sensitivity, as reported by Zaharatos et al. (21). However, the MIC_{90} of gentamicin was 128 μg/ml, showing low sensitivity, in contrast to the results of Brown et al. (3). Brown et al. obtained MIC using the agar plate dilution method, but we used the E-test, and the difference may have been due to methodological differences. Since a high resistance to β-lactams such as ampicillin and cefuroxime was noted, β-lactamase production by the test isolates was investigated using the disc method. β-Lactamase production was detected in 51.7% of the test isolates (data not shown), and it was in agreement with results reported by Brown et al. (3). Since *Methylobacterium* strains were multidrug resistant, it will also be necessary to elucidate the tolerance mechanisms.

As mentioned above, since the classification of *Methylobacterium* species is unclear in many regards, it is thought that a reconsideration of the taxonomy based on gene techniques will be even more necessary from now on.

From the fact that *Methylobacterium* species which are pathogenic bacteria of opportunistic infection inhabit hospital tap water in Japan, careful follow-up study is necessary to draw a conclusion that tap water can be used in stead of sterilized water for handwashing before surgical operations.

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IV. 研究成果の刊行物・別刷

２．レジオネラの病原性に関する研究

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Characterization of *Legionella pneumophila pmiA*, a Gene Essential for Infectivity of Protozoa and Macrophages

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The ability of *Legionella pneumophila* to cause pneumonia is dependent on intracellular replication within alveolar macrophages. The Icm/Dot secretion apparatus is essential for the ability of *L. pneumophila* to evade endocytic fusion, to remodel the phagosome by the endoplasmic reticulum (ER), and to replicate intracellularly. Protozoan and macrophage infectivity (*pmi*) mutants of *L. pneumophila*, which include 11 *dot/icm* mutants, exhibit defects in intracellular growth and replication within both protozoa and macrophages. In this study we characterized one of the *pmi* loci, *pmiA*. In contrast to the parental strain, the *pmiA* mutant is defective in cytopathogenicity for protozoa and macrophages. This is a novel mutant that exhibits a partial defect in survival within U937 human macrophage-like cells but exhibits a severe growth defect within *Acanthamoeba polyphaga*, which results in elimination from this host. The intracellular defects of this mutant are complemented by the wild-type *pmiA* gene on a plasmid. In contrast to phagosomes harboring the wild-type strain, which exclude endosomal-lysosomal markers, the *pmiA* mutant-containing phagosomes acquire the late endosomal-lysosomal markers LAMP-1 and LAMP-2. In contrast to the parental strain-containing phagosomes that are remodeled by the ER, there was a decrease in the number of ER-remodeled phagosomes harboring the *pmiA* mutant. Among several *Legionella* species examined, the *pmiA* gene is specific for *L. pneumophila*. The predicted amino acid sequence of the PmiA protein suggests that it is a transmembrane protein with three membrane-spanning regions. PmiA is similar to several hypothetical proteins produced by bacteria with a type IV secretion apparatus. Importantly, the defect in *pmiA* abolishes the pore-forming activity, which has been attributed to the Icm/Dot type IV secretion system. However, the mutant is sensitive to NaCl, and this sensitivity is abrogated in the *icm/dot* mutants. These results suggest that PmiA is a novel virulence factor that is involved in intracellular survival and replication of *L. pneumophila* in macrophages and protozoan cells.

Legionella pneumophila is the causative agent of Legionnaires' disease (29, 50). The ability of *L. pneumophila* to grow within mammalian cells, such as monocytes and alveolar macrophages, is essential for pathogenicity in humans (1, 42). Protozoa are reservoirs of *L. pneumophila* in natural environments, which play a crucial role in bacterial ecology and transmission to humans (38, 54, 63). Growth of *L. pneumophila* within amoebae enhances invasion of epithelial cells and macrophages (20). Amoeba-grown *L. pneumophila* becomes resistant to various stress conditions, such as acid, oxidative, osmotic, and heat stresses (4), and becomes more resistant to antibiotics (11). These phenotypic modulations of *L. pneumophila* may contribute to bacterial survival in harsh environmental conditions and to invasion and replication within human phagocytic cells.

The Icm/Dot type IV secretion apparatus is a major virulence system in *L. pneumophila*, since it is essential for evasion of endocytic fusion and remodeling of the phagosome in a suitable replicative niche (69, 80). The Icm/Dot system is also essential for early activation of caspase-3 in the host cell, which does not result in apoptosis until late stages of the infection (6, 83). Activation of caspase-3 results in cleavage of the Rab5 effector rabaptin-5, and this cleavage may be involved in the ability of *L. pneumophila*-containing phagosome to evade endocytic fusion and to be remodeled by the endoplasmic reticulum (ER) (55). *L. pneumophila* utilizes the Icm/Dot type IV secretion apparatus for injection of bacterial effector proteins into the host cell, leading to the creation of unique vacuoles suitable for intracellular growth (22, 58). Several proteins that are substrates of the Icm/Dot secretion apparatus, such as LepAB, LidA, RalF, and SidC, have been identified (18, 23, 47, 57). Although the functions of these substrates are unclear, it is thought that they modulate host signal transductions to establish the replicative vacuoles, allowing the organism to survive and replicate within phagocytic cells. At 15 min postinfection, *L. pneumophila* is contained in a unique phagosome with a membrane thinner than the plasma membrane, and it is surrounded by mitochondria and the ER (1, 2, 5, 41, 75–77). The *L. pneumophila*-containing phagosome excludes endosomal and lysosomal markers. In contrast, phagosomes containing *icm/dot* mutants acquire endosomal and lysosomal markers, and the mutants are unable to replicate intracellularly (64, 67, 80). Interestingly, calnexin, Rab1, and Sec22b are acquired by phagosomes containing the wild-type strain shortly after

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bacterial uptake (24, 44). Upon formation of this unique replicative niche, the organism replicates in the phagosome. During late stages of the infection, the phagosomal membrane becomes disrupted, and the bacteria escape into the cytoplasm, where the last rounds of replication are completed prior to lysis of the host cell membrane and bacterial egress (7, 51–53). Interestingly, within gamma interferon-activated macrophages, the *L. pneumophila*-containing phagosome fuses to lysosomes, and it is not remodeled by the ER (66).

Among the components of the Icm/Dot secretion apparatus, IcmT is essential for pore formation-mediated egress from the host cell upon termination of intracellular replication, in addition to a function required for phagosome biogenesis and evasion of lysosomal fusion (7, 51, 52). IcmQ has pore-forming capacities involving insertion into host cell membranes to form a channel for translocation of effectors from the bacterium into the host cell (25).

Loci other than the *icm/dot* genes are required for intracellular replication of *L. pneumophila*. The stationary-phase sigma factor RpoS and the stress-induced protease/chaperone HtrA are essential for multiplication within *Acanthamoeba polyphaga* (34, 59). The Rep helicase is required for replication within human macrophages and epithelial cells (31, 37). The type II secretion system of *L. pneumophila* is required for intracellular infection (33, 61, 62). The *pilD* gene, which encodes the prepilin leader peptidase, is required for assembly of both a type IV pilus (72) and a type II secretory apparatus, and it is also required for bacterial replication within amoebae and human macrophages (46). The LetA/LetS two-component regulatory system is required for infection of macrophages (30, 35, 48). The *csrA* gene is involved in regulation of the bacterial switch from the replicative form to the transmissible form and is essential for intracellular growth within both macrophages and amoebae (28, 56). Iron acquisition and assimilation are key factors for *L. pneumophila* virulence and intracellular growth (39, 79). The *lvgA* and *ptsP* genes of *L. pneumophila* affect colonization of the lungs and spleens of guinea pigs (26, 40). Therefore, numerous loci other than the *icm/dot* genes play major roles in the ability of *L. pneumophila* to replicate intracellularly and cause disease.

We previously isolated 89 insertion mutants of *L. pneumophila* that exhibit defects in cytotoxicity, intracellular survival, and replication within both U937 macrophage-like cells and *A. polyphaga*. These mutants were collectively designated *pmi* (protozoan and macrophage infectivity) mutants (32). Although 11 of the mutants have insertions within the *icm/dot* genes, most of the mutated genes of these strains have not been identified.

In this study, we characterized one of the *pmi* mutant strains, GB112, and identified the mutated gene, *pmiA*. The *pmiA* gene is localized outside the two genomic regions that include the *icm/dot* genes. The *pmiA* gene likely encodes an *L. pneumophila*-specific virulence factor required for intracellular survival and growth within macrophages and protozoan cells. The defect in intracellular survival is associated with trafficking of the *pmiA* mutant-containing phagosomes through the default endosomal-lysosomal degradation pathway. The predicted amino acid sequence suggests that the gene product is a transmembrane protein. Functional analyses using mutants and gene complementation indicated that the mutation does not eliminate sodium sensitivity, which is one of the phenotypes dependent on the Icm/Dot secretion apparatus, but abrogates the pore-forming activity, which is dependent on a functional Icm/Dot secretion apparatus.

MATERIALS AND METHODS

Bacterial strains and media. The *Legionella* strains used in this study were *L. pneumophila* serogroup 1 strain AA100 (3), *L. pneumophila* Philadelphia 1 JR32 (65), *L. pneumophila* Philadelphia 1 Lp02 (12), *L. pneumophila dotA* mutant LELA3118 (65), *L. pneumophila icmT* mutant GS3011 (70), *L. pneumophila icmT* mutant AA100kmT (52), *L. pneumophila* serogroup 5 strain GTC297, *L. pneumophila* serogroup 6 strain GTC748, *L. pneumophila* serogroup 7 strain GTC750, *Legionella bozemanii* GTC298, *Legionella micdadei* GTC299, *Legionella dumoffii* GTC303, *Legionella brunesis* GTC515, and *Legionella gratiana* GTC699. The GTC strains were obtained from the Gifu Type Culture Collection at Gifu University Graduate School of Medicine, Japan. The *Legionella* strains used in this study were grown on buffered charcoal-yeast extract (BCYE) plates or in buffered yeast extract (BYE) broth.

Cell culture. Macrophage-like U937 cells were maintained in RPMI 1640 (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum (ICN Biomedicals, Aurora, Ohio), as described previously (32). Axenic *A. polyphaga* was cultured as adherent cells in PYG medium. All cells were cultured under a humidified atmosphere containing 5% CO_2 and 95% air at 37°C, as described previously (32).

Intracellular bacterial growth assay. U937 cells (1×10^5 cells per well in 96-well flat-bottom plates [Falcon, Becton Dickinson, Franklin Lakes, NJ]) were allowed to differentiate into macrophages by incubation for 48 h with 50 ng ml^{-1} of phorbol 12-myristate 13-acetate (Sigma, Tokyo, Japan) in RPMI 1640 containing 10% fetal calf serum. *A. polyphaga* cells were grown in a 96-well plate at a density of 1×10^5 cells per well in PYG medium. The bacterial strains were grown in BYE medium to an optical density at 550 nm (OD_{550}) of 2.0 to 2.2 (post-exponential phase). The differentiated U937 cells or *A. polyphaga* cells were infected with the bacteria at a multiplicity of infection (MOI) of 10 in triplicate cultures. The plates were spun at $250 \times g$ for 20 min to bring the bacteria into contact with the host cell monolayer. The time at the end of this centrifugation was designated zero time. Then the monolayer was incubated for 1 h at 37°C to allow bacterial infection. At the end of the infection period, the cells were washed three times with an appropriate culture medium, and then they were incubated for 1 h at 37°C in culture medium containing 50 μg ml^{-1} gentamicin to kill the extracellular bacteria. The cells were washed again, and then incubation was continued in the culture medium. After several different times, cell lysis was performed by hypotonic treatment for U937 cells or by treatment with 0.04% Triton X-100 for *A. polyphaga*. Aliquots of the cell lysates were immediately diluted and plated on BCYE plates for enumeration of the intracellular bacteria. The number of intracellular bacteria was expressed as the number of CFU/ml (36, 52).

Cytopathogenicity of *L. pneumophila* for U937 cells and *A. polyphaga*. Infection of U937 cells with *L. pneumophila* strains in the post-exponential phase was performed in triplicate in 96-well tissue culture plates with 1×10^5 cells/well at an MOI of 10 for 1 h, and infection of *A. polyphaga* was performed in triplicate in 24-well tissue culture plates with 5×10^5 cells/well at an MOI of 10 for 1 h. After 23 h, the numbers of viable cells were determined by using the Alamar Blue (TREK Diagnostic Systems, Westlake, Ohio) assay for U937 cells and the trypan blue dye assay for *A. polyphaga*, and the degrees of cytopathogenicity for the two types of cells were expressed as described previously (32).

Cloning and sequencing the chromosomal junction of Kan insertions in the GB112 mutant. Genomic DNA from the GB112 mutant was digested with EcoRI, and the fragment containing the Kan cassette was ligated to EcoRI-digested pBC-SK$^+$ (Stratagene, La Jolla, Calif.). The cloned plasmid was digested with EcoRI and probed with pUC-4K (Pharmacia, Piscataway, N.J.) by Southern blotting to confirm the fidelity of the cloning. This plasmid was designated pGB112. pGB112 was digested with XhoI to obtain chromosomal fragment GB112 flanking half-Kan cassette. This fragment was ligated to pBC-SK$^+$, and the resulting plasmid was designated pGB112-XHOI. pGB112-XHOI was used for sequencing analysis with a T7 primer of pBC-SK$^+$. The sequence of approximately 360 nucleotides at the junction of the insertion was determined and used in database searches of the *Legionella* Genome Project at the Columbia Genome Center.

Construction of a GB112 complemented strain. Amplification of lpg1728 (*pmiA*) was performed with primer GB112-F3, complementary to a region 136 nucleotides upstream of the lpg1728 start codon (5′-GTTGATGATATGGGG

GCTG-3'), and GB112-R3, complementary to a region 138 nucleotides downstream of the lpg1728 stop codon (5'-CTCCGACAGGATAATCAGGA-3'). A 1,189-bp fragment was amplified by PCR with a Gene Amp PCR 9600 system (Perkin-Elmer, Yokohama, Japan). This fragment was cloned into the pGEM-T Easy system (Promega, Madison, Wis.). A NotI-digested fragment containing this region was subcloned into pBC-SK$^+$. The recombinant clone was designated pGB112C. pGB112C was introduced into the GB112 strain by using a Gene Pulser II (Bio-Rad, Hercules, Calif.) as recommended by the manufacturer to generate complemented strain GB112C-5.

Sequencing of *pmiA* gene. Sequencing of the whole *pmiA* region of the AA100 strain, using pGB112C, was carried by Hokkaido System Science Co., Ltd. Sequence alignments and comparisons were performed with GENETYX-MAC 8.0 (Software Development Co., Ltd.) and National Center for Biotechnology Information (blastp) programs, respectively.

Confocal laser scanning microscopy. Samples used for immunofluorescence analysis were prepared by the method described previously (36). In brief, U937 cells were cultured and then allowed to differentiate on coverslips in the wells of a six-well culture plate (Falcon, 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 1 h to kill the extracellular bacteria. At different times after the initiation of infection (see below), the coverslips were fixed in 4% paraformaldehyde for 15 min at room temperature, and then nonspecific binding sites were blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min. The cells were then permeabilized with 0.5% Triton X-100 in PBS for 10 min. The nuclei of both the bacteria and the U937 cells were stained with TO-PRO-3 iodide (Molecular Probes, Eugene, Oreg.) for 1 h. For colocalization experiments with endosomal markers and ER markers, 1 h of infection and 1 h of gentamicin treatment, followed by 2 h and 4 h of incubation, respectively, in culture medium without gentamicin were performed. Thus, 4-h or 6-h postinfection samples were examined. To label lysosomal and late endosomal compartments, serial 1-h incubations were performed with mouse anti-human LAMP-1 (H3B3) or LAMP-2 (H4B4) monoclonal antibodies (1:10 dilution; Developmental Studies Hybridoma Bank, University of Iowa) and Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) (1:100 dilution; Molecular Probes). To label ER proteins, serial 1-h incubations were performed with mouse anti-KDEL monoclonal antibodies (StressGen Biotechnologies, Victoria, Canada) and Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes). In this experiment, extracellular bacteria were labeled by incubation with rabbit anti-*L. pneumophila* serum (1:10 dilution; Denka Seiken, Tokyo, Japan) and Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:100 dilution; Molecular Probes) before permeabilization. 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, Calif.). Samples were observed with a Carl Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss, Germany).

Transmission electron microscopy. For examination of infected amoebae by transmission electron microscopy, monolayers were infected by *L. pneumophila* strains in six-well plates at an MOI of 10 for 1 h, followed by three washes. At 6 h postinfection, the infected monolayers were washed with 0.1 M Sorenson's phosphate buffer and then incubated for 45 min in 0.1 M Sorenson's phosphate buffer containing 3.5% glutaraldehyde, pH 7.4, at 4°C. Fusion between lysosomes of the *L. pneumophila* phagosomes was determined by examination for the presence of the lysosomal enzyme acid phosphatase, as described previously (13, 51). The monolayers were washed three times with 0.1 M Sorenson's phosphate buffer and three times with 0.1 M acetate buffer (pH 5) and incubated with an acid phosphatase-specific substrate, β-glycerolphosphate (0.1 M acetate buffer, 2 mM β-glycerolphosphate as the substrate, and 1.2% lead nitrate as the capture metal), for 1 h at 37°C. After three washes in 0.1 M acetate buffer (pH 5) and Sorenson's phosphate buffer (5 min each), infected cells were postfixed with 1% OsO_4 in the same buffer for 45 min. Samples were dehydrated and processed as described previously (31). Sections were stained with uranyl acetate and lead citrate and examined with an Hitachi H-7000/STEM electron microscope (Hitachi, Inc., Japan) at 80 kV, as described previously (31).

Southern blotting. Genomic DNA was isolated from *L. pneumophila* strains using an AquaPure genomic DNA isolation kit (Bio-Rad). Samples of genomic DNA were digested with EcoRI, separated by electrophoresis in 0.8% agarose gels, and transferred to Hybond-N nylon membranes (Amersham Biosciences, Buckinghamshire, England). pGB112C was used as a DNA probe. Labeling of DNA probes and detection of signals were performed by using the ECL direct nucleic acid and detection system (Amersham Biosciences).

Contact-dependent pore formation assay. Contact-dependent pore formation in membranes was determined by examining hemolysis of sheep red blood cells (sRBCs) by *L. pneumophila* at an MOI of 10 after 2 h of bacterial contact, as described previously (45). The release of hemoglobin from the lysed red blood cells was measured by spectrophotometry at 415 nm.

Sodium sensitivity assay. Bacteria were grown in BYE broth until the post-exponential phase (OD_{550}, 2.0 to 2.2). The OD_{550} of bacterial cultures were adjusted to 1.0 to equalize the number of bacteria. Dilutions were plated on BCYE plates in the presence or absence of 0.6% NaCl. The ratio of the plating efficiency with 0.6% NaCl to the plating efficiency without 0.6% NaCl was calculated for each strain.

Nucleotide sequence accession number. The sequence of the whole *pmiA* region of the AA100 strain has been deposited in the DDBJ database under accession number AB193439.

RESULTS

Characteristics of intracellular survival, replication, and cytopathogenicity of the GB112 mutant in U937 cells and *A. polyphaga*. We showed previously that the GB112 *pmi* mutant exhibits a defect in intracellular growth within both macrophages and protozoa (32). To ensure that the GB112 mutant had no other mutations, we decided to reconstruct the Kan insertion in the GB112 mutant in the wild-type strain. A DNA fragment containing the insertion and the flanking chromosomal regions was cloned from GB112 genomic DNA and introduced into wild-type strain AA100 by homologous recombination, as described previously (73). The fidelity of the allelic exchanges was confirmed by Southern hybridization (data not shown). For all the studies described in this paper, we show data only for the reconstructed mutant, which for simplicity was designated GB112.

Since many virulence-associated phenotypes have been shown to be induced at the postexponential phase (15), we examined the phenotype of the reconstructed GB112 mutant for intracellular growth and cytopathogenicity for host cells using bacteria grown to the postexponential phase. We first examined the cytopathogenicity for U937 human macrophage-like cells using Alamar Blue assays and the cytopathogenicity for *A. polyphaga* using trypan blue. The data showed that the cytopathogenicity of the GB112 mutant for both U937 macrophages and *A. polyphaga* was diminished compared to that of parental strain AA100 (Fig. 1A and B).

The intracellular growth of the GB112 mutant was less than that of wild-type strain AA100 in U937 cells. The number of GB112 mutant bacteria within U937 cells was approximately 1/10 the number of AA100 bacteria from 24 h to 48 h postinfection (Fig. 1C). In contrast, the GB112 mutant was severely defective in survival and replication within *A. polyphaga*. At 24 h postinfection, there were no detectable viable GB112 bacteria in *A. polyphaga*, while AA100 showed robust replication by 24 h (Fig. 1D). This result indicated that the GB112 mutant bacteria were killed in *A. polyphaga*.

Intracellular trafficking of the GB112 mutant within U937 macrophages and *A. polyphaga*. Survival and replication of *L. pneumophila* within mammalian and protozoan cells are totally dependent on evasion of fusion of the bacterial phagosome to lysosomes (51, 64, 74, 82). In addition, bacterial replication has also been shown to occur in a phagosome that is remodeled by the rough endoplasmic reticulum within macrophages and protozoa (1, 2, 5, 41, 75–77). Since the GB112 mutant was defective in survival in macrophages and amoebae, we hypothesized that intracellular trafficking of the mutant was likely altered in both types of host cells. To test this hypothesis, we examined colocalization of the bacterium-containing phagosomes with

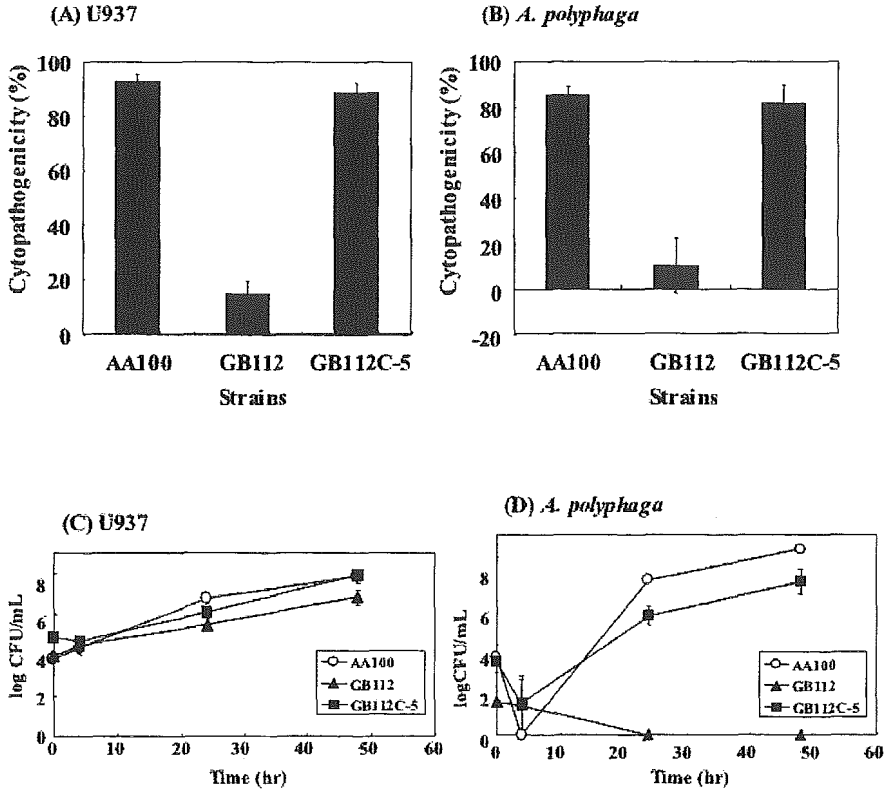

FIG. 1. Cytopathogenicity and intracellular replication of *L. pneumophila* strains in U937 macrophage-like cells and *A. polyphaga*. U937 macrophages or *A. polyphaga* cells were infected with bacteria at an MOI of 10 for 1 h, followed by gentamicin treatment to kill extracellular bacteria, and the cells were then incubated for 24 h. The viability of host cells was determined by the Alamar Blue (for U937 cells) (A) or trypan blue (for *A. polyphaga*) (B) method. The following *L. pneumophila* strains were used: AA100 (wild type), GB112 (*pmiA* deficient), and GB112C-5 (*pmiA*-complemented GB112). The percentage of killed cells after bacterial infection was normalized to the percentage of uninfected cells, which was considered 100% viable cells. (C and D) Intracellular growth kinetics of *L. pneumophila* GB112 strains within U937 macrophage-like cells and *A. polyphaga*, respectively. Infection of the monolayers was performed exactly as described above for the cytopathogenicity assay, except that at the end of the 1-h infection period the monolayers were treated with gentamicin for 1 h to kill extracellular bacteria. The intracellular bacteria were recovered at several times postinfection, and the number of viable cells was determined by enumeration of the CFU. All experiments were done three times in triplicate; the data are the data from one representative experiment, and the error bars indicate standard deviations.

the late endosomal-lysosomal markers LAMP-1 and LAMP-2 in U937 cells by confocal laser scanning microscopy showing only LAMP-2 staining (Fig. 2A). Approximately 70% of the phagosomes containing the GB112 mutant colocalized with LAMP-1 and LAMP-2 (Fig. 3). In contrast, only 10 to 25% of the phagosomes containing parental strain AA100 colocalized with LAMP-1 and LAMP-2 (Fig. 2A and 3). The phagosomes harboring heat-killed *L. pneumophila* as a positive control colocalized with both LAMP-1 and LAMP-2, as expected (Fig. 2A and 3).

We examined the presence of ER-associated proteins in the phagosomes at 6 h postinfection, using an antibody that recognizes the KDEL amino acid sequence, which is the signal for ER retention (Fig. 2B). Approximately 60% of the GB112 mutant-containing phagosomes did not acquire the KDEL marker. In contrast, more than 70% of the AA100-containing phagosomes acquired the KDEL marker (Fig. 2B and 3). Phagosomes harboring heat-killed AA100, as a control, did not acquire the KDEL marker, as expected. Thus, the defect in survival and replication of the GB112 mutant within U937 macrophages was associated with acquisition of the late endosomal-lysosomal markers LAMP-1 and LAMP-2 and with a reduction in the frequency of colocalization with the ER.

Since the GB112 mutant was severely defective in replication within *A. polyphaga*, we examined whether this defect was associated with fusion of the GB112-containing phagosomes to lysosomes. We examined the presence of the lysosomal enzyme acid phosphatase in the bacterium-containing phagosomes by electron microscopy, as described previously (51). The data showed that only 7% of the phagosomes containing AA100 contained acid phosphatase at 6 h postinfection (Fig. 4A and D). In contrast, 82% of the phagosomes harboring the GB112 mutant contained acid phosphatase (Fig. 4B and D). For the *icmT* null mutant, which was used as a positive control, ~60% of the phagosomes acquired acid phosphatase (Fig. 4C and D), which is consistent with previous observations (51, 52). On the basis of these findings, we concluded that the severe defect of the GB112 mutant in survival and replication in *A. polyphaga* was associated with the fusion of the GB112 mutant-containing phagosomes to lysosomes.

Identification of the mutated gene in the GB112 mutant. We cloned the DNA fragment containing the Kan insert and flank-

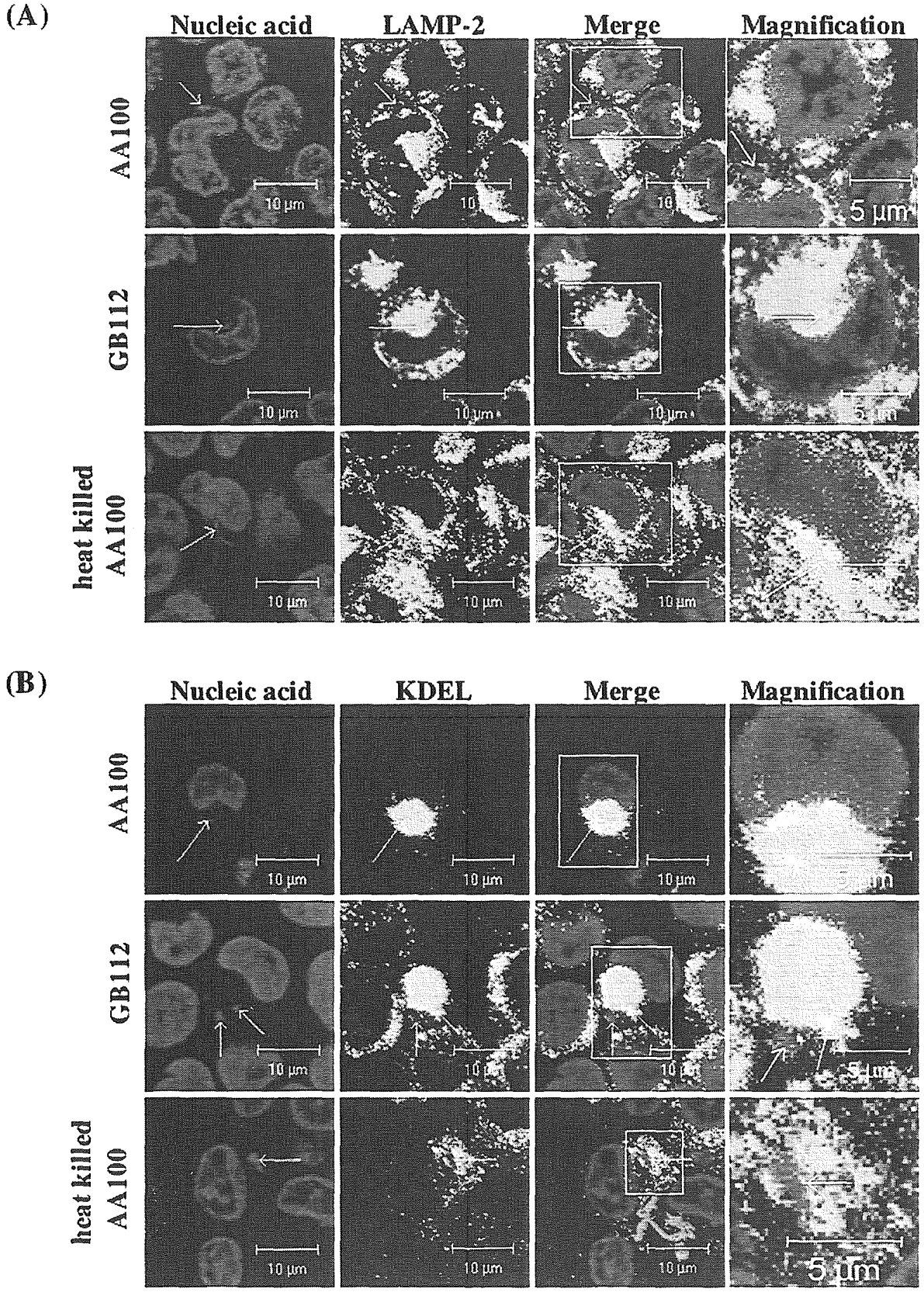

FIG. 2. Representative confocal microscopy images of colocalization of the bacterial phagosomes with LAMP-2 (A) and ER proteins (B) within U937 cells infected by *L. pneumophila* AA100 (wild type) or GB112 (*pmiA* deficient). The DNA of intracellular and extracellular bacteria, as well as the U937 nucleus, were stained with TO-PRO-3 (blue). LAMP-2 and the KDEL marker were visualized with secondary antibodies conjugated to Alexa 488 (green). Extracellular bacteria were visualized with secondary antibodies conjugated to Alexa Fluor 546 (red). The arrows indicate intracellular *L. pneumophila*. The magnified portions are indicated by the squares in the merge panels.

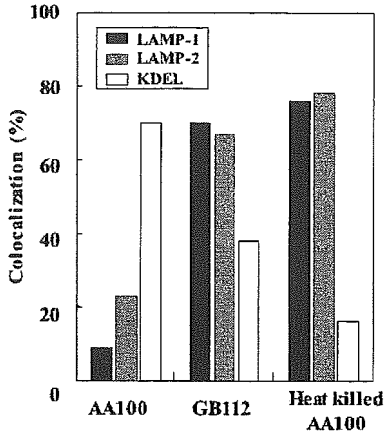

FIG. 3. Quantitation of colocalization of LAMP-1, LAMP-2, and ER proteins with the phagosomes containing *L. pneumophila* strains. Phagosomes containing AA100 (wild type), GB112 (*pmiA* deficient), or heat-killed AA100 (control) were examined for the presence of LAMP-1 and LAMP-2 at 4 h postinfection and for proteins with the ER retention signal (KDEL motif) at 6 h postinfection using confocal laser scanning microscopy.

ing sequences from the chromosome of the GB112 mutant. The sequence of the DNA fragments at the junction of the chromosomal DNA and the insert was analyzed. Using the BLAST program at the website of the *Legionella* Genome Project for the Philadelphia 1 strain (http://genome3.cpmc.columbia.edu/~legion/index.html) (19), we identified an open reading frame (ORF) that was 915 bases long (lpg1728) in the genome sequence that was interrupted by the insertion in the GB112 mutant. This ORF was also present in the complete genome sequences of two other *L. pneumophila* strains, Paris and Lens (17). We designated the lpg1728 ORF *pmiA* (accession no. AB193439) (Fig. 5A). The *pmiA* gene was located outside the *icm/dot* regions and was 1.1×10^6 bp downstream from *icm/dot* region I (*icmVWX-dotABCD*) and 1.45×10^6 bp upstream from *icm/dot* region II (*icmTSRQPO-lphA-icmMLKEGCDJB-tphA-icmF* or *dotMLKJIHGFENO*).

There are five ORFs, three upstream and two downstream of lpg1728 (Fig. 5A). Since the predicted directions of transcription of all six ORFs were the same, it is possible that the Kan insertion has a polar effect on expression of the two ORFs downstream of *pmiA*. However, a 1.19-kb PCR-generated fragment containing only *pmiA* (lpg1728) was sufficient to complement the GB112 mutation (strain GB112C-5) for the defects in intracellular growth and cytopathogenicity for U937 macrophages and *A. polyphaga* (Fig. 1). Thus, the defect of the GB112 mutant in intracellular survival and trafficking is due to the defect in *pmiA*.

Topology analysis using the TopPred program (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html; Institut Pasteur, France) suggested that the predicted PmiA protein has three transmembrane domains (Fig. 5B). Following the putative transmembrane domain near the N terminus, there is a predicted long hydrophilic region (SOSUI program [http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html]) (Fig. 5C). These results suggested that PmiA might be a transmembrane protein.

We examined the gene distribution of *pmiA* in several species of the genus *Legionella* by genomic Southern hybridization, using low-stringency conditions for hybridizations (see Materials and Methods). The *pmiA* gene was present in all *L. pneumophila* strains regardless of the serogroup, but it was not detected in any other *Legionella* species examined (Fig. 6). This result suggested that *pmiA* might be specific for *L. pneumophila*.

PmiA is essential for contact-dependent pore formation. The pore-forming activity has been shown to be dependent on a functional Icm/Dot type IV secretion system (45). Many Icm/Dot proteins that are predicted to be structural components of the Icm/Dot secretion apparatus are essential for the pore-forming activity (21, 45, 51, 52). We examined the pore-forming activity of the GB112 mutant, using contact-dependent hemolysis of sRBCs. The *dotA* and *icmT* mutants were used as controls, since both of these mutants are defective in pore-forming activity. The GB112 mutant was completely defective in pore-forming activity, similar to the *dotA* and *icmT* mutants, as well as heat-killed bacteria (Fig. 7A). Importantly, the defect of the GB112 mutant was fully complemented by the wild-type *pmiA* gene on a plasmid (Fig. 7A). These results indicated that the PmiA protein is essential for the contact-dependent pore-forming activity.

***pmiA* mutation does not affect NaCl sensitivity.** *L. pneumophila* virulent strains have been shown to be sensitive to sodium chloride (16, 65). It has been proposed that the sodium sensitivity is likely to be dependent on the function of an intact Icm/Dot secretion system because *icm/dot* mutants are resistant to high concentrations of sodium ions. It has been speculated that a functional Icm/Dot secretion apparatus is leaky to NaCl, which may explain why mutants defective in the secretion apparatus are resistant to salt (81). We examined the sodium sensitivity of the GB112 mutant and compared it to that of other *icm/dot* mutants. The GB112 mutant was similar to the wild-type strain in terms of sensitivity to 0.6% sodium chloride, whereas the *dotA* mutant was resistant to NaCl (Fig. 7B).

DISCUSSION

The hallmark of Legionnaires' disease is the intracellular survival and growth of *L. pneumophila* within alveolar macrophages. It is thought that in natural aquatic environments, *L. pneumophila* is a parasite of protozoa (38, 54, 63), and the infected protozoa are important as a source of infection of humans by *L. pneumophila* (10, 27).

The major virulence *icm/dot* gene clusters of *L. pneumophila*, which encode components of type IV secretion systems, are required for intracellular growth and killing of human macrophages (14, 49, 60, 65, 67–69, 78, 80). The *icm/dot* genes of *L. pneumophila* are also required for intracellular growth within amoebae, as well as within human macrophages (71). However, many genes other than the *icm/dot* genes are also required for intracellular replication (26, 28, 30–40, 46, 48, 56, 59, 61, 62, 79). It is plausible that different sets of genes are utilized in a coordinated manner with the *icm/dot* genes. It is also possible that different pathways of pathogen-host interaction are host cell specific.

In this study, we characterized the GB112 mutant. This mutant has been identified as one of the *pmi* mutants (32). Our confocal laser scanning and transmission electron microscopic

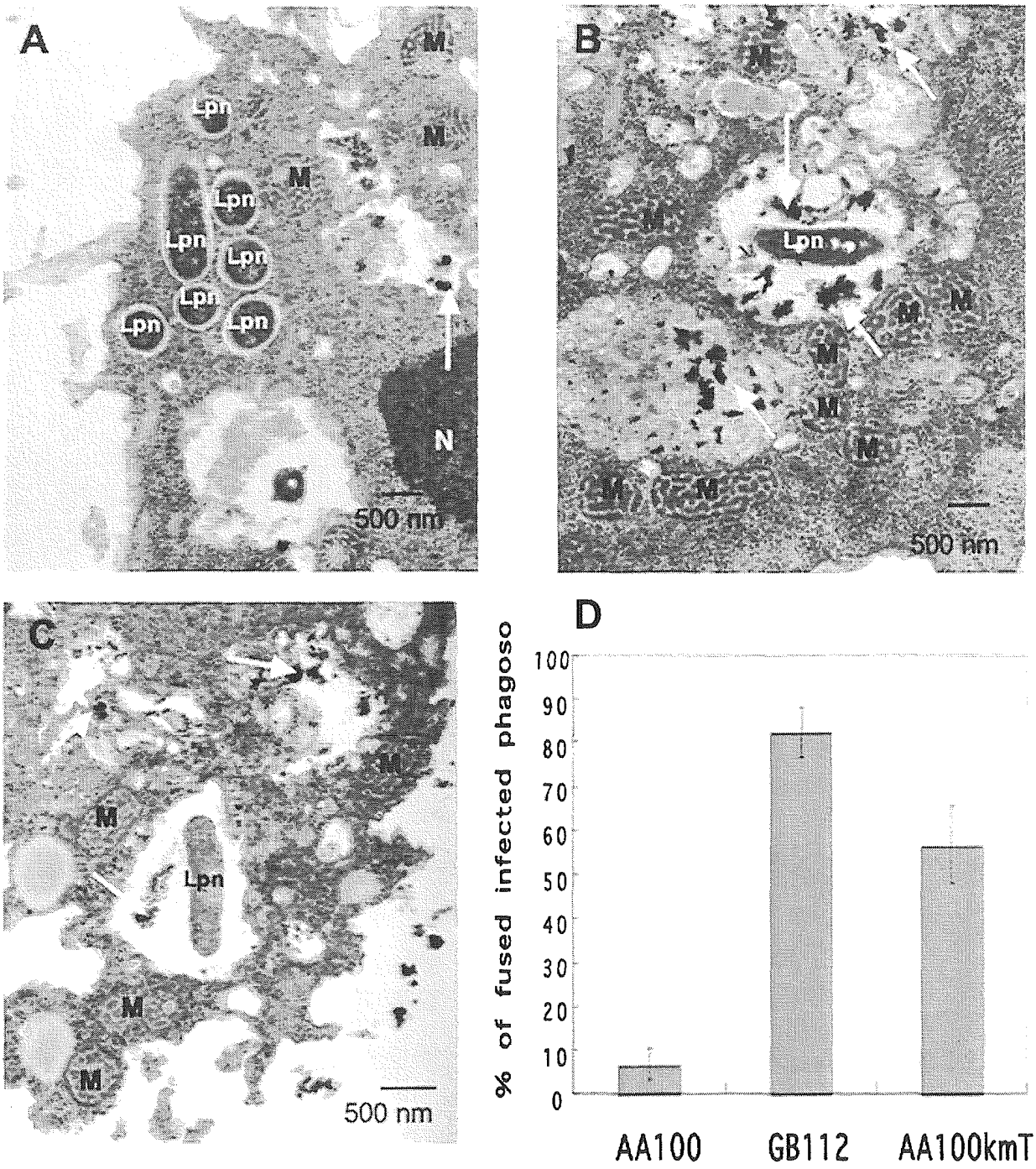

FIG. 4. Fusion of phagosomes containing the GB112 mutant in *A. polyphaga* to lysosomes. Localization of lysosomal acid phosphatase in phagosomes within *A. polyphaga* was determined at 6 h postinfection. (A to C) Representative electron micrographs of *L. pneumophila*-infected *A. polyphaga* at 6 h postinfection. Signals representing acid phosphatase were detected as electron-dense lead nitrate, as indicated by the arrows. Tight phagosomes containing wild-type strain AA100 (A) were negative for acid phosphatase. Phagosomes containing GB112 (B) or AA100KmT (C) (*icmT* deficient) exhibited positive signals for acid phosphatase. (D) Quantitative results obtained by examining 150 *Legionella*-containing phagosomes for the presence of acid phosphatase. Lpn, *L. pneumophila*; M, mitochondrion; N, nucleus; phagoso, phagosomes. The experiment was done three times in triplicate; the data are the data from one representative experiment, and the error bars indicate standard deviations.

studies showed that phagosomes containing the GB112 mutant are defective in evasion of acquisition of late endosomal and lysosomal markers at early stages of infection of both U937 macrophages and *A. polyphaga*. Thus, *pmiA* is involved in inhibition of fusion of the bacterium-containing phagosomes to lysosomes in both types of host cells. Interestingly, the *pmiA* mutant is severely defective in protozoa but exhibits a less severe defect in intracellular growth in the U937 human mac-

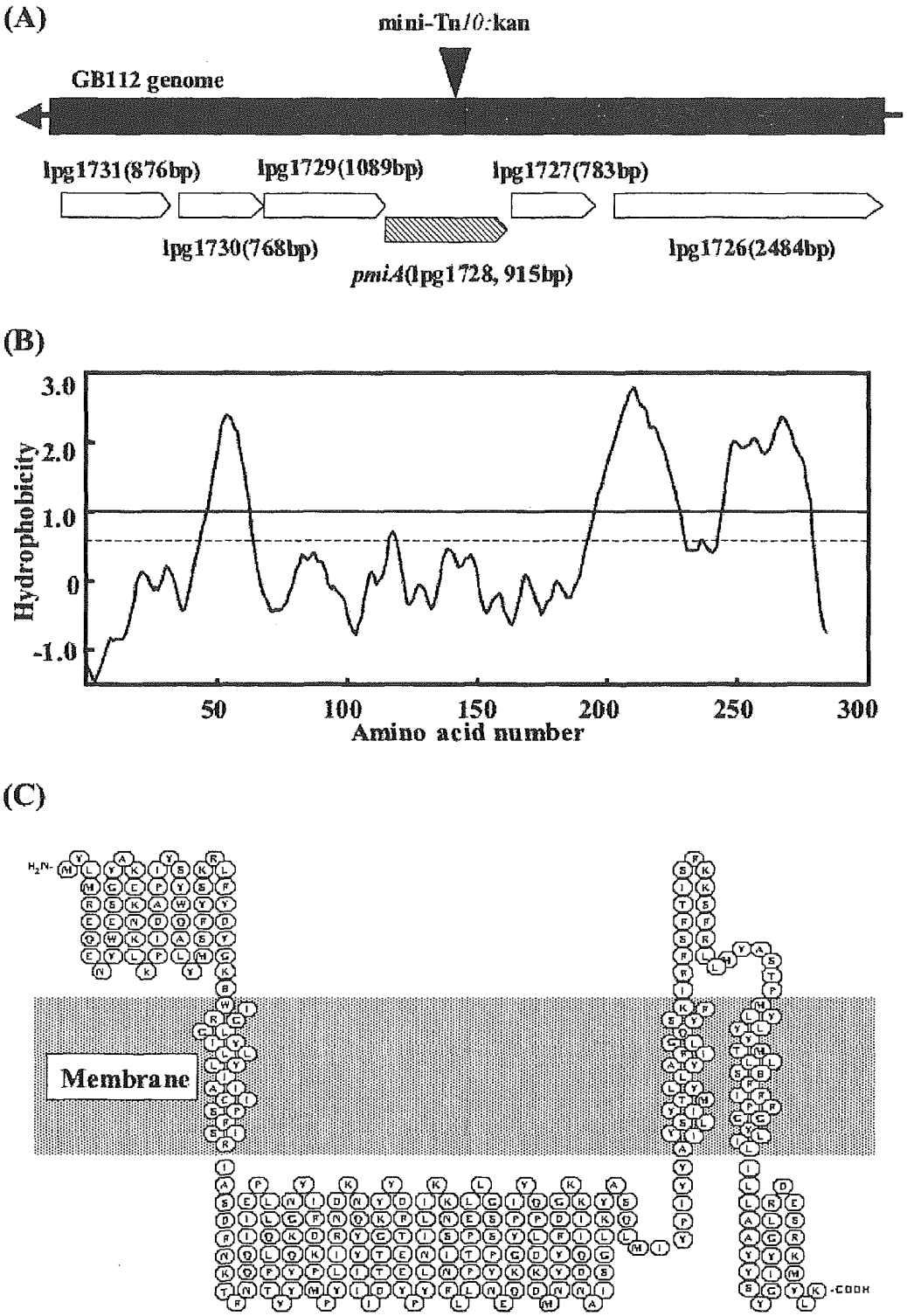

FIG. 5. Genetic and structural analysis of PmiA. (A) Genetic organization of the mutated region in the GB112 mutant. Mini-Tn*10*::*kan* was found to be inserted into a putative 915-bp open reading frame (lpg1728), *pmiA*. In the flanking regions, there are five ORFs up- and downstream of *pmiA* (lpg1731, lpg1730, lpg1729, lpg1727, and lpg1726), whose direction of transcription was the same as that of *pmiA*. (B) Hydropathy profile of the predicted PmiA protein amino acid sequence based on a Kyte-Doolittle analysis with a default window of 21. The solid and dashed lines indicate the cutoff values for certain and putative transmembrane segments, respectively. Negative values indicate relative hydrophilicity. (C) Secondary structure of PmiA predicted by the SOSUI program.

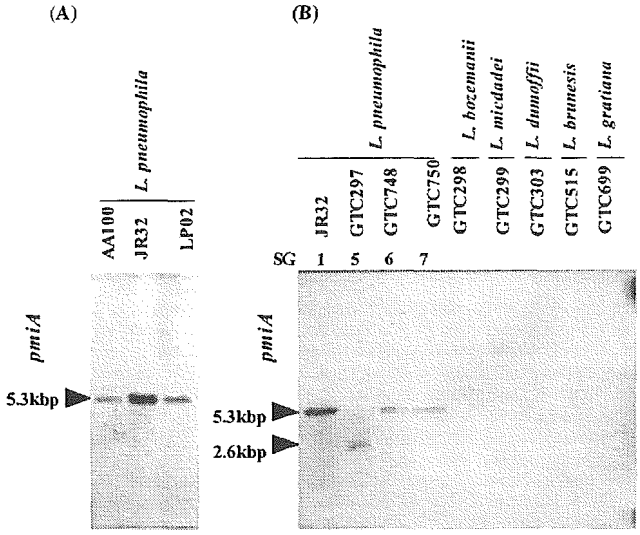

FIG. 6. Southern blot analysis of the *pmiA* gene in *L. pneumophila* serogroup 1 strains (A) and in several serogroups of *L. pneumophila* and other *Legionella* species (B). *pmiA* was detectable in four different serogroups of *L. pneumophila* but was absent from other *Legionella* species, including *L. bozemanii*, *L. micdadei*, *L. dumoffii*, *L. brunesis*, and *L. gratiana*.

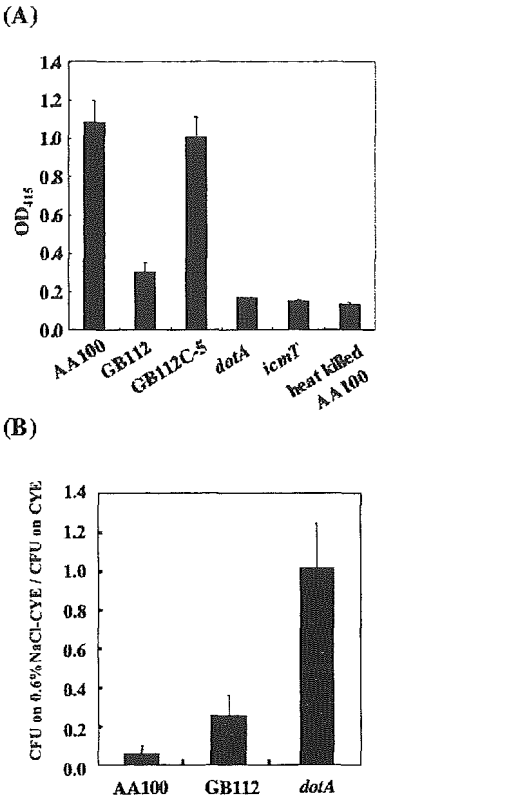

FIG. 7. PmiA is essential for the pore-forming activity but is not required for NaCl sensitivity of *L. pneumophila*. (A) The pore-forming activities of *L. pneumophila* mutant GB112 (*pmiA*), LELA3118 (*dotA*), and GS3011 (*icmT*) were tested by contact-dependent hemolysis of sRBCs. GB112C-5 is a *pmiA*-complemented strain of GB112. Wild-type strain AA100 and heat-killed AA100 were used as positive and negative controls, respectively. (B) NaCl sensitivity of the GB112 mutant. The data are expressed as the ratio of the colony-forming activity after plating on 0.6% NaCl–BCYE agar plates to the colony-forming activity after plating on regular BCYE plates. The AA100 (wild type) and *dotA* strains were used as NaCl-sensitive and NaCl-resistant controls, respectively. The experiments were done three times in triplicate; the data are the data from one representative experiment, and the error bars indicate standard deviations.

rophage cell line. Although the intracellular growth of the *pmiA* mutant within U937 cells shows a partially defective phenotype, LAMP-1 and LAMP-2 colocalize with phagosomes containing the *pmiA* mutant. The acquisition of these late endosomal and lysosomal markers by the *pmiA* mutant-containing phagosomes is correlated with the defect in intracellular survival and replication. It is interesting that despite the colocalization of LAMP-1 and LAMP-2 with phagosomes containing the *pmiA* mutant within U937 macrophages, the bacteria that were able to establish a replicative niche replicated in these cells. It has been reported that the *dotA* and *dotB* mutants reside in a nonlysosomal LAMP-1-positive compartment within mouse-derived bone marrow macrophages (43). The *pmiA* mutant might reside in the same type of phagosome as *dotA* and *dotB* mutants within macrophages.

The *pmiA* gene encodes a putative transmembrane protein that has three membrane-spanning domains based on hydropathy and membrane topology analysis. Interestingly, PmiA has a distinct long hydrophilic region between the transmembrane domains. This region has high similarity to hypothetical proteins of other bacteria possessing type IV secretion systems, such as *Rickettsia* species and *Helicobacter pylori*. A comparison using the protein-protein BLAST (blastp) program revealed that the region between amino acids 36 and 269 of PmiA, which covers the entire long hydrophilic region and the neighboring two transmembrane regions, exhibits similarity (20% identity and 40% similarity) to the corresponding part of hypothetical protein RP489 (accession no. Q9ZD57) of *Rickettsia prowazekii* (9). Interestingly, the corresponding hypothetical protein of *Rickettsia* species is predicted to be a transmembrane protein, and the secondary structure of the whole protein is also similar to that of PmiA (data not shown). The region between amino acids 68 and 155, which covers most of the long hydrophilic region, exhibits similarity (29% identity and 48% similarity) to hypothetical protein jhp0336 (accession no. C71944) of *H. pylori* (8). At least five ORFs both up- and downstream of *pmiA*, which have the same transcriptional direction as *pmiA*, exhibit no similarity with any genes of these bacteria. The genomic positions of the genes encoding each of the corresponding hypothetical proteins of these pathogens are far from the genetic loci that encode the type IV secretion apparatus (data not shown). The functions of these proteins in intracellular survival and replication and whether there is any relationship to the type IV secretion apparatus have not been reported.

We demonstrate here that PmiA is involved in the pore-forming activity which is attributed to the Icm/Dot type IV secretion system (45). The loss of the pore-forming activity in many *icm/dot* mutants, including *dotA*, *icmT*, *icmQ*, and *icmR* mutants, has been demonstrated previously (45, 51, 52). Recently, it has been shown that the IcmQ protein is localized on the bacterial surface shortly after contact with a macrophage

(25). It has been suggested that pore formation in the mammalian cell membrane is caused by membrane insertion of IcmQ after its translocation to the bacterial surface (25). It is possible that PmiA is a component of the Icm/Dot secretion apparatus involved in export of IcmQ.

In summary, we show here that a defect in *pmiA* results in a defect in survival and replication of *L. pneumophila* in U937 macrophages and protozoa. Interestingly, the *pmiA* mutant exhibits a severe defect in intracellular growth within protozoa, in contrast to the partial defect in intracellular growth within macrophages. This defect is associated with trafficking of the *pmiA* mutant-containing phagosome through the endosomal-lysosomal pathway in macrophages and protozoa. The severe defect in the pore-forming activity of the *pmiA* mutant, in addition to a defect in its intracellular trafficking, suggests a potential contribution of the PmiA protein to export of Icm/Dot substrates. However, this potential role of PmiA in export of Icm/Dot substrates must be demonstrated directly.

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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

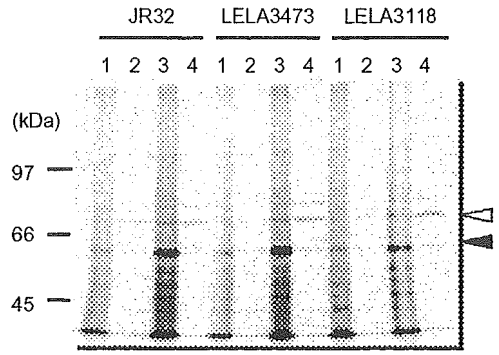

Fig. 1. Comparison of the heat shock response between the wild type and *icm/dot* mutant strains of *L. pneumophila* in vitro. Wild type strain JR32 and two *icm/dot* mutants, LELA3473 (*icmR* mutant) and LELA3118 (*dotA* mutant), were grown to the mid-log phase at 28 °C. After the addition of Tran^{35}S 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

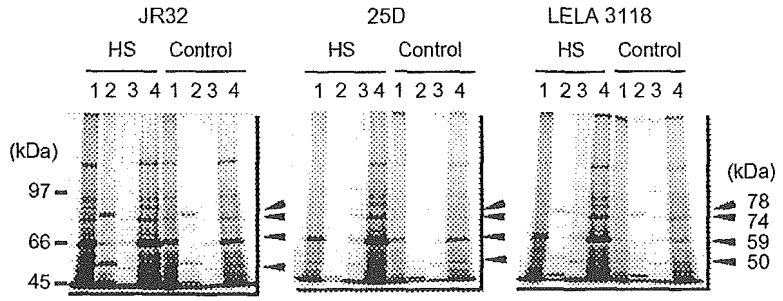

Fig. 2. Subcellular localization of heat shock proteins. JR32 (A), 25D (B), and LELA3118 (C) were grown to the mid-log phase at 28 °C. After Tran^{35}S label had been added, incubation was performed at 28 °C (control) or 42 °C (heat shock; HS) for 1 h. Lane 1, whole bacterial proteins; lane 2, secreted 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

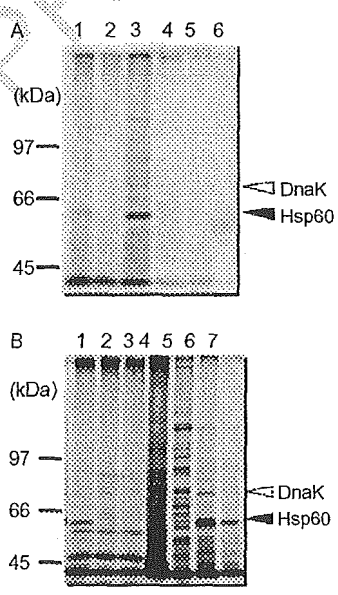

Fig. 3. Difference in the protein expression profiles of JR32 within different host cells. Open and solid arrowheads indicate the bands of DnaK and Hsp60, respectively. (A) Whole bacterial protein expression by intracellular JR32 within U937 macrophage-like cells. Lane 1, in vitro heat shock at 42 °C 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.

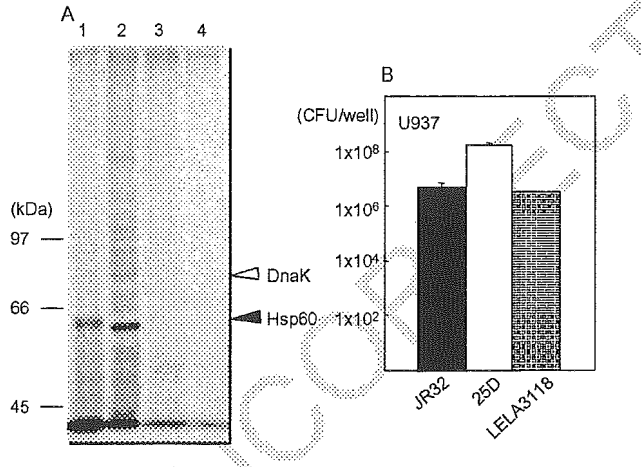

Fig. 4. Intracellular protein expression of the wild type and intracellular growth-deficient strains within U937. (A) Bacterial protein expression profiles of the wild type and intracellular growth-deficient strains within U937. Lane 1, JR32 with in vitro heat shock at 42 °C for 1 h; lane 2, JR32 within 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).

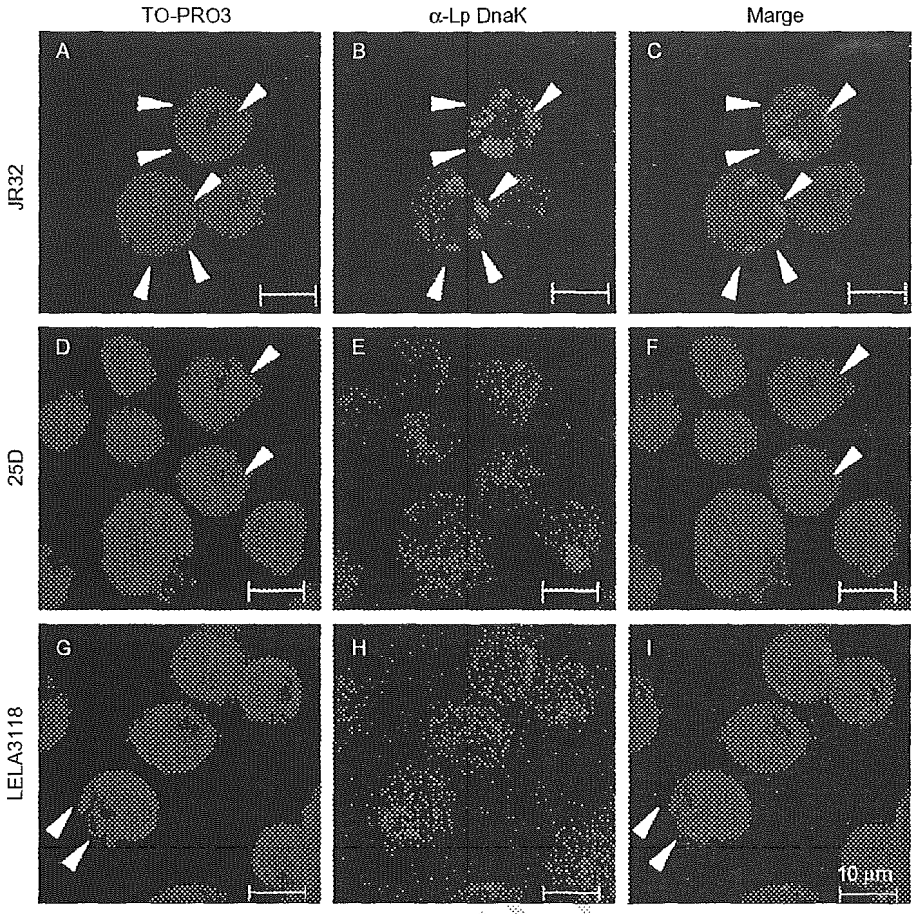

Fig. 5. Detection of the expression of *L. pneumophila* DnaK within U937 cells at 12 h post-infection. JR32 (A, B, C); 25D (D, E, F); LELA3118 (G, H, I). TO-PRO-3 (blue) stains the nucleic acid of both intracellular and extracellular bacteria as well as the U937 nucleus. *L. pneumophila* DnaK was visualized 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