Original Article

Phenotypic Function of *Legionella pneumophila* Type I-F CRISPR-Cas

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Abstract

Objective   CRISPR-Cas protects bacteria from exogenous DNA invasion and is associated with bacterial biofilm formation and pathogenicity.

Methods   We analyzed the type I-F CRISPR-Cas system of *Legionella pneumophila* WX48, including *Cas1, Cas2-Cas3, Csy1, Csy2, Csy3*, and *Cas6f*, along with downstream CRISPR arrays. We explored the effects of the CRISPR-Cas system on the in vitro growth, biofilm-forming ability, and pathogenicity of *L. pneumophila* through constructing gene deletion mutants.

Results   The type I-F CRISPR-Cas system did not affect the *in vitro* growth of wild-type or mutant strains. The biofilm formation and intracellular proliferation of the mutant strains were weaker than those of the wild type owing to the regulation of type IV pili and Dot/Icm type IV secretion systems. In particular, *Cas6f* deletion strongly inhibited these processes.

Conclusion   The type I-F CRISPR-Cas system may reduce biofilm formation and intracellular proliferation in *L. pneumophila*.

Key words: *Legionella pneumophila*; CRISPR-Cas; Biofilm; Intracellular proliferation

INTRODUCTION

*L*egionella is a Gram-negative bacterium. *Legionella* is widely found in freshwater environments, moist soil and compost, showers, hot tubs, plumbing networks, air-conditioning systems, and other natural and artificial water environments close to areas inhabited by humans. *Legionella* spreads from these environments to humans *via* aerosols and infects lung macrophages, causing Legionnaires' disease[1,2]. More than 70 *Legionella* species have been identified, 25 of which can cause disease. *Legionella pneumophila* is one of the most pathogenic *Legionella* species and has 16 serotypes; serotype 1 accounts for 70%–90% of human *Legionella* infections[3,4]. The antibiotics used to treat Legionnaires' disease mainly include macrolides and fluoroquinolones[5,6].

CRISPR-Cas is an adaptive immune system that protects bacteria and Archaea from invasive mobile genetic agents, particularly phages. The CRISPR-Cas systems are broadly categorized into two classes, six types, and 44 subtypes according to the *Cas* protein content and locus arrangement[7-10]. Class 1 systems are divided into types I, II, and IV, all of which

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employ multiprotein effector complexes to interfere with the invasion mechanisms of mobile genetic elements. Class 2 systems are classified into types II, V, and VI, all of which use single effector proteins for interfering with invasive mobile genetic agents[11]. The CRISPR-Cas system enables adaptive immunity through a three-stage process: adaptation, expression, maturation, and targeted interference[12-14]. CRISPR-Cas systems widely occur in nature, being found in approximately 50% and 90% of bacterial and archaeal genomes, respectively[15,16], and their occurrence does not correlate with ecological niche or phylogenetic branch. The type I CRISPR-Cas system is the most common, which uses a CRISPR RNA (crRNA)-bound multiprotein complex called the CRISPR-associated complex in antiviral defense to identify the target. This multiprotein complex uses nuclease *Cas3* to cleave the target[12,17]. However, only a few type I CRISPR-Cas system systems have been characterized, such as type I-E in *Escherichia coli (E. coli)*[18,19]. Many *Vibrio* species have miniature I-F systems comprising tniQcas5cas7cas6f, which is associated with TN7-like transposons. Miniature I-F CRISPR-Cas systems identify Tn7-like transposons and target them at their insertion sites[20–22]. The type I-Fv CRISPR-Cas system from *S. putrefaciens* provides *E. coli* with heterogeneous protection against lambda phage infection[21].

The CRISPR-Cas system has been thoroughly investigated in several bacterial species, including numerous human pathogens, such as *Staphylococcus epidermidis, Streptococcus pneumoniae, Yersinia pestis, Klebsiella pneumoniae,* and *Salmonella* spp[23–29]. The CRISPR-Cas system is the core adaptive immune mechanism through which microorganisms resist phage invasion. The type I-C, I-F, and II-B CRISPR-Cas systems protect against exogenous genetic elements through sequence-specific cleavage (DNA or RNA targets)[30]. The understanding of the functions of the CRISPR-Cas have expanded from classical genome defense to nonclassical regulatory fields, such as gene expression regulation and epigenetic modifications. The II-B-type CRISPR-Cas system is dominant in *Legionella pneumophila*[31], and the nuclease activity of *Cas2* is crucial for *L. pneumophila* growth within amoeba hosts[32,33]. The heterogeneous distribution, dynamic evolution, and the type I-F CRISPR-Cas system enable *L. pneumophila* to adapt to the environment[34]. The CRISPR-Cas spacer sequence library suggests that the CRISPR-Cas system enables continuous defense against Microviridae phages[35]. However, the

biological functions of the type I-F CRISPR-Cas system in *L. pneumophila* have not been explored.

We assessed the functional phenotypes of the type I-F CRISPR-Cas system in *L. pneumophila* to explore the relationship between *L. pneumophila* and CRISPR-Cas. Our results lay the foundation for discovering the biological functions of the CRISPR-Cas system in *L. pneumophila*.

MATERIALS AND METHODS

Bacterial Strains and Culture

The type I-F environmental strain, *L. pneumophila* WX48, was used as the wild-type (WT) strain. All *Legionella* strains were cultured on buffered charcoal yeast extract (BCYE) agar plates (Oxoid) at 37 °C under 5% CO_2 or in N-(2-acetamido)-2-aminoethanesulfonic acid-buffered yeast extract broth at 37 °C. *E. coli* DH5α was used for cloning *via* culturing in Luria-Bertani medium containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol at 37 °C.

Mutant and Complementation Plasmid Construction

Legionella strains (WX48) from aquatic environments with complete I-F type CRISPR-Cas system were screened and identified using whole-genome sequencing data. CRISPR-Cas system loci were annotated using CRISPRCasFinder, a web-based tool for computationally predicting CRISPR arrays and associated *Cas* proteins, with the default parameters.

The *L. pneumophila* deletion mutants were generate by separately amplifying the upstream and downstream fragments of *Cas1, Cas2-Cas3, Csy1, Csy2, Csy3, Cas6f*, and CRISPR arrays with homologous arms from *L. pneumophila* WX48 genomic DNA using polymerase chain reaction (PCR) with the appropriate primers. The primers are listed in Supplementary Table S1. Overlapping PCR was used to fuse the upstream and downstream fragments; the product was ligated to the pGEM-T Easy vector *via* TA cloning to generate pT-Cas/CRISPR constructs. The constructs were enzymatically digested using SmaI.

A kanamycin resistance (Km) cassette generated from pUT-mini-Tn5 Km was inserted into the upstream and downstream fusion fragments to generate pT-Cas/CRISPR-Km *via* enzymatic digestion and ligation. These pT-Cas/CRISPR-Km constructs were digested with NotI. Fragments carrying the

disrupted Cas/CRISPR region were cloned into the NotI site of the allelic exchange vector PLAW344, generating PLAW344-Cas/CRISPR-Km. PCR amplification and sequencing were conducted using specific primers for each gene to confirm the recombinant plasmid was successfully constructed.

Recombinant plasmids carrying *Cas* and CRISPR arrays were introduced into competent cells of the original strain through electroporation. Transformants were selected on BCYE agar supplemented with 50 µg/mL kanamycin. Subsequently, the transformants were cultured on BCYE agar containing kanamycin and 5% sucrose at 37 °C under 5% CO_2 for approximately 5 days to screen for Δ*Cas1*, Δ*Cas2-3*, Δ*Csy1*, Δ*Csy2*, Δ*Csy3*, Δ*Cas6f*, and ΔCRISPR mutant strains. PCR amplification and sequencing were performed using specific primers for each gene, and successful construction of the mutants was confirmed.

We constructed a plasmid for the complementation of the *Cas* and CRISPR mutant strains by amplifying the target *Cas* genes and CRISPR array from the genomic DNA extracted from the WT strain. The product was digested with the corresponding enzymes and cloned into a PMMB207 plasmid. Finally, the plasmids were transferred into the corresponding competent cells *via* electroporation, and the transformants were cultured on BCYE agar containing chloramphenicol and sucrose to screen for Δ*Cas1*, Δ*Cas2-3*, Δ*Csy1*, Δ*Csy2*, Δ*Csy3*, Δ*Cas6f*, and ΔCRISPR deletion strains. Positive colonies were confirmed by PCR and sequencing. The results of whole-genome sequencing (Illumina NovaSeq 6000 platform, 150-bp paired-end reads) with comparative genomic analysis revealed no detectable single-nucleotide polymorphisms (SNPs) or indels between the mutant and WT genomes, confirming the absence of secondary mutations.

Extracellular Growth Assay

We monitored extracellular growth by inoculating *L. pneumophila* into BYE broth, and incubating the culture at 37 °C overnight with shaking at 200 rpm. The culture was then quantitatively transferred to 50 mL of fresh broth, and the optical density at 600 nm (OD600) was measured using a spectrophotometer (NanoPhotometer NP80 Touch, Germany). The initial OD600 was 0.2. The cultures were incubated again at 37 °C under shaking at 200 rpm, and the OD600 was measured every 6 h until the growth stabilized. Each experiment was performed in triplicate.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was evaluated following the EUCAST recommendations and manufacturer's instructionsthe. The minimum inhibitory concentration (MIC) of the antibiotic (Liofilchem, Teramo, Italy) against the complement, deletion, and WT strains was evaluated using the E test strip. Briefly, we prepared a bacterial suspension and adjusted its concentration to 0.5 McFarland standard. A sterile cotton swab was dipped into the bacterial solution, which was then evenly applied to the BCYE agar surface, and an E test strip was placed at the center. The agar was incubated at 37 °C for 48 h, after which the MIC of the E test strip was determined. *L. pneumophila* ATCC 33152 was used as the control in all tests. Each experiment was conducted in triplicate.

Biofilm Formation Assay

Fresh *Legionella* colonies on BCYE agar were suspended in BYE broth, and the OD600 of the suspension was adjusted to 0.3. Next, 200 µL of this suspension was added to each well of a 96-well microplate. The plate was then incubated at 37 °C under 5% CO_2 for 3 days. We then added 40 µL of 0.25% crystal violet or 1.25% safranin to each well. The plate was allowed to stand at 25 °C for 15 min, after which the plate was washed three time with 300 µL of ddH$_2$O and dried in air. *L. pneumophila* biofilms were visible as purple rings after crystal violet staining or red rings after safranin staining around the walls of the inoculated wells. We then added of 300 µL of 30% acetic acid to each well, and the plate was incubated for 15 min until the crystal violet or safranin dissolved. Finally, 200 µL of the sample was transferred to a new microtiter plate; the samples were then treated with crystal violet and read at OD600, or the samples treated with safranin were read at OD530 using a Synergy H1 plate reader (Biotek). Each experiment was conducted in triplicate.

Adhesion, Invasion, and Intracellular Growth Assays

A pure culture of fresh *Legionella* was resuspended in phosphate-buffered saline (PBS), and the *Legionella* concentration was adjusted to a McFarland standard of 2.0 (1×10^8 CFU/mL). The bacterial suspension was diluted 10-fold in DMEM-H and used to infect the mouse macrophage line J774 (1×10^5 cells/mL) at a multiplicity of infection of 100. The infected cells were incubated at 37 °C for 1 h under 5% CO_2. The cells were then washed twice

with PBS to remove extracellular bacteria, and 1 mL of sterile water was added. The cell suspension was then transferred to a 1.5 mL centrifuge tube. A series of 10-fold dilutions of the cell suspension were plated on BCYE agar, and the bacterial colonies that developed were counted. We measured the invasive strength of the bacteria by removing the extracellular bacteria *via* washing the cells with PBS three times, and 1 mL of the medium with 100 μg/mL of gentamicin was added to each well. The wells were incubated for 1 h to kill extracellular bacteria. The cells were washed again with PBS, and the number of intracellular bacteria was determined as described above in this section.

This infection process was repeated for an intracellular growth assay. The infected cells were incubated at 37 °C for 1 h under 5% CO_2 and then washed three times with PBS to remove extracellular bacteria. Next, 1 mL of DMEM-H was added to each well, and the bacterial colonies proliferating within the J774 cells were counted every 24 h for three consecutive days. The total number of colony-forming units (CFU) formed after coating the BCYE agar with the cell suspensions was also calculated.

Cell Infection Status

Cell smears were fixed on glass slides with methanol, stained with carbonate fuchsin for 5 min, gently washed with water, stained with 8% malachite green for 1 min, rinsed again, and air-dried for 1 min. The slides were dried and then observed under a light microscope. We randomly selected 100 fields of view under a microscope, counted the total number of cells and the number of bacteria-infected cells in each field, and calculated the ratio of bacteria-infected cells to the total number of cells.

Quantitative Real-Time PCR

Total RNA was reverse-transcribed into complementary DNA (cDNA) using a SuperScript III First-Strand Synthesis System for qRT-PCR analysis. cDNA was amplified using quantitative real-time PCR (qRT-PCR) with SYBR Green Real-time PCR Master Mix (Takara, Japan). The gene expression levels were relatively quantified using the $2^{-\Delta\Delta Ct}$ method. *GyrB* was used as the internal control. Supplementary Table S1 presents the primers used for qRT-PCR.

Statistical Analysis

We assessed the statistical significance of our data using Student's *t*-test and one-way analysis of variance with Dunnett's multiple comparison tests. $P < 0.05$ was considered as indicating significance.

RESULTS

CRISPR-Cas Loci in L. pneumophila WX48

The type I-F CRISPR-Cas system of *L. pneumophila* WX48 was analyzed using CRISPRCasFinder[34]. Figure 1 shows that the type I-F system consists of six *Cas* genes, namely, *Cas1, Cas2-Cas3, Csy1, Csy2, Csy3*, and *Cas6f*, along with a downstream CRISPR array with a conserved repeat sequence (5'-TTTCTAAGCTGCCT GTACGGCAGTGAAC-3').

Bacterial Growth

L. pneumophila WT as well as its Δ*Cas1*, Δ*Cas3-Cas2*, Δ*Csy1*, Δ*Csy2*, Δ*Csy3*, Δ*Cas6f*, ΔCRISPR, and complementation mutant strains were grown in BYE broth. Their standard growth curves were plotted. The proliferation of the deletion mutants, complementation mutants, and WT strain was determined after their incubation at 37 °C for 48 h. The growth trends were consistent among all the strains, indicating that the CRISPR-Cas system did not affect the growth of *L. pneumophila* (Figure 2).

Antimicrobial Susceptibility
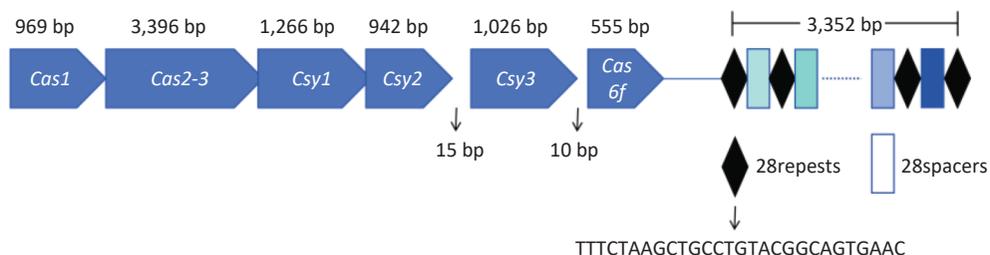
The antimicrobial susceptibilities of WT, deletion

Figure 1. Type I-F CRISPR-Cas locus of *L. pneumophila* WX48. Horizontal arrows indicate locations and orientations of *Cas1, Cas2-3, Csy1, Csy2, Csy3*, and *Cas6f.* Diamonds represent repeat sequences. Rectangles represent spacer sequences.

mutants, and complementation mutant *L. pneumophila* strains were evaluated. The deletion mutant strains were slightly less sensitive to erythromycin than the WT and complementation mutant strains, with their MICs decreasing from 0.75 in the WT 0.125 or 0.5. Although the MIC values fluctuated, the sensitivity remained within this range. The MIC of the deletion and complementation mutant strains did not substantially differ from those of the WT strain for the other antibiotics (Table 1).

Biofilm Formation Ability

We used crystal violet staining to evaluate the impact of *Cas* gene deletion on the biofilm-forming ability of *L. pneumophila* mutants. The biofilm formation ability of the Δ*Cas6f* mutant was weaker than that of the WT. Introducing the complete *Cas6f* gene restored the biofilm formation ability of the

mutant to that of the WT strain, indicating that Δ*Cas6f* impacts biofilm formation ability. The biofilm formation ability of the other deletion mutant strains remained similar to that of the WT strain (Figure 3A). We obtained similar results when we measured biofilm formation ability using safranin staining (Figure 3B).

Adhesion and Invasion Ability

We assessed the adhesion and invasion abilities of the WT, deletion mutants, and complementation mutants of *L. pneumophila* using J774 cells. The adhesion or invasion abilities of these strains did not substantially differ (Figure 4). Therefore, the CRISPR-Cas system may not affect the adhesion and invasion abilities of *L. pneumophila.*

Intracellular Growth
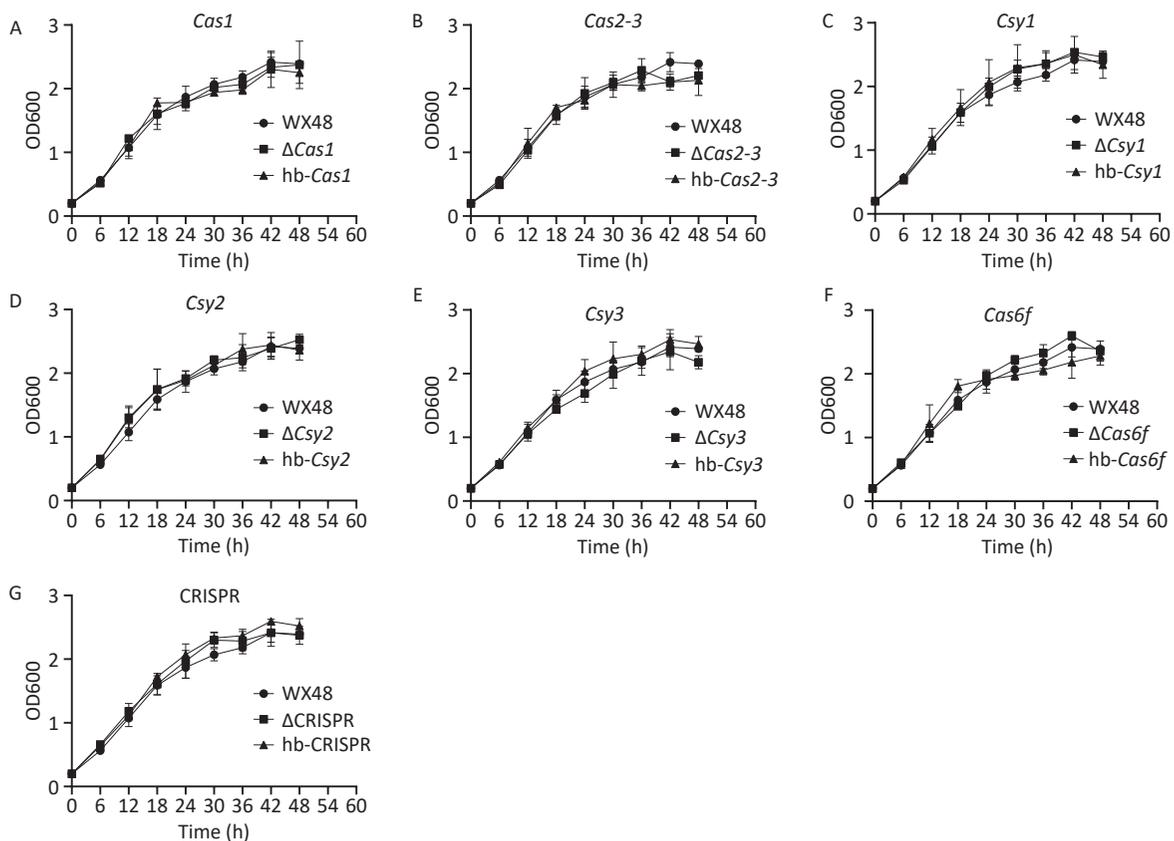
We evaluated the effect of the CRISPR-Cas

Figure 2. *In vitro* growth curves of various *L. pneumophila* strains. WX48: wild-type *L. pneumophila* strain; Δ*Cas1*, Δ*Cas2-3*, Δ*Csy1*, Δ*Csy2*, Δ*Csy3*, Δ*Cas6f,* and ΔCRISPR: mutant strains lacking *Cas1, Cas2-3, Csy1*, *Csy2*, *Csy3*, *Cas6f*, and the CRISPR gene, respectively. hb-*Cas,* hb-*Cas2-31*, hb-*Csy1,* hb-*Csy2,* hb-*Csy3,* hb-*Cas6f*, and hb-CRISPR: *Cas1-, Cas2-3-, Csy1-, Csy2-, Csy3-. Cas6f-*, and CRISPR-complemented strains, respectively. *In vitro* growth curves of (A) WX48, Δ*Cas1*, and hb-*Cas1*; (B) WX48, Δ*Cas2-3*, and hb-*Cas2-3*; (C) WX48, Δ*Csy1*, and hb-*Csy1*; (D) WX48, Δ*Csy2*, and hb-*Csy2*; (E) WX48, Δ*Csy3*, and hb-*Csy3*; (F) WX48, Δ*Cas6f*, and hb-*Cas6f*; (G) WX48, ΔCRISPR, and hb-CRISPR.

Table 1. MICs of various antibiotics for WT and mutant strains of *L. pneumophila*

Drug	MIC range (mg/L)	ECOFF (mg/L)	ATCC 33152	WT (WX48)	Cas1		Cas2-3		Csy1		Csy2		Csy3		Cas6f		CRISPR	
					ΔCas1	hb-Cas1	ΔCas2-3	hb-Cas2-3	ΔCsy1	hb-Csy1	ΔCsy2	bb-Csy2	ΔCsy3	hb-Csy3	ΔCas6f	hb-Cas6f	ΔCRISPR	hb-CRISPR
Erythromycin	0.032–2	1	0.5	0.75	0.5	0.75	0.125	0.75	0.5	0.75	0.5	0.75	0.5	0.75	0.5	0.75	0.5	0.75
Rifampicin	0.004–0.032	0.032	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
Levofloxacin	0.064–1	0.5	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
Moxifloxacin	0.25–1	1	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Azithromycin	0.038–8	1	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Tigecycline	1–16	16	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ciprofloxacin	0.25–2	1	0.5	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Clarithromycin	0.064–1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Doxycycline	1–8	8	0.75	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Note. MIC, minimum inhibitory concentration; ECOFF, epidemiological cutoff.

system on *L. pneumophila* proliferation in human alveolar macrophages by infecting J774 cells with WT, deletion mutants, and complementation mutants of *L. pneumophila* in vitro and assessing their proliferative ability. The numbers of the WT strain, as well as those of the Δ*Cas1*, Δ*Cas2-3*, Δ*Csy1*, Δ*Csy2*, Δ*Csy3*, ΔCRISPR, and complementation mutants, within the macrophages increased gradually over time, indicating that *Cas* gene deletion did not affect the intracellular proliferation ability of *L. pneumophila* (Figure 5A–E, G). The intracellular survival ability of the Δ*Cas6f* strain was lower on the first day than that of the WT strain, and the intracellular proliferation ability of the Δ*Cas6f* strain was markedly lower than that of the WT strain on days 2 and 3. The complete *Cas6f* gene was introduced into the Δ*Cas6f* mutant strain, which restored its intracellular growth capacity to that of the WT strain (Figure 5F). Thus, *Cas6f* deletion may affect the intracellular proliferation of *L. pneumophila*.

Micrographs of Gimenez-Stained J774 Cells

We used Gimenez staining to visualize the bacterial growth in J774 cells infected with WT, deletion mutants, and complementation strains of *L. pneumophila*. The quantities of the WT, Δ*Cas1*, Δ*Cas3-Cas2*, Δ*Csy1*, Δ*Csy2*, Δ*Csy3*, ΔCRISPR, and complementation strains within the cells gradually increased over time, and their growth trends were consistent. Although the intracellular quantity of *Cas6f* also gradually increased over time, its intracellular bacterial load was markedly lower than that of the WT and complemented strains. This result is consistent with the results reported in Section 3.6, indicating that deleting *Cas6f* reduces the intracellular proliferative ability of *L. pneumophila* (Figure 6). We randomly selected 100 fields of view under a microscope and counted the number of cells infected with bacteria in these fields. The proportion of infected cells in the WT and mutant strains (Δ*Cas1*, Δ*Cas3-Cas2*, Δ*Csy1*, Δ*Csy2*, Δ*Csy3*, and ΔCRISPR), along with complemented mutants, consistently increased with infection time, similar to the results described above in this section (Figures 7A–J, M–O). However, the proportion of cells infected with the Δ*Cas6f* strain began to decrease after the first day compared with those in the WT and complemented strains. The curve depicting the infection ratio (infected/total cells) flattened for Δ*Cas6f* (Figure 7K, L, O).

mRNA Expression Levels of Genes Related to Intracellular Proliferation and Biofilm Formation

We analyzed the expression levels of genes related to intracellular proliferation and biofilm formation, specifically the CRISPR-Cas, Dot/Icm secretion, and pili systems, using qRT-PCR.

The type I-F CRISPR-Cas system consists of six *Cas* genes and a CRISPR array, with the *Cas* gene constituting an operon headed by *Cas1*. The CRISPR-Cas system genes of *L. pneumophila* regulated each other, and deleting any of these genes affected the expression levels of their downstream genes. *Cas1*, *Csy1*, and *Cas6f* were individually deleted; the expression levels of the upstream and downstream genes of these three deletion mutants were considerably lower compared with those of the WT. The expression level of the *Cas1* gene was slightly higher in the *Cas2-3* deletion mutant, whereas the expression levels of other genes were lower compared with those in the WT. The expression levels of the *Cas2-3* and CRISPR genes of the *Csy2*

deletion mutant, whereas the expression levels of other genes were lower compared with the WT. The expression levels of *Cas1* and *Cas2-3* did not differ compared with the WT, but those of other genes were lower. CRISPR deletion did not affect the expression levels of *Cas1*, *Cas2-3*, and *Csy3* in the mutant strain, whereas those of the other genes were substantially downregulated compared with the WT (Figure 8).

We analyzed the structural gene expression levels of the Dot/Icm type IV secretion system (T4SS) in *Cas6f* because its intracellular proliferation was notably lower than that in the WT. The expression levels of *DotB*, *DotC*, *DotD*, *DotV*, *DotW*, *IcmX*, *IcmB*, *IcmS*, *IcmF*, and *LvgA* were considerably lower in the *Cas6f* strain than in the WT strain. Therefore, *Cas6f* deletion may reduce the expression levels of the most structural genes of Dot/Icm T4SS, reducing the intracellular Δ*Cas6f* proliferation (Figure 9F). In addition, although the intracellular proliferation of Δ*Cas1*, Δ*Cas2-3*, Δ*Csy1*, Δ*Csy2*, Δ*Csy3*, and ΔCRISPR was not affected compared with that of the WT, the
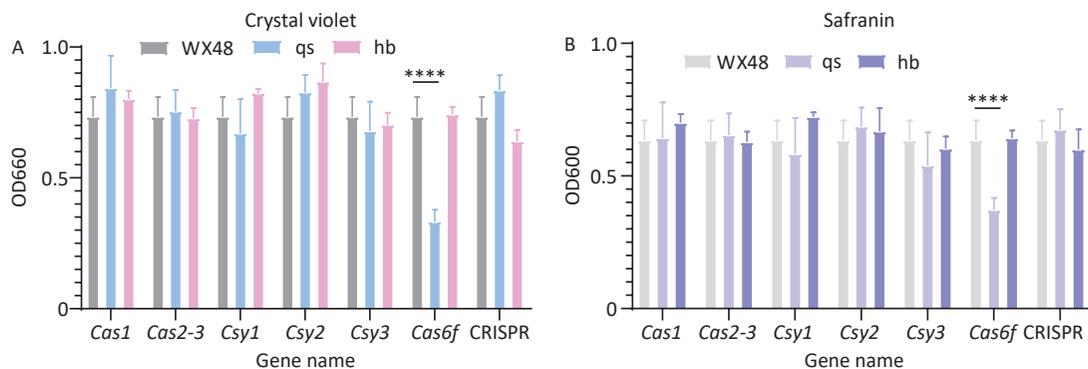

Figure 3. The biofilm formation ability of *L. pneumophila* was measured using crystal violet staining. qs: Δ*Cas*/ΔCRISPR mutant strains; hb: complementation strains. $^{****}P < 0.0001$. (A) Crystal violet staining and (B) safranin staining were used to measure the amount of biofilm formed by bacteria.
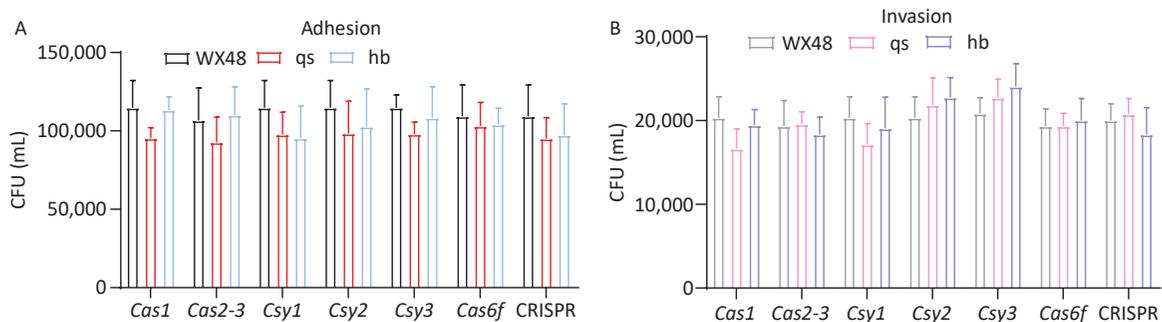

Figure 4. Adhesion and invasion of J774 cells by various *L. pneumophila* strains. (A) The adhesion ability of each strain. Black (WX48), red (qs), and blue (hb) representing the wild-type, deletion mutant, and complemented strains, respectively. (B) The adhesion ability of each strain. Gray (WX48), pink (qs), and purple (hb) represent the wild-type, deletion mutant, and complemented strains, respectively.

expression levels of the individual structural genes of the Dot/Icm T4SS were slightly lower (Figures 9A–E, G), which may have been due to the mutual

regulation among the *Cas* genes in the CRISPR-Cas system.

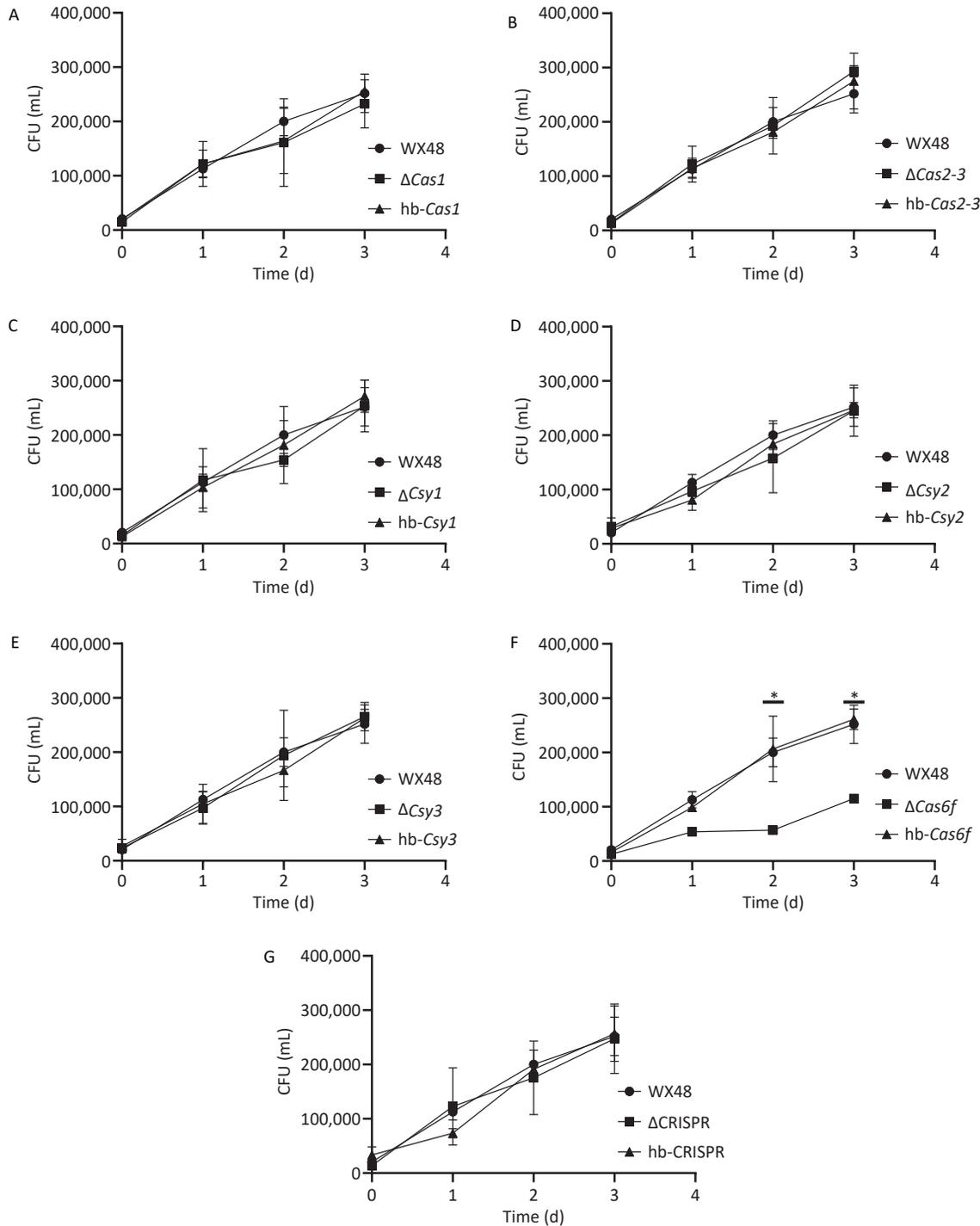Flagellin may enhance the ability of *Legionella* to

Figure 5. Proliferation ability of CRISPR-Cas deletion and complemented mutants of *L. pneumophila* in J774 mouse macrophages. $^*P < 0.05$. The proliferation ability of (A) WX 48, Δ*Cas1*, and hb-*Cas1*; (B) WX 48, Δ*Cas2-3*, and hb-*Cas2-3*; (C) WX 48, Δ*Csy1*, and hb-*Csy1*; (D) WX 48, Δ*Csy2*, and hb-*Csy2*; (E) WX 48, Δ*Csy3*, and hb-*Csy3*; (F) WX 48, Δ*Cas6f*, and hb-*Cas6f*; (G) WX 48, ΔCRISPR, and hb-CRISPR.

adhere to and invade host cells, increasing intracellular growth. Therefore, we examined the flagellin expression levels in each mutant strain. The expression of flagellin was moderately downregulated in ΔCsy1, ΔCsy2, ΔCsy3, and ΔCRISPR; upregulated in ΔCas1; and unchanged in ΔCas3-Cas2 and ΔCas6f compared with that In the WT strain (Figure 9H). This finding suggests that the reduced intracellular proliferative capacity of the ΔCas6f strain is unrelated to flagellin.

Deleting *Cas6f* led weakened the biofilm formation ability compared with that of the WT; therefore, we analyzed the expression levels of the structural genes in the type IV pili system. The expression levels of type IV pili-associated genes (*pilB*, *pilD*, *pilA*, *pilQ*, *pilN,* and *pilM*) were substantially downregulated in the *Cas6f* mutant compared with those in the WT (Figure 10F).

Complementation with an intact *Cas6f* gene restored their expression levels to WT levels. These findings suggest that *Cas6f* deletion contributes to altering biofilm formation through modulating pilus-related gene networks. In addition, although the ΔCas1, ΔCas2-3, ΔCsy1, ΔCsy2, ΔCsy3, and ΔCRISPR deletions did not affect biofilm formation, the expression levels of the individual structural genes of T4P were slightly lower than those in the WT (Figure 10A–E, G), which may have been due to the mutual regulation of biofilm formation among the *Cas* genes in the CRISPR-Cas system.

DISCUSSION

The genomes of many prokaryotes, such as *L. pneumophila*, contain CRISPR arrays and *Cas* genes that enable their resistance to foreign genetic
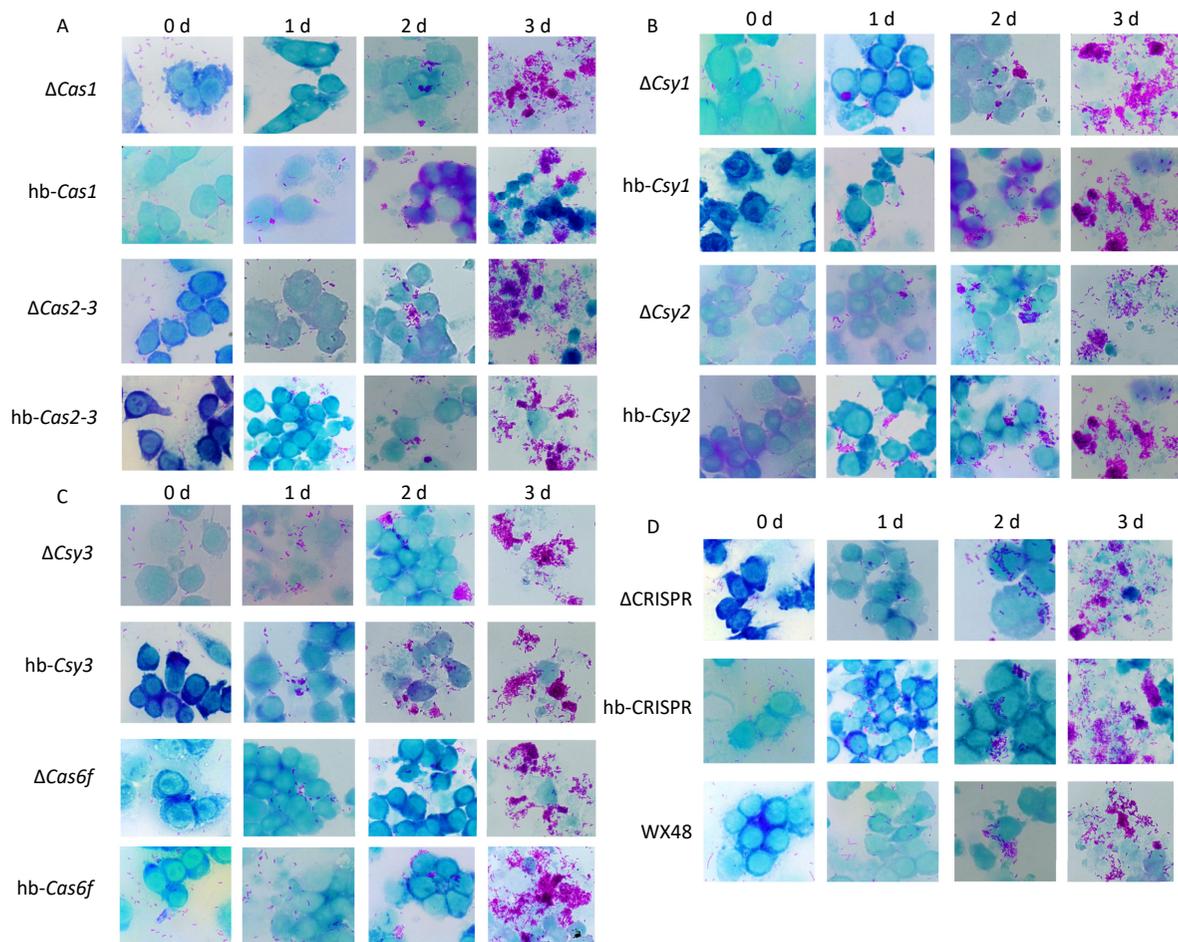

Figure 6. Micrographs of Gimenez-stained monolayers of J774 cells from 0 to 3 days after infection with various *L. pneumophila* strains. Magnification: 100×. Microscopic images of (A) ΔCas1, hb-*Cas1*, ΔCas2-3, and hb-*Cas2-3*; (B) ΔCsy1, hb-*Csy1*, ΔCsy-2, and hb-*Csy2*; (C) ΔCsy3, hb-*Csy3*, ΔCas6f, and hb-*Cas6f*; (D) ΔCRISPR, hb-CRISPR, and WX48.

factors. Some bacteria use *Cas* proteins in their CRISPR/Cas systems to target their own genes, increasing their virulence during host cell invasion[36,37]. The pathogenesis of *Legionella* has been extensively studied, and the bacterial factors

that promote intracellular infection and virulence have been identified[38,39]. However, relatively few studies have focused on the nonclassical functions of CRISPR-Cas in *Legionella*. *Cas2* in *L. pneumophila* has nuclease activity and is essential for intracellularly
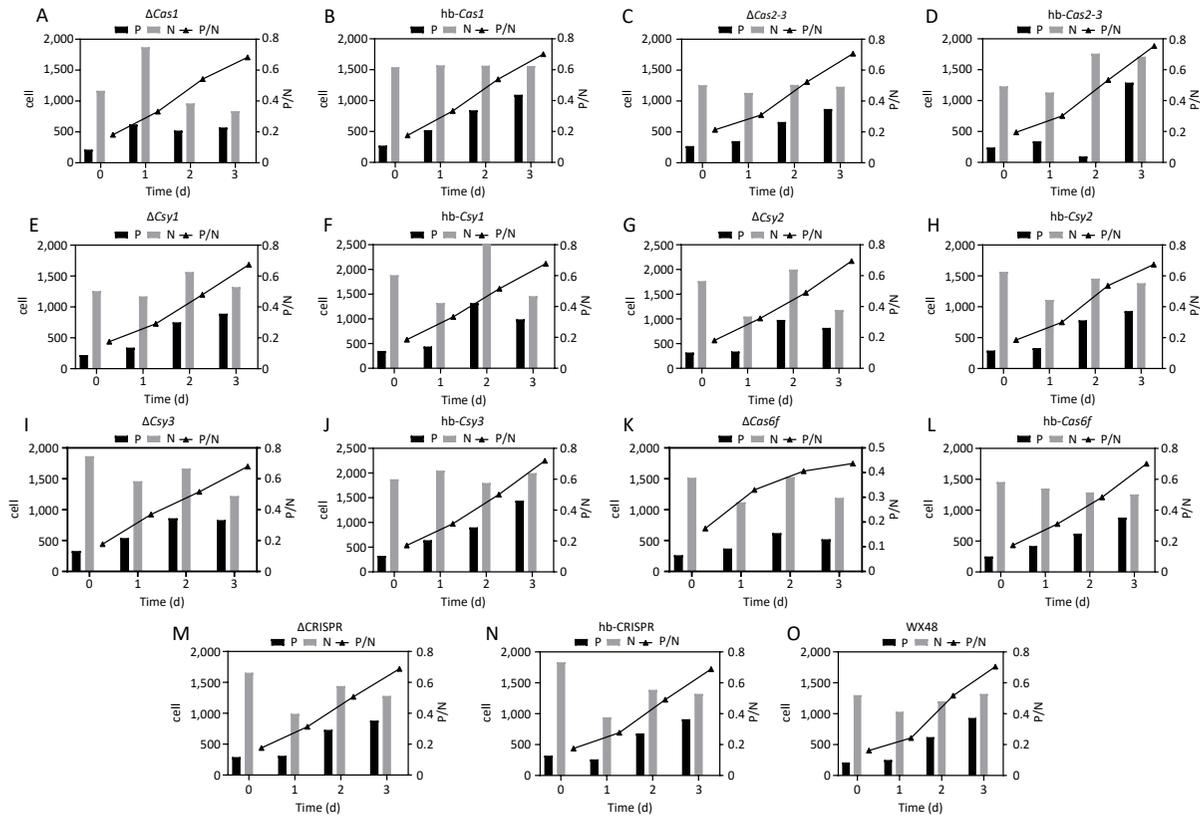
Figure 7. Cell infection status of various *L. pneumophila* strains from 100 microscopic fields. P: The number of bacteria-infected cells in a single field of view. N: The total number of cells in a single field of view. P/N: The ratio of cells infected with bacteria to the total number of cells in a single field of view. All values are presented as averages. The cell infection status of (A) ΔCas1, (B) hb-Cas1, (C) ΔCas2-3, (D) hb-Cas2-3, (E) ΔCsy1, (F) hb-Csy1, (G) ΔCsy2, (H) hb-Csy2, (I) ΔCsy3, (J) hb-Csy3, (K) ΔCas6f, (L) hb-Cas6f, (M) ΔCRISPR, (N) hb-CRISPR, and (O) WX48.
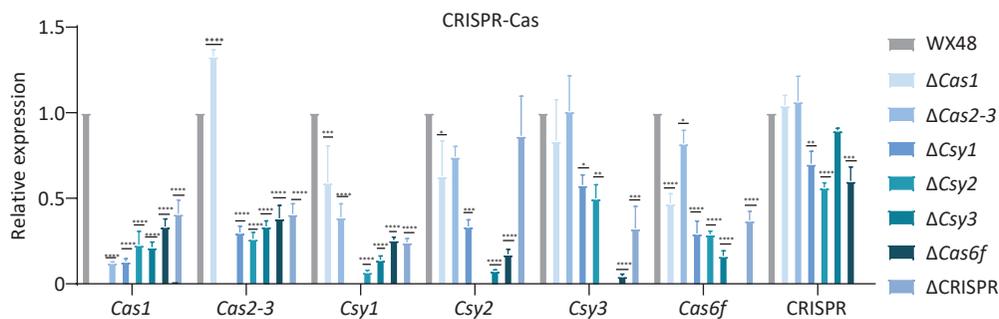
Figure 8. Expression levels of CRISPR-Cas system-related genes in *L. pneumophila* CRISPR-Cas deletion strains. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. WX48 served the control group. *Cas1, Cas2-3, Csy1, Csy2, Csy3, Cas6f, and* CRISPR: the expression levels of the *Cas1, Cas2-3, Csy1, Csy2, Csy3, Cas6f,* and *CRISPR* genes in the deletion mutants, respectively.

infecting amoeba cells[32,33]. In addition, *Cas2* is associated with thermotolerance[40]. The type II-B CRISPR-Cas system plays a crucial role in regulating the drug resistance and intracellular proliferation of *L. pneumophila*[31]. We evaluated the effects of the CRISPR-Cas system on the *in vitro* growth, drug resistance, biofilm formation, and intracellular proliferation of *L. pneumophila* by deleting its type I-F CRISPR-Cas genes. The type I-F CRISPR-Cas system of *L. pneumophila* regulates the expression of structural genes of the T4P system and Dot/Icm T4SSs, affecting the biofilm formation and intracellular proliferation of *L. pneumophila*.

Therefore, the CRISPR-Cas system may be involved in regulating *L. pneumophila* virulence.

The gene distribution of the type I-F CRISPR-Cas system in *Legionella* was reported[34]. We analyzed the gene distribution of the type I-F CRISPR-Cas system using CRISPRCasFinder, and the results were similar to those of previous studies[34]. We also analyzed the relative expression levels of CRISPR-Cas-related genes using qRT-PCR. Deleting each *Cas* gene in the type I-F CRISPR-Cas system affects the expression levels of upstream and downstream *Cas* genes. This finding indicates that deleting one gene in the CRISPR-Cas system affects the expression
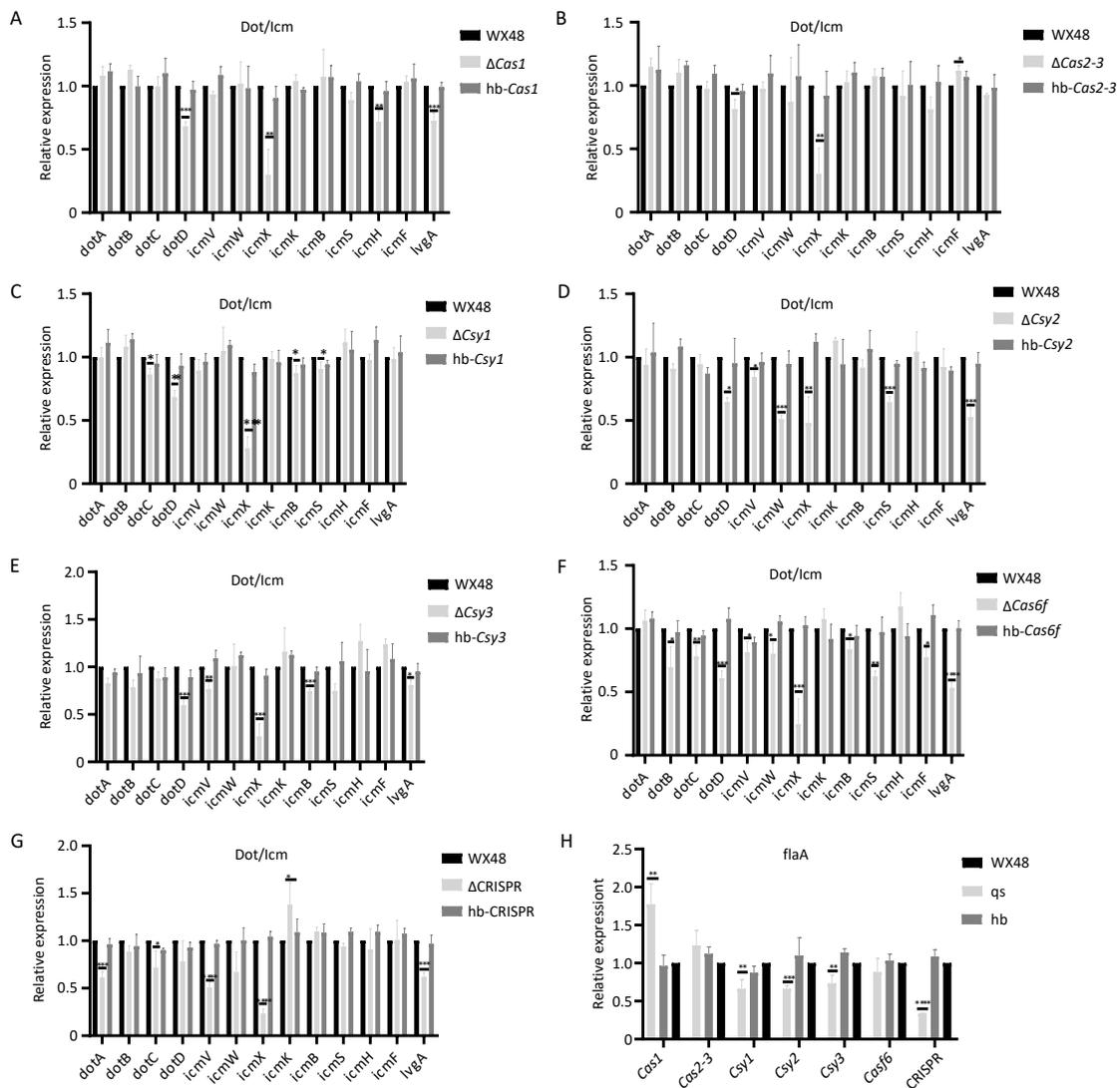

Figure 9. Expression levels of structural genes related to the Dot/Icm type IV secretion system in mutant strains. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Expression of structural genes related to the Dot/Icm type IV secretion system in (A) WX48, ΔCas1, and hb-Cas1; (B) WX48, ΔCas2-3, and hb-Cas2-3; (C) WX48, ΔCsy1, and hb-Csy1; (D) WX48, ΔCsy2, and hb-Csy2; (E) WX48, ΔCsy3, and hb-Csy3; (F) WX48, ΔCas6f, and hb-Cas6f; (G) WX48, ΔCRISPR, and hb-CRISPR. (H) Expression of the *flaA* flagellin gene.

levels of other genes in the system.

The CRISPR-Cas system, in addition to the defense function of exogenous nucleic acids, plays a role in regulating endogenous gene expression and host cell physiology. Biofilm production and colonization may be important for the environmental spread, survival, and pathogenesis of *L. pneumophila*[11,41,42]. Moreover, the type I-C CRISPR-Cas system in *Streptococcus* mutant strains may be associated with biofilm formation, and *Cas3* deletion may hinder biofilm formation[43]. The biofilm formation ability of a WT *Salmonella* strain with type I-E CRISPR-*Cas3* was a stronger than that

of the Δ*Cas3* mutant. Thus, *Cas3* may be involved in regulating bacterial biofilm formation[44]. In this study, the biofilm formation ability of the Δ*Cas6f L. pneumophila* mutant with a type I-F CRISPR-Cas system was weaker than that of the WT, whereas the biofilm formation ability of the complemented strain with the complete *Cas6f* gene was stronger than that of Δ*Cas6f*. These findings collectively demonstrate that the CRISPR-associated gene *Cas6f* is involved in modulating biofilm formation in *L. pneumophila*.

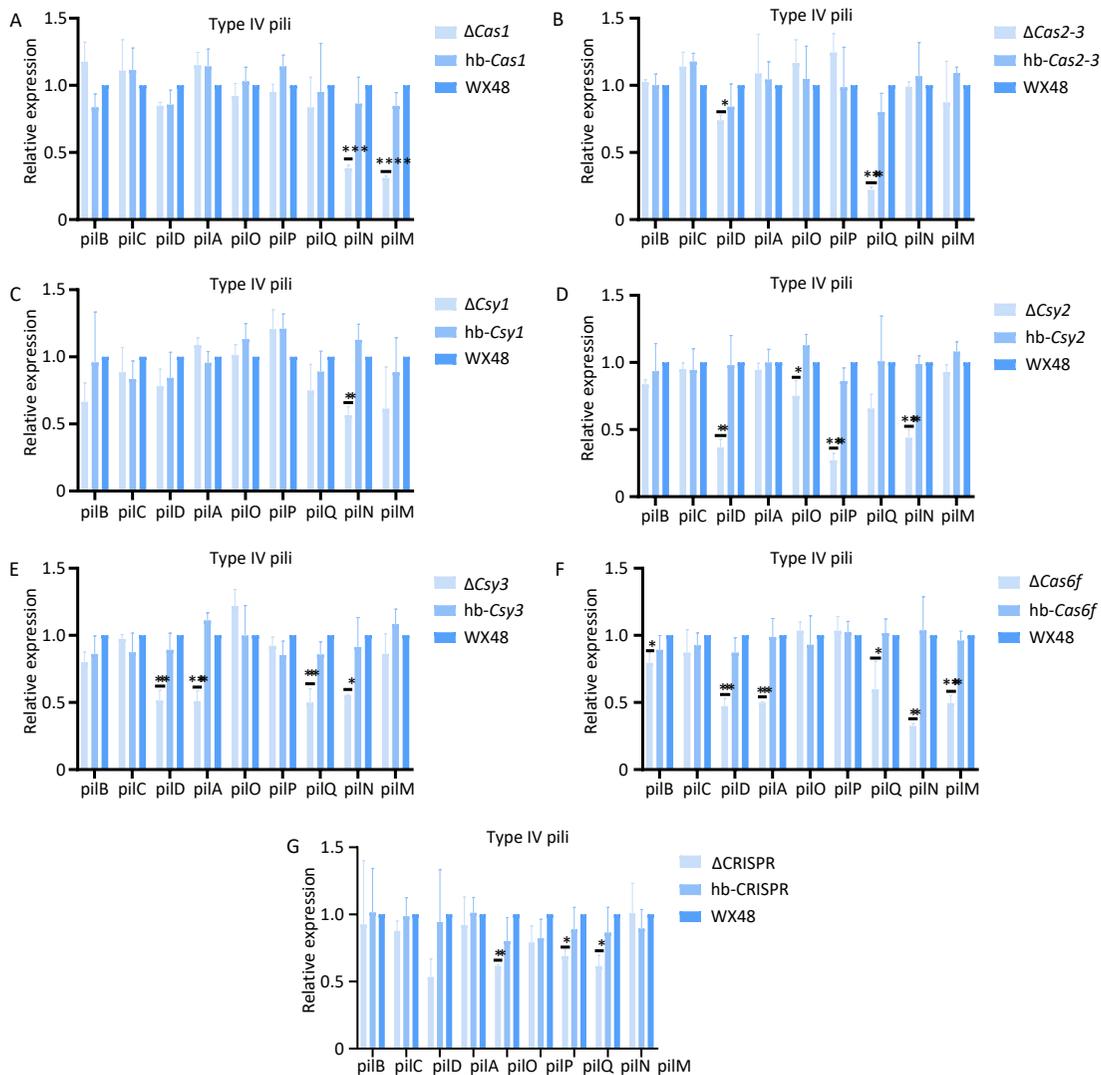The T4P system influences the biofilm formation and surface movement of *Clostridioides difficile*[45,46].

Figure 10. Expression levels of structural genes related to the type IV pili system in mutant strains. *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Expression of structural genes related to the type IV pili system in (A) WX48, Δ*Cas1*, and hb-*Cas1*; (B) WX48, Δ*Cas2-3*, and hb-*Cas2-3*; (C) WX48, Δ*Csy1*, and hb-*Csy1*; (D) WX48, Δ*Csy2*, and hb-*Csy2*; (E) WX48, Δ*Csy3*, and hb-*Csy3*; (F) WX48, Δ*Cas6f*, and hb-*Cas6f*; (G) WX48, ΔCRISPR, and hb-CRISPR.

The type IV pili (T4P) system mediates key processes, such as bacterial twitching motility, DNA uptake, and biofilm formation. *PilA* is a core structural protein that is directly involved in assembling and functionally regulating the pilus[45,47]. Therefore, we analyzed the effects of regulating *Cas* genes using the CRISPR-Cas system on the gene expression levels of the T4P system. *Cas6f* deletion led to a reduction in the expression levels of most genes in the T4P system. Thus, *Cas6f* may be indirectly involved in regulating of biofilm formation in *L. pneumophila*. Although deleting other genes impacted the expression levels of the individual structural genes of the T4P system, the biofilm formation ability did not notably differ compared with that of the WT strain, indicating that these genes are not essential for biofilm formation. Alternatively, these genes may maintain phenotypic stability through other unknown mechanisms that were not observed in our experiments.

The pathogenesis of *L. pneumophila* relies on the Dot/Icm type IV secretion system, which likely reflects its extensive engagement with the cellular processes of the host during the evolution of bacterial virulence[38,48]. Dot/Icm T4SS is crucial for *Legionella*-containing vacuole biogenesis and intracellular replication as well as a key virulence factor in the intracellular survival and pathogenicity of *Legionella*. A single *L. pneumophila* cell can transfer more than 300 proteins to the host cells *via* Dot/Icm T4SS effectors, which manipulate host cell functions to benefit the bacterium. This process is essential for the growth and intracellular transport of human macrophages and amoebas[49-52]. In this study, the Δ*Cas1*, Δ*Cas2-3*, Δ*Csy1*, Δ*Csy2*, Δ*Csy3*, and ΔCRISPR strains showed an ability to proliferate in macrophages that was similar to that of the WT strain; however, the proliferation ability of Δ*Cas6f* was much weaker. Moreover, *L. pneumophila* containing the complete type I-F CRISPR-Cas system exhibited a higher capacity to proliferate in macrophages than those lacking *Cas6f*. This suggests that *Cas6f* is involved in regulating intracellular proliferation. *Cas6f* forms a tight complex with cleaved crRNA products in *Pseudomonas aeruginosa*, which plays a functional role within the CRISPR pathway beyond pre-crRNA cleavage[53]. The *Cas6f* (also known as *Csy4*) in the type I-F CRISPR-Cas system is an endonuclease that binds with high affinity to repetitive stem-loop structures in pre-crRNAs by cutting these structures at the base to produce mature crRNAs[21,54,55,56]. Our qRT-PCR results demonstrated that deleting *Cas6f* decreased

the expression levels of most genes related to intracellular proliferation in T4BSS of *L. pneumophila* compared with those in the WT. Although deletion of other genes affected the expression levels of individual structural genes in the T4BSS secretion system, the intracellular proliferation did not change. Therefore, we speculate that *Cas6f* is the main factor causing changes in intracellular proliferation via affecting the expression levels of Dot/Icm type IV secretion system structural genes, whereas the other genes do not affect intracellular proliferation.

The type I-F CRISPR-Cas system regulates the *flaA* expression levels. Notably, although the expression level of *flaA* in the Δ*Cas6f* mutant did not differ from that of the WT, the intracellular proliferation capacity of the Δ*Cas6f* mutant was considerably lower, suggesting that this phenotype is independent of the flagellin pathway. Flagellin enhances the adhesion to and invasion of host cells by *L. pneumophila*; however, this protein is not necessary for the intracellular proliferation of the bacteria[57]. In summary, the CRISPR-Cas system may affect the expression of the type IV secretion and the type IV pili (T4P) systems, causing changes in the intracellular proliferation and biofilm formation of *L. pneumophila*, with *Cas6f* playing an important role in this process.

CONCLUSION

The virulence mechanisms of *Legionella* have been extensively studied. The classical and nonclassical functions of the CRISPR-Cas system have been examined. We observed that the type I-F CRISPR-Cas system influenced the biofilm formation and intracellular proliferation of *L. pneumophila* and regulated the expression levels of the structural genes of the Dot/Icm T4SS and T4P systems, altering the pathogenicity of *L. pneumophila*. This finding suggests that the CRISPR-Cas system plays an important role in the virulence of *L. pneumophila*. This study expands our understanding of the biology of the CRISPR-Cas system and provides new targets for further understanding the pathogenic mechanisms of *Legionella*.

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