physically blocking the binding of RNA polymerase (Fig. 6). However, a basal level of expression is allowed, since binding is intermittent. Occurrence of several kinds of damages results in single stranded DNA (ssDNA), either directly or through processing of lesions or strand breaks. Several monomers of RecA are recruited, forming filaments with ssDNA and turning to an active form. Interaction of the ssDNA-RecA complex with LexA induces the auto-cleavage of the repressor. The resulting polypeptides lose capacity of DNA binding, allowing RNA polymerase to have access to the promoters. Two classical genes controlled by SOS is *recA* and the repressor itself, providing an off-switch to the system: as soon as the lesions are repaired, levels of ssDNA-RecA filaments start to drop; without interaction with activated RecA, the level of intact LexA protein rises; and promoters once again are occluded by LexA dimer binding, eventually shutting the whole system down.

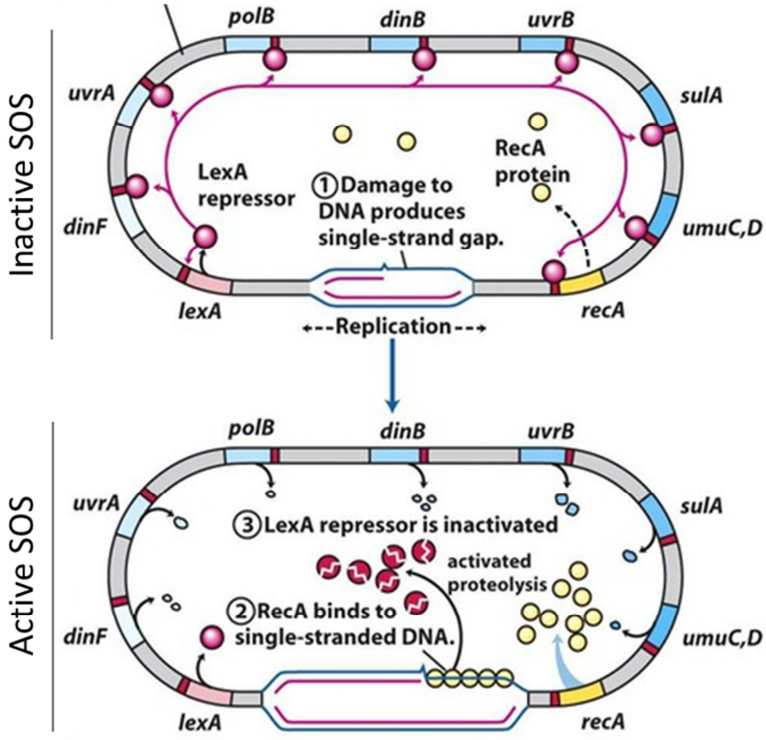

Figure 6. SOS induction as characterized in *E. coli*. While the system is inactive, the LexA repressor is bound to the promoter of several genes, including itself and *recA* (1). Once there is any damage in the DNA causing ssDNA, RecA is recruited to these regions (2). The interaction between LexA and the filament RecA-ssDNA cause the auto-cleavage of the repressor, releasing the expression of the controlled genes (3). Modified from Lehninger Principles of Biochemistry, fifth edition.

1.4.3. LexA repressor

The SOS regulator belongs to Family S24 of serine-proteases, which mechanism involves a catalytic dyad Ser_{119}-Lys_{156} in *E. coli* (Slilaty and Little, 1987). The structure of the protein is divided in two regions. The amino-terminal domain, formed by three alpha-helices and two beta-barrels, is responsible for DNA binding. The carboxy-terminal domain, composed only by beta-barrels, is where dimerization and catalysis occur. The carboxy-terminal domain exists in two distinct conformations, cleavable and non-cleavable. In the non-cleavable form, Lys_{156} is protonated and exposed to the solvent. In addition, the targeted peptide bond Ala_{84}-Gly_{85} is distant from the active site. On the cleavable conformation the loop containing this specific peptide bond moves closer to the active site (Luo et al., 2001). The new hydrophobic environment created around Lys_{156} drops the pK of its lateral group, allowing the residue to lose a proton and act as a general base to Ser_{119}. The transfer of the positive charged Lys from the solvent to a hydrophobic cleft is an important energetic barrier. As so, LexA only undergoes self-cleavage when the cleavable form is stabilized by the interaction with activated RecA (Butala et al., 2009). The same mechanism hold true for other members of the Family S24, as phage lambda regulators cI/Cro, which are also cleaved in contact with activated RecA (Little, 1984). Binding to DNA is mediated by helices 2 and 3 from the amino-terminal region, which form a helix-turn-helix domain. LexA dimers interact with imperfect palindromes (sometimes direct repeats) in the DNA major groove. SOS boxes are greatly variable throughout the bacterial taxa due to the lack of conservation in the LexA DNA-binding domain from different groups (Mazon et al., 2004).

1.4.4. SOS response consequences

Even with high diversity of SOS regulons among bacteria, they usually play a role in aspects of DNA repair, replication, recombination and regulation of cell cycle events (Storz and Hengge, 2010). Cell division inhibition activated by SOS leads to a classical filamented phenotype (Friedberg et al., 2006; Trusca et al., 1998). The main DNA repair systems activated by SOS are recombination and excision repair. Moreover, it induces damage

tolerance mechanisms, such as alternative polymerases. These are part of the Y family of polymerases, capable of translesion synthesis of DNA on damaged sites where the replicative polymerase gets stalled. DNA polymerases V (*umuCD*) and IV (*dinP*) have low fidelity (Goodman, 2002; Neeley et al., 2007; Ohmori et al., 2001), and the last is associated with increasing occurrences of -1 frameshifts (McKenzie et al., 2001).

These mutator phenotypes associated with the up-regulation of *dinP* and *umuCD* can lead to important consequences for pathogens, due to the potential for developing antibiotic resistance and increased persistence inside the host. Other relevant aspect in the medical point of view is the induction of prophages and integrons directly through LexA or cI/Cro. Some classical examples of SOS-dependent induction of toxins are the expression of Shiga toxin in *E. coli* during treatment with beta-lactams (Wagner et al., 2002; Zhang et al., 2000) and cholera toxin in *Vibrio cholera* (Quinones et al., 2005). An additional advantage of the SOS system for pathogens is the dissemination of virulence factors and antibiotic resistance genes, through the increased mobility of prophages (Abella et al., 2007; Dibbens et al., 1992; Harrison and Gabriel, 1983; Singletary et al., 2009), integrons (Cambray et al., 2010; Guerin et al., 2009) and pathogenicity islands (Hacker and Kaper, 2000; Schmidt and Hensel, 2004; Ubeda et al., 2007).

1.4.5. DNA damage response in leptospires

Despite the significance of DNA damage response for adaptation, virulence and diversity of pathogens, little is known about how leptospires handle genotoxic stress. A pioneer work by Stamm *et al.* (Stamm and Charon, 1988) evaluated the sensitivity of several strains of leptospires to UV-C and MMC. All bacteria had the capacity of photo reactivation, although *L. interrogans* strains were extremely sensitive to both agents when compared to free-living *L. biflexa* and *L. illini*. The reasons for this difference were never addressed. However, regulation of DNA damage repair pathways was shown to be important for saprophytic and pathogenic species. Knocking out *recA* from *L. biflexa* resulted in a considerably higher sensitivity to genotoxic stress (Kameni et al., 2002). Moreover, DNA

repair systems seem to be essential for maintenance of virulence in *L. interrogans* even during normal growth. The most remarkable difference in gene expression profile of a virulent strain of serovar Lai in comparison to a attenuated derivative is up-regulation of genes in the DNA replication and repair functional category (Zhong et al., 2011). The first superficial study on SOS response in leptospires was a characterization of LexA binding to the *recA* promoter in *L. interrogans* serovar Lai (Cuñé et al., 2005). A SOS box was determined, and a SOS response composed solely by *recA* proposed.

1.5. Objectives

The aim of this thesis was to characterize the SOS system of *L. interrogans* serovar Copenhageni. Specifically to: (*i*) analyze the behavior of this bacterium to UV-C irradiation; (*ii*) assess the functionality of a putative second LexA repressor; (*iii*) identify the genes controlled by SOS and the sequence responsible for LexA recognition; (*iv*) evaluate the global response to genotoxic stress; (*v*) identify the DNA repair pathways present in *L. interrogans* serovar Copenhageni and their role during SOS activation.

1.6. Structure of the thesis

Our findings investigating the SOS and general DNA damage response in *L. interrogans* will be presented in the three following chapters.

Chapter 1 ("*Leptospira interrogans* serovar Copenhageni harbors two *lexA* genes involved in SOS response") presents the overall response to UV-C-induced damages in the leptospiral genome and a brief characterization of the *lexA*-like gene LIC12654, exclusive of the serovar Copenhageni. UV-C irradiation of serovar Copenhageni led to massive death and filamentation of the survival cells. After 12h, it was observed a peak in expression of some common SOS genes: *lexA1, lexA2, recA, recN* and *dinP*. This was concomitant with LexA1 and LexA2 intact protein levels decrease. LexA1 was able to bind to the promoter regions of these five genes, while LexA2 would bind to its own promoter region. LexA1 binding was though to happen through versions of the $TTTGN_5CAAA$ palindrome, while LexA2 would bind to the $TTGTAN_{10}TACAA$.

Chapter 2 (“Integrated analysis of *Leptospira interrogans* expression reveals induction of mobile genetic elements and repression of virulence genes during genotoxic stress”) explores further the response of serovar Copenhageni to genotoxic stress and the role of LexA1 in regulation of expression. LexA1 was found to bind to 25 sites in the genome using chromatin immunoprecipitation coupled with massive parallel sequencing (ChIP-seq). These enriched sequences were used to redefine the leptospiral SOS box to an asymmetrical imperfect palindrome CTNARYAYYTGTNTAG. Site-associated genes were involved in various processes, including prophage mobilization, cell growth, motility, mutagenesis and some putative virulence factors, in addition to previously identified targets (shown in Chapter 1). Genotoxic stress also influenced the expression of 18% of leptospiral genes, as assessed by microarrays. Increase of expression was observed in transposon and prophage genes, in addition to DNA repair pathways. On the other hand, motility, general metabolism and almost all virulence genes were repressed. These findings are discussed as mechanisms to adapt and evolve potential new characteristics, including virulence.

Intrigued by the sensitivity of serovar Copenhageni to UV-C and conscious of the complexity of the DNA damage response in leptospires, we performed a comparison of DNA repair-related genes of leptospira species with genomes available in public databases. In addition, we assessed the induction of several DNA repair pathways during infection of susceptible animals with *L. interrogans* serovar Copenhageni. The Chapter 3 (“Genomic survey and expression analysis of DNA repair genes in the genus *Leptospira*”) shows more diversity and redundancy of repair genes in *L. interrogans* than in saprophytic (*L. biflexa*) or obligatory pathogens (*L. borgpetersenii*). Direct repair, nucleotide excision repair (NER) and homologous recombination in leptospires have several characteristics typical of Gram-positive, naturally transforming bacteria. Other interesting finding was the huge impact of LGT in providing tools for repair, as seen in direct and excision repair, non-homologous end joining (NHEJ) and also the SOS response. Finally, key players from excision repair, NHEJ and SOS response were shown to be induced during infection.

2. CHAPTER 1

Leptospira interrogans serovar Copenhageni harbors two *lexA* genes involved in SOS response

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Leptospira interrogans serovar Copenhageni Harbors Two *lexA* Genes Involved in SOS Response

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Abstract

Bacteria activate a regulatory network in response to the challenges imposed by DNA damage to genetic material, known as the SOS response. This system is regulated by the RecA recombinase and by the transcriptional repressor lexA. *Leptospira interrogans* is a pathogen capable of surviving in the environment for weeks, being exposed to a great variety of stress agents and yet retaining its ability to infect the host. This study aims to investigate the behavior of *L. interrogans* serovar Copenhageni after the stress induced by DNA damage. We show that *L. interrogans* serovar Copenhageni genome contains two genes encoding putative LexA proteins (*lexA1* and *lexA2*) one of them being potentially acquired by lateral gene transfer. Both genes are induced after DNA damage, but the steady state levels of both LexA proteins drop, probably due to auto-proteolytic activity triggered in this condition. In addition, seven other genes were up-regulated following UV-C irradiation, *recA*, *recN, dinP*, and four genes encoding hypothetical proteins. This set of genes is potentially regulated by LexA1, as it showed binding to their promoter regions. All these regions contain degenerated sequences in relation to the previously described SOS box, TTTGN $_{5}$CAAA. On the other hand, LexA2 was able to bind to the palindrome TTGTAN $_{10}$TACAA, found in its own promoter region, but not in the others. Therefore, the *L. interrogans* serovar Copenhageni SOS regulon may be even more complex, as a result of LexA1 and LexA2 binding to divergent motifs. New possibilities for DNA damage response in *Leptospira* are expected, with potential influence in other biological responses such as virulence.

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Introduction

Leptospira interrogans is one of the etiologic agents of leptospirosis, a worldwide disease with important economic and public health consequences, in particular to developing tropical countries [1,2]. The leptospires can infect a wide range of mammalian species that compose their natural reservoir, colonizing the kidneys, and being shed in the urine during the whole life of these animals [3]. There are nine pathogenic species of *Leptospira*, divided in more than 260 serovars [4]. In Brazil, the majority of the leptospirosis cases in humans is the result of infection with serovar Copenhageni [1]. In spite of its social and economic impact, the molecular mechanisms of *Leptospira* pathogenesis are still poorly understood, as a consequence of the difficulties in their genetic manipulation. Particularly, *L. interrogans* serovar Copenhageni remains one of the serovars most refractory to genetic transformation and only two mutants were so far obtained by targeted mutagenesis [5,6].

L. interrogans can survive in water or mud for weeks, after which they are still able to infect the host. These leptospires are exposed to a wide spectrum of DNA-damaging agents, from sun radiation and heavy metals to oxidative stress and antibiotics [7,8]. One of the most important mechanisms employed by bacteria to deal with stress induced by DNA damage is the SOS response. This regulatory network controls DNA repair, error prone DNA replication, cell division and mobilization of phages and transposable elements in *E. coli*

[9-12]. The expression of these genes is repressed by LexA, which dimerizes and binds to operators in their promoters at regions called SOS boxes [13,14]. The induction of the SOS response is triggered by genomic structure alterations that generate single-stranded DNA (ssDNA), which is sensed by the cells during replication. RecA recognizes and interacts with these damaged regions, acquiring an active conformation and playing a role as a co-factor in the self-cleavage reaction of LexA. The cleavage generally occurs in a peptide bond flanked by Ala-Gly residues near the center of the protein sequence, disrupting LexA dimerization, which in turn, reduces DNA binding and allows transcription initiation [15-18]. Once the damage is repaired, the level of activated RecA drops, and newly synthesized LexA (whose expression is usually under its own regulation) binds to SOS boxes again, returning the system to the non-induced state [11].

The *lexA* gene is found in most bacterial taxa, with few exceptions. However, the set of genes directly repressed by LexA diverges substantially. This is in part consequence of the great degree of sequence heterogeneity among SOS boxes. The sequence variation is a result of the low conservation in amino acid residues in the DNA binding domain of LexA among different groups of bacteria [19]. Although the majority of bacteria has only one copy of *lexA*, genomic sequences of some organisms underwent duplication or lateral gene transfer, resulting in two genes coding LexA proteins regulating different sets of genes [20-22]. As a consequence, the SOS response is a very unique and complex regulatory network, with a remarkable flexibility of LexA-regulated genes.

The SOS response has important consequences for bacterial physiology and for virulence mechanisms in pathogenic organisms [23,24]. All leptospiras sequenced to date harbor one copy of the *lexA* gene. Previous work reported that the LexA protein from *L. interrogans* serovar Lai (LA1447) [25] has activity of a transcriptional repressor, acting only on *recA* gene expression. Moreover, the identified SOS box palindrome (**TTTG**CTATA**CAAA**) was found only upstream of the *recA* gene. As such, *L. interrogans*, along with *Thermotoga maritima*, would be among the rare organisms in which LexA does not regulate its own transcription [26]

In this study we show that the DNA damage induced by UV-C irradiation triggered the SOS response in *L. interrogans* serovar Copenhageni. Analyzing the bacterium genome, we found a second *lexA* gene (*lexA2*) within a prophage-like region rich in genes encoding hypothetical proteins. Following the stress induced by UV-C irradiation, *L. interrogans* displayed filamentation and both LexA repressors were depleted, presumably as a consequence of self-cleavage. The expression levels of both *lexA* genes, as well as those of other seven genes, were increased from eight up to 12 hours after the UV-C treatment. LexA1 was able to bind to the promoter sequences of *recA* and *recN*, and competition assays indicate its binding to the promoters of the remaining UV-C induced transcripts, including the one containing *lexA1*. Not all the genes showing UV-C induction do have the exact previously described SOS box in their promoter regions, but alignment of these sequences showed the presence of imperfect palindromic dyads that could be LexA1 binding sites. On the other hand, LexA2 showed specific binding only to a sequence upstream of its own transcriptional unit. Therefore, the physiological response of *L. interrogans* serovar Copenhageni may be even more complex than in other bacteria, as LexA1 appears to have some flexibility to recognize degenerated SOS sequences.

Material and Methods

In silico *analysis*

All sequences used were obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) and National Center for Biotechnological Information (NCBI) databases. The secondary structure predictions were made by PsiPred [27], and search for structural domains in hypothetical protein sequences by HHPred (http://hhpred.tuebingen.mpg.de). Finally, the search for putative SOS box motifs was carried out by using the tool "genome scale DNA-pattern", available from RSAT (http://rsat.ccb.sickkids.ca). The output returned all palindromes present in the upstream region of the genes (nucleotides +20 to -250 from the start codon).

Phylogenetic analysis

A total of 48 protein sequences (Table S1) were obtained through BLAST searches using either LexA1 or LexA2 as query. MUSCLE [28,29] alignments were used to infer phylogenetic trees, constructed using maximum likelihood analysis with WAG substitution model in PhyML [30]. The robustness of the trees was assessed by aLRT [31]. Node support was assessed as the posterior probability from two independent runs, with four chains of 200,000 generations each (sampled at intervals of 100 generations with a burn-in of 1000 trees).

Bacterial strains and growth conditions

L. interrogans serovar Copenhageni Strain FioCruz L1-130 and other serovars (Australis, Autumnalis, Bataviae, Canicola, Pomona, Pyrogenes, Hardjo) and *L. borgpetersenii* serovar Hardjobovis were obtained from Faculdade de Medicina Veterinária e Zootecnia (Universidade de São Paulo, Brazil), while the genomic DNA from serovars Smithi and Naam were obtained as described by da Silva et al. [32]. The growth and virulence maintenance were carried out according to da Silva et al. [33]. *E. coli* DH5α and BL21(DE3) Star pLysS were used for cloning and expression procedures, respectively. *E. coli* cells were grown at 37°C in LB medium containing the appropriate antibiotics.

UV-C irradiation, survival curves and visualization

Virulent *L. interrogans* serovar Copenhageni L1-130 was cultivated until density of approximately $4x10^8$ cells/ml. Bacteria were transferred to 140mm diameter Petri dishes, conserving a thin layer of culture, and exposed for increasing times to a germicidal lamp (254 nm, rate 1 $J.m^{-2.}$ s^{-1}). After treatment, the same volume of fresh medium was added to stimulate cellular division and the culture was incubated at 30°C in the dark. Surviving bacteria were counted 24 hours post-treatment using

a Petroff-Hausser counting chamber and survival frequency was calculated as the ratio of irradiated to non-irradiated cells. Cells were visualized by fluorescence microscopy after labeling with DAPI (4',6-diamidino-2-phenylindole) [34] and measured using ImageJ software (http://rsbweb.nih.gov/ij). For RNA extraction, cells were collected 4, 8, 12 and 28h following UV-C exposure, as well as their non-treated counterparts, immediately frozen in liquid nitrogen and stored at -80°C until use.

Recombinant protein expression and purification

All enzymes cited in this section were obtained from Fermentas (USA), and used according to the manufacturer instructions. The coding regions of *lexA1* (LIC12305) and *lexA2* (LIC12654) were codon-optimized for expression in *E. coli* (Genscript, USA). These sequences were cloned in the expression vector pAE [35] and the recombinant proteins were over-expressed in *E. coli* BL21(DE3) Star pLysS as described elsewhere [36]. The cells were harvested by centrifugation, ressuspended in 50 mM Tris-HCl (pH 6.3), 150 mM NaCl and lysed in a French press (Thermo Spectronic). The supernatant was applied to a 1 cm-diameter column containing 3 ml Ni^{2+}-charged chelating Sepharose (GE Healthcare Life Sciences, USA). The proteins were eluted with 400 mM imidazole, which was removed by dialysis. Purified proteins were visualized by Coomassie blue staining after separation by 15% SDS-PAGE.

Western blot

For immunoblotting, 20 ng of purified proteins and 40 µg or 150 µg of leptospiral extracts were separated by 15% SDS-PAGE and transferred to Hybond-P Polyvinylidene Difluoride (GE Healthcare Life Sciences, USA) membranes. Incubations and detection were carried out as described elsewhere [36], using anti-LexA1 in 1:5000 dilution, anti-LexA2 in 1:1000 and anti-LipL32 [37] in 1:5000. Anti-LexA2 serum was incubated with 200ng/µl purified LexA1 for 2h prior to use, to decrease cross-reactivity.

DNA purification and PCR

Genomic DNA (gDNA) of *Leptospira* was isolated using DNAzol (Invitrogen), following the manufacturer instructions, and quantified by NanoDrop (Thermo Scientific, USA) spectrophotometer. It was used as positive control in RT-PCR experiments (see below).

RNA manipulation and quantitative PCR

Total RNA was prepared using Trizol (Invitrogen, USA) according to manufacturer instructions and treated with DNaseI (Fermentas, USA) to avoid gDNA contamination. Purified RNA was quantified by NanoDrop. Next, 1 µg of RNA was used as template for the complementary DNA (cDNA) synthesis by the reverse transcriptase M-MuLV (New England Biolabs, USA), using random hexamers. Reverse transcriptase-PCR (RT-PCR) to assess the transcription organization of *lexA1* and *lexA2* vicinities were carried out using 1 µl of 1:5 cDNA as template, for 40 cycles. Quantitative PCR (qPCR) was performed with SYBR Green Master Mix (Applied Biosystems, USA), using 1 µl of 1:100 cDNA in 12 µl reactions. Reactions were set at the default profile of Applied Biosystems 7300 Real-Time PCR System (2min at 50°C and 10min at 95°C, followed by 40 cycles of 15s at 90°C and 1min at 60°C). A posterior dissociation cycle (15s at 96°C, 20s at 60°C, 15s at 90°C and 15s at 60°C) was added to discard the existence of any contaminating product. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method, using the 16S as internal control. Each experiment was repeated three times, with biological replica. Data were analyzed through GraphPad Prism5, where the variance was assessed by one way ANOVA and significance of differences by Dunnett post-test. Oligonucleotides used in these experiments are compiled in Table S2.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed using DIG Gel Shift Kit (Roche), following the manufacturer instructions. Probes amplified by PCR (Table S2) were purified by GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, USA), quantified by NanoDrop and labeled with a terminal DIG. Alternatively, probes were labeled with [γ ^{32}P ATP] by T4 Polynucleotide Kinase (Fermentas) and column purified (GenElute PCR Clean-Up Kit, Qiagen). Binding reactions were carried out in ice, in binding buffer provided in the kit, using poly[d(A-T)] as unspecific competitor. Solutions containing 1.55 fmol of labeled probes were incubated with 40 µg leptospiral extracts or 80 ng purified LexA2 for 20min. In antibody blockade assay, extracts were incubated with 1 µl of anti-LexA1 or preimmune sera for 30min in ice prior to the addition of the probe. In competition assays, non-labeled probes were added to the binding reaction after the labeled one, in 200 fold excess (310 fmol). Mixtures were loaded onto a 5% non-denaturing 0.5x TBE gel pre-run at 80 V for 90min. DNA-protein complexes were separated at 80 V for 150min at 4°C and transferred to a Hybond-N (GE Healthcare, USA) nylon membrane using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Germany) in TBE 0.5x for 90min at 5 V. Detection followed the manufacturer instructions, and the membranes were exposed to photographic films (Hyperfilm, ECL GE Healthcare, USA).

Results

L. interrogans serovar Copenhageni genome harbors a second *lexA* gene

Analysis of the genome of *L. interrogans* serovar Copenhageni [38] revealed the presence of a second homologous gene for *lexA*, LIC12654. For clarity, we named it *lexA2*, whereas the gene identical to *lexA* from *L. interrogans* serovar Lai [25] was named *lexA1* (LIC12305). The LexA2 predicted amino acid sequence exhibits very low similarity with the known LexA proteins, sharing 28% of amino acid identity to LexA1. Nevertheless, the predicted secondary structure shows both DNA-binding and serine-protease domains compatible with LexA-like protein structure [16,39] (Figure 1). The catalytic residues Ser and Lys (indicated with arrowheads in Figure 1), located at the carboxy-terminal domain and typically 37 amino acids apart, are at positions 130 and 166 in LexA2, respectively, while the scissile peptide bond is probably located

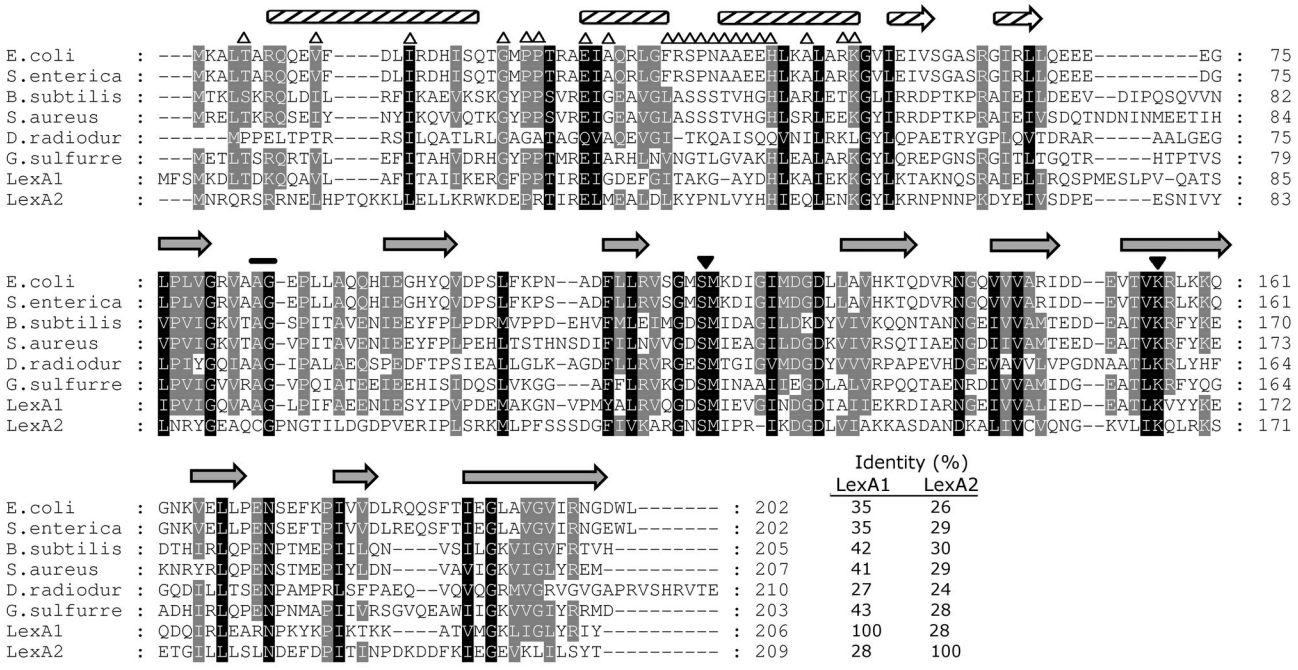

Figure 1. Comparison of LexA amino acid sequences. Amino acid sequence alignment and secondary structure prediction were carried out using *E. coli* LexA as reference. The amino-terminal region is composed of three helixes (striped rectangles) and β strands 1 and 2 (striped arrows), while the carboxy-terminal is composed of nine β strands (grey arrows). Arrowheads indicate the catalytic residues, and the bar indicates the residues flanking the scissile peptide bond. Open triangles represent residues that interact with DNA. The percentage of identity of each sequence to either *L. interrogans* serovar Copenhageni LexA proteins is indicated.

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in Cys_{92}-Gly_{93} (indicated with a bar in Figure 1). The helix-turn-helix structure of the amino-terminal domain of LexA2 is conserved. However, from the 21 amino acid residues potentially involved in DNA binding in this domain (labeled with Δ in Figure 1), 16 are different between LexA1 and LexA2. Since this is the domain responsible for the SOS box recognition, it is conceivable that both proteins must regulate different sets of genes.

LexA2 coding sequence was possibly acquired through lateral gene transfer

The remarkable differences in amino acid sequence of LexA2 raised the question if it was acquired through lateral gene transfer. This hypothesis was tested through phylogenetic analyses (Figure 2). The multiple alignments were used to construct phylogenetic trees with maximum-likelihood algorithm. The distribution of the evolutionary distances and the tree topology reveals a long phylogenetic distance among the two *L. interrogans* serovar Copenhageni LexA repressors. The LexA2 protein grouped in a distinct clade with sequences from marine metagenomes, while LexA1 clustered with orthologous from other leptospires. Therefore, both coding sequences did not evolve together.

Comparative genomic organization of genes *lexA1 and lexA2* vicinities

The *lexA*1 and *lexA*2-containing regions are located within the large inversion which differentiates serovars Lai and Copenhageni genomes [40]. The *lexA1* gene vicinities of both genomes are identical, sharing 99% of nucleotide identity. This region is enriched with genes encoding peptidases and stress response proteins (Figure 3A). Besides the S24 peptidase *lexA1*, LIC12303 is annotated as a S41 peptidase and LIC12302 as a M22 peptidase. The hypothetical protein LIC12304 shows a structure similar to the xenobiotic response element (XRE) family of transcriptional regulators; *czcB* is a heavy metal efflux pump; and LIC12307 is a TolC superfamily transporter protein. The genomic organization, exhibiting less than 43 bp intergenic spaces, indicates a structure of an operon (Figure 3A) [41]. To investigate this hypothesis, primers were designed to amplify across intergenic regions of *L. interrogans* serovar Copenhageni cDNA. The result showed amplicons for all gene pairs, from LIC12308 to *pssA* (Figure 3B), suggesting the occurrence of an mRNA spanning this entire region. The *lexA*2 gene lies within a prophage-like region rich in genes encoding hypothetical proteins. The genome context of *lexA*2 resembles the remnant of an ancient phage infection, which has been subject of mutational decay and rearrangements leading to losses of most of the prophage

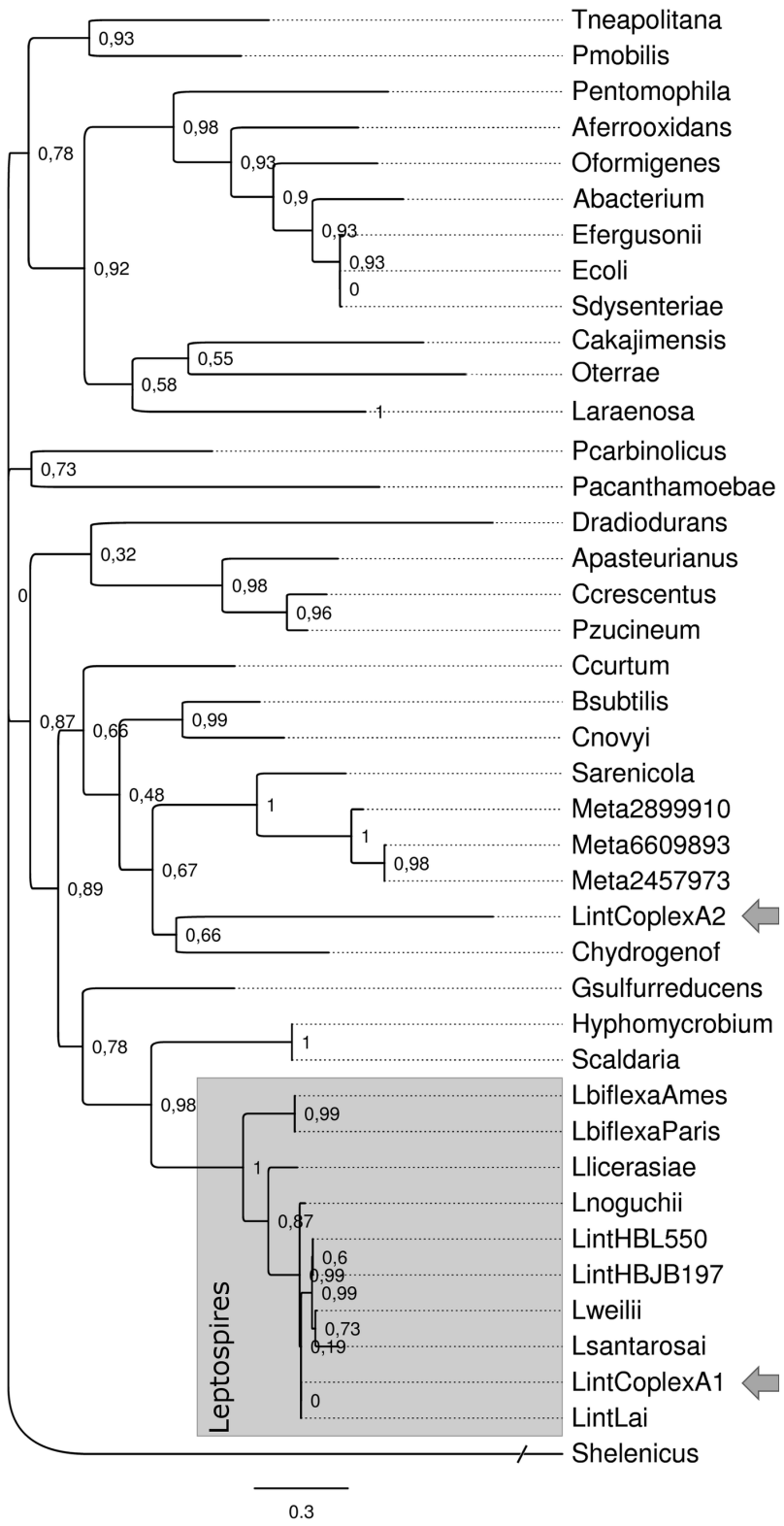

Figure 2. Phylogenetic analysis of LexA. Phylogenetic analysis was performed using LexA amino acid sequences from several bacteria. The leptospiras clade is highlighted by a grey box, and the sequences of the LexA proteins present in *L. interrogans* serovar Copenhageni are indicated with arrows. Local bootstrap values are attached to the internal nodes. Species code description and sequences used are compiled in Table S1.

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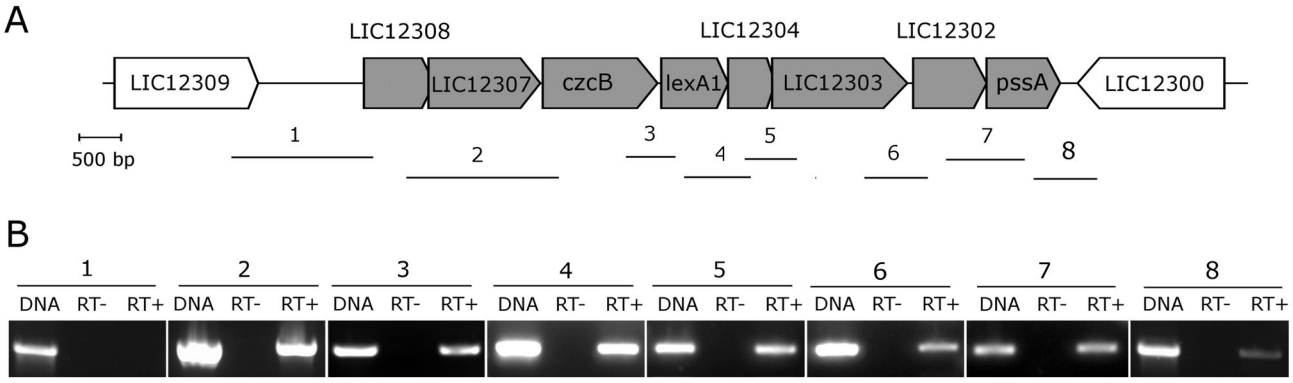

Figure 3. Genomic and transcriptional organization of the *lexA1* region. (A) Schematic representation of the *lexA1* genomic region. The arrows indicate the direction of transcription. The fragments amplified by the primer pairs used for the RT-PCR analysis are indicated by numbered lines below the genes. (B) Composite image of agarose gels from resulting RT-PCR reactions, using either genomic DNA (DNA), RNA (RT-) or cDNA (RT+) as templates. The numbers refer to the respective fragments shown in (A).
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genes. This mosaic architecture also harbors insertion sequence (IS) elements interrupting genes as compared to the equivalent region of serovar Lai (Figure 4A). BLASTX searches revealed the presence of remnants of a phage-integrase gene (Figure 4A) but no duplication or any potential site of insertion could be detected. RT-PCR analysis showed that all the genes surrounding *lexA*2 are probably expressed in normal culture conditions as three different transcripts: LIC12650-12652, LIC12653 and lexA2-LIC12655 (Figure 4B). While LIC12650 and LIC12651 encode phage-related genes, LIC12655 encodes a peptidase of the M28 family. The peculiar nature of this region suggests that both serovars Copenhageni and Lai at some point harbored these similar regions that have evolved apart mostly because of multiple events of insertions and rearrangements. We also investigated the presence of *lexA2* region in the genomes of other serovars and species of *Leptospira* that have been sequenced. MEGABLAST searches at the Whole-genome shotgun contigs database (wgs) at GenBank (Figure S1), together with tentative PCR amplification of *lexA2* using non-sequenced serovars DNA (Figure S2), revealed that this region is highly specific to Copenhageni.

Leptospira displays filamentation after UV-C irradiation

To assess how *L. interrogans* serovar Copenhageni behaves after the stress induced by DNA damage, cells were exposed to increasing UV-C doses. A dose of 4.5 $J.m^{-2}$ was sufficient to kill 50% of the cells, whereas after 18 $J.m^{-2}$, only 10% of the cells survived (Figure 5A). Additionally, persistent UV-C-irradiated cells exhibited filamentation when compared to non-irradiated ones. Cultures were stained with DAPI after 24h of incubation and visualized by fluorescence microscopy. Since the genomic DNA in spirochetes occupies the whole intracellular space [34], the cells were homogeneously stained throughout their length (Figure 5B). Individual bacteria in treated cultures were measured and compared with those from the non-irradiated sample (NI). The average size of at least 200 cells in each experiment was obtained, and it was observed increase in length with higher UV-C doses (Figure 5C). Statistically, these data sets display significant difference, with $P_{value}<0.001$. To better compare the elongated population among the different treatment conditions, the bacterial length values were categorized. Length values corresponding to each of the NI culture quartiles were used to determine size categories. The fraction of persistent cells from the irradiated cultures that were included in the longest category changed from 25% in NI to 46% in the lower UV-C dose, and to 61% in the higher dose (Figure 5C). This is the first observation of filamentation in *L. interrogans* following the stress induced by DNA damage.

Depletion of LexA repressors following UV-C irradiation

Filamentation of bacteria suggests the leptospiral cell division is arrested after UV-C treatment. To assess whether lepstospires are eliciting the SOS response to deal with this stress, the presence of intact LexA repressors in NI and irradiated cells with 4.5 $J.m^{-2}$ was evaluated (Figure 6). For this, we produced recombinant LexA1 and LexA2 (Figure S3), as well as polyclonal antisera against these proteins. Anti-LexA1 serum was LexA1-specific, whereas anti-LexA2 showed some cross-reaction with recombinant purified LexA1 (Figure S4). The cells were harvested 12h after irradiation, since the expected duplication time for *L. interrogans* is 8-12h [42]. Both proteins were detected by western blot in the non-irradiated culture, though LexA1 was barely detectable in the irradiated extract, while LexA2 was not (Figure 6). The lower band is presumably one of the LexA2 fragments resulting from its cleavage. As a control, LipL32, an immunodominant leptospiral lipoprotein [37], did not show changes after UV-C irradiation. These results suggest that both repressors are undergoing the self-cleavage responsible for SOS response induction.

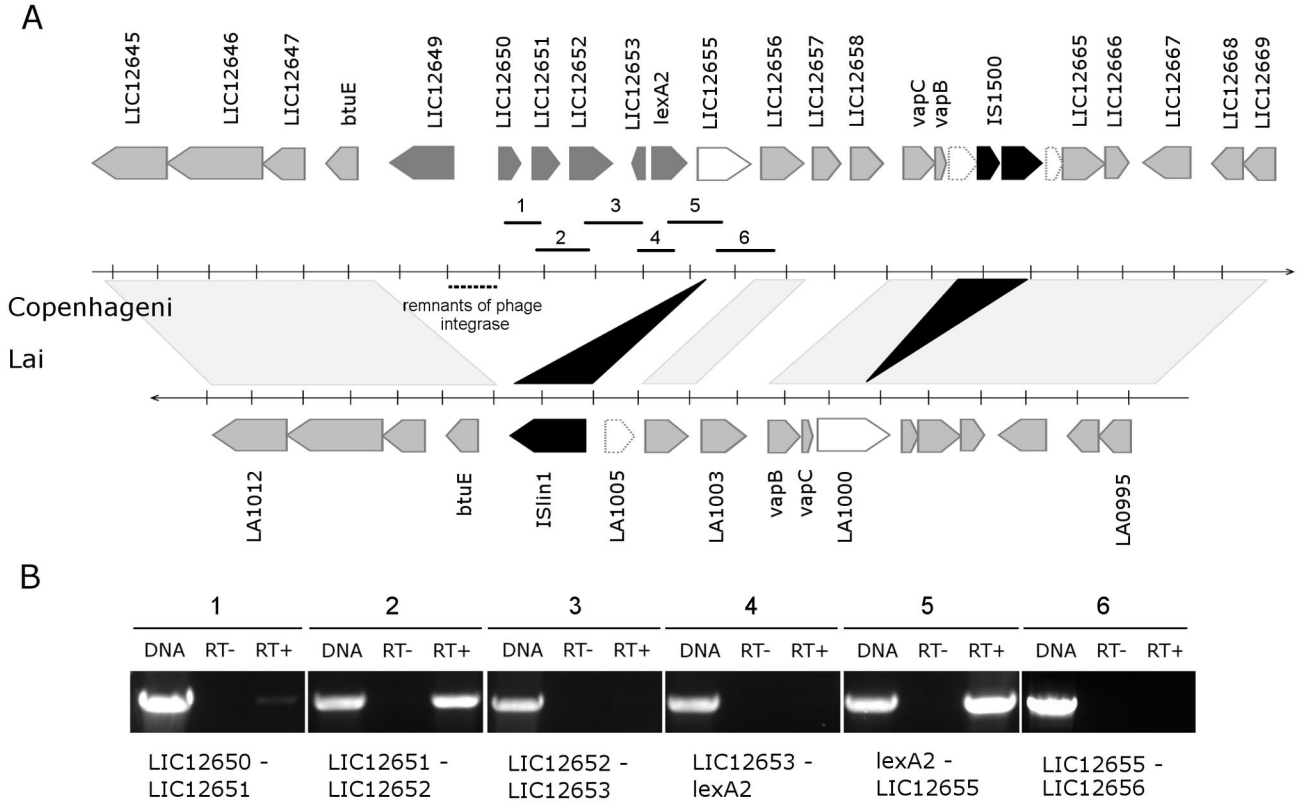

Figure 4. Genomic and transcriptional organization of the *lexA2* region. (A) Schematic representation of the *lexA2* genomic region from *L. interrogans* serovar Copenhageni (upper) compared to the equivalent region of serovar Lai (lower). Arrows represent predicted genes and transcription orientation. Light grey arrows represent genes orthologous between genomes, dark grey genes that are specific to Copenhageni and black arrows indicate genes encoding transposases. The white arrows represent genes with truncated versions in Lai genome (traced arrows) by insertion of IS elements. Remnants of a phage integrase are indicated by a traced line. The numbered bars below the genes indicate the amplified fragments corresponding to the primer pairs used in the RT-PCR analyses. (B) RT-PCR reactions, using either genomic DNA (gDNA), RNA (RT-) or cDNA (RT+) as templates, and primers flanking intergenic regions. The numbers refer to the respective fragments shown in (A).

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Some leptospiral genes potentially involved in DNA repair and lesion tolerance are UV-C inducible

We searched for genes associated with LexA regulon and DNA damage stress induced by UV-C in *L. interrogans* serovar Copenhageni. The *recA* gene expression was examined in different times after UV-C exposure (Figure 7). The up-regulation of recA started 8h and reached its maximum 12h after UV-C exposure. The same assay was performed for *lexA1*, *lexA2* and other genes whose expression is generally UV-C regulated in other organisms (Table 1 and Figure 7): the recombination-involved gene *recN*, the DNA polymerase IV (*dinP/dinB*), responsible for translesion DNA synthesis and *uvrA*, the excinuclease subunit A from the nucleotide excision repair (NER). To avoid false positives, genes were considered differentially expressed only if their fold change following UV-C irradiation had strong statistic support. Except for the non-induced *uvrA*, all genes showed the same pattern of transcriptional regulation, with the difference that *recN* induction started sooner, after 4h. The similarity among the expression profiles is an indication of a co-regulation mechanism.

In an attempt to identify other genes that could be required following UV-C induced stress, 21 genes involved in DNA repair and members of the SOS regulon in other organisms [43-45] had their expression levels measured. None of the new genes tested was induced by UV-C (Table 1). Surprisingly, the homologues of two cell division inhibitor proteins, *sulA* from *E. coli* [46] and *maf*, from *B. subtilis* [47] did not respond to UV-C. In addition, a bioinformatic search was performed for the described SOS box palindrome **TTTG**N $_5$**CAAA** from *L. interrogans* serovar Lai [25] in the upstream region of all annotated genes in the genome of *L. interrogans* serovar Copenhageni. Seventy genes were detected, including nine identical ISlin1 transposases (Table S3). As expected, the exact palindrome was found in *recA* promoter region, in addition to *recN*, and genes encoding some permeases, oxidoreductases, and others. From this set, 11 genes were

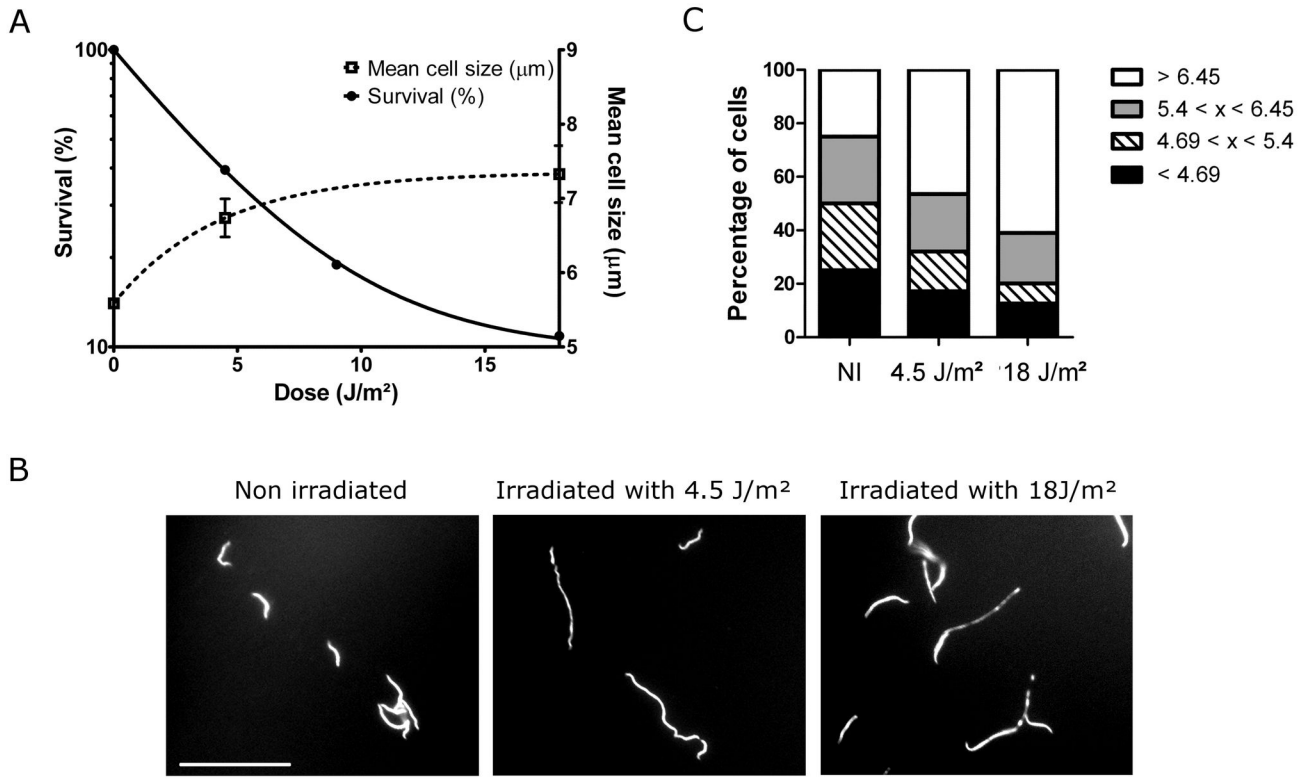

Figure 5. Phenotypic analyses of *L. interrogans* serovar Copenhageni after UV-C irradiation. (A) Relationship between survival after treatment with increasing doses of UV-C (left axis) and the mean size of the persistent cells (right axis). Cultures of bacteria were treated with increasing doses of UV-C, diluted in fresh medium and incubated during 24h in the dark. Surviving bacteria were counted and the frequency was calculated as the ratio of irradiated to non-irradiated cells. (B) Fluorescent microscopy of leptospires in cultures treated or not with UV-C, after staining with DAPI (1000x magnification). The bar represents 20 μm. (C) Frequencies of the size categories from treated and non-treated samples. Categories were determined by the size values (expressed in μm) that divided the non-irradiated culture in its four quartiles. All the data represent the average of three independent experiments.

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tested by qPCR, but also failed to increase their expression after UV-C irradiation (Table 1).

Analyses of *lexA1 and lexA2* vicinities reveal other UV-C inducible genes

The gene expression pattern after UV-C exposure was also evaluated for the *lexA1* and *lexA2* genomic regions. Interestingly, the genes in *lexA1* operon exhibits distinct regulation pattern after UV-C treatment. While *lexA*1 and LIC12304 showed more than eight-fold increase in expression, LIC12303 barely reached the cutoff (Figure 8A). The other genes did not display significant expression change in irradiated sample relative to the non-irradiated one. Thus, this profile suggests the presence of an alternative promoter within the *czcB-lexA1* intergenic region, which may be bound by LexA1, in contrast with previous findings [25]. In the case of *lexA2*, we have already shown that the six genes in the vicinity of *lexA2* are probably expressed in three different transcripts (Figure 4). These transcriptional units became more evident during analysis of induction after UV-C irradiation by qPCR (Figure 8B). The LIC12649 and LIC12650-12652 transcripts did not show significant fold change in expression, whereas LIC12653 and *lexA2*-LIC12655 were both up regulated by more than six fold (Figure 8B). Since these genes are oriented in opposite directions, they probably share an SOS box.

Correlation between UV-C-up-regulation and LexA1 binding

We performed EMSA to test whether the recombinant LexA1 was able to bind to the UV-C up-regulated gene promoters, using the *recA* promoter as positive control (recAup). As purified LexA1 did not show DNA-binding activity (data not shown), we tested if total cell extract contains any factor with this activity (Figure 9A). The incubation of *recA* and *recN* promoters with leptospiral extract revealed the formation of protein/DNA complex, as mobility shifts were obtained (Figure 9A). The specificity of the protein/DNA complex was confirmed by competition with 200 fold excess of unlabeled DNA fragment identical to the probe binding assay, resulting in abolishment of the complex (Figure 9A). To ascertain if LexA1 was present in

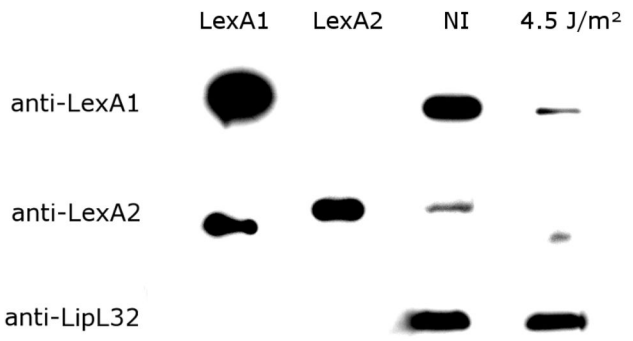

Figure 6. Presence of LexA1 and LexA2 repressors in *L. interrogans* extracts. Immunoblotting of total cell extract and purified LexA proteins was probed with antisera against LexA1 (1:5000 dilution), anti-LexA2 preincubated with 200 ng/µl LexA1 (1:1000 dilution) and anti-LipL32 (1:5000 dilution). The blots contained 20 ng of recombinant purified LexA1 and LexA2. In LexA1 and LipL32 blots, 40 µg of cell extracts from non-irradiated (NI) or irradiated (4.5 $J.m^{-2}$) cultures were used, whereas for detection of LexA2, 150 µg.
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the protein/DNA complex, we incubated the extract with anti-LexA1 prior to the addition of the probe (Figure 9A). The formation of the complex was completely abolished, indicating the recognition of LexA1 by the antibody, whereas pre-immune serum was not able to inhibit binding. This result confirms the binding of LexA1 to two of the UV-C induced gene promoters. Our analysis proceeded with competition binding experiments to address if LexA1 also binds to *lexA1*, *dinP* and *lexA2* promoters, as they were UV-C induced (Figure 9B). To decrease background problems, [γ-^{32}P] ATP-labeled *recA* promoter was used as probe. The LexA1/recAup complex was disrupted when unlabeled DNA fragments containing the promoters from *recA*, *recN*, *lexA1*, *lexA2* and *dinP* were added to the binding reaction in 200-fold excess. Promoters from all UV-C up-regulated genes were extremely effective in abolishing the protein/DNA complex in this concentration. On the other hand, sequences containing the previously predicted LexA1 box upstream of non UV-C-induced genes did not abolish the complex. It is important to emphasize that total cell extract contains many different DNA-binding proteins, and it is possible to have non-specific binding to the DNA probe. In these situations, the protein/DNA non-specific complexes migrate very slowly and diffusely, and are not abolished by the specific competitor [48]. Therefore, the complexes disrupted in our EMSAs are probably specific to LexA1 protein.

The exact SOS box described for serovar Lai [25] was found only in the promoter regions of *recA* and *recN*. However, our results point to LexA1 binding to its own promoter, as well as *lexA2* and *dinP* upstream sequences, suggesting that there is some flexibility of sequence for LexA1 binding. Alignment of these promoters revealed the presence of similar versions of the TTTGN $_5$CAAA palindrome in all of the UV-C induced genes (Figure 9C). The *lexA1* gene has a modified SOS motif, TTTAttcttAAAA. This palindrome could be responsible for gene repression, even with two mismatches. The same could be true for *dinP*, which harbors one possible SOS motive containing two substitutions, TTCGaaattGAAA. For *recN*, beyond the palindrome TTTGgaagaCAAA identified by our bioinformatics searches, there is a modified palindromic motif located within the gene predicted promoter: TTTGtatagCAAT. The alignment also shows that from the eight positions of the putative SOS box, only four are conserved: two thymines in one half and two adenines in the other half of the palindrome (Figure 9C).

The *lexA2* promoter region is bound by both LexA repressors

Gel mobility shift assays were done using purified recombinant LexA2 to test its ability to bind to promoter sequences of UV-C induced genes. The recombinant protein was incubated with labeled DNA segment of its own promoter (lexA2up), and the LexA2/DNA complex was retarded in the gel (Figure 10A). Formation of the upper complex was abolished by the presence of unlabeled upstream sequences from *lexA2*, but not from the other UV-C induced genes. Therefore, these results show the specific binding of LexA2 to its own promoter. The *lexA2* gene possesses a TTTAaatgtCAAG motif in its promoter region, possibly responsible for LexA1 binding. In addition, we identified two other palindromes, potential motifs for LexA2 binding: ATTCN $_{13}$GAAT (box 1) and TTGTAN $_{10}$TACAA (box 2) (Figure 10B). Formation of LexA2/lexA2up complex was not competed out by the ATTCN $_{13}$GAAT motif. On the other hand, the fragment containing the TTGTAN $_{10}$TACAA motif disrupted the complex formation (Figure 10C). A more efficient complex disruption was obtained with longer sequences flanking the palindrome. Accordingly, LexA2 probably binds to the motif **TTGTA**TGCAATGTCT**TACAA**, localized between -146 and -127 of *lexA2* coding sequence, adjacent to its putative promoter (-124 to -96) (Figure 10C).

Discussion

The present work revealed that *L. interrogans* is capable of responding to DNA damage through a coordinated and reversible mechanism, the SOS system. In these situations, the triggered SOS response is not only involved in DNA repair, but also influences antimicrobial resistance spread, general stress response and induction of virulence factors in organisms as uropathogenic *E. coli*, *Salmonella enterica* and *Vibrio cholerae* [10,49,50]. These mechanisms probably also occur in *L. interrogans*, and the full characterization of the DNA damage response is the first step to identify them. The SOS induction in *L. interrogans* serovar Copenhageni after UV-C treatment was confirmed by LexA1 and LexA2 depletion 12h after UV-C exposure (Figure 6), probably a consequence of auto-proteolysis promoted by RecA. In addition, UV-C induced cell filamentation, as seen in several bacteria under stress [10]. This is the first documentation of *L. interrogans* filamentation induced by DNA damage. We could not determine the gene responsible for blocking the cell division, since the genes encoding both cell division protein orthologs present in *L. interrogans* serovar Copenhageni, *sulA* and *maf*, showed no increase in expression after UV-C exposure.

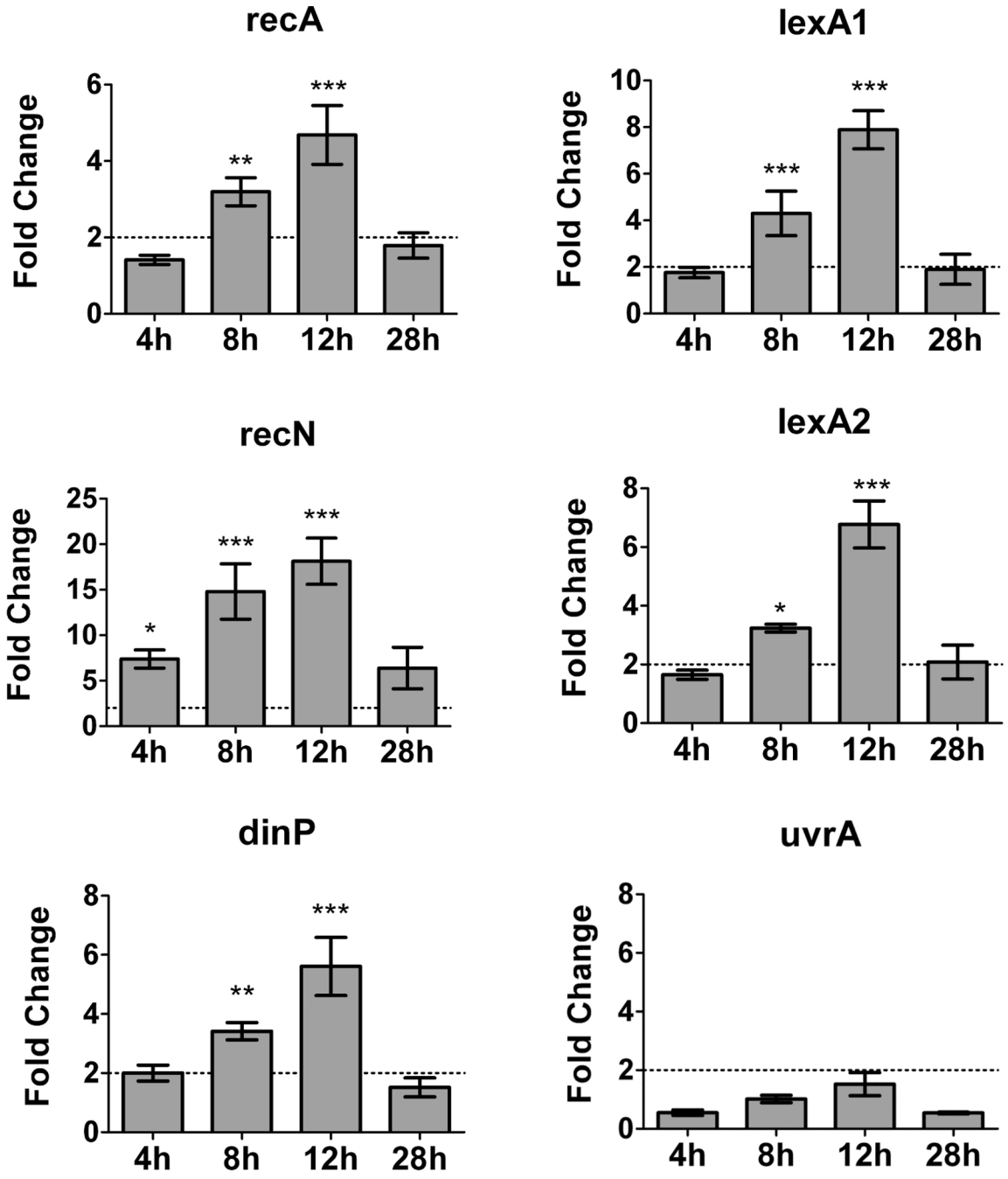

Figure 7. Expression kinetics of *L. interrogans* serovar Copenhageni genes in response to UV-C. The *Leptospira* culture was irradiated with 4.5 J.m^{-2}, kept in the dark, and the RNA samples were obtained at the indicated time points. The fold change corresponds to gene expression in the irradiated sample *versus* the non-irradiated one at each time point, analyzed by qPCR. The 16S was used as normalizer. Error bars represent the standard deviation of the average of three independent experiments. Samples showing significant changes are indicated by *** (P value<0.001), ** (P value>0.01) or * (P value>0.05).

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L. interrogans serovar Copenhageni has 10% survival after 18 J.m^{-2} (Figure 5A). For comparison, the serovar Pomona has this level of survival following exposure to approximately 7.5 J.m^{-2} [51], and the saprophytic *L. biflexa*, near 20 J.m^{-2} [52]. The obligatory pathogen *Borrelia burgdorferi* is even more sensitive and it displays 10% of survival with an UV-C dose of 8 J.m^{-2} [53]. According to these results, *L. interrogans* serovar

Table 1. Leptospiral genes tested for UV-C response.

ORF	Gene	Annotation	Putative SOS box	Fold Change
UV-C responsive*				
LIC11745	*recA*	Recombinase A	**TTTG**CTATA**CAAA**	6.28 ± 1.23
LIC11620	*recN*	DNA repair protein	**TTTG**TCTTC**CAAA**	19.85 ± 0.47
LIC12305	*lexA1*	LexA repressor	**TTT**ATTCTTA**AAA**	8.38 ± 1.78
LIC12304		hypothetical protein		9.34± 1.31
LIC12303		hypothetical protein		2.80± 0.26
LIC12654	*lexA2*	LexA repressor	**TTT**AAATGT**CAA**G	6.76 ± 1.96
LIC12653		hypothetical protein		6.81 ± 1.58
LIC12655		hypothetical protein		5.65 ± 1.64
LIC13052	*dinP*	DNA polymerase IV	**TT**C**G**AAATTG**AAA**	5.60 ± 2.20
Non UV-C responsive*				
LIC10265	*ISlin1*	Transposase	**TTTG**GCGAT**CAAA**	0.69 ± 0.04
LIC10344	—	Anti-sigma factor antagonist	**TTTG**ATTTC**CAAA**	0.52 ± 0.06
LIC10362	—	Hypothetical protein	**TTTG**CTCTC**CAAA**	0.58 ± 0.19
LIC10491	*acrB*	Acriflavin resistance	**TTTG**TGTTT**CAAA**	0.31 ± 0.10
LIC10647	—	SoxW family	**TTTG**AAGAT**CAAA**	0.80 ± 0.09
LIC10867	—	CopG/DNA-binding domain	**TTTG**CTTCG**CAAA**	0.46 ± 0.06
LIC10881	—	TonB dependent porin	**TTTG**AAAGT**CAAA**	0.73 ± 0.83
LIC11702	*dnaG*	DNA primase	**TTTG**TTGGA**CAAA**	0.58 ± 0.11
LIC11925	—	Terminase GpA (phage)	**TTTG**CGATT**CAAA**	0.83 ± 0.18
LIC12993	—	High affinity receptor for IgE Fc	**TTTG**TTTTT**CAAA**	0.52 ± 0.21
LIC13395	—	OsmC-like protein	**TTTG**GACAT**CAAA**	1.03 ± 0.41
LIC11717	*uvrA*	Excinuclease ABC subunit A	—	1.26 ± 0.72
LIC12941	*uvrB*	Excinuclease ABC subunit B	—	0.84 ± 0.48
LIC11756	*uvrC*	Excinuclease ABC subunit C	—	1.49 ± 0.64
LIC11624	*uvrD*	Excinuclease ABC subunit D	—	1.53 ± 0.19
LIC11148	*ruvA*	DNA helicase subunit A	—	1.45 ± 0.08
LIC12811	*ruvB*	DNA helicase subunit B	—	1.32 ± 0.76
LIC12885	*ruvC*	Endodeoxyribonuclease	—	1.92 ± 1.00
LIC12112	*ssb*	Single-stranded DNA binding protein	—	0.58 ± 0.15
LIC13064	*tag*	3-methyladenine DNA glycosylase I	—	0.96 ± 0.91
LIC12362	*alkA*	3-methyladenine DNA glycosylase	—	1.01 ± 0.66
LIC11759	*nth*	Endonuclease III	—	0.43 ± 0.21
LIC10016	*maf*	Cell division Inhibitor	—	1.15 ± 0.89
LIC10837	*sulA*	Cell division Inhibitor	—	0.71 ± 0.06
LIC12245	*hfq*	Host factor-1 (RNA degradation)	—	0.47 ± 0.17
LIC12017	*clpB*	ATP-dependent protease	—	0.34 ± 0.08
LIC10222	*dnaE*	DNA polymerase III subunit alpha	—	0.69 ± 0.13
LIC12109	*dnaB*	Replicative DNA helicase	—	0.61 ± 0.19
LIC11339	*phr*	Deoxyribodipyrimidine photolyase	—	0.72 ± 0.25

Fold change is relative to expression in the non-irradiated sample, and 16S gene was used as normalizer. The *recA* gene SOS box palindrome is indicated in bold letters.

*. After one-way ANOVA and Dunnet post-test, genes with $P_{value} < 0.001$ were considered UV-C responsive.

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Copenhageni has a relatively high resistance to UV-C, comparable to that showed by a free-living spirochete.

The *lexA2* gene is located in an ancient prophage-like region rich in genes encoding hypothetical proteins (Figure 4). Although some of these ORFs are also present in serovar Lai, *lexA2* seems to be exclusive to serovar Copenhageni (Figure S1 and Figure S2). Furthermore, our phylogenetic analysis points out to an event of horizontal gene transfer (Figure 2). Despite the differences in amino acid sequence, the predicted secondary structure of LexA2 shows the necessary features for its activity as a transcriptional repressor. The catalytic residues are correctly placed, as well as the scissile peptide bond, flanked by Cys and Gly (Figure 1). This is not the usual Ala-Gly scissile peptide bond, found in all characterized LexA proteins. However, this substitution still allowed the *E. coli* LexA repressor to undergo autolysis, probably due to similar sizes of side chains in alanine and cysteine [54].

The best way to investigate the role of LexA2 would be the characterization of *lexA1* and *lexA2* mutants. However, *L. interrogans* serovar Copenhageni remains one of the serovars most refractory to genetic transformation. Nevertheless, since only a minor proportion of the DNA transferred from an organism to another is likely to be established in the recipient genome [55], the maintenance of *lexA2* in *L. interrogans* serovar Copenhageni suggests it may have an important role in the bacterium.

The consequence of RecA-dependent proteolysis of LexA is the de-repression of SOS-regulated genes [11]. In this work we identified nine genes that were upregulated following UV-C treatment. They are divided in six transcriptional units (*lexA1*-LIC12304, LIC12303, LIC12653, *lexA2*-LIC12655, *recA, recN* and *dinP*) (Figure 8 and Table 1). All of them presented the same pattern of expression, and their mRNA levels reached a maximum 12h after irradiation. The timing agrees with the expected duplication time, since the attempt of DNA replication is necessary to activate RecA [56]. Twenty-eight hours after the induction the system returned to its normal configuration and no increase on transcripts levels was observed (Figure 7). This coincident pattern of expression corroborates the hypothesis of these genes sharing the same transcriptional control.

In our hands, purified LexA1 protein was unable to bind to promoter sequences of UV-C-induced genes. Thus, we used crude cell extracts in our EMSAs (Figure 9). This approach tries to overcome the problems of working with cell-free

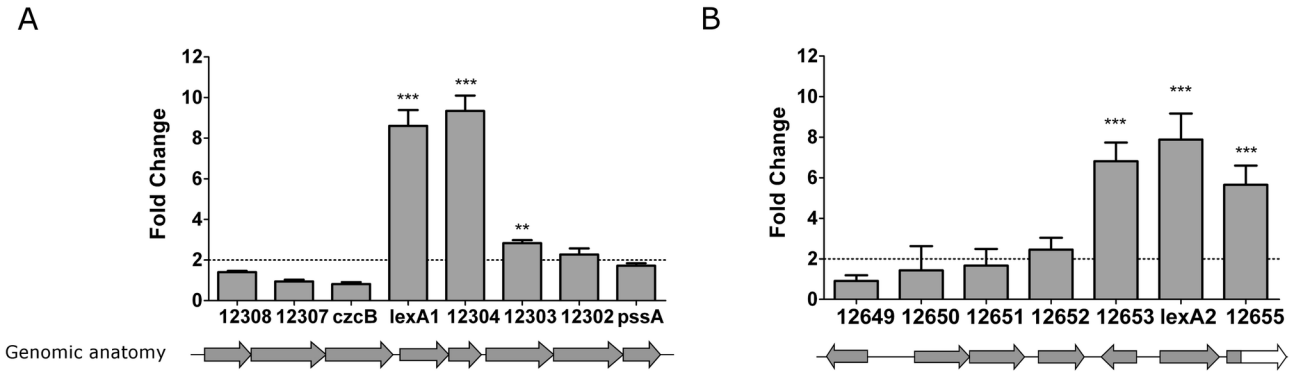

Figure 8. Expression of transcripts in (A) *lexA1* and (B) *lexA2* genomic regions post-treatment with UV-C. The graphic represents the fold change in gene expression in the irradiated sample *versus* the non-irradiated one 12h after irradiation with 4.5 J.m^{-2} and analyzed by qPCR. Genes showing significant changes are indicated by *** (P value<0.001) or ** (P value>0.01). Below, the genomic organization is indicated.

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systems, where the choice of buffer, component concentrations and lack of additional proteins could limit the ability to detect potential LexA1 binding sites [57]. Our results show the formation of protein/DNA complex for *recA* and *recN* promoters (Figure 9A). An antibody blockage reaction confirmed that LexA1 was responsible for the observed shifts. Preincubation of the protein with a specific antibody favors the inhibition of DNA/protein complex formation, leading to the disappearance of the shifted band rather than supershifting it [58]. We used competition binding experiment to address if LexA1 also binds to the promoters of the remaining UV-C-induced genes (*lexA1*, *lexA2* and *dinP*). The disruption of LexA1/recAup complex confirmed the binding affinity of LexA1 to the corresponding upstream sequences (Figure 9B). Therefore, we concluded that LexA1 has binding affinity to the promoters of UV-C-induced genes. The *L. interrogans* serovar Copenhageni LexA1 regulon includes at least genes involved in DNA repair (*recA* and *recN*), DNA damage tolerance (*dinP*), both *lexA* orthologs and four genes encoding hypothetical proteins. The previous SOS motif identified for *Leptospira* LexA [25] is found upstream of *recA* and *recN* genes. However, degenerated sequences are found upstream of *lexA1*, *dinP* and *lexA2* genes (Figure 9C), suggesting that there is some flexibility of sequence for LexA1 binding.

The expression analysis after UV-C treatment in *lexA1* genomic region suggests the presence of an additional internal promoter, upstream of *LexA1*, since only *lexA1* and the downstream genes LIC12304 and LIC12303 were upregulated (Figure 8A). Therefore, this gene cluster may contain additional regulatory sequences, generating transcripts of various lengths. In addition, the hypothetical transcriptional regulator LIC12304 may control other genes, increasing the response complexity.

The *lexA2* gene was also induced by UV-C irradiation (Figure 7), in addition to LIC12655 and LIC12653 (Figure 8B). While LIC12655 encodes a putative M28 peptidase, LIC12653 encodes a hypothetical protein. LexA2 has the ability of binding to its own promoter region, recognizing a SOS box different than that recognized by LexA1, indicated by the EMSA experiments (Figure 10). This could be a result of the different amino acid composition in their respective DNA binding domains (Figure 1). Similarly to what was observed in this work, *Xanthomonas axonopodis* LexA1 binds to *lexA1* and *lexA2* promoters, but LexA2 binds only to its own promoter [59]. This divergences may be consequence of relaxed selection of the extra regulators, and it is probably associated with very small regulons in these cases [60].

Although *recN* was not initially described as a potential member of the leptospiral SOS regulon, it possibly harbors two SOS boxes: the same palindrome found at *recA* promoter, and another one containing a mismatch (Figure 9C). Therefore, this gene may be tightly regulated through multiple binding sites. Leptospiral *recN* expression is induced by UV-C before any other gene analyzed, and reaches almost 20 fold increase after 12h of UV-C treatment (Figure 7 and Table 1). In addition, RecN and RecA may have important roles in the maintenance of virulence, since in *L. interrogans* serovar Lai their expression is enriched in a virulent strain when compared to a virulence-attenuated one [61].

Surprisingly, various genes involved in DNA repair were not UV-C induced (Table 1). Among the *uvr* group (*uvrA, -B, -C, -D*), *uvrA* usually belongs to the SOS regulon, as well as *ruvAB* and *ssb*, although their repression by LexA is not unanimous through different taxa [8]. The genes encoding for proteins involved in Base Excision Repair (BER), responsible for removing small, non-helix-distorting base lesions from the genome, were not induced as well (Table 1). Possibly the basal level of expression of these genes is sufficient to maintain the genome integrity, which would explain the lack of UV-C induction. Taking into account the *L. interrogans* lifestyle, and the amount of environmental pressure this bacterium undergoes either inside or outside the host, a constitutive expression of Nucleotide Excision Repair (NER), a very flexible and versatile DNA repair pathway that removes helix distorting lesions from the genome, and BER genes makes sense [62]. In addition, the recombination repair (which includes RecA and

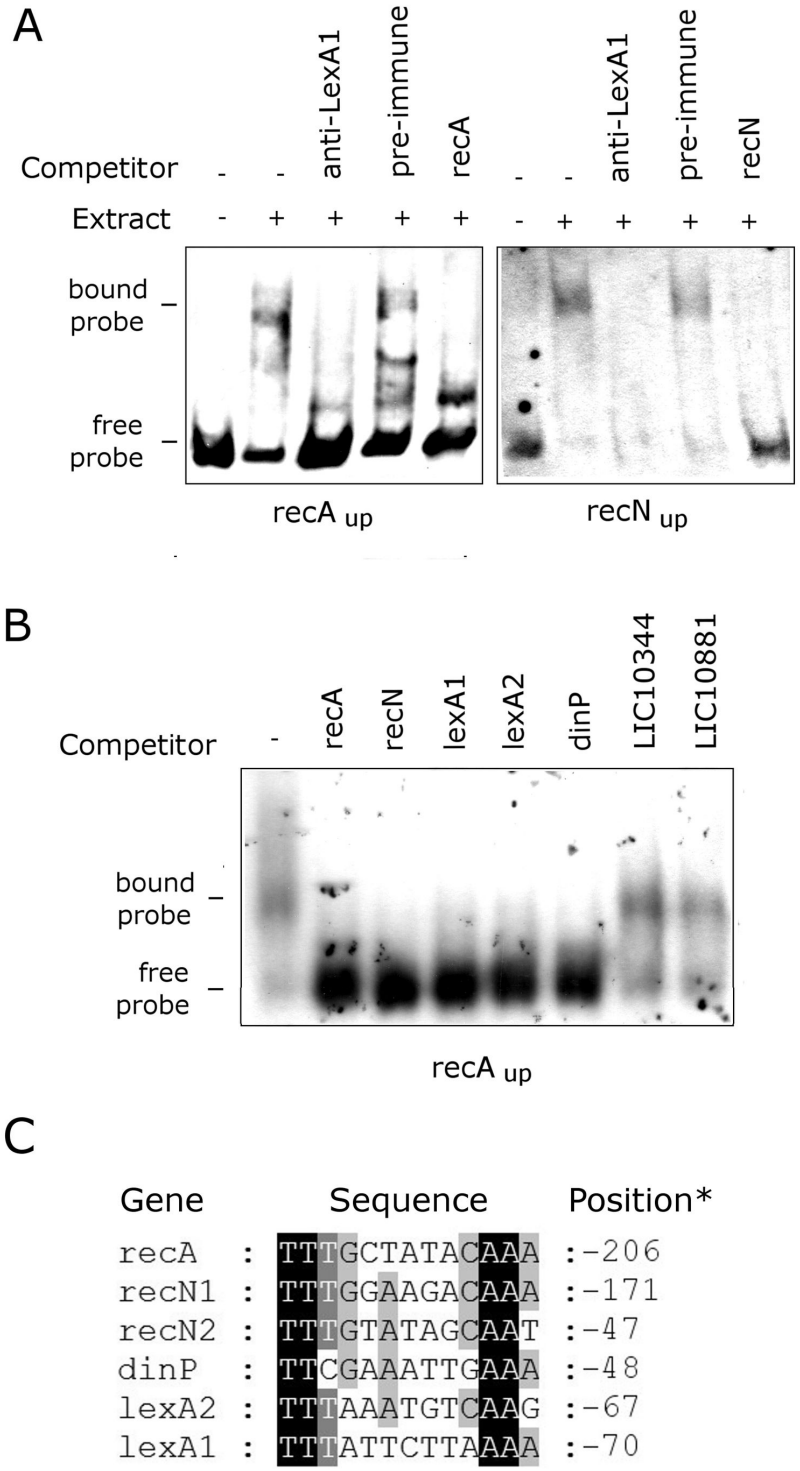

Figure 9. Analysis of LexA1 binding. (A) LexA1 binding assays (40 µg cell extract) with 1.55 fmol DIG-labeled probes corresponding to the upstream sequences of *recA* ($recA_{up}$) and *recN* ($recN_{up}$). Competitors were anti-LexA1 or pre-immune sera (1 µl each), as well as non-labeled probes in 200 fold excess to the labeled ones. Bound and free labeled probes are indicated. (B) LexA1 competition assays, done as in (A), but with 1.55 fmol [γ-^{32}P] ATP-labeled $recA_{up}$. After the binding reaction with protein extracts (40 µg), 200 fold excess of non-labeled fragments were added, corresponding to the upstream sequences of the indicated genes. (C) Alignment of the putative SOS box sequences from UV-C-induced promoter genes. * Distance of the central nucleotide in the palindrome to the initial codon.

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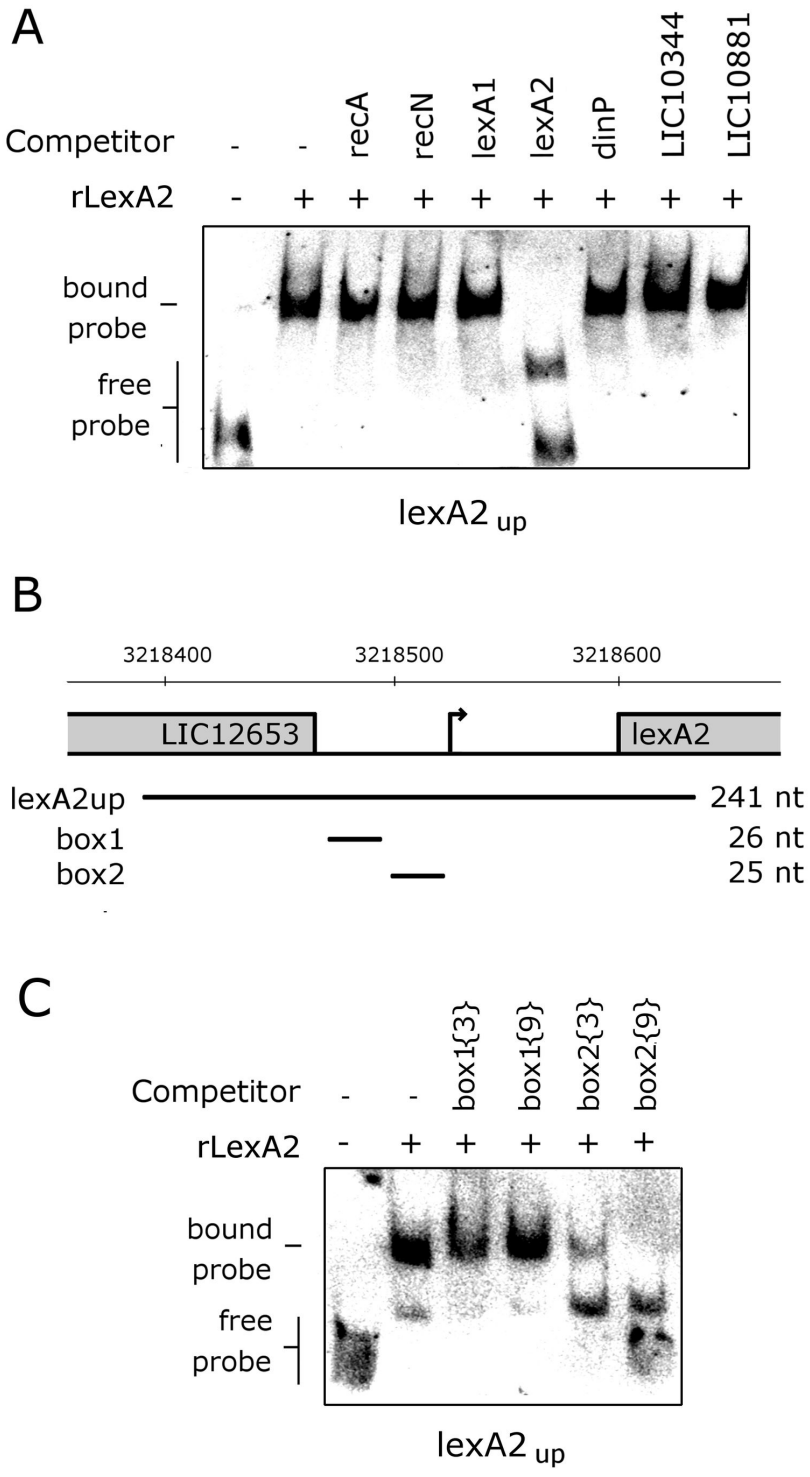

Figure 10. Analysis of LexA2 binding. (A) LexA2 binding assays were carried out with 80 ng of purified recombinant protein, using 1.55 fmol DIG-labeled $lexA2_{up}$ as probe. For the competition assays, 200 fold excess of unlabeled probes was added to the binding reaction. (B) Scheme representing the fragments used for LexA2 binding experiments. Genomic coordinates and size of each fragment (in nucleotides) are indicated. Box 1 indicates the palindrome ATTCN $_{13}$GAAT, and box 2, TTGTAN $_{10}$TACAA. The putative *lexA2* promoter is indicated by an arrow. (C) Competition assays, where 200 fold excess of unlabeled probes was added to the binding reactions, corresponding to the two putative binding sites contained in lexA2up. The number of nucleotides flanking the palindromes are indicates in braces ({3} or{9}).

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RecN) may play a major role in DNA damage response in leptospires, dealing with the additional DNA lesions generated after UV-C treatment.

Trying to extend the known SOS regulon of *L. interrogans*, we performed a search for the TTTGN $_5$CAAA palindrome [25] in the upstream regions of the entire *L. interrogans* serovar Copenhageni genome (Table S3). Some of the resulting genes had their expression levels tested after UV-C irradiation, including one of the nine identical ISlin1 transposases. The SOS response is known to regulate several transposable elements, such as integrons and transposons [63,64]. Interestingly, none of the tested genes exhibited increase in their expression after UV-C irradiation (Table 1). One explanation for this difference is that sequences outside the operator region may affect the interaction of LexA with operator bases *in vivo*. A similar situation was observed for LexA repressor from *B. subtillis* [65]. Also, we cannot exclude the possibility of an accessory protein playing a role in the LexA1/ promoter complex formation.

In *M. tuberculosis*, it was found that LexA was capable of binding to sites with up to three mismatches to the original SOS box [66]. *Leptospira* LexA1 may be capable of binding to the degenerated sequences through the interaction with other proteins present in the extract. The sequence originally considered here is the one present specifically at the *recA* operator [25], though the EMSA results (Figure 9A) show that LexA1 repressor is also capable of binding to somewhat different palindromes. SOS boxes from some organisms are rather variable, as for *Petrotoga miotherma*, in which the consensus GANTN $_6$GANNAC permits a variety of binding sequences [26]. This may be the case of *L. interrogans* serovar Copenhageni. We have few genes to evaluate a new SOS box, but it is possible that nucleotides positioned adjacent to the palindrome, or inside the spacer, play an important role in LexA1 binding.

In this study, we expanded the knowledge on the DNA damage response of *L. interrogans* serovar Copenhageni and on the SOS regulon. UV-C exposure caused the up-regulation of at least nine genes, including *lexA1* and *lexA2*, a second lexA repressor also involved in the SOS response. We were able to show a correlation between the UV-C-dependent increase in the expression of these genes and LexA1 binding to their upstream sequences by EMSA. The depletion of LexA1, as a consequence of self-cleavage triggered by DNA damage, would release it from the promoters and allow the accessibility of RNA polymerase apparatus. Moreover, *lexA2* promoter is also bound by LexA2. In this regard, we were able to characterize the motif (TTGTAN $_{10}$TACAA) responsible for LexA2 binding at the *lexA2* promoter. The existence of two functional LexA repressors indicates a more complex DNA damage response in leptospires than previously imagined. Still, a more specific approach is needed to identify the definitive leptospiral SOS box, and other LexA1 or LexA2 regulated genes.

Supporting Information

Figure S1. Comparison of *lexA2* region between sequenced Leptospiras. Scheme representing MEGABLAST searches against Leptospiras genome sequence projects used the whole genome shotguns contigs database (wgs) at GenBank. The regions shown (red strands) are those with alignment score greater than 200, relative to the region in *L. interrogans* serovar Copenhageni.
(PDF)

Figure S2. Presence of *lexA1* and *lexA2* in the genome of different leptospires detected by PCR. The reactions used 20ng of genomic DNA, and the primers were designed for serovar Copenhageni, according to Table 1. The negative reaction was carried without template DNA. The upper panel corresponds to *lexA1*, while lower panel, to *lexA2*. All amplicons had the expected molecular size corresponding to 621 bp for *lexA1* and 630 bp for *lexA2*.
(PDF)

Figure S3. Expression and purification of 6xHis tagged recombinant LexA1 and LexA2. Both proteins were expressed in soluble form in *E. coli* BL21(DE3) Star pLysS after IPTG-induction. The soluble fractions of the extracts were clarified by filtration and used as column input. After washes, the proteins were eluted by 400 mM imidazole.
(PDF)

Figure S4. Titration and specificity of anti-LexA1 and -LexA2 sera. Anti-sera were generated by intraperitoneal immunization of five BALB/c mice with 10 µg of purified protein in $Al(OH)_3$. The immunizations were performed weekly in four doses and mice were bled by the retrorbital plexus one week after the last dose. (A) Sera titration following the protocol by Hauk et al. (2005), comparing pre-immune and immune sera. Continuous line with squares corresponds to anti-LexA1, while discontinuous line with circles corresponds to anti-LexA2; triangles mark the pre-immune serum. (B) Cross-reaction analyses. The continuous line represents anti-LexA1, and the discontinuous one represents anti-LexA2. Squares stand for coating with purified LexA1, and circles, LexA2.
(PDF)

Table S1. LexA amino acid sequences used for phylogenetic analysis. It is shown the complete species name for each code used in Figure 2, with phylum classification, in addition to the GenBank accession number of the correspondent protein sequences.
(PDF)

Table S2. Oligonucleotides used in this study.
(PDF)

Table S3. Presence of the TTTGN $_5$CAAA palindrome in upstream sequences of *L. interrogans* serovar Copenhageni genes.

(PDF)

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Author Contributions

Conceived and designed the experiments: LSF PLH RMAC. Performed the experiments: LSF JBS JSM LM. Analyzed the data: LSF CBMV RMAC. Contributed reagents/materials/ analysis tools: ZMM SAV MVM. Wrote the manuscript: LSF CBMV MVM PLH RMAC.

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3. CHAPTER 2

Integrated analysis of *Leptospira interrogans* expression reveals induction of mobile genetic elements and repression of virulence genes during genotoxic stress

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ABSTRACT

The DNA damage response of the human pathogen *Leptospira interrogans* serovar Copenhageni was assessed, integrating high throughput data from ChIP-seq and DNA microarrays to determine the role of the SOS regulator LexA1. LexA1 could act as either as an activator or repressor, binding near or inside the coding region of at least 25 genes. LexA1 ChIP-seq peaks shared an imperfect asymmetric palindrome of 16bp, representing the updated leptospiral SOS box. SOS regulon followed the same pattern of expression as the general genotoxic stress response, except for virulence-related genes. UV-C caused a massive rearrangement of several aspects of bacterial physiology, decreasing energy metabolism and translation, in addition to genes related to virulence and motility. On the other hand, mutagenesis and DNA repair, although not expected for bulky DNA lesions, were up-regulated. The most striking feature of this response was the massive up-regulation of two prophages. Our findings point to an expression profile shift from cell growth and virulence to mutagenesis and repair, emphasizing the fundamental role of DNA damage response as a highway for adaptation and evolution.

INTRODUCTION

Leptospira interrogans colonizes the kidney of rats and other mammals, being shed in the urine and surviving for weeks in water and mud until it infects the next host. Bacteria can infect humans through abrasions on skin or in contact with mucous membranes (1). Leptospira invade the bloodstream and can target eyes, kidneys, lungs and liver, causing a variety of symptoms (2, 3). The majority of cases is mild, but up to 10% of patients can develop Weil's syndrome, with acute renal failure, thrombocytopenia, uveitis and pulmonary hemorrhage (4). During infection, antibiotics and oxidative bursts from macrophages can attack directly or indirectly the bacterial DNA. In the environment, leptospires can encounter several other DNA damaging agents (5, 6).

Genetic stability is essential for survival, proper distribution of chromosomes and persistence in the host. When the bacterial DNA is attacked, most bacteria induce the SOS

response, which orchestrates DNA repair and tolerance of remaining lesions. The system is elicited by single stranded DNA (ssDNA) generated by replication fork stall or enzymatic processing of DNA broken ends (5). The controller of SOS is the LexA transcriptional repressor, which binds to promoter regions of genes, blocking their transcription (7). In the presence of unprotected ssDNA, RecA is recruited and activated. In this form, RecA is capable of inducing autocleavage of LexA and other transcriptional repressors, such as λ phage CI repressor. The cleaved repressor no longer binds to DNA, freeing the promoter regions of controlled genes, thereby allowing their increased expression. Since LexA controls its own expression, as soon as the levels of ssDNA (and consequently, activated RecA) drop, enough LexA is produced to shut down the system (7, 8). Although the SOS regulon core is considered to focus in DNA repair, bacteria can use it to better adapt to its environment, regulating mutagenesis and virulence factors expression, such as Shiga-like toxins and type III secretion system of virulence-associated factors in enterohemorrhagic *Escherichia coli* (9-11). Pathogenic bacteria also employ the SOS response to disseminate mobile elements, as prophages and pathogenic islands. This makes the SOS response an important target against virulence and antibiotic resistance (12, 13).

Despite the importance of SOS for pathogens, little is known about how leptospires coordinate mutagenesis and DNA repair. *L. interrogans* serovar Copenhageni, the most frequent causative of human leptospirosis in Brazil, has a second exclusive LexA repressor, LexA2, probably acquired by lateral gene transfer (LGT) (14). However, the main controller of SOS response in this organism is LexA1, shown to bind to both *lexA1* and *lexA2* promoters and at least other three transcripts (containing *recA*, *recN* and *dinP*), all up-regulated after UV-C irradiation (14). In spite of identification of some virulence genes (15-19), the mechanisms *L. interrogans* uses for successful infection are not yet fully understood, and difficulties in genetic manipulation are a major challenge that must be transposed. The first step to overcome such difficulties was sequencing important members of the *Leptospira* genus. Comparative genomics and reverse vaccinology explored new insights from the

genome of pathogenic, intermediate and saprophytic strains (20-23). Transcriptional profiles during some important environmental aspects, as temperature, osmolarity and contact with host (24-28) were also assessed. At present, more effort is being applied to integrate this knowledge and to understand determinants for virulence and adaptation, with pathogenomics and comparative proteomics comparing virulent to attenuated strains (29, 30).

In this work, combining gene expression profile by microarrays and ChIP-seq, the knowledge on mechanisms employed by *Leptospira* to deal with DNA insults was expanded. The known LexA1 regulon comprises at least 25 genes, including genes involved in prophage mobility, cell growth and putative mutagenic and virulence factors. Unexpectedly, DNA repair genes from nucleotide excision repair and homologous recombination were not regulated by LexA1. Mapping and analysis of all LexA1 binding sites allowed us to better define the leptospiral SOS box. Transcriptional profiling during response to DNA damage confirms the induction of SOS regulon and shows striking similarity to the response to the antibiotic ciprofloxacin. Mobile genetic elements are highly induced, and some components of the structural maintenance of the chromosome and mismatch repair are also up-regulated. In contrast, cellular growth, mobility and virulence determinants are repressed. These findings suggests *L. interrogans* turns down virulence and controllably increases chromosome instability during genotoxic stress, leaving room for genotypic and phenotypic variations that could increase fitness of daughter cells.

MATERIAL AND METHODS

Growth conditions and UV-C exposition. L. interrogans serovar Copenhageni Fiocruz L1-130 was cultivated at 30℃ in supplemented EMJH medium (Difco) without agitation (31). The strain was frequently re-isolated from experimentally infected hamsters to maintain its virulence (32). We used the UV-C irradiation protocol as described (13). Briefly, exponential culture of bacteria were exposed (IR) or not (NI) to a UV-C germicidal lamp (254nm), to a dose known to kill 50-60% of cells (5 $J \cdot m^{-2}$). After treatment, the same volume of fresh medium was added to both cultures to stimulate cellular growth and they were incubated at

30℃ for 12h. Next, cells were harvested by centrif ugation (6000xg, 15min) and pellets were immediately frozen in liquid nitrogen and stored at -80℃ until use. All procedures were performed in the dark. This procedure was repeated for four different biological samples.

ChIP-seq. Cross-linked lysates preparation and immunoprecipitation were performed as described by Lin & Grossman (33), with some modifications. Growing cells were harvested by centrifugation (6000xg, 15min), washed and resuspended in formaldehyde 2% final concentration. After 40 or 60min incubation at room temperature, glycine was added and cross-linked cells were lysed. DNA present in lysates was sheared by sonication (30s pulse, 15% amplitude) to fragments between 200 to 600bp and visualized in a BioAnalyzer 2100 (Agilent). Aliquots from lysates ("total" samples) were saved for later analysis. Immunoprecipitations were performed with 1:100 final concentration of anti-LexA1 mouse serum (14), followed by incubation with Protein G Sepharose resin (GE Healthcare). DNA from elution ("IP") and total samples were purified by QIAquick PCR Purification Kit (Qiagen) and used for PCR or high-throughput sequencing.

High-throughput sequencing analysis. Both pairs of IP and total samples (40 and 60 min of crosslinking) were selected for 200-400pb fragments and sequenced in a HiSeq 2500 (Illumina) platform. At least 17 million 40-nt reads were obtained for each sample (Table S1). These reads were aligned to both *L. interrogans* serovar Copenhageni chromosomes (NC_005823.1 and NC_005824.1) using Bowtie2, allowing no mismatches. Reads aligned to the reference genome ranged from 88-94%. The number of reads at each chromosomal position was normalized to the total number of reads of the sample. The reads were extended in silico to the estimated average length of 250bp. The peak-calling tool from CisGenome (34) was used to define enriched regions in each IP sample, using the total sample as background control. The parameters were: -e 250 -b 5 -c 2 -maxgap 25 –minlen 50 –bw 2. Only regions with fold enrichment of at least 2-fold, present in both IP samples and absent from total samples were selected. Aligned reads and peak-calling data were deposited into GEO (accession number to be defined). Graphs shown are for 60min cross-

link sample pair, since it presented more number of reads and it is representative for both treatments. De novo motif search was performed in MEME (35) with 100bp from the center of the peaks, allowing any number of motif occurrences. A reverse search in the peak sequences using the final matrix was performed by FIMO (35), with a cutoff P-value of 0.01.

DNA microarrays analysis. RNAs from UV-C irradiated and non-irradiated samples were purified using RNEasy mini kit with RNA Protect, as described by the manufacturer (Qiagen). RNA quality was assessed by BioAnalyzer (Agilent), with RNA integrity numbers (RIN) greater than 9. Complementary DNA (cDNA) synthesis and labeling were performed with FairPlay III Microarray Labeling Kit following manufacturer's instructions (Agilent), but using only Cy-3. Samples were hybridized to custom gene expression slides for *L. interrogans* serovar Copenhageni, containing eight arrays of 15k spots. Images were captured by Agilent DNA Microarray Scanner Bundle and extracted with Agilent Feature Extraction software version 9.5.3. Microarray intensity data were converted into $\log_2$ and normalized by Quantile. Student t-test analysis was used to identify probes differentially expressed at 12 hours post-irradiation compared to non-irradiated samples and probes representing the same gene were collapsed by taking the probe with lowest P-value. Gene Set Enrichment Analysis (GSEA) was used to determine statistically differentially expressed functional categories. Expression data was deposited in GEO (accession number to be defined).

RNA isolated from mouse infected kidney. The samples used in this study were collected previously (32). Briefly, mice were infected intraperitoneally with virulent leptospira; after five days of infection, organs were collected and total RNA extracted. Total cDNAs from kidneys of six C3H/HeJ susceptible mice were used to test gene expression analysis.

qPCR. All oligos used are listed in Table S2. All qPCR experiments were replicated three times for each biological sample. For validation of ChIP conditions, the amount of DNA correspondent to the *rrs* gene (16S, not bound by LexA1) and *recA* promoter (bound by LexA1) amplified in both IP and total samples was quantified. Fold enrichment was calculated by the ratio $(recA_{IP}/rrs_{IP})/(recA_{total}/rrs_{total})$. ChIP conditions used in this work allowed

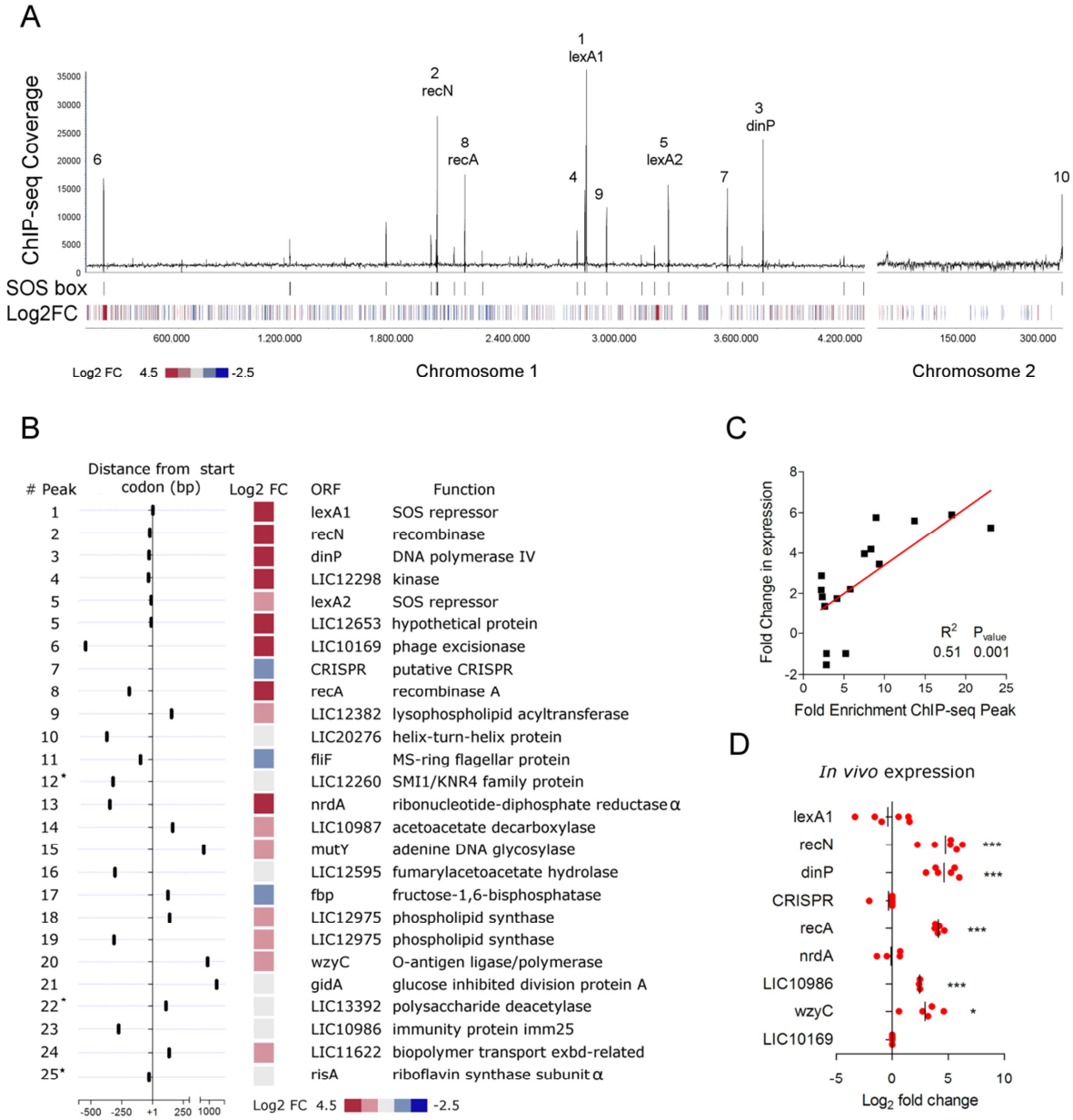
enrichment of *recA* promoter between 3.5- to 6-fold. For validation of microarray data and estimate of expression during infection, fold change was calculated by the $2^{-\Delta\Delta Ct}$ method, with rrs coding region as normalizer. Values from IR cultures or infected kidneys were compared to a normal culture. Any value from the animal assay exceeding one absolute deviation around the median (median-MAD method) (36) was considered an outlier and discarded from analysis. P-values were calculated by two-tailed Student's t-test.

RESULTS

This work aimed to determine the SOS regulon from the pathogenic *Leptospira interrogans* through the identification of whole-genome binding sites of the LexA1 transcriptional repressor by ChIP-seq. Analysis and integration of these data with gene expression profile after UV-C irradiation, through microarrays, gave us an unprecedented integrated view of the gene network controlling repair, tolerance and adaptation responses upon UV-C induced DNA damage in *L. interrogans* (Fig. 1).

Global identification of LexA1 binding sites

Our previous study suggested a flexible nature of LexA1 binding to DNA, hampering the computational search for SOS regulated transcripts. In order to get a better picture of the SOS regulon in *L. interrogans*, ChIP-seq experiments were performed. As described in the Methods section, the IP and two independent total samples (40 and 60 min of crosslinking) were analyzed. The 17 million 40-nt reads for each sample were aligned to both *L. interrogans* serovar Copenhageni chromosomes. LexA1 was associated to 24 loci scattered throughout chromosome 1, and to one site near the origin of chromosome 2. The 10 most enriched binding sites are indicated in Fig. 1A. Enrichment of 11 peak regions in relation to background was validated by qPCR (ChIP-qPCR in Table S3). Peaks were mapped in relation to the putative start codon of a protein-coding gene, and the majority was localized from +5 to -500 (Fig. 1B).



# Peak	ORF	Function
1	lexA1	SOS repressor
2	recN	recombinase
3	dinP	DNA polymerase IV
4	LIC12298	kinase
5	lexA2	SOS repressor
5	LIC12653	hypothetical protein
6	LIC10169	phage excisionase
7	CRISPR	putative CRISPR
8	recA	recombinase A
9	LIC12382	lysophospholipid acyltransferase
10	LIC20276	helix-turn-helix protein
11	fliF	MS-ring flagellar protein
12*	LIC12260	SMI1/KNR4 family protein
13	nrdA	ribonucleotide-diphosphate reductase α
14	LIC10987	acetoacetate decarboxylase
15	mutY	adenine DNA glycosylase
16	LIC12595	fumarylacetoacetate hydrolase
17	fbp	fructose-1,6-bisphosphatase
18	LIC12975	phospholipid synthase
19	LIC12975	phospholipid synthase
20	wzyC	O-antigen ligase/polymerase
21	gidA	glucose inhibited division protein A
22*	LIC13392	polysaccharide deacetylase
23	LIC10986	immunity protein imm25
24	LIC11622	biopolymer transport exbd-related
25*	risA	riboflavin synthase subunit α

Figure 1. Overview of the results from LexA1 ChIP-seq and transcriptional profiling after UV-C-induced DNA damage by DNA microarrays in *L. interrogans* serovar Copenhageni. (A) ChIP-seq coverage is plotted on the y-axis versus chromosomal position on x-axis. Identified LexA1 binding sites (SOS boxes) are indicated in the upper row, while the differential expression data is shown in the lower row. Genes significantly altered after UV-C irradiation (P-value < 0.05) are color-coded according to the $\log_2$ fold change in relation to a non-treated sample. The top 10 enriched peaks are indicated by their numbered rank and previously known targets of LexA1 are named. (B) Genes directly associated with LexA1 binding sites. Each peak is presented from the most to the less enriched, with the distance from the LexA1 binding site to the next coding region graphically represented. Peak-associated genes and their induction during SOS response, as assessed by microarrays, are shown. Asterisks indicate peaks containing two closely localized binding sites. (C) Correlation between ChIP-seq peak fold enrichment and $\log_2$ fold change after DNA damage of the nearest gene. (D) Level of expression of selected LexA1 targets in leptospires infecting kidneys from C3H/HeJ susceptible mice. The values from qPCR of infecting bacteria were normalized to the 16S rRNA control and compared to *in vitro* cultured leptospires. The gene expression profile is expressed as the $\log_2$ of the fold change between the two conditions. The line indicates the median.

Each peak was assigned to a single transcript, except for peak #5 (localized in the intergenic region between LIC12653 and *lexA2*, coded on different strands) and peaks #18 and #19 (respectively upstream and inside the 5' end of LIC12975 coding region) (Table S3). Peak-to-gene correspondence confirmed that ChIP-seq identified the previously known targets of LexA1, almost all of them with DNA-related functions: *lexA1*, *lexA2* and LIC12653, *recA*, *recN* and *dinP* (14) (Fig. 1). They are all high ranked, suggesting a stronger or more frequent binding to LexA1. Identified targets are indicated in Fig. 1 and Table S3 had their functional categories identified by GSEA. Surprisingly, DNA repair mechanisms are not overrepresented and unexpected categories, as intermediary metabolism, fatty acid and small molecules biosynthesis, tRNA modification, motility and cell structure are regulated by SOS. Another intriguing finding was the binding of LexA1 to prophage related genes. A brief description of such transcripts is presented below.

Novel DNA metabolism-related genes. In addition to the previously identified LexA1 targets, three other genes related to DNA metabolism were found. MutY (adenine DNA glycosylase) was associated with a ChIP-seq peak in the 3' region of its coding region. MutY removes adenine mispaired with 8-oxoguanine (8-oxo-G), the most abundant oxidative lesion from G:C rich genomes. The *nrdA* gene, coding for the subunit alpha of ribonucleotide-diphosphate reductase (RNR), is also part of the LexA1 regulon. RNR catalyzes de novo synthesis of dNTPs, playing a critical role in regulation of total rate of DNA synthesis. LIC20276 has a conserved helix-turn-helix domain, indicating a DNA-binding function.

Prophage-related genes. We have previously shown LexA1 binding to the promoter region of genes LIC12653 and *lexA2*, located within a prophage-like region (14). Here we identified a second prophage-related gene component from LexA1 regulon, the phage excisionase LIC10169 (peak #6). Both phage integration into and excision from the bacterial chromosome requires an integrase. However, while integrase alone is able to perform the first, the last reaction also requires the excisionase (37).The peak #7 is located in a putative CRISPR (clustered regularly interspaced short palindromic repeats) region, responsible for

the bacterial adaptive immunity against phage infection (38). A complete Family I-C Cas (CRISPR associated genes) cluster is located more than 1kb downstream of the peak.

Intra-specific competition. LIC10986, associated with peak #23, is possibly an immunity protein lacking its cognate polymorphic toxin (39). These proteins protect the cell from toxins produced and released by bacteria of close-related strains or species. In contrast, peak #22 maps at the 5' end of LIC11622, which codes for a putative ExbD transporter, component of a system for receptor-dependent transport across the membrane. This protein and its homologue TolR are involved in the uptake of colicins B and M (40), the most studied category of bacteriocins.

Metabolism and motility. Several genes involved in central metabolism, specifically biosynthesis of phospholipids (peaks #9, 18 and 19) and intermediary metabolism (peaks #14, 16 and 17), were associated with LexA1 binding sites. Peak #11 mapped upstream of *fliF*, the first gene of an operon containing genes related to synthesis and function of flagella.

Putative virulence factors. The *gidA* gene (peak #21) contains a LexA1 binding site in the center of its coding region. It has a role in tRNA modification and modulates post-transcriptionally virulence factors in several pathogens (41, 42). Two genes involved in synthesis and modification of the membrane and the peptideoglycan are also bound by LexA1. LIC13392 codes for a polysaccharide deacetylase that catalyzes the N- or O-deacetylation of acetylated peptidoglycan (43, 44), evading mammalian hydrolases as lysozyme (45). This is an important evasion mechanism not only for Gram-positives, but also in Gram-negatives as *Helicobacter pylori* (44). The antigen-O polymerase WzyC, a previously non annotated protein in *L. interrogans* serovar Copenhageni, performs the last step in the formation of lipopolysaccharide (LPS), extremely important for gram-negative pathogen infections. In leptospira, the O-antigen chain length is known to change under in vivo conditions (46). LexA1 ChIP-seq peak #20 maps in the center of its 2.4kb coding region. Finally, *risA* codes for subunit alpha of riboflavin synthase and may be related to activation of mucosal-associated invariant T-cells (47).

De novo motif search updates leptospiral SOS box

The LexA1 binding site consensus derived from ChIP-seq data is an imperfect palindrome of 16nt (Fig. 2, inset). The motif was found in 20 from 25 input sequences, with an E-value less than 10^{-6}. The motif was detected in the remaining sequences (from peaks #2, 9, 19, 21 and 24) after a reverse search with the final matrix (represented by the logo in Fig. 2, insert). Three peak sequences contained two binding sites, namely #12, 22 and 25, with three to 15 nucleotides between them (Fig. 1B and Table S3). Variations of the original SOS box described for leptospira, TTTGN$_5$CAAA, identified at *recA* promoter by DNase I footprinting (48), were present in the LexA1-binding upstream regions of *lexA1, lexA2, recN* and *dinP* (14). Although the ChIP-seq-resulting consensus differs from this palindrome, both overlap in the *recA* promoter (Fig. 2). The coincidence of location with a binding site identified by a different method corroborates the motif found in the present work. In addition, this updated SOS box is also present in all probes capable of binding to LexA1 in EMSA (14).

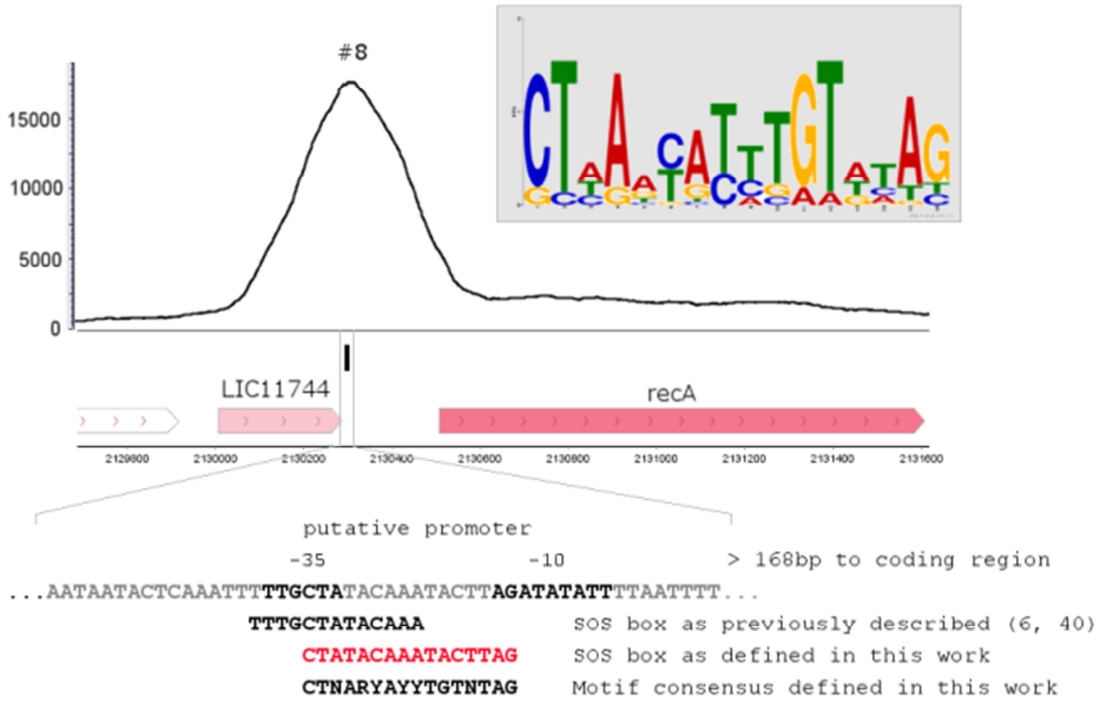


Figure 2. ChIP-seq-derived LexA1 binding motif. Detail of the *recA* promoter region, indicating the overlap between them. ChIP-seq and expression data are presented as in Figure 1. Logo (insert) for the PSWM generated by the *de novo* search for a motif present in the peak sequences performed by MEME. This motif had an E-value of less than 10^{-6}. The consensus is presented in IUPAC code: N = any base, R = A or G, Y = C or T.

Global transcriptional profiling during UV-C DNA damage response

Following identification of the leptospiral SOS regulon, we sought to evaluate the whole DNA damage response, including aspects independent of or indirectly controlled by LexA1. Growing cells were exposed to a dose of UV-C capable to kill half of the population (14) and RNAs collected 12 hours after treatment were used for microarrays, comparing levels of expression to non-treated samples. The P-value cut-off was 0.05. UV-C irradiation altered expression of 18% of genes, either increasing or decreasing in relation to untreated samples. The UV-C-induced differential expression of 37 genes was assessed independently by qPCR, and it positively correlates with microarray data (R^2=0.71, P-value < 0.001, Fig. S1). The major functional categories affected by UV-C stress were identified by GSEA (Fig. 3A).

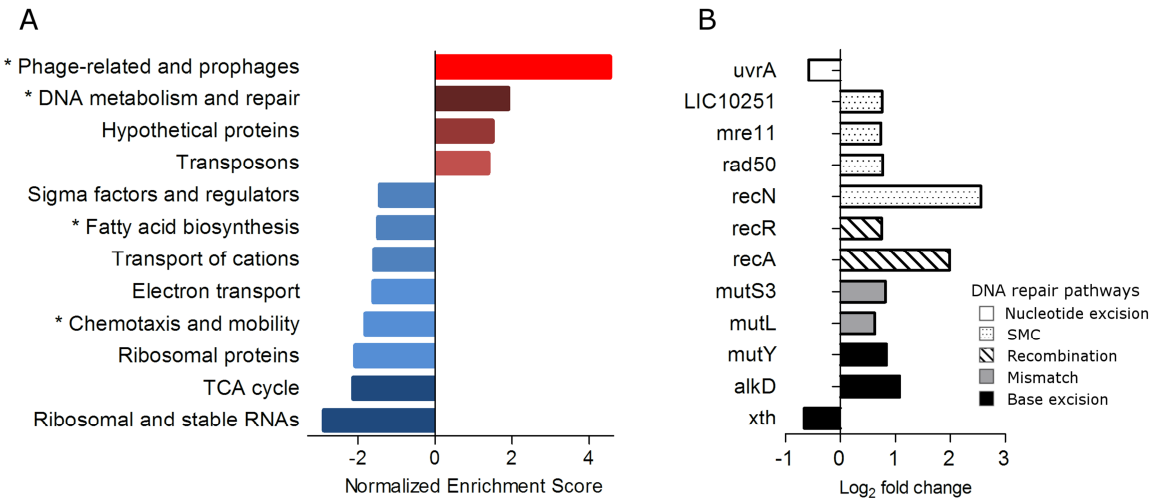


Figure 3. Enriched functional categories in differentially expressed genes after UV-C in *L. interrogans* serovar Copenhageni. (A) Genes displaying altered expression upon UV-C DNA damage were ranked and analyzed through GSEA. Red bars to the right represent up-regulated categories, and blue bars to the left, down-regulated ones. Asterisks mark categories represented in the LexA1 regulon. (B) Differences in expression of genes contained in the DNA repair category. Values are indicated as $\log_2$ fold change, as assessed by microarray data.

Mobile genetic elements. The most striking feature of UV-C DNA damage-induced expression profile was the enrichment of prophage-related and transposon categories (Fig. 3A and 4). Nine transposases, from at least four different families (IS1533, IS1501, IS3 and ISlin1), are induced around two-fold. However, from the 46 genes in the prophage functional category, 43 were not only up regulated, but were at the top most differentially expressed genes after UV-C irradiation (Table S5). These genes are all components of two prophages:

prophage 1 (PP1) comprises approximately 22kb and 28 genes, ranging from LIC10136 to LIC10189, while prophage 2 (PP2) spans 13kb between LIC12600 and LIC12616 (Fig. 4). Besides head and tail structural genes, PP1 possesses two cI/Cro repressors, responsible for lysogeny control, and a late gene regulator D (GpD, LIC10181). Moreover, an integrase (LIC10167), a LexA1-regulated excisionase (LIC10169) and a Gam-like rolling-circle replication protein (LIC10165). This protein protects recombination intermediates from RecBCD, allowing both phage rolling circle replication (49) and bacterial non-homologous end joining (50) to occur. Since the serovar Copenhageni genome lacks a Ku homologue (Martins-Pinheiro *et al.*, manuscript in preparation), protein necessary for non-homologous end joining, this prophage gene can add one more possibility for repair (50,51). PP1 also harbors a glycosyl hydrolase (LIC10172) and a pore-forming protein (LIC10186) for lysis of the host cell. The existence of a phospholipase A2 (PLPA2, LIC10163) could provide some advantages for the bacteria during mammalian infection (52), since it is involved in hemolysis, inflammation and tissue invasion as in other pathogenic Gram-negative bacteria (53). At least eight genes in PP2 are structural, and a GpD (LIC12608) is also present. It harbors a plasmid maintenance toxin system (LIC12609) and a phage tail protein (LIC12611) similar to *pblA*, used by *Streptococcus mitis* to bind to platelets (52).

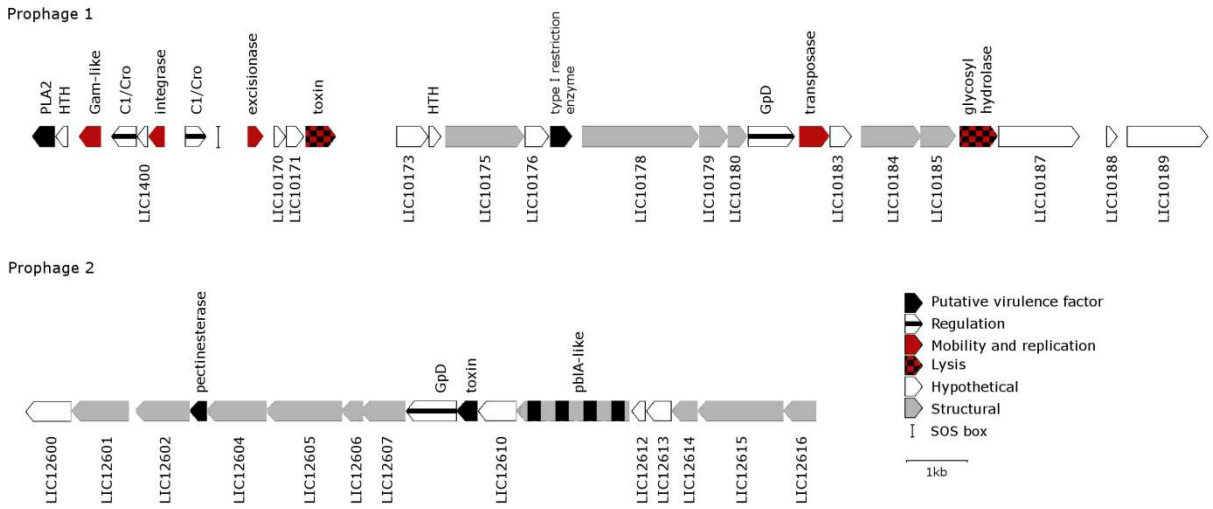


Figure 4. Genomic context of two highly expressed prophages found in *L. interrogans*. For each prophage, putative functional categories are indicated by color. In prophage 1, the SOS box corresponds to the peak #8.

PP1 and PP2 lack important components required for transcription and DNA packaging, indicating both are disrupted. This was corroborated by lack of their excision from the chromosome in UV-C treated or non-treated cells. For this, total DNA recovered from treated and non-treated cultures was analyzed by PCR to search for expected products of chromosomal excision and formation of circular forms (Fig. 5).

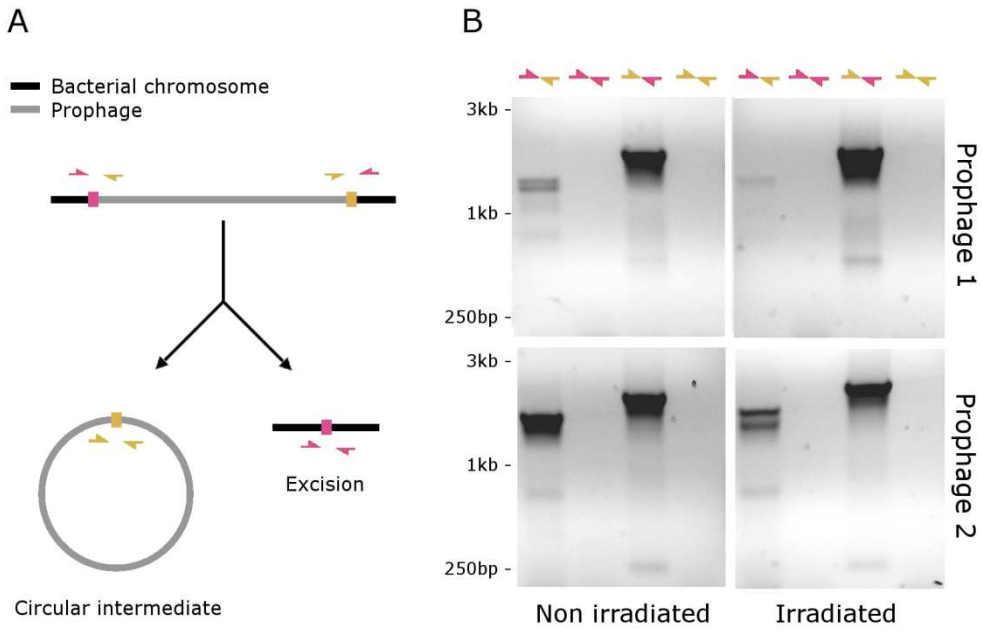


Figure 5. Prophages excision analysis. (A) Experimental design. For each prophage, two sets of oligonucleotides were used, targeting the bacterial chromosome (pink) or the prophage region (yellow). Combining oligonucleotides with the same color would only generate amplicons if there was excision of the prophage from the chromosome (pink) and the existence of a circular intermediate (yellow). (B) Isolated gDNA from irradiated or non-irradiated cultures were used as template for PCR with the sets of nucleotides. The reactions indicated by mixed colors should always be able to amplify products, while the ones indicated by same-color arrows would only provide products if there was excision of the prophages.

DNA repair and metabolism. The second most enriched category belongs to DNA metabolism. Removal of UV-C induced damages usually requires the nucleotide excision repair (NER). However, none of the NER components had their transcripts up-regulated or were even down-regulated, as *uvrA* (Fig. 3B). Instead, DNA damage induced few components of other repair systems, such as the DNA-glycosylases *mutY* (LIC11614) and *alkD* (LIC12552), involved in removal of oxidative and alkylating damages, respectively. The mismatch repair genes *mutS3* and *mutL* were up-regulated after DNA stress induction, as well as the structural maintenance of the chromosome (SMC) and recombination repair

components *recA, recN rad50*, *Mre11* and *recR*. SMC proteins interact with DNA free ends and are involved in chromosomal rearrangements (54, 55). This scenario suggests little DNA lesion removal but higher propensity of chromosome rearrangement. The up-regulation of both subunits of DNA gyrase (LIC1005 and LIC1006) reinforces this hypothesis (Table S4).

Cell growth, virulence and mobility. Most of the transcriptional changes induced by genotoxic stress was down-regulation of cell growth, general transcription and motility (Fig. 3A and Table S4). Several sigma factors, as *rpoD*, (the sigma factor responsible for general transcription in exponentially growing cells) were down-regulated. This correlates with decrease in expression of categories related to translation (ribosome proteins and ribosome RNA) and central metabolism (TCA cycle, electron transport and fatty acid biosynthesis). On the other hand, sigma N is possibly activated. The *rpoN* activator LIC11549, absolutely required for sigma N function, showed increase in expression. This sigma factor is responsible for transcription of genes involved in nitrogen metabolism and virulence factors, suggesting a shift in the metabolism under DNA stress situation. According to this, genes involved in flagella biosynthesis and function were down-regulated, probably affecting motility. Flagellins are the most down-regulated genes, and both *flaA*-1 and *flaA*-2 were repressed, being the most down-regulated genes (Table S4). These flagellins are two of the few confirmed virulence factors in leptospira (17). Other virulence factors are *katE* (16), LIC20153 (56) and the chaperones *htpg* (LIC20044) and *clbP* (LIC12017). LIC11574, LIC12341 (57), LIC20172 and *ligB* (58) are defined adhesins. All these factors were also down-regulated after DNA damage (Table S4).

Expression of direct LexA1 target genes during genotoxic stress

Expression levels of 15 LexA1 target genes were validated by qPCR, adding expression information for two regions that were not annotated before: *wzyC* coding sequence and CRISPR region (UV-C induction qPCR in Table S3). All expression data assessed by qPCR agreed with microarray data, except for *nrdA* and *dinP*, which did not achieve an acceptable P-value in microarrays. In these cases, we considered the qPCR results, since this technique

is more sensitive; on the others, we considered the values derived from microarrays. Higher values in peak fold enrichment positively correlates with an increased expression of the associated gene upon SOS induction (Fig. 1C), suggesting peak rank indicates binding in a larger fraction of cells, rather than stronger interaction with LexA1. As expected for a transcriptional repressor effect, most of the genes directly controlled by LexA1 was up-regulated after DNA damage. This was true for the previously known targets and for some interesting genes as *nrdA*, excisionase LIC10169, *exbD*-like LIC11622 and *wzyC* (Fig. 1B). However, all genes associated with two motifs (LIC12260, LIC13392 and *risA*) did not present alteration in expression after DNA damage. This can be caused by persistence of LexA1 dimers in the region even when the repressor levels drop. Surprisingly, three peak-associated genes with repressed expression during SOS response were found: CRISPR, *fliF* and *fbp* (Fig. 1B), corroborating the microarray analysis.

Induction of SOS regulon components during infection

Our analyses portrayed an expression pattern for LexA1 direct targets in face of DNA damage. To assess whether these components are important for leptospires during infection, expression of some SOS genes in kidneys of the susceptible C3H/HeJ mouse were evaluated. We used total mRNA material from infected kidneys during the acute phase of the disease (fifth day) (32). The main regulator of SOS, *lexA1*, did not have significant difference in expression, along with other UV-C-induced genes as *nrdA* and excisionase LIC10169 (Fig. 1D). On the other hand, *recA, recN, dinP and wzyC* were up-regulated during infection. The same stands for the putative immunity protein LIC10986, not altered upon *in vitro* DNA damage.

DISCUSSION

Stress in bacteria is usually linked to adaptation, regardless of being transient (in expression levels) or permanent and heritable (alterations in genetic material). In case of DNA damage, the response can include repair and tolerance, but also mutagenesis and virulence (7). These alterations can be dependent or independent of the SOS system. In this

work, we investigated the SOS composition of *L. interrogans* serovar Copenhageni and its relation to the transcriptomic response induced by UV-C. Genotoxic stress elicited the SOS response and caused an enormous rearrangement in the expression landscape, with alteration in mRNA levels for 18% of genes.

The SOS regulon in different organisms varies considerably, and this is in part explained by the non-conserved nature of SOS boxes. These sequences follow roughly the phylogenetic relationships between groups of bacteria (6, 59). Although the SOS box in some organisms is constituted by direct repeats, or asymmetrical non-palindromic sequences, the majority of LexA repressors recognizes a palindrome (6, 60-62). This explains the bias in previous works in leptospira (14, 48). The investigation of the probable SOS motif in the close serovar Lai, through DNaseI protection assays in the promoter of *recA,* identified a perfect palindrome that was considered the leptospiral SOS box ($TTTGN_5CAAA$) (48). Later analysis showed binding of LexA1 to degenerated versions of the above palindrome in promoters from other genes (14). ChIP-seq is a powerful tool to identify DNA targets of transcriptional regulators. Using it in conjunction with *de novo* motif search, the SOS box from leptospira was updated to the imperfect, asymmetric palindrome CTNARYAYYTGTNTAG. The correspondent sequence in the *recA* promoter is contained in the protected region in Lai, and present in LexA1 bound promoters identified previously by electrophoretic motility shift assay (14, 48). This motif was found in all enriched sequences from the LexA1 ChIP-seq, correspondent to the 25 genes described here composing the direct LexA1 regulon.

Study of the SOS regulon from *E. coli, B. subtilis* and some other Gram-positive bacteria pointed to the hypothesis of an ancestral SOS core gene set composed by *recA*, *uvrA*, *ruvAB* and *recN* (6). However, the discovery of a LexA-induced gene cassette, more widespread than the above complicated the notion of a SOS core gene set (62). In most bacteria, SOS favors DNA repair pathways, mainly NER. Our findings indicate that, in *L. interrogans* serovar Copenhageni, few DNA repair genes are components of this RecA/LexA-dependent

response. Existence of a second DNA damage response pathway, RecA-independent, was observed for *M. tuberculosis* (63, 64) and this possibility is not excluded for leptospira. The newly identified SOS component, *mutY*, links SOS response to oxidative DNA damage repair. While classical UV-C DNA damage repair does not seem to be favored by SOS response in leptospira, mutagenic mechanisms might have a special role. Up-regulation of *dinP* (Pol IV) and *nrdA* (RNR) are indicative of this strategy. Pol IV (*din P*) is important for replication upon a stalled replication fork, but potentially mutagenic, since it can incorporate any base at the lesion site, even in undamaged DNA (7, 65). In *E. coli, nrdA* expression increases up to three fold after UV-C irradiation, causing a 10-fold raise in dNTP pool. This increase in RNR generated a “dNTP mutator” phenotype, characterized by decrease in fidelity of the replicative polymerase (66). The post replicative mismatch repair (MMR), up-regulated following genotoxic stress in *L. interrogans*, might act over the mispaired bases generated during the mutagenic pathway. These data indicate that LexA1 do not control a specific DNA damage response in *L. interrogans*, but a network able to deal with general genotoxicity, including oxidative lesions and replicative errors. The increase in SMC and recombination systems may be directly related to the activation of mobile genetic elements, in order to avoid over destabilization of the chromosome.

As expected for a LexA repressor activity, the majority of LexA1 targets increased their expression after 12h of UV-C irradiation. Curiously, three targets displayed a slight down-regulation after UV-C. One of them was the CRISPR region downstream of ChIP-seq peak #7. CRISPR/Cas is a bacterial immune system evolved against foreign genetic elements such as plasmids and phages (67). Cas genes expression remained constant, though, as also seen after treatment with antibiotics (68), higher temperatures (69) or in lepstopires inside the peritoneum of rats, in dialysis membrane chambers (28). Regulation of the CRISPR/Cas system in leptospira may rest in the pre-crRNA levels, which after processed will be used as guide for Cascade (38). The decreased levels of pre-crRNA transcript can be enough to lead to an increased risk for mobility and infection by phages. LexA acting as an

activator is not common: *Caulobacter crescentus* and *Rhodobacter sphaeroides* are known exceptions. In the first, LexA activates expression of two SOS genes (70), while in the latter, a dual role in the *recA* promoter was documented (71).

In this context, at the same time this bacterial immune system is down regulated under direct control of LexA1, several transposons and two prophages were heavily up-regulated. Both prophages are under tight regulation in *L. interrogans*: while they are entirely activated in the conditions tested in this study and after ciprofloxacin treatment (68), they are repressed in leptospires in dialysis membrane chambers (28). In other situations, some components seem to be individually controlled. For example, LIC12603 (pectinesterase) increases expression after treatment with serum (68). The pore-forming toxin LIC10172 is strongly expressed in blood, liver and kidney of infected hamsters, while LIC12611 (*pblA*-like) is up-regulated in blood and liver, but down-regulated in kidney (29). However, in our conditions, the prophage genes tested (LIC10168, LIC10169, LIC12608, LIC12609) did not alter their expression *in vivo*, despite the strong induction after *in vitro* DNA damage.

Our data suggest prophage expression is result of a broad RecA-dependent response controlled by the SOS system. Both prophages possess GpD regulators (LIC10181 and LIC12608), usually responsible for regulation of phage late promoters from structural and lysis modules (72, 73). GpD are under control of cI repressors, present in PP1 (LIC10166 and LC10168). cI/Cro are Family S24 peptidases, as LexA repressors: they share the same characteristics of DNA binding and self-cleavage upon interaction with activated RecA (74). In serovar Lai, the orthologous of gene LIC10168 was shown to bind to the operator shared by its own gene and the divergently transcribed gene cassette from LIC10167 to LIC10163 (75). While regulation of the rest of the prophage genes is probably through RecA-cI/Cro, LIC10169 (excisionase) is controlled by LexA1. Increased levels of excisionase in relation to integrase favor excision. The lower expression of LIC10169 (2.8 fold) in relation to LIC10167 (15.32 fold) may be responsible for stability of the prophages in our conditions.

Both prophages may force their preservation in the genome through the presence of putative addiction modules (76): LIC10172 codes for a pore-forming toxin, while LIC12609, a plasmid killer toxin. Both may be accompanied by an anti-toxin coded by one of the hypothetical protein genes. In addition, some encoded factors could bring benefits to the bacteria. The Gam protein could add NHEJ to the DNA repair toolbox, while LIC10163 is the only phospholipase A2 in *L. interrogans* genome. Finally, PlbA (LIC12611) may be used as an adhesin as in *S. mitis* (52).

While mobile genetic elements and mutagenic DNA repair genes are increased in response to genotoxic stress, three important aspects of cell biology are down-regulated: general metabolism, virulence and motility. The great rearrangement in metabolism is probably consequence of alterations in sigma factors and other regulators. Most of them are down-regulated, while few activators increase expression. Almost every aspect related to cell growth is repressed, including cell division. Although we could not determine the mechanisms for DNA damage-dependent cell division inhibition in *L. interrogans*, our group had already documented filamentation in response to SOS induction in this organism (14). This is probably a strategy to slow down replication until the lesions are repaired (7). Motility, an important aspect for pathogenesis, is also probably heavily affected, since genes related to flagella function and also chemotaxis are down-regulated. In addition, almost every known or putative virulence factor identified until now is unchanged or down-regulated. Some examples are virulence genes *katE* (16), *flaA*-1, *flaA*-2 (16) and *clpB* (77), and the colonization-required LIC20153 (56), all reduced more than two-fold after DNA damage. The same is true for genes required for uptake by macrophages and binding to the extracellular matrix, *ligB* and LIC20172 (58). The only putative virulence factors increased in expression during this stress are RecA-dependent, coded inside the prophages. LIC10172 (toxin) and LIC12611 (*pblA*-like) are both mutated in an attenuated strain of serovar Lai and expressed during hamster infection (29).

Although the response to UV-C in Leptospira seems quite general, it is specific for DNA damage and replication stress. This profile of increased expression in such specific DNA repair genes combined with decreased cell growth and motility is not seen in the response to physiologic osmolarity, serum, DMCs or various antibiotics (25, 28, 68, 78). However, it has a similar profile to the response to ciprofloxacin, a DNA gyrase inhibitor indicated for infections caused by Gram-negative bacteria (68, 79). In *L. interrogans* as well in *P. aeruginosa*, it causes up-regulation of *recA* concomitant with increase in DNA repair, prophages and mutagenesis, in addition to decreased cell growth, motility, virulence and ciprofloxacin-specific repair genes (*recG*, *ruvABC*) (68,80,81).

In our infection model of leptospirosis, using a mice deficient in TLR4 (32), immunity protein-coding gene LIC10986 remained constant during *in vitro* DNA damage response, but it was up-regulated during infection. Since it is possibly a defense against bacteriocins, this can be a reflection of increased intra and interspecific competition between bacteria inside the host. The O-antigen polymerase is up-regulated in kidney. The tendency of *L. interrogans* serovar Lai during infection of a susceptible host is to lower the O-antigen content in comparison with colonization of a carrier (46). In contrast, LPS and O-antigen assembly genes show moderate increase in expression when in contact with macrophages (24), suggesting a tight regulation of O-antigen levels upon different environmental cues. Finally, *lexA1* and *nrdA* did not alter their expression *in vivo*, as prophage genes. These differences in induction may be a result of partial activation of the SOS system, additional regulation of their expression, different timing for transcription during infection or a combination of all alternatives.

In this work, integrating powerful techniques for transcription factor target assessment and transcriptional profiling, we provided an unprecedented view of DNA damage response in *L. interrogans* and the direct role of LexA1 in its regulation. The LexA1 regulon involves several aspects of the cell biology, including cell growth, mutagenesis and DNA repair. Surprisingly, LexA1 may be playing a dual regulatory role, acting also as an activator. General genotoxic

stress response follows the LexA1 targets trend, going further than just repair. The global expression profile is shifted from cell growth and virulence towards DNA repair, mutagenesis and mobile genetic elements. DNA damage may decrease the ability to adhere, colonize and cause disease, but stimulates variation inside the affected population, probably causing a boost in genetic diversity and evolution that can bring long term benefits to the pathogen population.

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4. CHAPTER 3

Genomic survey and expression analysis of DNA repair genes in the genus *Leptospira*

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ABSTRACT

Background: Leptospirosis is an emerging and worldwide zoonosis with important economic and public health consequences. The disease is caused by pathogenic leptospires that can infect a large range of animal species that compose their natural reservoir. The genus *Leptospira* comprises saprophytic (*L. biflexa*), pathogenic (*L. interrogans*) and host-dependent (*L. borgpetersenii*) members. DNA repair pathways are the main mechanisms for DNA safeguarding, and are able to remove virtually all kinds of lesions.

Results: Here we present an *in silico* search for DNA repair pathways in the genus *Leptospira* based on genomic information. The genus exhibits some distinct and unexpected characteristics, as the existence of an apparent redundant mechanism for repair of alkylated nucleobases, a new *MutS*-like gene and a shorter version of UvrD. Although gram-negative, *Leptospira* spp. resembles *B. subtilis* for the presence of PcrA, two RecQ paralogs and two SSB proteins; the latter is considered a feature shared by naturally transformed bacteria. Surprisingly, we did not find a significant reduction in the number of DNA repair-related genes in the host-dependent species. The pathogenic leptospires were enriched for genes dedicated to base excision repair and their evolutionary history reveals a remarkable importance of lateral gene transfer (LGT) events. The relevance of such DNA repair pathways was assessed through the identification of mRNA levels of some genes during infection in animal model.

Conclusions: The assessment of DNA repair genes through genome comparison in the genus *Leptospira* resulted in new insights into the evolutionary history of DNA processing. LGT had huge impact in the genome dynamic of the genus, providing genes for several pathways. The up-regulated expression of specific DNA repair genes, including the components of the SOS regulon, during infection in animal model validates the critical role of DNA-repair mechanisms for the complex interplay between the host and the pathogen.

Keywords: *Leptospira*, DNA repair, bacterial genomics, SOS response, alkyltransferase, nucleotide excision repair, base excision repair.

BACKGROUND

The genus *Leptospira* belongs to the order Spirochaetales, and the genetically based classification indicates the existence of at least 19 species (13 pathogenic and six saprophytic). The *Leptospira* species are categorized into 24 serogroups and 250 serovars, based on the expression of surface-exposed lipopolysaccharide (LPS) [1]. The pathogenic leptospires are the etiologic agents of leptospirosis, an emerging and worldwide zoonosis with important economic and public health consequences, in particular to developing tropical countries [2]. The leptospires can infect a great range of animal species that compose their natural reservoir, colonizing the kidneys and being shed in the urine during the whole life of these animals [3]. Humans are considered incidental hosts, and the infection occurs through direct contact with infected animal urine or indirectly through contaminated water.

The molecular mechanisms of *Leptospira* pathogenesis are still poorly understood. However, this scenario is changing due to the availability of genomic information. The whole-genome sequences of the pathogenic *L. interrogans* serovars Lai and Copenhageni, and *L. borgpetersenii* serovar Hardjo, as well as two strains of the free-living *L. biflexa* are driving the knowledge of their host-pathogen relation. For example, it became clear that the restricted pathogenic life-style from *L. borgpetersenii* results from the detriment of some genes necessary for environment sensing as well as metabolite transport and utilization [4]. On the other hand, *L. interrogans* does not show this level of genome reduction, and, as a consequence, the bacteria can survive in water or mud for weeks, after what they are still able to infect the host [5, 6].

As any living organism, leptospira has to deal with endogenous and exogenous agents that can cause DNA alterations, disturbing several metabolic processes [6]. DNA repair pathways are the main mechanisms for DNA safeguarding, and are able to remove virtually all known kinds of lesions. The remaining lesions that are not removed and block the progress of replication forks during DNA replication are tolerated by the cells through DNA damage tolerance pathways.

The DNA repair capacity of *Leptospira* was barely addressed until now. Both *L. interrogans* and *L. biflexa* were shown to have a functional photo reactivation DNA repair mechanism. However, the pathogen was extremely sensitive to both UV-C irradiation and mytomicin-C treatment [7]. The capacity to repair UV-C lesions was also assessed in *L. biflexa* through a knockout of *recA* [8], responsible for recombination and control of the SOS response. The mutant grew poorly and was more sensitive to damage. Recently, our group showed SOS response activation following UV-C irradiation in *L. interrogans* serovar Copenhageni. Surprisingly, the typical DNA repair genes were not regulated by LexA [9]. In addition, low expression of DNA repair genes in the virulence-attenuated *L. interrogans* serovar Lai IPAV strain may be responsible for the several genetic variations in comparison to its highly virulent ancestral strain, 56601. This finding highlights the importance of genomic stability in maintaining the virulence of *L. interrogans* [10, 11].

This work presents a thorough bioinformatics search for DNA repair pathways in the genus *Leptospira* based on genomic information. The genes involved in DNA repair are, in general, part of the cell core metabolism and they maintain a strong similarity in different bacterial genomes. However, this search revealed intriguing differences in *Leptospira*. Leptospires have an unusual number of genes involved in reversion and removal of alkylated lesions and for members of the UvrD family. So far, these activities appear to be the most extensive among bacteria. In addition, the DNA repair core genes from saprophytic and pathogenic bacteria differed. Pathogenic leptospires were enriched for genes dedicated to base excision repair, responsible for the removal of small, non-helix-distorting base lesions from the genome. Lateral gene transfer events appear to be responsible for most of these acquisitions and provide evidence for the genome dynamic of the genus. The relevance of some DNA repair pathways and the SOS response was validated through *in vivo* expression analysis during leptospirosis in animal model. DNA repair genes showed up-regulation only in kidneys from susceptible mice during the acute phase of the infection. On the other hand, components from SOS regulon were up-regulated in both intermediate and susceptible mice.

METHODS

Identification of DNA repair genes

BLAST similarity searches using the DNA repair genes were first performed with *Escherichia coli* as seed, or *Bacillus subtilis, B. cereus, Mycobacterium tuberculosis and Caulobacter crescentus.* The searches were conducted against the non-redundant (nr) protein sequence database (BlastP) [12] for each complete leptospira genome: *L. biflexa* serovar Patoc str. 'Patoc1 (Paris)' (NC010602.1/NC010843.1/NC010844.1) and str. 'Patoc 1 (Ames) (NC010842.1/ NC010845.1/NC010846.1); *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (NC005823.1/NC005824.1) and serovar Lai str. 56601 (NC004342.2/NC004343.2); *L. borgpetersenii* serovar Hardjo-bovis strain JB197 (NC008510.1/NC008511.1) and str. L550 (NC008508.1/NC008509.1). Results were considered only if E-value were lower than 10^{-4}. Candidate genes were confirmed both by sequence similarity searches (BlastP) and domain analysis (Pfam [13] and HHpred [14]). Comparison tables show homologous proteins for only one strain per species, unless there were differences between strains.

Phylogenetic analyses

Protein sequences of genes were aligned using ClustalX 2.0 [15] and all gaps were eliminated. The phylogenetic reconstructions were conducted in Mega5 [16] with Maximum Likelihood (ML) and Distance Based Neighbor-Joining (NJ). Similar topologies were found for both algorithms employed, but only one is displayed. Bootstrap assessment of tree topology (1000 replicates) was generated but only bootstrap values greater than 50% are shown.

Animals and *Leptospira* strain

Samples used in this study were collected previously [17]. Briefly, virulent *L. interrogans* serovar Copenhageni was cultured in liquid Ellinghausen McCullough Johnson and Harris (EMJH) medium at 30^{o}C under aerobic conditions. After 5 days of culture, bacteria were counted in Petroff-Hausser chamber and suspensions were used to infect mice. Mice strains C3H/HeJ and C3H/HePas and hamsters were provided by Instituto Butantan (Brazil).

Experimental infection of animals

We used the same material from kidneys, during the acute phase, i.e. five days after mice infection as described [17]. The strains of C3H/HeJ and C3H/HePas mice were infected intraperitoneally with 1 x 10^7 leptospires and the remaining five animals of each strain were kept uninfected as control. In parallel, four 3-4 weeks old hamsters were infected with the same dose to confirm the virulence of the bacteria. Samples of six mice were collected at days 0 (uninfected animals) and 5 after inoculation. The samples were kept at -80^{o}C until analysis. The protocol used in this study was approved by the Ethical Committee for Animal Research of Butantan Institute (number: 560/08).

RNA manipulation and quantitative PCR

The organs of mice were collected in liquid nitrogen and kept to -80^{o}C, as previously described [17]. Total RNA was isolated using Trizol reagent (Invitrogen). An aliquot of 2 μg RNA of each sample was transcribed to cDNA using RevertAid^{TM} H Minus Reverse Transcriptase (Fermentas). The oligonucleotides sequences for *lexA1*, *recA*, *recN*, LIC12362 (*alkA2*), LIC11717 (*uvrA*) and LIC13064 (*tag*) genes were previously described [15]. Oligonucleotides sequences for LIC12677 (*DNA lig*) genes were: forward AGCTCTCTTCAAACCGGAAC and reverse CTGTGTATCCTTTCGCCATCT; LIC10238 (*alkA1*) forward CCTTCGCCCGAACAGATTT and reverse GCCTCAGCGATACGTTTGAT, LIC13402 (*ada*) forward TTCTCTCTAAACCTCAAACCACTT and reverse CGGAGTCATCCCTTCAATCTTTA; and *16S* forward TTCAGTTGGGCACTCGTAAG and reverse CGTGTGTTGCCCTAGACATAA. The qPCR reaction was carried out with Syber Green Master Mix (Applied Biosystems, USA) using 100 ng of cDNA for samples of kidney and 10 ng for samples of bacteria from EMJH cultured 5 days at 30^{o}C. The PCR thermal cycle conditions were described previously [17], performed in an Applied Biosystems 7300 Real-Time PCR System. Cycle threshold (Ct) values for specific genes were normalized to the Ct values of the leptospiral 16S mRNA gene and expression fold change compared to gene expression levels in vitro EMJH cultured Leptospira. The relative levels of mRNA of

each selected gene were analyzed using $2^{-\Delta\Delta CT}$ method [18]. Real-time PCR reactions were performed in triplicate and the results represent the data of two individual experiments. Any values outside one absolute deviation around the median (MAD-median method) were considered outliers [19]. P-values were calculated through two-tailed Student's t-test. Statistics and plotting of data were done in Prism software (GraphPad).

RESULTS AND DISCUSSION

The genome of some saprophytic and pathogenic leptospires was searched for open reading frames with significant similarity to genes known to play roles in DNA repair and/or tolerance. An overview of the genes present in each pathway is presented in Fig. 1. The activation of some DNA repair pathways was tested in experimental intermediate and susceptible mice models through the detection of corresponding transcripts by qRT-PCR.

Direct repair

The direct repair converts DNA damage to its original state in an error-free single-step reaction, and can be performed by photolyases or alkyltransferases (Fig. 1).

Photo reactivation

Photolyases require light absorption to revert the pyrimidine dimers induced by UV irradiation [20]. The presence of photolyase-encoding genes in leptospire genomes has a direct correlation with their respective life-style: *L. borgpetersenii*, which is not a free-living organism, has no single orthologous, while *L. interrogans,* which is also a free-living organism besides being pathogenic, presents a photolyase family II gene (*phrb*), which is closer to eukarya photolyases and to cryptochromes (Fig. 2A, shaded). In addition to *phrb*, *L. biflexa* possesses a *phra*-like from *Rhodobacter sphaeroides* (Fig. 2A, asterisks), which expression is up-regulated in response to singlet oxygen and hydrogen peroxide [21]. The regulation of *L. biflexa* phra-like may be similar, since it is upstream of a gene coding for catalase. Leptospires also present an additional photolyase, encoded by *splB*. In *B. subtilis*, it repairs an exclusive UV lesion from spores, when the DNA is highly compacted [22].

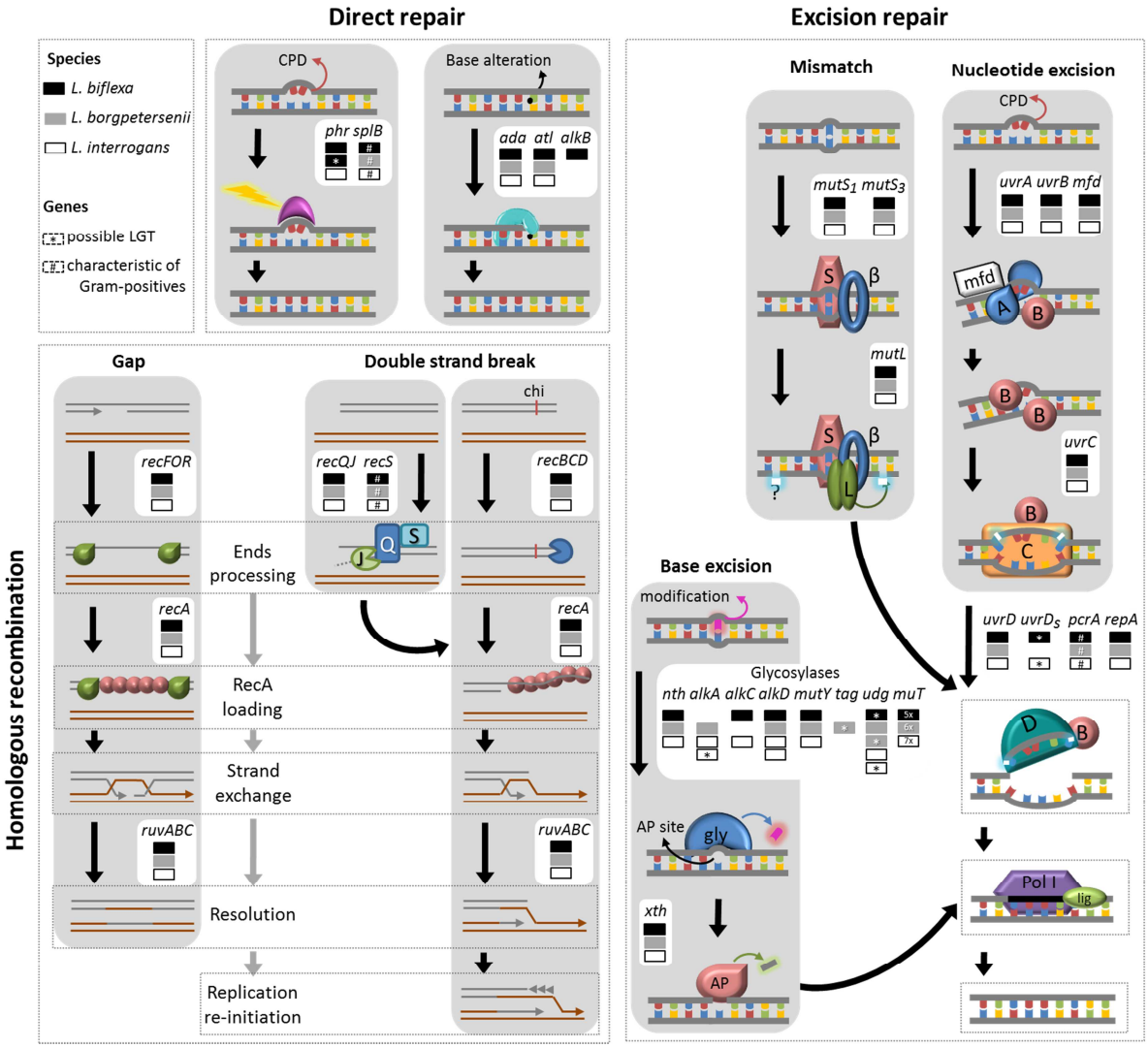

Figure 1. Main DNA repair pathways present in *Leptospira*. In every pathway, the genes required for each step are shown, with black, gray or white boxes representing genes identified in *L. biflexa, L. borgpetersenii* or *L. interrogans*, respectively. Asterisks indicate genes possibly acquired by LGT events and hashtag, genes found in Gram-positives. The direct repair is carried out by photolyases (*phra, phrb, splB*) or alkyltransferases (*ada*, *atl* and *alkB*). Excision repair requires *uvrABC* and in some cases *mfd* for NER; mono (*alkA, alkC, alkD, mutY, tag, udg or mutY*) or bifunctional (*nth*) glycosylases and AP endonucleases (*xth*) for BER; and *mutSL*, interacting with the β clamp for MMR. The excision repair pathways are finished by one of *uvrD, uvrDs, pcrA* or *repA*, DNA polymerase I and DNA ligase. Finally, homologous recombination requires RecFOR, RecBCD or RecJQ/S to process DNA end and load RecA. The products from *ruvABC* resolve the Holliday junctions.

Non-enzymatic Alkyl transfer

Alkylated damages generate a variety of adducts with different potentials of genotoxicity. They can be repaired through the alkyltransferases coded by *ada* and *ogt* genes. Their products, O^6-alkylguanine-DNA alkyltransferase I and II (O^6 AGT I and O^6 AGT II), respectively, act as a single agent to remove alkyl groups in a non-enzymatic suicide

mechanism [23, 24]. While *ogt* is absent from *Leptospira*, all species have *ada* and *atl* (alkyltransferase-like) (Fig. 1), whose identities were determined by phylogenetic analysis (Fig. 2B). The ATL represents a novel class of DNA repair proteins, homologues of alkyltransferases lacking the alkyl acceptor cysteine [25]. The absence of intrinsic alkyltransferase, glycosylase or endonuclease activities suggests their interaction with other DNA repair proteins to remove adducts [26, 27], maybe shunting the damaged DNA into the nucleotide excision repair pathway [28].

Excision repair

This is a repair mechanism able to remove various different kinds of damaged bases and allows the synthesis of the correct nucleotide sequence in an error-free manner (Fig. 1).

Base excision repair (BER)

It protects the genome from small modifications into nitrogenated bases from DNA, spontaneous or caused by oxidation such as that induced by macrophages. The excision of the damaged base needs the sequential activity from DNA glycosylases, apurinic/apyrimidinic (AP) endonucleases and exonuclease [30], all present in *Leptospira* (Fig. 1). There are two kinds of DNA glycosylases: the monofunctional, which catalyze only base excision, and bifunctional, which also contain a lyase activity. Leptospires show a very diverse group of monofunctional enzymes (*alkA, alkC, alkD, mutY, tag, udg and mutY*), present in different number in pathogenic and saprophytic species. On the other hand, they contain only one bifunctional DNA glycosylase (*nth*).

Orthologous for *alkA* are absent from *L. biflexa*, while there are one orthologous for *alkA* in *L. borgpetersenii* and two in *L. interrogans* (Fig. 1 and Table S2). The secondary structure inferred for LIC10238 (AlkA1) and LIC12362 (AlkA2) is in agreement to the known structure of the protein from *E. coli* and *B. subtilis* [31, 32] (Fig. 3A). The D237N mutation is known to decrease the enzyme activity, although the bacterium shows the same resistance to the methylation agent methyl methanesulfonate as the wild type [32]. Both *alkA* homologs from *L. interrogans* contain a substitution on the corresponding residue, and their level of

activity remains to be evaluated. The product of LIC10238/LA0281 gene (marked as AlkA1) clusters with other pathogenic leptospires, while LIC12362/LA1370 (AlkA2, shaded in Fig. 3B) clusters with a very distant group, suggesting its acquisition by LGT. Lateral gene transfer might also explain the presence of *tag* only in *L. interrogans*, which is flanked by transposases (Fig. S1).

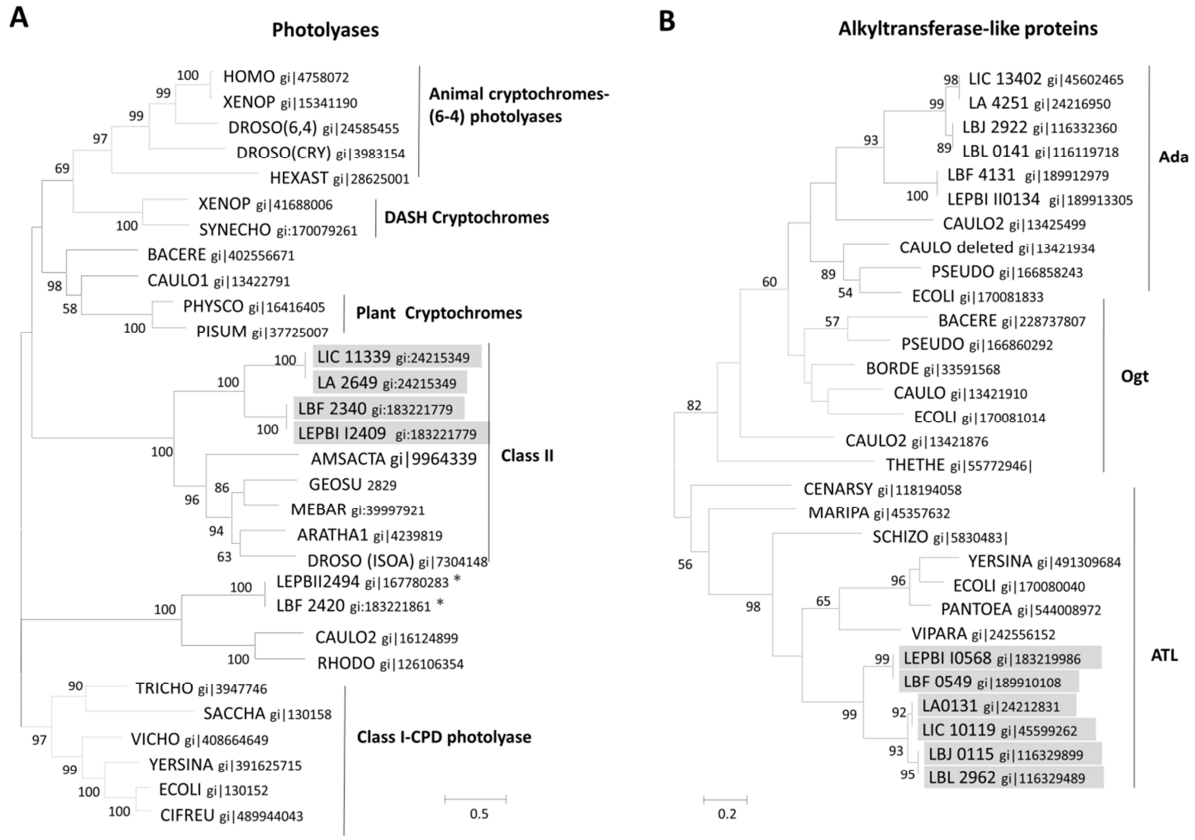

Figure 2. Phylogenetic relationships among photolyases (A) and putative alkyltransferase proteins (B). (A) ClustalW alignments were used for generating the phylogenetic tree using maximum likelihood inference. The common photolyases from *L. biflexa, L. interrogans* and close homologs are shaded. The *phra*-like from *L. biflexa* is highlighted by asterisks. Functional groups are indicated: animal cryptochromes and 6-4 photolyase; DASH cryptochromes; plant cryptochromes; class II photolyase; Phra-like and class I photolyase. (B) Relationships among alkyltransferase proteins were inferred using the maximum likelihood method. Functional groups are Ada, Ogt and ATL. The corresponding *Leptospira* orthologous are shaded. For both analyses, the bootstrap consensus trees were inferred from 1000 replicates and only bootstrap support levels ≥ 50% are shown. The accession codes of the proteins analyzed are presented after the abbreviated names of organisms. The same methods apply to the next figures.

All species have one *alkD*, although *L. interrogans* presents an additional homolog (Fig. 1). *L. borgpetersenii*, on the other hand, is the sole lacking an *alkC* homolog. AlkC and AlkD 3-methyladenine DNA glycosylases are specific for removal of alkylated bases. These recently described proteins in *B. cereus* share no sequence similarity with any other glycosylase and thus define novel families of alkylbase DNA glycosylases [33].

Although uracil-DNA glycosylases (UDG) are present in all leptospires, the occurrence of two UDGs is exclusive to pathogenic leptospires. This enzyme deals with uracil in DNA, resulting mainly from deamination of cytosine [34]. The UDGs from pathogenic and saprophytic leptospires belong to different groups (Fig. 4). The saprophytic bacteria have family 1 UDG, which are the most efficient. On the other hand, both UDGs from pathogenic *Leptospira* belong to family 4, found in thermophilic organisms. LIC10548 clusters with UDGs from other spirochetes, suggesting that this is the original orthologous for the genus, while LIC13102 and LEPBII0821 were acquired through a LGT event. We could not assign a family for LIC13102 neither comparing its domains nor by phylogenetic analysis (Fig. 4).

The genus *Leptospira* showed remarkable differences in number of MutT-like proteins (Table S2). We found four, six and seven proteins from this family in *L. biflexa, L. borgpetersenii* and *L. interrogans*, respectively. They are able to hydrolyze the oxidative-induced 8-oxo-(d)GTP to the corresponding nucleoside monophosphate, avoiding their mutagenic incorporation into the DNA [35-37]. The enrichment of orthologous genes in the pathogenic species might be associated with the oxidative burst promoted by host macrophages. Accordingly, Luo and colleagues [38] identified a virulent-attenuated mutant for one nudix-hydrolase in Lai serovar (LA3977) present in all three species.

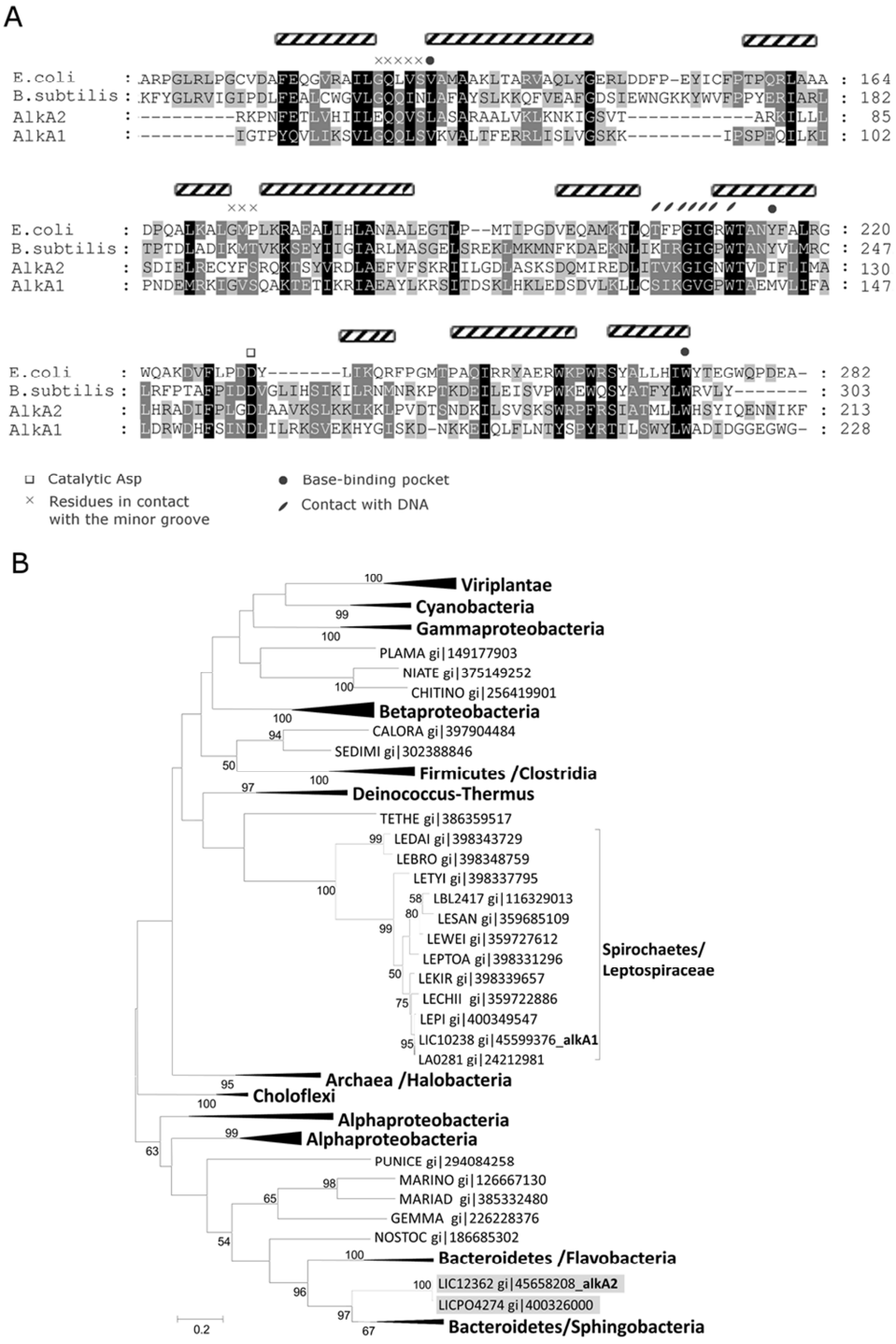

Figure 3. Comparison of AlkA amino acid sequences and phylogenetic analysis. (A) Amino acid sequence alignment and secondary structure prediction of LIC10238/LA0281 (AlkA1) and LIC12362/LA1370 (AlkA2) were carried out using *E. coli* and *B. subtilis* AlkA as reference. The proteins are composed of 10 alpha helices (striped rectangles). The residues responsible for contact with DNA, forming the base-binding pocket and the catalytic Asp are indicated. (B) Phylogenetic analysis of AlkA was performed as described in Figure 1. The *Leptospira* clade is indicated by the bracket. AlkAs present in *L. interrogans* serovar Copenhageni are named alkA1 and alkA2 and the sequences exclusive to *L. interrogans* are shaded.

Nucleotide excision repair (NER)

This pathway, composed of UvrA, UvrB, endonuclease UvrC and helicase UvrD, is responsible for removing any lesion causing distortion in the double helix [39]. Polymerase I and ligase proteins complete the repair [39]. Transcription-coupled nucleotide excision repair (TC-NER), requiring the TRCF protein (transcription repair coupling factor, *mfd* gene), permits faster repair of the transcribed strand in active genes [40]. This pathway is also complete in *Leptospira* (Fig. 1).

Leptospires present four different UvrD-like proteins. UvrD is a member of the superfamily 1A helicase, which includes PcrA, found in Gram-positives, and Rep, encoded by Gram-negative bacteria [41]. Their domains are shown in Fig. 5, with the phylogenetic analysis that revealed the identity of all three helicases. In addition, there is a shorter version of UvrD, with homologues only in *L. biflexa*, *L. interrogans*, *Leptonema illini* and *Turneriella parva* (Fig. 5). To our knowledge, leptospire is the first organism that shows this apparent redundancy for such helicases and their functional interplay is subject for future studies. UvrD is required for releasing the excised oligonucleotide from the post-incision complex in both NER and MMR (below). The functional activity of the new short UvrD helicase is still an enigma and future experimental studies are required. In *E. coli*, the truncated form of UvrD obtained through deletion of 40 or 102 residues for the C-terminal region shows reduced affinity for single-stranded DNA, but retains helicase activity on a variety of substrates [42].

Mismatch repair (MMR)

MutS and MutL proteins are responsible for preventing mutations that arise from errors of replication or recombination, ensuring the fidelity of DNA replication and repair [43,44]. All species of *Leptospira* present *mutS* and *mutL* genes, in addition to a *mutS3* gene (Fig. 1), member of a MutS subfamily with restricted distribution [45]. The *mutH* gene, present in *E. coli* but not common among other groups of bacteria, had no corresponding homologue.

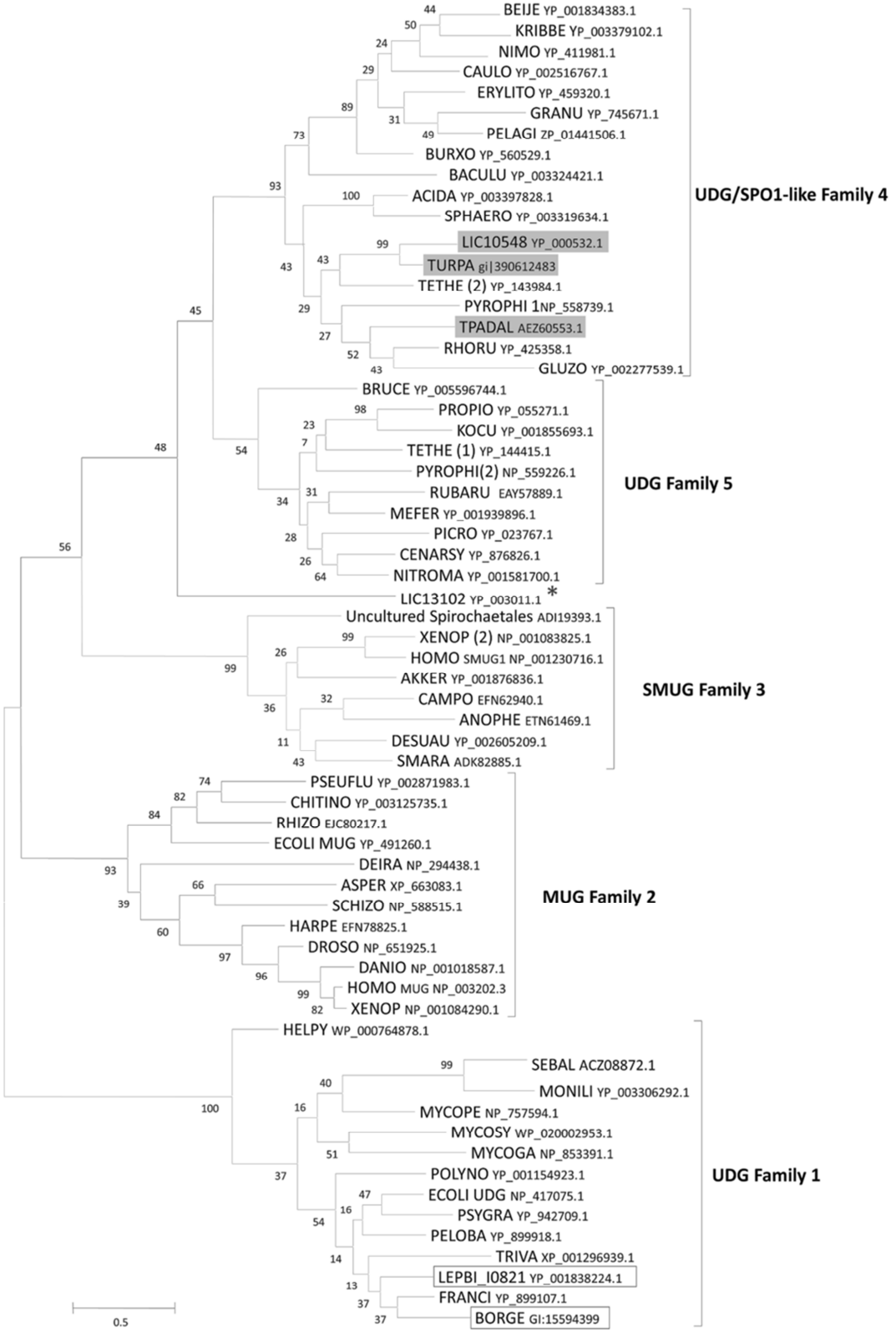

Figure 4. Phylogenetic analysis of leptospiral UDGs. The branches indicate functional groups: UDG/SPO1-like Family 4; UDG Family 5; SMUG Family 3; MUG Family 2 and UDG Family 1. Proteins from the spirochetes *T. pallidum*, *T. parva* and LIC10548, from *L. interrogans*, were grouped in the same clade (UDG/SPO1-like Family 4) and are shaded. LIC13102 is indicated by asterisk. The proteins from *L. biflexa* and *B. bourgdorferi* clustered with UDGs from Family 1 and are in rectangles.

Recombinational repair

Recombinational repair is essential to all living organisms, as it deals with double-strand break (DSB) damages, single-strand gaps and it restores the blocked or destabilized replication fork [34]. In most bacteria these processes are performed by homologous recombination (HR) apparatus (Fig. 1).

Homologous recombination (HR)

Present in all leptospires, it involves genetic exchanges between two DNA molecules sharing an extended region of nearly identical sequence (Fig. 1). There are two main pathways, the RecBCD and RecFOR, and an alternative one, RecQJ, acting in HR [46]. Apart from all these components, leptospires also possess two orthologous for the RecA DNA-binding modulator *ssb*, a characteristic of naturally transformable bacteria [47]. Zeng *et al*. [49] identified one of the *ssb* orthologous (LA1676) as component of the extracellular proteome, opening the possibility for a new function for SSB protein in leptospira physiology. Most bacteria express only one RecQ, from the auxiliary DNA end-resection pathway, whereas multicellular organisms express two or more [51]. *B. subtilis* an additional shorter version named RecS [52], with limited distribution. Both probably act in combination with RecJ in repair [53]. Leptospires express both the long and short versions (Fig. 1).

The SMC (structural maintenance of chromosomes) family members Mre11-Rad50 and RecN proteins are also present in leptospires (Table S3). The Mre11-Rad50 proteins stabilize DNA ends at a DSB [54]. A recent work on the transcriptional profile of leptospires in contact with the host showed significantly up-regulated mRNA levels of Mre11-Rad50 [55].

Non homologous end joining

When a homologous chromosome is not available, DSBs can be repaired through a highly mutagenic pathway, the non-homologous end joining (NHEJ) [56]. Bacterial NHEJ consists of a Ku homolog and an ATP-dependent ligase. ATP-dependent ligase is found only in *L. interrogans* (Table S3), in addition to a Gam-like protein, a phage-derived protein capable of assuming the functions of Ku. This suggests their acquisition through LGT.

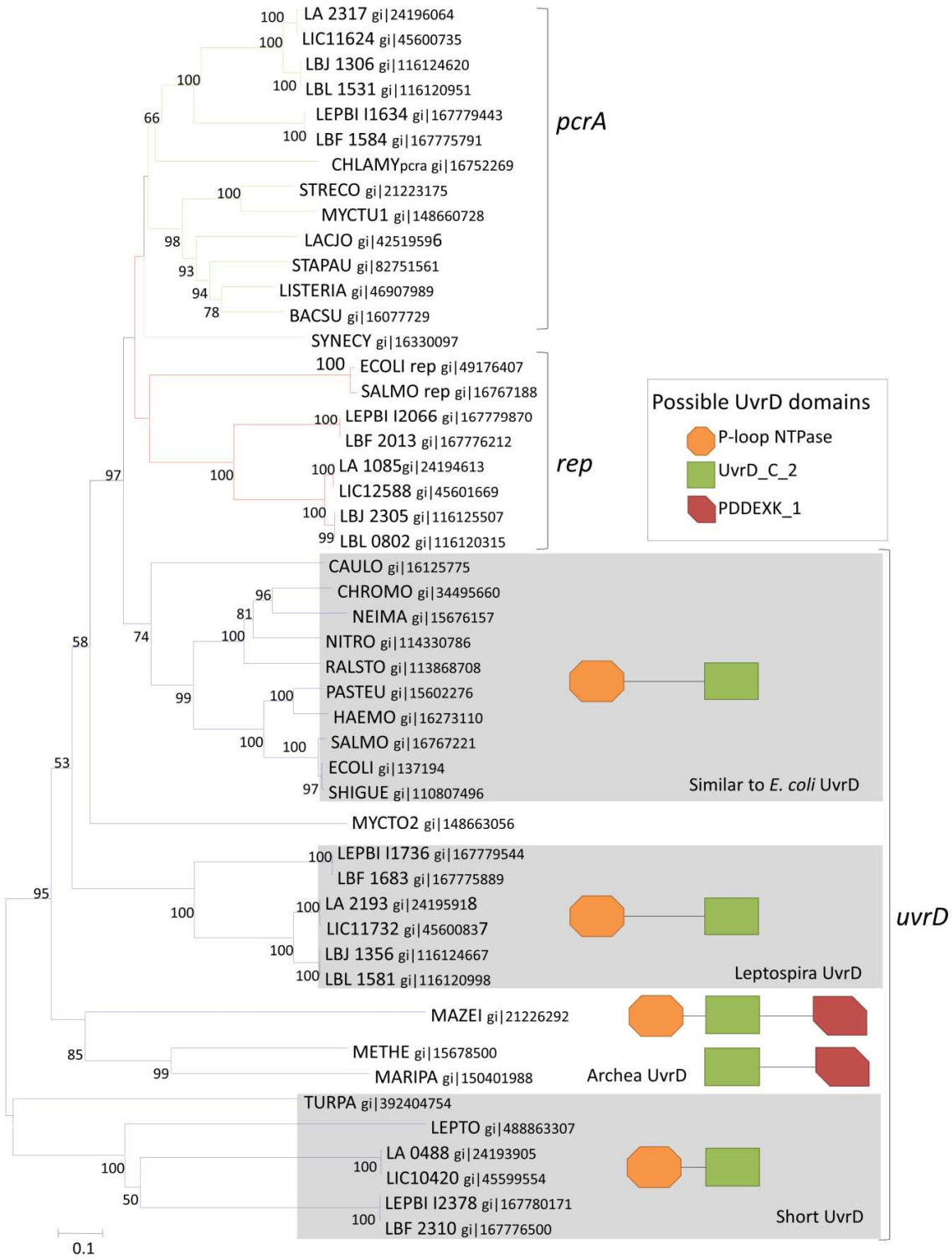

Figure 5. Phylogenetic tree for UvrD and related proteins. Schematic representations of domain structure present in UvrD and UvrD-like proteins are shown: the yellow boxes characterize P-loop NTPase domains; the green box shows the C-terminal domain of UvrDC2; the PDDEXK domain (rose box) characterizes members from a new nuclease superfamily [50]. *L. biflexa and interrogans*, together with the Spirochetes *Leptonema illini* and *Turneriella parva* have a shorter UvrD helicase. The *Leptospira* members with this shorter UvrD have four genes coding for uvrD/rep helicase family. The accession codes of the proteins analyzed are presented after the abbreviated names of organisms.

DNA damage tolerance

We found a very limited distribution of Y-family translesion synthesis (TLS) polymerases among leptospires (Table S4). These enzymes have the ability to bypass lesions that block progression of the replicative DNA polymerase. The *E. coli* PolV, *umuDC* gene product, is absent in leptospires, as well as the error-prone processing cassette expressing a second copy of the catalytic subunit of Pol III, *dnaE2*, *imuA and imuB* [57, 58].

Pol IV, coded by *dinP*, is present in *L. biflexa* and *L. interrogans* (Table S4). *L. borgpetersenii* lost this orthologous probably due to genomic rearrangements, since a transposase insertion is found in its corresponding locus (data not shown). The absence of TLS polymerases can lead to reduced survival under such stressing situations [59]. *dinP* is part of the SOS regulon from *L. interrogans* and it is up-regulated following the stress induced by UV-C [9]. In UPEC (uropathogenic *E. coli*) infections, DNA polymerase IV was critical for the survival of UPEC during genome instability driven by the host inflammatory assault [60]. Finally, *L. biflexa* encodes an archaea-like B family DNA polymerase (Table S4). This family was described as inactive since it contains disrupted versions of the essential sequence motifs for the catalytic function [61].

Other repair related proteins

SOS system

The SOS is an inducible and coordinated pathway comprised by a variable set of genes, and it is regulated by LexA, the repressor, and RecA, the sensor of ssDNA [34]. All *Leptospira* have both *lexA* and *recA* genes, suggesting a functional SOS system. However, the components of SOS regulon might diverge among the genus, as some of the identified regulated genes in *L. interrogans* are absent or in different genomic configuration in the other species. The serovar Copenhageni present an exclusive second *lexA*, acquired by LGT [9]. This adds complexity to the SOS regulon, more intricate than previously thought [62]. More than 25 genes are under direct control of LexA1 (Fonseca *et al*., manuscript in preparation).

Genes responsible for UV-induced lesion removal (*uvr* genes) are not up-regulated following UV exposition in *L. interrogans* [15], but do in *L. biflexa* (Renata Costa, results not shown). We and others [7, 9] showed the striking difference of UV-sensitivity among *L. biflexa* and *L. interrogans*. This difference in gene expression regulation is responsible for the higher sensitivity of *L. interrogans* to this environmental mutagenic agent.

DNA repair genes expression during experimental leptospirosis

Previous studies showed a different expression profile of some inflammatory mediators in the leptospirosis-susceptible C3H/HeJ and in its intermediate parental lineage, C3H/HePas, following inoculation with virulent *L. interrogans* serovar Copenhageni [17, 63].

The transcripts from *alkA1* and *alkA2*, *ada*, *tag*, *uvrA* and *ATP dependent DNA ligase* were tested for expression *in vivo* in relation to *in vitro*. With the exception of *ada* and *alkA1*-LIC10238 (data not shown), which were barely expressed in both situations, all tested genes showed up-regulation in the infected organ from the leptospirosis-susceptible mice (Fig. 6). In contrast, this did not happen in the intermediate mice, suggesting their requirement may not be associated with colonization, but with the development of disease. These findings suggest that assaults to leptospire DNA are introduced during infection, and specific DNA repair mechanisms are recruited. Importantly, genes acquired by LGT like *alkA2* and *ATP dependent DNA ligas*e also have roles in it.

The LexA1 targets *recA* and *recN* were up-regulated in both susceptible and intermediate mice (Fig. 6), indicating SOS activation in kidney is not associated with immunological competence, but with the environmental change from EMJH to organ. SOS activation was also observed for UPEC pathogenesis, consistent with DNA damage induction by the host intracellular environment [64]. Our results point to similar destabilization of *Leptospira* genome during kidney infection. In addition, since *uvrA* is not a component of the *L. interrogans* SOS regulon [15], the *uvrA* up-regulation during infection implies a distinct and specialized transcriptional control of NER to deal with DNA insults introduced during infection.

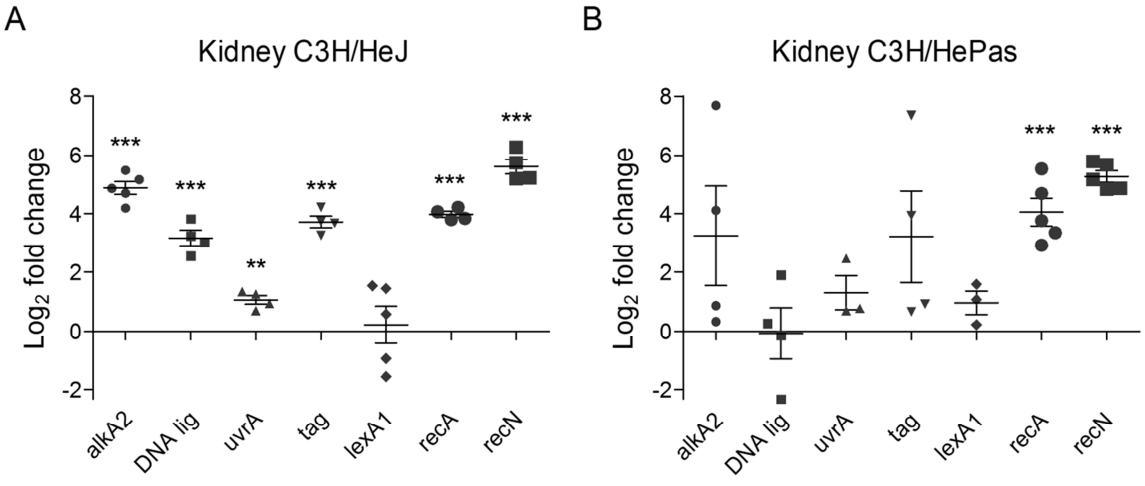

Figure 6. *In vivo* DNA repair-related genes expression. mRNAs from kidneys of intermediate (C3H/HePas) and susceptible (C3H/HeJ) mice inoculated with *L. interrogans* serovar Copenhageni was extracted. The corresponding cDNA was used as template for quantitative PCR analysis of a*lkA2* (LIC12362), *ada* (LIC13402); *tag* (LIC13064); *uvrA* (LIC11717), *ATP dependent DNA ligase* (LIC12677), *lexA1* (LIC12305), *recA* (LIC11745) and *recN* (LIC11620). The Ct values were normalized to the 16S rRNA control and compared to the *in vitro* cultured (EMJH) leptospires. The gene expression profile is expressed logarithmically as the $\log_2$ of the fold change between the two conditions. Experiments were performed in groups of three mice and were repeated on two separate occasions. The outlier values (calculated by MAD-median method) were excluded from the analyses. Two-tailed Student's t-test P values are shown as *** for P<0.001, ** for P<0.01 and * for P<0.05.

CONCLUSIONS

The present work assessed the DNA repair-related genes in *Leptospira*. The set of DNA repair genes diverges considerably from model organisms, what renders it as an interesting organism for future studies. Some examples are the new *MutS*-like and the shorter version of *UvrD*, restricted to the genus and to the family, respectively. In addition, although gram-negative, leptospires share some orthologous from gram-positive bacteria involved in direct repair, NER and HR.

Leptospira has an unusual number of genes related to the reversion or removal of alkylation lesions, even in the genome-reduced *L. borgpetersenii*. Moreover, *L. interrogans* possesses the highest number of genes encoding alkyl DNA-glycosylases. These unique genes showed a distinct evolutionary history compatible with LGT.

The extent of lateral transfer of DNA repair genes into the genus was unexpected. Most of the differences among the pathogenic and free-living bacteria were consequence of

gene gain rather than gene loss, since the pathogenic *L. interrogans* is more enriched in DNA repair genes. The massive occurrence of LGT events is reflected in several aspects of DNA repair, since it provided novel and additional components for direct and excision repair, tolerance and SOS response.

The up-regulation of DNA repair and SOS components during experimental leptospirosis suggests that DNA integrity is important for the pathogen to be able to colonize its host. Future studies will provide insights to understand how DNA repair affects the lifestyle of *Leptospira*.

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5. GENERAL CONCLUSIONS

Leptospires are assumed to be at the base of the phylogenetic tree of spirochetes, themselves being one of the oldest groups of bacteria. They are known by their genetic stability, believed to be a result of their slow growth, which provides fewer opportunities for genetic changes. Nevertheless, the pathogenic strains exhibit more variation between strains than the basal saprophytic leptospires. Recognizing the importance of stress responses in providing means for genetic variability in bacteria, we studied the effects of UV-C irradiation in *L. interrogans* serovar Copenhageni, duration and impact of the SOS system in the general DNA damage response and assessed the DNA repair toolbox of pathogenic and saprophytic leptospires.

During the development of this work, we identified two different SOS boxes for LexA1, and a possible SOS box for LexA2. The use of *in vitro* low throughput approaches as the ones applied by us and others to identify the former LexA1 SOS box (such as DNase I footprinting assay and EMSA) can provide important information about specific DNA-protein interactions. However, the update of the SOS box achieved by *de novo* motif search on ChIP-seq-derived data shows how high throughput analysis allows a better understanding of the complexity of biological systems.

Taken together, our findings suggest DNA damage response can be a powerful source of genetic variation for leptospires, in SOS-dependent or independent pathways. The main effectors of these changes seem to be mobile genetic elements, which orchestrate gene gain and gene loss in the genus. For instance, transposons and prophages influence dramatically the DNA repair tools available, and even the SOS response itself. Prophages supplied a Gam-like protein that could act with ATP-dependent DNA ligase and allow non-homologous end joining in *L. interrogans;* a second LexA repressor exclusively to serovar Copenhageni is found among remnants of an ancient prophage; and several hypothetical proteins which are induced during genotoxic stress. The slight repression of CRISPR RNA during DNA damage response, as assessed by qPCR, could also facilitate entrance of

foreign bacteriophage DNA. The SOS response has a paramount role in this induction, since several of these players seems to be controlled by LexA1.

In addition, we identified almost all DNA repair pathways in the genus, and some with great redundancy in *L. interrogans*. However, low doses of UV-C leads to high mortality rates in serovar Copenhageni. This is probably consequence of the combination of low constitutive expression levels of the majority of DNA repair genes and the lack of induction, or even down-regulation, of genes dedicated to the repair of UV-C-induced lesions.

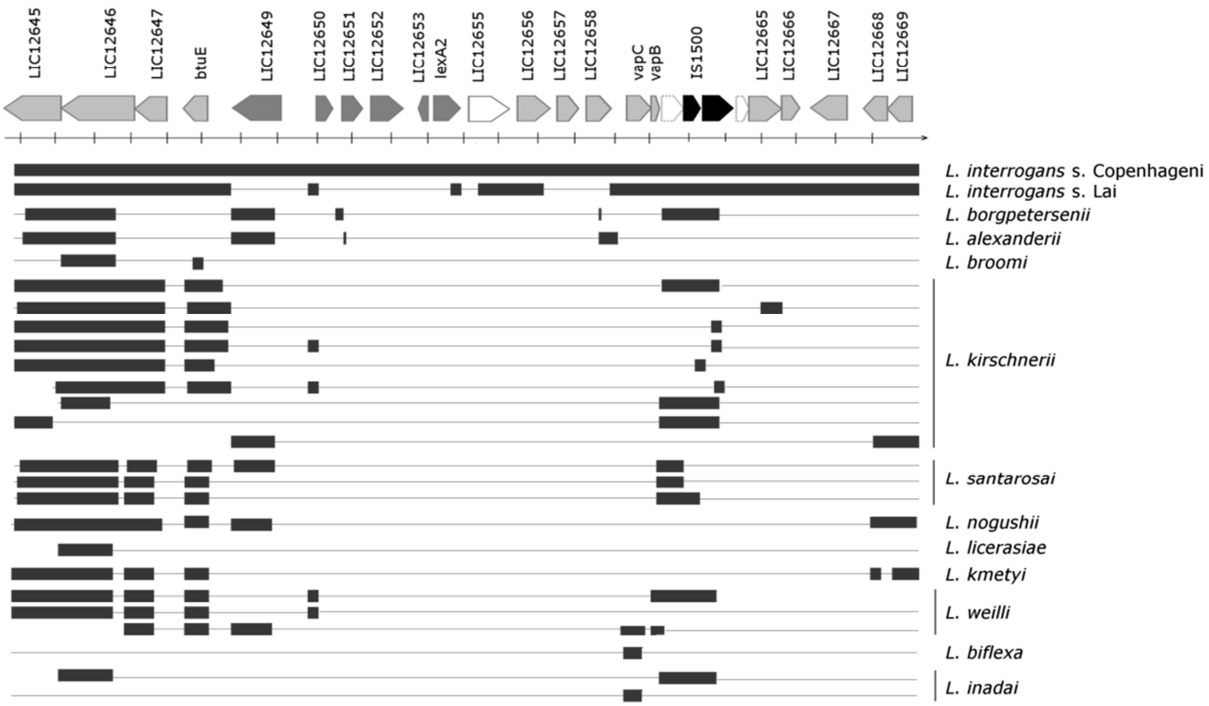
Finally, we observed *L. interrogans* serovar Copenhageni shutting down the majority of its virulence genes during the DNA damage response, except for some putative virulence factors with RecA-dependent control of expression. Nevertheless, the same response seems to increase mutagenesis and genomic rearrangements alongside with recombination and structural maintenance of the chromosome components, suggesting an organized induction of genetic variability. We propose *L. interrogans* pauses its virulence mechanisms and allow these variations to occur, when subpopulations of daughter cells can better adapt to the environment or even develop different mechanisms for colonization, invasion and pathogenesis.

6. APPENDICES

6.1. Appendices – Chapter 1

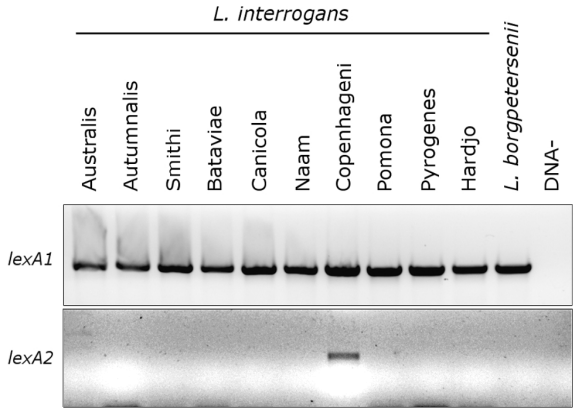
Supplementary figures from Chapter 1. Supplementary tables are available at http://dx.doi.org/10.1371%2Fjournal.pone.0076419.

Figure S1



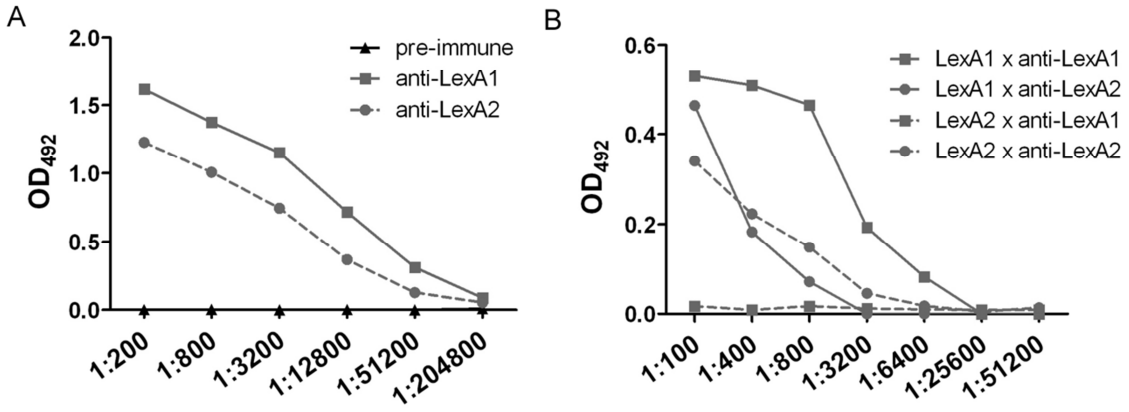
Comparison of *lexA2* region between sequenced leptospires. Scheme representing MEGABLAST searches against leptospire genome sequence projects used the whole genome shotguns contigs database (wgs) at GeneBank. The regions shown (red strands) are those with alignment score greater than 200, relative to the region in *L. interrogans* serovar Copenhageni.

Figure S2



Presence of *lexA1* and *lexA2* in the genome of different leptospires detected by PCR. The reactions used 20ng of genomic DNA, and the primers were designed for serovar Copenhageni, according to Table 1. The negative reaction was carried without template DNA. The upper panel corresponds to *lexA1*, while lower panel, to *lexA2*. All amplicons had the expected molecular size corresponding to 621 bp for *lexA1* and 630 bp for *lexA2*.

Figure S4

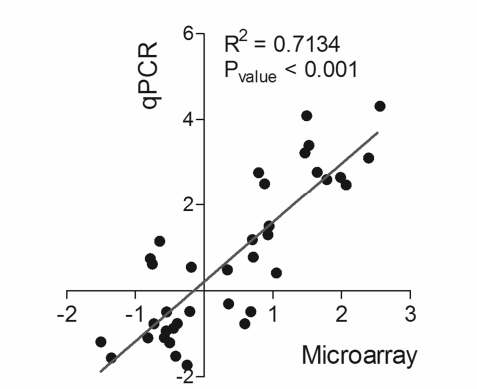


Titration and specificity of anti-LexA1 and -LexA2 sera. Anti-sera were generated by intraperitoneal immunization of five BALB/c mice with 10 µg of purified protein in $Al(OH)_3$. The immunizations were performed weekly in four doses and mice were bled by the retrorbital plexus one week after the last dose. (A) Sera titration following the protocol by Hauk *et al*. (2005), comparing pre-immune and immune sera. Continuous line with squares corresponds to anti-LexA1, while discontinuous line with circles corresponds to anti-LexA2; triangles mark the pre-immune serum. (B) Cross-reaction analyses. The continuous line represents anti-LexA1, and the discontinuous one represents anti-LexA2. Squares stand for coating with purified LexA1, and circles, LexA2.

6.2. Appendices – Chapter 2

Supplementary figures and tables from Chapter 2.

Figure S1



Validation of microarray expression data. Correlation between gene expression fold change after DNA damage as assessed by qPCR or microarrays. It was only plotted points with P value < 0.05.

Table S1. LexA1 ChIP-seq data features

Sample		**Total reads**	***Reads* aligned to reference genome (%)**		
			zero time	1 time	> 1 time
40min cross-link	Total	17056735	0.38	94.47	5.15
	IP	18875232	8.01	87.42	4.57
60min cross-link	Total	17813614	0.45	94.38	5.17
	IP	19771542	6.63	88.77	4.6

Table S2. Oligonucleotides used in this study

Oligo	Target	Sense oligo sequence	Reverse oligo sequence	Amplicon (bp)
	coding region			
16S	rrs	CATGCCGCGGTGAATACGTT	CGACTTCACCCCCTTCACGA	101
lexA1	lexA1	TTGTGGCCTGAACGGGAAGT	GGCAAAGGGTGCCTACGATC	131
lexA2	*lexA2*	GGCGACCCGGTAGAACGAAT	TGCAATCACCAAGTCCCCATCT	129
recN	recN	GAGAATGTGGGCGGGATGGA	GTTGTGAACTTCGGCGAGCA	104
dinP	dinP	GAAACGCGCAAAATCATTCATGT	CAGCCGAAACAACCGATCTACTATT	133
LSF19/20	LIC12298	TCGAACGCTTCTTCCAGATAA	TGCAAGAATCAGCAAATTACCA	130
LIC12653	LIC12653	TCCGGATTCGATCTCGGGA	GTCAATGACTCGAGGTGAGTTTGT	122
LSF51/52	LIC10169	CCGCTCGATACTTAAATCTTTCG	CCTTATCCAAATCTCCAACCATAAA	120
recA	recA	TTGATTCTGTGGCGGCTTTGG	TCTAAGCGCCTGGGACATGAG	101
LSF25/26	fliF	GTCGATCCAGAACAAAGACAAG	GTCGAACTGTGTTTCGGTAAAT	120
LSF23/24	nrdA	AGGACAGAAGGCTGGATTG	CAAGGCCCATTTGTAGGTTATG	147
LSF29/30	LIC12975	CGAGTTACACCGGATTCTCTTT	GGGAATACTCGATTCTTTCGATTTC	138
LSF31/32	LIC11622	GACACAGATCCGGTTCCTTTAG	AACTTGCAGTTCCATCTCCTC	121
	ChIP-peak			
lexA1up2	*lexA1*	AGGAGCAGAGATCGTTTCTA	CAGGTCTTTCATACTAAACAAC	124
lexA2up1	*lexA2*	TTTCCTCTGGAAGGATCGTA	CAATTAAAGCAATGGGACA	115
LSF57/58	CRISPR	TTTCTTAGTTTCTCGGGCTCTAAA	TGCTTCCCAGTAGCTTTGC	117
LSF01/02	LIC10169	GGTGAAACTTGAAAGTCGTATTC	AACCCAAACGATTTAGGACAA	200
recAup1	recA	GATTCTTTCTCAATAATACT	ATGAATCGATTTCAATTT	121
LSF05/06	lic12382	ACCAAATCTATGAACGACTTGATTG	CGTAGAATCAATATTGGAGACGAAAG	186
LSF35/36	fliF	GGGATTCTCTCTACGATCTCTCT	CTCGTATCGGAACTACCGTATTG	131
LSF07/08	nrdA	CTGTTAATCACTGGGTCGCT	ACGATAATCGGATTTCTGTTGA	186
LSF41/42	lic12975	ACCCTTGCACATCTTTATCTCA	CCCAGGTTTCAGATCTGCTT	138
LSF09/10	wzyC	ACTGGCTAATCCAAAGATCAATTTC	CAAAGTTGTTCTTCTTTCTGGATCA	190
LSF11/12	LIC11622	TGGGAACTGTGCTTGGAATTA	CCATTTCAGCAATCGTGTCTTC	177

Table S3. Complete information on the ChIP-seq peaks and LexA1-controlled genes.

# Peak	Chromosomal		Score		ChIP-qPCR (60min cross-link)*	Predicted SOS box**	Distance from downstream	Peak-associated genes	Possible protein function	Log2FC: UV-C induction (microarray)***	Log2FC: UV-C induction (qPCR) ***
	Begining	End	40min cross-link	60min cross-link							
1	2780131	2780251	23.07	35.51	2.97 ± 1.1	CTACTAAGTTGTTTAG	1	lexA1	SOS repressor	2.4	3.1
2	1982311	1982431	18.27	27.54	-	GGTATAATTTGTTTAG	-23	recN	recombinase	2.56	4.31
3	3724351	3724471	13.67	21.18	-	CATTACAAATGTTTAG	-29	dinP	DNA polymerase IV	-	2.48
4	2772631	2772751	9.36	16.23	2.55 ± 0.02	CTAAACAGATATTTAC	-34	LIC12298	kinase	1.79	2.12
5	3218551	3218671	8.95	14.20	-	CTAATCATGTACTTAG	-12	lexA2	SOS repressor	0.79	2.75
5	3218551	3218671	8.95	14.20	-	CTAATCATGTACTTAG	-28	LIC12653	hypothetical protein	1.64	2.76
6	198931	199051	8.30	13.05	2.55 ± 0.02	CTATATATATGTTTAG	-545	LIC10169	phage excisionase	2.07	2.47
7	3533911	3534031	8.15	12.12	4.7 ± 0.1	CTTAGCGTTTGTTGAG	?	CRISPR	putative clustered regularly interspaced short palindromic repeats	-	-0.15
8	2130271	2130391	7.50	11.60	4.84 ± 0.3	CTATACAAATACTTAG	-191	recA	recombinase A	1.99	2.65
9	2888611	2888731	5.76	9.56	3.3 ± 0.8	ACATTTGTATGCCAAC	204	lic12382	lysophospholipid acyltransferase	-	1.15
10#	348625	349064	5.46	7.89	-	ACATACAAGTACTTAG	-373	lic20276	helix-turn-helix domain protein	-	-
11	1709491	1709611	5.19	8.47	4.7 ± 0.5	ATACACAGATGTTAAG	-99	fliF	MS-ring flagellar protein	-	-0.32
12	2730691	2730811	4.65	6.63	-	CTAACCACTGGTAATG caa CTACACAAATCACGAG	-322	lic12260	hypothetical protein	-	-
13	1949251	1949371	4.52	6.57	1.7 ± 0.4	CTCAATTTTTGTATAG	-348	nrdA	ribonucleotide-diphosphate reductase alpha	-	1.76
14	1194811	1194931	4.13	5.83	-	ACTATCATGTGTTTAG	164	lic10987	acetoacetate decarboxylase	0.81	-
15	1976551	1976671	3.54	5.03	-	CTAATCCTGTGATTAG	923	mutY	adenine DNA glycosylase	0.84	-
16	3144511	3144631	2.96	4.06	-	CTGTACAAATATCAAG	-304	lic12595	fumarylacetoacetate hydrolase	-	-
17	2072911	2073031	2.91	4.05	-	CTACACAAACATTGAG	184	fbp	fructose-1,6-bisphosphatase	-0.79	-
18	3613291	3613411	2.84	4.71	1.94 ± 0.6	CAAAGTATTGGATCTG	186	lic12975	phospholipid synthase	0.84	0.81
19	3612811	3612931	2.83	4.69	-	ATCATTCTACTTTTAG	-290	lic12975	phospholipid synthase	0.84	0.81
20	2223130	2223364	-	2.61	1.97 ± 0.6	GTAAACATTGGTTTAT	940	wzyC	O-antigen ligase/polymerase	-	0.44
21	4273580	4273844	2.47	2.56	-	CCACACCTTTGTGAAC	1256	gidA	glucose inhibited division protein A	-	-
22	4157131	4157251	2.39	2.55	-	CTTCACAAAGATTGAG ttctggaattccgg CTTATTACCCGTATAG	109	lic13392	polysaccharide deacetylase	-	-
23	1193731	1193851	2.21	3.05	-	CTGTATAAACGTTAAG	-275	lic10986	immunity protein imm25	1.03	-
24	1984531	1984651	2.17	2.54	4.71 ± 0.5	GTGAGGCGTTGATTAC	-136	lic11622	biopolymer transport exbd-related	-	1.12
25	3074311	3074431	2.04	2.87	-	GTAAACATCCGTGCAC ctcccaaatatcttt CCCGACACTTGTTTTC	-32	risA	riboflavin synthase subunit alpha	-	-

* Fold enrichment in relation to *rrs* coding region

** for peaks containig more than one SOS, both are shown, with the nucleotides separating them in lowercase

*** shown only P-value < 0.05

located at chromosome 2

Table S4. Top 25 genes with known function (excluding phage-related genes) with altered expression 12h after UV-C as assessed by DNA microarrays.

Symbol	Description		
Top 25 up-regulated genes with known function		**P-value**	**Log2FC**
recN	DNA repair protein	0.003	2.561
lexA	LexA1 repressor	0.020	2.396
lemA	lipoprotein	0.003	2.363
recA	recombinase A	0.012	1.986
LIC12599	alpha/beta fold family hydrolase	0.002	1.883
LIC10373	lipoprotein	0.028	1.704
LIC13120	diguanylate phosphodiesterase	0.012	1.583
fumC	fumarate hydratase	0.004	1.553
LIC13192	histidine kinase response regulator hybrid protein	0.033	1.538
LIC12297	RadC	0.031	1.528
LIC10378	ParR family transcriptional regulator	0.016	1.473
LIC12253	lipoprotein	0.046	1.464
LIC11207	lipoprotein	0.020	1.389
gyrA	DNA gyrase subunit A	0.035	1.333
fliP	flagellar biosynthesis protein FliP	0.033	1.333
dbp	peptide ABC transporter substrate-binding protein	0.042	1.279
LIC12808	histidine kinase sensor protein	0.019	1.246
LIC10022	arsenate reductase	0.017	1.213
LIC13321	thermolysin homolog precursor	0.002	1.203
LIC13250	apolipoprotein n-acyltransferase	0.035	1.185
LIC10157	hypothetical protein	0.029	1.180
LIC10005	DNA gyrase subunit B	0.008	1.177
LIC11467	RCC1	0.040	1.165
LIC12457	chemotaxis protein	0.049	1.152
ech3	enoyl-CoA hydratase	0.025	1.140
Top 25 down-regulated genes with known function			
flaB	flagellin protein	0.005	-2.183
LIC11890	flagellin protein	0.013	-1.932
flgJ	flagellum-specific muramidase	0.007	-1.603
flaA-1	flagellar filament sheath protein	0.009	-1.306
lig	NAD dependent DNA ligase	0.042	-1.294
LIC12925	citrate synthase	0.004	-1.249
LIC11680	alginate o-acetyltransferase	0.031	-1.237
flgE	hook protein	0.001	-1.219
LIC10562	lipoprotein	0.040	-1.216
LIC11852	O-acetylhomoserine (thiol) lyase	0.004	-1.213
encK	O-methyl transferase	0.002	-1.209
wcaJ	UDP-glucosyltransferase	0.011	-1.199
LIC10875	molybdopterin oxidoredutase membrane subunit	0.049	-1.198
cysT	sulfate ABC transporter permease	0.033	-1.193
LIC11093	glycosyl transferase	0.018	-1.187
sbp	periplasmic sulfate-binding protein	0.042	-1.184
LIC10900	adenylate/guanylate cyclase	0.043	-1.160
LIC10553	hydrolase	0.018	-1.156
lpxC	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	0.018	-1.144
leuA2	2-isopropylmalate synthase 2	0.018	-1.139
LIC10628	lipoprotein	0.021	-1.122
LIC13006	lipoprotein	0.037	-1.105
panE2	2-dehydropantoate 2-reductase	0.038	-1.104
LIC12079	ABC transporter ATP-binding protein	0.046	-1.102
mraY	phospho-N-acetylmuramoyl-pentapeptide-transferase	0.020	-1.099

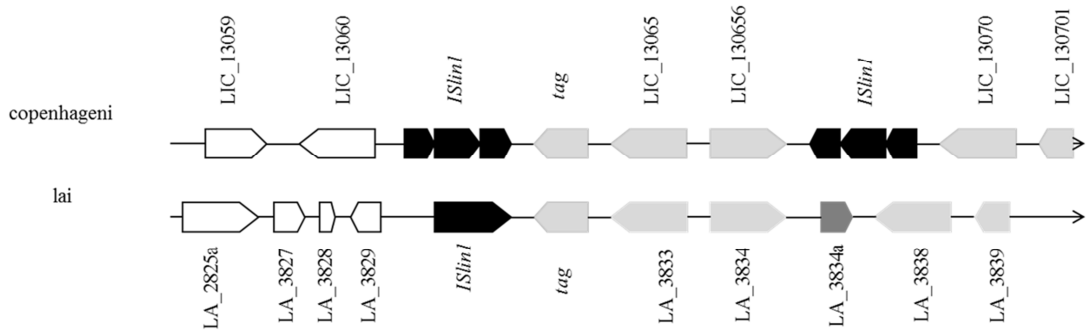
Table S5. Prophage genes annotation and relative expression 12h after UV-C as assessed by microarrays

Symbol	Description	P-value	Log2FC
Prophage 1			
LIC10163	phospholipase A2	0.00018	3.263691
LIC10164	DNA-binding protein	0.00035	3.624575
LIC10165	Mu Gam-like protein	0.00023	3.469367
LIC10166	C1 repressor	6.22E-05	3.989203
LIC10167	phage integrase	4.10E-05	3.93743
LIC10168	C1 repressor	9.41E-05	2.066484
LIC10169	phage excisionase	0.00173	1.494869
LIC10170	hypothetical protein	9.32E-05	3.250165
LIC10171	hypothetical protein	0.00056	3.131419
LIC10172	pore-forming toxin	0.00074	1.765498
LIC10173	hypothetical protein	9.80E-05	3.408187
LIC10174	hypothetical protein	0.00017	3.985362
LIC10175	phage tail sheath protein	0.00013	4.193307
LIC10176	phage-related protein	0.00023	3.917526
LIC10177	Type I restriction enzyme	0.00016	3.627451
LIC10178	phage-related protein	6.67E-05	3.885735
LIC10179	phage tail protein	0.00018	3.817646
LIC10180	phage tail protein with LysM domain	8.98E-05	3.790418
LIC10181	phage late control gene D protein	0.00035	3.079053
LIC10182	phage baseplate assemply protein	0.00011	3.364402
LIC10183	hypothetical protein	0.00068	3.269313
LIC10184	phage baseplate assembly protein J-like	3.11E-05	2.960475
LIC10185	hypothetical protein	0.00053	2.743043
LIC10186	glycosyl hidrolase	0.00019	4.16465
LIC10187	hypothetical protein	0.00013	3.074527
LIC10188	hypothetical protein	0.00096	2.867868
LIC10189	hypothetical protein	0.00617	2.169985
Prophage 2			
LIC12600	hypothetical protein	0.00104	3.11097
LIC12601	phage tail collar protein	0.00016	2.948577
LIC12602	phage tail collar protein	0.0003	3.014243
LIC12603	pectinesterase	0.00025	3.239405
LIC12604	phage tail protein	0.00054	3.755167
LIC12605	phage baseplate J protein	0.00038	3.52092
LIC12606	putative phage protein	0.00025	3.644773
LIC12607	phage baseplate assembly protein V	6.71E-05	3.463424
LIC12608	Late control gene D protein	0.00013	3.26978
LIC12609	plasmid killer toxin	0.00079	3.541799
LIC12610	hypothetical protein	1.72E-05	3.33374
LIC12611	phage tail protein *plbA*-like	0.00016	3.331647
LIC12612	hypothetical protein	3.89E-05	3.837924
LIC12613	hypothetical protein	7.05E-05	3.660122
LIC12614	putative phage protein	0.00015	3.693915
LIC12615	putative phage protein	0.00023	3.577897
LIC12616	minor capsid protein	0.0002	3.362965

6.3. Appendices – Chapter 3

Supplementary figures and tables from Chapter 3.

Figure S1



Genomic organization of the *tag* region. Schematic representation of the *tag* genomic region from *L. interrogans* serovar Copenhageni (upper) compared to the equivalent region of *L. interrogans* serovar Lai (lower). Arrows represent predicted genes and transcription orientation. Light grey arrows represent genes orthologous between genomes and in the same synteny, black arrows indicate genes encoding transposases. White arrows represent orthologous genes that are in different synteny due to the rearrangement in serovar Lai. The dark grey arrow represents an ORF that is specific to Lai.

Table S1. Putative DNA repair of *Leptospira* for direct repair. Each ORF is identified by the corresponding locus tag.

Genes	*L. biflexa*	*L.interrogans*	*L.borgpetersenii*
phra-like *	LBF2420	−−−	−−−
*photolyase b**	LBF2340	LIC11339	−−−
splB	LBF0608	LIC10195	LBJ0162
*ada**	LBF4131	LIC13402	LBJ2922
alkB	LBF3071	−−−	−−−
*atl**	LBF0549	LIC10119	LBJ0115

* Genes that had their identity confirmed by phylogenetic analysis.

Table S2. Genes encoding putative repair proteins for Excision Repair in *Leptospira*. Each ORF is identified by the corresponding locus.

Genes	*L. biflexa* Ames	*L. interrogans* Copenhageni	*L. borgpetersenii* JB197
Base excision repair			
alkA *	−−−	LIC10238; **LIC12362**	LBJ0662
alkC	LBF0595	LIC11639	−−−
alkD	LBF2342	LIC12552; LIC11252	LBJ1745
mutY	LBF1233	LIC11614	LBJ1296
Tag	−−−	**LIC13064**	−−−
Ung or udg *	**LBF0791**	LIC10548; **LIC13102**	LBJ2712; **LBJ0598**
MutT-Nudix motif	−−−	LIC12191	−−−
MutT-Nudix motif	−−−	LIC10257	LBJ2602
MutT-Nudix motif	LBF0166	LIC13264	LBJ0192
MutT-Nudix motif	pseudo	LIC20067	pseudo
MutT-Nudix motif	LBF2015	LIC12589	LBJ2308
MutT-Nudix motif	LBF3172	LIC10106	LBJ0097
MutT-Nudix motif	LBF3212	LIC13180	LBJ0418
endonuclease III (nth)	LBF0987	LIC11759	LBJ1856
exonuclease III (xth)	LBF1776	LIC11452	LBJ1669
Nucleotide excision repair			
uvrA	LBF1784	LIC11717	LBJ1403
uvrB	LBF0814	LIC12941	LBJ2353
uvrC	LBF2022	LIC11756	LBJ1853
Mfd	LBF1770	LIC11455	LBJ1666
uvrD *	LBF1683	LIC11732	LBJ1356
pcrA *	LBF1584	LIC11624	LBJ1306
repA *	LBF2013	LIC12588	LBJ2305
Short uvrD *	LBF2310	LIC10420	−−−
Mismatch repair			
mutL	LBF2096	LIC12514	LBJ2225
mutS1	LBF0802	LIC11774	LBJ1867
mutS3 **	LBF0324	LIC13389	LBJ2911

* Genes that had their identity confirmed by phylogenetic analysis.
**Genes specific to Leptospiraceae.
Bold: Genes possibly obtained by LGT events.

Table S3. Putative proteins related to recombinational repair in Leptospira spp. Each ORF is identified by the corresponding locus tag.

Genes	*L. biflexa* Ames	*L. interrogans* Copenhageni	*L. borgpetersenii* JB197
HR			
recA	LBF2374	LIC11745	LBJ1344
recB	LBF5019	LIC12686	LBJ0684
recC	LBF5020	LIC12688	LBJ0685
recD	LBF5018	LIC12685	LBJ0683
recF	LBF0006	LIC10003	LBJ0006
recG	LBF0338	LIC13150	LBJ0400
recJ	LBF1255	LIC11066	LBJ1989
recO	LBF2037	LIC12104	LBJ1202
recR	LBF3348	LIC13476	LBJ3003
recQ	LBF0300	LIC12961	LBJ0296
recS	LBF2428	LIC12689	LBJ0686
ruvA	LBF1447	LIC11148	LBJ1987
ruvB	LBF0505	LIC12811	LBJ0761
ruvC	LBF3203	LIC12885	LBJ0544
ssb1	LBF2873	LIC10564	pseudo
ssb2	LBF2045	LIC12112	LBJ1194
SMC			
recN	LBF1580	LIC11620	LBJ1302
mre11	LBF2807	LIC10252	LBJ0659
rad50	LBF2806	LIC10251	LBJ0658
NHEJ			
ATP dependent ligase	−−−	**LIC12677**	−−−

Bold: Genes possibly obtained by LGT events.

Table S4. *Leptospira* genes related to DNA damage tolerance. Each ORF is identified by the corresponding locus tag.

Genes	*L. biflexa*	*L. interrogans*	*L. borgpetersenii*
Y-family TLS			
dinP	LBF4145	LIC13052	−−−
B-family			
DNA pol II	**LBF2104**	−−−	−−−

Bold: Genes possibly obtained by LGT events.

7. GENERAL REFERENCES

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8. CURRICULUM

PERSONAL INFORMATION

Name: Luciane Schons da Fonseca
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EDUCATION

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Universidade Federal do Rio Grande do Sul, Porto Alegre, RS; 2009.

OCCUPATION

Ph.D. student fellow, FAPESP; September/2009 to January/2015.
Visiting Ph.D. student fellow at Massachusetts Institute of Technology, January to July/2013.

PUBLICATIONS (Complete articles and Abstracts in Conferences)

Articles

FONSECA, LS; DA SILVA, JB; MILANEZ, JS; VITORELLO, CBM; MOMO, L; MORAIS, ZM; VASCONCELLOS, SA; MARQUES, MV; HO, PL; DA COSTA, RMA. *Leptospira interrogans* serovar Copenhageni Harbors Two *lexA* Genes Involved in SOS Response. Plos One, v. 8, p. e76419, 2013.

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