# Identification and Subcellular Localization of the *Legionella pneumophila* IcmX Protein: a Factor Essential for Establishment of a Replicative Organelle in Eukaryotic Host Cells

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The gram-negative respiratory pathogen *Legionella pneumophila* infects and grows within mammalian macrophages and protozoan host cells. Upon uptake into macrophages, *L. pneumophila* establishes a replicative organelle that avoids fusion with endocytic vesicles. There are 24 *dot/icm* genes on the *L. pneumophila* chromosome required for biogenesis of this vacuole. Many of the Dot/Icm proteins are predicted to be components of a membrane-bound secretion apparatus similar to type IV conjugal transfer systems. We have been investigating the function of *L. pneumophila dot/icm* gene products that do not have obvious orthologs in other type IV transfer systems, since these determinants could govern processes unique to phagosome biogenesis. The *icmX* gene product falls into this category. To understand the role of the IcmX protein in pathogenesis, we have detailed interactions between an *L. pneumophila icmX* deletion mutant and murine bone marrow-derived macrophages. These data demonstrate that *icmX* is required for biogenesis of the *L. pneumophila* replicative organelle. Immunoblot analysis indicates that the *icmX* gene product is a polypeptide with an estimated molecular mass of 50 kDa. The IcmX protein was localized to the bacterial periplasm, and periplasmic translocation was mediated by an N-terminal *sec*-dependent leader peptide. A truncated IcmX product was secreted into culture supernatants by wild-type *L. pneumophila* growing extracellularly in liquid media; however, transport of the IcmX protein into eukaryotic host cells was not detected. Proteins similar in molecular weight to IcmX were identified in other *Legionella* species by immunoblot analysis using a monoclonal antibody specific for *L. pneumophila* IcmX protein. From these data, we conclude that the IcmX protein is an essential component of the *dot/icm* secretion apparatus, and that a conserved mechanism of host cell parasitism exists for members of the *Legionellaceae* family.

The respiratory pathogen *Legionella pneumophila* is a facultative intracellular bacterium that can grow within human alveolar macrophages (29). When virulent *L. pneumophila* bacteria come in contact with host macrophages, they induce the formation of pores in the macrophage membrane (31) and enter a vacuole that evades fusion with late endosomes and lysosomes (28, 46). Phagosomes containing *L. pneumophila* mature into specialized organelles that support intracellular growth (14, 27). Events that permit *L. pneumophila* to alter endocytic trafficking of the phagosome in which it resides are mediated through the actions of products encoded by the *dot/icm* genes (5, 35, 49, 55).

Phagosomes containing *L. pneumophila dot/icm* mutants traffic to lysosomes as efficiently as and with kinetics similar to vacuoles harboring dead or avirulent microorganisms (46). The inability of *L. pneumophila dot/icm* mutants to modulate phagosome trafficking is manifested as a severe defect in growth and survival within macrophages (3, 5, 48, 50), as well as a pronounced reduction in virulence (18, 35). Interestingly, when these mutants reside in the same vacuole as wild-type *L. pneumophila*, they are capable of replicating within this compartment (14). Thus, the *dot/icm* genes are not required for

intracellular growth per se, but play a fundamental role in creating a nutrient rich organelle that is isolated from endocytic traffic.

How the *dot/icm* gene products control trafficking of phagosomes containing *L. pneumophila* is unclear. It has been postulated that the *dot/icm* genes encode a transport apparatus that exports protein molecules into the host cell which directly affect phagosome maturation (45, 51, 56). Evidence in support of this hypothesis comes from DNA sequence analysis indicating that a number of the *dot/icm* products are similar to components of type IV secretion systems. Recently, it was shown that 14 of the *dot/icm* products have significant sequence similarities to proteins in the transfer region of the IncI plasmid colIb-P9 (53). Thus, these *dot/icm* products and their col1b-P9 counterparts are likely orthologous components of a type IV secretion apparatus with a common ancestral progenitor.

Endogenous substrates secreted by the *dot/icm* apparatus have eluded detection; however, the observation that conjugal transfer of plasmid RSF1010 from *L. pneumophila* to other gram-negative bacteria requires several of the *dot/icm* gene products demonstrates that this apparatus has transport activity (49, 55). It seems unlikely that the transfer of genetic material from *L. pneumophila* into the host cell plays a fundamental role in preventing fusion of the phagosome with lysosomes, since this process happens either during or immediately after bacterial uptake (46, 57). It is apparent that further analysis of the numerous *dot/icm* proteins is required to understand the function of these genes and determine how

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TABLE 1. *Legionella* strains used in this study

| Strain | Parent strain | Important trait(s) | Reference |
|---|---|---|---|
| CR24 | LP02 | *rpsL thyA* | 5 |
| CR26 | JV374 | *rpsL thyA ΔdotB* | 55 |
| CR39 | LP01 | *rpsL* | 5 |
| CR51 | LP02 | *rpsL thyA ΔdotA* | 14 |
| CR58 | LP01 | *rpsL ΔdotA* | 47 |
| CR157 | LP01 | *rpsL ΔicmW* | 58 |
| CR174 | CR157 | *rpsL ΔicmW* (pIcmWM45) | 58 |
| MM101 | LP01 | *rpsL ΔicmX* | This study |
| *L. pneumophila* SG1 | ATCC 33152 | Serogroup 1 | 9 |
| *L. pneumophila* SG2 | ATCC 33154 | Serogroup 2 | 9 |
| *L. pneumophila* SG3 | ATCC 33155 | Serogroup 3 | 9 |
| *L. bozemanii* | ATCC 33217 | | 36 |
| *L. gratiana* | ATCC 49413 | | 6 |
| *L. micdadei* (Pittsburgh) | ATCC 33204 | | 42 |
| *L. micdadei* (Tatlock) | ATCC 33218 | | 25 |

they regulate biogenesis of phagosomes in which *L. pneumophila* resides.

To understand *L. pneumophila* pathogenesis and elucidate the molecular mechanisms that bacteria employ to alter vesicle trafficking, we have been focusing on *dot/icm* genes that are not present in other type IV secretion systems. Our hypothesis is that these genes may encode factors that are either exported by the bacteria or tailor a specific function of the type IV apparatus that is directly related to phagosome trafficking. Gene products encoded within the *icmWXYZ* operon appear to be unique to the *L. pneumophila dot/icm* system. Recently, we demonstrated that the *icmW* gene encodes a protein that is not required for the formation of pores in the macrophage plasma membrane upon contact, even though it is necessary for phagosome trafficking and intracellular growth (58). It was postulated that IcmW could be a chaperone that pilots protein molecules to the Dot/Icm apparatus for directed secretion through the pore formed in the host cell plasma membrane.

In this study, we extend our molecular and genetic analysis of the *icmWXYZ* gene cluster. Our results demonstrate that *icmX* is required for *L. pneumophila* phagosome trafficking and intracellular growth. Translational gene fusions and immunoblot analysis indicate that the *icmXYZ* region encodes a single protein, IcmX, located in the bacterial periplasm. From these data, we conclude that the IcmX protein is an essential component of the Dot/Icm transporter.

## MATERIALS AND METHODS

**Bacterial strains and media.** The *L. pneumophila* strains used in this study (Table 1) were grown on charcoal-yeast extract (CYE) plates or in ACES [*N*-(2-acetamido-2-aminoethanesulfonic acid)]-buffered yeast extract (AYE) broth as described previously (19, 47). As required, antibiotics were added to *L. pneumophila* media at the following concentrations: streptomycin, 100 μg ml$^{-1}$; kanamycin, 20 μg ml$^{-1}$; and chloramphenicol, 10 μg ml$^{-1}$. *Escherichia coli* strains were grown on L-agar plates or in L broth, and antibiotics were added to growth media at the following concentrations as required: ampicillin, 100 μg ml$^{-1}$; kanamycin, 40 μg ml$^{-1}$; and chloramphenicol, 25 μg ml$^{-1}$.

**Cell culture.** U937 cells (American Type Culture Collection) were cultured in RPMI 1640 with 10% fetal bovine serum (Gibco). U937 cells were differentiated with phorbol 12-myristate 13-acetate (Sigma) for 48 h as described elsewhere (43). Bone marrow-derived macrophages were cultured from female A/J mice (Jackson Laboratory) as described elsewhere (10).

**Plasmid constructions.** The in-frame *icmX* deletion was constructed by joining 5′ and 3′ *icmX* DNA fragments together in the gene replacement vector pSR47S (39). The 5′ DNA fragment was generated by PCR amplification using a forward primer (5′-GG**GAGCT**CCTCTTACGATCCTTGATCC-3′) and a reverse primer (5′-CC**ACGCGT**GGCCAAGGCCAGTTTAGGTAA-3′). The 3′ DNA fragment was generated by PCR amplification using a forward primer (5′-CC**ACGCGT**CCCACTGCTGATTTTTCAAGCT-3′) and a reverse primer (5′-CC**TCTAGA**GCCGGGGAATGATTTAAACCAT-3′). *Mlu*I restriction sites that were incorporated into primers are in boldface. The 5′ *icmX* DNA product was digested

with *Mlu*I and *Xba*I. The 3′ *icmX* DNA product was digested with *Mlu*I and *Sac*I. The DNA fragments were ligated into the vector pSR47S, which had been digested with *Xba*I and *Sac*I. The resulting plasmid contains an *icmX* deletion allele that has the coding region for IcmX replaced by a *Mlu*I restriction site. All pSR47S plasmid constructs were propagated in *E. coli* DH5α (λ*pir*). This strain encodes the RK6 π protein, which is required for plasmid replication (32).

The *icmX* deletion was introduced onto the chromosome of *L. pneumophila* CR39 by allelic exchange. The deletion plasmid was mated from *E. coli* into CR39 as previously described (47). Mating mixtures were plated on CYE containing kanamycin and streptomycin to select for *L. pneumophila* that had integrated the deletion plasmid onto the chromosome. Kanamycin-resistant colonies were then plated on CYE containing 5% sucrose to select for bacteria that had lost the plasmid. Sucrose-resistant colonies were screened by PCR using primers that flank the *icmX* gene to identify clones that had incorporated the deletion. Incorporation of the deletion was further confirmed by digesting the PCR products with *Mlu*I. Several Δ*icmX* clones were assayed for growth in U937 cells, and all were incapable of intracellular replication. Clone MM101 was selected for future studies.

To construct plasmid pMM1.3, the *icmX* gene was amplified from *L. pneumophila* CR157 by using a forward primer (5′-GG**GAGCT**CCAATAACCCTTGCCTGTAC-3′) and a reverse primer (5′-CC**TCTAGA**GCCGGGGAATGATTTAAACCAT-3′). The PCR product was digested with *Sac*I and *Xba*I and then ligated into the broad-host-range cloning vector pMMB207 (40), which had been digested similarly. The resulting plasmid contains the *icmX* gene and the promoter for the *icmWX* operon but lacks the *icmW* gene.

To construct plasmid pMM5, the *icmX* gene was amplified from strain CR157 by using a forward primer (5′-CC**GAATTC**TCTTTCTCACCCAATAACC-3′) and a reverse primer (5′-GG**CCATGG**CTTGCTCAGAAGGAGAGCCTTG-3′). The *Nco*I site in the reverse primer will fuse the last codon of *icmX* in frame with the M45 epitope tag present in the vector pSB616 (16). The PCR product was digested with *Eco*RI and *Nco*I and then ligated into pSB616, which had been digested with the same enzymes. The *icmXM45* allele was removed from this plasmid using enzymes *Eco*RI and *Sal*I. This fragment was ligated into pMMB207, which had been digested with the same enzymes. The resulting plasmid, pMM5, contains the *icmXM45* allele and the *icmWX* promoter.

Plasmid pCR2-1, which produces the IcmX48-PhoA protein, was constructed by first generating a PCR using a forward primer (5′-GG**GAGCT**CCTCTTACGATCCTTGATCCTG-3′) and a reverse primer (5′-CCGTCTAGATTTTCCCATCTGCGCCGG-3′). The PCR product was digested with *Eco*RV and *Sac*I and then ligated into the vector pDot450::PhoA (47), which had been first cut with *Xba*I, blunted with Klenow enzyme, and then cut with *Sac*I. Plasmid pCR3140, which expresses the IcmX399-PhoA protein, was constructed by generating a PCR product using the same forward primer and the reverse primer 5′-CCG**TCTAGA**CCATTGTTGGTTTGGTTGTC-3′. This PCR product was digested with *Sac*I and *Xba*I and then ligated into the vector pDot450::PhoA, which had been digested with the same enzymes. Plasmid p*icmX*1231::*phoA* was constructed using the same forward primer and the reverse primer 5′-CCG**TCTAGA**CTGTAGCAGGGGATGCGT-3′. This PCR product was also digested with *Sac*I and *Xba*I and cloned into the vector pDot450::PhoA, which had been digested with the same enzymes.

**Intracellular growth assay.** Growth of *L. pneumophila* in U937 cells and bone marrow-derived macrophages was determined by using a previously described standard intracellular growth assay (47). Confluent monolayers of bone marrow-derived macrophages or phorbol ester-treated U937 cells in 24-well tissue culture dishes were infected with *L. pneumophila* at a multiplicity of infection (MOI) of 0.1. The bacteria were harvested in early stationary phase by either growth for 16 to 18 h in AYE broth or isolation from a heavy region of growth on a CYE-agar plate 48 h after inoculation. The infected macrophages were incubated at 37°C

and 5% $CO_2$ for 1 h and washed three times with phosphate-buffered saline (PBS) to remove extracellular bacteria. Fresh tissue culture medium was added prior to further incubation. At the appropriate time points, macrophages were lysed in sterile distilled $H_2O$, and dilutions were plated on CYE-agarose plates.

**Macrophage permeability assay.** Formation of pores in macrophage membranes upon contact with *L. pneumophila* was determined as described previously (31). *L. pneumophila* strains were grown for 48 h on CYE-agar plates and opsonized with polyclonal rabbit anti-*L. pneumophila* antibody (1:2,000) prior to infections (antibody provided by Ralph Isberg, Tufts University). Bacteria were added at the indicated MOI to $1.5 \times 10^5$ murine bone marrow-derived macrophages that were plated on coverslips in 24-well tissue culture dishes. The tissue culture plates were centrifuged at $150 \times g$ for 5 min at room temperature and then incubated for 1 h at 37°C in 5% $CO_2$. The coverslips were inverted onto 5 μl of PBS containing ethidium bromide (25 μg ml$^{-1}$) and acridine orange (5 μg ml$^{-1}$). Acridine orange enters all cells, but ethidium bromide is excluded from cells with an intact plasma membrane. Pore-forming activity was measured as the percentage of total cells that stained positive for ethidium bromide. Overlapping color images were recorded for each coverslip using a Zeiss LSM510 confocal microscope and 10× objective. Each image was saved as a TIFF file and exported into NIH Image 1.62 for analysis. The cell counting macro for NIH Image 1.62 was used to determine the number of macrophages that stained positive with ethidium bromide in each image.

**Immunofluorescence microscopy.** *L. pneumophila* was grown overnight to saturation at 37°C in AYE broth. The bacteria were washed and resuspended in PBS (optical density at 600 nm [$OD_{600}$] = 1.0). *L. pneumophila* at an MOI of 2 to 10 was added to $10^5$ mouse bone marrow-derived macrophages plated on coverslips in 24-well tissue culture dishes. The tissue culture plates were centrifuged at $150 \times g$ for 5 min at room temperature and incubated for 1 h at 37°C in 5% $CO_2$. Extracellular bacteria were removed by washing multiple times with PBS. Fresh medium was added to each well, and the plates were returned to the incubator for 1 or 8 h. Cells were fixed for 20 min at room temperature in PBS containing 2% paraformaldehyde and 4.5% sucrose. The cells were washed with PBS containing 4.5% sucrose (PBS-S) and permeabilized in ice-cold methanol for 10 s. Coverslips were blocked in PBS containing 2% goat serum (Gibco) and 4.5% sucrose (PBS-GS) at room temperature for 1 h. Lysosomes and late endosomes were stained with the rat monoclonal antibody (MAb) 1D4B (1:100) specific for LAMP-1 (11), followed by fluorescein isothiocyanate-labeled goat anti-rat secondary antibody (1:500). All antibody washes were in PBS-S, and all antibody dilutions were in PBS-GS. Coverslips were treated with RNase A (100 μg ml$^{-1}$ [Roche]) at 37°C for 30 min, and then bacterial and macrophage DNA was stained with 0.05 μg of propidium iodide (Sigma) ml$^{-1}$ in PBS at 37°C for 15 min. Coverslips were washed in PBS and mounted onto 1 μl of mounting medium (90% glycerol, 10% PBS, 1 mg of phenylenediamine ml$^{-1}$) on glass slides. Bacterial phagosomes were imaged using a Zeiss LSM510 confocal microscope. TIFF files were transferred, and images were labeled using Adobe Illustrator 7.0.

**Immunoblot analysis.** N-terminal glutathione *S*-transferase (GST) fusion proteins were constructed with the IcmX and DotA proteins. A DNA fragment encoding the C-terminal 100 amino acid residues of the DotA protein was generated by PCR using the forward primer 5′-CG**GAATT**CCGGAATCTTTT GGTCAAG-3′ and the reverse primer 5′-GG**GTCGAC**TGAATGTTATTCGG GAGGTGG-3′. The fragment was digested with *Eco*RI and *Sal*I and then ligated into the similarly digested vector pGEX-KG (23). The GST-DotA fusion protein was expressed in *E. coli* and affinity purified on glutathione-Sepharose 4B as recommended by the manufacturer (Pharmacia Biotech). The GST-IcmX fusion protein was constructed by cloning an *icmX* restriction fragment into the vector pGEX-KG (23). The in-frame fusion junction was created by ligating the internal *Eco*RV site in *icmX* to the *Eco*RI site in pGEX-KG after a Klenow reaction was performed to fill in the 5′ *Eco*RI overhang. The resulting GST-IcmX fusion protein was insoluble when expressed in *E. coli* and was isolated from inclusion bodies as described elsewhere (24). These GST fusion proteins were used for all animal immunizations (24). Mouse MAbs were produced by the Cell Culture and Hybridoma Facility at State University of New York at Stony Brook. Antibodies from the supernatants of cloned hybridomas were screened by enzyme-linked immunosorbent assay using plates coated with the GST-DotA and GST-IcmX fusion proteins. Clones 5.1 and 11.29 were found to be specific for the IcmX protein, clones 2.29 and 37.29 were found to be specific for the DotA protein, and clones 99 and 107 were found to be specific for the GST protein. Rabbit polyclonal antibodies against IcmX were produced by the Yale University Animal Resources Center Immunization Service using the same antigen.

For immunoblot analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing *E. coli* or *Legionella* protein extracts in Laemmli sample buffer (33) were transferred to Immobilon P membranes (Millipore) in transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS, 20% methanol [pH 8.3]) at either 20 V for 12 h or 100 V for 1 h. The membranes were blocked in BLOTTO (PBS, 5% nonfat dry milk, 0.1% Tween 20) for 1 h at room temperature and incubated overnight with the designated primary antibodies at the following dilutions: rabbit polyclonal IcmX antibody (1:10,000), IcmX MAb 5.1 (1:10), M45 MAb 45 (1:1,000) (41), and DotA MAb 37.29 (1:1,000). Horseradish peroxidase conjugated anti-rabbit and anti-mouse secondary antibodies (Zymed) were used at a dilution of 1:2,000. Renaissance chemiluminescnece reagent (NEN) was used for antibody detection.

**Cell fractionation.** Subcellular localization of the IcmX protein was conducted using *L. pneumophila* cells harvested from 50-ml cultures that were grown to saturation in AYE broth at 37°C. Bacterial cells were disrupted, and lysates were fractionated into soluble and membrane-associated proteins as detailed previously (47). Briefly, bacterial membranes were isolated by centrifugation onto a 60% sucrose cushion and then washed in a high-salt buffer (10 mM HEPES, 5 mM $MgSO_4$, 0.5 M NaCl [pH 7.4]). Inner membrane proteins were solubilized by treating the washed membranes in a Triton X-100 buffer (10 mM HEPES, 5 mM $MgSO_4$, 2% Triton X-100 [pH 7.4]). Outer membrane proteins and Triton X-100-insoluble debris were isolated by centrifugation at $100,000 \times g$ for 30 min. Proteins from an estimated $10^8$ *L. pneumophila* cells were separated by SDS-PAGE (12% gel), and IcmX protein in each fraction was identified by immunoblot analysis using MAb 5.1. The scanned immunoblots were analyzed using NIH Image 1.62 to determine the relative amounts of immunoreactive product in each lane.

To determine whether IcmX is secreted into culture supernatants, 50-ml cultures of *L. pneumophila* were grown in AYE broth at 37°C. Exponentially growing bacteria taken from a CYE-agar plate were used to inoculate the cultures (starting $OD_{600}$ = 0.2). Bacterial growth was monitored by measuring the $OD_{600}$ of the cultures at regular intervals. After each $OD_{600}$ reading, an aliquot of liquid culture containing $10^9$ bacterial cells was removed. Bacterial cells were isolated by centrifugation at $8,000 \times g$ for 15 min at 4°C. The clarified culture supernatants were centrifuged at $15,000 \times g$ for 20 min at 4°C to remove any remaining bacterial cells or insoluble debris. Proteins in the culture supernatants were precipitated by adding ice-cold 100% trichloroacetic acid (TCA) to a final concentration of 12%. After incubation on ice for 1 h, the TCA precipitate was pelleted at $15,000 \times g$ for 30 min at 4°C. Whole cell bacterial pellets and TCA-precipitated supernatant proteins were resuspended in 200 μl of Laemmli buffer and boiled for 5 min, and 20 μl of each sample was separated by SDS-PAGE (12% gel). IcmX protein was detected by immunoblot analysis using a rabbit polyclonal antibody specific for IcmX.

To determine whether the IcmX protein is secreted upon contact with eukaryotic host cells, murine bone marrow-derived macrophages were infected and fractionated as described previously (15, 58). Briefly, a confluent monolayer of murine bone marrow-derived macrophages in 100-mm-diameter tissue culture dishes were infected with *L. pneumophila* at an MOI of 50 in 2 ml of RPMI medium without fetal bovine serum. The infected macrophages were incubated at 37°C in 5% $CO_2$ for 2 h to allow bacterial uptake. After infection, the tissue culture medium was removed. To separate extracellular bacteria from proteins in the tissue culture supernatant, the collected medium was centrifuged at $15,000 \times g$ for 15 min. The pellet, which contains extracellular bacteria, was resuspended in 50 μl of PBS. Proteins in the tissue culture supernatant were precipitated with TCA (12% final), pelleted, and resuspended in 50 μl of PBS. The macrophage monolayer was lysed on ice in 2 ml of Hanks' balanced salt solution (Gibco) containing 0.1% Triton X-100 and a 1:1,000 dilution of a protease inhibitor cocktail (Sigma P-8340). The macrophage lysate was transferred to a microcentrifuge tube and treated with RNase A (10 μg ml$^{-1}$) and DNase I (10 μg ml$^{-1}$) for 15 min at room temperature. Intracellular bacteria and Triton X-100-insoluble material were pelleted by centrifugation at $15,000 \times g$. The pellet was resuspended in 50 μl of PBS. Triton X-100-soluble proteins, which would presumably include factors secreted or translocated by the bacteria upon contact with host cells, were precipitated in TCA (12% final), and pelleted at $15,000 \times g$. The TCA pellet was resuspended in 50 μl of PBS; 50 μl of 2× Laemmli buffer was added to each fraction. Samples to be probed for IcmX protein were boiled for 5 min before loading, and samples to be probed for DotA protein were incubated at 37°C for 5 min prior to being loaded. A 20-μl aliquot of each sample was separated by SDS-PAGE (12% gel) for immunoblot analysis. IcmX protein was identified in each fraction by immunoblot analysis using a polyclonal antibody specific for IcmX, and the DotA protein was identified using the MAb 37.29.

**Alkaline phosphatase assay.** Plasmids pCR2-1, pCR3140, and p*icmX*1231::*phoA* were transformed into the *E. coli phoA* mutant strain CC118 (30) and *L. pneumophila* strains CR24 (*thyA*), CR26 (*thyA* Δ*dotB*), and CR51 (*thyA* Δ*dotA*). The resulting bacterial strains were grown in liquid broth. Alkaline phosphatase activity was determined by using the substrate Sigma 104 and measuring production of the colorimetric reaction product as described previously (47).

**Biotin labeling.** *L. pneumophila* strain CR174 was grown to early stationary phase in AYE broth at 37°C. CR174 expresses the IcmWM45 epitope-tagged protein, which has been shown previously to be located in the bacterial cytoplasm (58). Bacterial cells were pelleted at $8,000 \times g$ for 15 min and washed in ice-cold PBS. To label bacterial proteins, $2 \times 10^9$ *L. pneumophila* cells were resuspended in 1 ml of PBS. Sulfosuccinimidyl-6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin; Pierce) was added at a final concentration of 10 μg ml$^{-1}$. The cells were incubated on ice for 30 min. Excess biotin was removed by three successive washes where the cells were pelleted at $15,000 \times g$ for 2 min and resuspended in 1 ml of ice-cold PBS. After the final wash, the cells were lysed in 100 μl of cracking buffer (1% SDS, 10 mM Tris, 1 mM EDTA [pH 8.0]) and boiled for 2 min. The lysates were cooled at room temperature, and 900 μl of ice-cold immunoprecipitation buffer (2% Triton X-100, 50 mM Tris, 1 mM EDTA, 150 mM NaCl [pH 8.0]) was added. The lysates were cleared by pelleting insoluble debris at $15,000 \times g$ for 10 min. TCA (12% final) was added to 400 μl of the cleared lysate, and the precipitated proteins were resuspended in 100 μl of

protein loading buffer. To isolate biotin-labeled proteins, 50 µl of streptavidin-agarose (Sigma) was added to 400 µl of the remaining cleared lysate, and the mixture was incubated overnight at 4°C on a rotating shaker. The streptavidin beads were pelleted for 30 s in a microcentrifuge, and the beads were washed three times in immunoprecipitation buffer. After the final wash, the beads were resuspended in 100 µl of protein loading buffer and boiled for 5 min. Then 20 µl of the TCA-precipitated lysate and 20 µl of the streptavidin agarose precipitate were separated by SDS-PAGE (12% gel). The IcmX protein was identified in each fraction by immunoblot analysis using a rabbit polyclonal antibody specific for IcmX, and the IcmWM45 protein was identified with MAb 45.

**Protease protection.** *L. pneumophila* strain CR174 was grown to mid-exponential phase ($OD_{600} = 2.0$) in AYE broth at 37°C. Bacterial cells were pelleted at $15,000 \times g$ for 5 min and washed with either a sucrose buffer (30 mM Tris, 20% sucrose [pH 8.0]) or a Tris-buffered saline solution (30 mM Tris, 100 mM NaCl, 1 mM MgCl$_2$ [pH 8.0]). For each time point, approximately $10^9$ washed bacterial cells were pelleted and resuspended in 100 µl of similar wash buffer. To permeabilize the outer membrane and peptidoglycan layer, EDTA (1 mM final) and lysozyme (10 µg ml$^{-1}$ final) were added to the bacterial cells in sucrose buffer. After a 10-min incubation period on ice, pronase (Sigma) was added to all tubes (0.05 mg ml$^{-1}$ final), and the reaction mixtures were incubated at room temperature. Reactions were stopped just before and 10, 30, and 60 min after the addition of pronase. The pronase digestions were terminated upon addition of 1.4 ml of 12% TCA to the reaction tube. TCA-precipitated proteins were collected for each time point and resuspended 400 µl of Laemmli buffer, and 20 µl of each fraction was separated by SDS-PAGE (12% gel) for immunoblot analysis. To demonstrate that IcmX protein in cellular extracts is protease sensitive, $10^9$ bacteria in sucrose buffer and Tris-buffered saline were lysed on ice by sonication for five bursts of 30 s each (Branson Sonifier 250 with microtip), and the resulting extracts were digested with pronase (0.05 mg ml$^{-1}$ final) for 60 min at room temperature. IcmX protein was identified by immunoblot analysis using a rabbit polyclonal antibody specific for IcmX.

**Identification of IcmX protein in other *Legionella* serogroups and species.** *Legionella* species and serogroups were obtained from the American Type Culture Collection. The bacteria were first passaged on CYE-agar plates and then grown overnight in AYE broth at 37°C to saturation. Approximately $10^9$ bacterial cells were pelleted, washed once in PBS, and lysed in 200 µl of Laemmli buffer. The bacterial extracts were separated by SDS-PAGE (12% gel), and IcmX-immunoreactive products were identified by immunoblot analysis using MAb 5.1. For nucleic hybridization studies, digested chromosomal DNA from each of the *Legionella* species and serogroups was probed using $^{32}$P-labeled DNA fragments encoding *dotA* (nucleotides 40 to 454), *icmX* (nucleotides 1 to 519), and *mip* (nucleotides 267 to 696) as described previously (12). To detect DNA fragments with at least 70% nucleotide identity, the filters were hybridized with the probes at 37°C in the presence of 5× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate)–18% deionized formamide and were washed at 50°C in 5.3× SSC containing 0.1% SDS.

## RESULTS

**Identification of the IcmX protein.** The *icmX* gene was initially reported to be a 420-bp open reading frame located within the *icmWXYZ* operon, and it was predicted to encode a protein of 140 amino acids (8). Recent sequence corrections suggest that the *icmXYZ* region contains a single gene, *icmX*, predicted to encode a 1,398-bp open reading frame that would produce a protein with a molecular mass of 50.6 kDa (GenBank accession no. U07354). To determine whether a 50.6-kDa IcmX protein is produced by this open reading frame, the M45 epitope tag was fused to the 3′ end of the putative 1,398-bp *icmX* gene. An epitope-tagged product with an estimated molecular mass of 50 kDa was identified by immunoblot analysis in *L. pneumophila* extracts expressing the *icmXM45* gene fusion encoded on plasmid pMM5 (Fig. 1, bottom). To identify an endogenously produced *icmX* product, MAbs were generated against a GST-IcmX fusion protein and used to detect a product with an estimated molecular mass of 50 kDa in *L. pneumophila* extracts (Fig. 1, top). To determine whether this product was encoded by the *icmX* gene, an in-frame chromosomal deletion of *icmX* was created in *L. pneumophila*, resulting in strain MM101. The 50-kDa protein identified by MAb 5.1 was not found in extracts prepared from the *L. pneumophila icmX* deletion mutant; however, the MAb did recognized the epitope-tagged IcmX-M45 protein (Fig. 1). As expected, the apparent molecular weight of the IcmXM45 protein was slightly larger due to the addition of 18 C-terminal
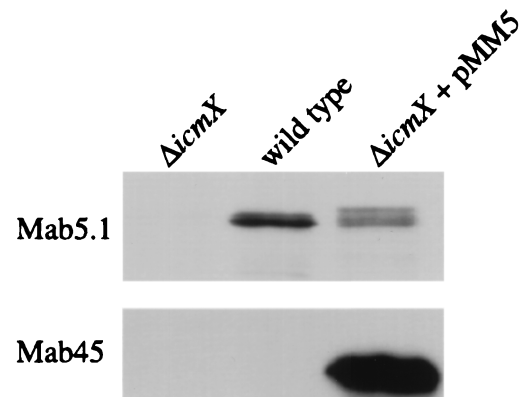


FIG. 1. The *L. pneumophila icmX* gene encodes a polypeptide with an estimated molecular mass of 50 kDa. Immunoblots of cellular extracts from *L. pneumophila* strains MM101 (Δ*icmX*), CR39 (wild type), and MM101 expressing the epitope-tagged IcmX-M45 protein (Δ*icmX* + pMM5) were probed with either MAb 5.1 specific for IcmX or MAb 45 specific for the M45 epitope tag. The gel region illustrated was located between protein standards with molecular masses of 56 and 42 kDa.

amino acid residues that comprise the M45 peptide. These data demonstrate that the *icmX* gene encodes a 50-kDa polypeptide, indicating that the most recent *icmX* sequence is correct (GenBank accession no. U07354).

**IcmX protein is required for phagosome trafficking and intracellular growth.** Previous studies suggest that *icmX* is essential for *L. pneumophila* pathogenesis (18, 35). To delineate the role of *icmX* during uptake and trafficking of phagosomes containing *L. pneumophila*, we first measured the capacity of MM101 to grow intracellularly. To complement the Δ*icmX* mutation in MM101, a DNA fragment encompassing the *icmWX* operon was amplified from the chromosome of *L. pneumophila* strain CR157, which has an in-frame deletion in the *icmW* gene (58). The plasmid containing this amplified DNA fragment, pMM1.3, contains the entire *icmX* gene transcribed by the endogenous *L. pneumophila* promoter for the *icmWX* operon but does not produce the IcmW protein.

We investigated whether the Δ*icmX* mutant, MM101, could replicate in mammalian phagocytic cells by measuring the CFUs recovered from infected host cells over 72 h. MM101 (Δ*icmX*) was not capable of replicating in either the human U937 macrophage-like cell line (Fig. 2A) or bone marrow-derived macrophages cultured from A/J mice (Fig. 2B). Intracellular survival of MM101 in murine bone marrow-derived macrophages paralleled that of an isogenic Δ*dotA* mutant. When plasmid pMM1.3 was introduced in *trans* to the chromosomal *icmX* deletion, intracellular replication of MM101 was restored to near wild-type levels in both U937 cells and murine macrophages. These data demonstrate that *icmX* is essential for intracellular growth of *L. pneumophila* within mammalian cells.

Virulent *L. pneumophila* will form pores in the plasma membrane of host cells upon contact (31). Mutations in several of the *dot/icm* genes have been shown to eliminate pore-forming activity (31, 58). To determine whether *icmX* plays an essential role in the formation of pores in the macrophage membrane, we examined whether macrophages infected with MM101 (Δ*icmX*) would exclude the membrane-impermeable dye ethidium bromide. Nuclear fluorescence after ethidium bromide staining was not observed for macrophages infected with MM101 at an MOI as high as 1,000 (Fig. 3). Thus, ethidium bromide is excluded from these cells, indicating that the Δ*icmX*
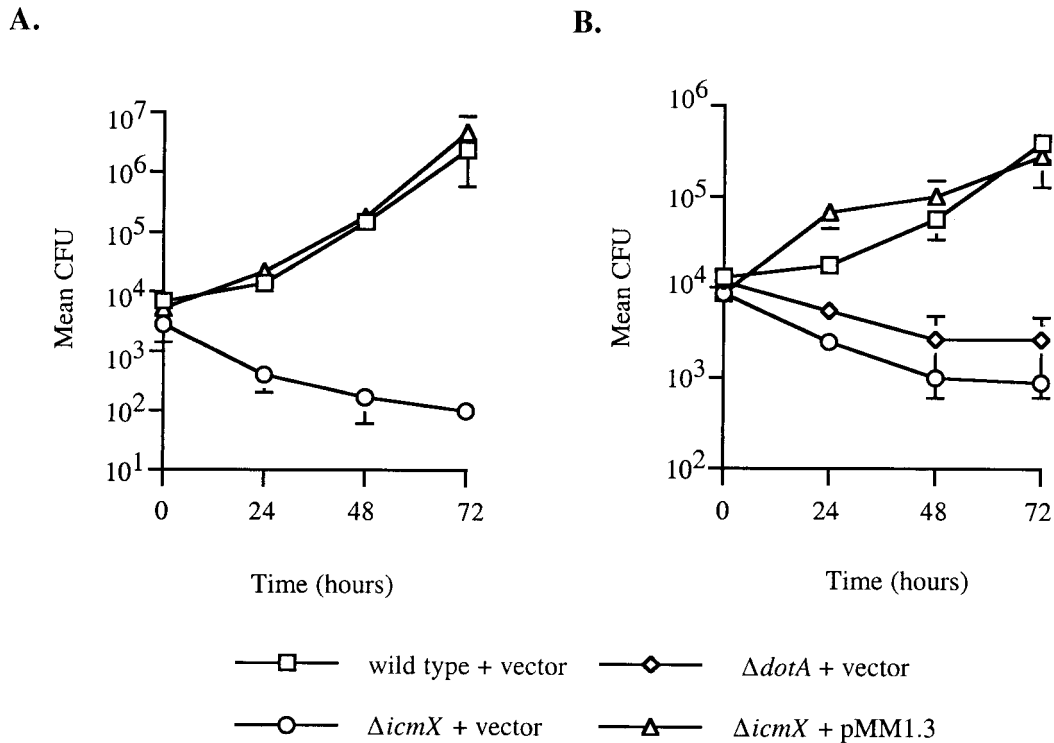
A.

B.



FIG. 2. The IcmX protein is required for replication of *L. pneumophila* within macrophages. Intracellular growth of *L. pneumophila* in differentiated U937 cells (A) or in mouse bone marrow-derived macrophages (B) was measured over 72 h. Host cells were infected with CR39 (wild type), CR58 (Δ*dotA*), and MM101 (Δ*icmX*) containing the cloning vector pMMB207 (vector) and with MM101 containing the *icmX* gene in *trans* (pMM1.3). Each data point represents mean CFU recovered from eukaryotic host cells in triplicate wells ± standard deviation.

mutant does not create pores in the macrophage plasma membrane. Pore-forming activity was fully restored to the Δ*icmX* strain when the *icmX* gene was returned in *trans* on the plasmid pMM1.3. A similar number of macrophages infected with MM101 (pMM1.3) were stained with ethidium bromide as macrophages infected with wild-type *L. pneumophila*. These

data demonstrate that *icmX* is required for the formation of pores in the membrane of eukaryotic host cells.

We examined bone marrow-derived macrophages infected with MM101 to determine whether *icmX* is required for inhibition of phagosome lysosome fusion and establishment of a replicative vacuole. Mouse bone marrow-derived macrophages
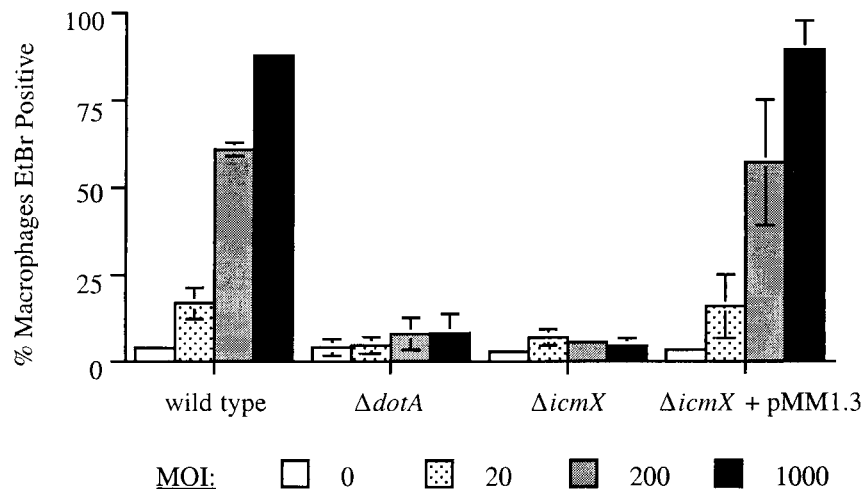


FIG. 3. *L. pneumophila* IcmX protein is required for the formation of pores in the plasma membrane of eukaryotic host cells. Bone marrow-derived macrophages were infected with *L. pneumophila* at MOIs of 0, 20, 200, and 1,000 for 1 h and stained with ethidium bromide and acridine orange. Fluorescence micrographs were used to calculate the percentage of macrophages that stained positive for ethidium bromide in random fields. Data shown are the average of three independent readings ± standard deviation. The *L. pneumophila* strains used were CR39 (wild type), CR58 (Δ*dotA*), MM101 (Δ*icmX*), and MM101 containing plasmid pMM1.3.
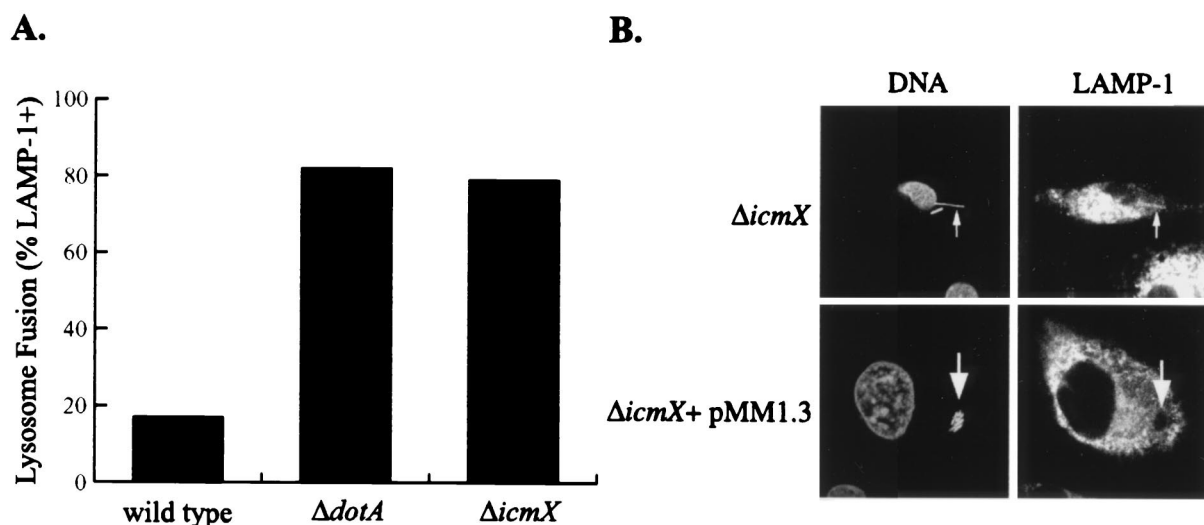
**A.**



**B.**



FIG. 4. *L. pneumophila* requires the IcmX protein to establish a replicative organelle that evades fusion with late endosomes and lysosomes. (A) Murine bone marrow-derived macrophages were infected with *L. pneumophila* for 1 h, and phagosomes were scored for the presence of LAMP-1. Results show that phagosomes containing wild-type *L. pneumophila* strain CR39 (wild type) were able to avoid fusion with LAMP-1-containing organelles, whereas phagosomes containing the mutant CR58 (Δ*dotA*) or MM101 (Δ*icmX*) accumulated LAMP-1. Data are the average of two independent experiments that did not differ by more than 10% in which 100 phagosomes were scored for each strain. (B) Macrophages were infected with *L. pneumophila* for 8 h and then stained with propidium iodide to label bacteria and macrophage nuclei and MAb (1D4B) specific for LAMP-1. *L. pneumophila* Δ*icmX* mutants were found as single rods inside phagosomes that stained positive for LAMP-1 (small arrows). The complemented *L. pneumophila* mutants (Δ*icmX* + pMM1.3) were localized to organelles containing multiple bacteria that did not exhibit appreciable levels of LAMP-1 staining on the surrounding membrane (large arrows).

were infected with *L. pneumophila* for 1 h. Fixed macrophages were stained with MAb 1D4B, specific for the late endosomal/lysosomal protein LAMP-1 (11). Bacterial and macrophage nuclei were labeled with propidium iodide. The stained macrophages were examined by confocal microscopy, and the percentage of bacterial phagosomes that had acquired LAMP-1 was determined. The majority of phagosomes containing MM101 (Δ*icmX*) were found to have intense circumferential LAMP-1 staining, whereas LAMP-1 was absent from most phagosomes containing wild-type *L. pneumophila* (Fig. 4A). To investigate whether an *icmX* mutant can form a replicative niche, murine bone marrow-derived macrophages were pulsed with MM101 and the complemented strain MM101 (pMM1.3) for 1 h. Infected macrophages were fixed after an 8-h chase and then stained with 1D4B and propidium iodide. MM101 (Δ*icmX*) was unable to establish replicative organelles. The Δ*icmX* mutants were found as single bacterial rods inside phagosomes that stained positive for LAMP-1 (Fig. 4B). In contrast, when the *icmX* gene was reintroduced on plasmid pMM1.3, the bacteria were found replicating inside large vacuoles that were devoid of LAMP-1 staining (Fig. 4B). These results were consistent in three independent experiments in which more than 500 infected macrophages were examined for each strain. From these data, we conclude that the IcmX protein plays an important role in the early signaling events that regulate trafficking of the *L. pneumophila* phagosome, processes that are essential for the establishment of a replicative niche in eukaryotic host cells.

**Bacterium-associated IcmX protein is located in the periplasm.** Predictions based on sequence analysis suggested that *icmX* encodes a *sec*-dependent N-terminal signal peptide that could mediate translocation of the protein across the bacterial inner membrane into the periplasmic space. To examine whether the IcmX protein is translocated across the inner membrane by a *sec*-mediated process, *E. coli* alkaline phosphatase gene fusions were constructed (26, 34). The *phoA* gene was ligated in frame to the *icmX* gene to create transla-

tional C-terminal alkaline phosphatase fusions at amino acid positions 48 and 399 of the IcmX protein. High levels of alkaline phosphatase activity were detected in *E. coli* expressing the IcmX48-PhoA protein and the IcmX399-PhoA protein (Fig. 5A). Plasmid p*icmX*1231::*phoA* has the *phoA* gene ligated to the *icmX* +1 reading frame, resulting in an out-of-frame fusion. Alkaline phosphatase activity above background levels was not detected in *E. coli* containing p*icmX*1231::*phoA* (Fig. 5A). To determine whether the Dot/Icm apparatus plays a role in translocation of the IcmX-PhoA hybrid proteins into the bacterial periplasm, the fusion plasmids were introduced into *L. pneumophila* strain CR24 and into isogenic Δ*dotA* (CR51) and Δ*dotB* (CR26) mutant strains. Alkaline phosphatase activities were over 100-fold above background in CR24, CR51, and CR26 expressing the IcmX48-PhoA protein and the IcmX399-PhoA protein (Fig. 5B). These data demonstrate that the IcmX protein has an N-terminal sequence that can mediate translocation of polypeptides across the bacterial cytoplasmic membrane by a process that does not require the Dot/Icm transporter, indicating that this is a *sec*-mediated transport event.

To determine whether IcmX protein expressed endogenously is translocated into the bacterial periplasm, intact *L. pneumophila* cells were labeled with sulfo-NHS-LC-biotin. This relatively small molecule can cross the bacterial envelope to gain access to proteins in the periplasm, but it cannot diffuse across the inner membrane to label cytoplasmic proteins (1, 7). *L. pneumophila* extracts were prepared after treatment of bacterial cells with sulfo-NHS-LC-biotin, and labeled proteins were precipitated on streptavidin-conjugated agarose beads. Immunoblot analysis of proteins precipitated on streptavidin-agarose indicate that the IcmX protein is accessible to sulfo-NHS-LC-biotin labeling, but the cytoplasmic IcmW protein is not biotin labeled (Fig. 6). These data demonstrate that the IcmX protein is translocated across the inner membrane into the bacterial periplasm in *L. pneumophila*.

To investigate the subcellular localization of the IcmX protein, we divided *L. pneumophila* extracts into soluble and mem-
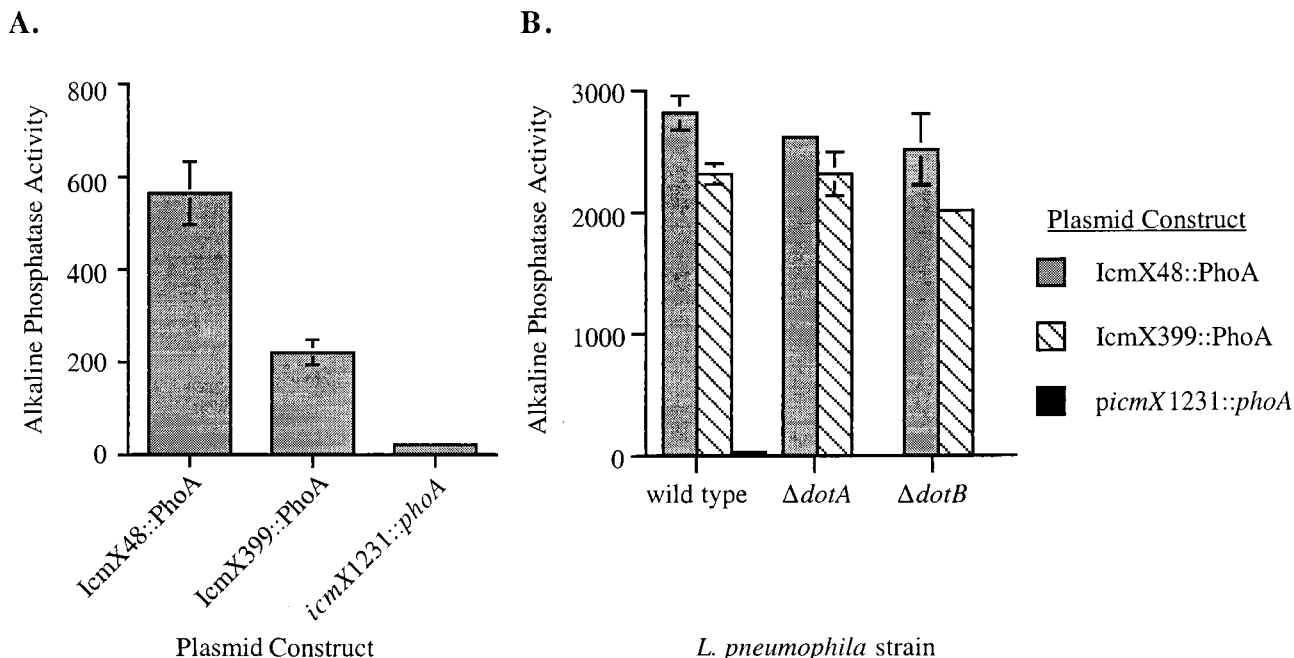
**A.**

**B.**



FIG. 5. The N-terminal domain of IcmX can direct translocation of alkaline phosphatase fusion proteins into the bacterial periplasm. (A) Alkaline phosphatase activity was measured for *E. coli* strain CC118 harboring plasmids pCR2-1 (IcmX48::PhoA), pCR3140 (IcmX399::PhoA), and p*icmX*1231::*phoA*. (B) Plasmids containing the *icmX::phoA* fusions were transferred into *L. pneumophila* strains CR24 (wild type), CR26 (Δ*dotB*), and CR51 (Δ*dotA*). Alkaline phosphatase activity for each fusion protein expressed in these isogenic *L. pneumophila* strains was determined. Data are the average of three independent assays ± standard deviation.

brane-associated fractions and then determined the amount of IcmX protein present in each fraction by immunoblot analysis (Fig. 7). An estimated 78% of the IcmX protein was soluble after bacterial lysis. The remaining IcmX protein was recovered in the bacterial membrane fraction and could be dissolved in 2% Triton X-100, which suggests that some of the IcmX protein is associated with the inner membrane. In agreement with previously published data (47), the DotA protein was found in the Triton X-100-soluble fraction when these fractions were probed with DotA-specific MAb 37.29 (data not shown).

One interpretation that can be drawn from the subcellular localization studies is that bacterial cell-associated IcmX protein resides primarily in the periplasmic space. Alternatively, IcmX protein may be located at the cell surface and associate weakly with the bacterial envelope. To distinguish between

these two possibilities, we subjected *L. pneumophila* cells to protease digestion and then measured the degradation of IcmX protein by immunoblot analysis. Cell-associated IcmX protein was degraded rapidly when the cells were pretreated with EDTA and lysozyme, agents that allow the protease to



FIG. 7. The IcmX protein in *L. pneumophila* is primarily soluble and not membrane associated. Proteins from *L. pneumophila* strain CR39 (wild type) and CR58 (Δ*dotA*) were separated into soluble and membrane-associated fractions. The bacterial lysates (whole cell), proteins in the cytoplasm and periplasm (soluble), proteins obtained after salt (0.5 M NaCl) and detergent (Triton X-100 [TX-100]) extraction of isolated membranes, and the Triton X-100-insoluble membrane proteins (pellet) were analyzed. The amount of IcmX protein in each fraction was determined by immunoblot analysis using MAb 5.1. Indicated on the right are the positions of molecular mass standards in kilodaltons.
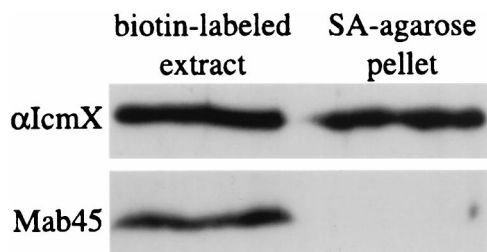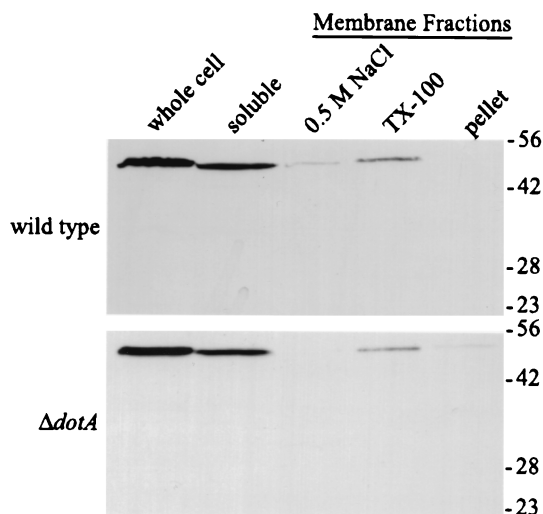


FIG. 6. The IcmX protein is translocated across the *L. pneumophila* plasma membrane. *L. pneumophila* strain CR174 was biotin labeled with the membrane-impermeable analog sulfo-NHS-LC-biotin. Biotin-labeled proteins were precipitated from the CR174 extract by using streptavidin-agarose. Immunoblots containing samples of total cell extract (biotin-labeled extract) and proteins precipitated on streptavidin-agarose (SA-agarose pellet) were probed using either a polyclonal antibody specific for IcmX or MAb 45, which will detect the cytoplasmic IcmWM45 protein.
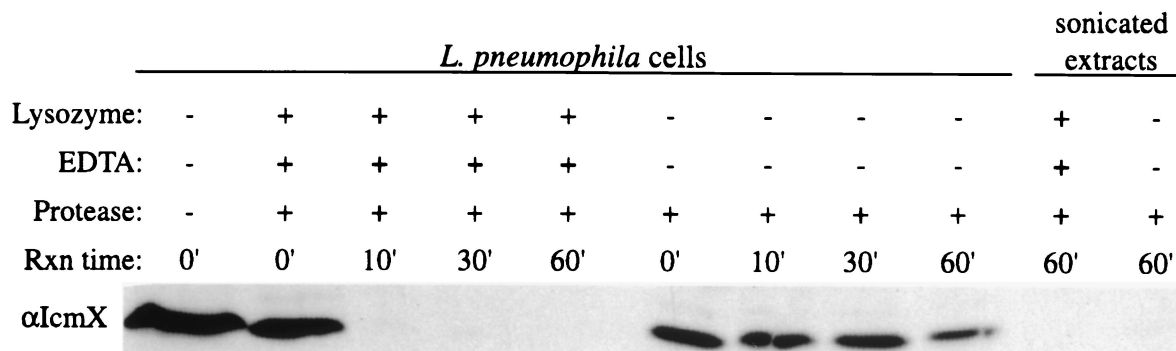
FIG. 8. Cellular IcmX protein is not exposed on the bacterial surface. *L. pneumophila* cells were suspended in either a sucrose buffer containing EDTA and lysozyme to make the cell envelope permeable or a Tris-saline buffer that will maintain outer membrane integrity. The bacterial cells were digested with pronase (Protease) for 0 to 60 min (Rxn time). Sonicated *L. pneumophila* extracts were prepared in these same buffers as a control for protease activity. Immunoblots containing samples from each reaction were probed with a polyclonal antibody specific for IcmX. The region of the blot containing the 50-kDa IcmX-immunoreactive product is shown (αIcmX).

enter the periplasmic space by selectively disrupting the outer membrane and peptidoglycan layer (Fig. 8). There was no detectable degradation of IcmX protein over the first 30 min in bacterial cells that were not treated with EDTA-lysozyme, which suggests that IcmX protein does not accumulate on the bacterial surface. Treatment of the bacterial cells with EDTA-lysozyme resulted in rapid proteolytic digestion of the polytopic inner membrane protein DotA but did not facilitate degradation of the cytoplasmic IcmW protein (data not shown). Based on these data, we conclude that cell-associated IcmX protein resides primarily in the bacterial periplasm.

**An IcmX fragment is secreted into culture supernatants during *L. pneumophila* growth in liquid broth.** It is possible that proteins secreted by the Dot/Icm apparatus will be present in supernatants isolated from broth-grown *L. pneumophila* cultures. To determine whether periplasmic IcmX protein is secreted by a *dot/icm*-dependent mechanism, culture supernatants were harvested during growth of wild-type and *dotA* mutant *L. pneumophila* in liquid broth. Immunoblot analysis of whole cell bacteria and culture supernatants indicate that full-length IcmX protein, with an estimated molecular mass of 50 kDa, is found associated with intact bacterial cells but is not
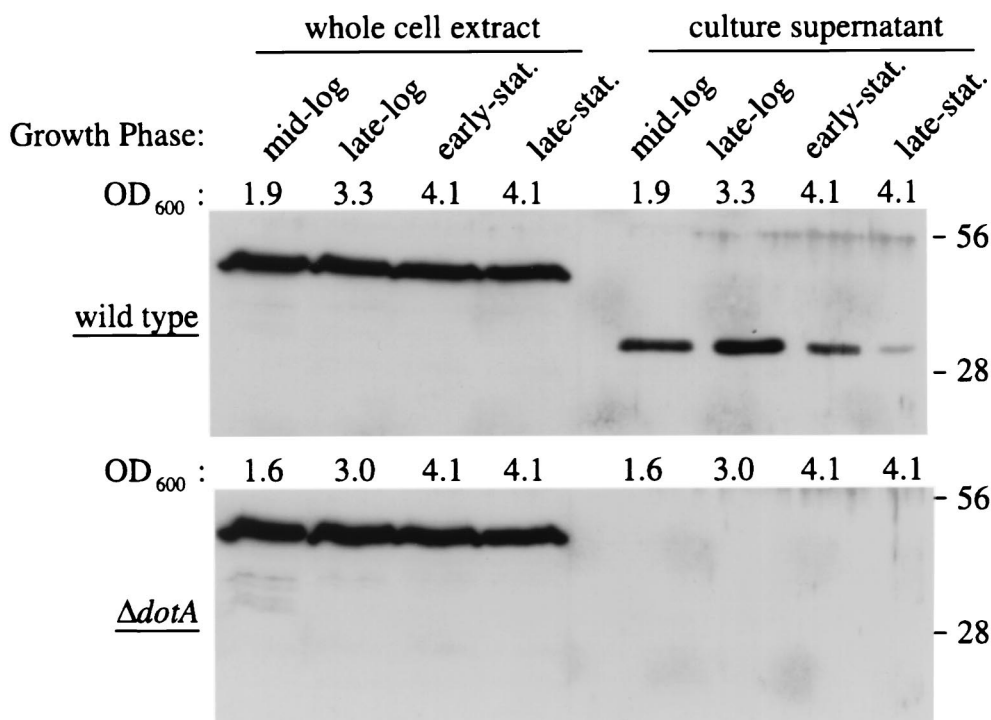


FIG. 9. Wild-type *L. pneumophila* secretes a truncated IcmX fragment. *L. pneumophila* strains CR39 (wild type) and CR58 (Δ*dotA*) were harvested during extracellular growth in liquid culture. The growth phase of bacteria in each sample was determined by plotting the OD$_{600}$ of the culture over time. Protein extracts were prepared from bacteria in mid-exponential, late exponential, early stationary (stat.), and late stationary phases of growth. Proteins were isolated from intact bacteria (whole cell extract) and the growth media (culture supernatant). Immunoblots containing these protein samples were probed with a polyclonal antibody specific for IcmX. The positions of molecular mass standards with masses of 56 and 28 kDa are indicated on the right.
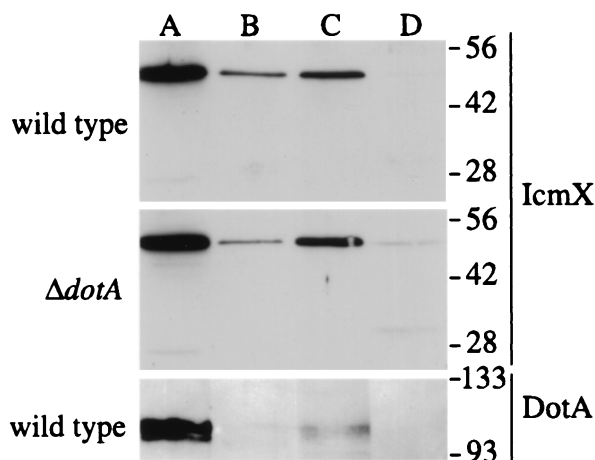
FIG. 10. Secretion of the IcmX protein is not enhanced upon *L. pneumophila* contact with eukaryotic host cells. Bone marrow-derived macrophages were infected separately with *L. pneumophila* strains CR39 (wild type) and CR58 (Δ*dotA*) for 2 h. After infection, fractions enriched for extracellular bacteria (lane A), proteins secreted into the tissue culture media (lane B), intracellular bacteria (lane C), and proteins secreted intracellularly (lane D) were collected (see Materials and Methods). IcmX and DotA protein levels were determined by immunoblot analysis using a rabbit polyclonal antibody specific for IcmX and the DotA-specific MAb 37.29.

present in culture supernatants (Fig. 9). A truncated IcmX-immunoreactive product was observed in wild-type *L. pneumophila* culture supernatants. This product was notably absent in supernatants isolated from *dotA* mutant cultures. The truncated immunoreactive product was never detected in culture supernatants from MM101 (Δ*icmX*) (data not shown). The IcmX-immunoreactive product became less abundant as the bacteria enter stationary phase. This decrease in protein level was likely due to degradation of the IcmX product by the potent zinc metalloprotease that accumulates in *L. pneumophila* culture supernatants (17). These data indicate that after translocation into the periplasm, the IcmX protein is processed and secreted by a process that requires the Dot/Icm apparatus.

**DotA-dependent secretion and translocation of IcmX is not detected upon contact with macrophages.** If secretion of the IcmX protein by the Dot/Icm apparatus is important for host cell parasitism, detection of this product may be enhanced upon *L. pneumophila* contact with macrophages. To determine whether *dot/icm*-dependent secretion and/or translocation of IcmX occurs during contact with host cells, monolayers of bone marrow-derived macrophages were infected with wild-type *L. pneumophila* and an isogenic *dotA* mutant for 2 h. Fractions enriched for proteins located in tissue culture supernatant, macrophage cytoplasm, extracellular bacteria, and intracellular bacteria were examined by immunoblot analysis using a polyclonal antibody specific for the IcmX protein (Fig. 10). The IcmX protein was found predominantly in fractions containing intact bacteria (Fig. 10, lanes A and C). There were no apparent differences in IcmX localization when fractions isolated from macrophages infected with wild-type *L. pneumophila* were compared with fractions isolated after infection with a Δ*dotA* mutant (Fig. 10). In contrast to extracellular growth in liquid broth, wild-type *L. pneumophila* did not secrete appreciable levels of the truncated IcmX product during host cell infection. When the fractions were probed for the inner membrane protein DotA, there was no detectable signal observed in the samples enriched for proteins secreted into the tissue culture supernatant or macrophage cytoplasm, even though there was detectable full-length IcmX protein in these same fractions (Fig. 10, wild type, lanes B and D). These data indicate that IcmX protein detected in lanes B and D was not derived from lysed *L. pneumophila* cells or intact bacteria. Thus, this pool of IcmX protein was released from the bacterial periplasm by a *dot/icm*-independent mechanism. We were unable to detect staining of the phagosome lumen, phagosome membrane, or macrophage cytoplasm when macrophages containing replicating *L. pneumophila* were stained with polyclonal or monoclonal IcmX antibodies and then examined by immunofluorescence microscopy (data not shown). These data suggest that targeted secretion of either full-length or the truncated IcmX product by the Dot/Icm apparatus may not be specifically induced upon bacterial contact with eukaryotic host cells.

**The IcmX protein is conserved among different *Legionellaceae* family members.** Homology searches indicate that the *icmX* product has no known counterparts in other secretion systems. To determine whether the IcmX protein is expressed by other *Legionella* isolates, several different *L. pneumophila* serogroups and other *Legionella* species were analyzed for the presence of IcmX protein by probing immunoblots with MAb 5.1. Immunoreactive products with a molecular mass similar to that of the *L. pneumophila* serogroup 1 IcmX protein were identified in all other *L. pneumophila* serogroups and *Legionella* species examined (Fig. 11). Nucleic acid hybridization studies using both the *L. pneumophila* serogroup 1 *icmX* and *dotA* genes as probes confirm that the *dotA/icmWX* region is present in these other *Legionella* serogroups and species (data not shown). These data indicate that an IcmX homolog is expressed by distantly related *Legionella* species, suggesting
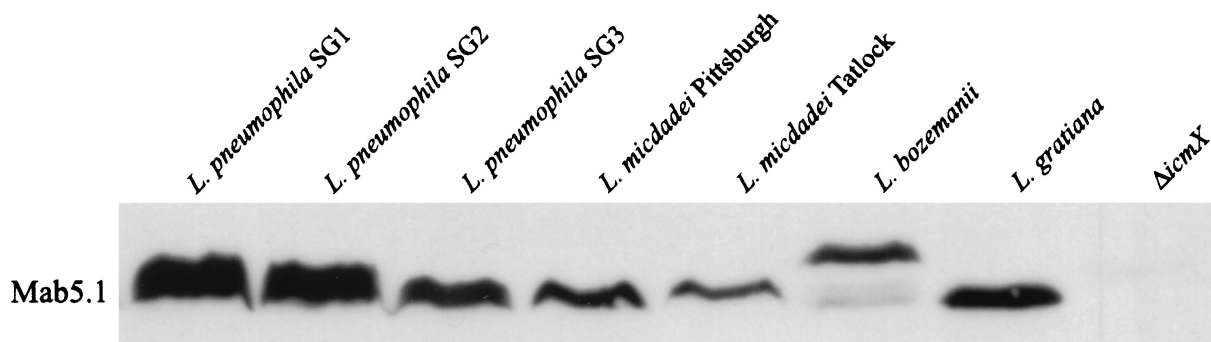


FIG. 11. A protein homologous to IcmX is expressed ubiquitously by *Legionellaceae* family members. Immunoblots containing whole cell protein extracts from various *L. pneumophila* serogroups and *Legionella* species were probed with MAb 5.1 specific for IcmX.

that the *dot/icm* secretion system is likely to play a role in host cell parasitism by all *Legionellaceae* family members.

## DISCUSSION

Modulation of phagosome biogenesis is an intracellular survival strategy that numerous microbial pathogens have adopted (38, 54). This process allows microorganisms to modify endocytic vacuoles into specialized organelles that will protect them from degradation and provide nutrients for intracellular growth. *L. pneumophila* is an interesting example of a facultative intracellular pathogen that can infect and multiply within evolutionarily diverse eukaryotic host cells (21, 52). In nature, freshwater protozoa are the principal reservoir for the bacteria (20). Aerosols containing *L. pneumophila* can result in human infections which are manifested by replication of the bacteria in alveolar macrophages (29, 37). The isolation of *L. pneumophila* mutants that are unable to replicate in eukaryotic host cells has identified a number of factors that play a role in pathogenesis (3, 5, 13, 22, 48). The *dot/icm* genes encode factors that enable *L. pneumophila* to alter trafficking of phagosomes formed upon uptake of the bacteria into macrophages (46, 57). Based on the capacity of *L. pneumophila* to replicate within different hosts, we would predict that the function of the *dot/icm* genes is to interfere with a fundamental cellular process that has been conserved across evolutionary boundaries. In an effort to understand how the *dot/icm gene* products can modulate biogenesis of the *L. pneumophila* phagosome, we have focused our attention on those products that are unique to *L. pneumophila* and are not found as components of other transport systems.

In this study, we identified the IcmX protein. Protein fusions and immunoblot analysis indicate that the *icmX* gene encodes a protein with an estimated mass of 50 kDa. These data confirm the most recent GenBank DNA sequence entry reporting that the *icmX* gene is 1,398 bp in length. We found that *L. pneumophila* mutants containing an in-frame deletion in the *icmX* gene were unable to replicate intracellularly and that bacterial growth in macrophages could be restored by introducing the *icmX* gene in *trans* to this mutation. These data demonstrate that *icmX* is essential for host cell pathogenesis. These findings agree with earlier reports demonstrating that *icmX* transposon insertion mutants were defective for replication in human monocytic cell lines (48) and were unable to cause pneumonia in a guinea pig model of disease (18).

Our results show that the IcmX protein is necessary for biogenesis of an organelle that supports *L. pneumophila* intracellular growth. *L. pneumophila icmX* deletion mutants were defective in the ability to form pores in the macrophage plasma membrane, and they reside in phagosomes that fuse with vesicles containing the late endosomal/lysosomal protein LAMP-1. These data indicate that the IcmX protein is required to modulate trafficking of the phagosome in which *L. pneumophila* resides. In a recent study we found that *icmX* mutants can replicate intracellularly if they reside in a vacuole formed by wild-type *L. pneumophila*, indicating that *icmX* is not required to assimilate nutrients from a niche that is permissive for growth (14). Thus, the *icmX* product is a factor that plays an essential role in the transmission of a signal to the host cell that modulates phagosome biogenesis.

C-terminal alkaline phosphatase protein fusions to the IcmX protein had phosphatase activity in both *E. coli* and *L. pneumophila*, and their activity was not dependent on a functional Dot/Icm transport apparatus. Sulfo-NHS-LC-biotin labeling experiments confirm that the IcmX protein is translocated across the bacterial inner membrane. Fractionation of *L. pneumophila* lysates determined that most of the bacterium-associated IcmX protein remains soluble and does not sediment with the inner and outer membranes. In addition, bacterium-associated IcmX protein was not accessible to extracellular proteases. From these data, we conclude that an N-terminal signal peptide mediates translocation of the IcmX protein into the bacterial periplasm by a *sec*-dependent process and that the bacterial-associated IcmX protein resides primarily in the periplasmic space.

Supernatants from wild-type *L. pneumophila* grown in liquid culture contain a truncated IcmX fragment. We have recently purified a protein from *L. pneumophila* with an estimated mass of approximately 30 kDa that is present in wild-type but not *dotA* mutant culture supernatants (H. Nagai and C. R. Roy, unpublished data). N-terminal amino acid sequence data obtained for this protein was 100% identical to amino acids 165 to 177 of the IcmX protein, indicating that this protein is the secreted IcmX-immunoreactive product identified in Fig. 9. Thus, the secreted IcmX product is a C-terminal fragment that results from processing events that remove the first 164 amino acid residues of the full-length IcmX protein. The IcmX 165-466 protein would have a calculated mass of 33.2 kDa, in close agreement with the mobility of the truncated IcmX product identified by immunoblot analysis. Interestingly, the VirB1 protein from *Agrobacterium tumefaciens* is secreted by an analogous mechanism (4). VirB1 has an N-terminal transmembrane domain that mediates translocation of the protein into the bacterial periplasm, where the protein becomes a substrate for the VirB secretion apparatus. Like IcmX, the secreted VirB1* product is a truncated C-terminal polypeptide. The first 172 amino-terminal residues are removed from VirB1 to generate the VirB1* product.

Secretion of the truncated IcmX protein could not be detected during infection of eukaryotic host cells. We cannot rule out the possibility that during eukaryotic host cell infection, the half-life of secreted IcmX protein is very short. This could limit our ability to detect secretion of IcmX protein into host cellular compartments by the Dot/Icm apparatus. Alternatively, the secreted IcmX protein could represent a spent form of the protein that is no longer needed by the bacteria. Future studies will focus on whether the secreted IcmX protein is required for host cell pathogenesis.

Immunoblot analysis indicates that other *Legionella* species express a protein homologous to IcmX from *L. pneumophila* serogroup 1. These data suggest that the Dot/Icm transport system has been conserved in other virulent *Legionella* species. There is evidence to suggest that *Legionella micdadei* creates a replicative organelle that is morphologically distinct from the *L. pneumophila* growth niche (2). It has also been reported that a number of *L. pneumophila* virulence traits that require the Dot/Icm apparatus, such as pore formation and evasion of phagosome lysosome fusion, are attenuated in strains of *L. micdadei* (30). In contrast, our data demonstrate that *L. micdadei*, one of the most distant *Legionella* species evolutionarily (44), expresses an IcmX homolog. In addition, we have identified genes with homology to *L. pneumophila dotA* and *icmX* in *L. micdadei* by using low-stringency nucleic acid hybridization techniques. These data suggest that functionally similar Dot/Icm secretion systems are expressed by most *Legionellaceae* family members. It is possible that putative Dot/Icm effector proteins secreted by these type IV-related transporters have divergent functions. This could explain the attenuation in virulence and differences in host cell interactions that have been observed for evolutionarily distant *Legionella* species. A more detailed understanding of how the Dot/Icm apparatus functions is certain to help elucidate the molecular mechanisms underlying these phenotypic variations.

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