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Topology of *Legionella pneumophila* DotA: an Inner Membrane Protein Required for Replication in Macrophages

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The *Legionella pneumophila dotA* gene is required for intracellular growth of the bacterium in macrophages. In this study, a structure-function analysis of the DotA protein was conducted to elucidate the role of this protein in *L. pneumophila* pathogenesis. Translational fusions of *dotA* to the *Escherichia coli phoA* and *lacZ* genes indicated that DotA is an integral cytoplasmic membrane protein with eight membrane-spanning domains. DotA contains two large periplasmic domains of approximately 503 and 73 amino acids and a carboxyl-terminal cytoplasmic domain of 122 amino acids. Protein fractionation studies were consistent with DotA residing in the inner membrane. An alkaline phosphatase fusion located 9 amino acids upstream from the C terminus of DotA still retained function and was able to restore intracellular growth when harbored by two *L. pneumophila dotA* mutants. A hybrid protein from which the carboxyl-terminal 48 amino acids of DotA were deleted was unable to complement the intracellular growth defect in the *dotA* mutants, indicating that this cytoplasmic region is required for function.

Legionella pneumophila is a gram-negative respiratory pathogen that grows within alveolar macrophages (24). In humans, *Legionella* infections can result in a pneumonia known as Legionnaires' disease or the flu-like syndrome Pontiac fever (13, 27). Bacteria unable to grow within macrophages have been shown to be avirulent in an animal model for disease (22).

Following uptake of *L. pneumophila* by macrophages, bacteria remain within membrane-bound phagosomes that have altered intracellular trafficking properties compared to those of phagosomes harboring nonpathogens (20). Phagosomes containing avirulent bacteria are normally acidified after uptake and fuse with lysosomal compartments inside the macrophage. The *L. pneumophila* phagosome, however, is not acidified and does not fuse with lysosomes (21, 23). Instead, *L. pneumophila* phagosomes recruit mitochondria to the cytoplasmic surface of the phagosome shortly after uptake and 3 to 6 h afterward associate with the rough endoplasmic reticulum in the cell (20, 38). This trafficking pathway results in a ribosome-studded compartment known as the replicative phagosome (20, 38). Formation of this compartment appears to be required for intracellular replication, although the molecular mechanisms used by the bacteria to establish this unique structure inside the cells are unknown.

Genetic studies have identified a cluster of genes required for intracellular replication of *L. pneumophila* (2, 5). Of the mutants isolated in one study, those exhibiting the most severe defects in intracellular growth were shown to be defective for formation of the replicative phagosome. These mutants could be complemented in *trans* by a single open reading frame called *dotA* (defective organelle trafficking) (3), predicted to encode a protein of 1,048 amino acids. Database searches failed to identify any regions of homology between DotA and other protein sequences. The *dotA* gene is located adjacent to the *icmWXYZ* operon on the *L. pneumophila* chromosome (5). Like *dotA*, mutations in the *icmWXYZ* region result in bacteria that are defective for growth inside macrophages (5, 32).

To begin to understand the role of DotA protein during intracellular growth of *L. pneumophila*, we detail here a molecular characterization of the *dotA* product. Hybrid proteins containing N-terminal *dotA* sequences and C-terminal *phoA* or *lacZ* sequences were used to determine the location and topological orientation of DotA in the bacterial cell (4, 30, 31). These studies show that DotA is an integral cytoplasmic membrane protein that traverses the membrane eight times. The finding that DotA is an inner membrane protein suggests that DotA works in association with other proteins to transmit a signal to the macrophage to direct the trafficking of the *L. pneumophila* phagosome.

MATERIALS AND METHODS

Strains and media. *L. pneumophila* LP02 (*thyA hsdR rpsL*) is a streptomycin-resistant thymine auxotroph derived from the serogroup 1 strain Philadelphia 1 (2). *L. pneumophila* LP03 (*thyA rpsL hsdR dotA03*) and LP053 (*thyA rpsL hsdR dotA053*) are *dotA* derivatives of LP02 unable to grow within macrophages (2). *L. pneumophila* strains were grown in AYE broth [1% yeast extract, 1% *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES; pH 6.9), 3.3 mM L-cysteine, 0.33 mM $Fe(NO_3)_3$] or on charcoal-yeast extract (CYE) plates (AYE containing 1.5% Bacto Agar, 0.2% activated charcoal) (11). When required, the following supplements were added to the media: thymidine at 100 μg/ml and streptomycin at 100 μg/ml.

Construction of gene fusions with *dotA*. The signal sequence-deficient *phoA* gene was removed from the plasmid pCH40 (18) as a *Pst*I fragment and ligated into the plasmid pSK(−) (Stratagene) at the *Pst*I site. Following electroporation into *Escherichia coli* DH5α (*recA1 endA1 gyrA hsdR17 supE44 thi-1 relA1* Δ(*lacIZYA-argF*)U169 *deoR*), recombinant clones were screened by restriction digestion. A plasmid, pSK40C, that had the 5′ end of the *phoA* gene adjacent to the *Bam*HI site in pSK(−), was identified. The *phoA* fragment was then removed from this plasmid and used to construct each of the *dotA*::*phoA* fusions in this study.

The plasmid pKB6 contains the *dotA* gene on an RSF1010 plasmid that can be transferred by conjugation from *E. coli* and then replicate in *L. pneumophila* (3). To construct the *dot309*::*phoA* fusion, pKB6 was digested with *Bam*HI and *Sal*I. The vector backbone encoding the N-terminal region of DotA was purified free from the 2.2-kb insert containing the 3′ portion of the *dotA* gene and ligated to a *Bam*HI-*Sal*I fragment encompassing the *phoA* gene from pSK40C. The ligation was electroporated into DH5α, and plasmids isolated from ampicillin-resistant clones were screened by restriction enzyme analysis to confirm the construction.

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The *phoA* gene was also ligated to the mutant *dotA053* allele present in the plasmid pKB14 (3) by the same approach.

Additional *dotA*::*phoA* fusions were constructed with *dotA* DNA fragments generated by primer-specific amplification with *Taq* DNA polymerase. The 5′ primer used in all reactions (5′-GGGAGCTCGGTGAGAAAGAATGAATAA ATTAGCT-3′) contained a *Sac*I restriction enzyme site followed by the predicted *dotA* translational initiation region and start codon. The 3′ primers were homologous to the junction of interest in the *dotA* gene, and all contained an *Xba*I site at the 5′ end of the primer. For instance, the 3′ primer used to amplify the *dotA* region used in the *dot50*::*phoA* fusion had the sequence 5′-CCGTCT AGAGTGCAATACACCATCGAC-3′. This combination of primers generated a PCR product encoding the first 50 amino acids in the N-terminal region of DotA flanked by 5′ *Sac*I and 3′ *Xba*I restriction sites. For each amplification reaction, 10 ng of pKB6 digested with *Sal*I was amplified for 20 cycles consisting of melting for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 2 min at 74°C. Amplified DNA product was purified with a QIAquick spin PCR kit (QIAGEN, Inc.) followed by digestion with *Sac*I and *Xba*I. The amplified *dotA* region was fused upstream of *phoA* by replacing the *Sac*I-*Xba*I fragment encoding the existing *dotA* region in p*dot309*::*phoA* and replacing it with the PCR-generated *dotA* fragment. To construct *dotA*::*lacZ* fusion plasmids, the *phoA* gene was removed from each *pdotA*::*phoA* plasmid by digestion with *Sma*I and *Sal*I and replaced with the *lacZ* gene removed from plasmid pMC1871 (7) as a *Sma*I-*Sal*I fragment. For construction of the *dotA* deletion fusions, a 3′ primer (5′-GGGAATTCCGCAATCAAAATCCTGGTG-3′) was used in conjunction with the 5′ primer above to generate a DNA fragment encoding the amino-terminal 611 amino acids of DotA. This DNA fragment was digested with the restriction enzymes *Eco*RI and *Sac*I prior to ligation. The carboxyl regions of the *dotA* deletions were generated with either a 5′ primer homologous to the *dotA* region encoding amino acids 845 to 850 (5′-CGGAATTCAATCGGATCAAG GAATAG-3′) or the 948-to-953 region (5′-CGGAATTCCGGAATCTTTTGG TCAAG-3′). These 5′ primers were used in conjunction with the appropriate 3′ primers described above to generate carboxyl-terminal fusions to the *dotA* gene. These DNA fragments were digested with *Eco*RI and *Xba*I prior to ligation. The amino-terminal and desired carboxyl-terminal *dotA* fragments were then ligated in frame with the *phoA* gene in p*dot309*::*phoA* vector as described above.

The *dotA*::*phoA* and *dotA*::*lacZ* plasmids were mated into *L. pneumophila* by triparental conjugations with the *E. coli* strains DH5α containing the fusion plasmid and MT607 (pRK600) (12) providing the broad-host-range transfer functions. The three strains were lightly patched onto CYE plates, and the plates were incubated for 4 h at 37°C. The conjugation mix was then swabbed into distilled water (dH_2O), and dilutions were plated onto CYE containing streptomycin to select against the *E. coli* donors and lacking thymidine to select *L. pneumophila* exconjugants. The fusion plasmids contain a copy of the thymidylate synthetase gene and are therefore able to restore growth of the *L. pneumophila thyA* recipients.

Alkaline phosphatase and β-galactosidase assays. *L. pneumophila* strains containing p*dotA*::*phoA* fusions were grown to saturation in AYE broth at 37°C and then diluted into fresh AYE to a final optical density at 600 nm (OD_{600}) of 0.2. The cultures were further incubated for 6 to 8 h at 37°C, and then isopropyl β-D-thiogalactopyranoside (1 mM) was added to induce expression of the fusion proteins from a 5′ ptac promoter. After 1 h, alkaline phosphatase activity was determined by pelleting 1 ml of bacteria and resuspending the cells in 1 ml of 0.1 M CAPS (3-[cyclohexylamino]-1-propanesulfonic acid [pH 11.0]) (1). The assay mixture consisted of 0.1 ml of bacterial cells, 0.9 ml of 0.1 M CAPS (pH 11.0), and 0.1 ml of Sigma 104 (0.4% in 0.1 M CAPS [pH 11.0]). The reaction mixtures were incubated at 37°C until a yellow reaction product was visible, at which time 0.1 ml of 1 M K_2HPO_4 was added to terminate the reaction. Bacteria were pelleted in an Eppendorf tube, and the OD_{420} of the supernatant was measured. Alkaline phosphatase units were determined by the formula (1,000 × OD_{420} of reaction)/[(minutes of reaction) (bacterial OD_{600})]. The same growth conditions were used for β-galactosidase assays, and β-galactosidase activities were determine as described by Miller (33).

Immunoblotting. Whole-cell *L. pneumophila* extracts were made from the identical broth cultures used to determine alkaline phosphatase activity. Bacteria (OD_{600} 1.0) were pelleted in a microcentrifuge, and the pellets were resuspended in 200 μl of sample buffer at 4°C (29). The whole-cell extract was then either boiled for 5 min or incubated for 1 h on ice prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following gel electrophoresis, the proteins were transferred onto Immobilon P (Millipore Corp.) membranes in transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS, 20% methanol [pH 8.3]) at 20 V for 12 h. Filters were washed in phosphate-buffered saline (PBS), blocked for 1 h in PBS containing 5% nonfat dried milk (Blotto), and probed for 1 h with a rabbit polyclonal anti-alkaline phosphatase antibody (1:1,000) in Blotto. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Zymed Laboratories, Inc.) was used as the secondary antibody, and the blot was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma) (16).

Rabbit antibodies against DotA were generated with a peptide corresponding to DotA amino acids 542 to 557 (Zymed Laboratories Custom Antibody Service). The resulting antiserum was affinity purified then diluted 1:100 for detection of DotA on Western blots by using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) and the ECL (Amersham) reagent to detect DotA product.

Cellular fractionation. Triton X-100 solubilization of membrane-associated proteins. *L. pneumophila* cultures (50 ml) were grown in AYE broth to mid-logarithmic phase (OD_{600} = 1.5) and pelleted at 5,000 × *g* for 10 min. All subsequent steps were performed at 4°C. The bacteria were resuspended in 0.5 ml of ice-cold 200 mM Tris HCl (pH 8.0) and 0.5 ml of ice-cold sucrose buffer (50 mM Tris HCl [pH 8.0], 1 M sucrose) was then added. Spheroplasts were made by adding 10 μl of 0.5 M EDTA (pH 8.0) and 10 μl of 10 mg of lysozyme per ml. The suspension was then diluted to 2 ml with the addition of 1 ml of dH_2O. After 30 min, $MgSO_4$ was added to a final concentration of 20 mM, and the spheroplasts were pelleted for 10 min at 5,000 × *g*. The cells were resuspended in 5 ml of 50 mM Tris HCl (pH 8.0) and lysed on ice by sonication with a Branson Sonicator model 250. Typically, three sonic bursts at 25 to 50% intensity (each 15 s in duration) were sufficient to lyse the bacteria. Unlysed cells were removed by centrifugation for 10 min at 5,000 × *g*. The supernatant containing both soluble and membrane-associated proteins was the total cellular protein extract (Fraction T). Cellular membrane was then pelleted at 100,000 × *g* for 1 h. Soluble proteins were removed and saved (fraction S). The cellular membrane fraction was gently resuspended in 1 ml of 50 mM Tris HCl (pH 8.0). Triton X-100 (10% solution) was slowly added to the membrane suspension to a final concentration of 1%, and the membrane solution was incubated on ice for 30 min. The suspension was centrifuged for 30 min at 100,000 × *g*, and the Triton X-100 soluble proteins were carefully removed (fraction I). The pellet was resuspended in 1 ml of 50 mM Tris-HCl (pH 8.0) (fraction O). For detection of DotA, equivalent volumes (20 μl of fractions T and S and 4 μl of fractions I and O) were diluted into Laemmli buffer and incubated on ice for 30 min. DotA was then identified on Western blots using anti-DotA antibody as described above. To detect *L. pneumophila* major outer membrane protein (MOMP), equivalent volumes of each fraction were diluted into Laemmli buffer, boiled for 5 min, and separated on an SDS-15% polyacrylamide gel. The proteins were stained with Coomassie brilliant blue, and MOMP was identified as an intense band at ~28 kDa (15).

The protocol used for sucrose gradient fractionation of *L. pneumophila* membranes was modified from the procedure described by Ishidate et al. (25). All sucrose solutions, for which values are given on a weight-per-weight basis, contained 10 mM HEPES and 5 mM EDTA (pH 7.6). For membrane isolation, 100 ml of bacteria was grown in AYE (OD_{600} = 0.8). The bacteria were pelleted at 5,000 × *g* for 15 min at 4°C and washed once in 10 ml of ice-cold 10 mM HEPES (pH 7.6). The final pellet was resuspended in 5 ml of ice-cold 20% sucrose containing 10 mM HEPES (pH 7.6), 2 μg of DNase per ml, and 10 μg of RNase per ml. The cells were disrupted by a single passage through a chilled French pressure cell at 10,000 lb/in^2. Unbroken cells were removed by centrifugation at 2,000 × *g* for 15 min at 4°C. EDTA (pH 8.0) was added to the lysate to a final concentration of 5 mM.

A crude membrane fraction was obtained by loading 1.5 ml of the bacterial lysate onto a two-step gradient consisting of 0.8 ml of a 60% sucrose cushion and 2.5 ml of 25% sucrose. The membranes were pelleted for 3.5 h at 40,000 rpm in an SW50.1 rotor at 4°C. A membrane layer visible on top of the 60% sucrose was extracted into a 1-ml syringe by puncturing the side of the tube with an 18-gauge needle. This crude membrane fraction was diluted to 25% sucrose with 10 mM HEPES (pH 7.6).

Isopyncnic sucrose density gradient centrifugation of the crude membrane was carried out on a gradient consisting of a 0.5-ml cushion of 55% sucrose and layers of 2.1 ml of 50, 45, 40, 35, and 30% sucrose. Approximately 0.8 ml of the crude membrane prep was placed on top of the gradient and centrifuged in an SW41 rotor at 37,000 rpm for 16 h at 4°C. Fractions (150 μl) were collected by gravity flow from the bottom of the centrifuge tube following puncture of the tube with an 18-gauge needle. Levels of the 28-kDa MOMP and the 65-kDa major cytoplasmic membrane protein (MCMP) were determined by Coomassie blue staining of SDS-15% PAGE gels containing 10 μl of each sucrose gradient fraction per lane. With an Apple Power PC computer and an integrated charge-coupled digital camera, images of the gels were captured and pixel intensities in the areas corresponding to the location of each protein were determined with NIH Image 1.54. Measurements of DotA levels were determined by immunoblot analysis of the fractions as described for DotA above; however, the fluorescent alkaline phosphatase substrate Attophos (JBL Scientific) was used for detection in these experiments. The washed blots were placed on top of 1 ml of Attophos substrate (1 mM Attophos in 2.4 M diethanolamine–0.057 mM $MgCl_2$ [pH 10.0]) on a sheet of plastic wrap. The blots were then scanned on a Storm 860 Imager (Molecular Dynamics) under the chemifluorescence mode and a PMT voltage of 650 V. DotA levels were then determined with ImageQuant software (Molecular Dynamics). Sucrose concentrations were measured for each fraction with a Zeiss refractometer.

Growth of *L. pneumophila* in U937 cells. U937 cells were passaged and differentiated with phorbol 12-myristate 13-acetate (Sigma) as described elsewhere (35). *L. pneumophila* strains were grown in AYE broth to early stationary phase. Approximately 5 × 10^9 bacteria were pelleted and resuspended in 1 ml of dH_2O (OD_{600} = 1). This suspension of bacteria was then diluted 1:20 into RPMI tissue culture media. The bacteria (2 μl) were then added to differentiated U937 cells (2 × 10^6 per well) in 24-well dishes to give a multiplicity of infection of about 0.25. The bacteria were incubated with the U937 cells for 1 h, and the U937 cell

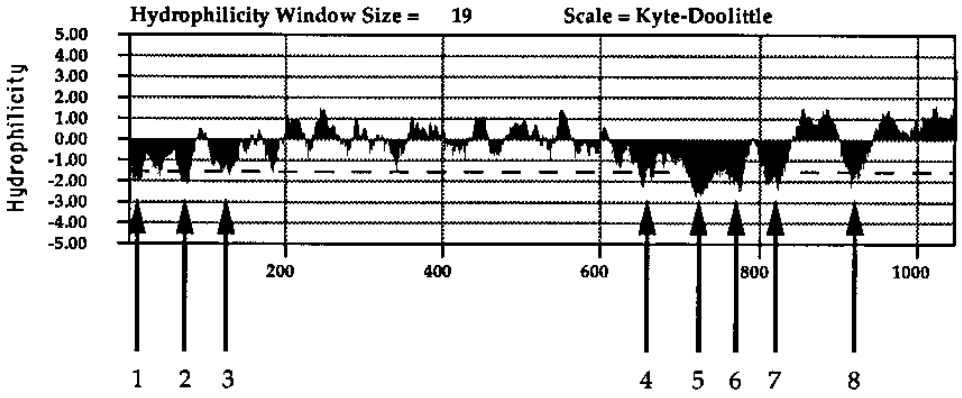

FIG. 1. Predicted DotA transmembrane domains. The hydropathic profiles of the DotA protein with the Kyte-Doolittle scale and a window size of 19 amino acids are shown. The eight regions in DotA predicted to contain transmembrane domains are indicated. The dotted line indicates the calculated hydrophobic threshold required for an α-helical transmembrane region.

monolayer was then washed several times with PBS to remove excess bacteria. Fresh tissue culture medium was added to the wells, and the U937 cells were maintained at 37°C in 5% CO_2. To measure intracellular replication of *L. pneumophila*, the bacteria were liberated by lysis of the U937 cells in dH_2O at various time points, and dilutions were plated onto CYE plates to measure CFUs.

RESULTS

DotA is an integral inner membrane protein with eight transmembrane domains. The DotA protein was postulated to associate with lipid bilayers based on a large hydrophobic domain in the C-terminal half of the protein (3). Further examination of the DotA amino acid sequence revealed that the DotA protein has structural characteristics of an integral cytoplasmic membrane protein. Eight amino acid regions in the DotA protein of sufficient length and hydrophobic potential to span the membrane were identified when the hydopathic profile of the protein was plotted with the Kyte-Doolittle scale and a window size of 19 (Fig. 1) (28).

To analyze whether the putative transmembrane domains identified in the DotA protein translocate regions of the protein across the cytoplasmic membrane, the signal sequence-deficient alkaline phosphatase gene (*phoA*) from *E. coli* was fused in frame to the *dotA* coding region at numerous C-terminal positions. To serve as a negative control, the *phoA* gene was ligated to the *dotA53* allele at the codon predicted to encode amino acid residue 309 in the *dotA* gene. The *dotA53* allele is defective for function due to a frameshift mutation resulting in an N-terminally truncated protein of only 230 amino acids. Alkaline phosphatase activity for each of the hybrid DotA::PhoA proteins was measured to assess translocation of the PhoA moiety to the periplasmic side of the cytoplasmic membrane.

The DotA::PhoA fusion results suggested that DotA contains distinct domains on opposite sides of the cytoplasmic membrane, as demonstrated by the oscillation in alkaline phosphatase activity as *phoA* fusions are extended from N- to C-terminal regions in the DotA protein (Fig. 2). A *phoA* fusion junction located at residue 50 in the DotA protein (Dot50::PhoA) had high alkaline phosphatase activity. This result is consistent with the predicted location of the first transmembrane domain (TM1) in DotA spanning amino acids 4 to 19 with the carboxyl-terminal portion directed toward the periplasm. The second DotA transmembrane domain (TM2) predicted to span amino acids 66 to 85 should return the protein to the cytoplasmic side of the inner membrane. The data showed that DotA::PhoA fusion junctions at amino acid position 100 or 119 exhibited low alkaline phosphatase activity, in agreement with the predicted location of TM2. DotA::PhoA fusion junctions at amino acid positions 150, 200, 309, 450, 546, 611, and 650 all had high alkaline phosphatase activities, indicating that a DotA transmembrane domain predicted to span amino acids 118 to 138 (TM3) is able to translocate a large region of the protein back across the cytoplasmic membrane.

The region in the DotA protein between amino acids 650 and 850 is very hydrophobic and is predicted to contain four transmembrane domains. A DotA::PhoA fusion junction at amino acid 700 results in low alkaline phosphatase activity, indicating that a transmembrane domain predicted to span amino acids 647 to 667 (TM4) returns this region of the DotA protein to the cytoplasmic side of the inner membrane. There is a transmembrane domain predicted to be present in the DotA protein from amino acids 719 to 739 (TM5) followed by another predicted transmembrane domain that spans amino acids 753 to 773 (TM6). Internal to TM5 and TM6 would be a 13-amino-acid periplasmic loop. Hybrid proteins containing DotA::PhoA fusion junctions within this periplasmic loop at amino acid position 750 or 755 did not exhibit alkaline phosphatase activity significantly above baseline, whereas a DotA::PhoA fusion constructed at amino acid position 760 resulted in alkaline phosphatase activity approximately sixfold above background. The Dot760::PhoA fusion contains the N-terminal 8 amino acids predicted to be present in TM6, which would interact with phospholipids on the periplasmic leaflet of the inner membrane. This interaction may stabilize the formation of the adjacent 13-amino-acid periplasmic loop aiding the translocation of the alkaline phosphatase moiety in the Dot760::PhoA fusion protein. These data suggest that TM5 requires a portion of TM6 to present the 13-amino-acid region to the periplasmic face of the inner membrane.

The alkaline phosphatase activities of PhoA hybrids constructed at DotA amino acids 765, 770, 774, and 793 were at baseline values, indicating that the predicted TM6 mediates translocation of these regions to the bacterial cytoplasm. The predicted transmembrane domain from amino acids 813 to 833 (TM7) oriented alkaline phosphatase to the periplasm for fusion junctions at DotA amino acids 863 and 900. The transmembrane domain from amino acids 906 to 926 (TM8) localizes the C terminus of DotA to the cytoplasm, as demonstrated by DotA::PhoA fusions made at amino acids 950, 1000, and 1039, all of which had low alkaline phosphatase activity.

With an antibody directed against the alkaline phosphatase

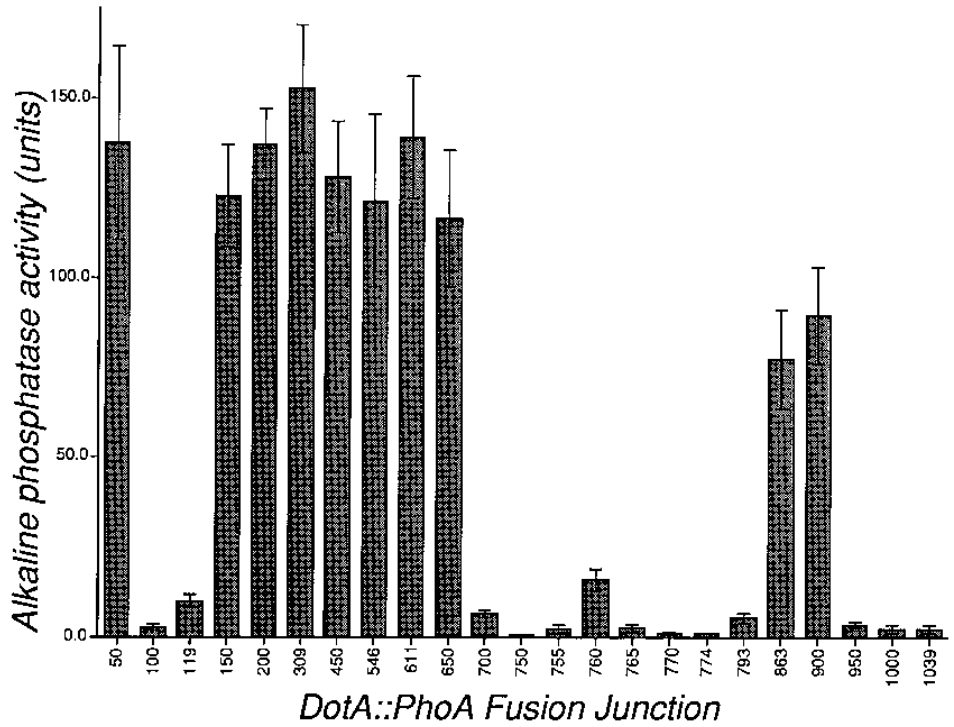

FIG. 2. Alkaline phosphatase activities of DotA::PhoA hybrids to analyze DotA topology. The DotA amino acid positions containing *phoA* gene fusions are indicated, along with the alkaline phosphatase activities measured in *L. pneumophila* cells expressing each fusion construct. Alkaline phosphatase activity was measured as described in Materials and Methods. Values are the averages of three independent assays, with error bars indicating standard deviations.

portion of the fusion proteins, Western blots of *L. pneumophila* strains solubilized in Laemmli buffer at 4°C were examined to verify that each of the DotA::PhoA fusions were being expressed. Full-length DotA::PhoA proteins were detected for all of the fusions (Fig. 3). Alkaline phosphatase fusions made to the large periplasmic region of DotA (fusions 150 to 650) appear to be more stable than fusions to other regions of the protein; however, this 2- to 3-fold increase in protein does not account for the greater-than-30-fold increase in alkaline phosphatase activity measured for these fusions in vivo. These data indicate that the large variations in activity measured for the different fusions were due to oscillations in the cellular localization of the alkaline phosphatase moiety of the hybrids. It should be noted that DotA::PhoA fusion proteins C terminal to TM4 were difficult to visualize on Western blots when the extracts were heated to 100°C in SDS buffer rather than 4°C prior to SDS-PAGE (data not shown), an observation that has been reported for a number of inner membrane proteins (6, 26, 36).

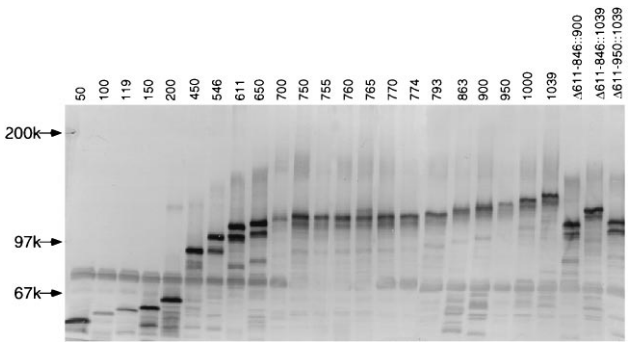

FIG. 3. DotA::PhoA fusions having low alkaline phosphatase activity are efficiently expressed. Whole-cell *L. pneumophila* extracts were separated on a 12% polyacrylamide gel and transferred to Immobilon P membranes. An anti-alkaline phosphatase antibody was used to detect the hybrid proteins. The amino acid position of the fusion junction for each DotA::PhoA protein being expressed is indicated. The positions of molecular mass markers (in kilodaltons) are given on the left.

To corroborate that fusions resulting in low alkaline phosphatase activity had fusion joints located in cytoplasmic domains of DotA, the *phoA* gene was replaced with an in-frame *lacZ* fusion. The resulting fusion proteins should exhibit high β-galactosidase activity only when the *lacZ* gene is fused to a cytoplasmic region of the DotA protein (30). The data show that for any given fusion junction within DotA, β-galactosidase activity is inversely proportional to alkaline phosphatase activity (Fig. 4).

DotA internal deletions resulting in altered membrane topology. To test the topology predictions of the C terminus of DotA, an internal deletion encompassing amino acids 611 to 846 was made to remove all transmembrane domains from this large hydrophobic region. According to the above data, this deletion should result in the removal of four transmembrane domains. Localization of regions of the protein C terminal to the deletion should not be affected by the removal of an even number of transmembrane domains. To determine the effect of this alteration on DotA protein topology, this deletion was introduced into DotA::PhoA fusions having junctions at residues 900, 1000, and 1039 in the wild-type DotA protein (Fig. 5). The DotAΔ611-846::PhoA fusion at residue 900 retained high alkaline phosphatase activity, indicating that localization of this region is not altered by the deletion. DotAΔ611-846 also failed to alter the activity of fusions with junctions at residues 1000 and 1039, which retained baseline levels of alkaline phos-

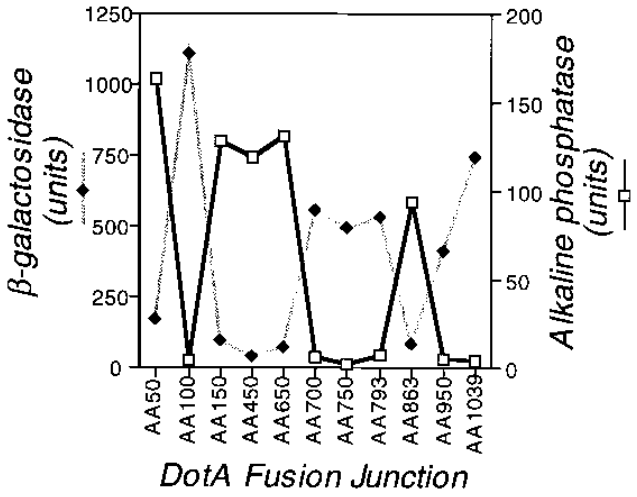

FIG. 4. Inverse relationship between activities of alkaline phosphatase and β-galactosidase hybrids. The DotA amino acid positions containing fusions to either alkaline phosphatase or β-galactosidase are indicated, along with alkaline phosphatase and β-galactosidase activity for each fusion as measured for *L. pneumophila* strain LP02.

phatase activity, in agreement with localization of the C-terminal domain of DotA in the bacterial cytoplasm.

The hydrophobic stretch in the DotA protein between amino acids 906 and 926 should encode the transmembrane region (TM8) required for cytoplasmic localization of the DotA C terminus. To verify this prediction, a deletion between residues 611 and 950 was constructed, resulting in the removal of five predicted transmembrane regions (TM4 to TM8). Alkaline phosphatase fusions at residues 1000 and 1039 of the wild-type DotA protein were constructed C-terminally to the deletions, and activity was determined. Both of the DotAΔ611-950::PhoA fusion proteins had alkaline phosphatase activities significantly higher than those of their counterpart DotAΔ611-846::PhoA fusion proteins (Fig. 5). Immunoblot analysis (Fig. 3) demonstrates that following the internal *dotA* deletions, expression of the fusion proteins in *L. pneumophila* was observed. The switch in alkaline phosphatase activity is, therefore, consistent with the model that DotAΔ611-950 removes an odd number of transmembrane regions, causing a switch in the localization of the PhoA moiety from a cytoplasmic to a periplasmic compartment.

The DotA protein has the fractionation properties of an inner membrane protein. Cellular fractionation studies were used to further characterize the localization of DotA. Logorithmically growing cells of *L. pneumophila* LP02 (*dotA*$^+$) and LP053 (*dotA053*) were lysed by lysozyme-sonication treatment, total cellular membrane was pelleted, and inner and outer membrane proteins were differentially extracted in Triton X-100 (37) and analyzed by Western blotting with an anti-DotA antibody. The DotA protein is clearly expressed in *L. pneumophila* LP02 but not in the *dotA* mutant LP053 (Fig. 6A), which is predicted to synthesize a protein that is truncated amino terminally to the DotA542-557 epitope recognized by the antibody (the prominent band seen below the 97-kDa marker is an unknown *L. pneumophila* protein that cross-reacts with the horseradish peroxidase-conjugated secondary antibody). In LP02, DotA protein is found exclusively in the membrane fractions. The majority of the DotA protein present in the membrane fraction becomes soluble following extraction of the membranes with 1% Triton X-100 (Fig. 6, lanes I and O, LP02). Protein profiles were also examined for each cellular fraction by Coomassie staining of the acrylamide gels (Fig. 6B). In contrast to DotA, the outer membrane protein MOMP remains insoluble following 1% Triton X-100 extraction (15). To further characterize the localization of the DotA protein, *L.*

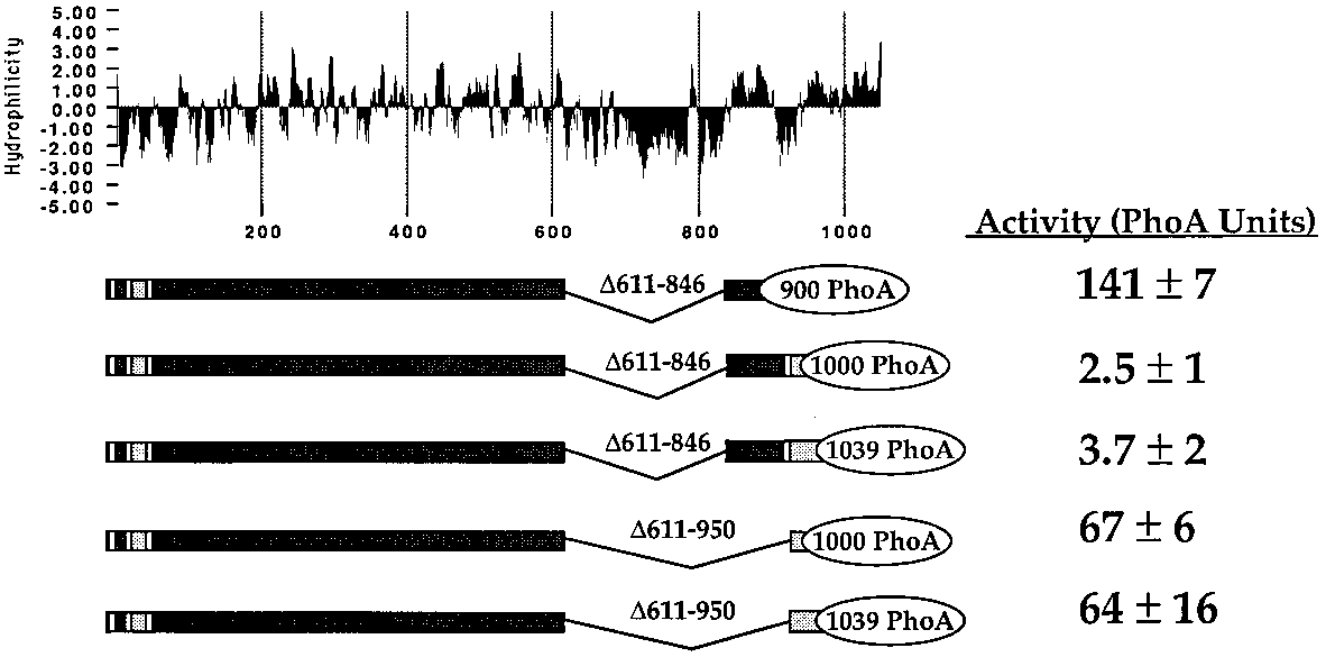

FIG. 5. Deletion of hydrophobic regions indicates the presence of a transmembrane region between residues 846 and 950. The hydropathic profile of the DotA protein is shown, along with a schematic representation of two internal DotA deletions containing C-terminal alkaline phosphatase protein fusions. Alkaline phosphatase activities and standard deviations are the result of measurements from three independent assays.

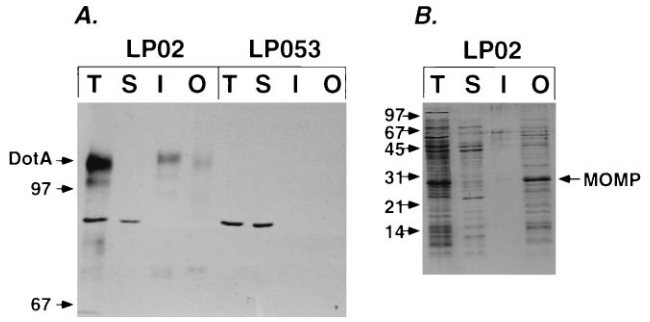

FIG. 6. DotA is in a Triton X-100-soluble membrane fraction. *L. pneumophila* extracts were fractionated as described in Materials and Methods. Lanes: T, total protein; S, soluble protein; I, Triton X-100-soluble membrane fraction; O, Triton X-100-insoluble membrane fraction. The positions of molecular mass markers (in kilodaltons) are given. The locations of DotA and MOMP are indicated. (A) Fractions were separated on an 8% polyacrylamide gel, transferred to Immobilon P, and probed with anti-DotA antibody. (B) Fractions were separated on a 15% polyacrylamide gel and Coomassie blue stained to visualize total proteins.

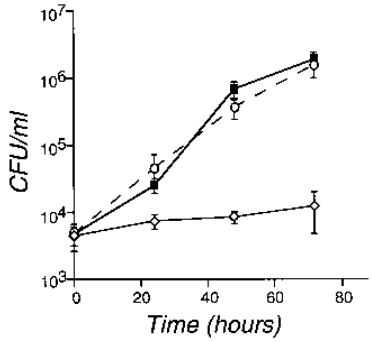

FIG. 8. Dot1039::PhoA has DotA activity. Intracellular growth of *L. pneumophila* LP053 (*dotA053*) containing plasmids expressing wild-type DotA protein (squares), Dot1039::PhoA fusion protein (circles), or Dot1000::PhoA fusion protein (diamonds) in U937 cells was measured (Materials and Methods).

pneumophila membranes were separated by centrifugation on a sucrose gradient (Fig. 7). In agreement with the Triton X-100 data, these results show that DotA and MOMP reside in distinct membrane fractions.

The DotA cytoplasmic C terminus is required for in vivo function. The plasmids encoding the DotA::PhoA hybrid proteins were mated into LP03 and LP053 to determine if intracellular growth of the bacteria could be restored in phorbol ester transformed U937 cell cultures (2). The Dot1039::PhoA protein was the only hybrid capable of restoring intracellular growth to LP03 (*dotA03* is a nonsense mutation that inserts a termination codon at amino acid 188) or LP053, whereas the plasmids containing an alkaline phosphatase fusion to DotA amino acid 309, 450, 546, 611, 793, 863, 900, 950, or 1000 or any of the five deletion constructs were all unable to complement these two chromosomal mutations (Fig. 8; data not shown). The Dot1039::PhoA protein was missing the nine C-terminal amino acids of DotA, yet restored intracellular growth to levels equivalent to that of the wild-type DotA protein (Fig. 8). In contrast, the Dot1000::PhoA protein, missing 48 C-terminal amino acids, was unable to complement the chromosomal *dotA* mutation (Fig. 8). The inability to restore intracellular growth with the Dot1000::PhoA hybrid indicates that the cytoplasmic C terminus has an important role in DotA function.

The plasmids expressing the DotA::PhoA hybrid proteins were also placed into *L. pneumophila* LP02 (*dotA*$^+$), and none had a detectable effect on intracellular replication (data not shown). These results indicate that introduction of multiple copies of each of the defective DotA proteins does not interfere with the function of the chromosomal *dotA* gene product.

DISCUSSION

The *dotA* gene of *Legionella pneumophila* is required for intracellular growth of the bacteria in macrophages and is also necessary for recruitment of host cell organelles to the phagosomal membrane (2, 3, 38). The molecular mechanisms employed by *L. pneumophila* to subvert the host-cell defenses and direct a distinctive phagosome trafficking pathway inside the cell are unknown. In this study, we have undertaken a molecular characterization of the *dotA* gene product. Our findings indicate that the *dotA* gene product has the topological properties of an integral cytoplasmic membrane protein. Protein fractionation studies are consistent with DotA residing primarily in the inner membrane. The DotA protein can be separated from the *L. pneumophila* MOMP by both solubilization of

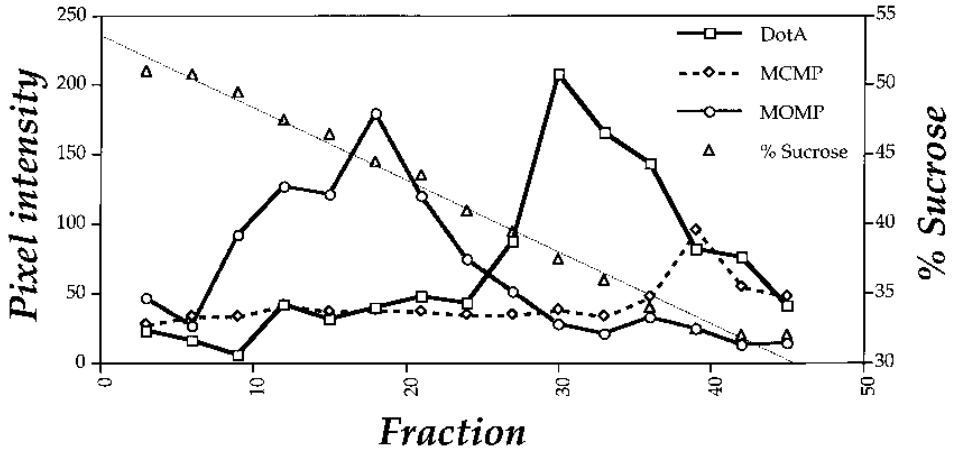

FIG. 7. Sucrose gradient fractionation of DotA protein. *L. pneumophila* membranes were separated by sucrose density centrifugation. Every third fraction was assayed for the outer membrane protein MOMP, the inner membrane protein MCMP, DotA, and sucrose density as described in Materials and Methods.

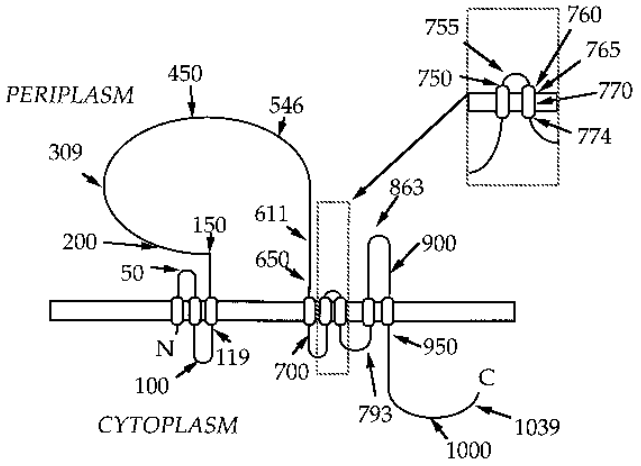

FIG. 9. Predicted DotA topology; a schematic of the proposed DotA inner membrane topology, along with the amino acid position of gene fusions used in these studies. Enlarged panel, locations of several fusions in the region between TM5 and TM6. The predicted amino acid positions of the DotA transmembrane domains are 4 to 19 (TM1), 66 to 85 (TM2), 118 to 138 (TM4), 647 to 667 (TM4), 719 to 739 (TM5), 753 to 773 (TM6), 813 to 833 (TM7), and 906 to 926 (TM8).

membranes in Triton X-100 and sedimentation of membranes following sucrose gradient centrifugation.

The *dotA* sequence and translated gene product show no obvious homologies to other sequences in the current databases, although our analysis suggests topological similarities to inner membrane transport proteins. By constructing sequential C-terminal PhoA protein fusions to DotA, the topological organization of the DotA protein within the inner membrane is predicted to contain eight transmembrane helices. These membrane-spanning regions are also predicted by the hydropathic characteristics of the protein (28) and by the TopPred II computer algorithm developed by von Heijne which uses the positive-inside rule favoring conformations that contain basic amino acid residues near the cytoplasmic face of the inner membrane (8, 39).

Based on studies of fusions to alkaline phosphatase and β-galactosidase, there are three transmembrane domains located in the N terminus of DotA between amino acids 1 and 150 (Fig. 9). These transmembrane domains are predicted to present a large periplasmic domain of 509 amino acids that contains 10 of the 12 cysteine residues found in the DotA protein, suggesting the presence of multiple disulfide bonds. This periplasmic domain is N terminal to a hydrophobic region in the DotA protein that is predicted to contain four transmembrane helices. The second periplasmic domain of significant size is located between amino acids 833 and 906. An eighth transmembrane domain exists that is required for the localization of the carboxyl-terminal 122 amino acids of DotA to the cytoplasm.

We failed to isolate *dotA*::*phoA* fusions between the fifth and sixth transmembrane domains (TM5 and TM6) that have high alkaline phosphatase activity. The periplasmic loop between TM5 and TM6 is predicted to be 13 amino acids in length, the shortest loop in the DotA protein. Proper localization of these 13 amino acids may require some form of cooperative interaction between TM5 and TM6. The Dot760::PhoA fusion protein contains eight amino acids of the predicted TM6 (Fig. 9) and is the only hybrid protein containing a fusion junction in this region that demonstrated alkaline phosphatase activity significantly above the baseline. These data suggest that the eight amino acids of TM6 may provide enough secondary structure for membrane insertion of TM5 and yet are not sufficient to mediate complete translocation of the protein back to the cytoplasm. An alternative explanation is that this region could be important for translocation of a substrate across the inner membrane and therefore be more fluid in its membrane interactions.

The predicted locations of transmembrane domains in DotA are similar to the arrangement of transmembrane domains in members of the ABC-type transporter proteins (17), which include the MalF protein. Although only about half of the size of DotA, MalF also has eight transmembrane domains, a large periplasmic domain between TM3 and TM4, and a cytoplasmic C terminus (4, 14). MalF is required for the transport of maltose from the *E. coli* periplasm to the cytoplasm. This process requires several additional proteins, the periplasmic maltose binding protein MalE, a second inner membrane component, MalG, and the cytoplasmic nucleotide-binding protein MalK (9, 10, 19, 34). However, the function of DotA does not appear to be at the level of importing an essential nutrient to bacteria once inside of macrophages. An *L. pneumophila* thymine auxotroph is defective for intracellular replication, as would be expected for a mutant defective in uptake or biosynthesis of a critical metabolite, and yet is able to form a replicative phagosome following uptake by macrophages in the absence of thymine (2). In contrast, *dotA* mutants are defective in the formation of replicative phagosomes (2, 3, 38), and their defect in targeting is apparent within 5 min following uptake of the bacterium into the macrophage (36a). The kinetics of this defect is, therefore, inconsistent with DotA protein being involved in a metabolic pathway.

The architectural similarity between DotA and ABC transporters suggests that DotA may also interact with other proteins as part of a complex required for intracellular targeting of the phagosome inside macrophages. The predicted DotA structure contains three spatially defined domains. The two periplasmic domains may interact with each other or mediate protein-protein interactions with either periplasmic or outer membrane-associated components.

The eight DotA transmembrane domains could interact to form a portal between the cytoplasm and the periplasm. The cytoplasmic domains of *dotA* may have a regulatory role, serving as a link between energy-transducing proteins in the cytoplasm and the extracellular milieu. Recent results from analysis of mutants defective for intracellular growth indicate that there is at least one protein predicted to have nucleotide binding capacity that may serve to transduce energy to a complex containing DotA protein (38a).

The *icmWXYZ* gene products are also attractive candidates for components that could work in association with DotA for the following reason. The *icmWXYZ* operon is found immediately adjacent to the *dotA* locus, and these genes are also required for growth of *L. pneumophila* inside macrophages (5, 32). It is clear that genes encoding pathogenic determinants in bacteria can often be found clustered either on the chromosome or on plasmids. Therefore, the proximity of the *icm* operon to *dotA* and the similar intracellular growth defects resulting from mutations in either region suggest that these genes may all encode a multiprotein system that is required for the establishment of a replicative niche once *L. pneumophila* cells are ingested by macrophages.

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