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Evidence that Dot-dependent and -independent factors isolate the *Legionella pneumophila* phagosome from the endocytic network in mouse macrophages

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Summary

***Legionella pneumophila* survives within macrophages by evading phagosome–lysosome fusion. To determine whether *L. pneumophila* resides in an intermediate endosomal compartment or is isolated from the endosomal pathway and to investigate what bacterial factors contribute to establishment of its vacuole, we applied a series of fluorescence microscopy assays. The majority of vacuoles, aged 2.5 min to 4 h containing post-exponential phase (PE) *L. pneumophila*, appeared to be separate from the endosomal pathway, as judged by the absence of transferrin receptor, LAMP-1, cathepsin D and each of four fluorescent probes used to label the endocytic pathway either before or after infection. In contrast, more than 70% of phagosomes that contained *Escherichia coli*, polystyrene beads, or exponential phase (E) *L. pneumophila* matured to phagolysosomes, as judged by co-localization with LAMP-1, cathepsin D and fluorescent endosomal probes. Surprisingly, neither bacterial viability nor the putative Dot/Icm transport complex was absolutely required for vacuole isolation; although phagosomes containing either formalin-killed PE wild-type or live PE *dotA* or *dotB* mutant *L. pneumophila* rapidly accumulated LAMP-1, less than 20% acquired lysosomal cathepsin D or fluorescent endosomal probes. Therefore, a Dot-dependent factor(s) isolates the *L. pneumophila* phagosome from a LAMP-1-containing compartment, and a formalin-resistant Dot-independent activity inhibits vacuolar accumulation of endocytosed material and delivery to the degradative lysosomes.**

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Introduction

Intracellular pathogens have evolved a variety of strategies to survive and replicate inside host cells. For example, *Toxoplasma* enters a phagosome that is completely removed from the endocytic network (Jones and Hirsch, 1972; Mordue and Sibley, 1997; Mordue *et al*., 1999b), whereas *Listeria*, *Shigella* and *Trypanosoma* each lyse their phagosomal membranes and replicate in the cytoplasm (Nogueira and Cohn, 1976; Finlay and Falkow, 1997). Phagosomes harbouring *Mycobacterium* do not merge with degradative lysosomes, but instead resemble a recycling/sorting early endosome (Sturgill-Koszycki *et al*., 1994, 1996; Clemens and Horwitz, 1995, 1996; Russell *et al*., 1996). In contrast, *Coxiella burnetti* and *Leishmania* thrive in the acidic, lysosomal compartment (Chang and Dwyer, 1976; Burton *et al*., 1978; Akporiaye *et al*., 1983; Heinzen *et al*., 1996).

Legionella pneumophila, the causative agent of the severe pneumonia Legionnaires' disease, replicates in macrophages within an unusual vacuole. After uptake, *L. pneumophila* resides in a phagosome that neither acidifies nor fuses with lysosomes (Horwitz, 1983a; Horwitz and Maxfield, 1984). Instead, the vacuole associates sequentially with smooth vesicles, mitochondria and endoplasmic reticulum (Horwitz, 1983b; Swanson and Isberg, 1995). After a 4–6 h lag period, the bacteria replicate for approximately 20 h before the host cell lyses, releasing a large number of pathogens.

The strategy used by *L. pneumophila* to evade fusion with macrophage lysosomes and establish a productive replication niche has not been discerned. Absence of fusion between lysosomes and *L. pneumophila* vacuoles aged 1–8 h has been documented clearly by a variety of microscopic techniques (Horwitz, 1983a; Berger and Isberg, 1993; Clemens and Horwitz, 1995; Swanson and Isberg, 1996). Similarly, the late endosomal proteins LAMP-1 and Rab7 are absent from *L. pneumophila* phagosomes of ages 5–150 min (Swanson and Isberg, 1996; Roy *et al*., 1998). Whether the early endosomal compartment interacts with this pathogenic vacuole has not been studied as extensively. Clemens and Horwitz (1992) have reported that, 3.5 min after formation, nascent *L. pneumophila* vacuoles are devoid of three proteins normally present on the plasma membrane and

early endosomes: alkaline phosphatase and MHC class I and MHC class II molecules. Yet, the phagosomal membrane retains two other plasma membrane proteins, complement receptor CR3 and 5′-nucleotidase. Thus, during phagocytosis of *L. pneumophila*, certain membrane proteins appear to sort rapidly such that the membranes which surround the bacterium are markedly different from the plasma membrane (Clemens and Horwitz, 1992). Therefore, *L. pneumophila* may resemble *Toxoplasma*, which establishes a vacuole that is completely separate from the endocytic pathway and whose membrane excludes particular classes of membrane proteins (Jones and Hirsch, 1972; Mordue and Sibley, 1997; Mordue *et al*., 1999a, b). Alternatively, *L. pneumophila* may be more similar to *Mycobacterium*, which persists within an early endosomal compartment that fails to mature (Sturgill-Koszycki *et al*., 1994, 1996; Clemens and Horwitz, 1995, 1996; Russell *et al*., 1996).

One mechanism that has been proposed for establishment of the unique *L. pneumophila* replication vacuole involves the Dot/Icm secretion system. More than 20 *dot* (defect in organelle trafficking) and *icm* (intracellular multiplication) genes are required by *L. pneumophila* both for growth in macrophages and for conjugal DNA transfer (Segal and Shuman, 1998; Segal *et al*., 1998; Vogel *et al*., 1998). A subset of these genes is similar to those encoding specialized secretion systems important for virulence of other pathogenic bacteria, namely the *Agrobacterium tumafaciens vir* complex (Christie, 1997), the *Bordetella pertussis ptl* system (Weiss *et al*., 1993) and the *Helicobacter pylori cag* complex (Censini *et al*., 1996). In phagocytes, *dot and icm* mutants fail to evade the endocytic pathway, as judged by their co-localization with endocytosed electron-dense colloids (Horwitz, 1987; Marra *et al*., 1992; Berger and Isberg, 1993), or with fluorescent probes (Swanson and Isberg, 1996; Wiater *et al*., 1998) or by the presence of the early and late endosomal protein Rab7 (Roy *et al*., 1998) or the late endosomal and lysosomal protein LAMP-1 (Swanson and Isberg, 1996; Andrews *et al*., 1998; Roy *et al*., 1998; Vogel *et al*., 1998; Zuckman *et al*., 1999). Accordingly, the Dot/Icm secretion system has been proposed to export effector molecules which act during phagosome biogenesis to prevent its fusion with lysosomes (Segal *et al*., 1998; Vogel *et al*., 1998).

The Dot/Icm transport system appears to be required for establishment of the replication vacuole, but not its maintenance. *L. pneumophila* that express *dotA* before infection, but not after, still replicate during the primary infection cycle (Roy *et al*., 1998). Furthermore, the intracellular growth defect of *dotA L. pneumophila* is complemented when a Dot/Icm-expressing, replication-defective auxotroph is present in the same phagosome (Coers *et al*., 1999). Also consistent with this hypothesis is the observation that exponential phase (E) *L. pneumophila* do not express a number of virulence traits, including evasion of phagosome–lysosome fusion (Byrne and Swanson, 1998). By this model, once a protected niche is established within a new host, virulent post-exponential phase (PE) *L. pneumophila* converts to a replicative form that downregulates virulence expression (Byrne and Swanson, 1998; Hammer and Swanson, 1999) and does not require *dotA* function (Coers *et al*., 1999).

Here, we sought to determine whether phagosomes harbouring *L. pneumophila* are isolated from the endocytic pathway or are stalled at an early stage of the endosome maturation pathway. We also investigated the effect of *L. pneumophila* viability, growth phase and Dot/Icm function on the biogenesis of its protective vacuole. Using a series of quantitative fluorescence microscopic assays, we compared the composition of phagosomes harbouring virulent *L. pneumophila* to those containing one of several different non-pathogenic particles as the vacuoles matured. Our data indicate that, during the first 4 h of infection, *L. pneumophila* resides in a vacuole that is isolated from the endocytic network. Surprisingly, the capacity to establish a vacuole that for several hours does not accumulate endocytic molecules nor fuse with degradative lysosomes was not strictly dependent on either *L. pneumophila* viability or Dot/Icm function.

Results

Phagosome acquisition of LAMP-1, a marker for late endosomes and lysosomes

LAMP-1 is an abundant membrane glycoprotein found primarily in late endosomes and lysosomes that has been proposed to protect these membranes from the acidic, proteolytic vacuole contents (Chen *et al*., 1988; Rabinowitz *et al*., 1992; Hunziker and Geuze, 1996). LAMP-1 acquisition by macropinosomes (Racoosin and Swanson, 1993) and by phagosomes containing killed *Staphylococcus aureus* or latex beads (Pitt *et al*., 1992; Rabinowitz *et al*., 1992; Desjardins *et al*., 1994) has been to used indicate fusion with late endosomes and lysosomes. In contrast, *L. pneumophila*-containing phagosomes aged 5–150 min do not acquire LAMP-1 (Swanson and Isberg, 1996; Roy *et al*., 1998). Therefore, we chose LAMP-1 as a marker to establish the kinetics of maturation of phagosomes containing either avirulent particles or virulent *L. pneumophila.*

Macrophages derived from the bone marrow of A/J mice were allowed to phagocytose particles for 5 min, the cells were incubated for an additional 0–60 min, then phagosome acquisition of LAMP-1 was analysed by immunofluorescence microscopy (Fig. 1). As expected (Byrne and Swanson, 1998), *L. pneumophila* obtained from E cultures, like avirulent particles, were delivered

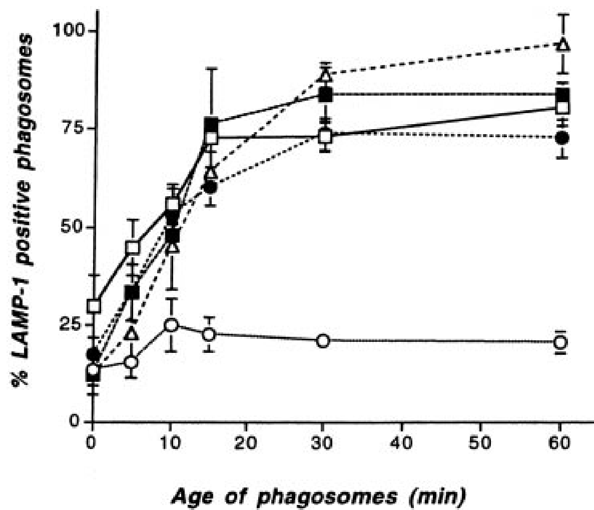

Fig. 1. Acquisition of LAMP-1 by phagosomes containing *L. pneumophila*. Macrophage phagosomes aged 0–60 min were analysed by immunofluorescence microscopy using LAMP-1-specific antibody as described in the *Experimental procedures*. Within 15 min of formation, phagosomes containing formalin-killed *E. coli* (open triangles), live (open squares) or formalin-killed E *L. pneumophila* (solid squares), or formalin-killed PE *L. pneumophila* (solid circles) acquired LAMP-1, whereas live PE *L. pneumophila* (open circles) did not. Shown are means and standard errors of the means determined for a minimum of 50 phagosomes in each of at least four separate experiments.

efficiently to the late endosomal compartment; within 15 min of phagocytosis, LAMP-1 co-localized with more than 60% of the phagosomes that contained *E. coli*, E *L. pneumophila* or E and PE *L. pneumophila* that had been killed with formalin. In contrast, more than 75% of phagosomes containing viable PE *L. pneumophila* did not acquire LAMP-1 within 5–65 min of formation, consistent with previous studies (Swanson and Isberg, 1996; Andrews *et al*., 1998; Roy *et al*., 1998; Vogel *et al*., 1998). Thus, when in the PE, viable *L. pneumophila* express factors which prevent phagosomal accumulation of LAMP-1, a late endosomal and lysosomal protein.

Phagosome acquisition of cathepsin D, a lysosomal protease

To measure the kinetics of phagolysosome formation, we analysed vacuole acquisition of cathepsin D, an acid protease that is predominantly lysosomal (Godbold *et al*., 1998). Macrophages were incubated for 5 min with live or formalin-treated E or PE *L. pneumophila,* then incubated for an additional 0–240 min before analysis by immunofluorescence microscopy (Fig. 2). Consistent with results obtained previously by the cryosection immunogold technique (Clemens and Horwitz, 1995), PE *L. pneumophila* did not co-localize with cathepsin D; by 4 h after infection, more than 75% of phagosomes bearing either viable or formalin-killed E *L. pneumophila* contained cathepsin D, whereas less than 10% of the phagosomes containing live PE bacteria did so. Surprisingly, PE *L. pneumophila* which had been killed by formalin retained the ability to evade lysosomes; even after 4 h, less than 20% of these vacuoles contained detectable cathepsin D. Therefore, based upon localization of late endosomal and lysosomal macrophage proteins, PE *L. pneumophila* appeared to express two distinguishable attributes that retard phagolysosome formation: a formalin-sensitive activity prevents accumulation of LAMP-1 and a formalin-resistant property blocks acquisition of cathepsin D.

The Dot/Icm secretion system of *L. pneumophila* is thought to export effector molecules that prevent phagosome–lysosome fusion (Segal and Shuman, 1998; Vogel and Isberg, 1999). In particular, DotA, a cytoplasmic membrane protein with eight transmembrane domains, is predicted to be a structural component of the Dot/Icm transport apparatus (Roy and Isberg, 1997); DotB, a protein homologous to a large family of nucleotide-binding proteins, including members of a number of conjugal transfer systems (Vogel *et al*., 1998), may provide energy required for assembly or activity of the complex. Because co-localization of *dot/icm* null mutants with lysosomal enzymes had not be examined previously, we compared the accumulation of cathepsin D by phagosomes bearing *dotA* or *dotB* null mutants with those containing either live or formalin-killed PE wild-type *L. pneumophila* (Fig. 2B and C). Surprisingly, few phagosomes containing *dotA* or *dotB* null mutants matured to the lysosomal stage. After 4 h of infection, only 22% of *dotA* and 14% of *dotB* mutant-bearing phagosomes contained cathepsin D, whereas 71% of the vacuoles containing E *L. pneumophila* co-localized with this lysosomal protein. Thus, the capacity of *L. pneumophila* to evade lysosomes did not appear to depend strictly on Dot/Icm function.

Dual labelling of phagosomes for late endosomal and lysosomal markers

To confirm that vacuoles harbouring formalin-killed or *dot* null mutant *L. pneumophila* do not progress to the lysosomal stage, this compartment was labelled by a second method, endocytosis of the soluble dye Texas Red-ovalbumin (TRov). Macrophages were infected synchronously with either wild-type or mutant bacteria, as described above, incubated for 1 h and then the late endosomal and lysosomal compartment was stained by immunofluorescence microscopy using an antibody specific for LAMP-1 (Fig. 3). As expected, within 1 h of phagocytosis, nearly 100% of polystyrene beads co-localized with both LAMP-1 and TRov. A similar pattern was observed for both live and formalin-killed E *L. pneumophila.* During this same period, phagosomes containing PE *L. pneumophila* rarely fused with either the

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late endosomes or the lysosomes. However, although approximately 85% of the phagosomes containing formalin-killed PE phase *L. pneumophila* acquired LAMP-1, less than 20% co-localized with TRov, indicating retarded phagolysosome development.

To test whether the *L. pneumophila* activity that blocks phagosome maturation was heat sensitive, we analysed the fate in macrophages of PE *L. pneumophila* that had been incubated at 80°C for 10 min (Fig. 3A). After 1 h of infection, heat-killed PE bacteria were degraded within phagolysosomes; more than 95% of these vacuoles had acquired both LAMP-1 and TRov. Similarly, heat

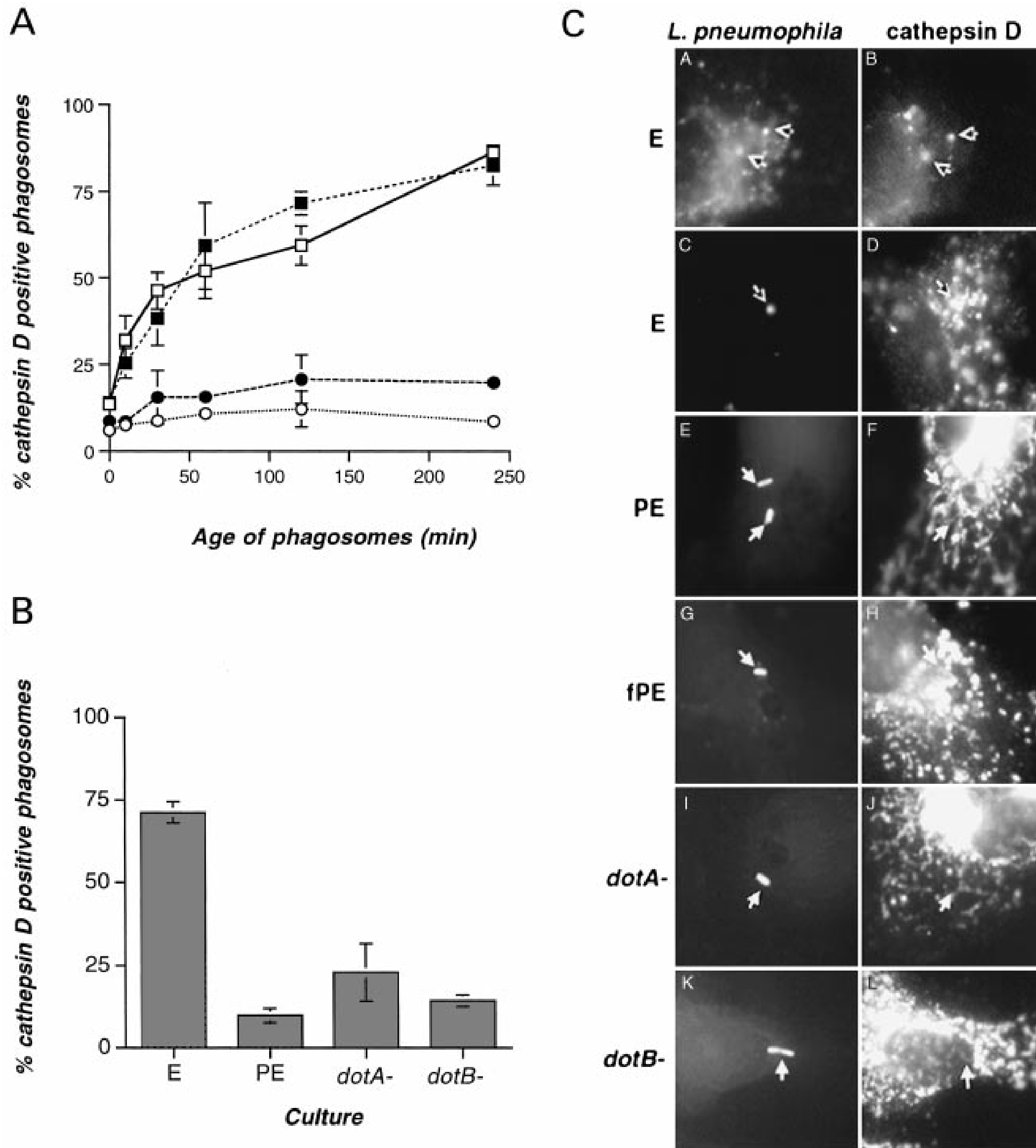


Fig. 2. Acquisition of cathepsin D by phagosomes containing *L. pneumophila*.
A. Macrophage phagosomes aged 5 min to 4 h were analysed by immunofluorescence microscopy using cathepsin D-specific antibody as described in the *Experimental procedures*. By 4 h, the majority of phagosomes containing live E (open squares) or formalin-killed E *L. pneumophila* (solid squares) acquired cathepsin D. In contrast, live (open circles) and formalin-killed (solid circles) PE *L. pneumophila* evaded fusion with the lysosomal compartment. Shown are the means and standard errors of the means determined for a minimum of 50 phagosomes in each of four separate experiments.
B. Even 4 h after infection, the majority of phagosomes harbouring live PE *dotA* or *dotB* mutant *L. pneumophila* do not acquire cathepsin D. Shown are the means and the standard errors of the means for at least 50 phagosomes determined in each of three separate experiments.
C. Immunofluorescence micrographs of cathepsin D co-localization with phagosomes containing *L. pneumophila*. Macrophages were infected for 4 h with carboxy-fluorescein-labelled *L. pneumophila,* then extracellular bacteria were stained with DAPI and lysosomes with cathepsin D-specific antiserum before microscopic analysis. Phagosomes containing E *L. pneumophila* acquire cathepsin D (A–D), whereas live (E and F) and formalin-killed PE wild-type (G and H) or live PE *dotA* (I and J) or *dot B* mutants (K and L) do not. Black arrows indicate phagosomes that were scored as positive; white arrows indicate phagosomes that were scored as negative.

treatment of formalin-killed PE phase bacteria destroyed their capacity to evade lysosome fusion. Thus, the activity that retards phagosome–lysosome fusion was heat labile, whether or not the bacteria had been formalin treated.

Consistent with our studies of cathepsin D localization (Fig. 2), a Dot-independent activity of *L. pneumophila* prevented fusion with TRov-labelled lysosomes, but not with a LAMP-1-containing compartment (Fig. 3). Although approximately 85% of the vacuoles containing *dotA* or *dotB* null mutants acquired LAMP-1, only ≈20% merged with TRov-labelled lysosomes. When phagosomes containing formalin-killed PE and *dotA* and *dotB* mutants were aged 18 h, then analysed for co-localization with lysosomes prelabelled with TRov, more had matured to phagolysosomes. Specifically, TRov was detected in approximately 65% of phagosomes containing formalin-killed PE, in 40% of *dotA* vacuoles and in 50% of *dotB* vacuoles (data not shown). Thus, PE *L. pneumophila* killed with formalin or lacking *dot* function still retarded phagolysosome maturation, but did not block it indefinitely. Taken together, the data indicate that evasion of lysosomes by *L. pneumophila* is a multicomponent process. A formalin-sensitive and Dot-dependent activity isolates the vacuole from a LAMP-1-containing compartment, whereas a formalin-resistant and Dot-independent activity retards progression to lysosomes, a compartment defined by the presence of cathepsin D and TRov.

Phagosome accumulation of Texas Red-ovalbumin, an exogenous soluble probe

We next determined whether vacuoles bearing pathogenic *L. pneumophila* interact with the early endosomal compartment. First, phagosome accessibility to soluble endocytic probes was analysed. Macrophages were infected synchronously, then incubated with the soluble endocytic tracer TRov for 25–145 min before microscopic analysis (Fig. 4). By 145 min after phagocytosis, TRov had accumulated in 75% or more of phagosomes either containing formalin-killed *E. coli* or containing live or formalin-killed E *L. pneumophila*. In contrast, not more than 25% of phagosomes containing either viable or formalin-killed PE *L. pneumophila* co-localized with the endocytic probe at any time period examined. Once again, phagosomes harbouring *dotA* or *dotB* null mutants resembled those containing formalin-killed PE *L. pneumophila*: 145 min after infection, less than 25% of either population had accumulated the fluorescent solute (Fig. 4B).

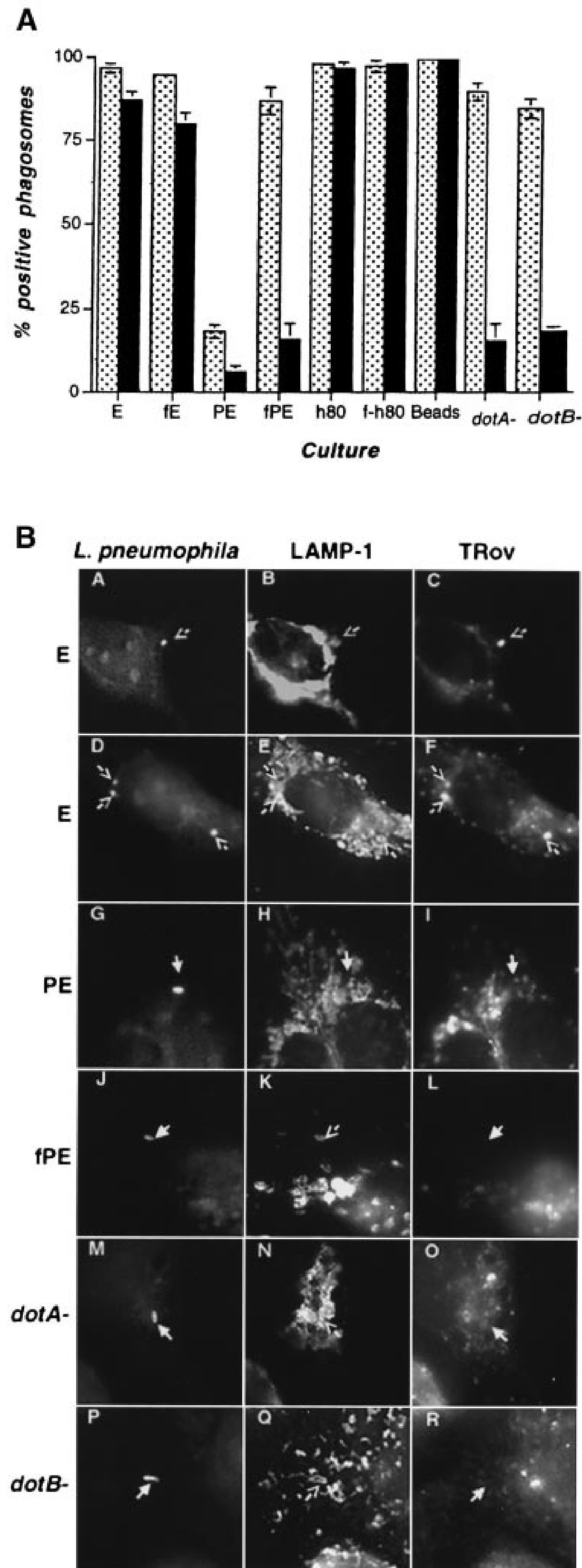
Phagosome accumulation of CM-DiI, an exogenous lipid probe

To assess by an alternative approach the interaction between phagosomes and the endocytic pathway, we used the fluorescent lipid CM-DiI. The macrophage plasma membrane was labelled by incubating cells for 5 min, then the fluorescent lipid was delivered to lysosomes during a subsequent 25 min incubation in medium without the dye. Next, labelled macrophages were infected with carboxy-fluorescein-labelled bacteria, phagosome maturation was allowed to proceed for 30 min, then cells were fixed and examined by fluorescence microscopy (Fig. 5A). Consistent with the results obtained by labelling lysosomes with cathepsin D (Fig. 2) or TRov (Fig. 3), more than 90% of latex beads and 80% of live or formalin-killed E phase *L. pneumophila* co-localized with the lysosomal lipid marker, whereas only 15% of live PE phase *L. pneumophila* did so. As reported above, formalin-killed PE *L. pneumophila* appeared isolated from endosomal traffic; only approximately 25% of these vacuoles accumulated CM-DiI.

Having confirmed that CM-DiI is a valid reporter of endosomal traffic (Mordue *et al*., 1999b), we next determined whether *L. pneumophila* phagosomes were accessible to endocytosed lipids. After a 1 h infection with carboxy-fluorescein-labelled bacteria, macrophages were treated with CM-DiI for 10 min, and then incubated for an additional 0–60 min before fixation and microscopic analysis (Fig. 5B). By 2 h after formation, nearly 75% of the phagosomes either containing formalin-killed *E. coli* or containing live or formalin-killed E *L. pneumophila* had fused with vesicles bearing the fluorescent lipid. In contrast, less than 25% of live or formalin-killed PE *L. pneumophila* co-localized with CM-DiI. Similarly, phagosomes aged 2 h that harboured either live PE *dotA* or live PE *dotB* mutants rarely accumulated the endocytosed fluorescent lipid (Fig. 5C). Thus, pathogenic *L. pneumophila* reside in a novel compartment that not only excludes LAMP-1 and lysosomal markers but also fails to accumulate by endocytosis exogenous solutes and lipids. Moreover, our data indicate that both Dot-dependent and Dot-independent factors are required to establish this protective vacuole.

Phagosome accumulation of transferrin receptor, a plasma membrane and early endosomal protein

The results described above indicate that virulent *L. pneumophila* establish a vacuole that is distinct from the endocytic network. To test this hypothesis more directly, we compared the maturation of phagosomes containing pathogenic and non-pathogenic particles by analysing the kinetics of acquisition of transferrin receptor, a marker for early endosomes and plasma membrane (van Renswoude *et al*., 1982; Klausner *et al*., 1983; Clemens and Horwitz, 1995, 1996; Sturgill-Koszycki *et al*., 1996; Mordue and Sibley, 1997). Macrophages were infected



synchronously, then incubated for an additional 0–60 min before analysis of transferrin receptor localization by immunofluorescence microscopy (Fig. 6). Immediately after infection, 31% of phagosomes containing polystyrene beads and 21% of those containing E *L. pneumophila* contained detectable amounts of transferrin receptor. In contrast, less than 10% of phagosomes containing virulent PE *L. pneumophila* co-localized with this marker for plasma membrane and early endosomes. Thus, consistent with their apparent inaccessibility to soluble and lipid probes, the lack of transferrin receptors indicated that nascent *L. pneumophila* vacuoles are distinct from the early endosomal compartment.

Accumulation of endosomal streptavidin–Alexa on biotinylated intracellular L. pneumophila

Formally, it remained possible that transferrin receptor or exogenously added soluble or lipid probes were present in the specialized *L. pneumophila* vacuole, but in quantities that were below the limit of detection of our assays. For example, the *L. pneumophila* vacuole could act like a recycling/sorting endosome which acquires and loses markers rapidly (Mukherjee *et al.*, 1997). To address this possibility, we applied a more sensitive streptavidin–biotin capture assay that was developed previously by Sheila Sturgill-Koszycki and David G. Russell (unpublished; see *Experimental procedures*). In brief, macrophages whose lysosomes were prelabelled with fluorescein dextran (Fdx) were infected with surface-biotinylated *L. pneumophila*, then fluorescent streptavidin was added to the culture medium and allowed to traffic through the entire endocytic network. Accordingly, if the *L. pneumophila*

Fig. 3. Interaction of phagosomes containing *L. pneumophila* with the late endosomes and lysosomes.
A. After macrophage lysosomes were prelabelled with TRov, the cells were infected for 1 h before extracellular and intracellular bacteria and late endosomal and lysosomal LAMP-1 were stained and analysed microscopically, as described in the *Experimental procedures*. Co-localization with LAMP-1 (stippled bars) and TRov (black bars) was scored for phagosomes containing live (E) or formalin-killed E *L. pneumophila* (fE), live (PE) or formalin-killed PE *L. pneumophila* (fPE), PE *L. pneumophila* incubated at 80°C for 10 min (h80), PE *L. pneumophila* that were formalin killed, then heat treated (f-h80), polystyrene beads (Beads), or live PE *dotA* (*dotA*) or *dotB* (*dotB*) L. *pneumophila*. Shown are the means and the standard errors of the means determined for a minimum of 50 phagosomes in each of at least three separate experiments.
B. Representative images of macrophages infected with live E (A–F) or PE wild-type (G–I), formalin-killed PE wild-type (J–L), or live PE *dotA* mutant (M–O) or *dotB* mutant *L. pneumophila* (P–R). Phagosomes containing E (A and D) co-localize with LAMP-1 (B and E) and TRov (C and F). In contrast, vacuoles containing live PE (G) do not co-localize with either LAMP-1 (H) or TRov (I). Phagosomes harbouring formalin-killed PE (J), live PE *dotA* (M) or *dotB* (P) mutants acquire LAMP-1 (K, N, Q), but not lysosomal TRov (L, O, R). Black arrows indicate phagosomes that were scored as positive; white arrows indicate phagosomes that were scored as negative.

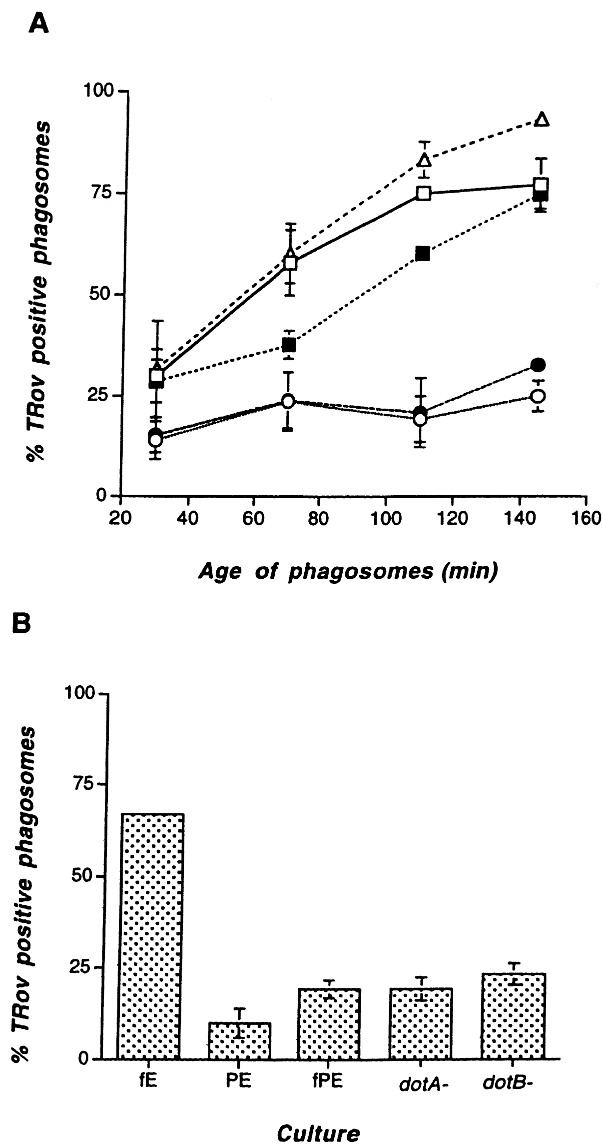


Fig. 4. Accumulation of endosomal solutes by *L. pneumophila* phagosomes.
A. Macrophages infected for 5 min with carboxy-fluorescein-labelled bacteria were incubated with TRov for 25–145 min, then preparations were fixed, extracellular bacteria were stained with DAPI and co-localization of phagosomes with TRov was scored. By 145 min after infection, the majority of formalin-killed *E. coli* (open triangles) and live E (open squares) or formalin-killed E (solid squares) *L. pneumophila* co-localized with TRov. In contrast, phagosomes containing live PE (open circles) or formalin-killed PE (solid circles) *L. pneumophila* did not accumulate the endosomal probe. Shown are the means and the standard errors of the means determined for a minimum of 50 phagosomes in each of at least three separate experiments.
B. Accumulation of TRov by phagosomes containing *dot* mutants or live or formalin-killed wild-type *L. pneumophila* 145 min after infection. Unlike phagosomes harbouring formalin-killed E *L. pneumophila*, vacuoles containing live or formalin-killed PE wild-type or containing live PE *dotA* or *dotB* mutant cells rarely accumulated the endosomal probe. Shown are the means and the standard errors of the means determined for a minimum of 50 phagosomes in each of three separate experiments.

vacuoles are accessible to the endocytic network, multiple fluorescent streptavidin molecules would bind avidly to the biotin molecules present on the bacterial surface, effectively amplifying the endosomal signal. Indeed, approximately 60% of E *L. pneumophila* vacuoles accumulated both streptavidin and Fdx (Fig. 7A and B). In contrast, approximately 80% of vacuoles harbouring PE *L. pneumophila* lacked both streptavidin and Fdx; likewise, neither marker was detected in more than 70% of the vacuoles that contained either formalin-killed PE or *dotA* or *dotB* mutant bacteria (Fig. 7A and B). Thus, viable PE *L. pneumophila* express both Dot-dependent and -independent factors to establish a vacuole that appeared to be completely inaccessible to the endocytic pathway for at least 4 h.

Discussion

L. pneumophila is an opportunistic pathogen that can establish infections within the lungs of some individuals, causing Legionnaires' disease, a severe pneumonia that can be fatal (Carratala *et al*., 1994; Marston *et al*., 1994). *L. pneumophila* survives in alveolar macrophages by evading phagosome–lysosome fusion (Horwitz, 1983a; Nash *et al*., 1984). To investigate the strategies used by *L. pneumophila* to establish a productive intracellular infection, we performed a series of quantitative immunofluorescence microscopy assays. Using a panel of antibodies against particular host cell proteins of the endocytic pathway and four soluble or lipid endosomal probes, we studied intracellular trafficking of *L. pneumophila* in macrophages. Our data indicate that post-exponential phase *L. pneumophila* express Dot-dependent and -independent factors to establish a vacuole that is isolated from the endocytic pathway (summarized in Fig. 8).

Previous studies have demonstrated that endocytic and host plasma membrane markers are absent from the *L. pneumophila* vacuole (Horwitz, 1983a; Clemens and Horwitz, 1992, 1993, 1995; Berger and Isberg, 1993; Swanson and Isberg, 1996; Roy *et al*., 1998; Wiater *et al*., 1998). Based upon immunogold electron microscopic localization of plasma membrane proteins in human mononuclear cells infected for 3.5 min with *L. pneumophila*, Clemens and Horwitz (1992) postulated that certain plasma membrane markers are sorted from the *L. pneumophila* phagosome during its formation. Consistent with previous reports, our data indicate that, during the first 4 h of infection, the *L. pneumophila* vacuole did not acquire proteins characteristic of the early endosome (transferrin receptor; Fig. 6), the late endosome (LAMP-1; Figs 1 and 3) or the lysosome (cathepsin D; Fig. 2). Moreover, from the time of formation until at least 4 h after infection, this organelle fails to accumulate membrane or solutes from the endocytic pathway (TRov, Figs 3 and 4;

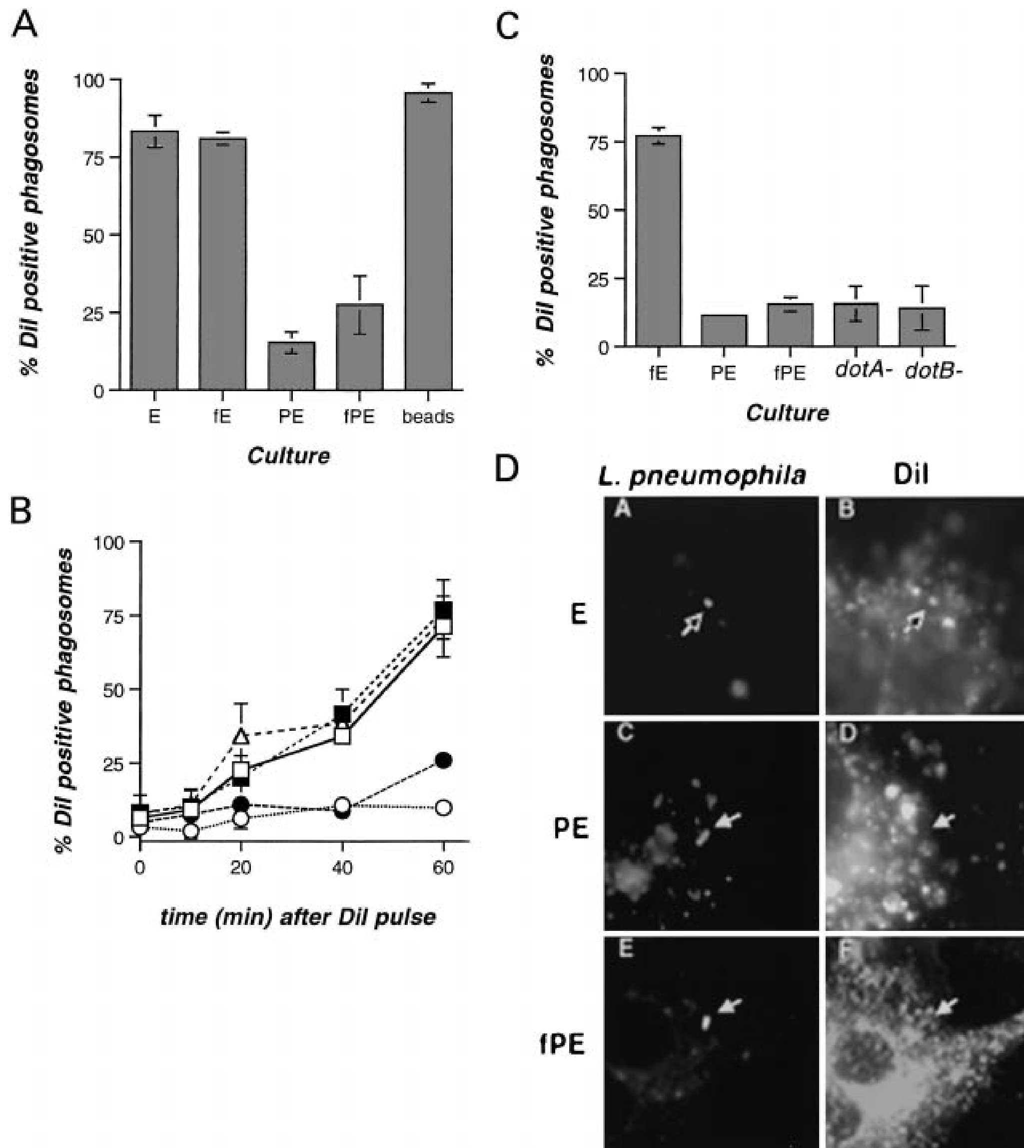


Fig. 5. Accumulation of a lipid endosomal probe within *L. pneumophila* phagosomes.

A. Macrophages whose lysosomes were prelabelled with CM-DiI were infected with *L. pneumophila* or PS beads for 5 min, then incubated for 1 h before fixation, staining of extracellular bacteria with DAPI and microscopic analysis of CM-DiI co-localization. The samples are designated as in Fig. 3A. Shown are the means and standard errors of the means determined from a minimum of 50 phagosomes in each of three separate experiments.

B. The plasma membrane of infected macrophage was labelled with the fluorescent lipid CM-DiI, then its co-localization with *L. pneumophila* was studied. After macrophages were infected with carboxy-fluorescein-labelled *L. pneumophila* for 1 h, cells were incubated with CM-DiI for 10 min, then an additional 0–60 min in label-free medium before fixation and staining of extracellular bacteria with DAPI. Phagosomes containing formalin-killed *E. coli* (open triangles) or live E (open squares) or formalin-killed E *L. pneumophila* (solid squares) readily accumulated DiI, whereas live PE (open circles) and formalin-killed PE *L. pneumophila* (solid circles) did so rarely. Shown are the means and the standard errors of the means determined from a minimum of 50 phagosomes in each of three separate experiments.

C. Accumulation of endosomal lipids by vacuoles containing *dot* mutants as described in B. Vacuoles containing live or formalin-killed wild-type PE *L. pneumophila* or containing live PE *dotA* or *dotB* mutants rarely acquired CM-DiI. Shown are means and standard errors of the means determined from a minimum of 50 phagosomes in each of at least three separate experiments. The samples are designated as in Fig. 3A.

D. Fluorescence micrographs of macrophages treated as described in B to analyse co-localization of CM-DiI with phagosomes containing *L. pneumophila*. Phagosomes containing E phase *L. pneumophila* accumulated endosomal CM-DiI (A and B), whereas those containing live PE (C and D) and formalin-killed PE bacteria (E and F) did not. Black arrows indicate phagosomes that were scored as positive; white arrows indicate phagosomes that were scored as negative.

CM-DiI, Fig. 5; streptavidin–Alexa, Fig. 7). Thus, to survive in macrophages, *L. pneumophila* appears to use a strategy similar to that of *Toxoplasma*, which triggers formation of a vacuole that is completely separate from the endocytic network (Mordue and Sibley, 1997; Mordue *et al.*, 1999a;b).

A classic electron microscopic study by Horwitz (1983b) revealed that the cytoplasmic face of *L. pneumophila* phagosomes aged 15 min or 60 min are decorated by smooth vesicles. The origin and function of these vesicles is not yet known. These vesicles could deliver nutrients from the endocytic pathway to the vacuolar bacteria. Alternatively, they could be docked vesicles whose fusion with the phagosome was aborted. Our analysis of phagosome dynamics using either antibody specific for transferrin receptor or each of three endosomal probes indicates that virulent, PE *L. pneumophila* reside in a vacuole that is inaccessible to endocytic traffic. Accordingly, it is unlikely that the population of smooth vesicles attached to the nascent phagosome indicate fusion with the endosomal network. The possibility remains that, immediately after bacterial entry, many vesicles bud from the *L. pneumophila* vacuole, remodelling the coiled phagosome (Horwitz, 1984). By this mechanism, *L. pneumophila* could shed host proteins critical for recognition or activation of the host membrane fusion machinery, thereby preventing phagolysosome maturation.

Surprisingly, vacuoles containing *L. pneumophila* that have been killed with formalin do not accumulate lysosomal markers or solutes from the endocytic pathway, but do acquire LAMP-1. Similarly, Bozue and Johnson (1996) documented that formalin-killed *L. pneumophila* evade lysosomes in amoebae. On the other hand, others have reported co-localization of formalin-killed *L. pneumophila* with acid phosphatase and endocytosed thorium dioxide (Horwitz, 1983a) and endocytosed rhodamine dextran (Wiater *et al.*, 1998). One critical confounding variable in these studies is the conditions used to culture *L. pneumophila.* We infected macrophages with broth cultures of post-exponential phase bacteria, a growth condition that induces virulence expression (Byrne and Swanson, 1998; Hammer and Swanson, 1999), whereas others typically analysed bacteria that had been cultured for 2–3 days on solid medium (e.g. Horwitz, 1983a; Wiater *et al.*, 1998), conditions that are likely to yield an asynchronous population of bacteria whose virulence expression has not been compared directly with synchronous PE broth cultures. Another possible explanation for the discrepant results is the relative sensitivity of electron and fluorescence microscopy, although a variety of endosomal probes were readily detected by fluorescence microscopy within phagolysosomes containing either degradable or non-degradable particles (e.g. Fig. 3A).

Our observation that *dotA* mutants evade lysosomes also contradicts the interpretation of a number of previous studies. As described above, explanations for this discrepancy may be our use of synchronous PE broth cultures or the relative specificity or sensitivity of the different methods used to label the endosomal compartment. For example, Berger *et al.* (1994) prelabelled U937 cells by endocytosis of ferric oxide, then analysed by electron microscopy phagosomes aged 6–8 h. Under these conditions, 37% of phagosomes containing Lp03 *dotA* mutant bacteria had fused with lysosomes, a value consistent with the 25% of Lp03 cells residing in vacuoles that contained the lysosomal protease cathepsin D (Fig. 2B). Using TRov co-localization as a marker for phagolysosome formation in mouse bone marrow-derived macrophages, Swanson and Isberg (1996) reported that one *dotA* mutant strain evaded the lysosomes, but a second *dotA* mutant strain did not. As both of these strains were generated by chemical mutagenesis, secondary mutations may affect their intracellular fate. Wiater *et al.* (1998) applied confocal microscopy to analyse phagolysosome formation in U937 cells whose endosomal pathway had been labelled with rhodamine dextran. In their study, the majority of the vacuoles harbouring *dot/icm* mutants fused with lysosomes within 30 min (Wiater *et al.*, 1998). However, the 40 h endosomal labelling protocol of Wiater *et al.* (1998) may have saturated the entire endosomal compartment with the fluorescent probe. Likewise, it is difficult to judge whether the protocol of Horwitz (1983a) labelled with thorium dioxide only the lysosomal compartment or also earlier

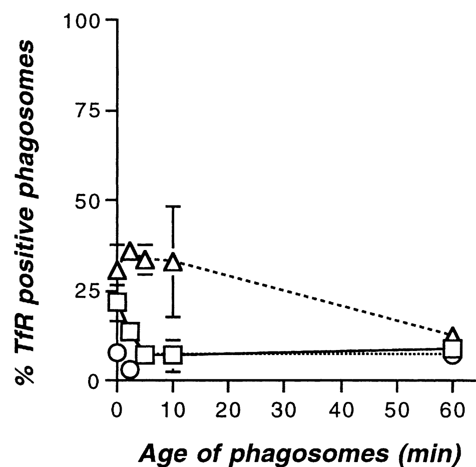
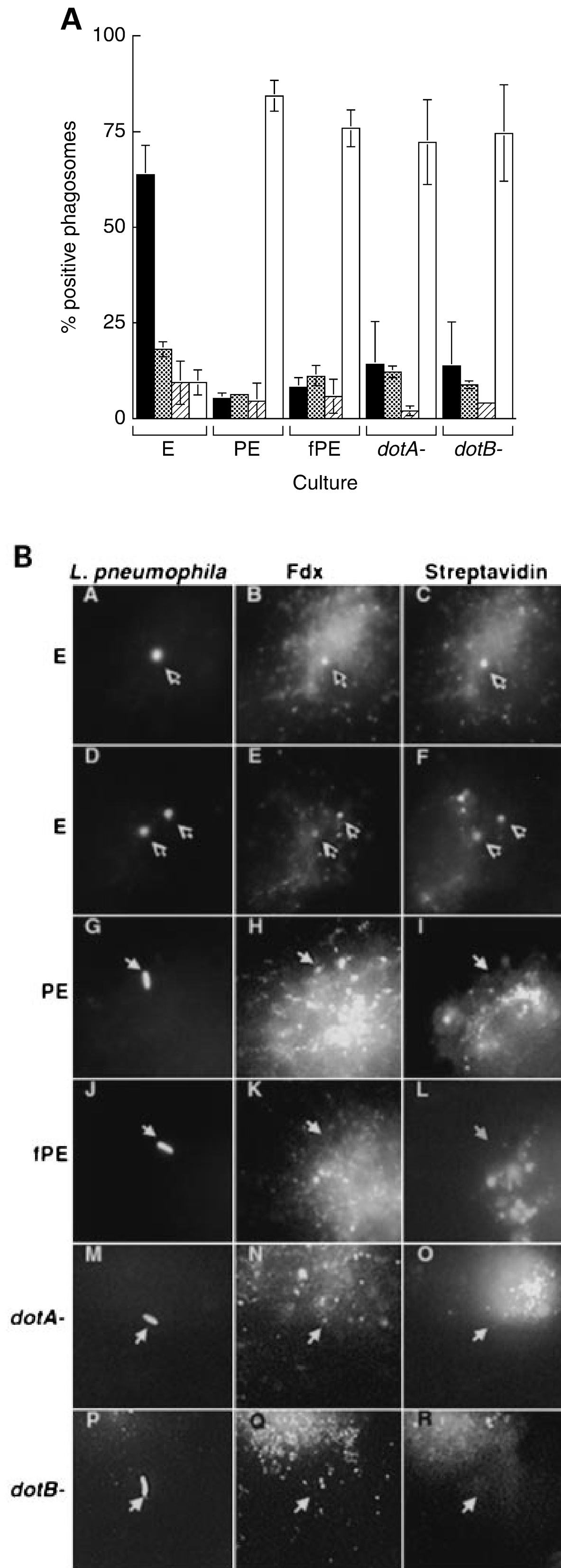


Fig. 6. Acquisition of transferrin receptors by phagosomes containing *L. pneumophila*. Macrophages which had phagocytosed for 5 min PS beads (open triangles) or E (open squares) or PE *L. pneumophila* (open circles) were incubated for an additional 0–60 min before analysis of transferrin receptor localization by immunofluorescence microscopy. Unlike PS beads and E *L. pneumophila*, live PE *L. pneumophila* reside in vacuoles that only very rarely acquired transferrin receptors. Shown are means and standard errors of the means determined from a minimum of 50 phagosomes in each of three separate experiments.



stages of the endosomal pathway. Finally, although LAMP-1 acquisition is often used to monitor maturation of *L. pneumophila* phagosomes (Andrews *et al*., 1998; Roy *et al*., 1998; Vogel *et al*., 1998; Zuckman *et al*., 1999), vacuoles may contain LAMP-1 but lack other endosomal characteristics, including cathepsin D, a lysosomal protease and accessibility to a variety of endosomal fluorescent probes (Figs 2–5 and 7). Taken together, our studies demonstrate that *L. pneumophila* factors required for evasion of lysosomal degradation can be separated experimentally into two PE components: (1) a Dot-independent, formalin-resistant activity blocks accumulation of lysosomal contents and (2) a Dot-dependent, formalin-sensitive activity interferes with acquisition of LAMP-1.

The Dot-dependent activity and the formalin-sensitive activity that are required by *L. pneumophila* to evade the endocytic pathway may be distinct. When PE *dotA* and *dotB* mutants are treated with formalin, twice as many bacteria are delivered to lysosomes, indicating an additive effect of formalin treatment and mutation of *dot* genes. Also, formalin-killed PE *Legionella* begin to lose their integrity 4 h after phagocytosis; by 18 h, more than 70% co-localize with lysosomal markers. In contrast, only a twofold increase in fusion of lysosomes with *dot* mutant-containing phagosomes was observed 18 h after infection, suggesting that the formalin-resistant factor(s) is different from and retains activity longer than the Dot-dependent factor(s) (B. Byrne, A. D. Joshi and M. S. Swanson, unpublished). Although both formalin-treated wild-type and *dot* mutant *L. pneumophila* retard phagolysosome formation, neither block maturation indefinitely. Likewise, more than 15 h after infection, when intracellular

Fig. 7. Accessibility of vacuoles containing biotinylated *L. pneumophila* to endocytosed streptavidin–Alexa and lysosomal fluorescein–dextran.

A. Macrophage whose lysosomes were prelabelled with Fdx were infected with biotinylated *L. pneumophila,* then incubated for an additional 30 min before addition of the endocytic probe Alexa 594 conjugated-streptavidin. Co-localization of the two probes with the phagosomes was analysed microscopically as described in the *Experimental procedures* and categorized as Fdx^+/streptavidin$^+$ (black bars), Fdx^+/streptavidin$^-$ (stippled bars), Fdx^-/streptavidin$^+$ (hatched bars), Fdx^-/streptavidin$^-$ (white bars). The samples are designated as in Fig. 3A. Shown are the means and the standard errors of the means determined from a minimum of 50 phagosomes in each of at least three separate experiments.

B. Fluorescence micrographs of the streptavidin–biotin capture assay described in A for phagosomes containing live E (A–F), live PE (G–I) or formalin-killed PE (J – L), and live PE *dotA* (M–O) or *dotB* mutants (P–R). Phagosomes occupied by live E *L. pneumophila* (A and D) co-localized with both lysosomal Fdx (B and E) and endocytosed streptavidin–Alexa (C and F). In contrast, phagosomes containing live PE (G) or formalin-killed PE wild-type (J), or live PE *dotA* (M) or *dotB* mutant cells (P) co-localized with neither lysosomal Fdx (H, K, N, Q) nor with endocytosed streptavidin–Alexa (I, L, O, R). Black arrows indicate phagosomes that were scored as positive; white arrows indicate phagosomes that were scored as negative.

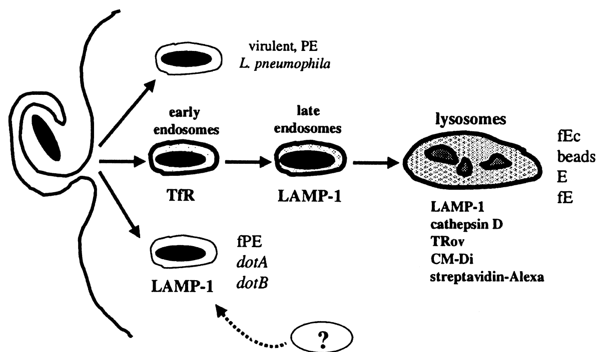


Fig. 8. Evidence that the *L. pneumophila* vacuole is isolated from the endocytic network, as described in the text. TfR, transferrin receptor; thick membranes indicate vacuoles that accumulated from the endosomal pathway the fluorescent probes TRov, CM-DiI and streptavidin–Alexa. Particles are polystyrene beads, formalin-killed *E. coli* (fEc), live exponential (E) or formalin-killed E *L. pneumophila* (fE), live post-exponential (PE) or formalin-killed PE *L. pneumophila* (fPE), and live PE *dotA* or *dotB* mutant *L. pneumophila.*

L. pneumophila are replicating, their capacity to block fusion with the endosomal compartment wanes (Sturgill-Koszycki and Swanson, 2000).

Vacuoles containing formalin-killed PE wild-type or viable *dotA* or *dotB* mutant *L. pneumophila* acquired LAMP-1, yet appeared separate from the endocytic network. The host autophagy pathway is unlikely to be the source of LAMP-1 as this marker is acquired within 15 min of phagocytosis, whereas ER envelopes the *L. pneumophila* vacuoles hours later (Swanson and Isberg, 1996). Epithelial cell vacuoles containing *Salmonella* also acquire some endosomal markers, but not others; this organelle readily accumulates the vacuolar ATPase, lysosomal membrane glycoproteins and acid phosphatase, but rarely lysosomal cathepsin D or horseradish peroxidase (Garcia-del Portillo and Finlay, 1995; Steele-Mortimer *et al*., 1999). Normally, LAMP-1 is targeted to the lysosomes by one of two routes (Hunziker and Gueze, 1996; Rohrer *et al*., 1996). LAMP-1, which has been delivered to the cell surface either directly from the trans-Golgi network or via recycling/sorting endosomes, is transported by the endocytic network to lysosomes. Biosynthetic or secretory vesicles from the trans-Golgi network also deliver proteins, including LAMP-1, to endosomes and then lysosomes. Recently, it has been proposed that, during biogenesis, phagosomes fuse with vesicles from the biosynthetic pathway before interacting with the endosomal network (Ullrich *et al*., 1999). Alternatively, we cannot rule out that vacuoles harbouring either formalin-killed PE wild-type or viable PE *dot* mutant bacteria readily receive and recycle endosomal material, but do not accumulate detectable quantities. Although our assays did include avirulent particles as positive controls for phagolysosome formation, a particle that is known to persist in an earlier endosomal compartment was not analysed. Therefore, additional experiments are required to determine the source of LAMP-1 that accumulates in phagosomes containing formalin-killed PE and *dot* mutant bacteria.

Evasion of lysosomes by *L. pneumophila* is likely to be mediated by a surface component, based on its requirement for a functional Dot/Icm secretion system and resistance to formalin treatment. Surface structures play a critical role in evasion of lysosomes by at least two other pathogens, *Mycobacterium and Leishmania*. Cell wall glycolipid cord factor, or trehalose dimycolate, of *Mycobacterium* is thought to inhibit phagosome–lysosome fusion by increasing fluidity and hydration of the membrane (Goren *et al*., 1976; Spargo *et al*., 1991; Crowe *et al*., 1994). Similarly, the promastigote form of *Leishmania* is covered with a thick glycocalyx composed primarily of a single molecule called lipophosphoglycan (LPG), which plays a critical role in the inhibition of phagolysosome formation (Desjardins and Descoteaux, 1997; Scianimanico *et al*., 1999). However, as promastigotes transform into the amastigote form, LPG production is downregulated and phagosome–endosome fusion is restored (reviewed by Turco and Descoteaux, 1992).

The intracellular pathways of *L. pneumophila* and *Leishmania* share a number of general features. Evasion of the lysosomes by both pathogens appears to be multifactorial, as judged by the fate of microbes that have been altered genetically or chemically (Fig. 3; Dermine *et al*., 2000). The DotA protein of *L. pneumophila*, like LPG of *Leishmania*, is required at the time of infection, but not for intracellular replication (Roy *et al*., 1998; Coers *et al*., 1999). In the exponential phase of growth, broth-grown *L. pneumophila* downregulate expression of a number of virulence factors (Byrne and Swanson, 1998); hence, phagosomes containing E *L. pneumophila* merge with the lysosomal compartment. Moreover, recent studies in our laboratory indicate that a similar pattern occurs in macrophages: as intracellular *L. pneumophila* convert from the PE phase to the replicative form, their vacuoles acquire several lysosomal characteristics (Sturgill-Koszycki and Swanson, 2000).

The apparent complexity of the *L. pneumophila* intracellular pathway motivates speculation on the evolution of this parasite of fresh water amoebae (reviewed by Fields, 1996) and opportunistic pathogen of alveolar macrophages. Presumably, ancestral legionellae were ingested and efficiently digested within amoebae phagolysosomes. In response to this considerable selective pressure, endosymbionts may have emerged by virtue of their expression of a formalin-resistant surface component that retards phagolysosome maturation and promotes survival. Later, acquisition by horizontal transmission of the genetically linked *dot/icm* loci may have permitted the emergence of pathogenic *L. pneumophila*

(Vogel and Isberg, 1999). Now endowed with the capacity to avoid the endocytic pathway for hours, *L. pneumophila* has a window of time sufficient to alter its physiology to exploit phagocytes as a replication niche. Accordingly, the complex intracellular life cycle of pathogenic *L. pneumophila* is the product of a relentless competition with the aquatic amoebae with which it evolves.

Experimental procedures

Cells

Bone marrow-derived macrophages were obtained from femurs of female A/J mice (Jackson Laboratory), as described previously (Swanson and Isberg, 1995). After a 6 day culture period in L-cell-conditioned medium, macrophages were collected by centrifugation, suspended in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (RPMI-FBS; Gibco BRL) and then 1.5×10^5 cells were plated per 12-mm-diameter circular coverslip. Experiments were performed with cultured macrophages, which were used within the next 1–2 days.

Every 2–3 weeks, *L. pneumophila* Lp02, a virulent thymine auxotroph derived from the serogroup 1 Philadelphia 1 strain that replicates efficiently in monocytic U937 cells (Berger and Isberg, 1993), human peripheral blood mononuclear cells and primary macrophages derived from the bone marrow of A/J mice (Swanson and Isberg, 1995), was colony purified from frozen stocks on *N*-(2-acetamido)-2-aminoethanesulphonic acid (ACES)-buffered charcoal–yeast extract agar supplemented with thymidine to 100 $\mu g\ ml^{-1}$ (CYET). For each experiment, *L. pneumophila* was inoculated into ACES-buffered yeast extract broth supplemented with thymidine to 100 $\mu g\ ml^{-1}$ (AYET) and cultured overnight, then subcultures were incubated for an additional 24 h to an optical density at 600 nm of 0.5–1 and 2.5–4 to obtain E or PE cultures respectively. Formalin-killed *L. pneumophila* and *Escherichia coli* were prepared by incubating as a suspension in 2% formalin in phosphate-buffered saline (PBS) for 15 min at room temperature with agitation. The cells were collected by centrifugation at 5000 r.p.m. for 5 min, then washed twice with PBS and once with RPMI-FBS. Heat-killed *L. pneumophila* were prepared by resuspending broth-grown bacteria in PBS and incubating in a heat block at 80°C for 10 min. Both the formalin treatment and the heat treatment killed 99.99% of bacteria, as determined by colony-forming units (cfus) before and after treatment.

Two previously characterized null mutant strains were also analysed: *dotA* mutant Lp03 (Berger *et al*., 1994) and the *dotB* null strain JV374 (a kind gift from Dr J. Vogel, Washington University, St. Louis). Control experiments showed that PE *dotA* and *dotB* avoided lysosomal degradation, whereas E *dotA* and *dotB* did not (data not shown). Therefore, only the more virulent PE *dot* mutants were analysed.

Polystyrene microspheres (beads) adsorbed to BSA were used as a positive control in some phagolysosome maturation experiments. Beads were incubated with 10 mg of BSA per ml of PBS at 4°C on a shaker overnight to allow non-specific adsorption of BSA, which promoted phagocytosis of the particles. Beads were then washed with PBS to remove excess BSA and stored at 4°C with 0.02% sodium azide until further use (Oh and Swanson, 1996).

Antibodies and reagents

Monoclonal antibody (mAb) 1D4B against late endosomal/lysosomal marker LAMP-1 was obtained from the Developmental Studies Hybridoma Bank of the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, IA; rabbit antiserum to cathepsin D was obtained from Dr Sadaki Yokota (Yamanashi Medical School, Japan); rat antiserum to transferrin receptor (R17.217.13) was obtained from Dr David Russell (Washington University, St. Louis); and rabbit serum specific for *L. pneumophila* was from Dr Ralph Isberg (Tufts University School of Medicine). Fluorescently conjugated secondary antiserum, 5(6)-carboxyfluorescein-*N*-hydrosuccinimide ester (FLUOS), Texas-Red dextran (TRdx; molecular weight 10 kDa), Texas-Red ovalbumin (TRov; molecular weight 10 kDa), chloromethylbenzamido derivative of DiI (CellTracker CM-DiI), 4′,6-diamidino-2-phenylindole (DAPI), streptavidin, Alexa 594-conjugated streptavidin and fluorescein dextran (Fdx; molecular weight 10 kDa) were obtained from Molecular Probes. Polystyrene microspheres (beads; 1 mm diameter) were purchased from Polysciences and sulphosuccinimidobiotin (EZ-Link; Sulfo-NHS-Biotin) was obtained from Pierce.

Infections

To perform synchronous *in vitro* infections, adherent macrophage cultures were placed on ice for 10 min to inhibit phagocytosis, then bacteria suspended in cold RPMI-FBS were added to attain a multiplicity of infection of approximately 12 for E and 6 for PE *L. pneumophila*. To synchronize the infections, the plates were then centrifuged at 400 *g* for 10 min at 4°C, then transferred to a 37°C water bath for 5 min to allow phagocytosis of the bacteria. Next, non-adherent bacteria were removed by washing the monolayer gently with cold RPMI-FBS three times. At each time point, coverslips were transferred to periodate–lysine–paraformaldehyde (McLean and Nakane, 1974) containing 4.5% sucrose. Control experiments showed that centrifugation and chilling of macrophages had no effect on trafficking of *L. pneumophila* (data not shown).

Interaction of L. pneumophila phagosome with the endocytic network

To determine whether phagosomes harbouring *L. pneumophila* fused with early endosomes, transferrin receptor, a resident of the plasma membrane and early endosomes (van Renswoude *et al*., 1982; Klausner *et al*., 1983), was localized by immunofluorescence microscopy. Macrophages infected for 5 min as described above were fixed after incubation for 0, 2.5, 5, 10 and 60 min. For all of the fluorescence staining procedures, PBS containing 2% goat serum and 5% sucrose was used as a diluent for antibodies and as a blocking buffer,

and preparations were washed with PBS containing 5% sucrose. Extracellular bacteria were stained with a rabbit antiserum specific for *L. pneumophila* diluted 1:2000 and Cascade-Blue-conjugated anti-rabbit serum diluted 1:1000. Next, the coverslips were permeabilized with cold methanol, and transferrin receptor was labelled using rat anti-transferrin receptor antibody diluted 1:2 and a 1:2000 dilution of Oregon Green-conjugated anti-rat serum. Finally, extracellular and intracellular bacteria were stained with rabbit antiserum against *L. pneumophila* and Texas-Red-conjugated anti-rabbit serum diluted 1:2000. Polystyrene beads served as a positive control for normal phagolysosome maturation. Macrophages were allowed to phagocytose beads during a 5 min pulse infection, after which the monolayer was washed three times. To label residual extracellular beads, 0.5 mg ml^{-1} Texas Red-dextran (TRdx) was added; this fluorophore is adsorbed rapidly onto extracellular beads after incubation at 4°C for 1 min (Oh and Swanson, 1996). The macrophages were washed three times, then incubated for the period indicated before fixation. Intracellular beads which were phagocytosed in the initial 5 min pulse infection were identified as phase-bright particles not stained with a rim of TRdx. The frequency of transferrin receptor acquisition was calculated by dividing the number of intracellular particles that co-localized with transferrin receptor by the total number of intracellular particles and then multiplying by 100. In all subsequent experiments, frequency of marker acquisition was calculated in a similar fashion.

The interaction between *L. pneumophila* and late endosomes and lysosomes was assessed by studying co-localization of the *L. pneumophila* phagosome with LAMP-1 (Chen *et al*., 1988). Briefly, after pulse infection, cells were incubated for an additional period of 5–60 min before fixation. Extracellular bacteria were labelled for 3 min with 0.1 mg of 4′,6-diamidino-2-phenylindole (DAPI) per ml of PBS, then the preparations were permeabilized by methanol extraction. Both extracellular and intracellular bacteria were then labelled as described above for transferrin receptor co-localization. LAMP-1 was stained by mAB 1D4B at a dilution of 1:5, followed by Oregon Green-conjugated anti-rat serum diluted 1:2000.

Cathepsin D, a soluble protease, served as a marker for macrophage lysosomes. For these experiments, bacteria were labelled before infection with carboxy-fluorescein, as described previously (Sturgill-Koszycki *et al*., 1994). The infected macrophages were then incubated at 37°C for 0–240 min before fixation. Extracellular bacteria were labelled with DAPI, and cathepsin D was localized using rabbit anti-cathepsin D antibody at a dilution of 1:250 followed by Texas Red-conjugated anti-rabbit serum diluted 1:2000. A vacuole was classified as positive for cathepsin D if its entire lumen was fluorescent, as seen for those containing degraded bacilli (Fig. 2), or if its entire periphery was rimmed with a fluorescence that was brighter than the adjacent cytosolic region, as was seen for the more rare vacuoles that contained intact bacilli. This stringent criterion probably biased the scoring in favour of negative vacuoles as, in the perinuclear region of the cell, the abundant lysosomes potentially obscured the fluorescence pattern of phagolysosomes.

To determine whether *L. pneumophila*-containing phagosomes diverge from the endocytic network, infected macrophages were dual labelled. Macrophage lysosomes were labelled by endocytosis of 50 μg of TRov per ml of RPMI-FBS for 20 min. Next, macrophages were incubated in label-free medium for an additional 20 min, then pulse infected and incubated for 1 h before fixation. The preparations were methanol extracted, and bacteria were labelled with rabbit antiserum specific for *L. pneumophila* followed by Cascade Blue-conjugated anti-rabbit antibody. LAMP-1 was stained as described above. Cell-associated bacteria were identified by phase-contrast microscopy, then analysed for co-localization with LAMP-1 and TRov.

Analysis of phagosome–lysosome fusion using fluid phase markers

To quantify fusion between *L. pneumophila*-containing phagosomes and lysosomes, macrophage lysosomes were prelabelled with either TRov or the lipid tracer CM-DiI. Co-localization of *L. pneumophila* phagosomes and TRov-labelled lysosomes was carried out essentially as described by Swanson and Isberg (1996). CM-DiI (Molecular Probes) was dissolved at 2 mg ml^{-1} DMSO and stored at −20°C. To examine lipid trafficking from macrophage plasma membrane, cells were incubated with 4 μg CM-DiI per ml of for 5 min at 37°C, washed three times to remove excess dye and incubated in RPMI-FBS for an additional 25 min to allow trafficking of the lipids to lysosomes (Mordue *et al*., 1999b). This protocol effectively removed labelled lipids from the plasma membrane. Monolayers were then pulse infected for 5 min with carboxy-fluorescein-labelled bacteria and incubated for an additional 25 min. Next, the cultures were fixed, and extracellular bacteria were labelled with DAPI before co-localization of intracellular bacteria with CM-DiI was scored.

Accessibility of L. pneumophila *phagosomes*

To determine whether *L. pneumophila* phagosomes fused with newly formed endosomes, TRov was used as a soluble endocytic probe. After macrophages were infected for 5 min with carboxy-fluorescein-labelled bacteria as described above, cultures were incubated with 300 μg of TRov per ml of RPMI-FBS for 25–145 min, monolayers were gently washed three times to remove free dye and then cells were fixed. Extracellular bacteria were labelled with DAPI as before, and preparations were scored for co-localization of TRov with intracellular bacteria. To study the accessibility of a lipid probe to *L. pneumophila*-containing phagosomes, monolayers were infected with carboxy-fluoresceinated bacteria for 5 min and then incubated in RPMI containing 1% FBS for an additional 55 min. To permit the dye to intercalate into the plasma membrane, the infected cultures were incubated with 4 μg of DiI per ml of RPMI containing 1% FBS at 37°C for 10 min. The macrophages were then washed, incubated in RPMI containing 1% FBS for 0–60 min and then fixed. Extracellular bacteria were labelled with DAPI, then co-localization of intracellular bacteria with CM-DiI was analysed microscopically.

Streptavidin capture assay

To assess accessibility of phagosomes to the endosomal pathway, we adapted a protocol developed previously by Sheila Sturgill-Koszycki and David G. Russell (unpublished). Approximately 1×10^9 bacteria were biotinylated by co-incubation with 1.5 mg biotin per ml of PBS at room temperature for 1 h. Biotinylation was stopped by the addition of 10 mM lysine, treated bacteria were washed three times to remove unbound biotin and then bacteria were added to macrophages whose lysosomes were prelabelled with fluorescein-conjugated dextran (Fdx). After the 5 min pulse infection, macrophages were incubated for 30 min to allow *L. pneumophila* phagosome maturation. After blocking the binding sites of the extracellular biotinylated bacteria by incubating for 5 min with an excess of unlabelled streptavidin (0.1 mg ml^{-1}), cultures were incubated with Alexa Fluor-conjugated streptavidin (0.1 mg ml^{-1}) for 30 min followed by an additional 30 min incubation in RPMI-FBS, a period sufficient for streptavidin to transit the endocytic network to the lysosomal compartment. Streptavidin in the endocytic network is trapped in phagosomes by biotinylated bacteria as a consequence of its high-affinity binding to biotin. The coverslips were then fixed and methanol extracted. Both intracellular and extracellular bacteria were labelled with rabbit anti-*L. pneumophila* serum and Cascade Blue-conjugated anti-rabbit serum. Phagosomes that contained streptavidin but not Fdx were interpreted to be endosomal but not lysosomal. Phagosomes that failed to acquire detectable quantities of either of the probes were interpreted as being isolated from the endocytic network.

Fluorescence microscopy

Samples were mounted in Pro-long Antifade (Molecular Probes) and observed using a Zeiss Axioplan 2 epi-fluorescence microscope equipped with a 100× Plan-Neo-fluar objective, numerical aperture of 1.3 and filters 487901, 487910 and 487900. In each experiment, at least 50 phagosomes were examined for each condition; data from at least three separate experiments are expressed as means ± standard errors of the means. Digital images were collected using a Spot camera (Diagnostics Instruments), and ADOBE PHOTOSHOP software was used to prepare the figures.

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