Inactivation of *clpB* in the Pathogen *Leptospira interrogans* Reduces Virulence and Resistance to Stress Conditions[▽][†]

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***Leptospira interrogans* is the causative agent of leptospirosis, which is an emerging zoonotic disease. Resistance to stress conditions is largely uncharacterized for this bacterium. We therefore decided to analyze a *clpB* mutant that we obtained by random transposon mutagenesis. The mutant did not produce any of the two isoforms of ClpB. The *clpB* mutant exhibited growth defects at 30° and 37°C and in poor nutrient medium and showed increased susceptibility to oxidative stress, whereas the genetically complemented strain was restored in ClpB expression and *in vitro* wild-type growth. We also showed that the *clpB* mutant was attenuated in virulence in an animal model of acute leptospirosis. Our findings demonstrate that ClpB is involved in the general stress response. The chaperone is also necessary, either directly or indirectly, for the virulence of the pathogen *L. interrogans*.**

Spirochetes belonging to the pathogenic *Leptospira* spp. are the causative agents of leptospirosis, which is an emerging disease with more than 500,000 annual severe cases worldwide (1). Leptospires colonize renal tubules of animal reservoirs and are excreted into the environment, where the bacteria can survive for weeks or even months in moist soil and water (37). Leptospirosis is usually transmitted to humans by contact of abraded skin or mucous membranes with water that is contaminated with the urine of infected animals. Leptospires then disseminate into the host, causing febrile illnesses and severe disease manifestations that can lead to death (16).

Like many other pathogenic bacteria, leptospires are subjected to environmental stresses during infection. Temperature constitutes an important signal in *Leptospira* spp., leading to alterations in gene transcription (20, 21, 32) and protein expression (3, 21). Stressful conditions may result in damage to intracellular proteins. Proteome analysis of *L. interrogans* has revealed that stress and heat shock proteins, including the chaperones DnaK, GrpE, and GroEL as well as the Hsp100/Clp chaperone ClpB, are upregulated at 37°C in comparison to 30°C, which is the optimal temperature for *Leptospira in vitro* growth (21).

The Clp chaperones and proteases are highly conserved across prokaryotes and are found in eukaryotes. Clp ATPases are divided into two main classes (35). Members of class I, which includes ClpA, ClpB, ClpC, ClpD, ClpE, and ClpL, contain two ATP nucleotide-binding domains or AAA+ (ATPases associated with various cellular activities) domains that have characteristic Walker A and Walker B nucleotide-binding motifs. Proteins of class II, which include ClpX and ClpY (HslU), are smaller and contain only one AAA+ domain. Most of these Clp ATPases (except ClpB) are associated with a proteolytic subunit (ClpP or ClpQ/HslV). These chaperones and proteases function mainly in the disaggregation, unfolding, and degradation of native as well as misfolded proteins. They have been shown to be involved in general stress responses in various bacteria.

In *Escherichia coli*, ClpB is a chaperone required for resistance to high temperature that assists DnaK, DnaJ, and GrpE in repairing damaged proteins (13, 36). A *clpB* mutant was discovered during a systematic search for insertional mutants obtained by random transposon mutagenesis in the pathogen *Leptospira interrogans* (24). In previous studies, *clpB* mutants of *Salmonella enterica* serovar Typhimurium, *Mycoplasma pneumoniae*, *Porphyromonas gingivalis*, *Listeria monocytogenes*, and *Francisella tularensis* have been shown to be relatively avirulent compared with their parental strains (5, 6, 15, 22, 38). In the present study, inactivation of the *clpB* gene of *L. interrogans* resulted in a mutant impaired in *in vitro* growth under stress conditions and exhibiting attenuation in virulence. Complementation with the full-length wild-type gene restored, at least partially, the stress survival and virulence phenotype to the *clpB* mutant, therefore demonstrating that ClpB is essential for survival under stress conditions and during *in vivo* infection. The only other described example of functional complementation in *L. interrogans* was obtained for the *loa22* mutant (34). This study should help us to understand the role of ClpB in leptospiral pathogenesis.

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▽ Published ahead of print on 5 July 2011.

MATERIALS AND METHODS

Leptospiral strains and culture conditions. The pathogens *L. interrogans* serogroup Canicola strain Kito and *L. interrogans* serovar Manilae strain L495 were used in this study. *Leptospira* organisms were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (9, 14) and on 1% agar-EMJH

TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)[a]	Reference
RTclb1	AACCAAAGTGATCGGACAGG	This study
RTclb2	ATACATGGAGGCTCATTCCG	This study
CLPBA	CGAGTGTAATCTTATTTTTTA	This study
998-1	CCGAAAAATTGGGAAATCCG	This study
ClpBascI	TTGGCGCGCCCGGGTGCAAAACATACTTTCAACC	This study
ClpBasc2	TTGGCGCGCCTAAAAAATAAGATTACACTCG	This study
SpcSmA	TCCCCCGGGGGCCCGAGCTTCAAGGAAGA	This study
SpcSmB	TCCCCCGGGGGAAAGTAAGCACCTGTTATTGC	This study
KS2R	GCACTTTCTGCTCCATTCAA	This study
KS2F	CGGCTTTCCTTCTAACAAAGG	This study
RTFLAB1	GAGAGAAACACCGAAGACGG	20
RTFLAB2	TGAATAGCAAGAACCCGGAT	20
dnaK1	CGATTACGAACGCGGTAAAT	This study
dnaK2	TTGGGGAGTGAATTCTCCTG	This study
hsp1	GATTAAACCTCTGGGCGACA	This study
hsp2	GGATGAGTTTCCCGTCTTCA	This study
LA	GGCGGCGCGTCTTAAACATG	23
LB	TTCCCCCCATTGAGCAAGATT	23
rt rpob1	ATGGAGCGGAACGTGTAGTC	This study
rt rpob2	CTTCGTTCGTTCCATGTCCT	This study

[a] AscI restriction sites are underlined.

plates at 30°C. When appropriate, spectinomycin or kanamycin was added to the culture medium at 50 μg ml⁻¹.

Identification of *clpB* mutants and construction of complemented strains. Random insertion mutagenesis was carried out with *L. interrogans* serogroup Canicola strain Kito, *L. interrogans* serovar Manilae strain L495, and a kanamycin-resistant *Himar1* transposon as previously described (24). Among the kanamycin transformants, we identified mutants with an insertion in *clpB* (LA1879) at positions 1852876 and 1853989 in the large chromosomes (nucleotide positions based on the genome of *L. interrogans* serovar Lai strain 56601) of strains L495 and Kito, respectively.

For complementation, *clpB* was amplified with primers ClpBasc1 and ClpBasc2 (Table 1) from *L. interrogans* strain Kito, and the amplified product, which was flanked by AscI restriction sites, was cloned into pCR2.1 (Invitrogen), thereby generating pCRclpB. The plasmid construct was verified by nucleotide sequencing. A spectinomycin resistance cassette was amplified with primers SpcSmA and SpcSmB and cloned into SmaI-digested pCRclpB. The resulting plasmid was then digested with AscI, thereby generating a 4.4-kb DNA fragment carrying the wild-type *clpB* gene and the spectinomycin resistance cassette. The DNA fragment was inserted into the AscI restriction site of the transposon carried by the conjugative plasmid pCJTKS1 (30). The plasmid was introduced into *clpB* mutants by conjugation as previously described (30). After 4 to 6 weeks of incubation, spectinomycin-resistant transformants were inoculated into liquid medium, and after DNA extraction, the transposon insertion site was identified by semirandom PCR (24). We selected one mutant, KS2 from strain Kito, exhibiting the additional transposon in an intergenic region between LA1293 and LA1294 of the large chromosome of *L. interrogans*. Two other mutants, KS1 and KS3, had the transposon inserted into LA2656 and LA0140. Another complemented strain, exhibiting the transposon in LA4340, was also obtained from the L495 *clpB* mutant strain (serovar Manilae).

Genomic DNAs were extracted from pellets of 20-ml cultures by using a QIAamp DNA blood minikit (Qiagen, Inc., Valencia, CA). Confirmation of genotypes was performed by using PCR with primer pairs 998-1-RTclb2 and KS2R-KS2F (Table 1), which target the flanking sequences of the insertion site of the transposon, and Southern blots of EcoRI-digested DNA were probed for hybridization with the kanamycin resistance cassette.

Western blotting. Expression of ClpB was verified by Western blotting. Polyclonal rabbit antiserum was prepared against *L. interrogans* ClpB (domain 158-334) by Proteogenix (Oberhausbergen, France). Protein concentrations of whole-cell lysates were determined by the Bradford protein assay (Coomassie Plus protein assay; Pierce). Ten micrograms of crude protein extract was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to Immobilon polyvinylidene fluoride (PVDF) membranes and probed with anti-*E. coli* ClpB (7) and *L. interrogans* ClpB antibodies at dilutions of 1:5,000 and 1:1,000, respectively. ImpL63 antiserum diluted 1:10,000 (a generous gift from Dave Haake) was also used as a loading control. The secondary antibody was anti-rabbit peroxidase, used at a dilution of 1:150,000. Blots were developed with a chemiluminescence kit (SuperSignal West Pico chemiluminescent substrate; Thermo Scientific, IL) according to the manufacturer's instructions.

Growth with limited nutrient concentrations and oxidative stress assay. To test for growth of *Leptospira* strains with limited nutrient concentrations, exponential-phase cultures of *L. interrogans* strains were inoculated (0.1 ml in 9 ml) in EMJH and EMJH diluted 1:2 in sterile water, and cultures were grown at 30°C under agitation. Cells were also grown in EMJH supplemented with 10% rabbit serum or M199 medium (PAN Biotech) with agitation at 37°C.

For disk diffusion assays, bacterial cells (approximately 10⁶ bacteria) were spread onto EMJH plates, and 6-mm- or 12-mm-diameter filter disks containing 20 and 50 mM hydrogen peroxide and 10 and 20 mM butyl peroxide were placed in the center of plates. The plates were incubated for 15 days at 30°C, and the diameters of the zones of inhibition created around the disks were measured. Plate assays were repeated at least three times, and similar results were obtained each time.

Heat shock assay. For heat shock assays, cultures were grown with agitation at 30°C until mid-exponential phase (optical density at 420 nm [OD₄₂₀], 0.2 to 0.3) and then transferred to 30 or 42°C for 1, 2, 4, or 6 h. Cells were then harvested for RNA extraction. One milliliter of TRIzol (Invitrogen) was added to a pellet from a 2-ml culture of leptospires, and RNA was purified and reverse transcribed as previously described (2). The expression of *flaB*, *rpoB*, *clpB*, *dnaK*, and *hsp10* was determined with the primer pairs indicated in Table 1. The PCRs were performed and analyzed with a CFX96 real-time PCR detection system (Bio-Rad). The fold change in the amount of target gene was normalized to the amount of 16S cDNA obtained with primers LA and LB (Table 1) and relative to the expression at time zero at 30°C by using the comparative threshold cycle (C_T) method [also called the $2(-\Delta\Delta C_T)$ method] (19).

Animal experiments. *L. interrogans* strains were tested for virulence in the gerbil model of acute leptospirosis. Groups of 28-day-old gerbils (Charles River Laboratories) were inoculated intraperitoneally with 10⁸ leptospires. Animals were monitored daily for clinical signs of leptospirosis (i.e., prostration, jaundice, etc.) and for survival for up to 21 days postinfection. Protocols for animal experiments were prepared according to the guidelines of the Animal Care and Use Committees of the Institut Pasteur.

Statistical analysis. Data were graphed and analyzed using GraphPad Prism 5.0c (GraphPad Software, San Diego, CA). Susceptibility to oxidative stress was statistically analyzed by one-way analysis of variance. Survival curves were compared using the log rank (Mantel Cox test) analysis. P values of <0.05 were considered to be significant.

RESULTS

Genome analysis of *clp* genes in *Leptospira* spp. The complete genome sequences of strains belonging to two pathogenic species, *L. interrogans* and *Leptospira borgpetersenii*, and one saprophytic species, *Leptospira biflexa*, were recently determined (4, 28, 31, 33). The genomes of the sequenced strains of *Leptospira* spp. possess *clpA*, *clpB*, *clpX*, *clpP*, *clpS*, *clpY*, and *clpQ*. *Leptospira* spp. also possess *clpC*, which is usually present in Gram-positive but absent in Gram-negative bacteria (Fig. 1). It has to be noted that *clpC* is also present in the spirochetes *Treponema pallidum* (TP0549) and *Borrelia burgdorferi* (BB0834). The genes of the Clp system are conserved in saprophytic and pathogenic *Leptospira* species and constitute part of the core genes of the genus *Leptospira* (31). ClpA, ClpB, ClpC, ClpX, and ClpY are proteins belonging to the Clp/Hsp100 family that carry domains typical of the AAA+ superfamily of ATPases. Amino acid sequence analysis predicted the presence of two well-conserved AAA domains in *L. interrogans* ClpA, ClpB, and ClpC and one AAA domain in ClpX and ClpY/HslU (Fig. 1; see Fig. S1 in the supplemental material). The Clp ATPase proteins ClpA, ClpC, and ClpX possess a
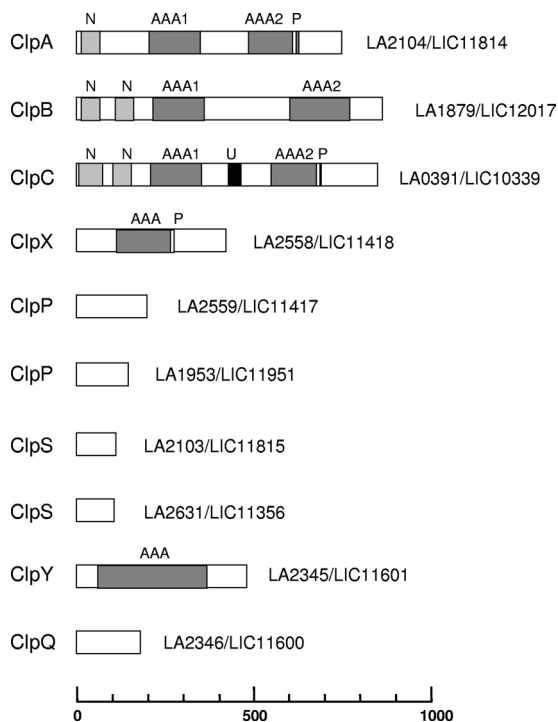
FIG. 1. Genome analysis of Clp chaperones and proteases in *Leptospira* spp. ClpA, ClpB, ClpC, ClpX, and ClpY belong to the family of Clp ATPases. The ATP domains of the AAA+ superfamily of ATPases are indicated (AAA). The P-loop domain (P), involved in the interaction with the ClpP peptidase, the N-terminal protein binding domain (N), and a domain resembling the UVRB/UVRC interaction domain (U) are also indicated. The genes in *L. interrogans* serovar Lai strain 56601 and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 are defined by their LA and LIC numbers, respectively.

ClpP recognition binding motif (also called a P-loop motif), (L/I/V)G(F/L), that allows association with the ClpP peptidase, resulting in a Clp proteolytic complex (see Fig. S1). ClpS is an adaptor protein binding to the ClpA N-terminal domain and altering the substrate specificity of ClpA. In *Leptospira* spp., *clpA* and *clpS* are adjacent to each other on the chromosome; another putative *clpS* gene was found elsewhere in the chromosome. No genes for adaptor proteins that target

E. coli ClpX (SspB, RssB, and UmuD) have been identified thus far in the *Leptospira* genomes. Although the sequences and functions of ClpA and ClpC are quite similar, ClpC can be distinguished from ClpA by a relatively longer spacer region between the two ATP-binding domains (Fig. 1; see Fig. S1). In addition, a UVR domain resembling the UVRB/UVRC interaction domain was identified in several ClpC proteins (12). The ClpB ATPases do not associate with ClpP and function as chaperones. Finally, ClpQ interacts with the ClpY chaperone, forming the ClpYQ (also known as HslUV) protease (17).

ClpB expression in *L. interrogans*. The *clpB* gene of *L. interrogans* encodes a protein of 860 amino acids which exhibits 95% and 62% similarity with orthologs in the pathogen *L. borgpetersenii* and the saprophyte *L. biflexa*, respectively. Among the pathogenic strains *L. interrogans* serogroup Canicola strain Kito, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Lai strain 56601, and *L. interrogans* serovar Manilae strain L495, the complete nucleotide sequence of *clpB*, including the promoter region (300 bp upstream of the start codon), exhibits 99% identity at the nucleotide level (data not shown).

In order to characterize ClpB properties in *Leptospira* spp., we assessed the effects of heat shock on the transcription of *clpB* in *L. interrogans* serogroup Canicola strain Kito (Fig. 2). Transcription of the *clpB* gene increased during a temperature shift from 30°C to 42°C, reaching a 3-fold increase after 2 h of exposure to 42°C. Transcriptional responses of the *L. interrogans dnaK* and *hsp10* genes to heat shock conditions were also measured by reverse transcription-quantitative PCR (RT-qPCR) and showed elevated expression of both genes. In contrast, the expression of the *rpoB* and *flaB* genes, which were included as controls to ensure reliability of the comparative C_T analysis, was unchanged (Fig. 2).

Construction of *L. interrogans* mutants. We previously obtained about 1,000 random mutants with characterized transposon insertion points in *L. interrogans* pathogenic strains (24). We pursued this work in the laboratory, and we recently identified one transposon mutant exhibiting an insertion in *clpB* (LA1879) (Fig. 3). The insertion of a single transposon in the mutant strain was confirmed by Southern blotting using a transposon-specific probe (data not shown).

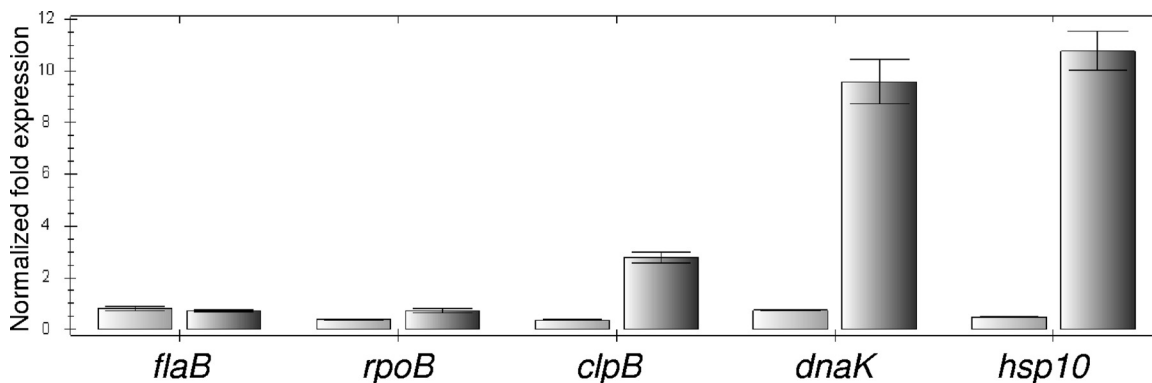Because there is no replicative plasmid vector available for

FIG. 2. Relative gene expression of *L. interrogans clpB*, *dnaK*, and *hsp10*. Exponential-phase cultures of *L. interrogans* were incubated at 30°C (left columns) and 42°C (right columns) for 2 h. Transcripts of the selected genes were measured by RT-qPCR, and the data were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method. The error bars indicate the standard deviations for three replicates.
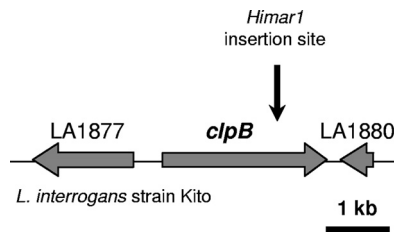
FIG. 3. Genetic locus of *clpB* in *L. interrogans*. The insertion site of *Himar1* in the chromosome of the *clpB* mutant in strain Kito is indicated.
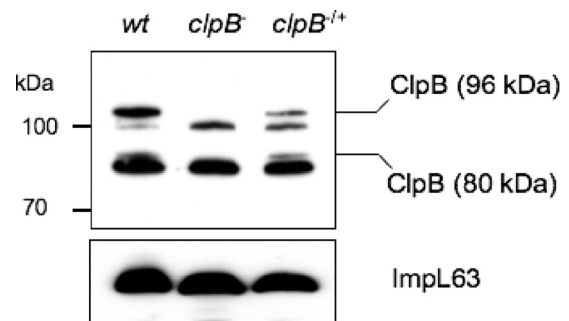

FIG. 4. ClpB in *L. interrogans* strains. Western analysis was performed with whole-cell extracts of wild-type (wt), *clpB* mutant (*clpB*⁻), and complemented *clpB* mutant (*clpB*⁻/⁺) strains grown at 30°C, using an antibody to *L. interrogans* ClpB. Data shown are from a single experiment and are representative of at least three experiments performed with similar results. ImpL63 antibody was used as a loading control in Western blots.

pathogenic *Leptospira* (16), we also generated a *clpB*-complemented mutant by insertion of the transposon carrying *clpB* and a spectinomycin resistance cassette. We identified the transposon insertion sites in several transformants and selected one strain, KS2, exhibiting the transposon in an intergenic region. PCR and Southern blotting confirmed the presence of the wild-type *clpB* gene in the wild-type and complemented strains (data not shown).

Western blots of whole-cell lysates of the *L. interrogans* wild-type strain, obtained by using antibodies raised against *L. interrogans* ClpB, showed several bands (Fig. 4). The use of *E. coli* ClpB antiserum showed similar results (data not shown). The *L. interrogans* ClpB protein (96 kDa) shares >60% similarity with *L. interrogans* ClpA (83 kDa) and ClpC (95 kDa), which are also members of the Hsp100/Clp family. This may explain the presence of more than one ClpB-reactive band. Interestingly, *E. coli* was found to use two transcriptional start sites to express two forms of ClpB, a 93-kDa and a 79-kDa protein (36). The *L. interrogans* ClpB protein could also be synthesized in two forms. Nucleotide sequence analysis of the *L. interrogans* clpB gene revealed a classical ribosome binding site (RBS) immediately upstream of at least three putative alternative start codons (ATG or TTG). These potential internal translation initiation sites should result in proteins with calculated molecular masses of 94, 89, and 80 kDa (instead of the 96-kDa mass for the full-length ClpB protein). Alignment of amino acid sequences showed that the alternative translational start of the 80-kDa polypeptide of *L. interrogans* ClpB coincides with amino acid 149 of *E. coli* ClpB, which is the start codon of the smaller form of *E. coli* ClpB (79 kDa) (36). Gene inactivation of *clpB* resulted in the disappearance of two bands (Fig. 4). Western blot analysis of the complemented strain with the ClpB antiserum detected two reactive bands similar to those for the wild-type strain (Fig. 4), further confirming the identity of the 96-kDa polypeptide and another smaller polypeptide (80 kDa) as ClpB.

***In vitro* phenotype.** The *L. interrogans* clpB mutant presented a similar motility and cell morphology to those of the wild-type strain (data not shown). However, analysis of the growth curves of the *clpB* mutant under normal growth conditions (i.e., aerobic conditions at 30°C) indicated that the lag phase of the growth curve was 48 h longer than that for the wild-type and complemented strains (Fig. 5). The *clpB* mutant did reach the maximum growth yield of the wild-type strain. The wild-type and complemented strains had similar cell growth kinetics in EMJH medium at 30°C. The capacity of the *clpB* mutant to grow under a variety of stress conditions was also examined.

No significant differences in growth between the complemented and parental strains were observed when cells were grown at 30°C with limited nutrient concentrations (diluted EMJH). Under similar conditions, the *clpB* mutant had greatly reduced growth (Fig. 5). Pathogenic *Leptospira* strains usually do not grow well in artificial medium at 37°C (10). In our hands, the Kito strain showed greatly reduced growth in EMJH, even with supplemented serum or tissue culture medium, at 37°C in comparison to that for cultures at 30°C. Under these conditions, the growth rate of the *clpB* mutant was dramatically reduced, sometimes with no evidence of multiplication, compared to those of the wild-type and complemented strains (Fig. 5).

To examine whether ClpB contributes to oxidative stress resistance, disk diffusion assays were carried out with hydrogen peroxide and butyl peroxide. The zone of inhibition for the *clpB* mutant was significantly larger than the zone of inhibition for the wild type, indicating that the *clpB* mutant is more susceptible to oxidative stress (Fig. 6). Additionally, complementation of the *clpB* mutant restored resistance to the wild-type level (Fig. 6). These results indicate that ClpB plays a major role in resistance to peroxides.

Together, our results show that both *in vitro* growth deficiencies and the lower resistance to heat and oxidative stresses in the mutant are due to inactivation of *clpB* and can be complemented by a transposon-carried *clpB* copy.

***In vivo* phenotype.** To determine if ClpB is important for the ability of *L. interrogans* to cause disease, gerbils were infected with 10⁸ organisms (three independent experiments with groups of 4 to 6 animals) of either the wild-type, *clpB* mutant, or complemented *clpB* mutant version of *L. interrogans* strain Kito. The *clpB* mutant demonstrated a complete loss of virulence, with no clinical signs of leptospirosis, during the 21-day follow-up period, while the wild-type strain caused death in 4 to 7 days after challenge (Fig. 7). The survival curve for the mutant was statistically different from the curve generated for gerbils infected with the wild type (P < 0.0001). Necropsy evaluation of gerbils infected with the *clpB* mutant did not show macroscopic lesions associated with leptospirosis (data not shown). The mutant phenotype could be complemented by reintroduction of *clpB*, with the log rank test between the
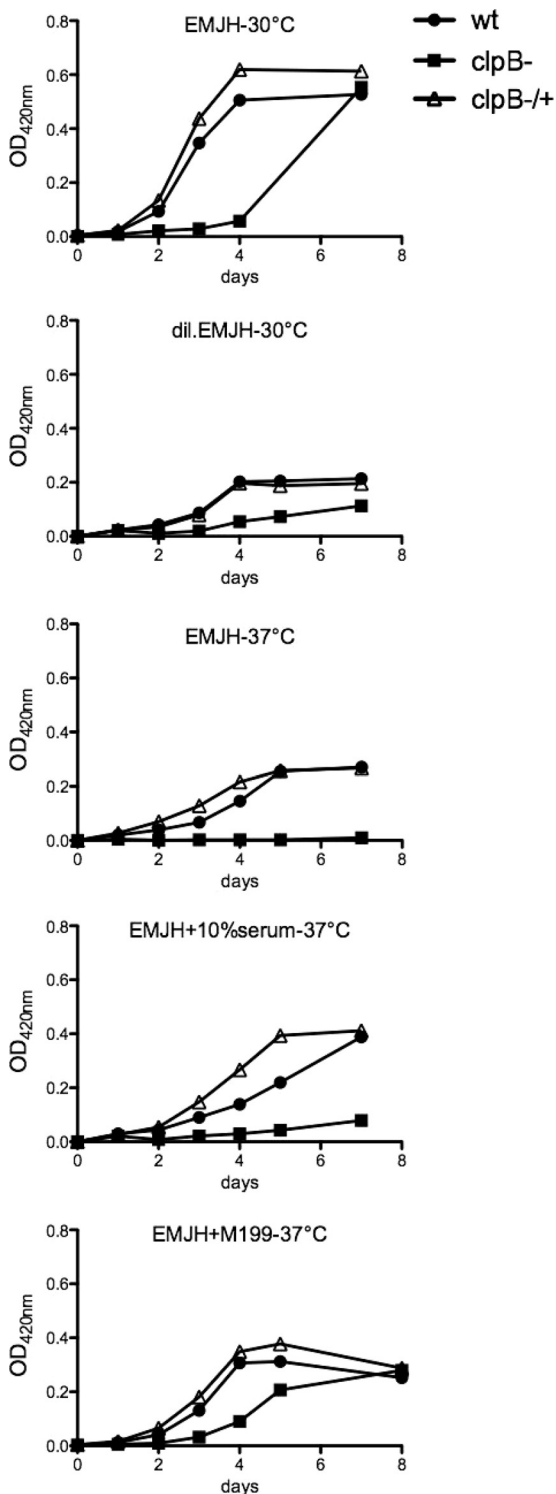
FIG. 5. Growth kinetics of wild-type, *clpB* mutant, and complemented *clpB* mutant strains of *L. interrogans*. Strains were grown in EMJH, EMJH supplemented with 10% rabbit serum, EMJH supplemented with 10% M199 medium, and diluted EMJH medium, and cultures were incubated at 30°C or 37°C with shaking. Growth was monitored by measuring the OD_{420}.

mutant and complemented strains giving a *P* value of 0.0027. The complemented strain caused death in 50% of the inoculated animals (Fig. 7). Our results showed that the lack of virulence of the *clpB* mutant could be complemented partially *in vivo* by expressing *clpB* from the *Himar1*-derived transposon, which is stably integrated into the chromosome of *L. interrogans* without antibiotic selection.

DISCUSSION

The Clp system is involved in the general stress response as well as the virulence of many pathogenic bacteria. Given the variety of environmental stresses that *L. interrogans* may encounter during its life cycle, it is likely that the Clp proteins play a role in stress survival and virulence.

Genome sequencing of *Leptospira* strains has enabled the identification of putative molecular chaperones and proteases. These include the ClpXP chaperone-protease, which is the most ubiquitous of the Clp proteases; ClpYQ, which is found in most proteobacteria and in certain Gram-positive bacteria; and ClpAP and its adaptor, ClpS, which are found in the Gram-negative proteobacteria. *Leptospira* spp. possess a double-membrane structure, including an outer membrane which contains lipopolysaccharide (LPS), and are therefore related to Gram-negative bacteria. In contrast to Gram-negative bacteria, which possess ClpA but not ClpC, *Leptospira* spp. were found to harbor both ClpA and ClpC, which are orthologs associated with ClpP and showing similar functions in other bacteria. Finally, like other organisms, including eukaryotes, *Leptospira* spp. possess a ClpB-encoding gene. Immunoblotting and sequence analysis indicated that two forms of ClpB may be produced in *L. interrogans*, like the case in *E. coli* (36). The biological role of the smaller ClpB form, which does not contain the substrate-interacting N-terminal domain, is not well understood. In *E. coli*, the two isoforms form heterooligomers that optimize the chaperone activity by synergistic cooperation (27).

A previous microarray study showed that *L. interrogans clpB* or LA1879/LIC12017 (misannotated *clpA*), together with other stress and heat shock genes, was upregulated 3.5-fold at 37°C in comparison to 30°C (20). This was also shown at the protein level (21). We confirmed that *clpB* is upregulated during heat stress. ClpB was also found to be downregulated in response to serum (29) and under iron-depleted conditions (11).

Only a few *L. interrogans* mutants showed attenuation in virulence in the animal model of leptospirosis. These avirulent mutants were disrupted in genes of the LPS biosynthesis locus (25) and in genes encoding a heme oxygenase (26), a lipoprotein, Loa22, of unknown function (34), or the flagellar motor switch protein FliY (18). However, only the attenuation of the *loa22* mutant was confirmed by complementation studies (34).

In this study, we isolated a mutant with an insertion in *clpB* in *L. interrogans* strain Kito. Our results suggest that ClpB plays an important role in *in vitro* growth and in responses to oxidative and temperature stresses in *L. interrogans*. Transformation of the *clpB* mutant with a construct to express wild-type ClpB restored the wild-type phenotype *in vitro*, confirming that all of the phenotypes described here for the *clpB* mutant were indeed due to the loss of function of this gene.

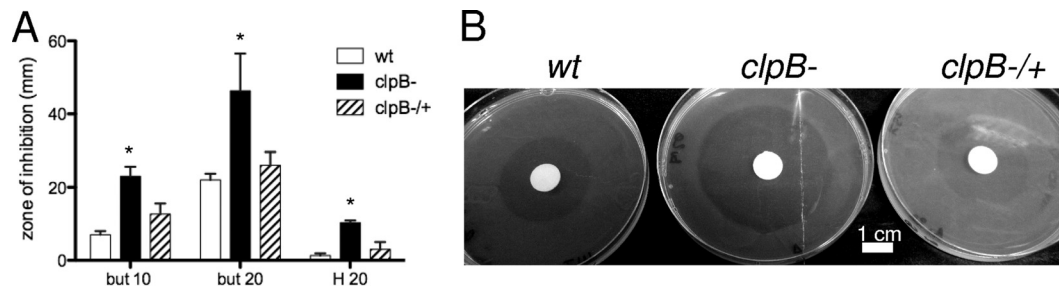The *clpB* mutant of *L. interrogans* strain Kito demonstrated

FIG. 6. Effects of hydrogen peroxide and butyl peroxide on *L. interrogans* strains. (A) Disk diffusion assays with 10 mM and 20 mM butyl peroxide (but) and 20 mM hydrogen peroxide (H), using the wild-type (wt), *clpB* mutant (*clpB⁻*), and complemented *clpB* mutant (*clpB⁻/⁺*) strains. Bars show the diameters of the zones of inhibition in millimeters. The means and standard deviations for three independent experiments are shown, with statistical significance indicated by asterisks ($P < 0.05$). (B) Plate assay with 20 mM butyl peroxide.

a loss of virulence. The attenuation of lethality in infected gerbils could be a consequence of downregulation of virulence factors, *in vivo* growth deficiency, or increased susceptibility to stress conditions. The attenuation of lethality was only partially restored in gerbils. The incomplete complementation of the *clpB* mutant may have been due to poor *in vivo* expression of *clpB* from the integrated *Himar1* transposon. Integration of the wild-type *clpB* gene into a chromosomal locus other than the original one may not allow optimal expression or regulation of *clpB* in complemented strains, as suggested by the level of expression of ClpB-reactive bands in Western blot analysis (Fig. 4). The only other example of complementation of an avirulent mutant of pathogenic *Leptospira* spp. also showed partial restoration of virulence, with the complemented strain being lethal in 57 to 80% of infected animals (34).

Interestingly, we recently identified another *clpB* mutant in the pathogen *L. interrogans* serovar Manilae strain L495 among random transposon mutants (24). Inactivation of *clpB* was therefore obtained in two distinct *L. interrogans* serogroups, i.e., serogroups Canicola (strain Kito) and Pyrogenes (strain L495). The L495 *clpB* strain exhibited growth defects at 37°C and at 30°C in poor nutrient medium, but not at 30°C in regular EMJH medium, in comparison to the parental and complemented strains. The susceptibility to oxidative stress of the *clpB* mutant was significantly higher than the susceptibility of both the wild-type and complemented cells (see Fig. S2 in the supplemental material). However, the *clpB* mutant of strain L495 did not show decreased virulence compared to the

wild-type strain in the animal model of leptospirosis (see Fig. S2). The reason for this difference of phenotypes between the *clpB* mutants of strains Kito and L495 is unclear. The *clpB* gene was not found to be duplicated in the genome of the *L. interrogans clpB* mutant (B. Adler, unpublished data). Differences in the regulation and/or expression of *clpB* may exist among clinical strains. Gene inactivation of *clpB* in strain L495 may also result in compensatory mechanisms, such as increased activity levels of Clp proteases or other chaperone machineries. The virulence of pathogens is partly due to their ability to survive in the host by escaping natural defense mechanisms. ClpB has been shown to be a key determinant in tolerance to oxidative stress conditions. Other mechanisms of resistance to oxidative stress, such as enzymatic degradation of peroxide, may also vary among pathogenic *Leptospira* strains (8), allowing persistence of virulence in the L495 *clpB* strain.

In conclusion, we demonstrated that ClpB, which is induced by heat stress, is required for growth in poor nutrient media and for resistance to oxidative and heat stresses. ClpB also appears to play a major role in virulence, as assessed using the Kito mutant. We believe that our results will provide insights for understanding the mechanisms of tolerance of pathogenic *Leptospira* spp. to stress conditions.
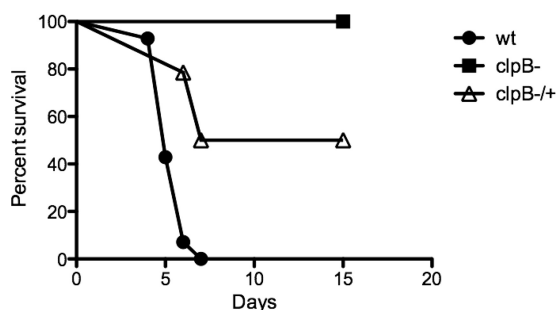
FIG. 7. Cumulative survival rates for gerbils infected with wild-type, *clpB* mutant, and complemented *clpB* mutant strains of *L. interrogans*. The survival curves ($n = 14$ animals) show the results for three independent experiments.

REFERENCES

1. **Abela-Ridder, B., R. Sikkema, and R. A. Hartskeerl.** 2010. Estimating the burden of human leptospirosis. Int. J. Antimicrob. Agents **36:**S5–S7.
2. **Aviat, F., L. Slamti, G. M. Cerqueira, K. Lourdault, and M. Picardeau.** 2010. Expanding the genetic toolbox for *Leptospira* species by generation of fluorescent bacteria. Appl. Environ. Microbiol. **76:**8135–8142.
3. **Beck, M., et al.** 2009. Visual proteomics of the human pathogen *Leptospira interrogans*. Nat. Methods **6:**817–823.
4. **Bulach, D. M., et al.** 2006. Genome reduction in *Leptospira borgpetersenii*

reflects limited transmission potential. Proc. Natl. Acad. Sci. U. S. A. **103:**14560–14565.

5. **Capestany, C. A., G. D. Tribble, K. Maeda, D. R. Demuth, and R. J. Lamont.** 2008. Role of the Clp system in stress tolerance, biofilm formation, and intracellular invasion in *Porphyromonas gingivalis*. J. Bacteriol. **190:**1436–1446.

6. **Chastanet, A., I. Derre, S. Nair, and T. Msadek.** 2004. *clpB*, a novel member of the *Listeria monocytogenes* CtsR regulon, is involved in virulence but not in general stress tolerance. J. Bacteriol. **186:**1165–1174.

7. **Chow, I. T., M. E. Barnett, M. Zolkiewski, and F. Baneyx.** 2005. The N-terminal domain of *Escherichia coli* ClpB enhances chaperone function. FEBS Lett. **579:**4242–4248.

8. **Corin, R. E., E. Boggs, and C. D. Cox.** 1978. Enzymatic degradation of H_2O_2 by *Leptospira*. Infect. Immun. **22:**672–675.

9. **Ellinghausen, H. C., and W. G. McCullough.** 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Am. J. Vet. Res. **26:**45–51.

10. **Ellinghausen, H. C. J.** 1973. Growth temperatures, virulence, survival, and nutrition of leptospires. J. Med. Microbiol. **6:**487–497.

11. **Eshghi, A., P. A. Cullen, L. Cowen, R. L. Zuerner, and C. E. Cameron.** 2009. Global proteome analysis of *Leptospira interrogans*. J. Proteome Res. **8:**4564–4578.

12. **Frees, D., K. Savijoki, P. Varmanen, and H. Ingmer.** 2007. Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria. Mol. Microbiol. **63:**1285–1295.

13. **Goloubinoff, P., A. Mogk, A. P. Zvi, T. Tomoyasu, and B. Bukau.** 1999. Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Proc. Natl. Acad. Sci. U. S. A. **96:**13732–13737.

14. **Johnson, R. C., and V. G. Harris.** 1967. Differentiation of pathogenic and saprophytic leptospires. J. Bacteriol. **94:**27–31.

15. **Kannan, T. R., O. Musatovova, P. Gowda, and J. B. Baseman.** 2008. Characterization of a unique ClpB protein of *Mycoplasma pneumoniae* and its impact on growth. Infect. Immun. **76:**5082–5092.

16. **Ko, A. I., C. Goarant, and M. Picardeau.** 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat. Rev. Microbiol. **7:**736–747.

17. **Kress, W., Z. Maglica, and E. Weber-Ban.** 2009. Clp chaperone-proteases: structure and function. Res. Microbiol. **160:**618–628.

18. **Liao, S., et al.** 2009. Inactivation of the *fliY* gene encoding a flagellar motor switch protein attenuates mobility and virulence of *Leptospira interrogans* strain Lai. BMC Microbiol. **9:**253.

19. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) methods. Methods **25:**402–408.

20. **Lo, M., et al.** 2006. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. Infect. Immun. **74:**848–859.

21. **Lo, M., S. J. Cordwell, D. M. Bulach, and B. Adler.** 2009. Comparative transcriptional and translational analysis of leptospiral outer membrane protein expression in response to temperature. PLoS Negl. Trop. Dis. **3:**e560.

22. **Meibom, K. L., et al.** 2008. The heat-shock protein ClpB of *Francisella tularensis* is involved in stress tolerance and is required for multiplication in target organs of infected mice. Mol. Microbiol. **67:**1384–1401.

23. **Merien, F., P. Amouriaux, P. Perolat, G. Baranton, and I. Saint Girons.** 1992. PCR for detection of *Leptospira* spp. in clinical samples. J. Clin. Microbiol. **30:**2219–2224.

24. **Murray, G. L., et al.** 2009. Genome-wide transposon mutagenesis in pathogenic *Leptospira* spp. Infect. Immun. **77:**810–816.

25. **Murray, G. L., et al.** 2010. Mutations affecting *Leptospira interrogans* lipopolysaccharide attenuate virulence. Mol. Microbiol. **78:**701–709.

26. **Murray, G. L., et al.** 2009. *Leptospira interrogans* requires heme oxygenase for disease pathogenesis. Microbes Infect. **11:**311–314.

27. **Nagy, M., et al.** 2010. Synergistic cooperation between two ClpB isoforms in aggregate reactivation. J. Mol. Biol. **396:**697–707.

28. **Nascimento, A. L., et al.** 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J. Bacteriol. **186:**2164–2172.

29. **Patarakul, K., M. Lo, and B. Adler.** 2010. Global transcriptomic response of *Leptospira interrogans* serovar Copenhageni upon exposure to serum. BMC Microbiol. **10:**31.

30. **Picardeau, M.** 2008. Conjugative transfer between *Escherichia coli* and *Leptospira* spp. as a new genetic tool. Appl. Environ. Microbiol. **74:**319–322.

31. **Picardeau, M., et al.** 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of Leptospira and the pathogenesis of leptospirosis. PLoS One **3:**e1607.

32. **Qin, J. H., et al.** 2006. Genome-wide transcriptional analysis of temperature shift in *L. interrogans* serovar Lai strain 5660. BMC Microbiol. **6:**51.

33. **Ren, S., et al.** 2003. Unique and physiological and pathogenic features of *Leptospira interrogans* revealed by whole genome sequencing. Nature **422:**888–893.

34. **Ristow, P., et al.** 2007. The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog. **3:**e97.

35. **Schirmer, E. C., J. R. Glover, M. A. Singer, and S. Lindquist.** 1996. HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem. Sci. **21:**289–296.

36. **Squires, C. L., S. Pedersen, B. M. Ross, and C. Squires.** 1991. ClpB is the *Escherichia coli* heat shock protein F84.1. J. Bacteriol. **173:**4254–4262.

37. **Trueba, G., S. Zapata, K. Madrid, P. Cullen, and D. Haake.** 2004. Cell aggregation: a mechanism of pathogenic *Leptospira* to survive in fresh water. Int. Microbiol. **7:**35–40.

38. **Turner, A. K., M. A. Lovell, S. D. Hulme, L. Zhang-Barber, and P. A. Barrow.** 1998. Identification of *Salmonella* Typhimurium genes required for colonization of the chicken alimentary tract and for virulence in newly hatched chicks. Infect. Immun. **66:**2099–2106.