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Differences in protein synthesis between wild type and intracellular growth-deficient strains of *Legionella pneumophila* in U937 and *Acanthamoeba polyphaga*

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Abstract

An important aspect of Legionnaires' disease is the growth of the causative agent, *Legionella pneumophila*, within infected host cells. Many proteins including stress proteins of *L. pneumophila* were strongly induced in a wild type strain that had been used to infect U937 human macrophage-like cells. In contrast, the expression of the proteins was much weaker within a protozoan host, *Acanthamoeba polyphaga*. The results suggested that active bacterial protein synthesis is required more within macrophages than within protozoa for adaptation of *L. pneumophila* to intracellular environments. The synthesis of these proteins was not observed in intracellular growth-deficient strains after infection in either type of host cells. The inability of protein synthesis in these strains is correlated with their inability of intracellular growth. Furthermore, on U937 infection, the synthesis of β-galactosidase encoded in an inducible reporter construct immediately ceased in the in intracellular growth-deficient strains after infection, while the wild type strain was able to synthesize it during the course of infection. These results suggested that the intracellular growth of *Legionella pneumophila* within macrophages requires active protein synthesis from an earlier stage of bacterial infection.

Keywords: *Legionella pneumophila*; Pathogenicity; Stress; Intracellular growth; β-galactosidase; Protein synthesis

1. Introduction

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular bacterium. The hHallmark of *L. pneumophila* pathogenicity is the ability to replicate within host cells, especially in human macrophages [1]. It is considered that this microbe survives within the host cells through resistance against intracellular microbicidal activity [2,3]. In natural environments, ubiquitous free-living amoebae are thought to provide an intracellular environment that permits the replication of this microbe [4]. In a project on the water samples from cooling towers that had been implicated in legionellosis through dissemination of *L. pneumophila*, bacteria and protozoa were isolated simultaneously from the same water source in all cases [5]. Another report has shown that legionellae isolates exhibiting pathogenicity in guinea pigs were able to replicate within the protozoan *Tetrahymena pyriformis* [6]. These reports suggested that intracellular environments within protozoa could provide favorable conditions for the growth of *L. pneumophila*. Thus, the microbes must be able to adapt themselves to a variety of intracellular microenvironments efficiently. To achieve this, bacteria may express different sets of genes appropriately in response to different intracellular microenvironments to facilitate their intracellular survival and growth. It can be reasonably expected that proteins whose expression is specifically regulated within mammalian intracellular compartments could constitute potential virulence factors.

The regulated expression of a number of bacterial proteins within host cells has been demonstrated for several intracellular parasites. The expression of more than 30 proteins of *Salmonella typhimurium* was up-regulated whereas that of 136 proteins was down-regulated upon infection in human macrophages [7]. The expression of heat shock proteins GroEL and DnaK has been shown to be up-regulated. In *Mycobacterium tuberculosis*, six proteins that are absent from bacteria during extracellular growth, with or without

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stress, were induced upon infection in human THP-1 cells [8]. It has also been shown that some macrophage-induced (MI) proteins play a critical role in the intracellular survival of *S. typhi* during infection [9].

In the case of *L. pneumophila*, Abu Kwaik et al. have reported that virulent strains showed the induction of expression of several proteins, including heat shock proteins GroEL and GroES, and global stress protein (GspA), during infection in macrophages [10–12]. It has also been demonstrated that the expression of HtrA/DegP, a stress-induced protease/chaperone homologue, is increased by 12,000- to 20,000-fold throughout intracellular growth in macrophages as compared with extracellular growth in vitro. The authors concluded that HtrA/DegP is indispensable for intracellular replication within mammalian macrophages and alveolar epithelial cells [13]. In addition, heat shock protein Hsp60 was found to be induced during intracellular growth in mouse L929 cells [14,15]. Furthermore, Hsp60 has been implicated in the bacterial attachment to and entry into epithelial HeLa cells [16].

As one of representative virulence factors of *L. pneumophila*, a type IV secretion apparatus, Icm/Dot, has been believed to deliver presumed effector molecules into the cytoplasm of host cells during phagocytosis resulting in the establishment of *L. pneumophila* replication vacuole [17–22]. Until now, the relation between the function of Icm/Dot system and bacterial proteins induced in intracellular environments (including stress-related proteins) towards the establishment of replicative phagosomes has not been investigated. Furthermore, it is not known how some stress-induced bacterial proteins may contribute to the virulence of *L. pneumophila*.

In the present study, we determined whether or not the expression of *L. pneumophila* stress-induced proteins differs between the intracellular environments of human macrophage cells line and protozoa. We also examined whether or not the stress-response differs between wild type and intracellular growth-deficient strains that are not able to grow intracellularly. We then investigated mechanisms underlying the differences of the stress responses of bacteria upon infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *L. pneumophila* strains used in this work were JR32, a streptomycin-resistant, restriction-negative mutant of *L. pneumophila* Philadelphia-1, which is a wild type strain [23]; LELA3118, a *dotA* mutant [23]; LELA3473, an *icmR* mutant [23]; and 25D, an in vitro attenuated strain that cannot grow intacellularly [24]. All *L. pneumophila* strains were grown on CYE medium [25] or in AYE broth [26].

2.2. Cell culture

Macrophage-like U937 cells were maintained in RPMI 1640 (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (ICN Biomedicals, Aurora, OH). Axenic *Acanthamoeba polyphaga* was cultured as adherent cells in PYG medium. All cells were cultured under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.3. Analysis of de novo protein synthesis after heat shock in vitro

The heat shock response in *L. pneumophila* was monitored by measuring the rate of incorporation of radiolabeled amino acids into newly synthesized proteins during heat shock stress. In brief, the *L. pneumophila* strains were grown to mid-logarithmic phase and an OD_{550} of bacterial cultures was adjusted to 0.6. Aliquots (2.5 ml) were transferred to sterile 50 ml conical tubes (Fisher Scientific, Pittsburgh, PA), and then the tubes were incubated at 28 °C with shaking. To each sample, 200 μCi of a ^{35}S-labeled methionine/cysteine mixture (Tran^{35}S label; ICN Biomedicals, Irvine, CA) was added. The samples were briefly mixed by vortexing, and then transferred to a 42 °C water bath with shaking or continuously incubated at 28 °C as a control. After 1 h, 250 μl of aliquots were removed and placed immediately on ice. Bacterial proteins were precipitated by adding ice-cold trichloroacetic acid (TCA) at a final concentration of 10% to each sample. Samples corresponding to an equivalent of bacteria were analyzed by SDS-PAGE (7% gel). The gels were dried under vacuum, and then the radiolabeled proteins were detected with a bio-imaging analyzer BAS-2000II (Fuji Film, Tokyo, Japan) and analyzed with Image Gauge software (Fuji Film). Total bacterial proteins were visualized directly on SDS-PAGE gel by staining with Coomassie Brilliant Blue R-250 (Merck, Darmstadt, Germany).

2.4. Immunoprecipitation

Immunoprecipitation (IP) of *L. pneumophila* DnaK was carried out using rabbit antiserum raised against *E. coli* DnaK kindly provided by Dr Chieko Wada (Kyoto University, Japan). Tran^{35}S label (200 μCi) was added to 2.5 ml of a mid-logarithmic phase bacterial culture (OD_{550} = 0.6). The bacterial culture was briefly mixed by vortexing and then incubated continuously at 28 °C or treated at 42 °C for 1 h. Three hundred micro liter of bacterial cultures were treated with ice-cold TCA at a final concentration of 10%, and precipitated proteins were resuspended in 1 ml of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS and 50 mM Tris–HCl, pH 8.0). Five micro liter of rabbit anti-*E. coli* DnaK serum (1:200 dilution) was added, followed by incubation for 1 h at 4 °C on a rotator. A 40 μl packed volume of Protein G-agarose (Roche, Mannheim, Germany) was added to the sample, and then the suspension was incubated overnight at 4 °C on a rotator. The immune complex was recovered by centrifugation at 1900*g* for 5 min, and then washed five times by centrifugation in 1 ml of RIPA buffer. After the final wash, the immune complex was re-suspended in 30 μl of RIPA buffer, and then 30 μl of SDS sample buffer (0.02% pyronine G, 1% SDS, 1% 2-mercaptoethanol, 20% glycerol, 10 mM Tris–HCl, pH 6.8) was added.

The samples were boiled for 5 min to denature and dissociate the antigen, followed by analysis by SDS-PAGE (7% gel).

2.5. Bacterial cellular fractionation after exposure to heat shock

Tran^{35}S label (200 μCi) was added to 2.5 ml of a mid-logarithmic phase bacterial culture ($OD_{550}=0.6$). The bacterial culture was briefly mixed by vortexing and then incubated continuously at 28 °C or treated at 42 °C for 1 h. Samples of 300 μl of bacterial culture were treated with ice-cold trichloroacetic acid (TCA) at a final concentration of 10% to precipitate whole cell proteins. Another 2.5 ml aliquot of culture was centrifuged at 550*g* for 15 min at 4 °C. The proteins in the supernatant were precipitated with TCA and this sample was referred to as the supernatant secreted proteins. On the other hand, the pellet of bacteria was disrupted by sonication (UD-210; Tomy, Tokyo, Japan). The lysate was centrifuged at 550*g* for 20 min at 4 °C to remove cell debris, and then the supernatant was centrifuged at 100,000*g* for 1 h at 4 °C. The pellet was referred to as the membrane fraction. TCA was added to the supernatant and the precipitate was referred to as the cytoplasmic protein fraction. Samples of each cellular fraction prepared from an equivalent number of bacteria were analyzed by SDS-PAGE (7% gel).

2.6. Detection of L. pneumophila stress proteins expressed within U937 and A. polyphaga cells

U937 cells (3×10^6 per well, FALCON six-well plate) (Becton Dickinson, Franklin Lakes, NJ) were differentiated for 48 h, using phorbol 12-myristate 13-acetate as described previously [10]. Differentiated cells were nonreplicative, adherent, macrophage-like cells. Differentiated U937 cells were infected with post-logarithmic phase *L. pneumophila*. A MOI (multiplicity of infection) of 10 was used for all strains for U937 cells. Heat-killed JR32 strains were phagocytosed in the ratio of 10 bacteria to 1 U937 cell. *A. polyphaga* was infected with post-logarithmic phase JR32 strains at a MOI of 50, and 25D and LELA3118 strains at a MOI of 100. Heat-killed JR32 strains were phagocytosed in the ratio of 50 bacteria to 1 *A. polyphaga* cell. The plates were spun at 250*g* for 20 min to synchronize the infection [21, 27–29], and the time point at the end of this centrifugation was designated as 0 h post-infection. After additional incubation for 1 h at 37 °C, the cells were washed three times with an appropriate culture medium, and then incubated in culture medium containing 50 μg ml^{-1} gentamicin and 100 μg ml^{-1} of cycloheximide to kill extracellular bacteria and inhibit protein synthesis by the U937 or *A. polyphaga* cells. At 12 h post-infection, 300 μCi of Tran^{35}S label was added and the proteins were metabolically labeled for 2 h. To harvest the intracellular bacteria that incorporated radioactivity, the infected monolayer was washed three times with culture medium and then the cells were lysed with water osmotically (U937) or with 0.05% TritonX-100 (*A. polyphaga*). Radiolabeled bacterial proteins were collected by TCA precipitation. The precipitated proteins were analyzed by SDS-PAGE (7% gel). The radioactivity of TCA-precipitated materials was equalized for each sample before application to the gel. To monitor the recovery of intracellular bacteria from each type of host cell under the conditions used for metabolic labeling, we followed the same procedure except for the addition of the labeled amino acids. For intracellular bacterial growth assay within U937 cells, we did the same procedure using a six well plate except the addition of the labeled amino acids and cycloheximide. Aliquots of the cell lysate were diluted and plated on CYE plates for enumeration of the intracellular bacteria.

2.7. Immunofluorescence microscopy for L. pneumophila-infected U937 cells

The recombinant fusion protein comprising *L. pneumophila* DnaK (^{Lp}DnaK) and a biotinylated protein was produced by subcloning the ^{Lp}DnaK gene into the PinPoint Xa-3 vector (Promega, Madison, WI) and expression in the *E. coli* JM109 strain. The fusion protein was purified on a column of SoftLink Soft Release Avidin Resin (Promega) by elution with 5 mM biotin dissolved in the cell lysis buffer (50 mM Tris–HCl, pH7.5, 50 mM NaCl, 5% glycerol) according to the technical manual. The protein was characterized by SDS-PAGE. Anti-recombinant *L. pneumophila* DnaK serum was obtained by immunizing a rabbit with the recombinant DnaK fusion protein following a standard immunization protocol using Freund's adjuvant. Samples for immunofluorescence were prepared according to the method previously described [29]. In brief, U937 cells were cultured and then allowed to differentiate on a cover slip in the well of a six-well culture plate (Becton Dickinson). The differentiated U937 cells were infected with *L. pneumophila* for 1 h. The cells were continuously incubated in culture medium containing gentamicin for 11 h. Thus, 12 h post-infection samples were examined. The cover slips were fixed in 4% paraformaldehyde for 15 min at room temperature, and then non-specific binding sites were blocked with 3% BSA in PBS for 30 min. The cells were then permeabilized with 0.5% TritonX-100 in PBS for 10 min. Intracellular *L. pneumophila* DnaK was detected by 1 h incubation with rabbit anti-recombinant *L. pneumophila* DnaK serum (1:10 dilution), followed by another 1 h incubation with Alexa Fluor 546-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) (1:100 dilution) at room temperature. The nuclei of both the bacteria and U937 cells were stained with TO-PRO-3 iodide (Molecular Probes) for 1 h. Between the incubations, the coverslips were washed three times with PBS for 5 min. The coverslips were then mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA). Samples were observed under a Carl Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss, Germany).

2.8. Protein synthesis from the reporter gene

Plasmid pAB-1, which has *lacZ* with an IPTG-inducible *tac* promoter [30], was introduced into *L. pneumophila* by electroporation using a Gene Pulser (BIO-RAD, Hercules,

CA) under the conditions of 2.3 kV with 25 μF capacitance. In order to examine the ability to express *lacZ*, bacteria that had been cultured in AYE until an OD_{550} of 0.3–0.5 (early-logarithmic phase) were induced with 1 mM IPTG for 30 min at 37 °C. The enzyme activity of β-galactosidase in the bacterial lysate was measured by quantifying the hydrolysis of *O*-nitrophenyl-β-D-galactopyranoside (ONPG), following Miller's method [31]. The enzyme activity was expressed as Miller's units, the activity being normalized per unit number of bacteria.

To monitor *lacZ* expression by intracellular bacteria, differentiated U937 cell monolayers were infected with *L. pneumophila* carrying pAB-1 at a post-logarithmic phase (OD_{550}=2.0–2.2). The plates were immediately centrifuged at 250*g* for 20 min at room temperature to facilitate contact between bacteria and U937 cells. The time point at the end of centrifugation was designated as 0 h post-infection. Cells were then incubated for 1 h at 37 °C in RPMI 1640 containing 10% FBS to allow internalization of the bacteria into the U937 cells. Then gentamicin (final concentration, 50 μg/ml) was added to kill extracellular bacteria, and the plates were continuously incubated for an appropriate period. After the indicated incubation periods (expressed as hours post-infection), 1 mM IPTG was added to the wells to induce *lacZ* transcription from viable intracellular bacteria, and then the cells were incubated for an additional 30 min at 37 °C. For the 0.5 h-sample, the bacteria were internalized for 30 min in the absence of gentamicin, and then IPTG induction was carried out in the presence of gentamicin. For the 0 h sample, the plate was centrifuged for 5 min and then brief 5 min incubation at 37 °C was performed. To minimize the contribution of extracellular bacteria, the U937 cell monolayer was washed three times in RPMI 1640 and then treated with 1 mM IPTG for 30 min. The cells were then lysed by adding Z buffer (60 mM Na_2HPO_4, NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.27% 2-mercaptoethanol). β-Galactosidase activity in the lysate was measured by quantifying the hydrolysis of ONPG in same manner as described above. The enzyme activity was expressed as units/well.

3. Results

3.1. Unimpaired responses of icm/dot mutant strains of L. pneumophila in the induction of heat shock proteins in vitro

Legionella pneumophila icm/dot gene products, which comprise the type IV secretion system, participate in creating an organelle that permits replication inside host cells such as macrophages and amoebae [32,33]. On the other hand, for most pathogenic bacteria, the expression of stress-induced proteins is induced for adaptation to environments in phagocytic cells [7–12]. To determine whether or not mutations in *icm/dot* genes affect the expression of stress-induced proteins *in vitro*, we employed heat shock at 42 °C. We compared the protein synthesis by wild type JR32 and two mutant strains, LELA3118 (*dotA*) and LELA3473 (*icmR*), by means of ^{35}S-Met/Cys metabolic labeling and SDS-PAGE. An obvious

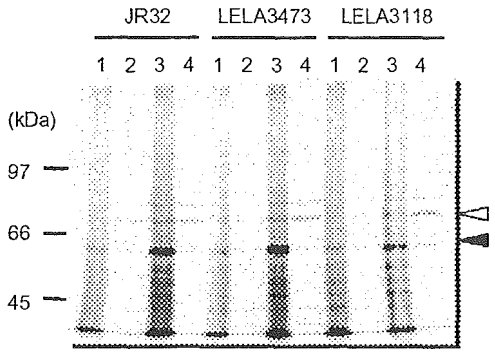

Fig. 1. Comparison of the heat shock response between the wild type and *icm/dot* mutant strains of *L. pneumophila* in vitro. Wild type strain JR32 and two *icm/dot* mutants, LELA3473 (*icmR* mutant) and LELA3118 (*dotA* mutant), were grown to the mid-log phase at 28 °C. After the addition of Tran^{35}S label, the bacteria were incubated at either 42 °C (heat shock) or 28 °C (control) for 1 h. Lane 1, whole bacterial proteins without heat shock; lane 2, immunoprecipitate (IP) of whole bacterial proteins without heat shock; lane 3, whole bacterial proteins with heat shock; lane 4, IP of whole bacterial proteins with heat shock. Open and solid arrowheads indicate the bands of DnaK and Hsp60, respectively.

increase in the radioactivity of whole bacterial proteins was observed after heat shock (Fig. 1, lanes 1 and 3). A 59 kDa protein, presumably Hsp60, and a 74 kDa protein were found to be strongly induced in whole cell lysates after heat shock. There was no difference in the protein expression pattern between the wild type and mutant strains. To specifically investigate DnaK protein as a representative heat shock protein and molecular chaperone, DnaK was immunoprecipitated from radiolabeled bacterial lysates using antiserum against *Escherichia coli* DnaK. The specific signal for DnaK appeared at 74 kDa (lanes 2 and 4). The induction of DnaK proteins was observed to be equal in the wild type and mutant strains upon heat shock.

3.2. Subcellular distribution of the proteins induced by in vitro heat shock in the wild-type and mutant strains deficient in intracellular growth

A whole cell-labeled lysate of JR32, LELA3118, or a spontaneous mutant 25D strain was separated into secreted (culture supernatant), cytoplasmic (including periplasm), and membrane fractions after heat shock treatment. The proteins in each fraction were analyzed by SDS-PAGE to determine the cellular localization of proteins induced by heat shock (Fig. 2). The majority of Hsp60 (59 kDa) was localized in the cytoplasmic fraction after heat shock treatment. The overall pattern of the subcellular distribution of Hsp60 was not different between the wild type and mutant strains. For JR32, a weak band representing Hsp60 was also observed for the membrane and secreted protein fractions. The localization of DnaK (74 kDa) appeared to be restricted to the cytoplasmic fraction. No difference in the subcellular distribution of DnaK was evident between the wild type and mutant strains. Additional bands of 50 and 78 kDa heat-induced proteins were observed for the secreted protein fraction of JR32. These

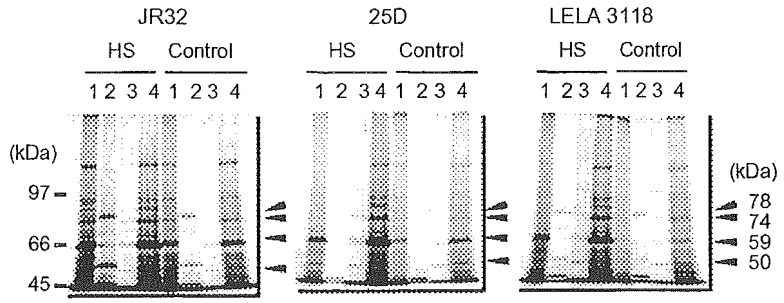

Fig. 2. Subcellular localization of heat shock proteins. JR32 (A), 25D (B), and LELA3118 (C) were grown to the mid-log phase at 28 °C. After Tran^{35}S label had been added, incubation was performed at 28 °C (control) or 42 °C (heat shock; HS) for 1 h. Lane 1, whole bacterial proteins; lane 2, secreted proteins into the culture supernatant; lane 3, membrane proteins; lane 4, cytoplasm and periplasm proteins. Open arrowheads on the right indicate the positions of protein bands of interest. The positions of molecular weight standards were shown on the left.

bands were hardly observed for 25D, and they were not strongly induced in LELA3118. These bands were not identified in this study.

3.3. Comparison of stress-induced protein expression of L. pneumophila in different intracellular environments: human macrophages and amoebae

We examined the protein expression profiles of *L. pneumophila* inside macrophages as human hosts and amoeba cells as environmental hosts. The bacteria in post-logarithmic phase were used for infection. The intracellular protein expression from 12 to 14 h post-infection was monitored as to 2 h pulsed incorporation of ^{35}S-Met/Cys into bacterial proteins, while host protein synthesis was inhibited by cycloheximide. Many proteins were up-regulated in JR32 within U937 cell lines (Fig. 3A), and the extent of protein induction was rather strong compared with in the case of heat shock in vitro. The synthesis of DnaK and Hsp60 was 2.7- and 4.2-fold greater (lane 3), respectively, than that after heat shock at 42 °C for 1 h (lane 1), on quantification analysis using BAS-2000II. In control experiments, no band was observed for U937 cells that had phagocytosed heat-killed bacteria (lane 4), U937 cells by themselves (lane 5), or bacteria exposed to RPMI 1640 tissue-culture medium (lane 6).

On the other hand, few bacterial proteins of the JR32 strain were observed within *A. polyphaga* (Fig. 3B). Although cycloheximide treatment has been performed to inhibit host protein synthesis, there was a considerable background of proteins from *A. polyphaga*. This background may be due to resistance to cycloheximide (lanes 3 and 4). Bacterial proteins specifically synthesized in amoebae appeared to be more limited (lane 1). Furthermore, the expression of DnaK and Hsp60 was 2.7- and 9.7 fold less (lane 1), respectively, than that after heat shock at 42 C for 1 h (lane 6), on quantification analysis using BAS-2000II. Considering the in vitro heat shock treatment as an internal standard, 7.1- and 41.0-fold more DnaK and Hsp60 were synthesized, respectively, in bacteria infecting U937 cells as compared with those infecting amoebae. In control experiments, the 59 kDa Hsp60 band was essentially absent for amoebae that had phagocytosed heat-killed bacteria (lane 2), amoebae by themselves (lane 3), and bacteria exposed to PYG amoeba culture medium (lane 5).

3.4. Expression of stress-induced proteins in wild type and intracellular growth-deficient L. pneumophila strains after phagocytosis in human macrophages

The strong induction of DnaK and Hsp60 synthesis in the JR32 wild type strain after infection in U937 cells was shown in Fig. 3. We compared the induction of these proteins in the

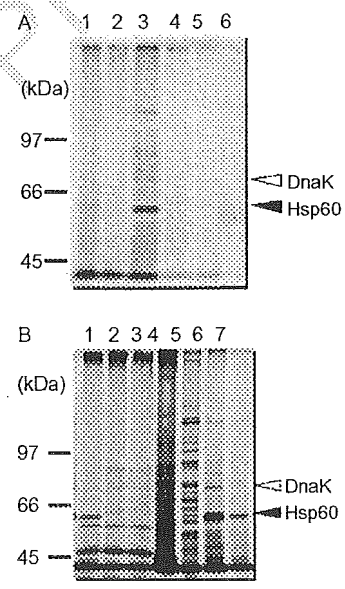

Fig. 3. Difference in the protein expression profiles of JR32 within different host cells. Open and solid arrowheads indicate the bands of DnaK and Hsp60, respectively. (A) Whole bacterial protein expression by intracellular JR32 within U937 macrophage-like cells. Lane 1, in vitro heat shock at 42 °C for 1 h; lane 2, in vitro control at 28 °C for 1 h; lane 3, within U937; lane 4, within U937 (heat-killed JR32 was phagocytosed); Lane 5, U937 lysate (without bacterial infection); lane 6, JR32 exposed to RPMI1640 tissue culture medium at 37 °C for 14 h. (B) Whole bacterial protein expression by intracellular JR32 within *A. polyphaga*. Lane 1, within *A. polyphaga*; lane 2, within *A. polyphaga* (heat-killed JR32 was phagocytosed); lane 3, *A. polyphaga* alone with cycloheximide treatment; lane 4, *A. polyphaga* alone without cycloheximide treatment; lane 5, JR32 exposed to PYG medium for protozoa culture at 37 °C for 14 h; lane 6, in vitro heat shock at 42 °C for 1 h; lane 7, in vitro control at 28 °C for 1 h.

mutant strains, 25D and LELA3118, using the same method. Regarding the intracellular growth phenotype, efficient replication of wild type JR32 strain and defects in intracellular growth of 25D strain and *dotA* mutant LELA3118 strain within U937 and *A. polyphaga* cells, was basically consistent with previous observations [23,34,35]. Although there was no difference in the response to heat shock stress in vitro between the wild type and mutant strains (Figs. 1 and 2), these mutant strains were not able to synthesize proteins including DnaK and Hsp60 (Fig. 4A, lanes 3 and 4). In contrast, wild type strain JR32 synthesized many proteins including DnaK and Hsp60 (lane 2). The radioactivity of all samples subjected to SDS-PAGE was equal. Furthermore, the MOI was chosen depending on the combination of bacterial strain and host cell so that the number of live bacteria at 14 h post-infection was almost equal (see Materials and methods). To confirm this in a particular experiment, intracellular bacteria were recovered at 14 h post-infection, and the number of colony forming units was determined. Equal numbers of bacteria were recovered from JR32- and LELA3118-infected U937 cells (Fig. 4B). In the case of 25D, the number of recovered bacteria was higher than those for the other two strains. This could be a reflection of experimental variation such as viability difference of bacteria or difference in the rate of bacterial internalization into U937 cells. And previous reports that the internalization rate of 25D strain into macrophage is slightly higher than that of JR32 strain, may support this evidence [24,36]. Cycloheximide treatment does not inhibit the bacterial growth [37]. Therefore, the lack of expression of stress-induced proteins in the mutant strains is not attributable to the elimination or killing of mutant bacteria in U937 cells at this time point.

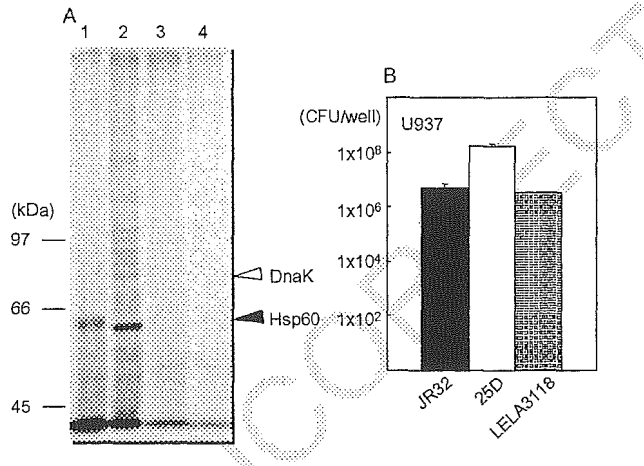

Fig. 4. Intracellular protein expression of the wild type and intracellular growth-deficient strains within U937. (A) Bacterial protein expression profiles of the wild type and intracellular growth-deficient strains within U937. Lane 1, JR32 with in vitro heat shock at 42 °C for 1 h; lane 2, JR32 within U937; lane 3, 25D within U937; lane 4, LELA3118 within U937. Open and solid arrowheads indicate the bands of DnaK and Hsp60, respectively. (B) The number of live intracellular bacteria at 14 h post-infection on a replicate experiment. Intracellular bacteria were recovered by cell lysis at 14 h post-infection, and the bacterial colonies were counted by inoculating the lysate onto a CYE plate. The experiments were performed in triplicate, and the data are shown as means ± SD.

We directly observed the protein expression induced intracellularly, using rabbit anti-recombinant *L. pneumophila* DnaK serum, by means of immunofluorescence. At 12 h post-infection, the signal representing DnaK proteins was co-localized with the intracellular bacteria in the case of JR32 that had been phagocytosed by U937 cells (Fig. 5). However, no expression of DnaK was seen for 25D or LELA3118 within U937 cells.

3.5. Expression of stress-induced proteins in wild type and intracellular growth-deficient L. pneumophila strains after phagocytosis in amoebae

The expression of stress-induced proteins of JR32 within amoebae was much weaker than that within macrophages (Fig. 3). We compared the protein expression in the wild type and mutant strains of bacteria after phagocytosis in *A. polyphaga* at 14 h post-infection by the same method as used for U937 cells. The synthesis of Hsp60 and DnaK in JR32 within amoebae was much weaker than in JR32 after heat shock. The synthesis of these proteins was not observed in the 25D and LELA3118 mutant strains (Fig. 6A). The MOI was also chosen so that the numbers of live bacteria at 14 h post-infection were equal (see Section 2). This was again confirmed by the fact that equal numbers of bacteria were recovered from JR32-, 25D- and LELA3118-infected *A. polyphaga* (Fig. 6B). These results may indicate that strong expression of any protein is not necessary for the growth of *L. pneumophila* within amoebae, unlike within macrophages.

3.6. Immediate cessation of protein synthesis in intracellular growth-deficient L. pneumophila strains after phagocytosis in human macrophages

It has been shown that phagosomes containing intracellular growth-deficient 25D and *icm/dot* mutant strains of *L. pneumophila* fuse with lysosome in early time point just after bacterial invading to host cells [3,35,38]. However, in our intracellular growth assay within U937 cells, the bacterial number of 25D and LELA3118 strain within the cells did not show notable decrease and was almost constant until at least 24 h post-infection, in contrast to active replication of wild type JR32 strain after 14 h post-infection (Fig. 7A). And the almost complete absence of bacterial protein synthesis in the mutant strains at 14 h post-infection made us suspect that there may be another sequence of events before or at the same time as phagosome/lysosome fusion leading to the complete cessation of protein synthesis. We examined the synthesis of proteins encoded by a reporter construct that had been exogenously introduced into bacteria. JR32, 25D or LELA3118 cells were transformed with the pAB-1 plasmid that had a *lacZ* reporter gene under the control of an IPTG-inducible promoter. Successful transformation was revealed by β-galactosidase activity after in vitro IPTG induction. The three strains exhibited similar levels of enzyme activity depending on the pAB-1 plasmid and IPTG induction (Fig. 7B).

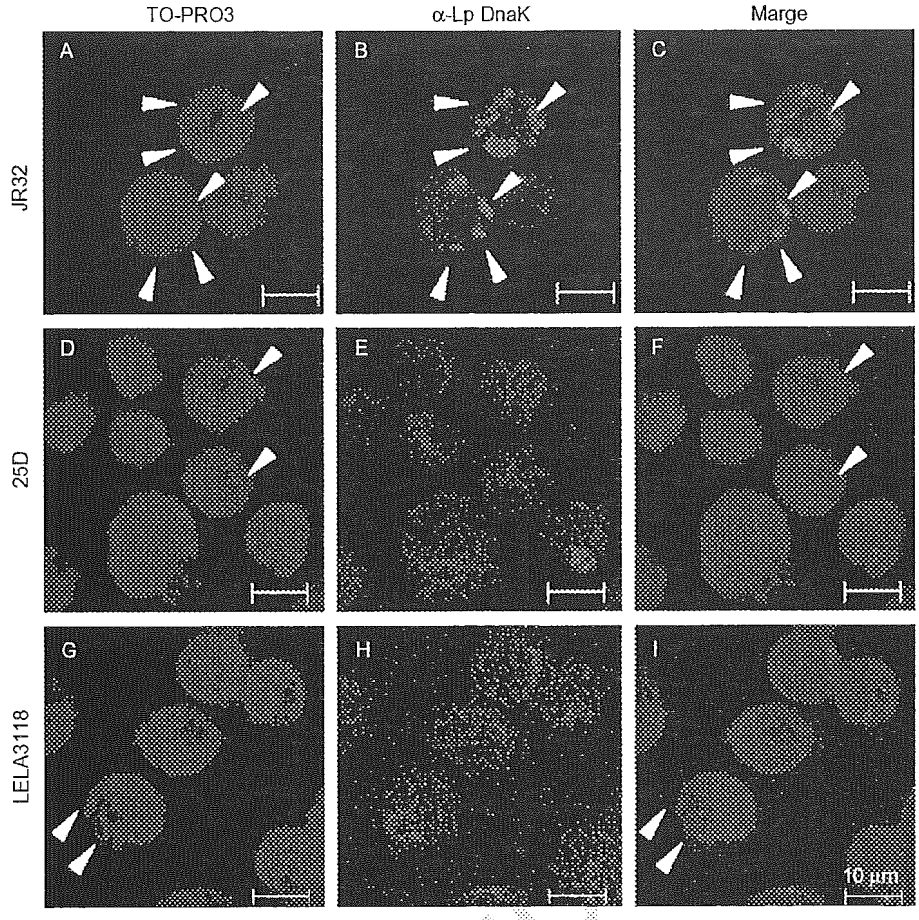

Fig. 5. Detection of the expression of *L. pneumophila* DnaK within U937 cells at 12 h post-infection. JR32 (A, B, C); 25D (D, E, F); LELA3118 (G, H, I). TO-PRO-3 (blue) stains the nucleic acid of both intracellular and extracellular bacteria as well as the U937 nucleus. *L. pneumophila* DnaK was visualized with rabbit anti-recombinant *L. pneumophila* DnaK serum and secondary antibodies conjugated with Alexa Fluor 546 (red). Arrowheads indicate intracellular *L. pneumophila*.

We then infected U937 cells with these bacteria carrying the reporter plasmid at a post-logarithmic phase. At several time points after infection, the infected U937 cells were treated with IPTG for 30 min, and then β-galactosidase activity in cell lysates was measured (Fig. 7C). For JR32, synthesis of the reporter protein was observed immediately after infection (time 0), and the protein synthesis increased with time, reaching a plateau at 4 h post-infection. In contrast, the level of β-galactosidase activity in 25D- or LELA3118-infected U937 cells was not only low in the initial phase but also decreased with time, reaching an undetectable level at 12 h post-infection. This phenomenon is consistent with the inability of the 25D and LELA3118 strains to produce bacterial proteins including stress proteins at 14 h post-infection in macrophages. When U937 cells treated with cycloheximide were used in this assay, the same result was obtained (data not shown).

4. Discussion

As a general phenomenon, pathogenic bacteria respond to and adapt to various local environmental conditions. In particular, the intracellular environments of macrophages may be hostile ones in which microbes are threatened by oxidative, acidic or microbicidal agents. One of the hallmarks of pathogenic bacteria is the ability to survive and replicate in such harsh environments. Each pathogenic bacterium should have unique and sophisticated mechanisms to adapt to severe environments. The bacterial intracellular protein expression system including stress response would be related to them. It is suspected that such an intracellular protein expression of pathogens might have some important biological function that contributes to their survival and replication within host cells.

Our first question in this study was whether *icm/dot* genes might have some effect on the expression of proteins induced by in vitro stress. After exposure to heat shock stress, no difference was observed in the protein expression profile between the wild type strain and *icm/dot* mutants (*icmR* and *dotA*). Spontaneous mutant 25D also showed the same pattern (data not shown). An immunoprecipitation study involving anti-DnaK antibodies also demonstrated that DnaK, which is a representative stress protein and chaperone, is expressed in the wild type strain and *icm/dot* mutants in the same way. Hsp60, another major stress protein, was also expressed with a similar

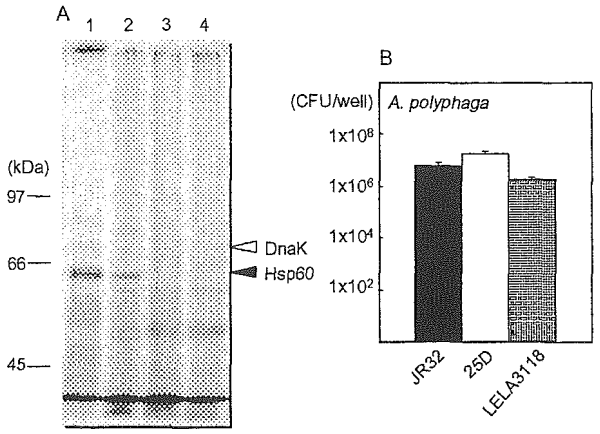

Fig. 6. Intracellular protein expression of the wild type and intracellular growth-deficient strains within *A. polyphaga*. (A) Bacterial protein expression profiles of the wild type and intracellular growth-deficient strains within *A. polyphaga*. Lane 1, JR32 with in vitro heat shock at 42 °C for 1 h; lane 2, JR32 within *A. polyphaga*; lane 3, 25D within *A. polyphaga*; lane 4, LELA3118 within *A. polyphaga*. Open and solid arrowheads indicate the bands of DnaK and Hsp60, respectively. (B) The number of live intracellular bacteria at 14 h post-infection on a replicate experiment. Intracellular bacteria were recovered by cell lysis at 14 h post-infection, and the bacterial colonies were counted by inoculating the lysate onto a CYE plate. The experiments were performed in triplicate, and the data are shown as means ± SD.

level within these strains. These results indicate that mutations of *icm/dot* genes probably do not affect the expression of whole cell proteins induced by heat shock. Regarding the presence of Hsp60 in the secreted protein fraction for the wild type strain, a previous immuno-electron microscopic study showed that heat-shocked *L. pneumophila* exhibited increased expression of the Hsp60 epitope on the cell surface and secreted Hsp60 molecules into the culture supernatant [39]. Our data were consistent with this finding, although the quantities of cell surface and secreted forms were considerably small.

DnaK chaperone participates in various cellular processes, including folding of nascent polypeptides, assembly and disassembly of multimeric protein structures, membrane translocation of secreted proteins and protein degradation. Especially in intracellular pathogens such as *Brucella suis* and *Listria monocytogenes*, DnaK is involved in and plays some critical role in intracellular replication [40–42]. Although the *L. pneumophila* DnaK gene has been cloned [43], its actual function is not known yet. We attempted to produce a *dnaK* mutant of *L. pneumophila* to examine its phenotypes, but failed. It is plausible that DnaK of *L. pneumophila* is essential for its survival.

We next investigated the stress-induced protein expression by *L. pneumophila* within host cells. One of our interests was whether there are any differences in the expression of bacterial proteins within different hosts. Because amoebae function as a reservoir for *L. pneumophila* in the natural environment [4,5], they may be less detrimental to the bacteria and provide relatively mild conditions for bacteria. In contrast, macrophages are major target cells during mammalian infection by *L. pneumophila* and should be able to fight against invasive bacteria. Hence we predicted that there might be differences in

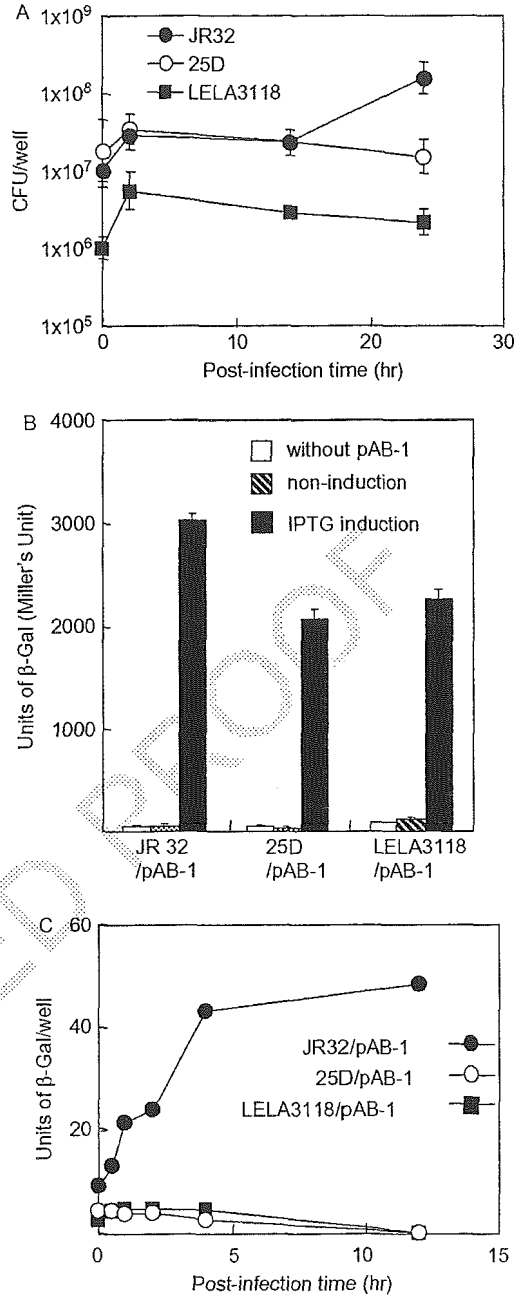

Fig. 7. Ability of bacterial protein synthesis determined with an inducible reporter construct. (A) Intracellular growth kinetics of *L. pneumophila* strains within U937 cells until 24 h post-infection. (B) Induction of β-galactosidase in *L. pneumophila* strains at the early logarithmic phase in culture by IPTG. The enzymatic activity from bacteria without pAB-1 (open column), one from bacteria with pAB-1 with no induction (hatched column), and one from bacteria with pAB-1 upon IPTG induction (filled column) are shown. The activity was normalized per the unit cell number, and the data are expressed as means ± SD for triplicate determinations. (C) Induction of β-galactosidase in intracellular *L. pneumophila*. U937 cells were infected with *L. pneumophila* strains with pAB-1. At several time points during post-infection (abscissa), β-galactosidase activity in the cell lysate was determined. The activity is expressed as units per well of tissue culture plates.

bacterial responses within different intracellular environments. *L. pneumophila* was strongly induced to produce a variety of proteins including heat shock proteins DnaK and Hsp60 within macrophages, whereas the quantity and variety of proteins expressed within amoebae were limited. These are novel data showing a difference of protein expression of *L. pneumophila* within macrophages or amoebae. These data may indicate that many of *L. pneumophila* proteins induced within macrophages might play some role in the survival of bacteria in macrophages, whereas such intense protein expression might not be needed within amoebae whose intracellular environments may be safe enough for *L. pneumophila*. It is conceivable that *L. pneumophila* might have originally evolved to become parasitic for protozoa.

Another interest was whether there are any differences in protein expression between wild type and intracellular growth-deficient strains within host cells. We used two types of mutants as intracellular growth-deficient strains; LELA3118, *dotA* genetic mutant, and 25D, a spontaneous mutant that was established by passage on suboptimal artificial medium [24]. Incredibly, de novo synthesis of bacterial proteins as revealed by metabolic labeling was markedly suppressed in both of the mutants in macrophages. This is confirmed by experiments using bacteria carrying a reporter construct encoding β-galactosidase. The ability of synthesis of β-galactosidase proteins was stopped in the mutant strains after infection in macrophages. This is also the first evidence of a difference in the protein expression pattern in vivo between wild type and intracellular growth-deficient strains despite the lack of a difference in the in vitro response to heat shock between them. We also confirmed that the same pattern of protein expression was observed in response to another form of in vitro stress, osmotic or oxidative, as was seen for heat shock on SDS-PAGE, and that there was no difference between an intracellular growth-deficient strain (LELA3118 or 25D) and the wild type JR32 strain (unpublished observation). These data may indicate that none of the stress imposed in vitro could precisely mimic the intracellular stress for bacteria. It is possible that the initial protein synthesis by bacteria upon infection in macrophages is involved in the subsequent stress response and protein synthesis at a later stage. This initial response may be regulated by some virulence factors including the type IV secretion system.

Early studies on vacuoles harboring *L. pneumophila* showed that phagosomes containing JR32 aged 0.5–6 h do not fuse with lysosomes, but the phagosomes containing 25D or LELA3118 do [35]. It has also been shown that the activity of NADPH oxidase, which produces reactive oxigen intermediates, affects the intracellular growth of *L. pneumophila* within macrophages at early phase of infection [44]. It is commonly believed that intracellular growth-deficient *L. pneumophila* are damaged immediately through the bactericidal reaction of host cells in intracellular compartments. However, our data indicated that the numbers of intracellular viable cells of mutant strains, 25D and LELA3118, did not decrease until at least 14 h post-infection, because the recovery of viable bacteria remained constant throughout this period (Fig. 7A). These data indicate that most cells of both mutant strains were not killed immediately after internalization by macrophages and were still viable until at least 14 h post-infection or probably for a longer period. This is supported by another report that *dotA* and *dotB* mutants reside in some non-lysosomal and LAMP-1 positive compartment within macrophages [45]. Taken together, these mutants likely remained alive with a suppressed-protein synthetic condition for at least 12 h.

In conclusion, we have shown a difference in the *L. pneumophila* intracellular stress response depending on the host cells. We have provided new evidence that intracellular growth-deficient *L. pneumophila* strains are not killed but forced to stop protein synthesis within macrophages immediately after internalization. They are viable without replication for some time in macrophages. Using bacteria carrying an inducible β-galactosidase construct, the immediate cessation of protein synthesis of intracellular growth-deficient strains upon internalization within macrophages, has been demonstrated. One could speculate that there might be a difference in the nature of phagosomes at the beginning between those containing wild type and intracellular growth-deficient strains. Only the former phagosomes may provide environments that allow bacteria to start protein synthesis. Recently it has been shown that RalF protein, which is one of Icm/Dot substrates, is translocated into host cells before bacterial internalization [46]. This evidence may suggest a possibility that the quality of phagosomes is determined at the beginning of bacterial infection.

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病態・病原性における最近のトピックス

菌側からみた新しい展開

～レジオネラのゲノム解読からみえてきたもの～

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はじめに

レジオネラ属菌は，グラム陰性の通性細胞内寄生菌でヒトに急性肺炎(レジオネラ肺炎，在郷軍人病)やインフルエンザ様の熱性疾患(ポンティアック熱)を引き起こす病原性をもっている．IDWR(Infectious Diseases Weekly Report Japan)の報告を集計すると，2002年1月より2004年12月までの3年間でレジオネラ症患者は471名にのぼる．平均すると1年間に157名の患者が発生している計算になる．

2005年4月現在で，レジオネラ属菌は50菌種が正式に命名されている．しかし，レジオネラ症患者から分離される菌種の90%以上は*Legionella pneumophila*で，その8割以上を血清群1に属する菌が占めている[1]．そのため多くの研究が*L. pneumophila*を使用して行われてきた．その結果，本菌が病原性を発揮するうえで最も重要な性質は，生体防御の第一線で働くマクロファージの殺菌に抵抗し，その中で増殖し，結果的にその細胞を殺す能力をもっていること(細胞内増殖能であること)が明らかとなった．細胞内増殖機構に関して精力的な研究が多くの研究室で行われ，表1に示したような遺伝子が本菌の細胞内増殖に関与する病原遺伝子として報告されている．

2004年に米国の研究グループ[2]とフランスの研究グループ[3]から本菌の全ゲノム配列の解析結果が相次いで発表された．全ゲノムの解読により，表1に示した既知の遺伝子以外に本菌の病原遺伝子である可能性の高い遺伝子(ORF)がゲノム上に多く発見された．本稿では，レジオネラのゲノム解析から得られた知見のうち，病原性に関与するものを中心に紹介する．

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■ゲノムの全体像

表2にゲノム解析に使用されたParis株，Lens株，Philadelphia-1株のゲノムの比較を示した．ゲノムサイズはいずれも約3.5Mbで大腸菌K-12株(4.6Mb)より約1Mbほど小さい．ゲノム配列のうち88～90%が遺伝子をコードしている領域として占められている．機能が不明な遺伝子は約43%を占め，これまでにゲノム配列が明らかにされたほかの菌の場合と同様である．*L. pneumophila*に特異的な遺伝子は約21%であり，菌株特異的な遺伝子がすべての遺伝子の10～14%を占めている．菌株特異的な遺伝子の割合はチフス菌では約2%，*Helicobacter pylori*では6～7%であり，本菌の菌株特異的な遺伝子の割合は非常に高い．ゲノム配列が決定された3株の*L. pneumophila*はすべて同じ血清群1に属しているにもかかわらず，菌株特異的な遺伝子が全体の1割以上を占めている事実は驚きである．自然形質転換[4]や染色体DNAの接合伝達[5]などにより遺伝子の水平伝播が活発に起こった結果かもしれない．

表1　既知の *Legionella pneumophila* 病原遺伝子とその産物/機能

機能	遺伝子	遺伝子産物/機能	報告文献
付着	*pil* E	Ⅳ型 線毛	*Infect Immun* 66：1768, 1998.
	omp S	主要外膜蛋白質(ポーリン)	*J Bacteriol* 174：914, 1992.
	hsp 60	60kDa 熱ショック蛋白質	*Infect Immun* 66：4602, 1998.
	lsp GH	Ⅱ型蛋白質分泌システムにより分泌される蛋白	*Infect Immun* 67：3662, 1999.
侵入および貪食	*fla* A	鞭毛	*Infect Immun* 69：2116, 2001.
	enh	侵入と貪食を促進する蛋白質	*Microbiolgy* 146：1345, 2000.
	icm/dot	Ⅳ型蛋白質分泌システム	*Mol Microbiol* 42：603, 2001.
酸素依存性殺菌に抵抗	*mip*	24kDa 塩基性蛋白質	*Infect Immun* 57：1255, 1989.
	sod C	Cu, Zn-SOD	*Jpn J Med Sci Biol* 49：168, 1996.
細胞内増殖	*icm/dot*	Ⅳ型蛋白質分泌システム	*Science* 279：873, 1998.
	ral F	Ⅳ型蛋白質分泌システム(Icm/Dot トランスポーター)により感染宿主細胞内へ分泌される蛋白質(エフェクター分子)	*Science* 295：679, 2002.
	lid A		*Mol Microbiol* 48：305, 2003.
	sid		*PNAS* 101：841, 2004.
	lep AB		*Science* 303：1358, 2004.
	lig A	870アミノ残基の蛋白質	*Int J Med Microbiol* 290：239, 2000.
細胞からの脱出(再感染)	*icm* T	ファゴゾーム膜にポア形成	*Mol Microbiol* 43：1139, 2002.
	rtx A	接触依存性の細胞溶解毒	*Infect Immun* 69：508, 2001.
遺伝子発現調節(分化)	*rel* A	ppGpp 合成酵素	*Mol Microbiol* 33：721, 1999.
	let A/*let* S	二成分制御系	*Mol Microbiol* 44：107, 2002.
	let E	二成分制御系で転写・翻訳を調節するRNA	*Mol Microbiol* 44：107, 2002.
	rpo S	静止期RNA ポリメラーゼ・シグマ因子	*Mol Microbiol* 40：1201, 2001.
	fli A	鞭毛特異的RNA ポリメラーゼ・シグマ因子	*J Bacteriol* 179：17, 1997.
	csr A	菌の細胞内分化を調節	*Mol Microbiol* 50：445, 2003.
アポトーシス(細胞傷害)	*icm/dot*	Ⅳ型蛋白質分泌システム	*Infect Immun* 70：1657, 2002.

表2　*Legionella pneumophila* 血清群1の3菌株におけるゲノムの比較

ゲノムの性質	菌株名		
	Paris 株	Lens 株	Philadelphia-1 株
サイズ(塩基対)	3,503,610	3,345,687	3,397,754
平均GC含量(%)	38.3	38.4	38
遺伝子をコードしている領域(%)	87.9	88	89.8
遺伝子(ORF)の総数	3,076	2,931	2,953
機能未知のORF(他菌の遺伝子と相同性なし)	1,354	1,320	
L. pneumophila に 特異的なORF	645	595	502
菌株に特異的なORFの数	428	280	347
rRNA オペロン(16S, 23S, 5S)の数	3	3	3
tRNA 遺伝子の数	43	43	43
プラスミドの数(サイズ)	1(131.9kb)	1(59.8kb)	1(45kb)

文献2, 3)より改変

■宿主細胞の機能を調節して細胞内増殖に関与する遺伝子群

図1は，*L. pneumophila* のマクロファージ内での動態を示したものである．*L. pneumophila* はマクロファージから伸びた偽足によって貪食される．菌はファゴソームの酸性化やリソソームとの融合を阻害することで殺菌に抵抗する．その後，菌を

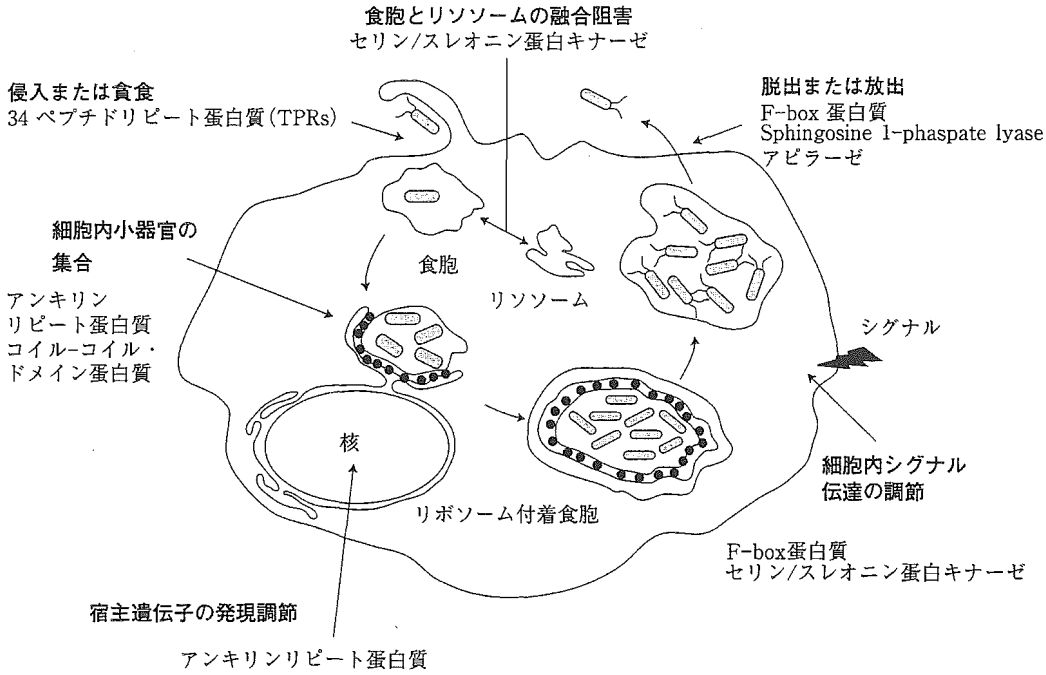

図1 *Legionella pneumophila* の細胞内動態と真核細胞類似レジオネラ蛋白質の役割(推定)
文献3)より改変

貪食したファゴソームの周りには滑面小胞体由来の小胞が集まってきて融合し，ファゴソーム膜は小胞体膜に置き換わる．変容したファゴソームには粗面小胞体由来のリボソームが集まってきて付着し，菌はリボソーム付着ファゴソーム内で増殖を開始する．また，細胞内で盛んに増殖中の菌(replicative form と呼ばれる)には鞭毛が観察されないが，細胞内増殖後期および感染細胞を殺して細胞外に出てきた菌(transmissible form と呼ばれる)には鞭毛が観察される．

強毒株の感染に伴うファゴソームとリソソームの融合阻害や，その後の細胞内小器官の集合は，菌が感染細胞の機能を調節していることを示している．菌は感染細胞を操り，自らが増殖するのに最適の環境を感染細胞自身に作らせるのである．レジオネラゲノム中には真核細胞の蛋白質と相同性の高い30の読み枠(ORF)が存在すること，また32のORFは真核細胞において蛋白質間の相互作用に関与することが知られている機能領域(ドメイン)をもっていることが明らかになった．これらの真核細胞類似蛋白が感染細胞に働きかけ，巧みにその細胞機能を調節して，菌の細胞内増殖を可能にしていると考えられる(表3，図1)．

1．Tetratrico peptide repeats(TPRs)をもつ蛋白質

TPRsは34アミノ残基が何度も(3～16回)繰り返されるモチーフである．このモチーフをもつ蛋白質は蛋白～蛋白間の相互作用のための場所(足場)を形成し，細胞内蛋白輸送，細胞周期の制御，転写抑制などに関与している[6]．レジオネラゲノム上にはこのモチーフを有するORFが5つ存在しており，2つ(EnhC, LidL)はすでに宿主細胞との相互作用が報告されている．残りの3つのORFも宿主細胞と相互作用していることが推察される．

2．セリン/スレオニンキナーゼ相同蛋白質

セリン/スレオニンキナーゼは細胞内シグナル伝達に関与する酵素である．この蛋白と相同性の高いORFがゲノム上に3つ存在する．結核菌もセリン/スレオニンキナーゼ相同蛋白を保有して

表3 *Legionella pneumophila* の主な真核細胞類似蛋白質とそれらの機能

モチーフ/ドメイン	ORF 数	機能	細胞内増殖過程での関与(推定)
34 ペプチド リピート(TRPs)	5	細胞内蛋白輸送，細胞周期調節，転写抑制	付着と侵入
セリン/スレオニンキナーゼ	3	細胞内シグナル伝達	ファゴソームとリソソームの融合
アンキリンリピート	20	膜蛋白質と細胞骨格のリンカー，転写調節	細胞内小器官の集合を誘導
F-box	3	蛋白間相互作用，細胞分裂の制御	細胞周期の制御と菌の細胞からの脱出
Sphingosine 1-phosphate lyase	1	細胞内シグナル伝達	細胞周期の制御と菌の細胞からの脱出
アピラーゼ	2	ATP 加水分解酵素	ATP の消費によるファゴソームの破壊

おり，この蛋白がファゴソームとリソソームの融合阻害に関与していることが報告されている[7]．レジオネラの場合も，これらの ORF がファゴソームとリソソームの融合阻害に関与している可能性は高い．

3．アンキリンリピートをもつ蛋白質

33 アミノ残基が 1 単位で連続して繰り返すモチーフで，膜蛋白質と細胞骨格をつなぐリンカー蛋白質として働く．また，NF-κB や GABP-β などの核内転写因子にもこのモチーフが認められる．アンキリンリピートをもつレジオネラの ORF は感染細胞の遺伝子発現を調節し，細胞骨格の変化を誘導することで細胞内小器官の集合を引き起こすのかもしれない．*Ehrlichia phagocytophila* のアンキリンリピートをもつ蛋白質(Ank A)は感染細胞の転写調節に影響を与えることが示されている[8]．

4．F-box 蛋白質

F-box 蛋白質は，基質蛋白と結合し，SCF 複合体(Skp1, Cullin-1, Rbx1 と種々の F-box 蛋白質からなる複合体)に基質を輸送するアダプター機能をもつ蛋白である．ユビキチン化反応による蛋白分解に重要な役割を果たしている．種々の蛋白の処理を担う蛋白質であることから，細胞の分裂や分化，細胞内シグナル伝達などに関与する[9]．菌はこの蛋白質を介して感染細胞の分裂や分化を止め，細胞活動に必要な栄養の消費を最小限に抑えて，余剰な栄養を自らの増殖のために搾取・利用するのかもしれない．*Agrobacteruim tumefaciens* の F-box 蛋白質である VirF 蛋白は感染細胞内に分泌され，宿主の蛋白質と相互作用することが報告されている[10]．

5．Sphingosine 1-phosphate lyase

細胞内シグナル伝達物質である sphingosine 1-phosphate を分解する酵素であり，細胞死に関係している[11]．新たな細胞への再感染のため，菌は適当な時期にこの酵素を菌体外に分泌し，感染細胞に細胞死を誘導して細胞外へ脱出するのかもしれない．

6．アピラーゼ

植物中に存在する ATP の加水分解酵素であり，原核生物ではこれまでに存在が知られていなかった酵素である．ヒトのアピラーゼ類似蛋白質は自己食胞(滑面小胞体膜が変性した細胞内小器官を取り囲んだ膜構造物のこと，この自己食胞にはやがてリソソームが融合し，細胞内小器官は消化分解される)に局在することが報告されている[12]．アピラーゼの局在部位とその酵素機能より考えて，本酵素は食胞などの膜構造物の運命を左右していることが予想される．レジオネラはアピラーゼを分泌することで自らを取り囲んでいる膜構造物，すなわち食胞の運命を握っていると思われる．

■他菌の病原遺伝子と相同性が高いレジオネラ遺伝子

ゲノム解読により，ほかの菌の病原遺伝子と高い相同性をもつ ORF をレジオネラがもつことも判明した．その主なものを表4に示した．

表4　新規に発見された *Legionella pneumophila* 病原遺伝子候補とその相同蛋白質

遺伝子*	既報の相同蛋白質	菌種	報告文献(モチーフ)
*lpg*1087	MviN	ネズミチフス菌	*J Bacteriol* 172：2608, 1990.
*lpg*2635	MviN	ネズミチフス菌	*J Bacteriol* 172：2608, 1990.
*lpg*0092	VacB	赤痢菌，腸管侵入性大腸菌	*J Biol Chem* 273：14077, 1998.
*lpg*1845	VacJ	赤痢菌	*Mol Microbiol* 11：31, 1994.
*lpg*1832	VirK	アグロバクテリウム，サルモネラ	*Mol Microbiol* 48：385, 2003. *Mol Microbiol* 6：2387, 1992.
*lpg*2822	BipA	腸管病原性大腸菌	*Mol Microbiol* 48：507, 2003.
*lpg*1488	表面抗原蛋白	マラリア原虫，赤痢アメーバ	(Sec-like coiled-coil domain)

*：文献2)による便宜上の遺伝子名

Salmonella typhimurium(ネズミチフス菌)のMviN蛋白質は内膜に局在し，この蛋白の欠損によりネズミチフス菌は弱毒化することが知られている．赤痢菌や腸管侵入性大腸菌のVacB蛋白質は3'-エキソリボヌクレアーゼ活性をもち，RNA分解作用を介して，菌の病原性発現を調節している．VacJ蛋白質は膜に局在するリポ蛋白であり，赤痢菌の細胞質内拡散に関与している．サルモネラやアグロバクテリウムのVirK蛋白質は感染細胞内に分泌され，感染細胞の蛋白質と相互作用する蛋白質である．前述のF-boxモチーフを分子内にもっている．腸管病原性大腸菌のBipA蛋白質はリボソーム結合型のGTP分解酵素で，鞭毛運動の制御を介して菌の付着や侵入に影響を与える．コイル—コイル・ドメイン(細胞内の小胞体輸送や膜と膜の融合に関与する蛋白質に多く見出される)をもったORFも発見された．このORFに相同性の高い蛋白質はマラリア原虫や赤痢アメーバも保有している．

また，*L. pneumophila* は約145の膜型，または分泌型の蛋白質分解酵素と加水分解酵素をゲノム上にコードしている．これらのいくつかは菌の細胞外脱出の際に働いている病原因子である可能性が高いと思われる．

おわりに

ゲノム解読が終了した細菌の多くが，真核細胞由来と思われる蛋白質を保有している．しかし，その数と種類の豊富さにおいて *L. pneumophila* 以上のものは見当たらない．おそらく *L. pneumophila* は自然宿主であるアメーバとの共進化の過程で，これらの遺伝子をアメーバから獲得し，マクロファージ内での増殖が可能になったのであろう．また，ゲノムの解読により，本菌は，重金属排出ポンプ遺伝子を複数もつことも見出された．アメーバが環境中から虫体内に取り込み，濃縮蓄積する鉛やカドミウムなどの重金属の曝露にレジオネラが抵抗するためかもしれない．コンピュータ中(*in silico*)でのゲノム情報の解析により，これまでの *in vitro, in vivo* での実験ではみえなかった多くのものがみえてきた．しかし，これらはあくまでも仮想的な解析であり，引き続き実験的にこれらを検証する必要がある．*in silico, in vitro, in vivo* が一体となったアプローチが不可欠である．今後のレジオネラ研究は，手探りの研究から対象を定めた計画的な研究に変貌していくことが予想される．

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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
VCS257	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*lacZ*-containing HindIII fragment from HMLD4001 in pBR322	This study
pHRO4	Tn*903*dII*lacZ*-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 PstI-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′-AATTTAA TCGCGGCCTCGAG-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 digoxigienin 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′-GGGAATTCGAGTAGATA CGAAGCAGGGT-3′) and djlA-2-PstI (5′-GGCTGCAGTTCCACCATAAAC GGACTACA-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′-GTG TGGAATTGTGAGCGGAT-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′-TTGGAG CCACTATCGACTAC-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 $150 \times g$ 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