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Legionella dumoffii DjlA, a Member of the DnaJ Family, Is Required for Intracellular Growth

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Legionella dumoffii **is one of the common causes of Legionnaires' disease and is capable of replicating in macrophages. To understand the mechanism of survival within macrophages, transposon mutagenesis was employed to isolate the genes necessary for intracellular growth. We identified four defective mutants after screening 790 transposon insertion mutants. Two transposon insertions were in genes homologous to** ***icmB*** **or** ***dotC*****, within** ***dot/icm*** **loci, required for intracellular multiplication of** ***L. pneumophila*****. The third was in a gene whose product is homologous to the 17-kDa antigen forming part of the VirB/VirD4 type IV secretion system of** ***Bartonella henselae*****. The fourth was in the** ***djlA*** **(for "*****dnaj*****-like** ***A*****") gene. DjlA is a member of the DnaJ/Hsp40 family. Transcomplementation of the** ***djlA*** **mutant restored the parental phenotype in J774 macrophages, A549 human alveolar epithelial cells, and the amoeba** ***Acanthamoeba culbertsoni*****. Using confocal laser-scanning microscopy and transmission electron microscopy, we revealed that in contrast to the wild-type strain,** ***L. dumoffii djlA*** **mutant-containing phagosomes were unable to inhibit phagosome-lysosome fusion. Transmission electron microscopy also showed that in contrast to the virulent parental strain, the** ***djlA*** **mutant was not able to recruit host cell rough endoplasmic reticulum. Furthermore, the stationary-phase** ***L. dumoffii djlA*** **mutants were more susceptible to H_2O_2, high osmolarity, high temperature, and low pH than was their parental strain. These results indicate that DjlA is required for intracellular growth and organelle trafficking, as well as bacterial resistance to environmental stress. This is the first report demonstrating that a single DjlA-deficient mutant exhibits a distinct phenotype.**

Legionella dumoffii was first isolated from cooling-tower water in 1979 (18) and later from a postmortem lung specimen in the same year (40) as an atypical *Legionella*-like organism. It was later classified by Brenner (11) as a new species, *L. dumoffii*. *Legionella* species are gram-negative, facultative intracellular parasites of freshwater amoebae in nature and are capable of growing within alveolar macrophages and epithelial cells after being accidentally transmitted to humans (22). The most common human pathogen in the genus *Legionella* is *L. pneumophila*, the causative agent of Legionnaires' disease (71). Humans contract the disease from contaminated environmental sources, primarily by aspiration of aerosolized water sources (22). After internalization by alveolar macrophages, *L. pneumophila*-containing phagosomes do not acidify (34) or fuse with lysosomes (33). Instead, the mitochondria, smooth vesicles, and rough endoplasmic reticula (RER) near these *L. pneumophila*-containing vacuoles are recruited, and *L. pneumophila* begins to multiply in this unique niche (32). This altered endocytic pathway is considered to be controlled by the Dot/Icm type IV protein secretion system (5, 17, 48, 55, 56, 74). The *dot/icm* genes are essential for the intracellular growth of *L. pneumophila* (5, 51, 60). The presence of the *dot/icm* loci in several species of *Legionella* was shown by Southern or PCR analysis (4, 36, 43); however, the contributions of these loci to the pathogenesis of other species have yet to be investigated.

L. dumoffii is the fourth or fifth most common pathogen causing Legionnaires' disease (8, 71). Some of proteins or factors which may promote *L. pneumophila* pathogenesis, such as flagella, catalase, and gelatinase, are also present in *L. dumoffii*. Several putative virulence factors—lipase, oxidase, and a zinc metalloprotease—are absent in *L. dumoffii* (6, 11, 52). *L. dumoffii* is capable of infecting and replicating within Vero cells and the human lung alveolar epithelial cell line A549 in vitro (41, 42). To elucidate the molecular mechanisms of the intracellular growth of this organism, we attempted to isolate the mutants that exhibited defective growth phenotypes in J774 mouse macrophage-like cells and A549 human type II alveolar epithelial cells by using transposon mutagenesis. We isolated four clones attenuated in virulence within mammalian cells by screening 790 derivatives with Tn*903*dII*lacZ* insertions. Two of four genes flanking the transposon insertions encode the proteins homologous to *L. pneumophila* IcmB and DotC (5, 51, 60), respectively. One gene has similarity to *virB5* (17-kDa antigen) in the VirB/VirD4 type IV secretion system of *Bartonella henselae* (14, 49, 59). The deduced protein encoded by a fourth gene showed homology to DjlA proteins (16). The DjlA homologue, a member of the DnaJ/Hsp40 family, was originally identified in *Escherichia coli* as a product of a hypothetical open reading frame (13, 72), and since then homologues have been identified in many other bacterial species, such as *Coxiella burnetti* (73), *Salmonella enterica* serovar Ty-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
DH5α	F^-*endA1 hsdR17 supE44D thi-1 recA1*Δ(*argF-lacZYA*)*U169*(ϕ80δ *lacZM15*) *gyrA96*λ$^-$	30
*VCS*257	DP50 *sup* F[*supE44 supF58 hsd53*(r_B m_B) *dapD8 lacY1 gln*V44 Δ(*gal-uvrB*)47 *tyrT58 gyrA29 tonA53* Δ(*thyA57*)]	Stratagene
L. dumoffii Tex-KL		ATCC 33343
HOLD254	Tex-KL *djlA*::Tn*903*dII*lacZ*	This study
HOLD491	Tex-KL *icmB(dotO)*::Tn*903*dII*lacZ*	This study
HMLD4001	Tex-KL 17-kDa antigen::Tn*903*dII*lacZ*	This study
HMLD4002	Tex-KL *dotC*::Tn*903*dII*lacZ*	This study
HOLD254-1	Tex-KL *djlA*::Tn*903*dII*lacZ*/pHRO18	This study
HOLD254-2	Tex-KL *djlA*::Tn*903*dII*lacZ*/pHRO25	This study
Plasmids		
pGEM-T Easy	Amp^r, *lacZ*, general cloning vector	Promega
pUC19	Amp^r, parental cloning vector	70
pBR322	*ori*R (ColE1); Amp^r Tc^r	New England Biolabs
pHC79	Wide-host-range pBR322 origin cosmid vector; Amp^r Tc^r	31
pLAW317	*rpsL* MCS[a] *oriT*(RK2) Cm^r*loxP ori*R(ColE1) Amp^r*loxP*	68
pLAW330	pLAW317::Tn*903*dII*lacZ tnpA*(Tn*903*) *oriR*(f1)	68
pMMB207	RSF1010 derivative, *lncQ lac I*^{q} Cm^r P*tac oriT*	47
pMMB207c	pMMB207 with 8-bp insertion in *mobA*; Mob	45
pHRO1	Tn*903*dII*lacZ*-containing HindIII fragment from HOLD254 in pBR322	This study
pHRO2	Tn*903*dII*lacZ*-containing BamHI fragment from HOLD491 in pBR322	This study
pHRO3	Tn*903*dII*lac*Z-containing HindIII fragment from HMLD4001 in pBR322	This study
pHRO4	Tn*903*dII*lac*Z-containing HindIII fragment from HMLD4002 in pBR322	This study
pHRO17	Amp^r; 4-kbp ScaI-EcoRI fragment containing *djlA* gene in pUC19	This study
pHRO18	4-kbp Pst-EcoRI fragment containing *djlA* from pHRO17 in pMMB207c	This study
pHRO24	PCR fragment of *djlA* cloned into pGEM-T Easy vector	This study
pHRO25	EcoRI-PstI fragment (1,155 bp) containing *djlA* from pHRO24 cloned into pMMB207c	This study

[a] MCS, multiple-cloning site.

phimurium, *Klebsiella pneumoniae*, and *Vibrio cholerae*. DjlA carries the J-domain characteristic of the DnaJ/Hsp40 family and is essential for interaction with the Hsp70 homologue, DnaK, by increasing its ATPase activity (67). Overproduction of DjlA stimulates colanic acid production in *E. coli* (15, 16, 27, 73). Analysis of the DjlA null mutant demonstrated that the gene was not essential for viability (16). Although DjlA homologue is present in *L. pneumophila* (10), the role of this gene in pathogenesis has yet to be determined.

In this study, we investigated the role of the *djlA* gene in avoidance of fusion with lysosomes and its role in organelle trafficking within macrophages and in bacterial resistance to environmental stresses such as oxidative products, high temperature, high salt concentrations, and acidic pH.

MATERIALS AND METHODS

Bacterial Strains, plasmids, and media. The bacterial strains and plasmids used in this work are described in Tables 1 and 2. The *L. dumoffii* Tex-KL strain and its derivatives were grown on buffered charcoal-yeast extract (BCYE) agar plates or in buffered yeast extract (BYE) broth. BYE broth was based on the formation of BCYE, but the charcoal and agar were omitted. *E. coli* DH5α (Toyobo Co., Ltd., Osaka, Japan) was used for the majority of the cloning experiments. As required, antibiotics were used at the following concentrations: kanamycin (KM), 30 μg/ml; chloramphenicol (CM), 5 or 20 μg/ml (for *L. dumoffii*); KM, 30 μg/ml; ampicillin (AMP), 50 μg/ml; CM, 20 μg/ml (for *E. coli*).

Cell culture. J774A.1 macrophages (JCRB9108), refered to as J774 in this paper, were derived from mouse macrophage-like cells. The cell line A549 (JCRB0076) was donated by the Health Science Research Resources Bank, Osaka, Japan. The cells were established from a human alveolar epithelial carcinoma and have characteristics of well-differentiated type II pneumocytes. J774 cells and A549 cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; Dainippon

TABLE 2. Strains of *Legionella* species used and their clinical relevance

Legionella strain	Source	Clinical relevance
L. pneumophila serogroup1 (ATCC 33153)	Human	Yes
L. pneumophila serogroup6 (ATCC 33215)	Human	Yes
L. dumoffii (ATCC 33343)	Human	Yes
L. longbeachae (ATCC 33462)	Human	Yes
L. micdadei (ATCC 33218)	Human	Yes
L. bozemanii (ATCC 33217)	Human	Yes
L. feelei (ATCC 35849)	Human	Yes
L. gormanii (ATCC 33297)	Soil	Yes
L. jordanis (ATCC 33623)	Water	Yes
L. quinlivanii (ATCC 43830)	Water	No
L. moravica (ATCC 43877)	Water	No
L. gratiana (ATCC 49413)	Water	No
L. geestiana (ATCC 49504)	Water	No
L. rubrilucens (ATCC 35304)	Water	No
L. worsleiensis (ATCC 49508)	Water	No
L. jamestowniensis (ATCC 35298)	Soil	No
L. adelaidensis (ATCC 49625)	Water	No

Pharmaceutical, Osaka, Japan). *Acanthamoeba culbertsoni* (44) was propagated at 28°C in 25-cm^2 flasks (Falcon) containing 8 ml of peptone yeast extract glucose (PYG) and AC buffer (PYG + AC) (9, 46).

DNA manipulation. Restriction enzymes and T4 DNA polymerase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan) and Toyobo Co., Ltd. (Osaka, Japan). Calf intestine alkaline phosphatase was purchased from New England Biolabs Inc. (Beverly, Mass.). PCR amplification was performed by using The Ready To Go PCR-Beads (Amersham Pharmacia Biotech, Piscataway, N.J.) or Ex-Taq polymerase (Takara, Kyoto, Japan). Oligonucleotides used for PCR amplification were purchased from Japan Flour Co., Ltd. (Tokyo, Japan). Plasmid DNA was isolated from *E. coli* and *L. dumoffii* by using the Wizard Plus Mini Prep (Promega, Madison, Wis.) or the alkaline lysis method (58). Chromosomal DNA of *L. dumoffii* was purified using the Genomic Prep cells and tissue DNA isolation kit (Amersham Pharmacia Biotech). Electroporations were performed with a Bio-Rad Gene Pulser, as recommended by the manufacturer. Purification of DNA fragments from agarose gels for subcloning or labeling was carried out with a GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech).

Transposon mutagenesis and construction of a bank of mutants. *L. dumoffii* was mutated with the Tn*903* derivative Tn*903*dII*lacZ*, as described previously (68). Tn*903*dII*lacZ* confers KM resistance (Km^r) and contains a 5′-truncated *lacZ* gene. Briefly, after electroporation of plasmid pLAW330, containing Tn*903*dII*lacZ*, into *L. dumoffii* Tex-KL, bacteria were incubated in BYE broth for 5 h at 37°C and plated onto BCYE-KM plates. Km^r transformants containing β-galactosidase activity were identified as blue colonies after the the plates were overlaid with 0.8% agar containing 0.6 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml. Km^r Cm^s colonies were saved as simple Tn*903*dII*lacZ* insertion mutants of *L. dumoffii*.

Southern hybridization. Chromosomal DNA from *L. dumoffii* strains was digested with HindIII, resolved on a 0.7% agarose gel in TBE buffer, and blotted onto a nylon membrane. DNA probes were prepared by random-primed labeling with digoxigenin-1 1-dUTP. The methods for prehybridization and hybridization and the washing conditions were the same as described previously (58), and the procedure for colorimetric detection of hybridized DNA was performed using the digoxigenin system (Roche Diagnostic Co., Indianapolis, Ind.).

Cloning and sequencing of the chromosomal junction of Tn*903*dII*lacZ* insertion in the mutants. Genomic DNA from the *L. dumoffii* mutants was digested with HindIII or BamHI and ligated to HindIII- or BamHI-digested pBR322. The ligation was used to transform DH5α, and the transformation mixture was plated on Luria-Bertani agar plates containing KM and AMP. Plasmid DNA was extracted, and the regions flanking Tn*903*dII*lacZ* were sequenced with the *lacZ* primer (5′-CCCAGTCACGACGTTG-3′) and the Km^r primer (5′-AATTTAATCGCGGCCTCGAG-3′), corresponding to the 5′ and 3′ ends, respectively, of Tn*903*dII*lacZ*.

Construction of plasmids for complementation. For wild-type *L. dumoffii* genomic library construction, the genomic DNA was isolated from *L. dumoffii* and partially digested with Sau3 AI, and fragments of about 40 kb were purified. The fragments were ligated to the BamHI-digested, calf intestinal alkaline phosphatase-treated cosmid vecter pHC79 (31). The ligation products were packaged, in vitro, using the GigapackII Gold packaging system (Stratagene). Packaged hybrid cosmids were then used to infect *E. coli* strain *VCS*257. Recombinant clones were screened for the presence of a 1,085-bp PCR product (254-45), amplified using primers 254-4 (5′-GCTTCTTCCTTTCCACCATAA-3′) and 254-5 (5′-AGGTAGGCCTTGGGCAATTA-3′), by colony hybridization techniques. The probes used for colony hybridization were labeled with the digoxigenin random-primed DNA-labeling system (Roche). About 1,000 recombinant clones from the library were plated on the Luria-Bertani–plus-AMP plates for screening. Several positive cosmid clones were identified. The 4-kb ScaI-EcoRI fragment containing 254-45 from one of these cosmid clones was cloned into HincII-EcoRI-digested pUC19 to generate pHRO17. The recombinant clone was confirmed to contain 254-45 by Southern blot hybridization. The 4-kb PstI-EcoRI fragment from pHRO17 was cloned into shuttle vecter pMMB207c digested with PstI and EcoRI to generate pHRO18. pMMB207c is a nonmobilizable derivative of pMMB207 containing an 8-bp insertion within the *mobA* gene (at base 3325) and replicates stably in *Legionella* spp. (45). pHRO18 was electroporated into HOLD254 to yield the complemented strain HOLD254-1. The DNA fragment containing the *djlA* gene was PCR amplified from plasmid pHRO17 by using primer pair djlA-1-EcoRI (5′-GGGAATTCGAGTAGATACGAAGCAGGGT-3′) and djlA-2-PstI (5′-GGCTGCAGTTCCACCATAAACGGACTACA-3′). EcoRI and PstI sites (underlined sequences) were incorporated into these primers, respectively. The 1,155-bp PCR product that was generated contained 158 bp upstream of the ATG codon of *djlA* and 72 bp downstream of the stop codon of *djlA*. This PCR product was ligated into the pGEM-T Easy vector (Promega), resulting in pHRO24. The 1,155-bp EcoRI-PstI fragment from pHRO24 was then cloned into EcoRI-PstI-digested pMMB207c, creating pHRO25. The *djlA* mutant of *L. dumoffii*, HOLD254, was transformed with pHRO25 by electroporation. One of the transformants containing the desired plasmid was designated HOLD254-2. The cloned *djlA* gene was sequenced by using the primer within pMMB207c (pMMB207c-1; 5′-GTGTGGAATTGTGAGCGGAT-3′) and the primer within the *djlA* gene (254-3; 5′-GCTGATGGGCTGGATAGCAA-3′).

DNA sequence analysis of the region surrounding the *djlA* gene. Primer pair djlA-3 (5′-AAGGATGGTAACTCTGACTCT-3′) and pHC79-2 (5′-TTGGAGCCACTATCGACTAC-3′) within the *djlA* gene and pHC79, respectively, were used to amplify the flanking region of the *djlA* gene from the cosmid clone containing *djlA* gene. This 4-kb PCR product and the 4-kb plasmid DNA within pHRO17 were sequenced using a primer walking technique. DNA-sequencing reactions were performed on plasmid templates with the CEQ DTCS-Quick Start kit (Beckman Coulter, Inc., Fullerton, Calif.) and the CEQ DNA analysis system (Beckman Coulter, Inc.). The nucleotide sequences and deduced amino acid sequences were compared to the GenBank database by using the programs BLASTX and BLASTP and also to the incomplete genomic database of *L. pneumophila* Philadelphia I (http://genome3.cpmc.columbia.edu/~legion/ngnp1033033). Motif searches were carried out using the Prosite program.

Intracellular growth assay. Growth of *L. dumoffii* in J774 cells and A549 cells was determined by using a previously described standard intracellular growth assay (43, 74). *L. dumoffii* strains were grown in BYE broth to the early stationary phase. Approximately 2×10^9 bacteria were pelleted, resuspended, and diluted (1:1,000) in RPMI 1640 tissue culture medium. The bacteria were then added to J774 cells and A549 cells (2×10^5 per well) in 24-well dishes to give a multiplicity of infection (MOI) of about 10. The infected cells were incubated at 37°C under 5% CO_2–air for 1.5 h and washed three times with phosphate-buffered saline (PBS) to remove extracellular bacteria. To measure bacterial internalization, 1 ml of sterile distilled H_2O was added to the wells to release intracellular bacteria from the host cells, and CFU were determined by plating dilutions on BCYE agar plates. To each of the remaining wells, 0.5 ml of fresh tissue culture medium was added. At 24-h intervals, the intracellular and extracellular bacteria in each well were combined, and the total CFU was determined by plating the dilutions onto BCYE agar plates. Infection of *A. culbertsoni* was carried out in an almost identical manner, except that bacteria were suspended in AC buffer and 0.05% Triton X-100 was added to release intracellular bacteria.

Assessment of phagosome-lysosome fusion by confocal microscopy. *L. dumoffii* strains were grown overnight to saturation at 37°C in BYE broth. They were added at an MOI of 25 to 50 to 8×10^4 J774 cells on glass coverslips in 24-well tissue culture plates. The plates were centrifuged at 150 × *g* for 5 min at room temperature and incubated for 20 min in 5% CO_2–air at 37°C. Extracellular bacteria were removed by washing three times with PBS, and fresh tissue culture medium was added to each well. The plates were returned to the incubator for 4 h. Cells were fixed for 15 min at room temperature in P-PFA (4% paraformaldehyde in 1 × PBS [pH 7.4]) (43, 74). Coverslips were immersed in PBS–0.1% Saponin for 5 min to permeabilize the cells and blocked with 5% FBS in PBS for 5 min. Lysosomes and late endosomes were stained with rat monoclonal antibody 1 D4B (1:100) specific for LAMP-1 or Ab1 93 (1:100) specific for LAMP-2, and the bacteria were stained with rabbit anti-*L. dumoffii* polyclonal antibody (1: 10,000) for 1 h. The cells were washed with blocking solution three times and incubated for 30 min with Cy3-labeled goat anti-rat secondary antibody (1:300) and Alexa488-labeled goat anti-rabbit secondary antibody (1:300). The coverslips were then washed three times with blocking solution. All antibody dilutions were performed with PBS containing 0.5% FBS and 0.1% Saponin. Coverslips were inverted onto 1 μl of mounting medium (50% glycerol) on glass slides (39). Fluorescence was viewed using a Radiance 2100 MP confocal microscope (Bio-Rad Laboratories, Richmond, Calif.). Alexa488- and Cy3-labeled secondary antibodies were purchased from Molecular Probes (Eugene, Oreg.). Rat monoclonal antibodies to LGP107 (mouse LAMP-1) and LGP96 (mouse LAMP-2) were purified from mouse liver lysosomal membranes, as described previously (23).

Quantification of phagosome-lysosome fusion by electron microscopy. To label cell lysosomes, J774 macrophages were incubated with bovine serum albumin (BSA)-conjugated colloidal 15-nm-diameter gold particles (BSA-gold) overnight, chased for 3 h, and pulsed with stationary-phase *L. dumoffii* strains at an MOI of 50 (19, 33). At 4 h postinfection, the cells were fixed and processed for electron microscopy as previously described (66). Briefly, infected macrophages were fixed with 2% glutaraldehyde and then with 1% OsO_4, dehydrated with ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl

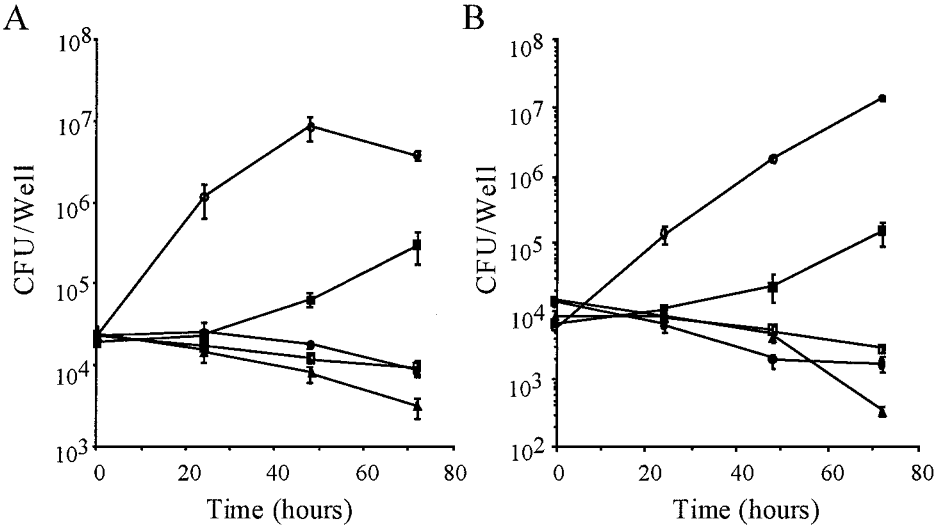


FIG. 1. Intracellular growth of *L. dumoffii* strains within J774 mouse macrophages (A) and A549 human epithelial cells (B). The formation of colonies (CFU per milliliter) was determined at the times indicated, in triplicate, for at least two independent experiments. Error bars indicate the standard deviations determined from samples taken from one experiment. Symbols: ○, *L. dumoffii* wild-type strain; ■, HOLD254; □, HOLD491; ●, HMLD4001; △, HMLD4002.

acetate followed by lead citrate and examined by electron microscopy in a JEM 2000EX instrument (JEOL, Ltd., Tokyo, Japan).

Examination of RER recruitment by transmission electron microscopy. J774 cells were plated in 90-mm-diameter petri dishes (2×10^5 cells/ml) and infected with stationary-phase *L. dumoffii* strains at an MOI of 20 for 8 and 24 h (32). Ultrathin sections were prepared as described above.

Assays for survival under stress conditions. *L. dumoffii* strains were grown for 2 to 3 days on BCYE agar plates and used to inoculate 4 ml of BYE medium. The bacteria were then grown at 37°C with aeration for at least 16 h. The initial CFU count was about 10^{10} per ml. Cells were divided into aliquots, centrifuged, and resuspended in equal volumes of 1 × M63 salts [22.0 mM KH_2PO_4, 40.2 mM K_2HPO_4, 14.6 mM $(NH_4)_2SO_4$ 500 nM $FeSO_4$ (pH6.5)]. One aliquot was used for measuring the untreated CFU. For heat shock, aliquots were transferred to 48°C and incubated for 60 min. For oxidative stress, aliquots were exposed to 10 mM H_2O_2 for 30 min. For osmotic shock, aliquots were exposed to 5 M sodium chloride for 30 min. For acid shock, aliquots were resuspended in 0.1 M citric acid (pH 3) for 5 min. Except for heat stress, the cells were incubated in a 37°C heat block. At the indicated time points, the cells were washed with 1 × M63 salts and serially diluted to determine the CFU on BCYE agar plates (29).

Detection of a *djlA* gene in other *Legionella* spp. The presence of *djlA* in 17 different strains of *Legionella* spp. was examined by PCR with the primer pair djlA-cons-1 (5′-ATAACAACCTGGTGGGGAAA-3′) and djlA-cons-2 (5′-TGGGCAATTAATTTATCTGGATG-3′), located in the transmembrane domain (TMD) and J domain within the *djlA* gene, respectively, which gave a 791-bp product. PCR was carried out by using chromosomal DNA from BCYE plate-grown bacteria as a template.

RESULTS

Isolation of intracellular growth mutants. Wild-type *L. dumoffii* Tex-KL was mutagenized with Tn*903*dII*lacZ* as described previously (57, 68). Plasmid pLAW330 containing Tn*903*dII*lacZ* was introduced into *L. dumoffii*, and 790 Km^r Cm^s mutants of *L. dumoffii* (HOLD strains 1 to 656 and HMLD strains 4004 to 4044 and 4048 to 4140) with various levels of β-galactosidase activity were isolated. The 790 mutants were individually screened for their ability to kill mouse macrophage-like J774 cells and human alveolar epithelial A549 cells. The mutants were grown for 2 days in 96-well tissue culture plates containing BYE medium. Then 5-μl samples of 2-day-old cultures of mutants were transferred to another 96-well tissue culture plate containing J774 cells or A549 cells. At each 24-h time point after infection, the monolayers were visually examined to determine the extent of killing of both J774 cells and A549 cells. From several assays, we isolated five mutants, based on their reproducible phenotypes. Southern blot analysis of the HindIII-digested genomic DNA of each of the five mutants probed with pLAW330 showed that four of them contained a single copy of the Tn*903*dII*lacZ* insertion and that these insertions were distributed in distinct locations within the chromosome of *L. dumoffii* (data not shown). For reasons not yet understood, one of the mutants showed no hybridization. Therefore, the four strains were chosen for further analysis. In vitro, the growth of these four mutants in BYE broth and on BCYE agar plates was similar to that of the wild-type strain (data not shown).

Intracellular growth phenotype of the mutants within J774 macrophages and alveolar epithelial cells. We examined the four candidates for their capacity to survive and to replicate within J774 macrophages and A549 epithelial cells. Bacterial CFU were determined over 3 days. The wild-type strain multiplied over 100-fold during the 3-day incubation period within J774 macrophages (Fig. 1A). HOLD254 showed a 1-log-unit increase after 3 days of incubation, whereas HOLD491, HMLD4001, and HMLD4002 did not grow during the incubation period in J774 cells. Within A549 epithelial cells (Fig. 1B), the wild-type strain increased approximately 1,000-fold over the 3-day period, while there was a 10-fold increase in the number of intracellular bacteria of HOLD254 over 3 days. For HOLD491 and HMLD4001, the number of CFU after 3 days of infection decreased 1 log unit to the initial number of CFU, and HMLD4002 was severely defective in intracellular survival (Fig. 1B).

Sequence analysis of the junctions of Tn*903*dII*lacZ* insertions. We cloned the HindIII fragment containing the Tn*903*dII*lacZ* insert and the flanking sequences of the mutants (HOLD254, HOLD491, HMLD4001, and HMLD4002). Using

TABLE 3. Sequence similarities of *L. dumoffii* genes responsible for intracellular multiplication[a]

Mutant strain	Homologous gene	Organism	% Identity	% Positive
HOLD254	*djlA*	*Legionella pneumophila*	61	73
HOLD491	*icmB/dotO*	*Legionella pneumophila*	89	95
HMLD4001	17-kDa antigen gene	*Bartonella henselae*	26	43
HMLD4002	*dotC*	*Legionella pneumophila*	85	92

[a] The values are taken from a Basic Local Alignment Search Tool for amino acid comparison (BLASTX program).

the primer within Tn*903*dII*lacZ*, we partially sequenced and analyzed them to identify the genes responsible for intracellular multiplication. The results are summarized in Table 3. Sequence homology searches against the Gen Bank database were done with these genes and corresponding proteins. HOLD254, HOLD491, and HMLD4002 contain insertions within the genes homologous to known *L. pneumophila* genes. The gene disrupted in HOLD254 is the *djlA* (for "*dnaJ*-like *A*") gene, encoding a member of the Hsp40 protein family, which has not been characterized in *L. pneumophila*. HOLD491 and HMLD4002 had a transposon insertion in their sequences similar to *icmB (dotO)* and *dotC*, respectively, identified as genes essential for intracellular growth in *L. pneumophila* (5, 51). HMLD4001 had an insertion within a gene whose product showed amino acid similarity to the 17-kDa antigen, VirB5, of *B. henselae*; the gene is located within the *virB* locus, which encodes a putative type IV secretion system together with the downstream *virD4* gene (14, 49, 59). Recently, Schulein and Dehio (59) also showed that VirB4 and VirD4, encoded by the *virB* and *virD4* loci of *B. tribucorum*, were required for establishing intraerythrocytic bacteremia.

Complementation of an *L. dumoffii djlA* mutant. DjlA is known to be a heat shock protein DnaJ/Hsp40 homologue. The virulence of the *djlA* mutant was compared with that of the wild-type strain and the *djlA*-complemented mutant in J774 macrophages, A549 epithelial cells, and *A. culbertsoni*. The *djlA* mutant showed only a 100-fold increase in intracellular replication within *A. culbertsoni* (Fig. 2C). As shown in Fig. 2, bacterial growth was fully restored in the complemented strains HOLD254-1 and HOLD254-2. The restoration of the wild-type-level of multiplication of the *djlA* mutant within these cells, achieved after complementation in *trans* with the cloned *djlA* gene, is proof of the important role of *djlA* in the intracellular growth of *L. dumoffii*.

Complete sequence and genetic structure of *djlA*. Figure 3A shows the organization around the *djlA* gene and the location of the Tn*903*dII*lacZ* insertion. The transposon insertion (Tn) was located in the J domain at the C terminus of the predicted protein, which was the defined feature of the DnaJ family of molecular chaperones (16, 27). Since the two genes (*waaA* and *orf1*) which flanked *djlA* were both oriented in the opposite direction from the *djlA*, we consider the *djlA* to be transcribed

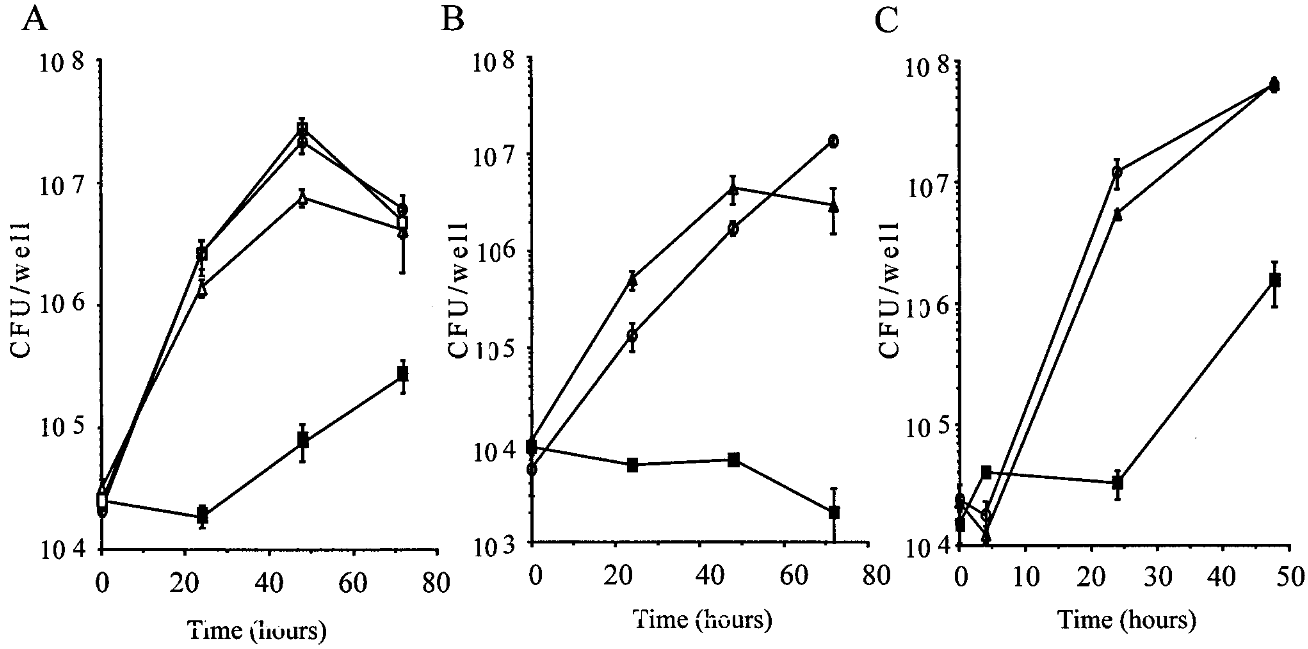


FIG. 2. Complementation of intracellular growth defects of *djlA* mutant HOLD254 in J774 macrophages (A), in A549 epithelial cells (B), and in *A. culbertsoni* (C). Growth was measured over 72 h (A and B) or 48 h (C). The data points and error bars represent the mean CFU/well for triplicate samples from a typical experiment (performed at least twice) and their standard deviations. Symbols: ○, *L. dumoffii* wild-type strain; ■, HOLD254; △, HOLD254-1 (*djlA*/pHRO18); □, HOLD254-2 (*djlA*/pHRO25).

monocistronically, and this transposon has no polar effect. The deduced amino acid sequence of *L. dumoffii* DjlA, together with *L. pneumophila* DjlA and *E. coli* DjlA, is presented in Fig. 3B. The putative *L. dumoffii djlA* gene encodes a protein of 302 amino acids with a predicted molecular mass of 35.33 kDa and an isoelectric point of 9.65. The protein size is similar to that of the *L. pneumophila* (296 amino acids) and *E. coli* (271 amino acids) proteins. *L. dumoffii* DjlA has 61% identity to *L. pneumophila* DjlA and 32% identity to *E. coli* DjlA (10, 16, 73). A potential TMD at the N terminus contains six glycines, spaced through the TMD at every three to five residues, which is similar to the structure of the TMD of *E. coli* (15, 16). There is a remarkable difference in the N terminus of DjlA protein between *E. coli* and *Legionella* spp. Clarke et al. (16) have demonstrated that *E. coli* DjlA is localized to the inner membrane and has a rare type III topology (i.e. N-out, C-in), with the N-terminal 6 to 8 residues located in the periplasm. *Legionella* spp. have longer stretches (15 residues) before the TMD structure, which are probably exposed in the periplasm. Another unique feature of *Legionella* DjlA is a glutamate-serine (QS)-rich spacer located before the J domain, instead of the glutamate-glycine (QG)-rich spacer of *E. coli* DjlA (Fig. 3B) (16). The cellular role of these QS- or QG-rich regions remain to be elucidated.

Quantification of endocytic maturation. To determine whether the *L. dumoffii* strains were able to inhibit endocytic maturation, we measured the colocalization of *L. dumoffii* phagosomes with endocytic markers LAMP-1 and LAMP-2. J774 macrophages were infected with postexponential phase *L. dumoffii* strains for 4 h (Fig. 4). The permeabilized cells were stained with monoclonal antibody 1D4B or Abl 93, specific for late endosomal and lysosomal proteins, LAMP-1 or LAMP-2. The *djlA* mutant was found in phagosomes that contained LAMP-1 (Fig. 4A), indicating that these vacuoles had fused with late endosomes, whereas, phagosomes containing wild-type *L. dumoffii* did not colocalize with LAMP-1 (Fig. 4A). When each *L. dumoffii* strain found in the phagosomes was scored for fusion with the late endosomal/lysosomal markers LAMP-1 and LAMP-2, approximately 80% of the wild-type bacteria were found in LAMP-1- and LAMP-2-negative phagosomes while 50 to 60% of the HOLD254 was found in LAMP-1- and LAMP-2-positive compartments (Fig. 4B). We also performed the same analysis for HOLD4002, the *dotC* mutant, and found that this mutant followed the same endocytic pathway as HOLD254, with 60 to 70% LAMP-1- and LAMP-2-positive (data not shown). We also conducted an assay of phagosome-lysosome fusion, at the ultrastructural level, using electron microscopy. BSA-gold was used as a pinocytic, fluid-phase marker of the endosomal-lysosomal pathway. BSA-gold was accumulated mainly in lysosomes after endocytosis of the conjugate-containing medium overnight at 37°C, followed by a chase period of 3 h at 37°C in conjugate-free medium as previously described (33). After a pulse with *L. dumoffii* strains and another chase for 4 h, electron microscopy counting of *L. dumoffii*-containing phagosomes that fused with BSA-gold-labeled lysosomes was performed to assess fusion (Fig. 5). Wild-type-strain-containing phagosomes did not fuse with BSA-gold-marked lysosomes (Fig. 5A). Quantitation showed that only 11.4% (24 of 210) of the phagosomes containing the wild-type strain fused with BSA-gold-marked lysosomes. On the other hand, 85% (187 of 220) of the phagosomes containing the *djlA* mutant strain accumulated BSA-gold (Fig. 5B). Thus, the *djlA* mutant was not able to evade phagosome-lysosome fusion.

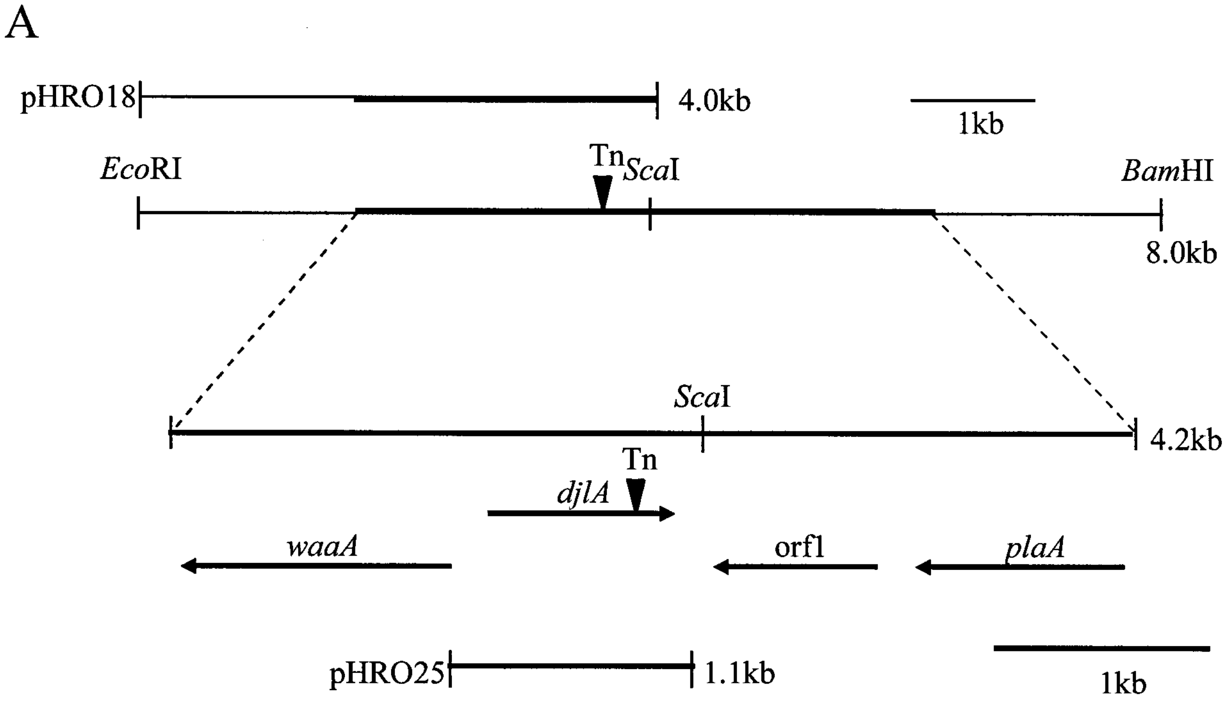
Recruitment of the RER. In mammalian macrophages and protozoa, *L. pneumophila* replicates intracellularly in specialized vacuoles surrounded by the RER of the host cells (25, 32). To determine the intracellular location of *L. dumoffii*, we examined J774 macrophages infected with wild-type and *djlA* mutant *L. dumoffii* by using transmission electron microscopy. At 8 h postinfection, the RER around 61 (37.2%) of 164 phagosomes containing wild-type strains were recruited (Fig. 6A) whereas we could not find any phagosomes containing the *djlA* mutant surrounded by RER or attached directly by ribosomes (0 of 153 phagosomes). This was also the case at 24 h (Fig. 6B and data not shown). Phagosomes containing *djlA* mutant cells appeared to harbor much debris, resulting from fusing lysosomes with these vacuoles, while phagosomes containing wild-type cells did not have any contents other than replicating *L. dumoffii* cells (Fig. 6). At 24 h postinfection, many phagosomes containing wild-type cells were broken and their inhabiting macrophages were lysed (data not shown).

Susceptibility of the *djlA* mutant to stress stimuli. In eukaryotic host cells, intracellular pathogens encounter hostile conditions such as toxic oxygen or nitrogen derivatives, intraphagosomal acidification, and harsh degradative enzymes (54, 62). As mentioned above, *djlA* is essential for intracellular growth of *L. dumoffii*. Thus, we examined whether the *djlA* mutant has an increased susceptibility to different environmental stresses. Since previous publications (12, 29) had demonstrated that *L. pneumophila* induces stress resistance in the stationary phase, *L. dumoffii* strains were grown to the stationary phase in BYE medium and subjected to acid shock, oxidative stress, osmotic stress, and heat shock (pH 3 for 5 min, 10 mM H_2O_2 for 30 min, 5 M sodium chloride for 30 min, and 48°C for 60 min, respectively). Compared to the wild-type strain, there was an elevated susceptibility to all stress conditions of the *djlA* mutant strain. There was an increase in the sensitivity of the mutant of 9.8-, 7.4-, 2.6-, and 1.6-fold on exposure to oxidative stress, osmotic stress, heat shock, and acid shock, respectively (Fig. 7). These results suggest that DjlA participated in the protection of *L. dumoffii* on exposure to environmental stress. In the *djlA*-complemented strain, in contrast, resistance to all stress stimuli was restored. The variability in the degree of complementation may result from the different expression of genes from the plasmid and the chromosome.

Presence of *djlA* in other *Legionella* spp. To determine whether *djlA* is also present in nonpathogenic *Legionella* species, PCR amplification with primers in the *djlA* gene was performed for 17 different *Legionella* strains. All the strains used in this experiment are listed in Table 2. The expected 790-bp band was observed in all *Legionella* strains tested except *L. jordanis* and *L. adelaidensis*, irrespective of whether the strain was pathogenic (data not shown). Thus, *djlA* is not unique to particular *Legionella* strains.

DISCUSSION

Legionella spp. are facultative intracellular bacteria that overcome host cell defenses. Although many studies have been



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B
                                    TMD
L. d MSLRDFFIITTWWGKIIGAFFGYLIAGPT-GAIFGLLVGNFFDRGLYNYFSNPHWLYYTEKRRAIQKIFFEA-TFLV  75
L. p MNLRDFFVITTWWGKILGAFFGYLTAGPV-GALFGILVGNFFDRGLVSYYSNPHWLYHAEKQRIVQKAFFEA-TFSI  75
E. c           MQYWGKIIGVAVALLMGGGFWGVVLGLLIGHMFDKAR----SRKMAWF-A-NQRERQ-AFF-ATTFEV  61

L. d MGHLAKADGRVSEQELDMAR-LFMDEMRLNGEQKTLAKHLFNEGKQSRFNLDSLLENLKKT--CKDNRDLLRLFI-D 148
L. p MGHVAKSDGRVSEQEISMAKSI-MNEMKLSKGQKDLAKRLFNEGKQADFNV-SL-ALIQLQRICKDNRDLLKLFV-D 148
E. c MGHLTKSKGRVTEADIHIASQL-MDRMNLHGASRTAAQNAFRVGKSDNYPLREKMRQ-FRSVCFGRFDLIRMFLEIQ 136

L. d IQYRAAQADG-LDSKKILLLDKIFSRLGFAPLHNQYRFYEDFGRSYSEPQYNTQEQP-QQSRQSQQSDSSSHSYSSY 223
L. p IQYRAAQVDG-LSSQKIHALDNIFTHLGFAPLHKQYRFYEDFG-SYFQQEQSKQHYHNQQEYKHT---SSSQG-QQG 219
E. c IQ--AAFADGSLHPNERAVLYVIAEELGI--SRAQFDQFLRMM----QGGAQFGGGYQQQT-------GGGNW-QQA 197
                                  J-domain
L. d SRYNYQPTKNNMDYAFALLEVSPKASKQEVKKAYRRLLSRNHPDKLIAQGLPQEMIKMANEKTQKIVKAYELICESKGW  302
L. p YKP-QSPPNTLA-HAFALLEVSPNANKQEVRRAYRRLLSRNHPDKLIAQGLPEEMIKLANDKTHQIMKAYELICETKGWX 296
E. c QRG---P--TLE-DACNVLGVKPTDDATTIKRAYRKLMSEHHPDKLVAKGLPPEMMEMAKQKAQEIQQAYELIKQQKGFK 271
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FIG. 3. Chromosomal arrangement of the region surrounding the *djlA* gene and sequence alignment of DjlA proteins. (A) At the top is a plasmid used for complementation studies (pHRO18) and an 8-kb region of the *L. dumoffii* cosmid clone including the *djlA* gene, along with the location of relevant restriction enzyme sites. The thick line represents the DNA region that we sequenced. Below these diagrams, the distance between the *djlA* gene and neighboring genes and the orientation and size of the transcribed genes are delineated by the arrows below the 4.2-kb sequenced region. Another plasmid used for complementation studies (pHRO25) is also shown. The site of the Tn*903*dII*lac*Z insertion (Tn) is indicated by the inverted arrowhead. The full names of the gene mapped are as follows: *waaA*, Kdo transferase gene; *djlA*, *dnaJ*-like A gene; *plaA*, lysophospholipase A gene. Orf1 is a putative open reading frame which showed no homology to known genes. (B) Sequence similarity of the predicted Dj1A protein of *L. dumoffii* (L.d, top line), *L. pneumophila* (L.p, middle line) and *E. coli* (E.c, bottom line). Amino acid residues conserved in the three sequences, appear in bold type. Gaps marked by dashes are introduced to reveal the maximal similarity among the sequences. The C-terminal J-domain and the N-terminal TMD are shown schematically above the sequences.

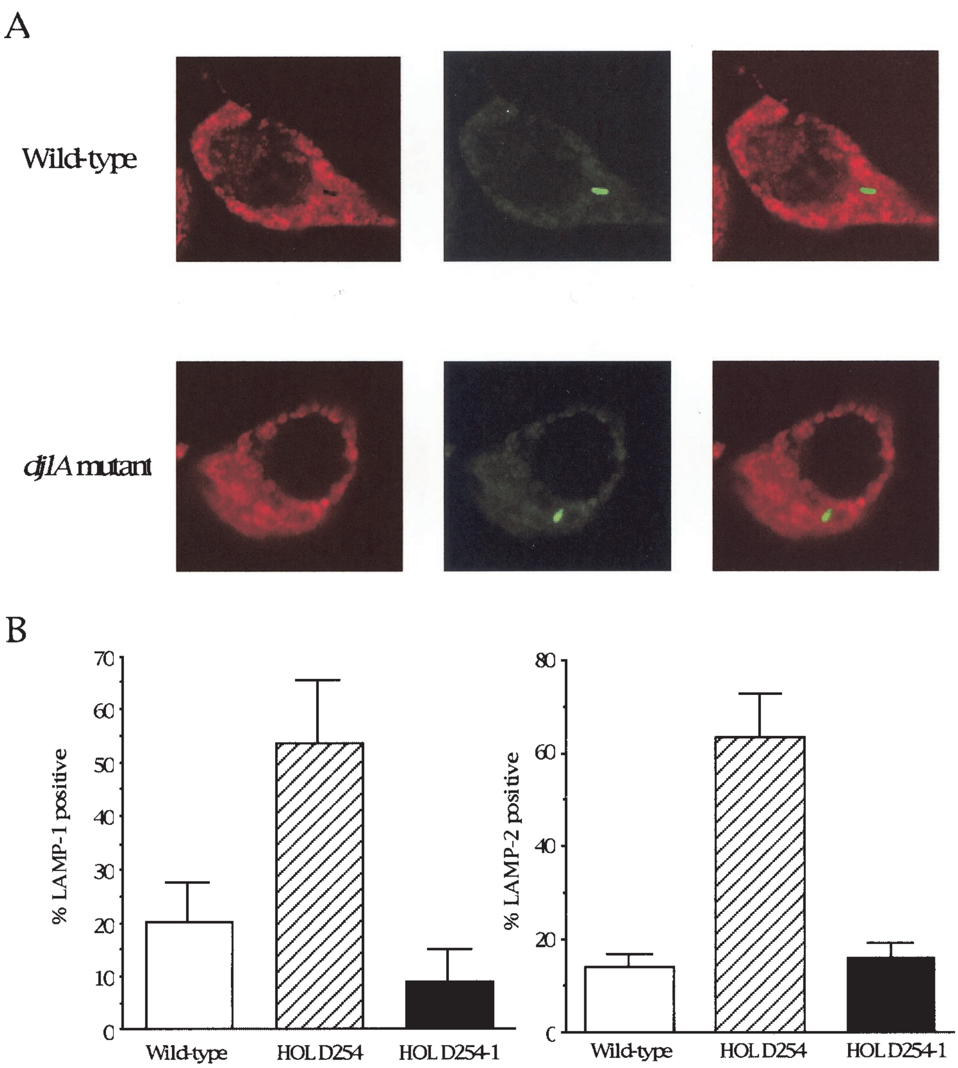

FIG. 4. Colocalization of the intracellular growth mutant with late endosomal/lysosomal marker LAMP-1 or LAMP-2 in J774 mouse macrophage cells by confocal laser-scanning microscopy. J774 macrophages were incubated with the *L. dumoffii* mutant or wild-type strain for 4 h. (A) Late endosomes and lysosomes stained with rat monoclonal antibody 1D4B, specific for LAMP-1, and Cy3-labeled anti-rat secondary antibody (red) are shown on the left. Bacteria stained with rabbit polyclonal antibody specific for *L. dumoffii* Tex-KL and Alexa488-labeled anti-rabbit secondary antibody (green) are shown in the middle. Merged images showing LAMP-1-positive bacteria (yellow) and LAMP-1-negative bacteria (green) are shown on the right. (B) Data were collected from about 100 intracellular bacteria in total. The percentage that is LAMP-1 or LAMP-2 positive was calculated by dividing the number of colocalizing intracellular bacteria by the total number of intracellular bacteria scored. The average and standard deviation described here were calculated from three coverslips per strain in two independent experiments.

undertaken to understand the intracellular life cycle of *L. pneumophila*, very few species other than *L. pneumophila* have been examined phenotypically. The aim of this study was to uncover how *L. dumoffii* survives and replicates in mammalian cells and to identify the genes of *L. dumoffii* needed for intracellular growth. We isolated 4 mutants that were defective in intracellular growth in macrophages and alveolar epithelial cells among 790 independently derived Tn*903*dII*lacZ* mutants of *L. dumoffii*. The defect in intracellular growth of these four mutants cannot be attributed to a defect in adherence or entry, because almost equal numbers of mutants and wild-type cells were present within mammalian cells at 0 h postinfection. Two of the four mutants had a transposon insertion in either the *dotC* or *icmB* homologues (5, 51, 60). The *dot*/*icm* genes are required for intracellular multiplication of *L. pneumophila* (5, 51, 60). Our results suggest that the *dotC* and *icmB* genes of *L. dumoffii* and *L. pneumophila* appear to perform similar functions. We propose that the *dot*/*icm* genes are involved in the pathogenesis of most *Legionella* species, since these genes are important in the intracellular growth of these distinct *Legionella* species.

One of the mutants defective in intracellular growth was shown to have a transposon insertion in the gene which had sequence similarity to the *djlA* gene (16). Cloning and sequence analysis of this gene revealed that the primary structure of *L. dumoffii* DjlA showed homology to other bacterial DjlA proteins (10, 16, 73). DjlA is the third DnaK cochaperone of *E. coli*, containing a J domain highly conserved in the DnaJ/

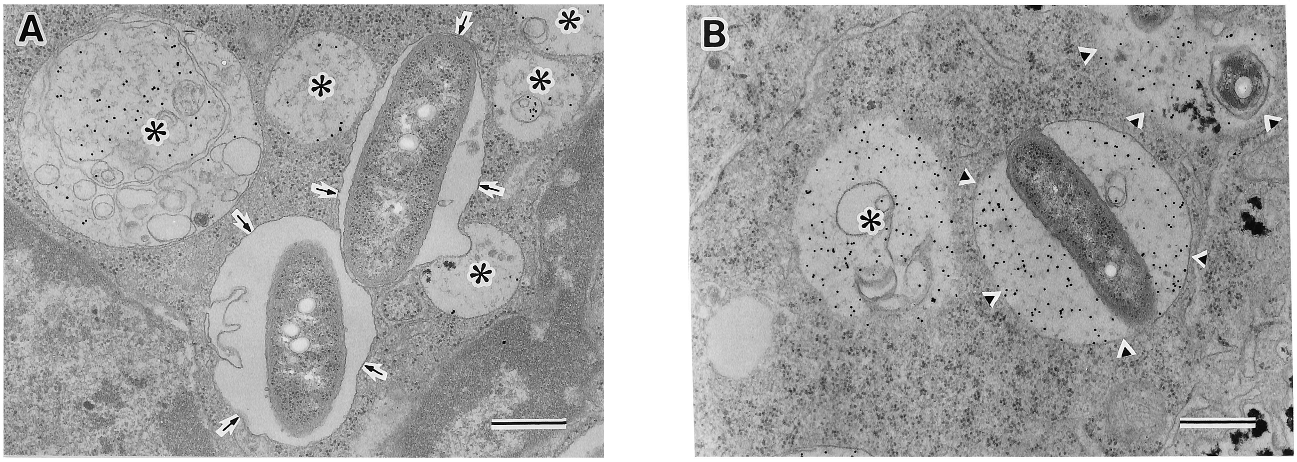

FIG. 5. Distribution of a lysosomal marker, BSA-gold, in phagosomes containing the wild-type strain or the *djlA* mutant strain. To label the lysosomal compartment, J774 cells were incubated with 15-nm BSA-gold overnight, washed, and then chased for 3 h. Cells were then infected with wild-type strain (A) or *djlA* mutant strain (B). At 4 h postinfection, the cells were fixed and processed for electron microscopy. Arrows in panel A indicate phagosomes containing no detectable gold; arrowheads in panel B indicate phagolysosomes containing BSA-gold; asterisks indicate, lysosomes containing BSA-gold. Bar, 0.5 μm.

Hsp40 family of molecular chaperones, including DnaJ and CbpA (16, 27, 65). CbpA is 39% identical to DnaJ along its entire length (64), while DjlA does not have any sequence similarity other than the J domain to DnaJ and CbpA in *E. coli* (26, 37). DjlA is unique in its structure and location in the DnaJ family. The J domain resides in the C terminus of DjlA but in the N terminus of other DnaJ family proteins. The N terminus of DjlA is integrated into the inner membrane through the single TMD, and the C-terminal J domain is located in the cytoplasm (16), while the whole of DnaJ and CbpA is localized in the cytoplasm. Moderate overproduction of *djlA* can trigger the synthesis of the colanic acid capsule in *E. coli*, mediated by the two-component regulatory system RcsC-RcsB, cooperating with DnaK and GrpE, but not DnaJ (15, 27, 37, 73). Unlike CbpA, DjlA could not adequately complement bacteriophage λ growth in a DnaJ-null background or restore bacterial growth above 40°C or below 16°C in the *dnaJ cbpA* null background in *E. coli* (15, 26, 37). The DjlA deletion mutant exhibits no apparent growth phenotype in *E. coli* (15, 16, 26). Thus, the true role of DjlA has been unclear.

We demonstrated that the *djlA* mutant of *L. dumoffii* exhibited a defective growth phenotype in mammalian cells and protozoan hosts. Phagosomes containing wild-type *L. dumoffii* excluded the late endosomal/lysosomal markers LAMP-1 and LAMP-2 and a lysosomal marker, BSA-gold, and were surrounded by RER in J774 macrophages, while *djlA* mutant-bearing phagosomes contained LAMP-1, LAMP-2, and BSA-gold and were not surrounded by RER (Fig. 4 to 6). It has been

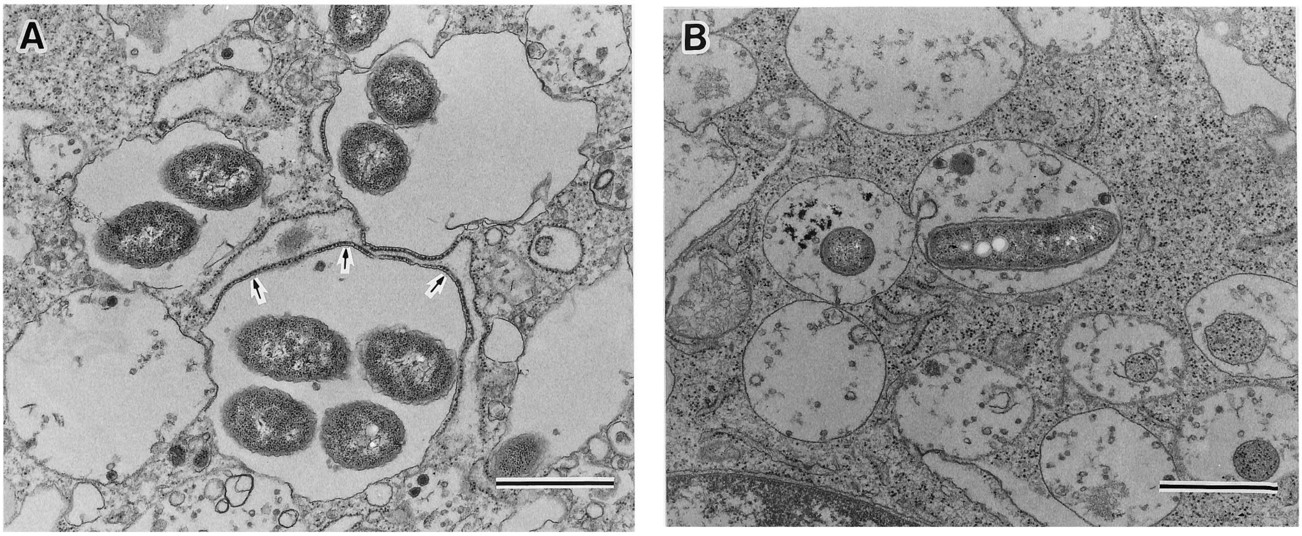

FIG. 6. Transmission electron micrographs of J774 mouse macrophages infected by the wild-type *L. dumoffii* (A) and the *djlA* mutant HOLD254 (B) at 8 h after infection. (A) Wild-type *L. dumoffii*-containing phagosomes were surrounded by RER (arrows). (B) HOLD254-containing phagosomes appeared to harbor much debris resulting from fusing lysosomes. Bar, 1.0 μm.

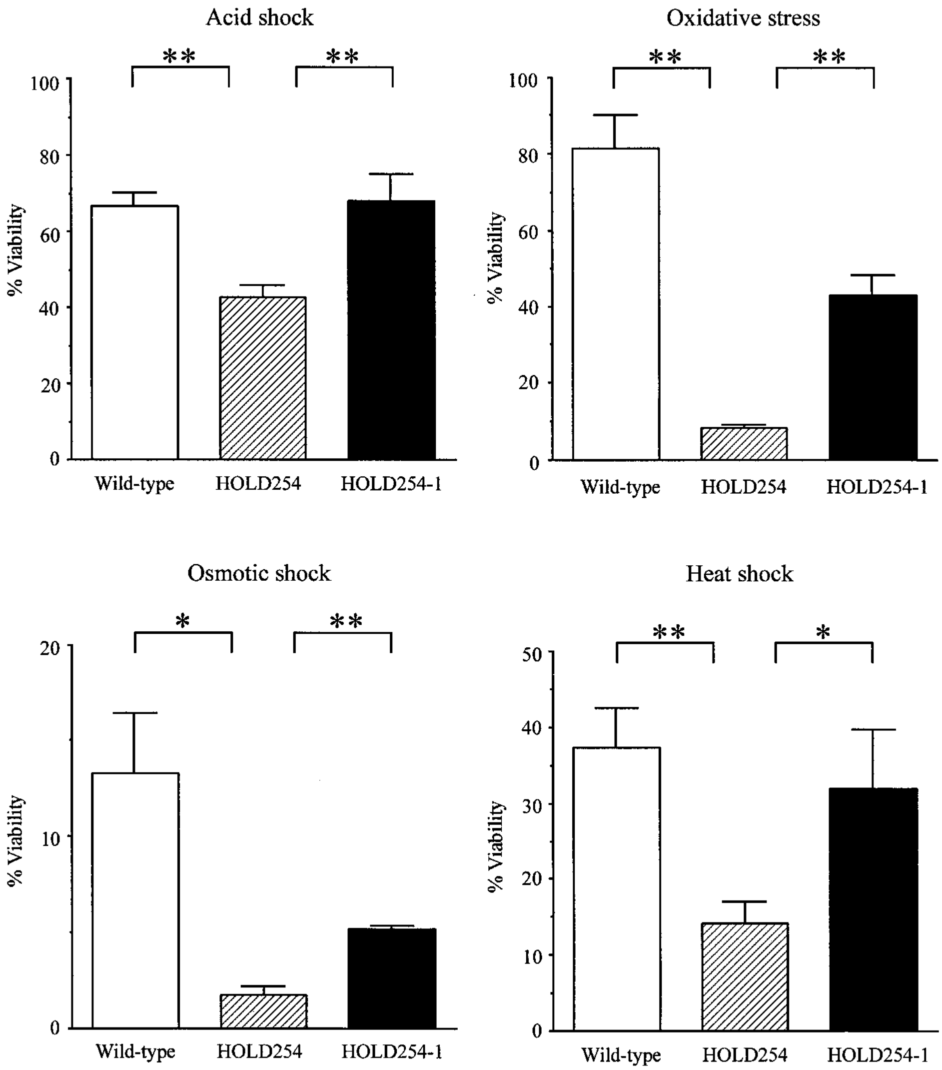

FIG. 7. Sensitivities of in vitro-grown stationary-phase wild-type *L. dumoffii* (open bars), the *djlA* mutant strain (hatched bars), and the *djlA* complemented strain (solid bars) to oxidative stress, osmotic stress, acid stress, and heat shock (10 mM hydrogen peroxide for 30 min, 5 M sodium chloride for 30 min, pH 3 for 5 min, and 48°C for 60 min, respectively). Stationary-phase cultures were exposed to each stress as described in Materials and Methods. The percentage of viable bacteria was calculated by dividing the CFU obtained from plating the bacteria onto BCYE agar plates following exposure to the indicated stress by the CFU of the bacteria obtained from plating the bacteria onto BCYE agar plates prior to exposure to the stress and multiplying by 100. Experiments were performed at least three times, and the results represent the mean and standard deviation. Results were analyzed for significance by analysis of variance and by a two-tailed, unpaired *t* test. Asterisks indicate significant differences between the *djlA* mutant and two other strains. (*, $P < 0.01$; **, $P < 0.001$).

reported that *L. pneumophila* is targeted into RER-surrounding phagosomes that do not fuse with lysosomes in mammalian cells (25, 33), while *L. micdadei* is targeted into RER-free phagosomes that are thought to fuse to lysosomes in mammalian cells (3, 36). Doyle et al. (20) reported that virulent *L. longbeachae*-containing phagosomes were surrounded by RER but avirulent *L. longbeachae*-containing phagosomes did not have RER. Our observations suggest that *L. dumoffii* might replicate in phagosomes which have not fused with lysosomes and are able to recruit host cell organelles, similar to that reported for *L. pneumophila*. The *djlA* mutant seemed to be intact (Fig. 5B), and no loss of CFU was observed during the infection (Fig. 1A and 2A). It is possible that the mutant bacteria are in either a late endosomal or a nondegradative lysosomal compartment, as described by Joshi et al. (35). The frequency of recruitment of *L. dumoffii* RER at 8 h is lower than that reported for *L. pneumophila* (32). We suspect that association with ER and avoidance of lysosomes by *L. dumoffii* is temporary, as shown for *L. pneumophila* (63).

Although the precise function of DjlA is unclear, it does not seem to play a direct role in intracellular trafficking. DjlA might contribute to folding or transportation of the proteins, such as Dot/Icm proteins, which play an important role in intracellular survival and growth. Most of the Dot/Icm proteins are located in the bacterial membranes, where they may associate to form a large transport complex, the type IV secretion apparatus (17, 43, 51, 60, 61). DjlA might cooperate with Dot/Icm proteins through their interaction in the membranes, since the N-terminal portion of DjlA is located in the cytoplastic membrane (16, 37). It has been reported that the two-component regulatory system, PhoP-PhoQ, of *Salmonella enterica* serovar Typhimurium plays an essential role in survival within macrophages (28). It is possible that DjlA promotes *L. dumoffii* to adapt to intracellular environments and to coordinate with the two-component signal transduction systems. In vitro, DjlA-deficient mutants showed an increased susceptibility to several stresses, including oxidative stress, that might be encountered by bacteria in mammalian cells. DjlA might protect the genes or proteins, including Dot/Icm and catalase-peroxidase (7), that are important for intracellular growth, from harmful stress in a direct or indirect manner. Several lines of evidence for the important role of stress proteins in intracellular growth and virulence have been reported for intracellular pathogens; these include DnaK of *Brucella suis* (38), ClpC and ClpP of *Listeria monocytogenes* (24, 54), Lon of *B. abortus* (53), and GsrA of *Yersinia enterocolitica* (69). In *L. pneumophila*, at least 30 proteins are included during the intracellular infection of macrophages and at least 13 of these proteins, including GroEL (Hsp60), GroES, and GspA, are also induced by several stress conditions in vitro (1, 2, 21). Recently, Pedersen et al. (50) demonstrated direct evidence for the role of the stress protein of *L. pneumophila*, HtrA, during intracellular growth in mammalian cells but not in protozoan cells. Our data indicated that DjlA plays an important role during intracellular growth in both mammalian and protozoan cells. Besides Dot/Icm proteins, stress proteins or molecular chaperones might play an important role in the intracellular growth of the *Legionella* species.

In conclusion, we showed the essential role of *L. dumoffii* Dot/Icm homologues and DjlA during the intracellular infection of mammalian cells and protozoa. The precise mechanism of DjlA involvement in intracellular multiplication, including interaction with DnaK, remains to be elucidated. Further investigation of specific substrates with which DjlA interacts will lead to a better understanding of the intracellular survival mechanism in the *Legionella* species.

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