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Rapid Escape of the *dot/icm* Mutants of *Legionella pneumophila* into the Cytosol of Mammalian and Protozoan Cells▽†

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The *Legionella pneumophila*-containing phagosome evades endocytic fusion and intercepts endoplasmic reticulum (ER)-to-Golgi vesicle traffic, which is believed to be mediated by the Dot/Icm type IV secretion system. Although phagosomes harboring *dot/icm* mutants are thought to mature through the endosomal-lysosomal pathway, colocalization studies with lysosomal markers have reported contradictory results. In addition, phagosomes harboring the *dot/icm* mutants do not interact with endocytosed materials, which is inconsistent with maturation of the phagosomes in the endosomal-lysosomal pathway. Using multiple strategies, we show that the *dot/icm* mutants defective in the Dot/Icm structural apparatus are unable to maintain the integrity of their phagosomes and escape into the cytoplasm within minutes of entry into various mammalian and protozoan cells in a process independent of the type II secretion system. In contrast, mutants defective in cytoplasmic chaperones of Dot/Icm effectors and *rpoS*, *letA/S*, and *letE* regulatory mutants are all localized within intact phagosomes. Importantly, non-*dot/icm L. pneumophila* mutants whose phagosomes acquire late endosomal-lysosomal markers are all located within intact phagosomes. Using high-resolution electron microscopy, we show that phagosomes harboring the *dot/icm* transporter mutants do not fuse to lysosomes but are free in the cytoplasm. Inhibition of ER-to-Golgi vesicle traffic by brefeldin A does not affect the integrity of the phagosomes harboring the parental strain of *L. pneumophila*. We conclude that the Dot/Icm transporter is involved in maintaining the integrity of the *L. pneumophila* phagosome, independent of interception of ER-to-Golgi vesicle traffic, which is a novel function of type IV secretion systems.

Legionella pneumophila is a gram-negative facultative intracellular pathogen that replicates within macrophages and monocytes (15, 16, 22, 40, 52). Bacterial replication within pulmonary cells in the alveoli is indispensable for the ability of the organism to cause disease, which is manifested as an acute pneumonia. Interestingly, no person-to-person transmission of *L. pneumophila* has ever been reported. *L. pneumophila* is ubiquitous in aquatic environments, where amoebae and protozoans are considered to be the environmental hosts and reservoirs for this intracellular pathogen. This host-parasite interaction plays a major role in bacterial ecology and pathogenesis (2, 54).

L. pneumophila has very similar intracellular fates within both mammalian and protozoan cells. Intracellular multiplication of *Legionella* in protozoans, such as *Acanthamoeba polyphaga*, and in macrophages requires the Dot/Icm type IV secretion system for biogenesis of the phagosome (34, 70). Most or all of the Dot/Icm structural proteins have been shown to be essential for biogenesis of the *Legionella*-containing phagosome (LCP) and for intracellular multiplication within both protozoan and mammalian cells (69, 74). The majority of the Dot/Icm proteins are involved in the assembly of a type IV secretion apparatus, which injects bacterial proteins into the cytosol of host cells (48, 56, 69, 74). Within both mammalian and protozoan cells, the LCP evades endocytic fusion (1, 42), intercepts early secretory vesicles exiting the endoplasmic reticulum (ER), and acquires proteins involved in ER-to-Golgi vesicle traffic, such as Rab1 and Sec22b (45). Within a few minutes of biogenesis, the LCP is remodeled into an ER-derived replicative vacuole (1, 29, 41, 44, 45, 72). The Dot/Icm system is also essential for modulation of proapoptotic and antiapoptotic events in the host cell (4, 5, 55). A few hours after internalization and formation of the ER-derived replicative organelle, bacterial replication is initiated. Both evasion of endocytic fusion and recruitment of early secretory vesicles as they exit the ER are controlled by the Dot/Icm type IV secretion system (1, 29, 41, 72). While the *L. pneumophila*-containing phagosome does not interact with the dynamic endocytic traffic, other species of *Legionella* seem to be trafficked differently. The *L. longbeachae*-containing phagosome interacts with the endocytic traffic, and its biogenesis exhibits some maturation within the endocytic pathway (9, 10). Recent studies have shown that within human macrophages, the *L. longbeachae*-containing phagosome is trafficked into a nonacidified late endosome-like phagosome that acquires the LAMPs and the M6PR late endosomal markers but excludes the vacuolar ATPase proton pump and lysosomal markers (9, 10). In addition, the *L. longbeachae*-containing phagosome is remodeled by the rough ER (RER), and bacterial replication occurs within the RER-remodeled late endosome-like phagosomes (9, 10). In contrast, the *L. micdadei* phagosomes are not remodeled by the RER (36).

It has been shown that the *dot/icm* mutants of *L. pneumophila* do not remodel their phagosomes into ER-derived vesi-

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TABLE 1. Strains used in this study

Strain	Species	Characteristic(s)	Reference(s)
AA100	*L. pneumophila*	Clinical isolate	34
Lp02	*L. pneumophila*	Derivative of Philadelphia-1	18
dotA	*L. pneumophila* (AA100 derived)	*dotA*::Km	34
dotApdotA	*L. pneumophila* (AA100 derived)	*dotA*::Km p*dotA*	19
icmT	*L. pneumophila* (AA100 derived)	*icmT*::Km	55a
icmTpTS	*L. pneumophila* (AA100 derived)	*icmT*::Km p*icmTS*	55a
icmS	*L. pneumophila* (AA100 derived)	*icmS*::Km	55a
icmR	*L. pneumophila* (AA100 derived)	*icmR*::Km	55a
icmW	*L. pneumophila* (AA100 derived)	*icmW*::Km	55a
icmQ	*L. pneumophila* (AA100 derived)	*icmQ*::Km	36a
icmJB	*L. pneumophila* (AA100 derived)	*icmJB*::Km	34, 36a
icmGCD	*L. pneumophila* (AA100 derived)	*icmGCD*::Km	34, 36a
dotDCB	*L. pneumophila* (AA100 derived)	*dotDCB*::Km	34, 36a
rpoS	*L. pneumophila*	*rpos*::Km, AA100 derived	3
rpoS	*L. pneumophila*	*rpos*::Km, Lp02 derived	38a
letA/S	*L. pneumophila*	*letA/S*::Km, Lp02 derived	39
letE	*L. pneumophila*	*letE*::Km, Lp02 derived	39
htrA	*L. pneumophila* (AA100 derived)	*htrA*::Km	59
Nu243	*L. pneumophila* (AA100 derived)	*pilD*::Km	62a
icmGCD-pilD		*icmGCD*::Km *pilD*::Km	34
Dh5α	*E. coli*	Wild type	

cles (18, 19, 43, 50, 55, 63, 68, 69, 71, 74, 76). However, studies have shown variable results for colocalization of the phagosomes containing the *dot/icm* structural mutants with lysosomes. Within a few minutes after internalization, phagosomes containing the *dot/icm* mutants colocalize with the late endosomal markers Lamp-1 and Lamp-2 (43, 55, 63, 68, 74). However, only 37% of Lamp-1-positive *dot/icm* mutant LCPs are *rab7* positive in mouse macrophages (63). Colocalization of the *dot/icm* mutant LCPs with lysosomal markers such as cathepsin D, as determined using both confocal and electron microscopy techniques, is variable, ranging from about 2% with ferric oxide, cathepsin D, and Trov to 75% with acid phosphatase (18, 19, 43, 50, 55, 68, 71, 75). Therefore, it is not clear whether the *dot/icm* mutants are routed along the endosomal-lysosomal degradation pathway. Contradictory observations about the trafficking of the *dot/icm* mutants through the endosomal-lysosomal degradation pathway remain to be clarified. First, the *dot/icm* mutant-containing phagosomes are not accessible to endocytosed materials (75), which is in contrast to their presumed trafficking through the endosomal-lysosomal pathway. Second, despite potential localization of the *dot/icm* mutants in lysosomes, it is surprising that they are not killed within macrophages for an extended period (up to 48 to 72 h). Third, isolating phagosomes harboring *dot/icm* structural mutants is very difficult compared to isolating the wild-type LCPs (28, 29; R. R. Isberg, personal communication). Importantly, studies to examine the integrity of the phagosomes harboring the *dot/icm* mutants have never been reported.

Here we show that the phagosomal membrane of the *dot/icm* mutants defective in the secretion apparatus is disrupted within minutes of bacterial entry, while other cellular membranes are intact. In contrast, mutants defective in cytoplasmic chaperones of specific Dot/Icm effectors and the *rpos*, *letA/S*, and *letE* regulatory mutants all have intact phagosomes. We confirmed disruption of the phagosomes harboring the *dot/icm* structural mutants by using multiple strategies: (i) at the ultrastructural level combined with enhanced staining of membrane proteins, (ii) by the lack of colocalization with CM-Dil-pulse-labeled plasma membranes; (iii) by colocalization with actin; and (iv) by the differential binding of antibacterial antibodies loaded into the cytosol of live macrophages. We conclude that the Dot/Icm secretion system is indispensable for maintaining the integrity of the LCP, which is a novel function for a type IV secretion system.

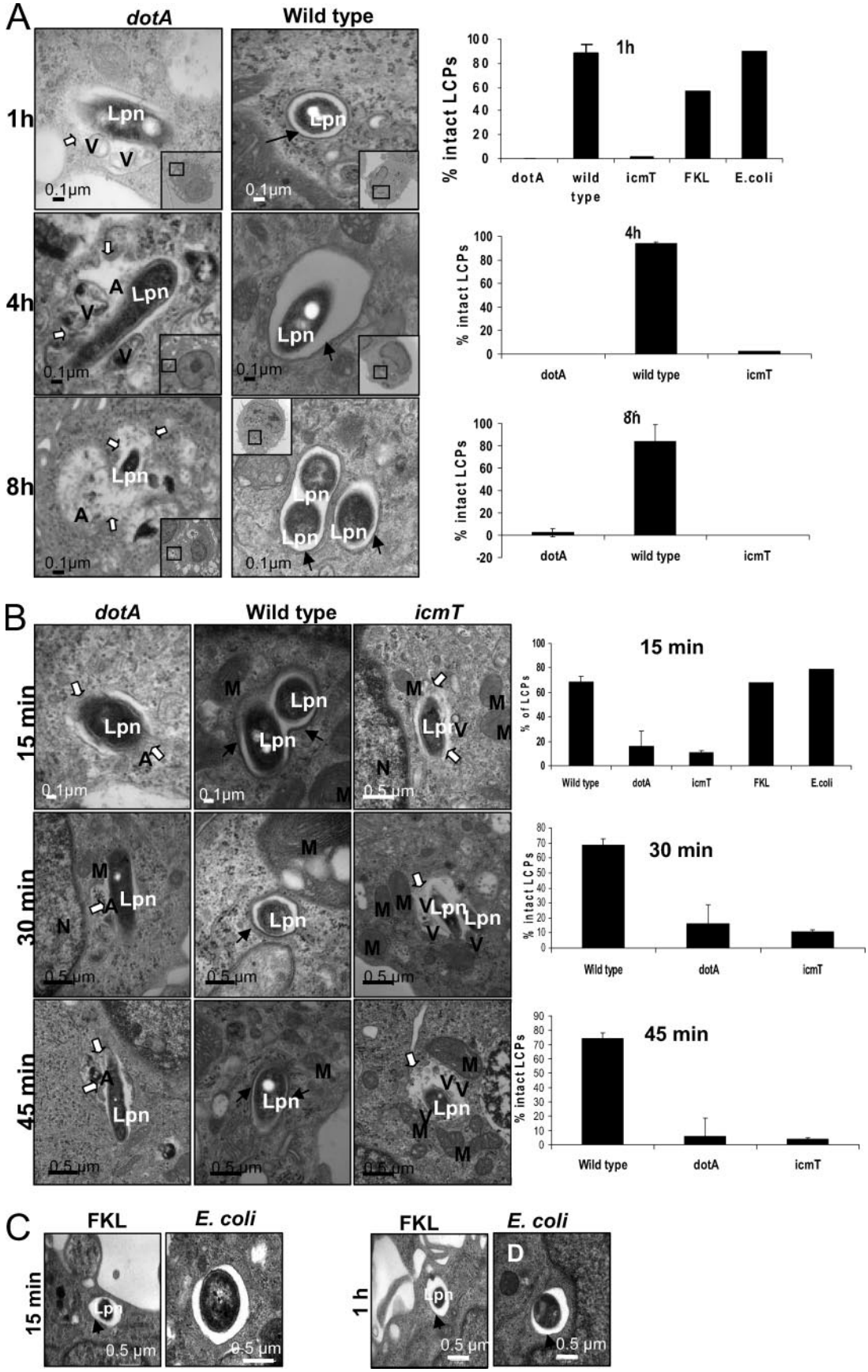
MATERIALS AND METHODS

Bacterial and cell cultures. The clinical isolate *L. pneumophila* serogroup I strain AA100 and its isogenic mutants, the Lp02 parental strain of *L. pneumophila* and its isogenic mutants (a gift from Joe Vogel, Washington University) (59, 77), and other strains are listed in Table 1. *Legionella* strains were grown on buffered charcoal-yeast extract plates supplemented with 5 μg/ml of chloramphenicol for *Legionella* strains when a green fluorescent protein-encoding plasmid or complementing plasmids were used. *Francisella tularensis* subsp. *novicida* strain U112 has been described previously (59, 77).

Maintenance of U937 macrophage-like cells and type A549 alveolar epithelial cells in RPMI medium with 10% serum and differentiation of U937 macrophages with phorbol 12-myristate 13-acetate (Sigma) were performed exactly as we described previously (36). *A. polyphaga* was maintained in peptone yeast glucose medium, as we described previously (34). Quiescent human monocyte-derived macrophages (hMDMs) were prepared from fresh peripheral blood monocytes obtained from healthy adult volunteers with no history of Legionnaires' disease or tularemia and were incubated for 3 days in ultralow attachment plates (Costar, Corning Inc., NY) to allow differentiation into macrophages, as we described previously (67). The concentration of fetal bovine serum was progressively decreased over 4 days to 1% in RPMI medium to obtain quiescent cells.

Infection experiments. A549 epithelial cells and differentiated U937 macrophages were used similarly in RPMI medium containing 10% serum. Trypsinization of A549 epithelial cells was performed with TrypLE Express (Gibco BRL, Gaithersburg, MD). *A. polyphaga* was incubated in peptone yeast glucose medium without glucose during the infection period. Cell monolayers were washed three times with the tissue culture medium prior to infection. Unless specified otherwise, all infections were carried out using 5×10^5 cells/ml at a multiplicity of infection (MOI) of 10 for 1 h, followed by washing the extracellular bacteria off, and the infected cells were incubated for the periods indicated below for each experiment. Pretreatment with brefeldin A (BFA) (10 μg/ml) was performed for 5 h, followed by infection, as described above.

Confocal and electron microscopy. For transmission electron microscopy (TEM) studies, six-well tissue culture plates containing 3 ml of a preparation containing 5×10^5 cells/ml were infected as described above. At different time


A
dotA
Wild type
1h
4h
8h
Lpn
V
A
0.1µm
% intact LCPs
1h
dotA
wild type
icmT
FKL
E.coli
4h
8h
B
dotA
Wild type
icmT
15 min
30 min
45 min
M
N
0.5 µm
% of LCPs
% intact LCPs
Wild type
dotA
icmT
FKL
E.coli
C
FKL
E. coli
D
0.5 µm

points after infection, the cells were fixed in 3.5% glutaraldehyde, dehydrated in alcohol, processed, and stained for TEM as we described previously (53). The cells were examined with a Philips CM-12 TEM.

To increase the contrast of the membrane proteins, 1% tannic acid was added during the fixation step in the glutaraldehyde solution (46, 61). The samples were then processed as described above. Preloading of the lysosomes with bovine serum albumin (BSA)-gold particles was done as we described previously (67). Briefly, BSA-gold particles (EMS, Washington, PA) were purified with PD-10 columns (Amersham Biosciences, Uppsala, Sweden) and resuspended in RPMI medium. Lysosomes were preloaded with BSA-gold particles, as we described previously (67). After three washes, the macrophages were infected for 1 h and were processed at the desired time points for TEM.

Phagosomes were determined to be disrupted when membrane disruptions were clearly visible in an irregular part of the membrane and vesicles, ribosomes, and organelles, such as mitochondria, were juxtaposed or touching the bacterium. Any visible disruption of the phagosome in a regular part of the membrane with no visible vesicles, ribosomes, and organelles juxtaposed or touching the organism were not included in our analyses. Importantly, all our samples for microscopy were coded by an individual who did not analyze the samples, and the codes were revealed only after final analyses of the samples. For data analyses, 100 infected cells from each of three grids obtained from three different blocks were examined. Each experiment was repeated two to four times (in general, three times), and the data shown below are representative of one experiment.

For confocal microscopy, 5×10^5 cells were allowed to adhere to circular glass coverslips (VWR) in 24-well culture plates. Infection of adherent cells using an MOI of 10 for 1 h and fixation and preparation of the cells for confocal microscopy were performed as previously described (55). The lipid membrane dye CM-Dil (2 μg/ml) and the actin dye phalloidin (2 U) were purchased from Molecular Probes and used according to manufacturer's recommendation. Anti-lipopolysaccharide monoclonal antibodies (MAbs) 3/1 (5) and polyclonal rabbit antiserum against *L. pneumophila* strain AA100 or *F. tularensis* (a gift from Karl Klose) were utilized to label the bacteria. MAbs against cathepsin D were purchased from BD Transduction Laboratories. The secondary antibodies (Molecular Probes) were AlexaFluor 594 conjugates. The cells were analyzed using an Olympus F500 laser scanning confocal microscope. For each sample, 100 infected cells from each of three different coverslips were analyzed. Each experiment was repeated three times, and the data shown below are representative of one experiment. On average, 15 to 20 1-μm-thick *z* serial sections of each image were captured and analyzed using Adobe Photoshop 6.0 (Adobe Photoshop, Inc.).

Loading the macrophage cytosol with antibacterial antibodies. The cytosol of live U937 macrophages was loaded with antibacterial antibodies using glass beads to "wound" the plasma membrane as described previously (51, 60). After 1 h of infection, cells were washed three times with phosphate-buffered saline, and 400 μl of antibacterial antibodies was added on the top of the coverslips, along with an aliquot containing 0.5 g of acid-washed and sterile glass beads (425 to 600 μm; Sigma). The beads were rolled over the cells 12 times. This treatment had no detectable effect on the viability of cells, as confirmed by trypan blue exclusion. The glass beads were immediately washed off with phosphate-buffered saline, and the cells were incubated at 37°C for 1 h to allow a sufficient time for the antibodies to bind. The cells were then fixed and processed with conjugated secondary antibodies. The controls were either cells subjected to the same treatment without the glass beads or cells fixed and permeabilized with 0.05% Triton X-100 for 15 min on ice.

Statistical analyses. All experiments were performed at least three times, and the data shown below are representative of one experiment. To analyze for statistical significant differences between different sets of data, the Student two-tailed *t* test was used, and the *P* value was obtained.

RESULTS

Ultrastructural analyses of disruption of *dot*/*icm* mutant-containing phagosomes. We utilized TEM to examine the integrity of the phagosomes of the *dotA* mutant and compare it to that of the wild-type strain during early stages of infection of U937 macrophages. When the integrity of the phagosome was examined by TEM at 1, 4, and 8 h postinfection, an LCP was considered intact when the membrane had no visible disruptions. An LCP was considered disrupted when apparent physical disruptions of the phagosomal membrane were present and/or when host cell cytoplasmic elements, such mitochondria, vesicles, and amorphous material, were next to the bacteria without a limiting membrane surrounding the organism. Surprisingly, at all time points, the *dotA* mutant phagosomes were disrupted and the mutant was cytoplasmic, while the wild-type strain was enclosed in intact phagosomes within U937 macrophages (Fig. 1A and Table 2). Intact cytoplasmic vesicles and amorphous material were visible in vicinity of the *dotA* mutant and in many cases were touching the bacteria (Fig. 1A). The size and/or the shape and location of the cytoplasmic material, such as ribosomes or vesicles, strongly suggested that they were host vesicles (Fig. 1). In contrast, other cellular membranes were intact. The presence of intact vesicles next to the bacteria and the integrity of other host cell membranes argued against the possibility of artifacts of sample processing for microscopy. Quantitative analysis of the integrity of the LCPs showed that at 1 h postinfection, none of the *dotA* mutant LCPs were intact, while 83% of the wild-type strain LCPs were intact ($P < 0.0001$, as determined by the Student *t* test) (Fig. 1A). At this time point, ~65% of the formalin-killed *L. pneumophila* (FKL) and ~90% of the *Escherichia coli* controls were surrounded by an intact phagosomal membrane (Fig. 1). Similar results were obtained at 4 and 8 h postinfection ($P < 0.0001$, as determined by the Student *t* test) (Fig. 1A).

To examine whether other structural components of the Dot/Icm secretion apparatus were also essential for maintaining the integrity of the LCP, we examined the integrity of the phagosomes of the *icmT*, *icmJB*, *icmGCD*, and *dotDCB* mutants by TEM at 1 h after infection of U937 macrophages. The data showed that more than 90% of the LCPs of all of these mutants were disrupted, in contrast to the wild-type strain LCPs ($P < 0.0001$, as determined by the Student *t* test) (Table 2; see Fig. S1 in the supplemental material). The defect of the *icmT* mutant was complemented by the wild-type gene (see below). Therefore, other *dot*/*icm* mutants defective in structural components of the type IV secretion apparatus were also defective in maintaining the integrity of their phagosomes.

Interestingly, even when they were examined at 15 to 45 min

FIG. 1. Phagosomes of the *dotA* and *icmT* mutants are disrupted within U937 macrophages. (A) Representative electron micrographs of LCPs of the *dotA* mutant and the wild-type strain at 1, 4, and 8 h after 1 h of infection at an MOI of 10. The whole cell is shown in each inset. (B) Representative electron micrographs showing the integrity of LCPs at 15 to 45 min postinfection. Quantitative analyses of the integrity of the LCPs for panels A and B are shown on the right. (C) Representative electron micrographs of controls, including FKL-containing LCPs and *E. coli* phagosomes at 15 min and 60 min postinfection. The white arrows indicate sites of disruption of the phagosomal membrane. The black arrows indicate sites where the phagosomal membrane is intact. Lpn, *L. pneumophila*; A, amorphous material; V, vesicle; M, mitochondrion. Some of the error bars are too small to be displayed by the software.

TABLE 2. Summary of the results of phagosomal disruption

Bacterium	Results with the following strategies and host cells							
	TEM disruption of phagosomes				TEM using tannic acid (U937 cells)	CM-Dil colocalization (U937 cells)	Binding to cytosolic antibodies (U937 cells)[a]	Actin colocalization (U937 cells)
	U937 cells	hMDMs	A549 cells	*A. polyphaga*				
AA100	−	−	−	−	−	+	−	−
dotA	+	+	+	+	+	−	+	+
icmT	+	+	+	+	+	−	+	
dotApdotA		−			−	+	−	−
icmTpTS					−	+	−	
icmS	−						+	
icmR	−							
icmW	−							
icmQ	−							
icmJB	+				+			
icmGCD	+				+			
dotDCB	+				+			
icmGCD-pilD	+				+			
pilD	−							
rpoS		−		−				
letA/S				−				
letE				−				
htrA	−				−	+	−	−
FKL	−					+	−	−
F. tularensis	+	+				−	+	

[a] Phagosome integrity assay using glass beads to load the macrophage cytosol with antibodies.

postinfection, the phagosomes of the *dotA* and *icmT* mutants were already disrupted, while the wild-type strain LCPs were intact ($P < 0.001$, as determined by the Student *t* test) (Fig. 1B). Importantly, 70 to 80% of the FKL and *E. coli*-containing phagosome controls were intact at 15 to 60 min postinfection (Fig. 1C and Table 2). Therefore, disruption of the *dotA* and *icmT* mutant LCPs occurred within minutes of bacterial uptake.

Integrity of the phagosomes at the ultrastructural level as determined using tannic acid. We were concerned that the lack of detection of a membrane around the *dotA* mutant may have been due to the possibility that there was a thin membrane with a low protein content that was not visualized by the staining technique used. To enhance staining of low-protein-content membranes, we used tannic acid (46, 61) and examined the phagosomal integrity at 1 h after infection of U937 cells. In these studies, in addition to *E. coli* and FKL, we included the *htrA* mutant as a control. The *htrA* mutant of *L. pneumophila* is a non-*dot*/*icm* mutant that fails to replicate intracellularly, and this defect is associated with colocalization of its phagosome with the late endosomal-lysosomal marker Lamp-2, similar to *dot*/*icm* mutants (59). Our data showed that only ~25% of the phagosomes of the *dotA* and *icmT* mutants were intact, whereas 96% of the LCPs of the wild-type strain were intact ($P < 0.001$, as determined by the Student *t* test) (Table 2; see Fig. S2 in the supplemental material). Vesicles and ribosomes were clearly seen touching the *dotA* mutant bacteria (see Fig. S2 in the supplemental material). Similar to the wild-type strain, 96, 90, and 76% of the *htrA* mutant, *E. coli*, and FKL, respectively, were in intact LCPs at 1 h after infection of U937 macrophages (Table 2; see Fig. S2 in the supplemental material). The difference between the *dotA* mutant and FKL was significant ($P < 0.01$, as determined by the Student *t* test). The integrity of the LCPs of the *dot*/*icm* mutants was restored upon complementation with their respective wild-type genes ($P < 0.01$, as determined by the Student *t* test) (Table 2; see Fig. S2 in the supplemental material).

Disruption of the *dotA* mutant phagosomes in other host cells. We examined the integrity of phagosomes of the *dot*/*icm* mutants within hMDMs, A549 type II alveolar epithelial cells, and *A. polyphaga*. At 6 h after infection of quiescent hMDMs, ~80% of the wild-type strain LCPs were intact, while all of the *dotA* mutant LCPs were disrupted ($P < 0.001$, as determined by the Student *t* test) (Fig. 2). Intact cytoplasmic vesicles and/or amorphous material were in close proximity to the *dotA* mutant bacteria that were not surrounded by an intact phagosomal membrane. Approximately 80 to 90% of the FKL- and *E. coli*-containing phagosomes, which were used as controls for phagosomes that fuse to lysosomes, were intact in quiescent hMDMs (Fig. 2). We utilized *F. tularensis* as a control for an intracellular pathogen that escapes from its phagosome into the cytoplasm within 1 to 6 h and is surrounded by a halo or a clear zone around the organisms when preparations are examined by regular TEM or cryo-TEM (66, 67). More than 90% of control phagosomes harboring the *F. tularensis* strain control were disrupted at 6 h postinfection, consistent with previous studies (66, 67). Similarly, at 6 h after infection of A549 cells, none of the *dotA* mutant LCPs were intact, while the wild-type bacteria were in intact LCPs (data not shown).

We also examined phagosome integrity in the protozoan host *A. polyphaga* at 5 and 30 min and 1 and 8 h after infection by the *dotA* and *icmT* mutants. The data showed that few or none of the *dotA* and the *icmT* mutants were in intact phagosomes at all the time points (data not shown). Taken together, these results show that disruption of the *dot*/*icm* mutant LCPs is not host cell dependent.

Pulse-labeling of the macrophage membrane during phagocytosis. There are many caveats for interpretation of disrup-

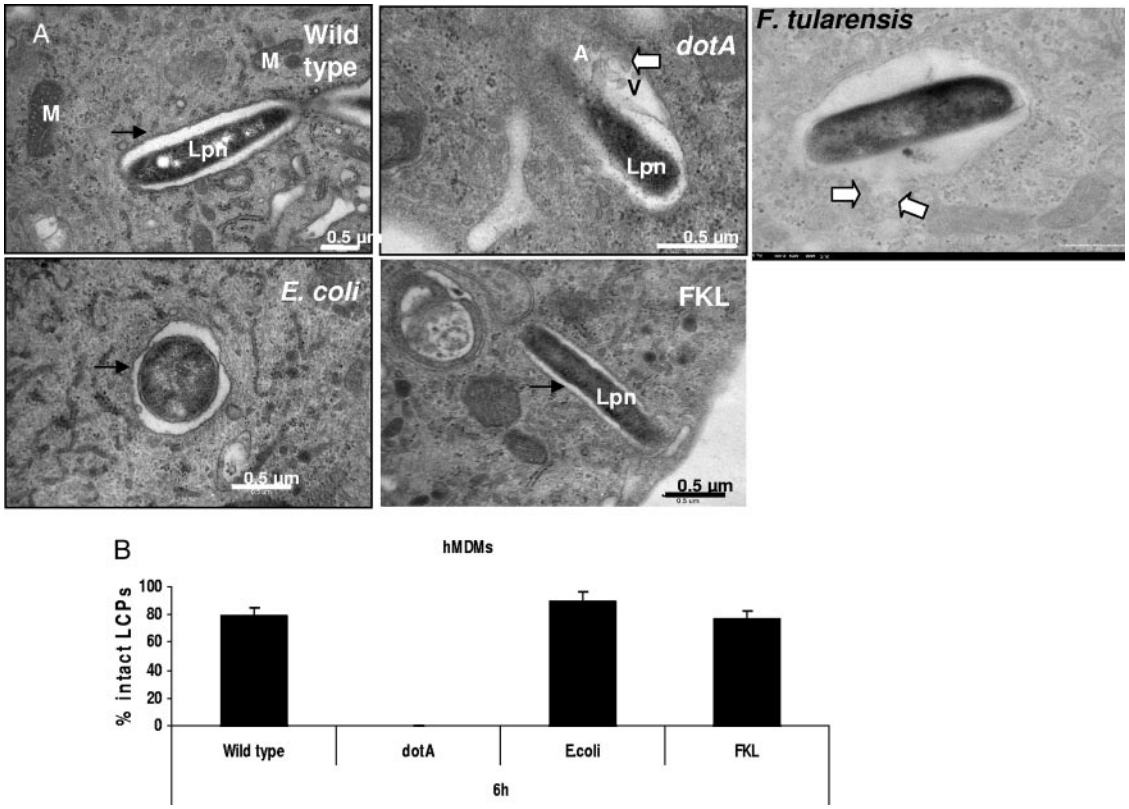


FIG. 2. Phagosomes of the *dotA* mutant are disrupted within hMDMs. (A) Representative electron micrographs of wild-type, *dotA* mutant, and *E. coli* phagosomes and FKL LCPs at 6 h after 1 h of infection of hMDMs at an MOI of 10. *F. tularensis* was included as a control for a cytoplasmic bacterium with a clear zone around the organism in the macrophage cytoplasm at 6 h postinfection. The intact portions of the LCP membrane are indicated by the black arrows. The white arrows indicate areas of disruption of the phagosomal membrane. Lpn, *L. pneumophila*; A, amorphous material; V, vesicle; M, mitochondrion. (B) Quantitative analyses of the integrity of the LCPs of the wild-type strain and the *dotA* mutant. The results are expressed as the percentages of intact LCPs.

tion of the phagosome by TEM, including artifacts of fixation and processing, and the plane of an ultrathin section may simply be the reason for the absence of part of the membrane surrounding the bacteria. We think that the plane of the sections may explain the observation that some of the wild-type phagosomes analyzed were missing part of the membrane and, therefore, were scored as disrupted. To minimize the caveats of the plane of the ultrathin sections, we analyzed three sections from three different blocks, and the samples were coded. To further support our findings of disruption of the *dot/icm* mutant-containing phagosomes, we resorted to different strategies. We utilized the lipophilic fluorescent dye CM-Dil to pulse-label the plasma membranes for 45 min during infection, followed by a 15-min chase to allow endocytosis of plasma membrane-derived vesicles to proceed. We examined colocalization of CM-Dil-labeled membranes with intracellular bacteria. The data showed that at 1 h after infection of U937 macrophages, ~70% of the wild-type strain colocalized with CM-Dil-labeled membranes (Fig. 3 and Table 2), while 82% of the *dotA* mutant did not colocalize with CM-Dil-labeled membranes ($P < 0.001$, as determined by the Student *t* test) (Fig. 3 and Table 2). In addition, 78% of the *icmT* mutants did not colocalize with CM-Dil-labeled membranes (Fig. 3 and Table 2). Importantly, the complemented *dotA* and *icmT* mutants colocalized with CM-Dil-labeled membranes (Fig. 3 and Table 2). Similar to the wild-type strain of *L. pneumophila*, ~70% of the *htrA* mutant and FKL colocalized with CM-Dil-labeled membranes (Fig. 3). In addition, 85% of the *E. coli* control colocalized with CM-Dil-labeled membranes (data not shown). Approximately ~65% of *F. tularensis* did not colocalize with CM-Dil-labeled membranes at 1 h postinfection, consistent with recent observations (59). These results showed that the type IV secretion system mutants do not colocalize with CM-Dil-labeled plasma membrane-derived phagosomal membranes.

Colocalization of the *dot/icm* mutants with actin. To confirm cytoplasmic localization of the *dotA* mutant, we used different strategies to label the cytoplasm of U937 macrophages. We used phalloidin to label the cytoplasmic protein actin, which is one of the most abundant cytoplasmic proteins within mammalian cells, in order to examine its colocalization with the wild-type strain and the *dotA* mutant. At 1 h after infection of U937 macrophages, 65% of the *dotA* mutant colocalized with actin, whereas only 18% of the wild-type strain colocalized with actin (Fig. 4 and Table 2). Similar to the wild-type strain, only ~20% of the complemented *dotA* mutant, the *htrA* mutant, and FKL colocalized with actin (Fig. 5 and Table 2).

We used other strategies to label the cytoplasm of the macrophages in order to confirm the cytoplasmic colocalization of the *dot/icm* mutants. These strategies included ectopic expres-

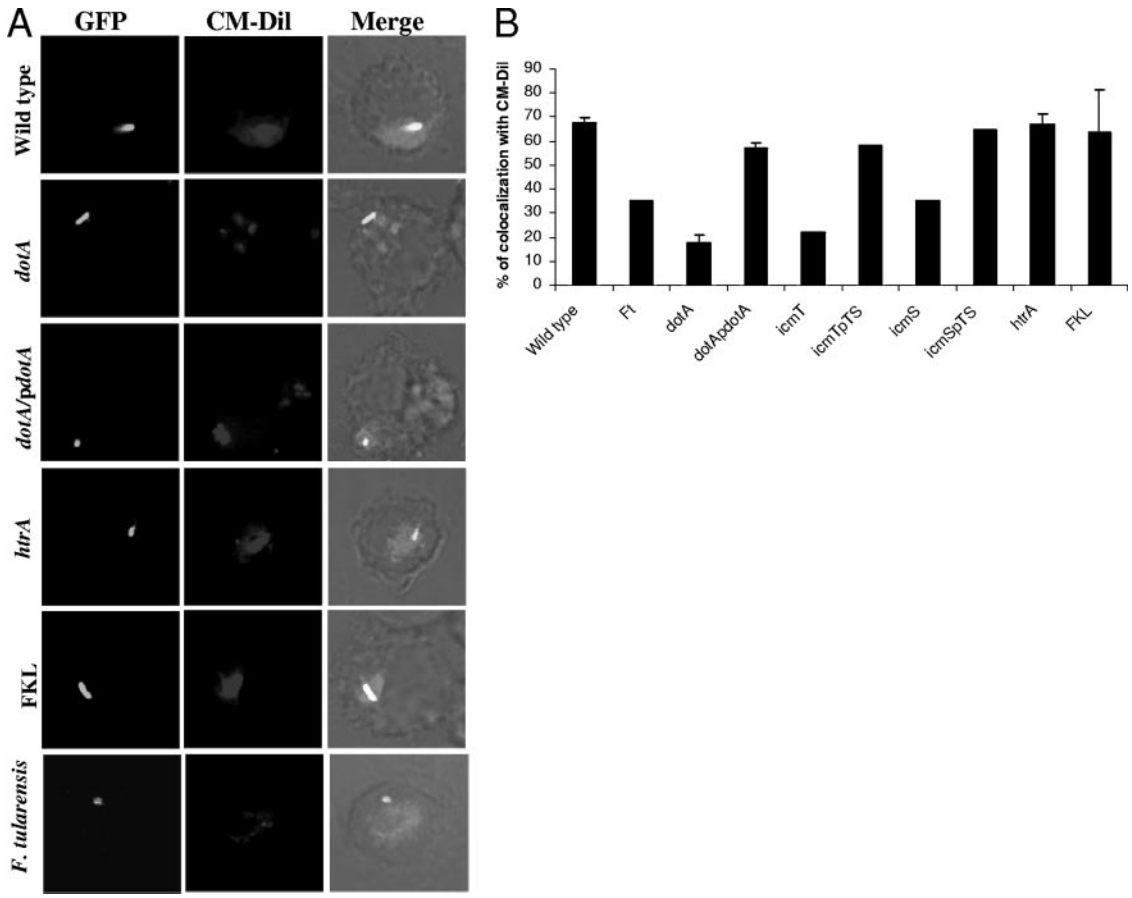


FIG. 3. *dot*/*icm* mutants are not surrounded by CM-Dil-pulsed-labeled membranes. (A) Representative confocal microscopy images of U937 macrophages infected by several strains at 1 h postinfection. The cells were pulsed-labeled with the lipophilic dye CM-Dil during 45 min of infection to label the plasma membrane, followed by a 15-min chase to allow endocytosis by CM-Dil-labeled plasma membrane. The images are stacks of 12 to 15 1-μm-thick *z* series of sections. (B) Quantitative analysis of colocalization with CM-Dil. Ft, *F. tularensis*. Some of the error bars are too small to be displayed by the software. GFP, green fluorescent protein.

sion of the red fluorescent protein or enhanced green fluorescent protein within the macrophage cytoplasm and the use of the cell tracker Red CMTPX, which passes freely through cell membranes but once inside the cell becomes cell impermeant (23). Unfortunately, although all the cells were labeled with these markers, the resolution of confocal microscopy was not sufficient to distinguish the wild-type strain from the *dot*/*icm* mutants, and the distribution of the enhanced green fluorescent protein and red fluorescent protein was not homogeneous throughout the cell.

Antibacterial antibodies loaded into the macrophage cytosol bind the *dot*/*icm* mutants. To confirm the lack of a limiting membrane surrounding the *dot*/*icm* mutants, we developed a fluorescence-based phagosome integrity assay to differentially label cytosolic bacteria or bacteria within compromised phagosomes versus bacteria harbored within intact phagosomes. To differentially label cytosolic bacteria, antibacterial antibodies were loaded into the cytosol of live U937 macrophages to determine whether the antibacterial antibodies can bind the *dot*/*icm* mutants but not the wild-type strain, the complemented mutants, or other bacteria that reside in phagosomes. To load the cytosol of U937 macrophages with MAbs, we utilized glass beads to "wound" the macrophage plasma membrane transiently in the presence of the MAbs (51, 60). This strategy results in transient "holes" in the plasma membrane that mammalian cells can repair within minutes after the membrane injury is stopped. Our results showed that only 14% of the wild-type strain of *L. pneumophila* bound the MAbs (Fig. 5 and Table 2), while ~65% of the *dotA* and *icmT* mutants bound the MAbs ($P < 0.001$, as determined by the Student *t* test) (Fig. 5). The defects in the integrity of the phagosomal membranes of the *dotA* and *icmT* mutants were complemented by their respective wild-type genes (Fig. 5 and Table 2). Similar results were obtained for the *dotA* isogenic mutant of the Lp02 parental strain of *L. pneumophila* ($P < 0.001$, as determined by the Student *t* test) (Table 2). In contrast, ~80% of the *htrA* mutant phagosomes, which colocalized with late endosomal-lysosomal markers, did not bind the MAbs (Fig. 5 and Table 2). As expected, all extracellular bacteria bound MAbs (Fig. 5). We utilized *F. tularensis* as a control for an intracellular pathogen that escapes from its phagosome into the cytoplasm between 1 and 6 h postinfection. The data showed that more than 95% of intracellular *F. tularensis* bound antibacterial antibodies loaded into the cytosol of live U937 macrophages at 6 h postinfection (Fig. 5). Several controls were included to ensure the reliability of this strategy. The cells repaired the "wounds"

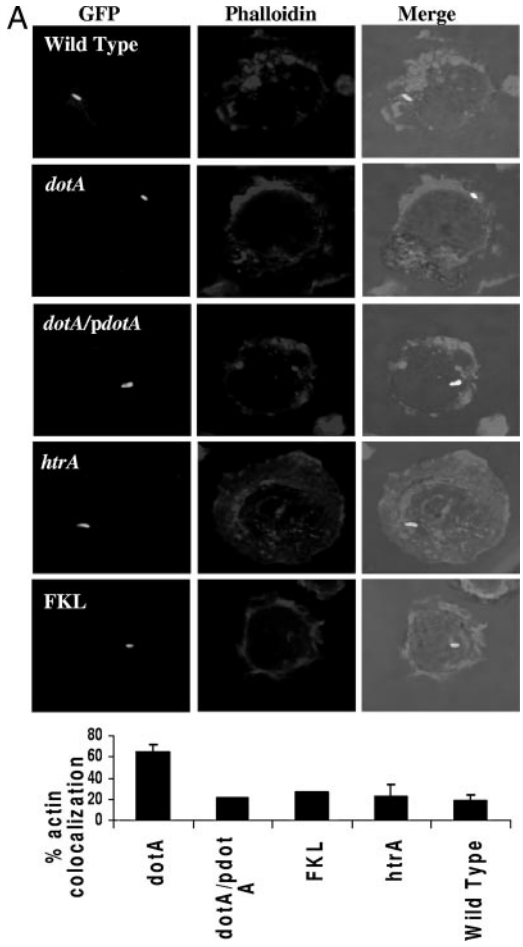


FIG. 4. Colocalization of the *dotA* mutants with the cytoplasmic protein actin within U937 macrophages. (A) Representative confocal microscopy images at 1 h after infection of U937 macrophages by the different strains and FKL. The cytoplasmic actin stain was phalloidin. The images are stacks of 12 to 15 1-μm-thick *z* series of sections. A quantitative analysis of colocalization of actin with the LCPs is shown below the images. Some of the error bars are too small to be displayed by the software. GFP, green fluorescent protein.

and became impermeable after ~3 min of incubation at 37°C following removal of the glass beads, as determined by trypan blue exclusion. When no beads were added, the *dotA* mutants did not bind the MAbs (Fig. 5). However, 90% of the wild-type bacteria in cells permeabilized with Triton X-100 bound the MAbs (Fig. 5).

Mutants defective in chaperones of the Dot/Icm effectors are localized within intact phagosomes. Mutants with mutations in *icmS*, *icmR*, *icmQ*, and *icmW* chaperones are defective in modulating phagosome biogenesis and do not intercept ER-to-Golgi vesicle traffic, and their LCPs fuse to lysosomes (26). Therefore, we examined whether these four genes were required for maintaining the integrity of the LCPs. We utilized different strategies to examine phagosome integrity. Our TEM data showed that in U937 cells infected for 1 h by the mutants, 45 to 90% of the LCPs of the mutants were intact, similar to the results for the wild-type strain (Table 2; see Fig. S3 in the supplemental material). Similarly, using the glass bead permeabilization technique to load antibacterial MAbs into the cytosol of U937 macrophages, the LCPs of the *icmS*, *icmR*, *icmQ*, and *icmW* mutants were intact at 1 h postinfection, similar to the results for the wild-type strain (Table 2). We conclude that IcmS, IcmR, IcmW, and IcmQ are not involved in maintaining the integrity of the LCP membrane.

Integrity of the phagosomal membrane of stationary-phase regulatory mutants. In mouse and human primary macrophages, the *rpoS* mutant of *L. pneumophila* is trafficked to a late endosome-like or lysosomal compartment and does not replicate (3, 13). Therefore, we examined the integrity of the phagosome harboring the *rpoS* mutant in hMDMs. Similar to the wild-type strain and the Dot/Icm chaperone mutants, the *rpoS* mutant bacteria were within intact phagosomes at 1 h after infection of hMDMs when preparations were examined at the ultrastructural level (Table 2). These results were also confirmed by using the glass bead permeabilization technique to load the cytoplasm with MAbs directed against the bacteria in hMDMs (Table 2). For this experiment, we utilized the AA100-derived and Lp02-derived *rpoS* mutants. Approximately 70 to 85% of the two *rpoS* mutant strains were found in intact LCPs (Table 2). Therefore, the RpoS stationary-phase transcription factor, which is essential for evasion of endocytic fusion within hMDMs, is not involved in maintaining the integrity of the LCP.

The global response two-component regulator of *L. pneumophila*, LetA/S, is involved in regulation of a phenotypic transition at the postexponential phase (13, 33, 39, 49), and LetE is also involved in this process (12). The *letA* mutant of *L. pneumophila* is severely defective in intracellular replication in *Acanthamoeba* (33, 49). Therefore, we examined the integrity of the phagosomal membrane of the *letA*, *letS*, and *letE* mutants in *A. polyphaga*. We showed that ~90% of the *letA*, *letS*, and *letE* mutant cells in *A. polyphaga* were within intact phagosomes when they were examined by TEM, similar to the results for the wild-type strain (Table 2; see Fig. S3 in the supplemental material). Taken together, these data show that regulatory proteins involved in the regulation of the phenotypic transition to the virulent form of *L. pneumophila* at the postexponential phase are not involved in maintaining the integrity of the LCP membrane.

Hydrolytic enzymes of the type II secretion system are not involved in disrupting the integrity of the phagosomal membrane. Since the type II secretion system of *L. pneumophila* is known to secrete many lytic enzymes, including proteases, lipases, and phospholipases (6–8, 30–32, 62), we examined whether the type II secretion system was involved in disruption of the *dot/icm* mutant phagosomal membranes. We used the *pilD* mutant Nu243, which is defective in the prepilin peptidase, and the *icmGCD-pilD* double mutant, which is defective in both type IV and type II secretion systems (62, 62a). Our data obtained from ultrastructural studies showed that the *icmGCD-pilD* double mutant was within disrupted phagosomes at 1 h after infection of U937 macrophages, while the *pilD* mutant was within intact phagosomes (Table 2). Therefore, the hydrolytic enzymes secreted through the type II secretion system are not involved in disruption of the phagosomes harboring the *dot/icm* structural mutants.

Interception of ER-to-Golgi vesicle traffic by the wild-type LCPs does not contribute to the integrity of the phagosomal membrane. The wild-type strain of *L. pneumophila* intercepts

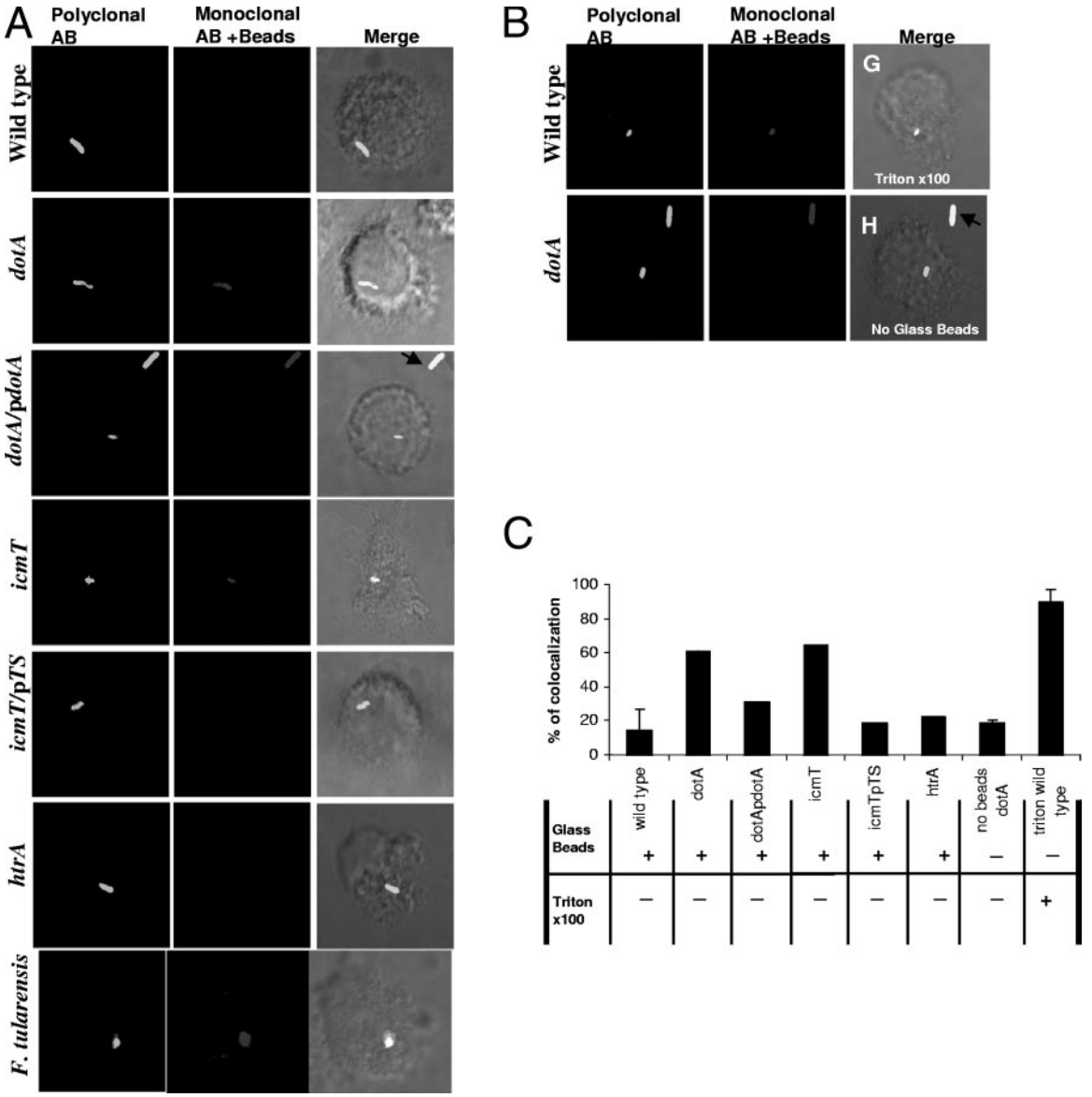


FIG. 5. Antibacterial antibodies loaded into the cytosol of live U937 macrophages bind the *dot*/*icm* mutants. (A) Representative confocal microscopy images at 1 h after infection of U937 cells by the different strains. After infection, specific antibacterial MAbs and glass beads were added to the live cells to permeabilize the plasma membrane in order to load the cytosol with the MAbs. The cells were fixed and processed for confocal microscopy. All bacteria were labeled either with a green fluorescent protein-containing plasmid or with a specific antibody used after fixation. Infection by *F. tularensis* at 6 h postinfection was included as a positive control for a cytoplasmic bacterium that bound the antibacterial antibodies loaded into the macrophage cytoplasm. (B) Controls for the glass bead loading technique. For the negative control (without beads), the cells were not permeable to the MAb and the *dotA* mutant showed only green fluorescent protein staining. For the positive control, the cells infected by the wild-type strain were permeabilized by Triton X-100, which permeabilized all membranes. Note that extracellular bacteria, indicated by the arrows, bound the MAbs. The images are stacks of 12 to 15 1-μm-thick *z* series of sections. (C) Quantitative analysis of binding of the antibacterial antibodies loaded into the macrophage cytosol. Some of the error bars are too small to be displayed by the software. AB, antibody.

ER-to-Golgi vesicle traffic within a few minutes postinfection in a Dot/Icm-dependent manner (1, 44, 45, 64, 72). Therefore, we examined whether interception of ER-to-Golgi vesicle traffic by the LCPs harboring the wild-type strain is involved in maintaining the integrity of the phagosomal membrane. We used BFA to block ER-to-Golgi vesicle traffic and examined its effects on the phagosome integrity of the wild-type strain (17, 44, 47, 73). Untreated or BFA-pretreated U937 macrophages were infected for 1 h, and phagosome integrity was examined by confocal microscopy using the glass bead loading technique for antibacterial antibodies. We confirmed the inhibition of recruitment of ER vesicles to the LCPs in BFA-treated cells by the lack of acquisition of the ER marker KDEL by the wild-type LCPs within BFA-treated U937 macrophages (Fig. 6). When preparations were examined for the effect of blocking ER-to-Golgi vesicle traffic on phagosome integrity, ~90% of

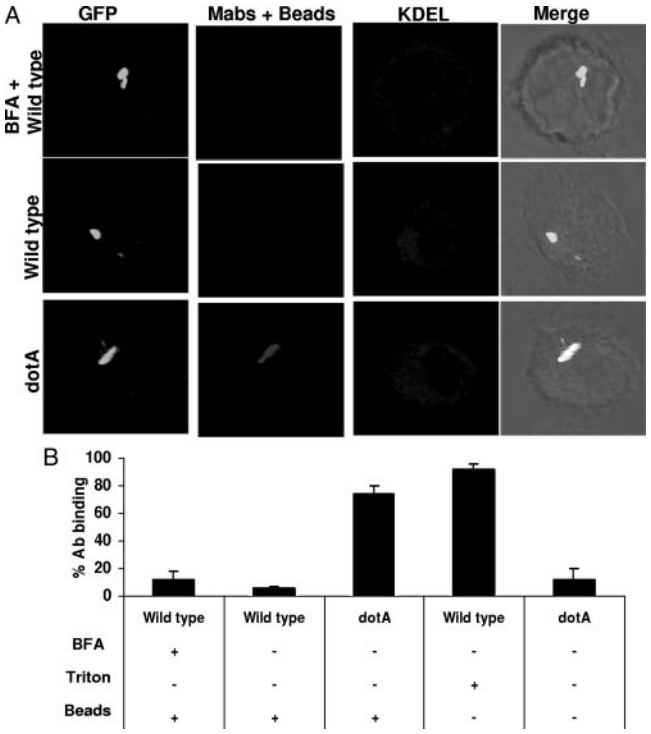


FIG. 6. Remodeling the LCPs of the wild-type strain by the ER does not contribute to the integrity of the LCPs. (A) Representative confocal microscopy images at 1 h after infection of U937 cells by the wild-type strain and the *dotA* mutant in untreated or BFA-treated cells. Phagosome integrity was determined using the glass bead permeabilization technique to load the macrophage cytosol with antibacterial MAbs, as described in the legend to Fig. 5. Note that in BFA-treated cells, the wild-type strain did not acquire the ER-derived marker KDEL. The images are stacks of 12 to 15 1-μm-thick *z* series of sections. The integrity of the LCPs was determined by the inability of the antibodies to bind the bacteria. (B) Quantitative analyses. For the negative control, the cells were incubated with MAb without glass beads, and for the positive control, the cells were permeabilized by Triton X-100. GFP, green fluorescent protein.

the wild-type bacteria were contained in intact phagosomes in both treated and untreated cells, in contrast to the *dotA* mutant (Fig. 6). We conclude that interception of ER-to-Golgi vesicle traffic by the wild-type strain LCP does not play a role in maintaining the integrity of the phagosomal membrane. Therefore, it is unlikely that the inability of the *dot/icm* structural mutants to intercept ER-to-Golgi vesicle traffic accounts for their inability to maintain the integrity of their phagosomes.

***dotA* mutant phagosomes do not colocalize with lysosomes.** Whether the *dot/icm* mutants' phagosomes colocalize with lysosomal enzymes is controversial. Therefore, we reexamined colocalization of the *dot/icm* mutant LCPs with lysosomes at the ultrastructural level, which is the most convincing strategy due to the high resolution. Lysosomes of hMDMs were preloaded with BSA-gold particles, followed by a chase prior to infection. The hMDMs were infected for 1 h with the *dotA* mutant, the complemented *dotA* mutant, the wild-type strain, or live *E. coli*. The data showed that 96% of the *dotA* mutant bacteria were cytoplasmic. The data also showed that ~80% of the *dotA* mutant bacteria did not colocalize with lysosomal contents (Fig. 6), despite the presence of abundant BSA-gold particles within lysosomes in the cells (Fig. 7, inset), consistent with previous observations (43). Approximately 80% of the complemented *dotA* mutants were in intact phagosomes, and only 9% of them contained gold particles (Fig. 7). Approximately 90% of the *E. coli*-containing phagosomes were intact, and ~70% were fused to lysosomes (Fig. 8). Similarly, 90% of the wild-type strain LCPs were intact, and only 8% contained BSA-gold particles, which was significantly different from the results for the *dotA* mutant ($P < 0.001$, as determined by the Student *t* test) (Fig. 7). Fusion of lysosomal vesicles to the *E. coli* phagosomes evident by physical contact and membrane fusions were repeatedly seen, but they were rarely seen for the *dotA* mutant, consistent with the lack of a contiguous phagosomal membrane.

Similar results were obtained with U937 macrophages in which lysosomes were preloaded with BSA-gold particles (Fig. 8). The data showed that 92% of the *dotA* mutant bacteria were cytoplasmic, and ~60% did not colocalize with BSA-gold particles (Fig. 8). For the FKL- and *E. coli*-containing phagosomes, ~90% were intact and the majority contained gold particles (Fig. 8). Approximately 70% of the complemented *dotA* mutants were in intact phagosomes, and only 1 to 2% of the phagosomes contained gold particles (Fig. 8). The wild-type strain-containing phagosomes were intact, and very few of them colocalized with BSA-gold particles (Fig. 8). Taken together, our results obtained with U937 macrophages and hMDMs showed that the majority of the *dotA* mutant phagosomes do not acquire lysosomal enzymes.

DISCUSSION

Trafficking of the *dot/icm* mutants is believed to follow the "default" endosomal-lysosomal pathway similar to that of inert particles or nonpathogens (63), which involves progressive stepwise maturation and fusions of the phagosome with endocytic and lysosomal vesicles. Here we show that mutants defective in structural components of the Dot/Icm transporter do not maintain the integrity of their phagosomes within a few minutes after entry into mammalian and protozoan cells. Some ultrastructural images show a clear zone or halo around the *dot/icm* mutants located in the cytoplasm, which may indicate that they are in a vesicle. This is very similar to the presence of a clear zone around *F. tularensis* cells after they escape into the cytoplasm (66, 67), as well as cells of the *sifA* mutant of *Salmonella* after they escape into the cytoplasm (20, 21, 25). Our observations of disruption of the *dot/icm* mutants' phagosomes are based on not one but four independent strategies (ultrastructural examination with enhancement of staining of membrane proteins, pulse-chase of CM-Dil-labeled membranes, colocalization with actin, and loading the cytosol of live cells with antibacterial antibodies). Although each of the strategies may have some caveats, the results of the combination of the four independent strategies show consistently that the *dot/icm* structural mutants are located in the host cell cytosol, while other mutants or controls that are trafficked within the endosomal lysosomal pathway are located within intact phagosomes.

Many studies have shown variable results for colocalization of the *dotA* mutant with lysosomal markers, ranging from about 20% with ferric oxide, cathepsin D, and Trov to 95%

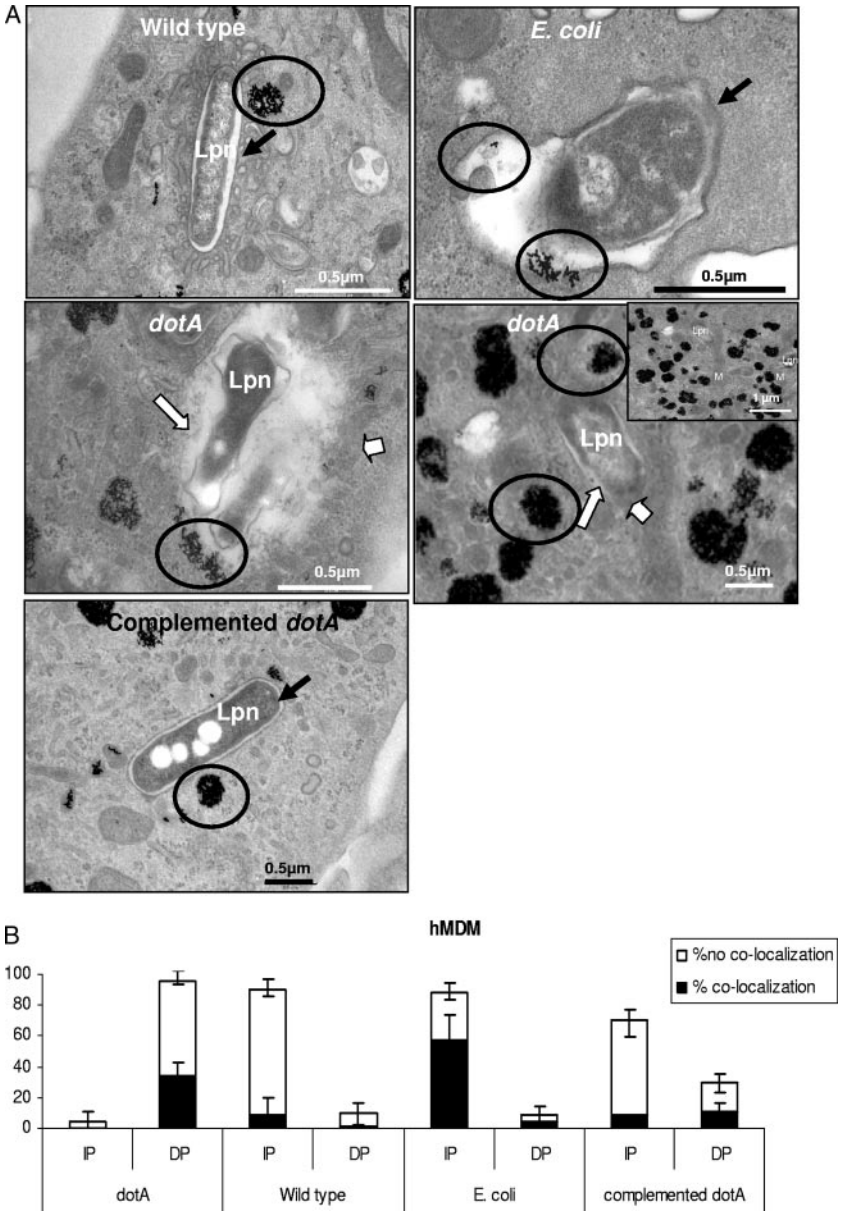


FIG. 7. Lysosomal vesicles of hMDMs preloaded with BSA-gold are in the vicinity of the *dotA* mutant in the cytoplasm at 1 h postinfection. (A) Representative images after infection by the different strains. Lysosomes of hMDMs were preloaded with BSA-gold particles and chased prior to infection by the different strains. (B) Quantitative analysis of the presence gold particles in the vicinity of the bacteria. The white arrows show sites of disruption of the phagosomal membrane. The black arrows indicate sites where the phagosomal membrane is intact. Lpn, *L. pneumophila*; A, amorphous material; V, vesicle; M, mitochondrion; IP, intact phagosomes; DP, disrupted phagosomes. The ellipses indicate areas where gold particles are present.

with thorium dioxide in both mice and human macrophages (18, 19, 43, 50, 55, 68). Joshi et al. reported exclusion of lysosomal markers from the *dotA* mutant phagosomes (43). In addition, only 37% of Lamp-1-positive *dot*/*icm* mutant LCPs are *rab7* positive in mouse macrophages (63). However, if the *dot*/*icm* mutants are not surrounded by a limiting membrane, how would they colocalize with endosomal-lysosomal markers? The presence of numerous host cell vesicles around the cytoplasmic *dot*/*icm* structural mutants may explain colocalization of the mutants with endosomal markers when preparations are examined by confocal microscopy, which has limited resolution power. Our ultrastructural data show that the majority of the *dotA* mutant bacteria, which are free in the cytoplasm and are surrounded by host cell vesicles, do not colocalize with lysosomal contents, consistent with many previous observations (18, 19, 43, 50, 55, 63, 68, 71, 75). Interestingly, it has been shown that lysosomes are recruited at the site of membrane injuries to repair the damaged membrane (65). It is not known whether the presence of lysosomes in the vicinity of the *dotA* mutant is due to an active mechanism of recruitment of the lysosomal vesicles in an attempt to repair the disruption of the phagosomal membrane.

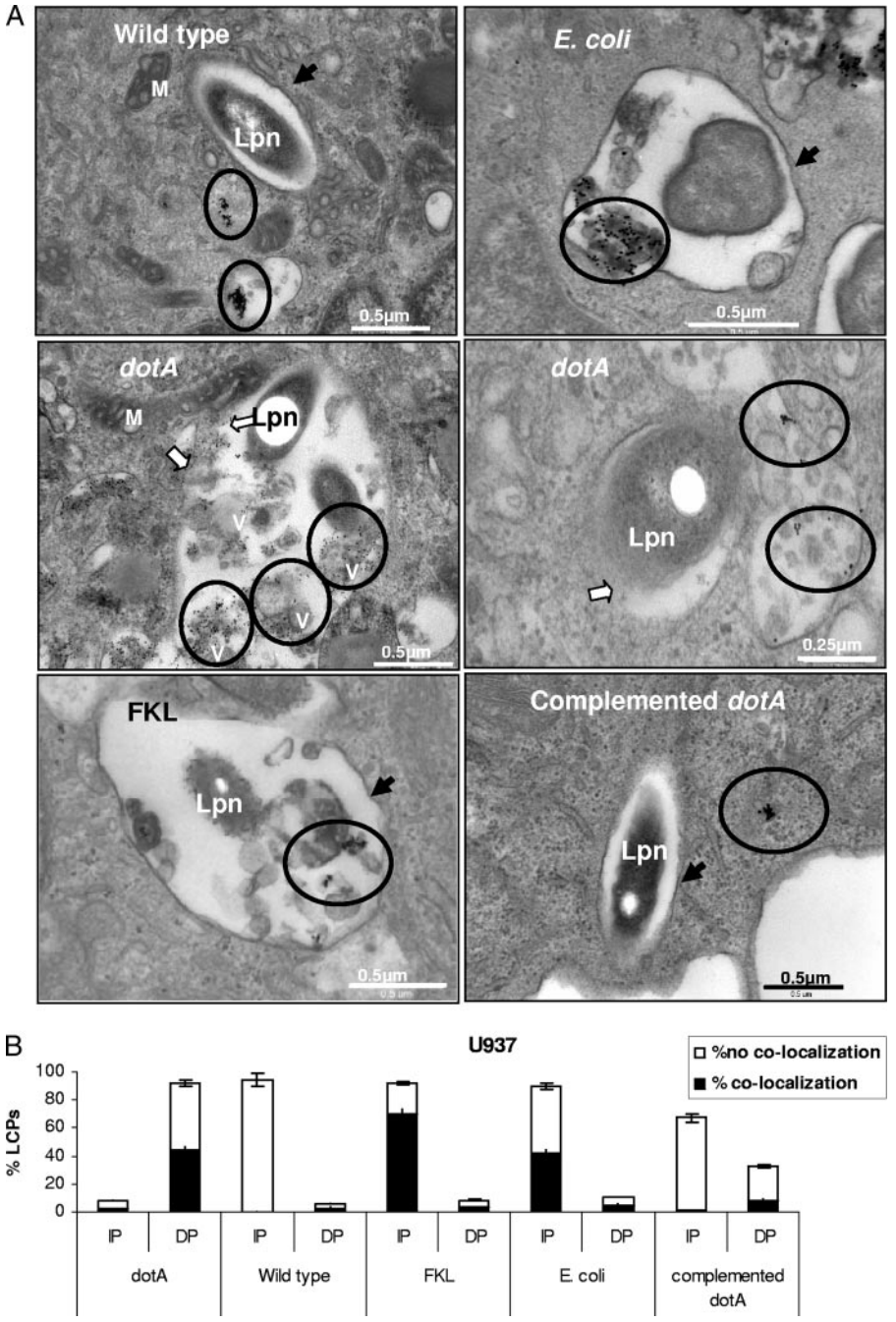


FIG. 8. Lysosomal vesicles of U937 macrophages preloaded with BSA-gold are in the vicinity of the *dotA* mutant in the cytoplasm at 1 h postinfection. (A) Representative images after infection by the different strains. Lysosomes of U937 macrophages were preloaded with BSA-gold particles and chased prior to infection by the different strains. (B) Quantitative analysis of the presence of gold particles in the vicinity of the bacteria. The white arrows indicate sites of disruption of the phagosomal membrane. The black arrows indicate sites where the phagosomal membrane is intact. Lpn, *L. pneumophila*; A, amorphous material; V, vesicle; M, mitochondrion; IP, intact phagosomes; DP, disrupted phagosomes. The ellipses indicate areas where gold particles are present.

The IcmS, IcmR, and IcmW proteins are nonstructural proteins involved in export of specific Dot/Icm effectors (14, 58). The *icmS*, *icmR*, *icmW*, and *icmQ* mutants are all defective in modulation of biogenesis of their phagosomes into an ER-derived compartment resistant to endocytic fusion, similar to *dot/icm* structural mutants (26). Our data show that in contrast to the *dot/icm* structural mutants, the *icmS*, *icmR*, *icmW*, and *icmQ* mutants have an intact phagosomal membrane. In addition, mutants with a defect in the RpoS, LetA/S, and LetE regulatory proteins that are required for intracellular replication in certain host cells (3, 11–13, 39) all have intact phagosomes in the host cells in which they fail to replicate. We also show that the type II secretion enzymes are not involved in disruption of the LCP membrane. Taken together, these data

confirm that only the Dot/Icm structural apparatus proteins are essential for maintaining phagosomal integrity.

Our findings may elucidate three unexplained observations about the intracellular fate of *L. pneumophila*. First, despite the presumed lysosomal localization of the *dot*/*icm* mutants determined in some studies, the mutants are not killed within macrophages, indicating that they are not in degradative phagolysosomes, which is consistent with previous observations (43, 75). Second, although the *dot*/*icm* mutant phagosome is thought to mature through the endosomal-lysosomal pathway, it is not accessible to endocytosed materials (43, 75), which is inconsistent with the presumed trafficking of the *dot*/*icm* mutants through the endocytic pathway. Third, the *dotA* mutant phagosomes are very difficult to isolate compared to the wild-type strain phagosomes (28; Isberg, personal communication). These observations can be explained by our finding that the *dot*/*icm* structural mutants are not trafficked through the endosomal-lysosomal pathway but rather are free in the cytoplasm due to their failure to maintain the integrity of their phagosomes.

The ability of the LCP to evade endocytic fusion is mediated by a *cis*-acting signal transmitted to the LCP to block its endocytic fusion, while other endocytic fusion events in the host cell continue to be functional (27). Importantly, coinfection of *dot*/*icm* mutants with the wild-type strain results in rescue of the *dot*/*icm* mutants for intracellular replication when they inhabit a communal phagosome with the wild-type strain (27). Our data indicate that the ability of the wild-type strain to maintain the integrity of the LCP is most likely a factor contributing to the rescue of the *dot*/*icm* mutants.

Goetz et al. have shown that upon microinjection of wild-type *L. pneumophila* into the cytoplasm of epithelial-type Caco-2 cells, the bacteria are unable to replicate in the cytoplasm (37). This can be due to one or two reasons; first, *Legionella* primarily infects alveolar macrophages in humans and animal models, and *L. pneumophila* is likely to behave differently in Caco-2 cells than in macrophages (35, 57). Therefore, it may be premature to extrapolate data from macrophages to Caco-2 cells. However, since the *L. pneumophila* wild type is unable to replicate within the cytoplasm of mammalian cells, it may not be surprising that the *dot*/*icm* mutants also fail to replicate.

The SifA protein, which is a translocated effector of the SPI2 secretion system of *Salmonella enterica* serovar Typhimurium, promotes the integrity of the *Salmonella*-containing vacuoles (21). The *sifA* mutants loose their surrounding *Salmonella*-containing vacuole membrane within murine macrophages and are released into the cytoplasm, where they are unable to replicate (20, 21, 25). Interestingly, the loss of phagosomal integrity of the *sifA* mutant is associated with overactivity of the molecular motor kinesin, and inhibition of dynein activity restores the integrity of the *sifA* mutant-containing vacuole (24, 38). It is possible that an effector(s) exported by the Dot/Icm secretion apparatus plays a role similar to that of the SifA protein to maintain the integrity of the phagosomal membrane. However, in silico analysis showed no similarity in the *Legionella* genome to the SifA protein or any of its domains. Since our data show that interception of ER-derived vesicles is not involved in maintaining the integrity of the wild-type strain phagosomes, it may be unlikely that a Dot/Icm type IV effector interacts with the microtubules to intercept ER-to-Golgi vesicle traffic.

In summary, we have shown that the *dot*/*icm* structural mutants are unable to maintain the integrity of their phagosomes and escape into the cytoplasm within a few minutes after entry into mammalian and protozoan cells. We have shown that the IcmS, IcmR, IcmW, and IcmQ nonstructural proteins are not required for phagosome integrity, nor are the stationary-phase regulators or the lytic enzymes secreted through the type II secretion system. We have also shown that interception of ER-to-Golgi vesicle traffic by the wild-type LCPs is not involved in maintaining the integrity of the phagosomal membrane of the wild-type strain. Our data show that the *dotA* mutant cytosolic bacteria do not colocalize with lysosomal enzymes. Our results show that the type IV secretion system is essential for maintaining the integrity of the LCPs, which is the first example of a role for a type IV secretion apparatus in maintaining the integrity of a bacterial phagosome. Our data caution that confocal microscopy studies on trafficking of the type IV secretion system mutants of other intracellular pathogens, such as *Coxiella*, *Brucella*, and *Bartonella*, should be supported by studies on the integrity of the phagosome.

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REFERENCES

1. **Abu Kwaik, Y.** 1996. The phagosome containing *Legionella pneumophila* within the protozoan *Hartmanella vermiformis* is surrounded by the rough endoplasmic reticulum. Appl. Environ. Microbiol. **62:**2022–2028.
2. **Abu Kwaik, Y., L.-Y. Gao, B. J. Stone, C. Venkataraman, and O. S. Harb.** 1998. Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis. Appl. Environ. Microbiol. **64:**3127–3133.
3. **Abu-Zant, A., R. Asare, J. E. Graham, and Y. Abu Kwaik.** 2006. Role for RpoS but not RelA of *Legionella pneumophila* in modulation of phagosome biogenesis and adaptation to the phagosomal microenvironment. Infect. Immun. **74:**3021–3026.
4. **Abu-Zant, A., S. Jones, R. Asare, J. Suttles, C. Price, J. Graham, and Y. A. Kwaik.** 2007. Anti-apoptotic signalling by the Dot/Icm secretion system of L. pneumophila. Cell. Microbiol. **9:**246–264.
5. **Abu-Zant, A., M. Santic, M. Molmeret, S. Jones, J. Helbig, and Y. Abu Kwaik.** 2005. Incomplete activation of macrophage apoptosis during intracellular replication of *Legionella pneumophila*. Infect. Immun. **73:**5339–5349.
6. **Aragon, V., S. Kurtz, and N. P. Cianciotto.** 2001. *Legionella pneumophila* major acid phosphatase and its role in intracellular infection. Infect. Immun. **69:**177–185.
7. **Aragon, V., S. Kurtz, A. Flieger, B. Neumeister, and N. P. Cianciotto.** 2000. Secreted enzymatic activities of wild-type and *pilD*-deficient *Legionella pneumophila*. Infect. Immun. **68:**1855–1863.
8. **Aragon, V., O. Rossier, and N. P. Cianciotto.** 2002. *Legionella pneumophila* genes that encode lipase and phospholipase C activities. Microbiology **148:** 2223–2231.
9. **Asare, R., and Y. Abu Kwaik.** 15 February 2007. Early trafficking and intracellular replication of Legionella longbeachaea within an ER-derived late endosome-like phagosome. Cell. Microbiol. doi:10.1111/j.1462-5822.2007.00894.x.
10. **Asare, R., M. Santic, I. Gobin, M. Doric, J. Suttles, J. E. Graham, C. D. Price, and Y. Abu Kwaik.** 2007. Genetic susceptibility and caspase activation in mouse and human macrophages are distinct for *Legionella longbeachae* and *L. pneumophila*. Infect. Immun. **75:**1933–1945.
11. **Bachman, M. A., and M. S. Swanson.** 2004. Genetic evidence that *Legionella pneumophila* RpoS modulates expression of the transmission phenotype in both the exponential phase and the stationary phase. Infect. Immun. **72:** 2468–2476.

12. **Bachman, M. A., and M. S. Swanson.** 2004. The LetE protein enhances expression of multiple LetA/LetS-dependent transmission traits by *Legionella pneumophila*. Infect. Immun. **72:**3284–3293.
13. **Bachman, M. A., and M. S. Swanson.** 2001. RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. Mol. Microbiol. **40:**1201–1214.
14. **Bardill, J. P., J. L. Miller, and J. P. Vogel.** 2005. IcmS-dependent translocation of SdeA into macrophages by the *Legionella pneumophila* type IV secretion system. Mol. Microbiol. **56:**90–103.
15. **Baskerville, A., A. B. Dowsett, R. B. Fitzgeorge, P. Hambleton, and M. Broster.** 1983. Ultrastructure of pulmonary alveoli and macrophages in experimental Legionnaires' disease. J. Pathol. **140:**77–90.
16. **Baskerville, A., R. B. Fitzgeorge, M. Broster, P. Hambleton, and P. J. Dennis.** 1981. Experimental transmission of legionnaires' disease by exposure to aerosols of *Legionella pneumophila*. Lancet **ii:**1389–1390.
17. **Becker, T., A. Volchuk, and J. E. Rothman.** 2005. Differential use of endoplasmic reticulum membrane for phagocytosis in J774 macrophages. Proc. Natl. Acad. Sci. USA **102:**4022–4026.
18. **Berger, K. H., and R. R. Isberg.** 1993. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. Mol. Microbiol. **7:**7–19.
19. **Berger, K. H., J. Merriam, and R. R. Isberg.** 1994. Altered intracellular targeting properties associated with mutations in the *Legionella pneumophila dotA* gene. Mol. Microbiol. **14:**809–822.
20. **Beuzon, C. R., S. Meresse, K. E. Unsworth, J. Ruiz-Albert, S. Garvis, S. R. Waterman, T. A. Ryder, E. Boucrot, and D. W. Holden.** 2000. *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. EMBO J. **19:**3235–3249.
21. **Beuzon, C. R., S. P. Salcedo, and D. W. Holden.** 2002. Growth and killing of a *Salmonella enterica* serovar *Typhimurium sifA* mutant strain in the cytosol of different host cell lines. Microbiology **148:**2705–2715.
22. **Bitar, D. M., M. Molmeret, and Y. Abu Kwaik.** 2004. Molecular and cell biology of *Legionella pneumophila*. Int. J. Med. Microbiol. **293:**519–527.
23. **Boleti, H., D. M. Ojcius, and A. Dautry-Varsat.** 2000. Fluorescent labelling of intracellular bacteria in living host cells. J. Microbiol. Methods **40:**265–274.
24. **Boucrot, E., T. Henry, J. P. Borg, J. P. Gorvel, and S. Meresse.** 2005. The intracellular fate of *Salmonella* depends on the recruitment of kinesin. Science **308:**1174–1178.
25. **Brumell, J. H., P. Tang, M. L. Zaharik, and B. B. Finlay.** 2002. Disruption of the *Salmonella*-containing vacuole leads to increased replication of *Salmonella enterica* serovar Typhimurium in the cytosol of epithelial cells. Infect. Immun. **70:**3264–3270.
26. **Coers, J., J. C. Kagan, M. Matthews, H. Nagai, D. M. Zuckman, and C. R. Roy.** 2000. Identification of icm protein complexes that play distinct roles in the biogenesis of an organelle permissive for *Legionella pneumophila* intracellular growth. Mol. Microbiol. **38:**719–736.
27. **Coers, J., C. Monahan, and C. R. Roy.** 1999. Modulation of phagosome biogenesis by *Legionella pneumophila* creates an organelle permissive for intracellular growth. Nat. Cell Biol. **1:**451–453.
28. **Conover, G. M., I. Derre, J. P. Vogel, and R. R. Isberg.** 2003. The *Legionella pneumophila* LidA protein: a translocated substrate of the Dot/Icm system associated with maintenance of bacterial integrity. Mol. Microbiol. **48:**305–321.
29. **Derre, I., and R. R. Isberg.** 2004. *Legionella pneumophila* replication vacuole formation involves rapid recruitment of proteins of the early secretory system. Infect. Immun. **72:**3048–3053.
30. **Flieger, A., S. Gong, M. Faigle, M. Deeg, P. Bartmann, and B. Neumeister.** 2000. Novel phospholipase A activity secreted by *Legionella* species. J. Bacteriol. **182:**1321–1327.
31. **Flieger, A., S. Gong, M. Faigle, S. Stevanovic, N. P. Cianciotto, and B. Neumeister.** 2001. Novel lysophospholipase A secreted by *Legionella pneumophila*. J. Bacteriol. **183:**2121–2124.
32. **Flieger, A., B. Neumeister, and N. P. Cianciotto.** 2002. Characterization of the gene encoding the major secreted lysophospholipase A of *Legionella pneumophila* and its role in detoxification of lysophosphatidylcholine. Infect. Immun. **70:**6094–6106.
33. **Gal-Mor, O., and G. Segal.** 2003. The *Legionella pneumophila* GacA homolog (LetA) is involved in the regulation of *icm* virulence genes and is required for intracellular multiplication in *Acanthamoeba castellanii*. Microb. Pathog. **34:**187–194.
34. **Gao, L.-Y., O. S. Harb, and Y. Abu Kwaik.** 1997. Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant hosts, mammalian and protozoan cells. Infect. Immun. **65:**4738–4746.
35. **Gao, L.-Y., B. J. Stone, J. K. Brieland, and Y. Abu Kwaik.** 1998. Different fates of *Legionella pneumophila pmi* and *mil* mutants within human-derived macrophages and alveolar epithelial cells. Microb. Pathog. **25:**291–306.
36. **Gao, L.-Y., M. Susa, B. Ticac, and Y. Abu Kwaik.** 1999. Heterogeneity in intracellular replication and cytopathogenicity of *Legionella pneumophila* and *Legionella micdadei* in mammalian and protozoan cells. Microb. Pathog. **27:**273–287.

36a. **Gao, L.-Y., O. S. Harb, and Y. Abu Kwaik.** 1998. Identification of macrophage-specific infectivity loci (*mil*) of *Legionella pneumophila* that are not required for infectivity of protozoa. Infect. Immun. **66:**883–892.

37. **Goetz, M., A. Bubert, G. Wang, I. Chico-Calero, J. A. Vazquez-Boland, M. Beck, J. Slaghuis, A. A. Szalay, and W. Goebel.** 2001. Microinjection and growth of bacteria in the cytosol of mammalian host cells. Proc. Natl. Acad. Sci. USA **98:**12221–12226.
38. **Guignot, J., E. Caron, C. Beuzon, C. Bucci, J. Kagan, C. Roy, and D. W. Holden.** 2004. Microtubule motors control membrane dynamics of *Salmonella*-containing vacuoles. J. Cell Sci. **117:**1033–1045.

38a. **Hales, L. M., and H. A. Shuman.** 1999. The *Legionella pneumophila rpoS* gene is required for growth within *Acanthamoeba castellanii*. J. Bacteriol. **181:**4879–4889.

39. **Hammer, B. K., E. S. Tateda, and M. S. Swanson.** 2002. A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. Mol. Microbiol. **44:**107–118.
40. **Harb, O. S., L.-Y. Gao, and Y. Abu Kwaik.** 2000. From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. Environ. Microbiol. **2:**251–265.
41. **Horwitz, M. A.** 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J. Exp. Med. **158:**1319–1331.
42. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
43. **Joshi, A. D., S. Sturgill-Koszycki, and M. S. Swanson.** 2001. Evidence that Dot-dependent and -independent factors isolate the *Legionella pneumophila* phagosome from the endocytic network in mouse macrophages. Cell. Microbiol. **3:**99–114.
44. **Kagan, J. C., and C. R. Roy.** 2002. *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. Nat. Cell Biol. **4:**945–954.
45. **Kagan, J. C., M. P. Stein, M. Pypaert, and C. R. Roy.** 2004. *Legionella* subvert the functions of Rab1 and Sec22b to create a replicative organelle. J. Exp. Med. **199:**1201–1211.
46. **Kalina, M., and D. C. Pease.** 1977. The probable role of phosphatidyl cholines in the tannic acid enhancement of cytomembrane electron contrast. J. Cell Biol. **74:**742–746.
47. **Lindmark, A., U. Gullberg, and I. Olsson.** 1994. Processing and intracellular transport of cathepsin G and neutrophil elastase in the leukemic myeloid cell line U-937—modulation by brefeldin A, ammonium chloride, and monensin. J. Leukoc. Biol. **55:**50–57.
48. **Luo, Z. Q., and R. R. Isberg.** 2004. Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. Proc. Natl. Acad. Sci. USA **101:**841–846.
49. **Lynch, D., N. Fieser, K. Gloggler, V. Forsbach-Birk, and R. Marre.** 2003. The response regulator LetA regulates the stationary-phase stress response in *Legionella pneumophila* and is required for efficient infection of *Acanthamoeba castellanii*. FEMS Microbiol. Lett. **219:**241–248.
50. **Marra, A., S. J. Blander, M. A. Horwitz, and H. A. Shuman.** 1992. Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. Proc. Natl. Acad. Sci. USA **89:**9607–9611.
51. **McNeil, P. L., and R. A. Steinhardt.** 2003. Plasma membrane disruption: repair, prevention, adaptation. Annu. Rev. Cell Dev. Biol. **19:**697–731.
52. **Molmeret, M., D. M. Bitar, L. Han, and Y. Abu Kwaik.** 2004. Cell biology of the intracellular infection by *Legionella pneumophila*. Microbes Infect. **6:**129–139.
53. **Molmeret, M., D. M. Bitar, L. Han, and Y. A. Kwaik.** 2004. Disruption of the phagosomal membrane and egress of *Legionella pneumophila* into the cytoplasm during the last stages of intracellular infection of macrophages and *Acanthamoeba polyphaga*. Infect. Immun. **72:**4040–4051.
54. **Molmeret, M., M. Horn, M. Wagner, M. Santic, and Y. Abu Kwaik.** 2005. Amoebae as training grounds for intracellular bacterial pathogens. Appl. Environ. Microbiol. **71:**20–28.
55. **Molmeret, M., S. D. Zink, L. Han, A. Abu-Zant, R. Asari, D. M. Bitar, and Y. Abu Kwaik.** 2004. Activation of caspase-3 by the Dot/Icm virulence system is essential for arrested biogenesis of the *Legionella*-containing phagosome. Cell. Microbiol. **6:**33–48.

55a. **Molmeret, M., O. A. T. Alli, M. Radulic, M. Susa, M. Doric, and Y. Abu Kwaik.** 2002. The C-terminus of IcmT is essential for pore formation and for intracellular trafficking of *Legionella pneumophila* within *Acanthamoeba polyphaga*. Mol. Microbiol. **43:**1139–1150.

56. **Nagai, H., J. C. Kagan, X. Zhu, R. A. Kahn, and C. R. Roy.** 2002. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. Science **295:**679–682.
57. **Neumeister, B., M. Faigle, K. Lauber, H. Northoff, and S. Wesselborg.** 2002. *Legionella pneumophila* induces apoptosis via the mitochondrial death pathway. Microbiology **148:**3639–3650.
58. **Ninio, S., D. M. Zuckman-Cholon, E. D. Cambronne, and C. R. Roy.** 2005. The *Legionella* IcmS-IcmW protein complex is important for Dot/Icm-mediated protein translocation. Mol. Microbiol. **55:**912–926.
59. **Pedersen, L. L., M. Radulic, M. Doric, and Y. Abu Kwaik.** 2001. HtrA homologue of *Legionella pneumophila*: an indispensable element for intra-

cellular infection of mammalian but not protozoan cells. Infect. Immun. **69:**2569–2579.
60. **Reddy, A., E. V. Caler, and N. W. Andrews.** 2001. Plasma membrane repair is mediated by Ca^{2+}-regulated exocytosis of lysosomes. Cell **106:**157–169.
61. **Roholl, P. J., W. Leene, M. L. Kapsenberg, and J. G. Vos.** 1981. The use of tannic acid fixation for the electron microscope visualization of fluorochrome-labelled antibodies attached to cell surface antigens. J. Immunol. Methods **42:**285–289.
62. **Rossier, O., and N. P. Cianciotto.** 2001. Type II protein secretion is a subset of the PilD-dependent processes that facilitate intracellular infection by *Legionella pneumophila*. Infect. Immun. **69:**2092–2098.
62a. **Rossier, O., S. R. Starkenburg, and N. P. Cianciotto.** 2004. *Legionella pneumophila* type II protein secretion promotes virulence in the A/J mouse model of Legionnaires' disease pneumonia. Infect. Immun. **72:**310–321.
63. **Roy, C. R., K. H. Berger, and R. R. Isberg.** 1998. *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol. Microbiol. **28:**663–674.
64. **Roy, C. R., and L. G. Tilney.** 2002. The road less traveled: transport of *Legionella* to the endoplasmic reticulum. J. Cell Biol. **158:**415–419.
65. **Roy, D., D. R. Liston, V. J. Idone, A. Di, D. J. Nelson, C. Pujol, J. B. Bliska, S. Chakrabarti, and N. W. Andrews.** 2004. A process for controlling intracellular bacterial infections induced by membrane injury. Science **304:**1515–1518.
66. **Santic, M., M. Molmeret, and Y. Abu Kwaik.** 2005. The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell. Microbiol. **7:**969–979.
67. **Santic, M., M. Molmeret, and Y. Abu Kwaik.** 2005. Modulation of the biogenesis of the *Francisella tularensis* subsp. *novicida*-containing phagosome in quiescent human macrophages and its maturation into a phagolysosome upon activation by IFN-gamma. Cell. Microbiol. **7:**957–967.
68. **Santic, M., M. Molmeret, and Y. Abu Kwaik.** 2005. Maturation of the *Legionella pneumophila*-containing phagosome into a phagolysosome within gamma interferon-activated macrophages. Infect. Immun. **73:**3166–3171.
69. **Segal, G., M. Purcell, and H. A. Shuman.** 1998. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* chromosome. Proc. Natl. Acad. Sci. USA **95:**1669–1674.
70. **Segal, G., and H. A. Shuman.** 1999. *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. Infect. Immun. **67:**2117–2124.
71. **Swanson, M. S., and R. R. Isberg.** 1996. Identification of *Legionella pneumophila* mutants that have aberrant intracellular fates. Infect. Immun. **64:**2585–2594.
72. **Tilney, L. G., O. S. Harb, P. S. Connelly, C. G. Robinson, and C. R. Roy.** 2001. How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. J. Cell Sci. **114:**4637–4650.
73. **Vago, R., C. J. Marsden, J. M. Lord, R. Ippoliti, D. J. Flavell, S. U. Flavell, A. Ceriotti, and M. S. Fabbrini.** 2005. Saporin and ricin A chain follow different intracellular routes to enter the cytosol of intoxicated cells. FEBS J. **272:**4983–4995.
74. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:**873–876.
75. **Watarai, M., I. Derre, J. Kirby, J. D. Growney, W. F. Dietrich, and R. R. Isberg.** 2001. *Legionella pneumophila* is internalized by a macropinocytotic uptake pathway controlled by the Dot/Icm system and the mouse *Lgn1* locus. J. Exp. Med. **194:**1081–1096.
76. **Wiater, L. A., K. Dunn, F. R. Maxfield, and H. A. Shuman.** 1998. Early events in phagosome establishment are required for intracellular survival of *Legionella pneumophila*. Infect. Immun. **66:**4450–4460.
77. **Zink, S. D., L. Pedersen, N. P. Cianciotto, and Y. Abu-Kwaik.** 2002. The Dot/Icm type IV secretion system of *Legionella pneumophila* is essential for the induction of apoptosis in human macrophages. Infect. Immun. **70:**1657–1663.

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