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Leptospira interrogans Catalase Is Required for Resistance to H_2O_2 and for Virulence

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Pathogenic *Leptospira* spp. are likely to encounter higher concentrations of reactive oxygen species induced by the host innate immune response. In this study, we characterized *Leptospira interrogans* catalase (KatE), the only annotated catalase found within pathogenic *Leptospira* species, by assessing its role in resistance to H_2O_2-induced oxidative stress and during infection in hamsters. Pathogenic *L. interrogans* bacteria had a 50-fold-higher survival rate under H_2O_2-induced oxidative stress than did saprophytic *L. biflexa* bacteria, and this was predominantly catalase dependent. We also characterized KatE, the only annotated catalase found within pathogenic *Leptospira* species. Catalase assays performed with recombinant KatE confirmed specific catalase activity, while protein fractionation experiments localized KatE to the bacterial periplasmic space. The insertional inactivation of *katE* in pathogenic *Leptospira* bacteria drastically diminished leptospiral viability in the presence of extracellular H_2O_2 and reduced virulence in an acute-infection model. Combined, these results suggest that *L. interrogans* KatE confers *in vivo* resistance to reactive oxygen species induced by the host innate immune response.

Leptospira is a diverse genus of spirochete bacteria spanning 20 species (5), including the saprophytic species *L. biflexa* and the pathogenic species *L. interrogans* (46). Leptospirosis, caused by pathogenic *Leptospira* spp., is a widespread zoonotic disease (62) that has emerged as an epidemic in urban slum settings (28). Leptospirosis in susceptible hosts, such as humans and hamsters (44), is manifested by a wide array of clinical signs, including fever and jaundice, with a potential for death from multiple organ failure (29). Rats are asymptomatic carriers and serve as a reservoir for pathogenic *Leptospira* species (54). In such maintenance hosts, leptospires colonize primarily renal tubules, from where they are shed into the urine and persist in freshwater until they gain access to a new mammalian host (29).

The induction of a host immune response generates a defensive oxidative burst (27). In leptospiral infections, the release of reactive oxygen species (ROS) was demonstrated *in vitro* with isolated rat Kupffer cells upon exposure to *Leptospira* (36), and *in vivo*, it has been shown that cattle diagnosed with leptospirosis display elevated levels of serum oxidative stress biomarkers (16). Together, these studies indicated that *Leptospira* is exposed to ROS during the infection process. During the host oxidative burst, ROS are generated from free radicals of molecular oxygen (superoxide). Reactive oxygen species can be generated through immune cells, including neutrophils and macrophages (45), which utilize both membrane and cytosolic proteins to generate oxygen radicals from molecular oxygen via the NADPH phagocyte oxidase pathway (1, 61). The generated oxygen radicals can then be used to produce other ROS, including hydrogen peroxide (H_2O_2), hydroxyl radicals, and hypochlorous acid, all of which mediate bacterial killing.

The molecular mechanism(s) underlying the killing of microorganisms via ROS is incompletely understood (18, 19). For bacteria, the primary cellular target of ROS was thought to be DNA, with extensive ROS-generated DNA damage resulting in a loss of bacterial viability (24). However, recent evidence for *Salmonella enterica* serovar Typhimurium demonstrated a requirement for the periplasmic superoxide dismutase (SodC), rather than the cytoplasmic enzyme counterparts SodA and SodB, for resistance to extracellular ROS, suggesting a noncytoplasmic target for ROS damage (10). Further evidence comes from the demonstration that membrane lipids are targeted by ROS in *Borrelia burgdorferi* (4), identifying one potential mechanism for the loss of spirochete viability during infection. Microbial pathogens have evolved various mechanisms to counter the effects of host-generated ROS, including the disruption of ROS delivery to the phagosome (60), the inhibition of ROS production (9), active DNA repair systems (23, 56), and the enzymatic detoxification of ROS (3, 20, 64). Microbial enzymes utilized for ROS detoxification include catalases (6) and peroxidases (50), which degrade hydrogen peroxide, and superoxide dismutases, which detoxify superoxide anions (57).

Catalases of pathogenic bacteria are important for optimal detoxification of H_2O_2 (15, 22), survival in macrophages (13), resis-

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tance to phagocyte-mediated killing (52), and virulence (3, 53, 59, 64). The pathogenic species *L. interrogans* displays catalase activity, while the saprophytic species *L. biflexa* displays predominantly peroxidatic activity (7), despite the fact that a *katG* homolog is present in *L. biflexa* (65). *L. interrogans* can degrade H_2O_2 at concentrations 50-fold higher than those tolerated by *L. biflexa* (8), consistent with a greater susceptibility of *L. biflexa* to H_2O_2-mediated killing (38). This suggests that *L. interrogans* has evolved an extensive H_2O_2 detoxification system which is absent from *L. biflexa*.

Additional evidence suggesting a role for catalase activity in virulence comes from a previous study which showed the upregulation of the catalase KatE by *L. interrogans* when exposed to "*in vivo*-like" conditions represented by bacterial growth in medium depleted of iron and containing 10% fetal bovine serum (17). This finding has been supported at the transcriptional level, where *katE* transcript levels were increased in response to an increase in temperature and the presence of serum (32, 47, 65). These results, together with the potential for the exposure of *Leptospira* to extracellular ROS during the infection process, prompted us to further characterize the leptospiral response to ROS. In this study, we demonstrate that KatE is a periplasmic catalase that enhances the resistance of *L. interrogans* to extracellular oxidative stress and is required for virulence in an acute animal model.

MATERIALS AND METHODS

***Leptospira* strains and culture conditions.** *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was isolated from a clinical sample in Salvador, Brazil (28). *L. interrogans* serovar Pomona L523 was originally isolated from a clinical sample in Australia and was obtained from the World Health Organization Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Australia. *L. interrogans* serovar Manilae strain L495 was originally isolated from a clinical sample in the Philippines and was provided by Nobuo Koizumi, National Institute of Infectious Diseases, Tokyo, Japan, and *L. biflexa* serovar Patoc strain Patoc1 (designated Paris strain) was obtained from a freshwater stream (2) and is stored and maintained at the National Reference Center of *Leptospira* (Pasteur Institute, Paris, France). All strains were cultured aerobically in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (26) at 29.5°C.

Catalase assay. Recombinant KatE (rKatE) was purified by using immobilized metal ion affinity chromatography as described previously (17). This recombinant protein preparation was estimated to have a purity of >95% and appeared as a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses. The enzymatic activity was measured by using a catalase assay kit (Cayman Chemical Company, Ann Arbor, MI) at room temperature according to the manufacturer's instructions, with the bovine catalase enclosed in the kit serving as a positive control. Recombinant KatE was added at final concentrations ranging from 0.23 ng/μl to 15 ng/μl. Catalase (KatE) inhibition experiments were conducted by adding 3-amino-1,2,4-triazol (ATZ) (USB Corporation, Cleveland, OH) to the assay buffer (100 mM potassium phosphate, pH 7.0) at a final concentration of 100 mM, prior to conducting the catalase assay. The formaldehyde control assay was performed concurrently according to the manufacturer's protocol. Catalase and formaldehyde standard assays were conducted using duplicate samples. The experiment was performed a total of three times, with comparable results; a representative result from one experiment is presented.

Bacterial oxidative stress conditions and inhibition assays. Leptospires were enumerated by using a Petroff-Hausser counting chamber (Fisher Scientific, Ottawa, Ontario, Canada) and an Eclipse 50i dark-field microscope (Nikon, Mississauga, Ontario, Canada). Bacteria were grown to $>4 \times 10^8$ bacteria/ml and diluted to 3×10^8 bacteria/ml in either EMJH medium (without H_2O_2) or EMJH medium containing H_2O_2 at a final concentration of 0.1 mM, 1 mM, or 10 mM, with or without 100 mM ATZ. Samples were incubated for 1 h at 37°C. Bacterial oxidative stress assays were also conducted, as described above, without ATZ for 10 min at 37°C. A total of 5×10^9 cells of strain L1-130 from each experimental group (no H_2O_2 exposure and 0.1, 1, and 10 mM H_2O_2 exposures) were collected by centrifugation at 2,000 × *g*, and the bacteria were flash-frozen in liquid nitrogen and stored at −20°C for subsequent immunoblot analyses. Exposures for 30 and 60 min were also tested and yielded similar results.

Bacterial viability assays. Triplicate samples of *Leptospira* bacteria exposed to the above-described conditions were dispensed into sterile Falcon 96-well flat-bottom polystyrene microplates (Becton Dickinson, Franklin Lakes, NJ) (250 μl/well). Fifty microliters of an alamarBlue (Biosource, Camarillo, CA) working solution, consisting of 0.4 ml alamarBlue stock solution plus 9.6 ml 0.1 M potassium phosphate buffer (pH 7.4), was added to each well and incubated at 30°C (the optimal temperature for leptospiral growth *in vitro*) in a humidified Hybaid chamber (ThermoFisher Scientific). In this assay, viable bacteria are able to reduce the nonfluorescent compound resazurin to the fluorescent compound resorufin (43). The chromogenic shift was measured by fluorescence using a 530- ± 25-nm filter for excitation and a 590- ± 20-nm filter for emission in a Synergy HT microplate reader (BioTek) at 0 and 4 h postincubation. Negative controls without bacteria were included. Percent survival was calculated at 4 h by subtracting initial readings (time zero) and the negative control and then by dividing by fluorescence values obtained from leptospires that had not been exposed to H_2O_2 and were grown in either EMJH medium or EMJH medium with 100 mM ATZ. Leptospiral viability was confirmed via dark-field microscopy, where motile bacteria were deemed viable. Data are representative of a single experiment with three replicates. Experiments were repeated at least two times, with similar results. Statistical analyses were conducted by using the two-tailed Student *t* test.

Protein fractionation. Bioinformatic analysis to predict the cellular location of KatE was performed by using pSORTb v3.0 (66). The experimental subcellular localization of KatE in *Leptospira* was determined by Triton X-114 solubilization of the outer membrane and subsequent fractionation into detergent (DET) and aqueous (AQ) phases, as described previously (21, 48, 68), except that fractionation was performed on approximately 4×10^{10} cells at a concentration of 5×10^9 bacteria/ml.

Immunoblot analyses. Bacterial cells exposed to conditions of oxidative stress and subcellular protein fractionation samples were resuspended in SDS-PAGE sample loading buffer, subjected to SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes, as described previously (17). Membranes were probed at room temperature with rabbit antiserum to either KatE (17), LipL21 (11), FlaA2 (30), or FlaA1 (12) at a 1:3,000 dilution for membranes containing the fractionated protein samples and at a 1:2,000 dilution for membranes containing protein samples from oxidative stress assays, and immunoreactivity was detected with a goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (Sigma-Aldrich Chemie GmbH, L'Isle d'Abeau Chesnes, Saint-Quentin Fallavier, France). Blots were developed with a chemiluminescent substrate (Super-Signal West Pico chemiluminescent substrate; Thermo Scientific, Illkirch, France) according to the manufacturer's instructions.

***Leptospira* insertion mutants.** Transposon mutagenesis was carried out on *L. interrogans* serovar Manilae strain L495 and *L. interrogans* serovar Pomona strain L523 with a kanamycin-resistant *Himar1* transposon, as described previously (39). Among the transformants, we identified an *L. interrogans* serovar Manilae mutant (m69) with an insertion into *katE* (LA1859) 384 bases into the 1,446-bp gene and an *L. interrogans* serovar Pomona mutant (mutant P3) with a transposon insertion 1,272 bases into the gene.

Genomic DNA was extracted from 20-ml cultures by using the QIAamp DNA blood minikit (Qiagen, Inc., Valencia, CA). Confirmation of the genotype was performed by using PCR with primers 69a (5′-GATACGGAAAGAGATCCGAG-3′) and 69b (5′-ATGATCTGAACACAAAACTTC-3′), which are located in the flanking sequences of the insertion site

of the transposon, and Southern blots of EcoRV-digested DNA were probed for hybridization with the kanamycin-resistant cassette.

Quantitative PCR was performed to test the effect of the transposon insertion on the transcription of the gene (*lic12031*) immediately downstream of *katE*. Wild-type strains of *L. interrogans* serovars Pomona and Manilae and the respective *katE* mutant strains were grown to the same density, and RNA was extracted as previously described (32), with the following modifications. Briefly, *Leptospira* bacteria were grown to 8×10^8 bacteria/ml, and 20 ml of each culture was centrifuged at 3,200 × *g*. Each pellet was resuspended in 1 ml TRIzol (Invitrogen, Saint-Aubin, Île-de-France, France), and RNA was purified according to the manufacturer's instructions. DNA digestion was conducted by the addition of 5 μl 10× Turbo DNase buffer (Ambion, Saint-Aubin, Île-de-France, France) and 1 μl Turbo DNase (Ambion) to the sample, followed by a 30-min incubation at 37°C. An additional 3 μl of Turbo DNase was added to the sample, followed by a 30-min incubation at 37°C. Turbo DNase was inactivated by the addition of 11 μl of DNase inactivation reagent (Ambion) to the sample and incubation for 5 min with occasional mixing. The sample was centrifuged at 10,000 × *g* for 2 min, and the supernatant was transferred into a new microcentrifuge tube.

First-strand cDNA synthesis was performed by using 1 μl RNA extract, 4 μl 5× iScript reaction mix (Bio-Rad, Marnes-la-Coquette, Île-de-France, France), 1 μl iScript reverse transcriptase (Bio-Rad), and 14 μl RNase-free H_2O (Bio-Rad). Thermal cycles of 25°C, 42°C, and 85°C for 5, 30, and 5 min, respectively, were performed by using a DNAEngine Peltier thermal cycler (Bio-Rad). A negative-control reaction to control for genomic DNA contamination of RNA samples in downstream quantitative PCR experiments was performed in the absence of reverse transcriptase.

Quantitative PCR analyses were performed with primer pair P1 (5′-TTTCGCATAATCTCCGTTCC) and P2 (5′-CTCCTCCGATCGTCTGAGTC) (amplifies bp 589 to 1837 of the *lic12031* open reading frame [ORF]). The *flaB1* transcript was used as a normalizing control and was amplified by primer pair fP1 (5′-GAGAGAAACACCGAAGACGG) and fP2 (5′-TGAATAGCAAGAACCCGGAT) (amplifies bp 187 to 287 of the *flaB1* [*lic11890*] ORF). Quantitative PCR was conducted by using 10 μl SsoFast EvaGreen Supermix (Bio-Rad), 5 pmol each primer, and 1 μl of either cDNA or the negative control lacking reverse transcriptase in a total volume of 20 μl. Thermal cycling was performed by using a C1000 Thermal Cycler CFX96 real-time system (Bio-Rad), with a cycling program of 1 cycle at 95°C for 3 min and 39 cycles at 95°C for 10 s, followed by 55°C for 30 s. A melting-curve analysis was performed to assess the amplification of a single amplicon per target by raising the temperature to 95°C for 10 s, followed by a 65°C to 95°C gradient with fluorescence measurements at 0.5°C increments. Triplicate quantitative PCR analyses were performed on each of 2 biological replicates. The Student *t* test was used to evaluate the differential expression of *lic12031* when *katE* mutant strains of *L. interrogans* serovars Manilae and Pomona were compared with their respective wild-type strains.

Hamster infections. *L. interrogans* strains were tested for virulence in the hamster model of acute leptospirosis. Male hamsters aged 28 days (Janvier) were inoculated intraperitoneally with 10^6 leptospires (50% infective dose [ID_{50}] of *L. interrogans* serovar Manilae of <100; $n = 8$) or 10^3 leptospires (ID_{50} of *L. interrogans* serovar Pomona of <100; $n = 10$), which reflects the standard in-house operating infectious doses for the two independent laboratories conducting the studies (30). Animals were monitored daily for clinical signs of leptospirosis and euthanized when moribund, in accordance with animal ethics requirements. In the countries involved in this study, the use of clinical disease symptoms as an experimental endpoint is required. Statistical analyses were performed by using Fisher's exact test.

RESULTS

Recombinant KatE displays catalase activity. Soluble recombinant KatE (rKatE) was expressed, purified, and utilized in a catalase-specific assay (Fig. 1) that measures the peroxidatic activity of catalase using methanol as an electron donor to decompose H_2O_2 into formaldehyde and water (25, 63). Other enzymes with peroxidatic activity do not utilize methanol to decompose H_2O_2 into formaldehyde (25), and therefore, any observed formaldehyde production can be attributed to the presence of catalase. The absorbance increased proportionally to the rKatE concentration, indicating the production of formaldehyde from methanol and H_2O_2 (Fig. 1A). The inclusion of 100 mM ATZ, a catalase inhibitor (37), diminished the absorbance relative to the rKatE concentration (Fig. 1A) but did not diminish the absorbance due to the formaldehyde concentration (Fig. 1B).

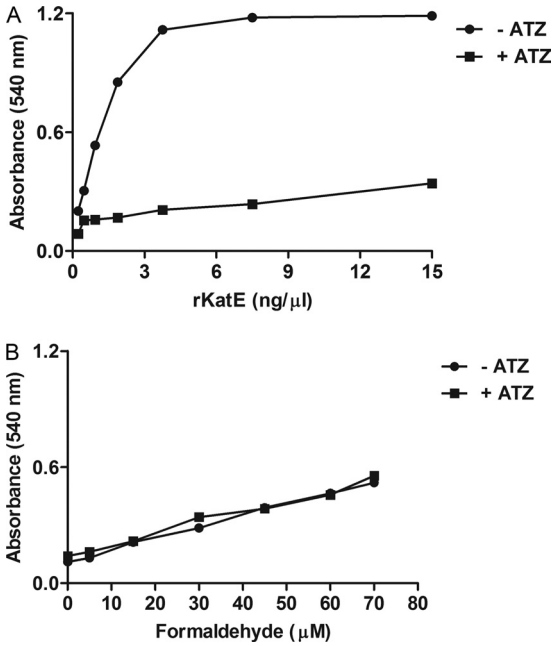

FIG 1 Recombinant KatE displays catalase activity. Recombinant KatE (rKatE) was analyzed in a catalase assay in the presence or absence of the specific catalase inhibitor ATZ (100 mM). (A) Increased absorbance with increasing rKatE concentrations in the absence of ATZ. No correlation was observed between the absorbance and the rKatE concentration in the presence of ATZ. (B) ATZ did not alter the reaction of formaldehyde standards with chromogen-producing compounds. Data are reported as the means from duplicate absorbance values at 540 nm for a single experiment.

H_2O_2-induced oxidative stress does not influence KatE expression. To test whether the expression of KatE was altered during the exposure of *Leptospira* to H_2O_2-induced oxidative stress, bacteria were exposed to H_2O_2 concentrations ranging from 0 to 10 mM. Total cellular protein was separated by SDS-PAGE and subjected to immunoblot analysis using antisera specific for KatE or FlaA1 (flagellar protein A1) (used as a loading control since it is constitutively expressed under various conditions [17, 32, 35]). KatE- and FlaA1-specific antisera recognized bands of molecular masses consistent with the predicted molecular masses of 54.7 kDa and 34.9 kDa for KatE and FlaA1, respectively (Fig. 2). Quantitative fluorescence immunoblot analyses of KatE expression in response to various H_2O_2 concentrations (normalized to FlaA1 expression) showed that KatE expression levels remained unchanged when cells were exposed to increasing H_2O_2 concentrations ($P > 0.5$ for all concentrations).

KatE is localized to the periplasmic space. Bioinformatic analysis using the subcellular localization prediction program

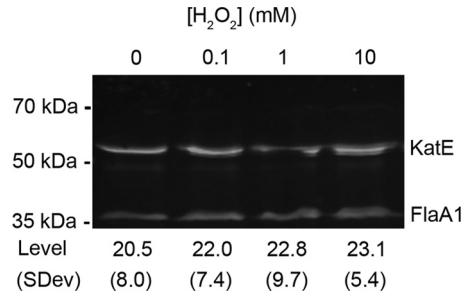

FIG 2 H_2O_2-induced oxidative stress does not affect KatE expression. Leptospires were exposed to 0, 0.1, 1, or 10 mM H_2O_2, and immunoblots were performed on total protein. Higher bands represent KatE, and lower bands represent FlaA1, with the latter being used as a loading control. The observed quantitative fluorescence levels are indicated, along with the standard deviations (SDev).

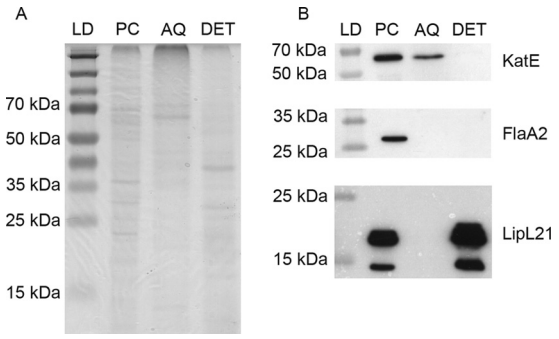

FIG 3 KatE localizes to the periplasmic space. Immunoblot analyses were conducted on *L. interrogans* fractionated protein samples. (A) SDS-polyacrylamide gel displaying the amount of protein loaded from each fraction. (B) Immunoblots demonstrating the reactivity of antisera with the indicated proteins in each fraction. Antisera to FlaA2 and LipL21 were used as controls to determine fraction purity. LD, protein ladder; PC, protoplasmic cylinder; AQ, aqueous phase; DET, detergent phase. The positions of molecular mass markers are indicated (in kilodaltons).

PSORTb predicted KatE to be located within the periplasm. To experimentally confirm this prediction, total cellular *L. interrogans* protein was fractionated to yield a protoplasmic cylinder (PC) containing cytoplasmic and inner membrane proteins, an aqueous (AQ) phase containing periplasmic proteins, and a detergent (DET) phase containing outer membrane proteins (21, 48, 68). Protein fractions were separated by SDS-PAGE (Fig. 3A) and subjected to immunoblot analysis. Antiserum to LipL21 reacted with a protein of approximately 20 kDa in the DET and PC phases (Fig. 3B), in agreement with previous observations showing that LipL21 fractionates with the PC and DET fractions (11). Antiserum to FlaA2 displayed reactivity with a band migrating at approximately 27 kDa in the PC fraction only (Fig. 3B), which is the expected fractionation result for a protein that associates with the flagellum core structure (30). Importantly, KatE-specific antiserum reacted with a band migrating at 52 to 55 kDa in both the AQ and PC phases, but no band was observed in the DET phase (Fig. 3B), consistent with the predicted localization of KatE to the periplasmic space.

Construction of *katE* transposon mutants and confirmation of the lack of transposon-induced downstream polar effects. *L. interrogans* transposon mutants disrupted in *katE* were constructed to assess the viability of these mutants under conditions of oxidative stress and during *in vivo* infection. Complementation in *L. interrogans* is extremely difficult and has been documented only twice (49, 67). We compensated for this lack of a genetic "knock-in" capability by constructing independent mutations in two different *L. interrogans* serovars (*L. interrogans* serovar Pomona mutant strain P3 and *L. interrogans* serovar Manilae mutant strain m69) and assessing if similar results were obtained with each of these mutants. To rule out the possibility of downstream polar effects of the *katE* (*lic12032*) transposon insertion, we compared the transcript levels of the gene immediately downstream of *katE*, *lic12031*, in the two independent mutants and their respective wild-type strains using quantitative PCR. These experiments indicated that *lic12031* transcription was not significantly different in the *katE* mutants compared to their wild-type strains (see Fig. S1 in the supplemental material).

***katE* enhances *L. interrogans* viability under conditions of oxidative stress.** *Leptospira* strains were exposed to various H_2O_2 concentrations and tested for viability at 4 h by using an alamarBlue survival assay. Wild-type *L. interrogans* strains displayed survival rates of 85 to 100% at 0.1 and 1 mM H_2O_2 and 30 to 50% at 10 mM H_2O_2 (Fig. 4A, C, and E) compared to *Leptospira* cells incubated in EMJH medium without exposure to H_2O_2. *L. biflexa* displayed survival rates of approximately 40, 10, and 2% (Fig. 4B) at 0.1, 1, and 10 mM H_2O_2, respectively, while *katE* mutant strains P3 and m69 showed survival rates of approximately 10, 5, and 2% (Fig. 4D and F) at 0.1, 1, and 10 mM H_2O_2, respectively, compared to growth in EMJH medium without exposure to H_2O_2. In the absence of H_2O_2, the wild-type and mutant strains displayed similar growth curves at 30°C (data not shown).

The inhibition of catalase activity with 100 mM ATZ severely reduced the viability of wild-type *L. interrogans* strains in the presence of H_2O_2 (Fig. 4A, C, and E) compared to *Leptospira* cells incubated in EMJH medium without H_2O_2 but in the presence of 100 mM ATZ. The presence of ATZ had a minimal effect on the survival of *L. biflexa* bacteria when exposed to H_2O_2 (Fig. 4B), consistent with the lack of a dependence of this species on catalase production. The survival of *L. interrogans* mutant strains upon H_2O_2 exposure in the presence of ATZ was slightly reduced to the background levels observed for the wild-type strains (Fig. 4D and F). No effect on viability was observed for bacteria in the presence of ATZ and in the absence of H_2O_2 (data not shown).

***katE* is required for virulence of *L. interrogans*.** To test the requirement for *katE* in leptospiral virulence, *katE* mutant strains of *L. interrogans* serovar Manilae (m69) and *L. interrogans* serovar Pomona (P3) were used in infection experiments for comparison to the wild-type strains. Virulence was assessed by using as an experimental endpoint the ability of the leptospiral strains to cause clinical disease symptoms. All hamsters injected with the wild-type strains exhibited signs of disease and were euthanized by day 19 postinfection, whereas all hamsters injected with the m69 or P3 mutant survived to 21 days postinfection and did not exhibit signs of disease (Fig. 5).

DISCUSSION

In this study, we investigated the responses of *Leptospira* species to oxidative stress conditions and, through insertional inactivation mutagenesis, characterized the role of the *L. interrogans* H_2O_2-detoxyifying enzyme KatE in protection from oxidative damage

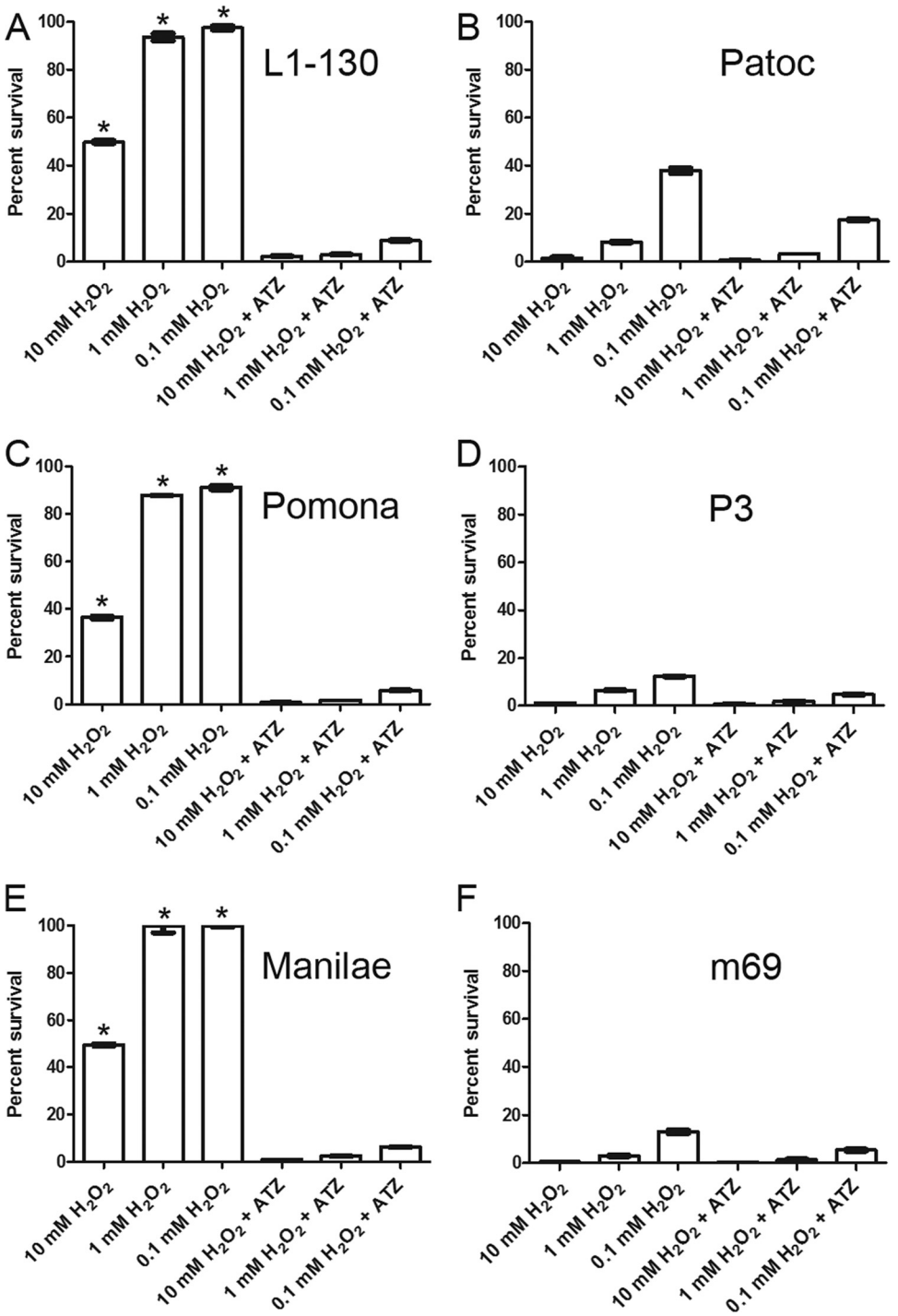

FIG 4 KatE enhances resistance to extracellular H_2O_2. Shown are percent survivals of *Leptospira* strains exposed to oxidative stress conditions in the presence or absence of the catalase inhibitor ATZ. (A) *L. interrogans* serovar Copenhageni; (B) *L. biflexa* serovar Patoc; (C) *L. interrogans* serovar Pomona; (D) *L. interrogans* serovar Pomona *katE* mutant strain P3; (E) *L. interrogans* serovar Manilae; (F) *L. interrogans* serovar Manilae *katE* mutant strain m69. Results are shown as means of triplicate values from a single experiment. Experiments were performed twice, with similar results. Statistical analyses were conducted by comparing the percent survival at a particular H_2O_2 concentration using the two-tailed Student *t* test under the following comparative conditions: (i) the presence and absence of ATZ, (ii) L1-130 versus Patoc, and (iii) wild type (*L. interrogans* serovar Pomona or Manilae) versus mutant (P3 or m69). Standard deviations are indicated, and significant differences between the means ($P < 0.0001$) are indicated by an asterisk, while the absence of an asterisk indicates no significant difference.

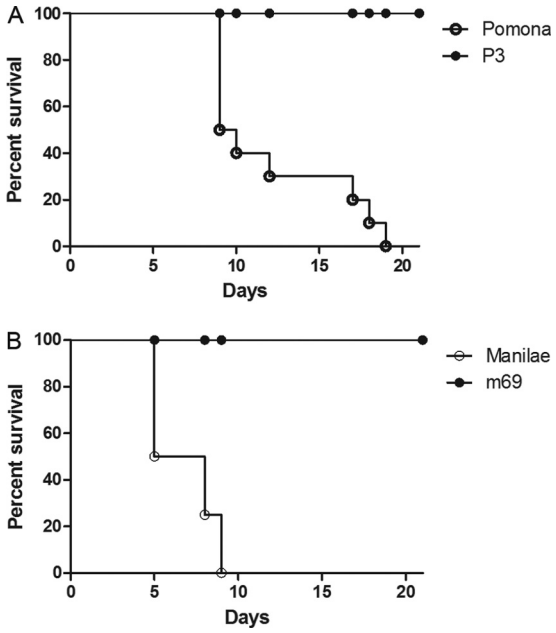

FIG 5 *Leptospira katE* mutants are attenuated in hamsters. All animals infected with wild-type strains of *L. interrogans* serovars Pomona (A) and Manilae (B) displayed disease symptoms, and all were euthanized by 19 days postinfection. Animals infected with mutant strains P3 (A) and m69 (B) did not display disease symptoms and exhibited 100% survival at 21 days postinfection ($P \leq 0.0007$ by Fisher's exact test).

and in leptospiral virulence. Here we showed that *L. interrogans* serovars Manilae and Pomona are more resistant to high H_2O_2 concentrations than the saprophyte *L. biflexa*, a finding in agreement with data from a previous report (38). Additionally, we demonstrated that leptospiral resistance to an H_2O_2-rich environment is mediated by KatE and that the loss of KatE expression leads to an attenuation of virulence for hamsters.

Through insertional inactivation in pathogenic *Leptospira* strains, seven mutants were previously shown to display attenuated virulence in animal models of infection, including mutations generated in the genes encoding an outer membrane lipoprotein, Loa22 (49); a heme oxygenase, HemO (41); a flagellum motor switch protein, FliY (31); two enzymes involved in lipopolysaccharide (LPS) biosynthesis (40); and the flagellar protein FlaA1 (30). Reduced virulence was achieved upon the inactivation of the gene encoding a putative outer membrane protease ClpB protein (34). Our identification of KatE as an additional virulence factor in *Leptospira* highlights both the dependence of this pathogen on a diverse repertoire of enzymes for survival and the requirement for a functional ROS resistance mechanism for full leptospiral pathogenesis.

In a previous quantitative proteomic study (17), we detected increased expression levels of KatE in *L. interrogans* serovar Copenhageni bacteria shifted to medium depleted of iron and supplemented with 10% fetal bovine serum to mimic conditions encountered within the host. Similar independent quantitative proteomic studies demonstrated increased expression levels of KatE in response to a shift in the temperature of *L. interrogans* from 29.5°C to 37°C (A. Eshghi and C. E. Cameron, unpublished observations), supporting data from a previous report showing that *katE* was upregulated more than 2-fold upon a shift of *L. interrogans* serovar Lai cells from environmental to physiological temperatures (32). Interestingly, Lo et al. did not observe an increase in *katE* expression levels within wild-type *Leptospira* cells under iron-limiting conditions (33), and similarly, in this study, we did not observe an increase in KatE expression levels upon the exposure of wild-type *Leptospira* bacteria to hydrogen peroxide-rich conditions. Combined, these studies suggest that in *Leptospira* temperature may serve as an environmental cue resulting in altered KatE expression, which differs from the requirement for H_2O_2 for the altered expression of ROS-detoxifying enzymes observed for *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (55). An elevated temperature would be an early indicator of a shift to a host environment, resulting in increased leptospiral KatE expression levels and, thus, preexisting protection from a subsequent host-induced oxidative burst response.

Cell fractionation experiments showed that KatE was present in both the aqueous phase and the protoplasmic cylinder, suggesting that this protein localizes, at least partially, to the periplasmic space. Cytoplasmic catalases are utilized for the detoxification of endogenous H_2O_2 produced from energy generation in the electron transport chain, while periplasmic catalases are utilized for the detoxification of exogenous H_2O_2 encountered during the oxidative burst response produced by host immune cells (42, 53). A periplasmic location for KatE may thus contribute to leptospiral survival upon infection by protecting periplasmic components against a host-derived oxidative burst response and is consistent with a previously reported observation that establishes the importance of periplasmic, but not cytoplasmic, superoxide dismutase for extracellular ROS resistance (10).

Further evidence supporting KatE-mediated extracellular H_2O_2 resistance comes from microarray analyses investigating iron-responsive genes. These investigations identified a peroxide stress regulator PerR homolog encoded by the *la1857* (*lic12034*) gene, which lies upstream of *katE*. The mutation of *la1857* resulted in a 4.3-fold increase in the *katE* expression level and an 8-fold increase in resistance to extracellular H_2O_2 (33); the *perR* mutant retained virulence in an animal model (39). To further delineate the critical role of KatE in leptospiral resistance to extracellular oxidative stress conditions, we conducted assays of survival upon exposure to conditions of H_2O_2-induced stress. The fact that leptospiral survival was significantly reduced when bacteria were exposed to 10 mM H_2O_2 in the presence of the specific catalase inhibitor ATZ, combined with the fact that the survival of the *katE* mutants was completely eliminated upon exposure to H_2O_2-rich conditions, demonstrates that bacterial survival under conditions of oxidative stress is dependent upon catalase activity. The approximately 50% survival rate of *L. interrogans* cells exposed to 10 mM H_2O_2 likely indicates that this concentration of H_2O_2 is on the maximal end of the oxidative stress conditions that *L. interrogans* can efficiently withstand. Together, these results indicate that KatE expression by *Leptospira* significantly enhances the ability of the pathogen to survive extracellular H_2O_2 toxicity.

An early study by Corin et al. (7) demonstrated that *L. interrogans* lysates can detoxify H_2O_2 concentrations that are 50-fold higher than those detoxified by *L. biflexa* lysates. In contrast, our study, which measured bacterial survival, rather than analyzing whole-cell lysates, suggested a range of 2- to 50-fold-higher resistance in *L. interrogans* than in *L. biflexa*. Corin et al. concluded that *L. interrogans* possesses this highly efficient H_2O_2 detoxification system to detoxify endogenous rather than exogenous H_2O_2 based

on the following two observations. First, *L. interrogans*, but not *L. biflexa*, contains cytochrome *d*, a heme-containing enzyme capable of catalyzing H_2O_2 to water and oxygen. Second, the extracellular locale of pathogenic *Leptospira* species would preclude exposure to high concentrations of extracellular H_2O_2, as would be encountered within a phagosome (7). However, a recent study using a zebrafish embryo system demonstrated that *L. interrogans* remains in host immune cells for up to 48 h and may use these cells as a means of dissemination to different tissues (14). This observation has been confirmed through an *in vitro* study demonstrating that *Leptospira* can survive within macrophages (58). It is therefore plausible that intracellular *Leptospira* bacteria may be exposed to high concentrations of exogenous H_2O_2 and other ROS while residing within host immune cells and that KatE expression may confer protection to *Leptospira* in this environmental context. Consistent with this hypothesis, *in vivo* studies conducted here with the two independent *katE* mutant strains *L. interrogans* serovar Manilae m69 and *L. interrogans* serovar Pomona P3 showed a strong attenuation of virulence for both mutants in an acute-infection model, demonstrating the essential nature of this enzyme for leptospiral virulence.

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REFERENCES

1. **Babior BM, Lambeth JD, Nauseef W.** 2002. The neutrophil NADPH oxidase. Arch. Biochem. Biophys. **397**:342–344.
2. **Babudieri B.** 1961. Studio serologico del gruppo Semaranga-Patoc di *Leptospira biflexa*, p 408–414. World Health Organization, Geneva, Switzerland.
3. **Bishai WR, Howard NS, Winkelstein JA, Smith HO.** 1994. Characterization and virulence analysis of catalase mutants of *Haemophilus influenzae*. Infect. Immun. **62**:4855–4860.
4. **Boylan JA, Lawrence KA, Downey JS, Gherardini FC.** 2008. *Borrelia burgdorferi* membranes are the primary targets of reactive oxygen species. Mol. Microbiol. **68**:786–799.
5. **Cerqueira GM, Picardeau M.** 2009. A century of *Leptospira* strain typing. Infect. Genet. Evol. **9**:760–768.
6. **Chelikani P, Fita I, Loewen PC.** 2004. Diversity of structures and properties among catalases. Cell. Mol. Life Sci. **61**:192–208.
7. **Corin RE, Boggs E, Cox CD.** 1978. Enzymatic degradation of H_2O_2 by *Leptospira*. Infect. Immun. **22**:672–675.
8. **Corin RE, Cox CD.** 1980. Characterization of leptospiral catalase and peroxidase. Can. J. Microbiol. **26**:121–129.
9. **Cowley SC, Myltseva SV, Nano FE.** 1996. Phase variation in *Francisella tularensis* affecting intracellular growth, lipopolysaccharide antigenicity and nitric oxide production. Mol. Microbiol. **20**:867–874.
10. **Craig M, Slauch JM.** 2009. Phagocytic superoxide specifically damages an extracytoplasmic target to inhibit or kill *Salmonella*. PLoS One **4**:e4975. doi:10.1371/journal.pone.0004975.
11. **Cullen PA, Haake DA, Bulach DM, Zuerner RL, Adler B.** 2003. LipL21 is a novel surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect. Immun. **71**:2414–2421.
12. **Cullen PA, et al.** 2005. Surfaceome of *Leptospira* spp. Infect. Immun. **73**:4853–4863.
13. **Das D, Bishayi B.** 2009. Staphylococcal catalase protects intracellularly survived bacteria by destroying H_2O_2 produced by the murine peritoneal macrophages. Microb. Pathog. **47**:57–67.
14. **Davis JM, Haake DA, Ramakrishnan L.** 2009. *Leptospira interrogans* stably infects zebrafish embryos, altering phagocyte behavior and homing to specific tissues. PLoS Negl. Trop. Dis. **3**:e463. doi:10.1371/journal.pntd.0000463.
15. **Elkins JG, Hassett DJ, Stewart PS, Schweizer HP, McDermott TR.** 1999. Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. Appl. Environ. Microbiol. **65**:4594–4600.
16. **Erdogan HM, et al.** 2008. Serum sialic acid and oxidative stress parameters changes in cattle with leptospirosis. Vet. Res. Commun. **32**:333–339.
17. **Eshghi A, Cullen PA, Cowen L, Zuerner RL, Cameron CE.** 2009. Global proteome analysis of *Leptospira interrogans*. J. Proteome Res. **8**:4564–4578.
18. **Fang FC.** 2011. Antimicrobial actions of reactive oxygen species. mBio **2**(5):e00141–11. doi:10.1128/mBio.00141-11.
19. **Fang FC.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. **2**:820–832.
20. **Fang FC, et al.** 1999. Virulent *Salmonella typhimurium* has two periplasmic Cu,Zn-superoxide dismutases. Proc. Natl. Acad. Sci. U. S. A. **96**:7502–7507.
21. **Haake DA, et al.** 1991. Changes in the surface of *Leptospira interrogans* serovar grippotyphosa during in vitro cultivation. Infect. Immun. **59**:1131–1140.
22. **Howell ML, et al.** 2000. AnkB, a periplasmic ankyrin-like protein in *Pseudomonas aeruginosa*, is required for optimal catalase B (KatB) activity and resistance to hydrogen peroxide. J. Bacteriol. **182**:4545–4556.
23. **Imlay JA, Linn S.** 1986. Bimodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. J. Bacteriol. **166**:519–527.
24. **Imlay JA, Linn S.** 1988. DNA damage and oxygen radical toxicity. Science **240**:1302–1309.
25. **Johansson LH, Borg LA.** 1988. A spectrophotometric method for determination of catalase activity in small tissue samples. Anal. Biochem. **174**:331–336.
26. **Johnson RC, Harris VG.** 1967. Differentiation of pathogenic and saprophytic leptospires. Growth at low temperatures. J. Bacteriol. **94**:27–31.
27. **Kawahara T, et al.** 2004. Role of nicotinamide adenine dinucleotide phosphate oxidase 1 in oxidative burst response to Toll-like receptor 5 signaling in large intestinal epithelial cells. J. Immunol. **172**:3051–3058.
28. **Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD, Jr, Riley LW.** 1999. Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. Lancet **354**:820–825.
29. **Ko AI, Goarant C, Picardeau M.** 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat. Rev. Microbiol. **7**:736–747.
30. **Lambert A, et al.** 2012. FlaA proteins in *Leptospira interrogans* are essential for motility and virulence but are not required for formation of flagellum sheath. Infect. Immun. **80**:2019–2025.
31. **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. doi:10.1186/1471-2180-9-253.
32. **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**:5848–5859.
33. **Lo M, et al.** 2010. Transcriptional response of *Leptospira interrogans* to iron limitation and characterization of a PerR homolog. Infect. Immun. **78**:4850–4859.
34. **Lourdault K, Cerqueira GM, Wunder EA, Picardeau M.** 2011. Inactivation of *clpB* in the pathogen *Leptospira interrogans* reduces virulence and resistance to stress conditions. Infect. Immun. **79**:3711–3717.
35. **Malmstrom J, et al.** 2009. Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. Nature **460**:762–765.
36. **Marangoni A, et al.** 2006. Production of reactive oxygen species and expression of inducible nitric oxide synthase in rat isolated Kupffer cells stimulated by *Leptospira interrogans* and *Borrelia burgdorferi*. World J. Gastroenterol. **12**:3077–3081.
37. **Margoliash E, Schejter A.** 1962. Kinetics of the irreversible inhibition of catalase by 3-amino-1,2,4-triazole in the presence of hydrogen peroxide

and catalase-hydrogen peroxide complex I hydrogen donors. J. Biol. Chem. **237**:2359–2363.
38. **Murgia R, Garcia R, Cinco M.** 2002. Leptospires are killed in vitro by both oxygen-dependent and -independent reactions. Infect. Immun. **70**: 7172–7175.
39. **Murray GL, et al.** 2009. Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. Infect. Immun. **77**:810–816.
40. **Murray GL, et al.** 2010. Mutations affecting *Leptospira interrogans* lipopolysaccharide attenuate virulence. Mol. Microbiol. **78**:701–709.
41. **Murray GL, et al.** 2009. *Leptospira interrogans* requires heme oxygenase for disease pathogenesis. Microbes Infect. **11**:311–314.
42. **Naclerio G, Baccigalupi L, Caruso C, De Felice M, Ricca E.** 1995. *Bacillus subtilis* vegetative catalase is an extracellular enzyme. Appl. Environ. Microbiol. **61**:4471–4473.
43. **Nakayama GR, Caton MC, Nova MP, Parandoosh Z.** 1997. Assessment of the Alamar Blue assay for cellular growth and viability in vitro. J. Immunol. Methods **204**:205–208.
44. **Nally JE, Chow E, Fishbein MC, Blanco DR, Lovett MA.** 2005. Changes in lipopolysaccharide O antigen distinguish acute versus chronic *Leptospira interrogans* infections. Infect. Immun. **73**:3251–3260.
45. **Nathan C, Shiloh MU.** 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc. Natl. Acad. Sci. U. S. A. **97**:8841–8848.
46. **Palaniappan RUM, Ramanujam S, Chang Y-F.** 2007. Leptospirosis: pathogenesis, immunity, and diagnosis. Curr. Opin. Infect. Dis. **20**:284–292.
47. **Patarakul K, Lo M, Adler B.** 2010. Global transcriptomic response of *Leptospira interrogans* serovar Copenhageni upon exposure to serum. BMC Microbiol. **10**:31. doi:10.1186/1471-2180-10-31.
48. **Pinne M, Haake DA.** 2009. A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of *Leptospira interrogans*. PLoS One **4**:e6071. doi:10.1371/journal.pone.0006071.
49. **Ristow P, et al.** 2007. The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog. **3**:e97. doi:10.1371/journal.ppat.0030097.
50. **Rodriguez-Lopez J, et al.** 2001. Mechanism of reaction of hydrogen peroxide with horseradish peroxidase: identification of intermediates in the catalytic cycle. J. Am. Chem. Soc. **123**:11838–11847.
51. Reference deleted.
52. **Srinivasa Rao PS, Yamada Y, Leung KY.** 2003. A major catalase (KatB) that is required for resistance to H_2O_2 and phagocyte-mediated killing in *Edwardsiella tarda*. Microbiology **149**:2635–2644.
53. **Steele KH, Baumgartner JE, Valderas MW, Roop RM, II.** 2010. Comparative study of the roles of AhpC and KatE as respiratory antioxidants in *Brucella abortus* 2308. J. Bacteriol. **192**:4912–4922.
54. **Sterling CR, Thiermann AB.** 1981. Urban rats as chronic carriers of leptospirosis: an ultrastructural investigation. Vet. Pathol. **18**:628–637.
55. **Storz G, Tartaglia LA, Ames BN.** 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science **248**:189–194.
56. **Suvarnapunya AE, Lagasse HA, Stein MA.** 2003. The role of DNA base excision repair in the pathogenesis of *Salmonella enterica* serovar Typhimurium. Mol. Microbiol. **48**:549–559.
57. **Tainer JA, Getzoff ED, Richardson JS, Richardson DC.** 1983. Structure and mechanism of copper, zinc superoxide dismutase. Nature **306**:284–287.
58. **Toma C, Okura N, Takayama C, Suzuki T.** 2011. Characteristic features of intracellular pathogenic *Leptospira* in infected murine macrophages. Cell. Microbiol. **13**:1783–1792.
59. **Tondo ML, Petrocelli S, Ottado J, Orellano EG.** 2010. The monofunctional catalase KatE of *Xanthomonas axonopodis* pv. citri is required for full virulence in citrus plants. PLoS One **5**:e10803. doi:10.1371/journal.pone.0010803.
60. **Vazquez-Torres A, et al.** 2000. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science **287**:1655–1658.
61. **Vignais PV.** 2002. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cell. Mol. Life Sci. **59**:1428–1459.
62. **Vinetz JM.**2001. Leptospirosis. Curr. Opin. Infect. Dis. **14**:527–538.
63. **Wheeler CR, Salzman JA, Elsayed NM, Omaye ST, Korte DW, Jr.** 1990. Automated assays for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity. Anal. Biochem. **184**:193–199.
64. **Wilson TM, de Lisle GW, Collins DM.** 1995. Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium bovis*. Mol. Microbiol. **15**:1009–1015.
65. **Xue F, et al.** 2010. Transcriptional responses of *Leptospira interrogans* to host innate immunity: significant changes in metabolism, oxygen tolerance, and outer membrane. PLoS Negl. Trop. Dis. **4**:e857. doi:10.1371/journal.pntd.0000857.
66. **Yu NY, et al.** 2010. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics **26**:1608–1615.
67. **Zhang L, et al.** 2012. The mammalian cell entry (Mce) protein of pathogenic *Leptospira* species is responsible for RGD motif-dependent infection of cells and animals. Mol. Microbiol. **83**:1006–1023.
68. **Zuerner RL, Knudtson W, Bolin CA, Trueba G.** 1991. Characterization of outer membrane and secreted proteins of *Leptospira interrogans* serovar Pomona. Microb. Pathog. **10**:311–322.