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A leptospiral AAA+ chaperone–Ntn peptidase complex, HslUV, contributes to the intracellular survival of *Leptospira interrogans* in hosts and the transmission of leptospirosis

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Leptospirosis caused by *Leptospira* is a zoonotic disease of global importance but it is considered as an emerging or re-emerging infectious disease in many areas in the world. Until now, the mechanisms about pathogenesis and transmission of *Leptospira* remains poorly understood. As eukaryotic and prokaryotic proteins can be denatured in adverse environments and chaperone–protease/peptidase complexes degrade these harmful proteins, we speculate that infection may also cause leptospiral protein denaturation, and the HslU and HslV proteins of *L. interrogans* may compose a complex to degrade denatured proteins that enhances leptospiiral survival in hosts. Here we show that leptospiral HslUV is an ATP-dependent chaperone–peptidase complex containing ATPase associated with various cellular activity (AAA+) and N-terminal nucleophile (Ntn) hydrolase superfamily domains, respectively, which hydrolyzed casein and chymotrypsin-like substrates, and this hydrolysis was blocked by threonine protease inhibitors. The infection of J774A.1 macrophages caused the increase of leptospiiral denatured protein aggresomes, but more aggresomes accumulated in *hslV* gene-deleted mutant. The abundant denatured leptospiral proteins are involved in ribosomal structure, flagellar assembly, two-component signaling systems and transmembrane transport. Compared to the wild-type strain, infection of cells in vitro with the mutant resulted in a higher number of dead leptospires, less leptospiral colony-forming units and lower growth ability, but also displayed a lower half lethal dose, attenuated histopathological injury and decreased leptospiral loading in lungs, liver, kidneys, peripheral blood and urine in hamsters. Therefore, our findings confirmed that HslUV AAA+ chaperone–Ntn peptidase complex of *L. interrogans* contributes to leptospiiral survival in hosts and transmission of leptospirosis.

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**Keywords:** hslU gene/hslV gene; HslUV chaperone–peptidase complex; infection/protein denaturation; *Leptospira interrogans*; survival/transmission

**INTRODUCTION**

Pathogenic *Leptospira* genospecies such as *Leptospira interrogans* are the causative agent of leptospirosis, a worldwide-prevailing zoonotic infectious disease.1 The disease has been endemic in populations of East and Southeast Asia, Oceania and South America.2–4 However, in recent years, the sporadic cases and small outbreaks of human leptospirosis have been frequently reported in many areas of Europe, North America and Africa,5–7 where leptospirosis is considered as an emerging or re-emerging infectious disease.8

At least 180 species of animals have been confirmed as the hosts of pathogenic *Leptospira* genospecies in different areas, but rodents, livestock and dogs have a most important role in transmission of leptospirosis by persistent excretion of the spirochete from their urine.9 Human individuals are infected by contact with water or wet soil that had been contaminated with *Leptospira*-containing animal urine.1,9 After invasion into humans through the skin and mucosa, the spirochete can promptly enter the bloodstream and then spread into internal organs such as lungs, liver and kidneys, where they cause tissue injury.10–11 The course of human leptospirosis varies from mild to rapidly fatal forms, including ‘flu-like’ clinical manifestations such as fever and myalgia, and lethal symptoms such as respiratory failure due to pulmonary diffusion hemorrhage and meningo, and renal failure caused by serious renal injury and jaundice.1,9–11 However, until now, the molecular and cellular basis of persistent *Leptospira* infection in host animals remains poorly understood.

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Infection results from an interaction between pathogens and their hosts. In the course of pathogen–host interactions, the hosts will generate an adverse environment such as high body temperature and high levels of intracellular reactive oxygen species and reactive nitrogen species during phagocytosis, which are assumed to cause irreversible denaturation of microbial proteins. When the accumulation of denatured proteins exceeds the degrading capacity of prokaryotic or eukaryotic cells, these abnormal proteins can form insoluble aggresomes that not only lose their biological function but can also cause cellular ageing and loss of cellular viability. A large number of the protein aggresomes can ultimately lead to the death of prokaryotes and eukaryotes. Therefore, we hypothesized that a timely degradation of the denatured proteins caused by adverse environmental factors during infection is a critical factor in the ability of pathogens to survive in hosts.

Chaperone–protease/peptidase proteolytic complexes are responsible for degradation of intracellular denatured proteins. Proteasomes, the 20S chaperone–protease complexes with two heptameric stacked rings, eliminate the denatured proteins in eukaryotes, but ATPase associated with various cellular activity (AAA+) superfamily domain-containing chaperone–peptidase complexes degrade the denatured proteins in both prokaryotes and eukaryotes. Until now, several bacterial ATP-dependent chaperone–peptidase complexes, such as the HslUV (ClpYQ), ClpAP and ClpXP of *Escherichia coli*, as well as CodXW, a homolog of HslUV, and ClpCP of *Bacillus subtilis*, have been characterized. Among the HslUV and CodXW complexes, the HslU or CodX protein is a chaperone responsible for recognition, unfolding and translocation of denatured proteins into the proteolytic chamber of HslV or CodW and providing energy by ATP for proteolysis, while the HslV or CodW protein is an ATP-dependent threonine or serine protease. However, role of the bacterial chaperone–peptidase complexes in degradation of intracellular denatured proteins due to infection has not been reported. Previous studies confirmed that PrcBA complex of *Mycobacterium tuberculosis*, a unique proteasome found in bacteria, is required for persistent infection in mice, and the depletion of PrcB segment attenuates the deleterious effect of persistent infection in the animal host. The chromosomal DNA of pathogenic *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai contains a pair of genes (NO LA2345 and LA2346) that code for protein products annotated as HslU and HslV. However, the roles of *L. interrogans* hslU and hslV genes during infection have not been characterized yet.

*L. interrogans* is the most prevalent pathogenic *Leptospira* genospecies in the world. Although many serogroups of *L. interrogans* are present in China, *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai is responsible for disease in over 60% of Chinese leptospirosis patients. On the other hand, macrophages but not neutrophils act as major infiltrating cells in biopsy samples from leptospirosis patients and *L. interrogans*-infected mice. In the present study, we therefore investigated the distribution of hslU and hslV genes in *L. interrogans* strains belonging to different serogroups and serovars that are prevalent in China, and then identified the HslU and HslV of *L. interrogans* strain Lai compose a ATP-dependent chaperone–peptidase complex by virtue of its ability to hydrolyze protein/peptide substrates and usage of different peptidase inhibitors. Subsequently, we identified the denatured proteins in the spirochete due to infection of murine macrophages. Moreover, we generated a hslU and hslV gene-deleted mutant to determine the role of the HslUV complex to decrease the level of denatured proteins of the spirochete during infection of macrophages or to enhance the viability and excretion of the spirochete in hamsters. The results of this study confirmed that the HslUV functions as an ATP-dependent chaperone–peptidase complex that required for survival of *L. interrogans* in hosts and transmission of leptospirosis.

**MATERIALS AND METHODS**

**Ethics statement**

All animals were handled in strict accordance with good animal practice as defined by the National Regulations for the Administration of Experimental Animals of China (1988-002) and the National Guidelines for Experimental Animal Welfare of China (2006-398). All the animal experimental protocols were approved by the Ethics Committee for Animal Experiment of Zhejiang University (Certificate NO: SCXK[zhe]2007-0030).

**Leptospiral strains and culture**

Ten pathogenic *L. interrogans* strains, serogroup Icterohaemorrhagiae serovar Lai strain Lai, serogroup Canicola serovar Canicola strain Lin, serogroup Pyrogenes serovar Pyrogenes strain Tian, serogroup Autumnalis serovar Autumnalis strain Lin4, serogroup Australis serovar Australis strain 65-9, serogroup Pomona serovar Pomona strain Luo, serogroup Grippotyphosa serovar Grippotyphosa strain Lin6, serogroup Hebdomadis serovar Hebdomadis strain 56069, serogroup Bataviae serovar Paidjan strain L37 and serogroup Sejroe serovar Wolfli strain L183, which serve as the standard strains in serological examination for human leptospirosis diagnosis, and two non-pathogenic saprophytic *Leptospira biflexa*, serogroup Samaranga serovar Patoc strain Patoc 1 and serogroup Andamanera serovar andamana strain CH-11, were provided by the Chinese National Institute for Control of Pharmaceutical and Biological Products in Beijing, China. All the leptospiiral strains were cultivated at 28 °C in Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium.

**Cell line and culture**

The murine macrophage-like cell line J774A.1 was provided by the Cell Bank of the Institute of Cytobiology in Shanghai, Chinese Academy of Sciences. The cell line was maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Gibco), 100 U/mL penicillin (Sigma, St Louis, MO, USA) and 100 µg/mL streptomycin (Sigma), in an atmosphere containing 5% CO2 at 37 °C.

**Animals**

Male Syrian hamsters (35 ± 2 g per animal, 3-week old) and New Zealand rabbits (3.0–3.5 kg/animal) were provided by the Laboratory Animal Center of Zhejiang University (Certificate NO: SCXK [zhe] 2007-0030).

**Primer**

The primers used in this study were synthesized by Invitrogen Co., Shanghai, China. The primer sequences are listed in Table 1.

**Detection of hslU and hslV genes in different leptospiiral strains**

Genomic DNA from each of the 12 leptospiiral strains was extracted using a Bacterial Genomic DNA Miniprep Kit (Axogen, Union City, CA, USA). By using a High Fidelity PCR Kit (TaKaRa, Dalian, China), the hslU and hslV genes in the DNA samples from the 10 strains of *L. interrogans* or the two strains of *L. biflexa* were detected by PCR with the primers hslU1-F/hslU1-R and hslV1-F/hslV1-R or hslU2-F/hslU2-R and hslV2-F/hslV2-R (Table 1) because of the large sequence difference at 5′ and 3′ ends of the two genes from *L. interrogans* and *L. biflexa* strains. The products were examined on 1.5% ethidium...
Expression and extraction of leptospiral recombinant HslU and HslV proteins

The sequenced pMD18-T plasmid was used to form pMD18-T^{hslU} and pMD18-T^{hslV} using a T-A Cloning Kit (Takara) for sequencing by Invitrogen Co.

Table 1 Sequences of primers used in this study

| Primer | Sequence (5′-3′) | Purpose | Size (bp) |
|--------|------------------|---------|-----------|
| hslU1  | F: CGC CAT ATG(NdeI)gca aat cat cca ata gac | L. interrogans hslU gene detection and expression | 1437 |
|        | R: CGC CTC GAG(XhoI)AAG AAT ATG CTG ACT CAG ATC | | |
| hslV1  | F: CGC CAT ATG(NdeI)CCA GAA AAT AAA ATT CTT TCT ACA | L. interrogans hslV gene detection and expression | 540 |
|        | R: CGC CTC GAG(XhoI)AAG AAT TTC TCT TAG GGT TAT ATG | | |
| hslU2  | F: ATG AGT TTC AAA ACA ATA CTT GCA | L. biflexa hslU gene detection | 1419 |
|        | R: TTA CAG GAA ACA ATT CAC GCA ACC | | |
| hslV2  | F: ATG GAA ACA ATT CAC GCA ACC | L. interrogans hslV mRNA detection | 534 |
|        | R: TTA TAA TTC TCT CTT CAA | | |
| hslU-3 | F: AAT AGA CCA CCA GGA ACT TAC ACC G | L. interrogans hslU mRNA detection | 127 |
|        | R: GGT TCT AAG AGC GAC TAC TGC | | |
| hslV-3 | F: GAT TCT TGG AAC TGG AGA TGT GAT T | L. interrogans hslV mRNA detection | 122 |
|        | R: CTT TGG GAA GAT TGG TAT GAT C | | |
| 16S    | F: CCT TCG TCG TCT AGC GTC AGT | 16S rRNA as an internal reference in qRT-PCR | 145 |
|        | R: CGC AGC CTG CAC TTG AAA CTA | | |
| U1     | F: CGC GCA TGG(SphI)CAA TCA AGC TCT ACA GGA TTG | 5′-homologous arm for hslUV gene deletion | 813 |
|        | R: CGC GTC GAC(SalI)GTC GAG CTC TTC TTC AAA | | |
| D1     | F: CGC GTC GAC(SalI)ATA AGT TTT TCT TCT ATG ATG AAA | 3′-homologous arm for hslUV gene deletion | 822 |
|        | R: CGC GAT ACC(KpnI)tag tca ata gcc aat gcc aat gca | | |
| P1     | F: CGC GTC GAC(SalI)TA TAC CCG AGC TTC TCA | figB gene promoter segment for Kan′ expression | 410 |
|        | R: tga tat tct cat ttg agg cat ATG GAA ACC TCC TTC ATT TAA | | |
| K      | F: tta aat gag gga gga ggg gcc ggg gcc gaa gaa gaa aat tca ggg | Kan′ segment for deletion of hslUV genes | 892 |
|        | R: CGC GTC GAC(SalI)CAC TAT CAG AGT GAC | | |
| U2     | F: CGC GCA TGG(SphI)GTT ACC AAT ACA TTG CTA ACC | 5′-homologous arm and hslUV genes for complementation | 3334 |
|        | R: CGC GTC GAC(SalI)TTA AAG AAT ATG CTT ACG | | |
| D2     | F: CGC GGT ACC(KpnI)ATA AGT TTT TCT ATG ATG AAA | 3′-homologous arm for hslUV gene complementation | 1350 |
|        | R: CGC GTC GAC(SalI)TTA CTT CTT TCT ATG CTT ATA TAA | | |
| P2     | F: CGC GTC GAC(SalI)TA TAC CCG AGC TTC TCA | figB gene promoter segment for Spc′ expression | 410 |
|        | R: cct taa aag tgc ggc ggc gtt ATG GAA ACC TCC TTC ATT TAA | | |
| S      | F: tta aat gag gga gga ggg gcc ggg gcc gaa gaa gaa aat tca ggg | Spc′ segment for selection of ΔhslUV mutant | 1235 |
|        | R: CGC GAT ACC(KpnI)AAC GGA TAA GAT AGC CAC TGG | | |
| C1     | F: GGA GAA TTT CAT TGG GTG | Confirmation of ΔhslUV mutant | 3177 |
|        | R: TTA ATC AAA AGC TAT AGA | | |
| C2     | F: ACG GAA TTT CTC CTG AAA ACA | Confirmation of ΔhslUV mutant | 6598 |
|        | R: TTA TCG TGT AAA CCG TCC AAA | | |

Abbreviations: forward primer, F; reverse primer, R; real-time fluorescence quantitative reverse transcription-qPCR, qRT-PCR.

Underlined nucleotides indicate the cleavage site of endonucleases. Letters in lowercase indicate the sequences for linking figB gene promoter of Borelia burgdorferi (pflgB) with Kan′ or Spc′.

bromide-pre-stained agarose gel after electrophoresis, and then cloned into pMD18-T plasmid to form pMD18-T^{hslU} and pMD18-T^{hslV} using a T-A Cloning Kit (Takara) for sequencing by Invitrogen Co.

Detection of HslU and HslV proteins in different L. interrogans strains

New Zealand rabbits were immunized intradermally on days 1, 14, 21 and 28 with 2 mg leptospiral HslU or HslV that pre-mixed with Freund’s adjuvant. Fifteen days after the last immunization, the sera were collected to separate the IgGs with ammonium sulfate precipitation plus a DEAE-52 column (Sigma) using 10 mM phosphate buffer (pH 7.4) for elution. The titer of each of the IgGs binding to the HslU or HslV was detected by immunodiffusion test.

Preparation of rHslU-IgG and rHslV-IgG

Freshly cultured 10 L. interrogans strains were precipitated by a 10 000g centrifugation at 4 °C for 30 min. After washing with phosphate-buffered saline (PBS, pH 7.4) and centrifugation again, (BioColor, Shanghai, China). The extracted rHslU or rHslV was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific). The expressed and extracted rHslU and rHslV were detected by SDS-polyacrylamide gel electrophoresis plus a Gel Image Analyzer (Bio-Rad, Hercules, CA, USA).

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the precipitated leptospires were suspended in distilled water for ultrasonic disruption using 4 W of continuous power for a total of 3 x 30 s on ice. The lysates were centrifuged at 3000g for 10 min (4 °C) to remove lepistolarial debris, and the supernatants containing total lepistolarial soluble proteins were collected for protein concentration determination as described above. Using 1:200 diluted rabbit anti-

rHslU or rHslV-IgG as the primary antibody and 1:3000 diluted horseradish peroxidase-labeled goat anti-rabbit-IgG (Abcam, Cambridge, UK) as the secondary antibody, two separate western blot assays were performed to detect HslU and HslV in the protein samples.

Extraction of leptosomal native HslU and HslV proteins

Native HslU and HslV proteins (nHslU and nHslV) of L. interrogans strain Lai were extracted using a Pierce Direct IP Kit (Thermo Fisher Scientific). Briefly, the rHslU-IgG or rHslV-IgG was equilibrated with the coupling buffer and then linked to the AminoLink-coupling resin to form rHslU- or rHslV-IgG-coupled resin. The total soluble leptosomal proteins from the spirochete that extracted as described above were dialyzed with the lysis/washing buffer for equilibration. Leptosomal proteins from the spirochete that extracted as described above were dialyzed with the lysis/washing buffer, and then its concentration was quantified by the Bio-Rad protein assay according to the manufacturer's instructions.

Detection of N-terminal cleavage of rHslV and nHslV

Previous studies confirmed that the HslV proteins from several bacteria were activated by cleavage to expose the catalytic threonine residue. Therefore, the N-terminal sequences of purified rHslV and nHslV were detected by Edman degradation-based N-terminal sequencing method using a protein sequence system (type PPSQ-33A, Shimadzu, Kyoto, Japan).

Prediction of functional domains in leptosomal HslU and HslV

L. interrogans strain Lai and L. biflexa strain Patoc I were selected for analysis of the chaperone and peptidase domains in HslU and HslV because of the high sequence identities of HslU and HslV genes among the pathogenic or saprophytic strains. The comparison of the leptosomal HslU and HslV with those of E. coli and Hemophilus influenzae in previous studies using NCBI Batch CD-Search tool.

Proteolysis assay

As the sequencing data indicated that the nucleotide and amino-acid sequences of hslU and hslV genes from the 10 L. interrogans strains were highly conserved (99.4%–100%), the rHslU and rHslV from L. interrogans strain Lai origin were used for determination of proteolytic activity as previously described. Briefly, the complexes of 100–800 nM rHslU and 100 nM rHslV (rHslU5rHslV) were mixed with 2 μM fluorescein isothiocyanate (FITC)-labeled casein (Thermo Fisher Scientific) in 100 μL of 100 mM Tris-HCl buffer (pH 8.0) containing 5 mM MgCl₂, 2 mM ATP, 20 mM phosphocreatine and 10 units/mL of creatine kinase for a 2 h incubation at 37 °C. Fluorescence intensity of the released FITC (485 nm excitation and 538 nm emission wavelengths) was detected by a fluorospectrophotometer (Molecular Devices, Sunnyvale, CA, USA). On the other hand, the rHslU5rHslV complexes (100–800/100 nM) were mixed with 100 μM 7-aminomethyl-4-coumarin (AMC)-labeled synthetic peptide substrates (ENZO Life Sciences, Farmingdale, NY, USA) in 100 μL of 100 mM Tris-HCl buffer containing 5 mM MgCl₂, 0.5 mM EDTA and 2 mM ATP (pH 8.0) for a 30 min incubation at 37 °C. Fluorescence intensity of the released AMC (380 nm excitation and 480 nm emission wavelengths) was detected by spectrofluorimetry as above. Among the five peptide substrates used, Suc-LLVY-AMC, Suc-AAF-AMC and Z-GGL-AMC were used for detecting chymotrypsin-like activity, while Boc-LRR-AMC or Z-LEL-AMC were applied for detecting trypsin-like or caspase-like activity. According to the results from the assays above, different concentrations of the compounds of 5–1250 nM rHslU or nHslU and 1–250 nM rHslV or nHslV (rHslU or nHslU/nHslV) were applied to determine the concentration–effect relationship during hydrolysis of FITC-casein, Suc-LLVY-AMC, Suc-AAF-AMC and Z-GGL-AMC. In the detection, single rHslV and rHslU were used as the controls.

Proteolysis inhibition test

According to the results of proteolysis assays as above, the complex of 500 nM rHslU and 100 nM rHslV (rHslU5rHslV) were pre-treated at 37 °C for 30 min with 20 μM MG132 (ENZO Life Sciences), an inhibitor of threonine, serine and cysteine proteases/peptidases, 5 μM 4-hydroxy-5-iodo-3-nitrophenylacetetyl-leu-leu-leu-1-nitrophenol sulfone (Calbiochem, Darmstadt, Germany) and 100 μM lactacystin (ENZO Life Sciences), the inhibitors of threonine proteases/peptidases, 20 μM leupeptin (ENZO Life Sciences), an inhibitor of serine and cysteine proteases/peptidases, and 1 mM E64 (ENZO Life Sciences), an inhibitor of cysteine proteases/peptidases, respectively. The subsequent experimental steps to detect the hydrolysis of FITC-casein, Suc-LLVY-AMC, Suc-AAF-AMC and Z-GGL-AMC substrates were the same as described above.

ATP-dependent proteolysis test

ATP-free reaction or the reaction buffer in which 2 mM ATP replaced with the same concentration of βγ-methylene-ATP (Sigma), a nonhydrolyzable ATP analog, was used to determine the dependence of proteolytic activity of lepistolporal rHslU5rHslV complex on ATP as described above.

Determination of Km and Kcat values

To determine the Km and Kcat values of leptosomal rHslU5rHslV complex, 0.5, 1, 1.5, 2, 2.5, 3 or 3.5 μM FITC-casein, or 60, 80, 100, 120, 140, 160, 180 or 200 μM Suc-LLVY-AMC, the preferred peptide substrate of the different rHslUV complexes confirmed in the proteolytic activity assay above, was mixed with the 500 nM rHslU and 100 nM rHslV in 100 μL of the reaction buffer. The subsequent experimental steps to detect the hydrolysis of the two substrates were the same as described above. The Km and Kcat values of rHslU5rHslV complex were calculated by double-reciprocal Line–weaver–Burk plot.

Isolation of intracellular leptospires from infected cells

Freshly cultured L. interrogans strain Lai was collected by a 10 000 g centrifugation at 4 °C for 30 min. The collected leptospires were counted under a dark-field microscope with a Petroff–Hauser counting chamber. The J774A.1 cells (1 x 10⁶ per well) were seeded in six-well culture plates for a 12 h pre-incubation in an atmosphere of 5% CO₂ at 37 °C. The cell monolayers were thoroughly washed with PBS and then infected with the spirochete (1 x 10⁸) at a multiplicity of infection (MOI) of 100 (100 leptospires per cell) for the indicated times. After washing with PBS and trypsinization, the cultures were centrifuged at 500 g for 5 min (4 °C). The precipitated cells were washed with PBS for three times followed by centrifugation as above to remove extracellular leptospires in the supernatants and then lysed with 0.05% NaTDC-PBS to release intracellular leptospires.
After a 5 min centrifugation at 500 g (4 °C) to remove cell debris, the lysates were centrifuged at a 10 000 g centrifugation at 4 °C for 30 min to precipitate intracellular leptospires. The leptosporal pellets were suspended in PBS for counting as above.

**Measurement of hslU- and hslV-mRNA levels during infection of cells**

The J774A.1 cell monolayers were infected with *L. interrogans* strain Lai at a MOI of 100 for 0.5, 1, 2, 4, 8, 12 or 24 h, and then the intracellular leptospires were collected as described above. Total RNAs from the leptospires were extracted using a TRizol Max bacterial RNA Isolation kit (Invitrogen) and a gDNA Eraser Kit (TaKaRa), and then the cDNAs from the total RNAs were synthesized using a PrimeScript RT Reagent Kit (TaKaRa). Using the cDNAs as templates, the hslU- or hslV-mRNA level was assessed by real-time fluorescence quantitative reverse transcription-PCR (qRT-PCR) using a SYBR Premix Ex-Taq Analyzer (Bio-Rad). In this assay, a lipoprotein (LipL41) of the spirochete was used as the control.10

**Generation of hslU and hslV gene-complemented mutant**

The segments of hslU and hslV gene-complemented plasmid from pGSBLe94 plasmid were amplified by PCR using the primers P2-F/P2-R and S-F/S-R (Table 1), and then the two segments were linked by a special PCR using the primers P2-F/S-R to form a plasmid for sequencing. Subsequently, two separate PCs were performed to amplify a 3334-bp arm-hslU-hslV arm segment and a 1350-bp arm segment from wild-type *L. interrogans* strain Lai using the primers U2-F/U2-R and D2-F/D2-R (Table 1), respectively. The pfgb-sp arm segments were digested with Sall and KpnI, SpHl and Sall, and KpnI and Sall endonucleases (TaKaRa), respectively, and then linked with T4 DNA ligase (TaKaRa) to form a 3' arm-hslU-hslV arm segment. The linking segment was inserted into the SpHl and KpnI sites in pUC19 plasmid to form a suicide plasmid pUC195arm-pfgb-kan-3' arm under action of T4 DNA ligase for sequencing again. The following steps such as preparation of the competent ΔhslUV mutant, transformation of the recombinant plasmid into the ΔhslUV mutant and selection of the hslU and hslV gene-complemented mutant (ΔhslUV) were performed in the ΔhslUV mutant. The strategy for generating the ΔhslUV mutant is summarized in Supplementary Figure S1A.

**Identification of ΔhslUV and CΔhslUV mutants**

Morphology, motility and growth of the ΔhslUV and CΔhslUV mutants were assessed by dark-field microscopy and spectrophotometry. The hslU and hslV gene deletion in the ΔhslUV mutant or the hslU and hslV gene complementation in the ΔhslUV mutant was determined by PCR using the primers C1-F/C1-R or C2-F/C2-R (Table 1) and sequencing. In addition, two separate western blot assays were performed to detect the HslU and HslV proteins in the ΔhslUV and CΔhslUV mutants as described above. In the experiments, wild-type *L. interrogans* strain Lai was used as the control.

**Detection of leptospiral denatured proteins during infection of cells**

The J774A.1 cell monolayers were infected with wild-type *L. interrogans* strain Lai, the ΔhslUV or CΔhslUV mutant at a MOI of 100 for 1, 2, 4, 8, 12 or 24 h, and then the intracellular leptospires were collected as described above. The insoluble denatured protein aggregates in the leptospires were detected using a ProteoStat Aggresome Detection Kit (ENZO Life Sciences). Briefly, the leptospires were fixed on Superfrost Plus glass slides (Thermo Fisher Scientific) with 4% paraformaldehyde at room temperature for 1 h. After thoroughly washing with PBS, the slides were treated with permeabilizing solution for 30 min on ice, and then stained with ProteoStat protein aggresome
dye and Hoechst 33342 cell nuclear dye at room temperature for 30 min in dark. After thoroughly washing with PBS again, the slides were examined under a laser confocal microscope (Olympus, Tokyo, Japan; 500 nm excitation and 600 nm emission wavelength for detection of the protein aggresome dye, and 350 nm excitation and 461 nm emission wavelength for detection of the nuclear dye), and the red fluorescence intensity reflecting the dead leptospires was quantified using MetaMorph software (Molecular Devices). In the detection, the wild-type strain and the two mutants without infection were used as the controls.

**Extraction of leptospiral denatured proteins during infection of cells**

According to the result of confocal microscopic examination, the J774A.1 cell monolayers were infected with wild-type *L. interrogans* strain Lai or the Δ*hslUV* mutant at a MOI of 100 for 8 or 12 h, and then the intracellular leptospires were collected as described above to extract leptospiral denatured proteins. Brieﬂy, the leptospires were suspended in 10 mM Tris-HCl buffer (pH 7.0) containing 1 mM EDTA and 1× protease inhibitor cocktail (Thermo Fisher Scientiﬁc) and then ultrasonically disrupted on ice as described above. The lysates were centrifuged at 8000 g for 30 min (4 °C), and the pellets were re-suspended in the Tris-HCl buffer for ultrasonic disruption again, followed by a 1000 g for 15 min (4 °C) to remove leptospiral debris. All the supernatants were combined for a 30 min centrifugation at 20 000 g (4 °C) to precipitate insoluble denatured protein aggresomes. The protein aggresomal pellets were suspended in 10 mM potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, 2% NP40 (Sigma) and 1× protease inhibitor cocktail (Thermo Fisher Scientiﬁc) by vigorous vortex for 5 min, followed by a 30 min centrifugation at 20 000 g (4 °C). The pellets were re-suspended in the potassium phosphate buffer for vortex again to allow the complete removal of contaminated membrane proteins. After were centrifuged at 20 000 g for 30 min (4 °C), the NP40-insoluble protein aggresomal pellets were suspended in a solution (7 M urea, 2 M thiourea, 4% CHAPS and 100 mM dithiothreitol), followed by a brief ultrasonic treatment for dissolve. Besides, the protein aggresomes from wild-type *L. interrogans* strain Lai and the Δ*hslUV* mutant without infection were also extracted as described above. Concentration of each the protein aggresome extracts was determined using a Bradford’s Protein Assay Kit (Bio-Rad).

**Identification of leptospiral denatured proteins during infection of cells**

The denatured aggregated proteins from wild-type *L. interrogans* strain Lai, the Δ*hslUV* or ΔΔ*hslUV* mutant during infection of J774A.1 cells were identiﬁed by liquid chromatography–mass/mass spectrometer (LC-MS/MS) after iTRAQ labeling. Brieﬂy, using an iTRAQ Reagent-labeling Kit (AB Sciex, Foster City, CA, USA), 100 μL each of the aggregated protein samples (200 μg proteins) was treated with 4 μL reducing reagent for 1 h at 60 °C and 2 μL cysteine-blockaging reagent for 10 min at room temperature, and then recovered with an ultra-filter by a 20 min centrifugation at 12 000g. The alkylated proteins were dissolved in 100 μL dissolution buffer and then treated with 4 μg trypsin at 37 °C overnight, followed by a centrifugation as above. A unit of 100 μg digested peptides in 50 μL dissolution buffer 5 were labeled with iTRAQ tags at room temperature for 2 h. After desalination with ZipTip agent, the labeled peptides were dried by lyophilization. The dried peptides were dissolved in 100 μL 2% acetonitrile-98% H2O (pH 10.0) and then separated by a 4.6 × 250 mm reversed phase Durashell C18 column in a high-performance liquid chromatographer (ABI) using 5–95% acetonitrile (pH 10.0) for gradient elution with 0.7 mL/min flow rate. The separated peptide fractions were collected for drying again. The peptide fractions were dissolved in 20 μL 2% methanol-0.1% methanoic acid. After centrifugation as above, the supernatant was separated by a 12 × 75 μm EASY-Spray C18 column using 4–95% acetonitrile–0.1% methanoic acid for gradient elution with 0.35 μL/min flow rate and each of the peptides was identiﬁed using a LC-MS/MS (ABI-5600) and ProteinPilot software.

**Bioinformatic analysis of leptospiral denatured proteins**

Among the LC-MS/MS-identiﬁed denatured aggregated proteins of wild-type *L. interrogans* strain Lai and the Δ*hslUV* mutant during infection of J774A.1 cells, those proteins with the abundance ≥ 2, and the quantitative increase ≥ 1.5-fold plus the P-value < 0.05 compared to the wild-type strain and mutant without infection were selected for KEGG pathway analysis and Gene Ontology analysis, including molecular function, biological process and cellular component subgene ontologies as previously described.

**Detection of viability of different leptospires from infected cells**

The J774A.1 cell monolayers were infected with wild-type *L. interrogans* strain Lai, the Δ*hslUV* or ΔΔ*hslUV* mutant at a MOI of 100 for 1, 2, 4, 8, 12 or 24 h, and the intracellular leptospires were collected as described above. Subsequently, the living or death of the leptospires was determined using a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA). Brieﬂy, the leptospires (1 × 106) were stained with SYTO 9, a green-fluorescent nucleic dye, and propidium iodide (PI), a red-fluorescent nucleic dye, for 15 min at room temperature, and then detected using a laser confocal microscope (Olympus) and a ﬂuorospectrophotometer (Molecular Devices; 485 nm excitation and 530 nm emission wavelength for SYTO 9, and 485 nm excitation and 530 nm emission wavelength for PI). The confocal microscopic data contained the images of SYTO 9-stained living (green) or PI-stained dead (red) leptospires, and the fold changes of red ﬂuorescence intensity for semi-quantification of the dead leptospires. The ﬂuorospectrophotometric data focused on the dead leptospiral percentages in the total leptospires. On the other hand, the leptospires (1 × 106) were serially diluted with EMJH liquid medium and then inoculated onto EMJH agar plates for a 3-week incubation at 28 °C to count leptospiral colony-forming units (CFU). In addition, the leptospires (1 × 106) were inoculated into EMJH liquid medium for a 7-day incubation at 28 °C and the counted using under a dark-ﬁeld microscope with a Petroff–Hauser counting chamber to investigate the difference of their growth and proliferation. In the CFU and leptospiral enumerations, the same number of wild-type *L. interrogans* strain Lai, the Δ*hslUV* and ΔΔ*hslUV* mutants from the cultures in EMJH liquid medium were used as the controls.

**Determination of virulence of different leptospires in hamsters**

Syrian hamsters (eight animals per group) were intra-peritoneally injected with 1 × 104, 105, 106, 107 or 108 of wild-type *L. interrogans* strain Lai, the Δ*hslUV* or ΔΔ*hslUV* mutant. Eight negative control animals were intra-peritoneally injected with the same volume of EMJH liquid medium. The animals were monitored twice daily and the death or survival of animals was recorded within 14 days after challenge for calculating 50% lethal dose by Warren’s probit analysis. Lung, liver and kidney samples from the animals on the days 3 and 7 after challenge were collected for histopathologic examination after hematoxylin and eosin staining.
Measurement of leptospiral loading in hamsters
Syrian hamsters (12 animals per group) were challenged with wild-type *L. interrogans* strain Lai, the Δ*hslU* or Δ*hslV* mutant (1×10⁶) as described above. Lung, liver and kidney samples from the animals on the days 3 and 7 after challenge were collected for observation of leptospiral loading in the tissues under a light microscope after silver staining.¹⁰ According to the general course of leptospirosis, the lung, liver and kidney samples (5 mg) on the days 3 and 7, the peripheral blood samples (0.5 mL) on the days 1 and 3, and the urine samples on the days 7 and 14 from the animals after challenge were collected for leptospiral quantification.¹¹ Briefly, the tissue specimens was homogenized in ice-bath and then suspended in 2 mL EMJH liquid medium. The suspensions as well as the peripheral blood samples were centrifuged at 250 g for 15 min (4 °C). A volume of 0.2 mL of each of the supernatants were inoculated into 2 mL EMJH liquid medium for a 24 h incubation at 28 °C in a rotator and then spread onto EMJH agar plates for a 3-week incubation at 28 °C for CFU counting. The urine samples were centrifuged at 250 g for 5 min to remove particulate matters, followed by a 30 min centrifugation at 10 000 g. The precipitated leptospires were suspended in PBS with 1/5 of 0.2 mL of each supernatant into 2 mL EMJH liquid medium for a 24 h incubation at 28 °C in a rotator and then spread onto EMJH agar plates for a 3-week incubation at 28 °C for CFU counting. The urine samples that collected at the 7 or 14 days during infection for silver staining and counting as described above.

Statistical analysis
Data from a minimum of three experiments were averaged and presented as mean ± s.d One-way analysis of variance followed by Dunnett’s multiple comparisons test were used to determine significant differences. Statistical significance was defined as *P* < 0.05.

RESULTS

### Extended distribution and expression of *hslU* and *hslV* genes in leptospiral strains

The PCR and western blot assay demonstrated that all of the 10 tested pathogenic leptospiral strains, belonging to 10 serovars in 10 serogroups of *L. interrogans*, possessed both the *hslU* and *hslV* genes and expressed both the *HslU* and *HslV* proteins (Figures 1A–1D). The nucleotide and amino-acid sequence of the *hslU* or *hslV* gene from the strains (GenBank accession NO: KR109192-109201 and KR109202-109211) were 99.4–100% and 99.8–100%, or 99.6–100% and 100% identical, respectively, compared with the same genes in GenBank (accession NO: NC_004342).²⁷ The two tested saprophytic leptospiral strains, belonging to two serovars in two serogroups of *L. biflexa*, also possessed the *hslU* and *hslV* genes (Supplementary Figures S2A and S2B), but their nucleotide or amino-acid sequence identities were low as 64.0–85.0% (GenBank accession NO: X622792-622795), compared to the 10 *L. interrogans* strains. The data suggest that the *hslU* and *hslV* genes are distributed extensively in different *Leptospira* strains and required by the spirochetes to respond to adverse environment.

### AAA+ chaperone and N-terminal nucleophile peptidase domains in leptospiral *HslU* and *HslV*

The bioinformatic analysis revealed that the *hslU* gene (NO LA2345) from *L. interrogans* strain Lai contains an AAA+ chaperone superfamily domain, including enzymatic active sites and polypeptide substrate-binding sites (Figure 1E).⁶⁶ Moreover, the *HslU* and *HslV* sequences from the spirochetes are similar to those from *E. coli* and *H. influenzae* (Figures 1F and 1G).²²,⁴⁷ In particular, the N-terminal sequencing results showed that 76% of the rHslV and 93% of the nHslV from *L. interrogans* strain Lai were cleaved to expose the catalytic threonine residue at N terminus. Although the sequence identities of *hslU* and *hslV* genes from the two *L. biflexa* strains were lower compared to that from the *L. interrogans* strains, the two genes contain AAA+ chaperone and Ntn peptidase superfamily domains, respectively (Supplementary Figure S2C–S2F). The data suggest that the products of leptospiral *hslU* and *hslV* genes compose a AAA+ chaperone–Ntn peptidase complex.

### Enzymatic activity of recombinant and native *HslU* and *HslV* complex

The SDS-polyacrylamide gel electrophoresis demonstrated that either the extracted r/nHslU or the r/nHslV from *L. interrogans* strain Lai showed a single band in gel (Supplementary Figure S3). The fluorospectrophotometric examination showed that the proteolytic activity of single rHslV was very low. However, the rHslU and rHslV complexes at M/M ratios of 1:81 (rHslU₄:rHslV₁) acquired the ability to hydrolyze the FITC-casein substrate and all the tested chymotrypsin-like peptide substrates (Z-GGL-AMC, Suc-LLVY-AMC and Suc-AAP-AMC), but the rHslU₈:rHslV₁ complexes provided the highest hydrolytic activity (Figure 2A). In particular, both the nHslU₈:nHslV₁ and rHslU₈:rHslV₁ complexes had a similar ability to hydrolyze all the four substrates, and their hydrolytic activity was persistently increased in a concentration-dependent manner (Figure 2B). Among the tested substrates, Suc-LLVY-AMC served as the preferred substrate (Figures 2A and 2B). However, both the rHslU₈:rHslV₁ and nHslU₈:nHslV₁ complexes did not hydrolyze the trypsin-like and caspase-like peptide substrates (Z-LLE-AMC and Boc-DLLE-AMC; data not shown). On the other hand, MG132, an inhibitor of threonine, serine and cysteine proteases/peptidases, and 4-hydroxy-5-iodo-3-nitrophenylacetyl-leu-leu-leu-vinylsulfone and lactacystin, the inhibitors of threonine proteases/peptidases, but not leupeptin, an inhibitor of serine and cysteine proteases/peptidases, and E64, an inhibitor of cysteine proteases/peptidases, blocked markedly the hydrolytic activity of rHslU₂:rHslV₁ complex (Figure 2C). When using ATP-free reaction buffer or replacing ATP in the buffer with βγ-methylene-ATP (AMP-PCP), a nonhydrolyzable ATP analog, the hydrolytic activity of rHslU₂:rHslV₁ complex was absent (Figure 2C).

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HslU and HslV proteins during infection of cells (Figures 3C–3F). The data suggest that the HslUV chaperone–peptidase complex is required by L. interrogans during infection of host cells.

Increase of leptospiral denatured proteins during infection of cells

The results about the identification of the ΔhslUV and CAhslUV mutants were shown in Supplementary Figure S4. The confocal microscopic examination revealed that denatured protein aggresomes in wild-type L. interrogans strain Lai of from the infected J774A.1 cells were markedly increased compared with the spirochete before infection, but the ΔhslUV mutant accumulated a significantly higher level of denatured protein aggresomes than the wild-type strain during infection (Figures 4A and 4B). The iTRAQ plus LC-MS/MS detection also revealed that the aggregated proteins in the wild-type strain after infection were higher than before infection (Figure 4C and Supplementary Table S1). In
particular, the ΔhslUV mutant accumulated a higher level of aggregated proteins than the wild-type strain during infection of cells or incubation in EMJH medium (Figure 4C and Supplementary Tables S1–S4). The data suggest that the infection of cells caused a significant increase of denatured proteins in L. interrogans and the HslUV chaperone–peptidase complex has an important role in removal of these abnormal proteins.

Functional classes of leptospiral denatured aggregated proteins during infection of cells

In wild-type L. interrogans strain Lai during infection of J774A.1 cells, the major denatured proteins in aggresomes have a role in the structure, assembly and function of ribosomes and flagella, which should therefore decrease the protein synthesis and motility of the spirochete (Figure 5A). Except for the ribosome- and flagellum-associated denatured proteins as above, the ΔhslUV mutant presented more significantly increased denatured aggregated proteins than the wild-type strain during infection of J774A.1 cells and the major increased denatured proteins in aggresomes are involved in the two-component signaling system and transmembrane transport (Figure 5B).

Decreased viability of the ΔhslUV mutant during infection of cells

The confocal microscopic examination showed that the number of dead wild-type, ΔhslUV or CΔhslUV mutant leptospires from the infected J774A.1 cells gradually increased during a 24 h infection, but the number of dead ΔhslUV mutant leptospires at 4, 8, 12 or 24 h of infection was significantly higher than for the wild-type or CΔhslUV mutant leptospires (Figures 6A and 6B). The fluorospectroscopic examination also confirmed that the percentages of dead ΔhslUV mutant at longer times of infection were significantly higher than for either the wild-type strain or CΔhslUV mutant (Figure 6C). The CFU enumeration showed that there were significantly fewer ΔhslUV mutant colonies recovered from the infected J774A.1 cells than the wild-type strain or CΔhslUV mutant (Figure 6D).
tendencies were found when the wild-type strain and the two mutants from the infected J774A.1 cells were compared for their ability to grow in EMJH liquid medium (Figure 6E). The data suggest that deletion of the hslU and hslV genes decreases the viability and survival of L. interrogans during infection of host cells.

**Figure 3**  Increase of leptospiral hslU and hslV gene expression during infection of cells. (A) Increase of hslU-mRNA in L. interrogans strain Lai during infection of J774A.1 cells for the indicated times, determined by quantitative reverse transcription-PCR (qRT-PCR). Bars show the mean ± SD of three independent experiments. The hslU-mRNA level in the spirochete from Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (before infection) was set as 1.0. *P < 0.05 vs the hslU-mRNA levels in the spirochete before infection or in incubation with RPMI-1640 medium at 28 or 37 °C. (B) Increase of hslV-mRNA in L. interrogans strain Lai during infection of J774A.1 cells for the indicated times, determined by qRT-PCR. Bars show the mean ± SD of three independent experiments. The hslV-mRNA level in the spirochete from EMJH medium (before infection) was set as 1.0. *P < 0.05 vs the hslV-mRNA levels in the spirochete before infection or in incubation with RPMI-1640 medium at 28 °C. (C) Increase of HslU protein expression in L. interrogans strain Lai during infection of J774A.1 cells for the indicated times, determined by western blot assay. The LipL41 protein, an outer membrane lipoprotein of L. interrogans, was used as the control. (D) Quantification of immunoblotting bands reflecting the HslU expression level during infection for the indicated times, assessed by gray scale determination. Statistical data from experiments such as shown in C. Bars show the mean ± SD of three independent experiments. The HslU expression level (gray scale value) from EMJH medium (before infection) was set as 1.0. *P < 0.05 vs the HslU expression levels in the spirochete before infection or in incubation with RPMI-1640 medium at 28 °C. (E) Increase of HslV protein in L. interrogans strain Lai during infection of J774A.1 cells for the indicated times, determined by western blot assay. The legend is the same as in C but for HslV detection. (F) Quantification of immunoblotting bands reflecting the HslV expression level during infection for the indicated times, assessed by gray scale determination. The legend is the same as in D but for HslV detection. *P < 0.05 vs the HslV expression levels in the spirochete before infection or in incubation with RPMI-1640 medium at 28 °C.

**Attenuated virulence of the ΔhslUV mutant in hamsters**

The ΔhslUV mutant showed lower virulence in infected hamsters. The 50% lethal dose values within 14 days after challenge was $1.24 \times 10^7$ leptospires for the ΔhslUV mutant, $0.92 \times 10^6$ leptospires for wild-type L. interrogans strain Lai and $0.96 \times 10^6$ leptospires for the CΔhslUV
mutant (P<0.05). The ΔhslUV mutant also provoked markedly weaker pathological injury in lungs, liver and kidneys of the infected hamsters than the wild-type strain or CΔhslUV mutant (Figure 7A). The data imply that the HslUV chaperone–peptidase complex is involved in the virulence of L. interrogans during in vivo infection.

Reduced leptospiral loading in tissues and ΔhslUV mutant excretion in urine

The number of leptospires in the lungs, liver and kidneys of hamsters infected with the ΔhslUV mutant was significantly lower than for wild-type L. interrogans strain Lai or the CΔhslUV mutant (Figure 7B). Similarly, significantly fewer leptospiral colonies were isolated from the lung, liver, kidney or peripheral blood samples when the hamsters were infected with the ΔhslUV mutant than the wild-type strain or CΔhslUV mutant (Figures 7B–7D). In particular, compared to the hamsters infected with the wild-type strain or CΔhslUV mutant, the animals infected with the ΔhslUV mutant presented significantly lower number of leptospires in urine (Figures 7E and 7F). The data suggest that the HslUV chaperone–peptidase complex has a role in the dissemination in vivo and transmission via urine of L. interrogans.

DISCUSSION

Prokaryotic microbes can be classified as parasites or saprophytes. The former needs to invade into hosts because the hosts provide all the conditions necessary for their growth and proliferation. However, this invasion (or infection) of host organisms inevitably leads to diseases of the hosts. The hosts, including human beings, have developed effective anti-infection strategies, such as acquired or innate immunity, but also enhancement of environmental conditions that are adverse for the pathogens.12 Conversely, the pathogens have developed strategies to counter the damage caused by the hosts.
Proteins have a crucial role in growth and proliferation of all organisms, but they can also be denatured or damaged in physiological and pathological processes, such as infection. The denatured proteins not only lose their biological function but also interfere with normal cellular metabolism and can ultimately cause cell death due to the accumulation of aggregated denatured proteins.

**Figure 5** Functional classes of leptospiral denatured proteins during infection of cells. (A) Function of the denatured proteins in aggresomes from wild-type L. interrogans strain Lai during infection of J774A.1 cells, determined by bioinformatic KEGG and Gene Ontology (GO) analysis. The abundance, increased folds and P-value for selecting the denatured proteins for analysis were 2.0, ≥1.5 and <0.05, respectively, compared to the strain before infection. (B) Function of the increased denatured proteins in aggresomes of the ΔhslUV mutant compared to wild-type L. interrogans strain Lai during infection of J774A.1 cells, determined by bioinformatic KEGG and GO analysis. The increased denatured proteins were observed only in the mutant with an over 1.5-fold increase than that before infection or found in both the mutant and wild-type strain but with a ≥1.5-fold increase in the mutant compared to the wild-type strain.
However, except for the PrcBA proteasome of *M. tuberculosis*, which is required for persistent infection in mice, the role of aggregated denatured proteins and proteolytic chaperone–peptidase complexes or proteasomes in pathogens during infection has not attracted much attention. Macrophage is the most powerful phagocyte of hosts in killing invaded pathogens including *L. interrogans* by phagocytosis. The pathogen-killing mechanisms of macrophages are involved in the cellular reactive oxygen species- and reactive nitrogen species-induced denaturation of bacterial proteins. Our recent study also confirmed that *L. interrogans* in macrophages could be killed by high level of reactive oxygen species. Rodent animals, the most important host of *L. interrogans*, have a critical role in transmission of leptospirosis by persistent discharge of leptospires in urine to contaminate environment. Therefore, we focused on the roles of protein denaturation and denatured protein elimination in survival of *L. interrogans* in murine macrophages after phagocytosis.

Our study revealed that all the tested *L. interrogans* strains possess both the *hslU* and *hslV* genes containing AAA+ chaperone superfamily domain and Ntn hydrolase superfamily domain as well as the

**Figure 6** For caption see next page.
expression of HslU and HslV proteins was significantly upregulated during infection of cells. Previous studies confirmed that the exposure of threonine residue at the N terminus of bacterial HslV was necessary for hydrolytic ability of Ntn peptidase/protease and E. coli could process the exogenous rHslV by cleavage to expose the catalytic threonine residue.22,47 Our sequencing data revealed that most of the process the exogenous rHslV by cleavage to expose the catalytic threonine residue.  

Peptidases in chaperone–peptidase complex or the proteasomes have been confirmed as endopeptidases or endoprotease that can be classified as chymotrypsin-, trypsin- or caspase-like types depending on their preference for hydrolytic substrates, which can be further divided into serine, threonine or cysteine peptidases/proteases based on their enzymatic active sites and hydrolytic mechanism.21,16 HslU, ClpAP, ClpCP and ClpXP have been identified as the AAA+ chaperone–peptidase complexes of E. coli, Helicobacter pylori and Staphylococcus aureus,22,23,52,53 but the HslV peptidase used the N-terminal Thr as the active site nucleophile for cleavage of denatured bacterial proteins while the ClpP peptidase degraded denatured bacterial proteins using a His–Asp–Ser catalytic triad.21,54 Moreover, precious studies proved the HslV in the HslU complex of E. coli or B. subtilis as a threonine or serine peptidase.22,24 In this study, both the recombinant and native HslU and HslV (r/nHslU and r/nHslV) complexes hydrolyzed casein, a universal substrate of proteases/proteases, but only hydrolyzed chymotrypsin-type peptide substrates and this hydrolytic ability was blocked by the threonine peptide/protease inhibitors (4-hydroxy-5-iodo-3-nitrophenylacetyl-leu-leu-leu-vinylsulfone and lacticin,).22,23 Besides, the ATP absence or the nonhydrolyzable ATP analog could inhibit the enzymatic ability of leptospiral rHslV complex. The data indicate that the HslU and HslV proteins of L. interrogans compose a chymotrypsin-like threonine-type ATP-dependent AAA+ chaperone–Ntn peptidase complex.  

Previous studies reported that bacterial proteins could be denatured during the adverse environmental conditions of infection, causing them to form insoluble aggresomes.13–15 Our study found that L. interrogans strain Lai produced much more denatured aggregated proteins during infection of macrophages than before infection, and the major aggregated proteins were involved in ribosomal protein synthesis and flagellar motility. Unlike the non-pathogenic saprophytic Leptospira species, pathogenic Leptospira species have a powerful invasive ability that allows them to invade into hosts and then diffuse form blood into internal organs,10,37,55 which are dependent on part on their flagellum-based motility.36,56 In particular, the deletion of L. interrogans hslU and hslV genes caused a significant increase of the denatured aggregated proteins during infection of macrophages, and the major aggregated proteins were involved in leptosomal sensing and protein export systems compared to the wild-type strain. Recent studies revealed that two-component signaling systems of bacteria could sense the biochemical signals in environment of hosts and then upregulate the synthesis and excretion levels of target proteins against the adverse conditions for survival.58 The data indicate that the infection of host cells acts as a stimulus to cause the increase of denatured proteins in L. interrogans and the leptosomal HslUV chaperone–peptidase complex may have a special role in maintaining the ability of the spirochete to sense and respond to the infectious environment.  

As our expected, the number of living leptospires of ΔhslUV mutant from the infected macrophages was significantly lower than that of the wild-type strain nearly in the whole process of infection. More importantly, the lower 50% lethal dose value and attenuated histopathological injury found in the hamsters infected with the ΔhslUV mutant compared to the wild-type strain may be also due to the decreased ability of the mutant to survive in the animals. In particular, compared to the wild-type strain, fewer leptospires of the ΔhslUV mutant were found in the urine of infected hamsters. As the transmission of leptospirosis from host animals to humans usually occurs indirectly through contact with Leptospira-infected animal urine contaminated soil or water,1,9 the HslUV chaperone–peptidase complex are involved in transmission of pathogenic Leptospira species from animals to humans. Taken together, our findings imply that the HslU and HslV proteins of L. interrogans form an ATP-dependent chymotrypsin-like threonine peptidase-type AAA+ chaperone–Ntn peptidase complex and this complex contributes to the survival and virulence of the spirochete during infection of hosts as well as the transmission of human leptospirosis. In addition, a recent review reported that bacterial proteolytic complexes can be expected as the effective therapeutic targets because of a large diversity between bacterial and mammalian proteolytic systems.59 Therefore, the
HslUV complex of pathogenic *Leptospira* species also has a potential as the target to develop novel drugs for treatment of human leprospirosis.

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Figure 7  Reduced histopathologic injury and invasion of ΔhslUV mutant in hamsters. (A) Attenuated histopathologic injury in the ΔhslUV mutant-infected hamsters, examined by microscopy after hematoxylin and eosin staining. The wild-type strain- or ΔhslUV mutant-infected hamsters showed the visible congestion (3 days), hemorrhage (7 days) and inflammatory cell infiltration (3 and 7 days) in lungs; inflammatory cell infiltration (3 and 7 days) and focal hepatocyte necrosis (7 days) in liver; and congestion and inflammatory cell infiltration (3 days), and serious congestion and focal necrotic tubular epithelial cell necrosis (3 and 7 days) in kidney. In contrast, the histopathologic injury in the ΔhslUV mutant-infected hamsters was markedly attenuated. (B) Decreased invasion in the lungs, liver and kidneys of ΔhslUV mutant-infected hamsters, assessed by microscopy after silver staining. The arrows indicate the leptospires in tissues. (C) Fewer colonies from the lung, liver and kidney samples of ΔhslUV mutant-infected hamsters, determined by CFU enumeration. *P<0.05 vs the CFU number of wild-type *L. interrogans* strain Lai and the CΔhslUV mutant. (D) Fewer colonies from the peripheral blood samples of ΔhslUV mutant-infected hamsters, determined by CFU enumeration. *P<0.05 vs the CFU number of wild-type *L. interrogans* strain Lai and the CΔhslUV mutant. (E) Decreased leptospiral loading in urine from ΔhslUV mutant-infected hamsters, examined by microscopy after silver staining. The urine samples were condensed by 50-fold (at 7 days during infection) and 5-fold (at 14 days during infection) in volume for leptospiral counting. (F) Decreased leptospiral numbers in the urine from ΔhslUV mutant-infected hamsters. Statistical data from experiments such as shown in E. Bars show the means±sd of three separate samples of at least five animals. *P<0.05 vs the leptospiral number in the urine samples from wild-type *L. interrogans* strain Lai- or the CΔhslUV mutant-infected animals.
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