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Membrane Vesicles Shed by *Legionella pneumophila* Inhibit Fusion of Phagosomes with Lysosomes

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When cultured in broth to the transmissive phase, *Legionella pneumophila* infects macrophages by inhibiting phagosome maturation, whereas replicative-phase cells are transported to the lysosomes. Here we report that the ability of *L. pneumophila* to inhibit phagosome-lysosome fusion correlated with developmentally regulated modifications of the pathogen's surface, as judged by its lipopolysaccharide profile and by its binding to a sialic acid-specific lectin and to the hydrocarbon hexadecane. Likewise, the composition of membrane vesicles shed by *L. pneumophila* was developmentally regulated, based on binding to the lectin and to the lipopolysaccharide-specific monoclonal antibody 3/1. Membrane vesicles were sufficient to inhibit phagosome-lysosome fusion by a mechanism independent of type IV secretion, since only ~25% of beads suspended with or coated by vesicles from transmissive phase wild type or *dotA* secretion mutants colocalized with lysosomal probes, whereas ~75% of beads were lysosomal when untreated or presented with vesicles from the *L. pneumophila letA* regulatory mutant or *E. coli*. As observed previously for *L. pneumophila* infection of mouse macrophages, vesicles inhibited phagosome-lysosome fusion only temporarily; by 10 h after treatment with vesicles, macrophages delivered ~72% of ingested beads to lysosomes. Accordingly, in the context of the epidemiology of the pneumonia Legionnaires' disease and virulence mechanisms of *Leishmania* and *Mycobacteria*, we discuss a model here in which *L. pneumophila* developmentally regulates its surface composition and releases vesicles into phagosomes that inhibit their fusion with lysosomes.

To exploit macrophages as a replication niche, *L. pneumophila*, *Coxiella burnetii*, and *Leishmania* spp. apply a similar strategy (69). When ingested, each pathogen differentiates from a transmissive form that inhibits phagosome-lysosome fusion for several hours to a cell type fit for intracellular replication (20, 35, 37, 63, 67). Differentiation of *L. pneumophila* can be modeled in broth, where exponential (E)-phase bacteria switch to the transmissive phenotype as their nutrient supply wanes (11). As *L. pneumophila* enters the postexponential (PE) phase, the alarmone ppGpp triggers a regulatory cascade mediated in part by the two component system LetA/LetS and the sigma factors RpoS and FliA to coordinate expression of motility, cytotoxicity, stress resistance, factors that inhibit phagosome-lysosome fusion, and other traits likely to promote transmission to a new host cell (55, 56).

To establish a replication niche in macrophages, *L. pneumophila* utilizes type IV secretion to deliver substrates that alter host membrane traffic (14, 75). Instead of merging with lysosomes, the *L. pneumophila* vacuole becomes surrounded by membranes derived from the early secretory pathway by a process that in A/J mouse macrophages resembles autophagy (3, 19, 34, 42, 70, 72). The pathogen persists for several hours within an ER-like compartment, where it differentiates to the replicative form. Nevertheless, mutants that lack a functional Dot/Icm type IV secretion system remain viable in mouse macrophages, presumably because their vacuoles lack several lysosomal features (39), although they are rich in the late endosomal and lysosomal protein LAMP-1 (16). Therefore, in addition to type IV secretion, transmissive *L. pneumophila* must possess other mechanism(s) to avoid delivery to digestive lysosomes.

To account for its inhibition of phagosome-lysosome by a mechanism that is independent of type IV secretion, we postulated that transmissive *L. pneumophila* expresses glycoconjugates on its surface that inhibit fusion with degradative lysosomes, based on several observations. First, viable E-phase or heat-killed PE-phase *L. pneumophila* is degraded in lysosomes, whereas PE bacteria that have been killed by formalin remain intact within nondegradative vacuoles that contain LAMP-1 but lack lysosomal markers (39). Second, many pathogens modify the glycoconjugates of their surface as a virulence strategy, including *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Campylobacter jejuni*, *Brucella suis*, *Salmonella* spp., and *Leishmania* spp. (26, 27, 74, 78). Moreover, two intracellular pathogens are known to regulate and shed surface glycoconjugants as a survival strategy. Infectious *Leishmania* promastigotes transfer surface lipophosphoglycan to phagosomal membranes, altering their biophysical properties and inhibiting fusion with lysosomes (36, 62, 73). Another abundant surface glycoconjugate that alters phagosome maturation is lipoarabinomannan of *M. tuberculosis* (6, 24). Gram-negative bacteria shed lipopolysaccharide (LPS) (25) and also vesicles derived from their outer membrane (5, 9, 32, 33, 41, 44, 65). Likewise, it has long been known that the surface of *L. pneumophila* is contiguous with numerous small vesicles (23).

A number of other observations link virulence of *L. pneumophila* to its LPS, the major component of the bacterial surface. The O chain of *L. pneumophila* LPS is unusually hydrophobic (43, 80), a feature common to synthetic particles

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that macrophages do not deliver to lysosomes efficiently (17, 59). Also, genetic phase variation of one strain correlated LPS composition with virulence in an animal model (51). Finally, *L. pneumophila* organisms are classified into 15 serotypes based on their LPS structures (22); however, ~80% of clinical isolates are serogroup 1 (29). The preponderance of serogroup 1 LPS among disease strains is especially striking given the extraordinary plasticity of the *L. pneumophila* genome (12, 13). Therefore, we tested the hypothesis that serogroup 1 *L. pneumophila* alter their LPS composition to inhibit phagosome-lysosome fusion in macrophages.

MATERIALS AND METHODS

Cell and bacterial cultures. Bone marrow-derived macrophages were obtained from femurs of female A/J mice (Jackson Laboratory) and cultured as described elsewhere (39). The *L. pneumophila* wild-type (WT) strain, Lp02, is a virulent thymine auxotroph derived from the serogroup 1 Philadelphia 1 strain that replicates efficiently in the primary macrophages from humans or A/J mice (70). Two mutants derived from Lp02 whose trafficking in macrophages is aberrant were also analyzed (28). Defective for type IV secretion (76), PE *dotA* mutants persist but do not replicate in a compartment rich in LAMP-1 but lacking lysosomal markers (7, 39). Since *L. pneumophila* requires the two-component regulatory system LetA/S to induce factors in the PE phase that inhibit phagosome-lysosome fusion, neither E-phase WT nor PE-phase *letA* mutant bacteria inhibit phagosome maturation efficiently, and most are degraded in lysosomes (4). For use as an additional avirulent control, *E coli* K-12 (ATCC 10798) was obtained from the American Type Culture Collection. For each experiment, *L. pneumophila* was inoculated into ACES-buffered yeast extract (Bacto; BD Diagnostic Systems) broth supplemented with thymidine to 100 μg ml^{-1} (AYET), cultured overnight to the E phase, and subcultured for an additional day to obtain cells in the E phase (optical density at 600 nm [OD_{600}] of 0.5 to 2.0) or the PE phase (OD_{600} of 3 to 4.2). PE-phase cells were analyzed <4 h after the cell density plateau, a period when the WT and *dotA* mutants were highly motile (11). CFU were enumerated on ACES-buffered charcoal-yeast extract agar supplemented with thymidine to 100 μg ml^{-1} (CYET).

SDS-PAGE and Western analysis of LPS. LPS prepared from PE-phase WT or *letA* mutant cultures by the method of Jurgens and Fehrenbach (40) was treated with proteinase K and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% matrix and stained with silver by the method of Dubray and Bezard that is optimal for carbohydrate analysis (21). Purified *Escherichia coli* O55:B5 LPS (Sigma) served as a control. Western analysis was performed with monoclonal antibody (MAb) 3/1 (31) after separation of 2 μg of LPS per lane in a 12% polyacrylamide gel and blotting onto nitrocellulose filter membranes as described previously (47). To stain proteins preferentially, the Silver Staining Plus protocol (Bio-Rad) was followed.

LPS yield. To determine the concentration of membrane vesicle preparations, LPS was quantified by the purpald dye colorimetric assay (48), which detects the two units of 3-deoxy-D-manno-octulosonic acid (KDO) that are present in the *L. pneumophila* LPS molecule (80). Purified KDO (Sigma) served as a standard, and the concentration of vesicles was expressed as the millimolar concentration of the LPS, considering there are two molecules of KDO per LPS molecule.

Lectin agglutination. All lectins (BSA, LOA, TP, WGA, UEA, VVA, PNA, ConA, LPA, BS-1, HAA, SBA, MMA, SNA, SJA, VAA, MPA, PTA, and AAA) were diluted in buffer as recommended by the supplier (E-Y Laboratories). Agglutination reactions were performed as described previously (38) in round-bottom microtiter wells. For the *Limulus polyphemus* lectin (LPA) agglutination experiments, the bacteria were collected by centrifugation at 5,000 × *g* for 5 min at the indicated culture densities and then resuspended in a buffer of 100 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 10 mM $CaCl_2$. To remove broth components, this step was repeated twice and then cell suspensions were adjusted to OD_{600} of 0.85 in buffer. To each test well, 50 μl of cell suspension was added to 50 μl of each lectin at either 240, 160, 80, 40, or 20 μg ml^{-1}, and then the samples were agitated on a rotary shaker at room temperature for 1 h. To monitor nonspecific aggregation, which was observed after longer incubations, two negative control samples that contained 50 μl of buffer and 50 μl of either lectin or cell suspension were analyzed in parallel.

Infection of macrophages. Infectivity is a gauge of the ability of *L. pneumophila* to contact, enter, and survive inside macrophages during a 2-h incubation and thus reflects evasion of lysosomal degradation (11). In brief, macrophages were infected in RPMI-fetal bovine serum (FBS) plus 100 μg of thymidine ml^{-1} (RPMI/FBS/Thy) with *L. pneumophila* strains at a multiplicity of infection of 3 by an initial centrifugation at 400 × *g* for 10 min and then incubated for an additional 10 min at 37°C, washed three times with warm RPMI to remove extracellular microbes, and incubated for an additional 110 min. The infectivity was calculated as follows: [(cell-associated CFU at 2 h)/(CFU added at 0 h)] × 100.

Hexadecane affinity. Bacterial attachment to hydrocarbons was performed with *N*-hexadecane essentially as described by Rosenberg et al. (60). Cultures of the densities indicated were adjusted to an OD_{600} of 0.2 and a final volume of 6 ml in 15-ml polystyrene tubes. The cells were collected by centrifugation at 5,000 × *g* for 5 min and then resuspended in 6 ml of phosphate-buffered saline (PBS; pH 7.4; Gibco). After the addition of 1 ml of hexadecane (Sigma), each sample was gently agitated at room temperature for 10 min and then held stationary for 10 min at room temperature to allow the aqueous and hydrocarbon phases to partition. The percentage of bacteria that remains in the aqueous phase after agitation with hexadecane was calculated as (100 − the OD_{600} of the cell suspension after agitation/OD_{600} of the cell suspension before agitation) × 100.

Enzyme-linked immunosorbent assay (ELISA) of LPS composition. Cells (400 μl) of different growth phases obtained from cultures of the OD_{600} indicated were collected by centrifugation at 5,000 × *g* for 15 min and then resuspended in 400 μl of PBS. In addition, the supernatants were transferred to another tube, clarified by centrifugation at 14,000 × *g* for 15 min and then 300 μl was removed for further analysis. The cells and the supernatant samples were each separately incubated at 95°C for 10 min and diluted to an equivalent OD_{600} of 0.2, and then 50 μl of each sample was transferred to 96-well microtiter plates, followed by incubation at 4°C overnight in a humidified chamber. Wells were washed three times with PBS-Tween 0.05% (PBS-T), and then samples were inhibited with 75 μl of 10% fetal calf serum in PBS-T (PBS-T-FCS) for 1 h at 37°C, washed once with PBS-T, and then incubated 90 min at 37°C with 50 μl of primary antibody (MAb 3/1 or MAb 8/5, an antibody that recognizes the core region of all serogroup 1 strains) in PBS-T-FCS. After three washes with PBS-T, the samples were incubated with anti-mouse secondary antibody conjugated to horseradish peroxidase diluted in PBS-T-FCS for 90 min at 37°C, washed three times with PBS-T, and developed with 50 μl of *o*-phenylenediamine/H_2O_2, and then the absorbance at 492 nm was determined.

Membrane vesicle purification. Membrane vesicles were isolated from bacterial culture supernatants according to a protocol adapted from Horstman and Kuehn (33). *E. coli* K-12 was cultured as described previously (32, 33). A colony of *L. pneumophila* was inoculated in 5 ml of AYET and incubated at 37°C overnight until reaching an OD_{600} of 2.0, subcultured into 50 ml of AYET overnight until reaching an OD_{600} of 2.0, and then subcultured into 500 ml of AYET in an Erlenmeyer flask and incubated overnight in an orbital shaker at 150 rpm until reaching an OD_{600} of 3.7, and then the cells were pelleted by centrifugation at 10,000 × *g* for 15 min. The culture supernatant was filtered through a membrane with 0.45-μm-pore-size pores, and the material from the supernatant was precipitated by the addition of ammonium sulfate to 70% final concentration, incubation at 4°C for 30 min, and then centrifugation at 10,000 × *g* for 15 min. The pellet was resuspended in PBS, and then ammonium sulfate was removed by dialysis against PBS (pH 7.4) overnight at 4°C. The samples were concentrated by using a filter that retains species >100 kDa (Amicon-Ultra; Millipore) and then adjusted to a volume of 7.6 ml with 45% Optiprep (Sigma). After transfer to a 38.1-ml ultracentrifuge tube, the samples were layered sequentially with the following Optiprep/PBS suspensions: 7.6 ml, 40%; 7.6 ml, 35%; 7.6 ml, 30%; and 7.6 ml, 25%. Density gradients were centrifuged at 100,000 × *g* for 3 h, and then fractions were removed sequentially from the top. Each fraction was dialyzed against PBS for >4 days, with changes of buffer every 12 h; concentrated by using a filter that retains species of >100 kDa (Amicon-Ultra, Millipore); and then adjusted to a final volume of 2 ml with PBS. In the 30% Optiprep fractions, the LPS yields were 2.4 mM (±1.1) for WT, 1.6 mM (±0.7) for *letA*, and 1.9 mM (±1.5) for *dotA* cells; the 35% Optiprep fractions contained 6.0 mM (±0.9), 3.7 mM (±2.4), and 2.2 mM (±1.0) LPS for the WT, *letA*, and *dotA* preparations, respectively, as calculated from four independent vesicle preparations.

Electron microscopy. After the removal of the Optiprep by dialysis against PBS, vesicles were concentrated by filtration with membranes of >100 kDa pore size and then resuspended in 10 μl of PBS. Aliquots were placed on 400-line/in. mesh grids, fixed with 1% glutaraldehyde, rinsed with 100 mM ammonium acetate, and visualized by negative staining with 1% uranyl acetate using a Philips CM-100 transmission electron microscope at the Microscopy and Image Analysis Laboratory of the University of Michigan Medical School. To calculate the size of the vesicles, digital images were captured on ITI IC-PCI frame grabber with Kodak 16 camera using AMT Advantage Software (version 2.25.5).

***Limulus polyphemus* lectin chromatography.** Two types of lectin affinity chromatography experiments were performed. The first approach compared the affinity of the LPA lectin for *L. pneumophila* PE-phase WT and PE-phase *letA* mutant bacteria. A mixture of 7×10^8 PE-phase WT kanamycin (Km)-sensitive and 7×10^7 PE-phase *letA* Km-resistant bacteria was incubated with 1 ml of LPA matrix for 2 h at 4°C. The mixture was poured into a chromatography column, and bacteria were eluted at 0.2 ml/min in a final volume of 5 ml. The CFU counts obtained before and after the column passage were determined by incubating aliquots on CYET agar with or without Km. The second approach compared vesicles released by PE-phase WT and *letA* mutant bacteria (see Fig. 4C). Material obtained from either the WT or mutant 30% Optiprep fraction was adjusted to a final concentration of 5 mM LPS in 1 ml of PBS (pH 7.4) with 5 mM Ca^{2+} and Mg^{2+}, added to a 1-ml suspension of LPA lectin covalently linked to agarose beads (EY Laboratories), and then rocked gently at 4°C for 3 h. Next, the material was loaded into a chromatography column (15 ml) and washed with 4 ml of PBS. Material was then eluted from the lectin matrix by a sequential series of washes at a flow speed of 35 μl per min^{-1} with 1-ml aliquots of PBS that contained either 50, 100, 150, or 200 mM *N*-acetyl-glucosamine, a sugar that inhibits *L. pneumophila* agglutination by LPA. Each fraction of eluate was dialyzed against PBS, and then its LPS concentration was quantified by using the purpald assay.

Phagosome-lysosome fusion. Delivery of phagocytosed polystyrene beads to lysosomes was quantified by fluorescence microscopy (68). To label the lysosomal compartment by endocytosis, macrophages were incubated at 37°C for 1 h with Texas Red ovalbumin as described previously (71) or with 5 mg of fluorescein-dextran (FDx) ml^{-1} (10,000 molecular weight; Molecular Probes) per ml of RPMI/FBS/Thy that contained penicillin and streptomycin (P/S). To allow the dye to traffic to the lysosomes, the macrophages were washed three times with warm RPMI/FBS/Thy and then incubated with RPMI/FBS/Thy for an additional 30 min. Polystyrene beads 1 μm in diameter (Polysciences) were added at a ratio of three beads per macrophage in the absence or presence of 5 mM LPS of membrane vesicles in RPMI/FBS/Thy/P/S. To synchronize phagocytosis, the microtiter plates were centrifuged at $400 \times g$ at 4°C for 10 min, and then the plates were incubated 10 min on a 37°C water bath for bead internalization. Next, extracellular vesicles and beads were removed by washing the monolayers twice with warm RPMI and then the samples were incubated for an additional 110 min at 37°C. To ensure any extracellular beads were eliminated from the analysis, macrophages whose lysosomes contained FDx were washed twice with warm PBS and once with PBS at 4°C and placed on ice for 5 min to inhibit endocytosis, and then the extracellular beads were labeled by incubating the cultures with 0.5 mg ml^{-1} Texas Red-dextran 10,000 MW (TRDx; Molecular Probes) per ml of PBS for 1 min at 4°C as described previously (59). After the samples were washed three times with warm PBS, the cells were fixed with periodate-lysine-paraformaldehyde (39) containing 4.5% sucrose, mounted onto slides, and examined by fluorescence microscopy. By this method, three populations of beads could be distinguished: beads that stained with TRDx were judged to be extracellular and were disregarded; beads that colocalized with FDx were scored as lysosomal; and beads that did not colocalize with either FDx or TRDx identified phagosomes whose maturation to phagolysosomes was inhibited. The ability of macrophages to bind or phagocytose beads was not appreciably affected by bacterial vesicles (data not shown). For each sample, at least 100 intracellular beads were scored in duplicate in at least three independent experiments.

The ability of vesicles attached to beads to inhibit phagosome-lysosome fusion was also quantified. To decorate beads with vesicles, protein G-carboxylate spheres, 1 μm in diameter (Polysciences), were first bound to MAb 3/1, an MAb specific for epitopes on the O-acetylated LPS of strain Philadelphia 1 (31). ELISA experiments verified that MAb 3/1 has similar affinity for PE-phase WT, *letA*, and *dotA* membrane vesicles (J. H. Helbig, unpublished data). In addition, although the MAb 3/1 epitope becomes less prevalent on vesicles shed in the PE phase (see Fig. 3B), the 185-nm-diameter vesicles retain sufficient reactivity for coupling to protein G-beads, as judged by SDS-PAGE (see Fig. 8B). Protein G-carboxylate beads were diluted to 1×10^5 to 10×10^5 per ml of PBS (pH 7.4) supplemented with 100 μg of BSA per ml and then incubated with 15 μg of MAb 3/1 hybridoma supernatant ml^{-1} for 1 h at 4°C. As a control for specificity, vesicles obtained from *E. coli* K-12 were bound to protein G-beads coated with 15 μg of Ab13626 ml^{-1}, an antibody specific for the K antigen (Abcam). Next, beads coupled to each MAb were washed five times in PBS and then incubated for at least 2 h with vesicles from the 30% Optiprep fractions at concentrations that ranged from 0.5 to 15 mg of LPS ml^{-1}. Vesicle-decorated beads in RPMI/FBS/Thy were added to macrophages whose lysosomes contained FDx; samples were then centrifuged at $400 \times g$ for 5 min and incubated at 37°C for 2 h. Finally, extracellular beads were stained for 5 min with TRDx at 4°C, and then phagosome-lysosome fusion was scored as described above. To test whether the vesicles were toxic to macrophages, vesicles from *E. coli* K-12 and from WT, *letA*, and *dotA L. pneumophila* strains were incubated with macrophages, and then the cell viability was quantified by the capacity of macrophages to reduce Alamar Blue (11).

To identify lysosomes by an independent method, we used Lysotracker, an acidotropic probe that accumulates in lysosomes. Macrophages were fed with the decorated beads for 1 h and then incubated with Lysotracker (1 μM; Invitrogen) in RPMI/FBS for 30 min. The cells were washed and incubated in fresh medium for 30 min before scoring. As a third marker for lysosomes, the soluble protease cathepsin D was localized by using goat anti-cathepsin D antibody as described previously (39). For each sample, at least 100 intracellular beads were scored in duplicate in at least two independent experiments by using vesicles obtained from independent purifications.

RESULTS

As *L. pneumophila* differentiates, its LPS profile changes. *L. pneumophila* broth cultures display many of the replicative and transmissive traits that are observed during growth in macrophages (56). In the PE phase, *L. pneumophila* inhibits phagosome-lysosome fusion, but E-phase cells do not (11, 39). To evaluate whether the developmental regulation of LPS could affect the fate of *L. pneumophila* in macrophages, we first tested whether transmissive PE-phase and replicative E-phase cells have different LPS profiles. In parallel, we analyzed the LPS profiles of PE-phase *letA* mutants, which are locked in the replicative form, and PE-phase *dotA* mutants, which traffic to a nontoxic late-endosomal compartment (Fig. 1A) (28, 39). A number of species of high molecular mass (45 to 78 kDa) were observed in multiple preparations of transmissive PE-phase WT and *dotA* LPS that were absent from replicative WT E-phase and PE-phase *letA* samples (Fig. 1B and C). Therefore, to a first approximation, LPS appeared to be another factor of *L. pneumophila* that is subject to developmental regulation.

As experimental tools to analyze growth phase regulation of *L. pneumophila* surface properties, we applied two other assays. Given that developmentally regulated lipophosphoglycan modifications affect *Leishmania* affinity for the lectin ricin (36, 62), we tested whether transmissive and replicative legionellae could be differentiated by lectin binding. Among a panel of 20 lectins whose carbohydrate specificities differ, *Limulus polyphemus* hemagglutinin (LPA) was found to mediate a robust, reproducible, and specific agglutination of transmissive *L. pneumophila* (Fig. 2A). At a concentration of 160 μg ml^{-1}, the lectin LPA specifically agglutinated cells of the transmissive phenotype (PE-phase WT and PE-phase *dotA* cells), but not replicative-phase cells (E-phase WT, PE- or E-phase *letA*, or E-phase *dotA* bacteria). Moreover, *L. pneumophila* binding to LPA correlated with its capacity to infect macrophages efficiently (Fig. 2A). As expected for this sialic-acid specific lectin (58), agglutination of *L. pneumophila* was inhibited by either 5 mM *N*-acetylneuraminic acid (sialic acid) or 50 mM *N*-acetyl-glucosamine (data not shown). The O polysaccharide of *L. pneumophila* LPS is a homopolymer of legionaminic acid, a sugar whose structure is similar to sialic acid (43). Accordingly, the lectin LPA most likely binds the O antigen expressed by transmissive-phase cells.

Affinity chromatography confirmed that LPA preferentially binds a developmentally regulated species on the surface of *L. pneumophila*. When a mixed suspension of 1 *letA* Km^r–10 WT Km^s bacteria was passed over an LPA column, the eluates were enriched for *letA* mutant cells. After one passage, the eluate

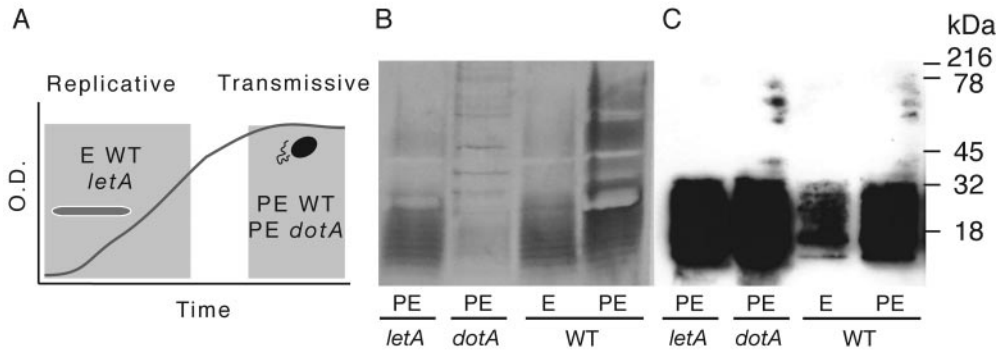

FIG. 1. The *L. pneumophila* LPS profile is developmentally regulated. (A) At the transition from E phase (OD_{600} of <2.0) to PE phase (OD_{600} of 3.0 to 4.0), WT and *dotA* mutant *L. pneumophila* differentiate to the transmissive form but *letA* regulatory mutants do not. (B) Equal amounts of protein-free LPS obtained from PE-phase *letA* and *dotA* mutants and E- and PE-phase WT *L. pneumophila* were separated by SDS–12% PAGE and silver stained. (C) Western analysis of *L. pneumophila* LPS samples using MAb 3/1. Molecular masses of standard protein markers are indicated at the right.

contained 10 *letA*:1 WT cells; after passage of the 10 *letA*:1 WT cell suspension, all of the eluted bacteria were *letA* mutants, as judged by their Km^r (>200 CFU scored; data not shown).

Bacterial adherence to hydrocarbons is another simple and rapid technique to separate cell populations according to their surface hydrophobicity and ionic character (1, 60). When aqueous suspensions of *L. pneumophila* were agitated with the hydrocarbon hexadecane, most PE-phase WT cells remained in the aqueous phase, whereas the majority of E-phase WT and E- and PE-phase *letA* mutants partitioned with the hydrocarbon, a pattern that correlated with their ability to survive ingestion by macrophages (Fig. 2A). Again, *dotA* type IV secretion mutants exhibited the same pattern as WT, indicating that hexadecane binding correlates with the capacity to inhibit delivery to lysosomes but not with the ability to avoid compartments rich in the late endosomal and lysosomal protein LAMP-1 (39).

As an additional test of the dynamic properties of the *L. pneumophila* surface, its composition was analyzed as transmissive phase broth cultures differentiated to the replicative form. Initially, PE-phase bacteria bound lectin but not hexadecane, and they infected macrophages efficiently; by 10 h after subculture into fresh broth, the WT bacteria displayed the opposite pattern (Fig. 2B). Thus, the ability of *L. pneumophila* to infect macrophages efficiently correlated with growth-phase-dependent modifications to its surface composition.

To investigate in more detail how *L. pneumophila* alters its surface during development, LPS composition was analyzed by ELISA with two MAbs. MAb 8/5 binds the LPS core that is common to all serogroup 1 strains (30), and MAb 3/1 recognizes an epitope that is associated with virulence (29, 49). Since many gram-negative bacteria shed LPS during replication (9), we analyzed both the cell surface and the culture supernatants by ELISA. The prevalence of the MAb 8/5 and 3/1 epitopes on the surface of *L. pneumophila* was constant throughout the life cycle (Fig. 3A). In contrast, relative to the MAb 8/5 epitope, the structure recognized by MAb 3/1 decreased markedly on the LPS that accumulated in the supernatant of PE-phase broth cultures (Fig. 3B). Therefore, concomitant with developmentally regulated changes to its surface character, *L. pneu-*

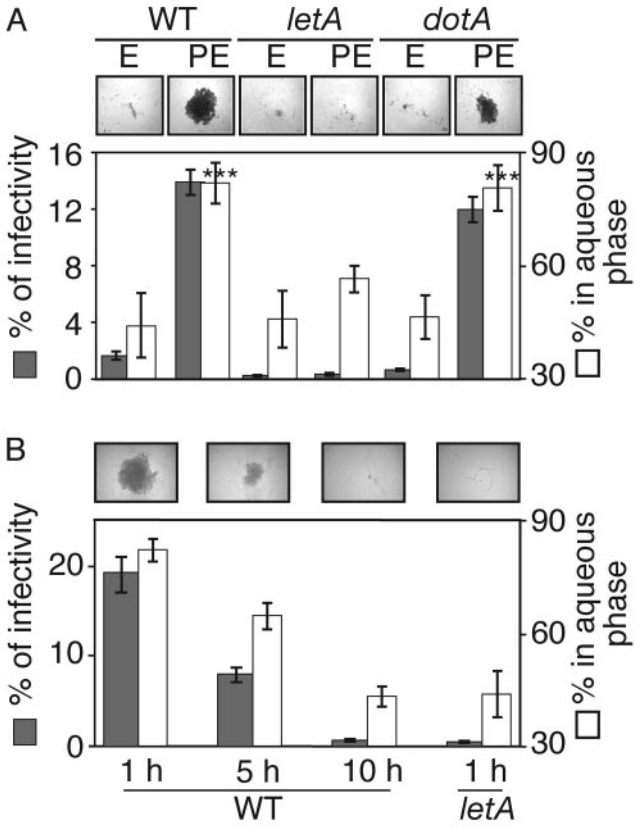

FIG. 2. Capacity of *L. pneumophila* to infect macrophages correlates with its surface properties. (A) Samples of E- or PE-phase WT, *letA* mutant, or *dotA* mutant *L. pneumophila* cultures were agitated with 160 μg of *Limulus polyphemus* lectin ml^{-1} for 1 h and then photographed. In parallel, samples from the same cultures were incubated for 2 h with macrophages, and then the fraction that entered and survived intracellularly was determined (▨). Another aliquot of each culture was analyzed to determine the fraction of bacteria that remained in the aqueous phase after agitation with hexadecane (□). (B) PE-phase WT and *letA* mutant bacteria were subcultured into fresh media and then incubated for an additional 1, 5, or 10 h before assaying agglutination by *Limulus polyphemus* lectin (photographs), infection of macrophages (▨), and repulsion from hexadecane (□). The mean percent ± the standard deviation was calculated from four independent assays.

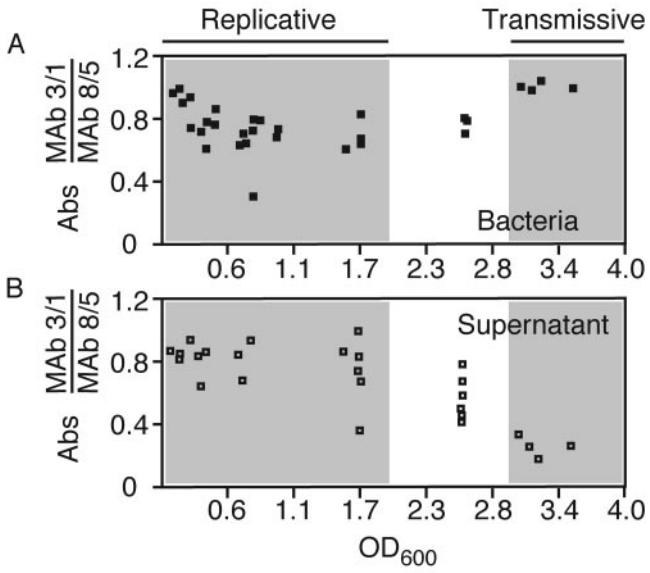

FIG. 3. *L. pneumophila* developmentally regulates an LPS epitope. Samples obtained from cultures at the densities shown (OD_{600}) were separated by centrifugation, and then the pelleted bacteria (A) and the supernatants (B) were bound to wells and probed with either MAb 8/5, which recognizes the core of LPS of all serogroup 1 strains, or MAb 3/1, which recognizes an LPS epitope associated with virulence. The amount of each MAb bound was then quantified by probing with a secondary antibody linked to horseradish peroxidase, and the results are expressed as a ratio. The culture densities at which replicative- and transmissive-phase cells are obtained are indicated by shading.

mophila alters the composition of LPS that is shed from its surface.

Legionella sheds membrane vesicles whose composition is regulated. One mechanism that could contribute to the dynamic character of the *L. pneumophila* surface and to the accumulation of LPS in culture supernatants is the release of outer membrane vesicles (9). Like other gram-negative bacteria, *L. pneumophila* does form small vesicles on its outer membrane (9, 23). Furthermore, after ingestion by macrophages, *L. pneumophila* LPS is detectable both in and near its vacuole (16). The pathogen also appeared to shed outer membrane material during growth in broth, since, compared to E-phase samples, WT PE-phase culture supernatants induced more robust coagulation of *Limulus polyphemus* lysates (E-Toxate), an indicator of LPS (data not shown). Therefore, to test whether *L. pneumophila* shed surface glycoconjugates in vesicle form, we analyzed material obtained from culture supernatants according to its buoyant density using a protocol adapted from Horstman and Kuehn (33).

Membrane vesicles obtained from PE-phase WT, *letA* mutant, and *dotA* mutant *L. pneumophila* and the reference strain *E. coli* K-12 (32, 33) were analyzed by electron microscopy, by SDS-PAGE, and by quantifying KDO (3-deoxy-*d*-manno-2-octulosomic acid), a carbohydrate component of *L. pneumophila* LPS (80). *L. pneumophila* vesicles were abundant in fractions 3 and 4, corresponding to 30%, and in fractions 5 and 6, corresponding to 35% (Fig. 4A and B). The average diameters of vesicles in these four fractions were 186 ± 83, 186 ± 83, and 181 ± 104 nm for WT, *letA*, and *dotA* vesicles, respectively ($n > 75$), whereas *E. coli* K-12 vesicle diameters averaged 124 ± 74 nm ($n > 75$).

To determine whether the vesicles contained developmentally regulated carbohydrates characteristic of transmissive *L. pneumophila*, their affinity for the lectin LPA was analyzed by chromatography. After binding to the lectin matrix, the peak of WT vesicles was released once the concentration of the eluate was increased to 150 mM *N*-acetylglucosamine (Fig. 4C). The *letA* mutant vesicles bound the LPA lectin less avidly, since most were released by 100 mM *N*-acetylglucosamine (Fig. 4C). Therefore, membrane vesicles exhibited a similar developmentally regulated lectin binding as observed for intact *L. pneumophila* (Fig. 2). Since legionaminic acid of the *L. pneumophila*

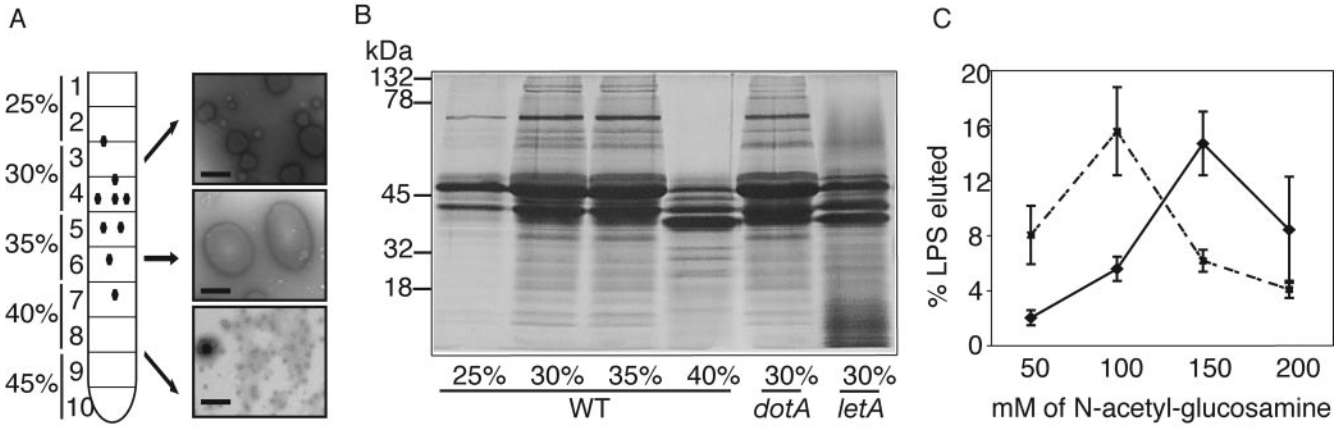

FIG. 4. The properties of *L. pneumophila* vesicles are developmentally regulated. (A) Membrane vesicles shed by WT *L. pneumophila* broth cultures were isolated by their buoyant density on an Optiprep gradient and visualized by negative staining. Bar, 100 nm. (B) Samples of vesicles obtained from the 25, 30, 35, or 40% Optiprep fractions (WT) or the 30% Optiprep fraction (*letA*, *dotA*) were separated by SDS–12% PAGE and visualized by Silver Staining Plus (Bio-Rad). The samples (20 μl) were normalized to 5 mM LPS equivalents as described in the text. (C) To compare the affinity of WT and mutant *L. pneumophila* membrane vesicles for *Limulus polyphemus* lectin by chromatography, the fraction of LPS eluted by the *N*-acetylglucosamine concentration shown for WT (solid line) or *letA* vesicles (dashed line) was determined by quantifying LPS in each fraction relative to the quantity initially loaded on the lectin matrix. The data shown were calculated from three independent experiments.

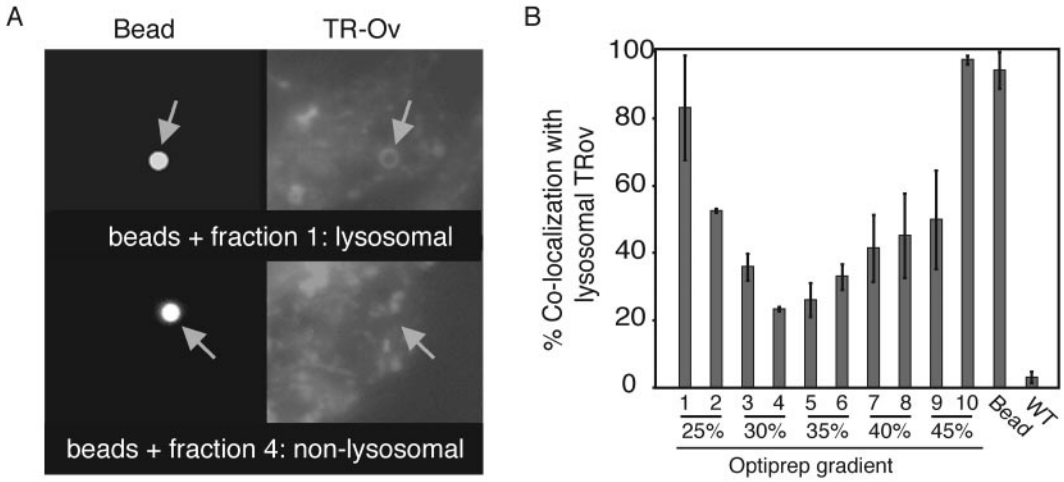

FIG. 5. Inhibition of phagosome-lysosome fusion by material obtained from PE-phase WT *Legionella* culture supernatants. (A) Representative fluorescence micrographs 1 h after macrophages whose lysosomes contain Texas Red-ovalbumin (TRov) were fed polystyrene beads (beads) in the presence of the fractions indicated. (B) Colocalization of beads with lysosomes was determined as illustrated in panel A. Beads alone serve as the positive control for phagosome-lysosome fusion (bead); PE-phase WT *L. pneumophila* served as the positive control for the inhibition of phagosome-lysosome fusion (WT). Fractions 1 and 2 correspond to 25%, fractions 3 and 4 correspond to 30%, fractions 5 and 6 correspond to 35%, fractions 7 and 8 correspond to 40%, and fractions 9 and 10 correspond to 45% Optiprep.

O antigen is most likely the ligand for the lectin LPA (80), the results of the lectin affinity experiments (Fig. 2A and 4C) and the LPS ELISA analysis (Fig. 3) are consistent; each supports the interpretation that *L. pneumophila* regulates the composition of the LPS that is shed from its surface.

Membrane vesicles inhibit phagosome-lysosome fusion independently of type IV apparatus. As a first test of whether membrane vesicles shed by WT *L. pneumophila* contribute to virulence, the capacity of each fraction to inhibit phagosome-lysosome fusion was analyzed. Macrophages whose lysosomes were labeled by endocytosis of Texas Red-ovalbumin were fed polystyrene beads in the presence or absence of vesicles (5 mM LPS, final concentration); after 1 h, colocalization of beads with lysosomes was evaluated by fluorescence microscopy (Fig. 5A). Fractions 1 and 10 had little effect on delivery of beads to lysosomes, whereas several other fractions markedly inhibited phagosome-lysosome fusion (Fig. 5B). Therefore, material that cofractionates with transmissive *L. pneumophila* membrane vesicles inhibits phagosome-lysosome fusion.

To analyze whether the inhibitory activity was mediated by a mechanism that requires either developmental regulation by LetA/S or type IV secretion, we pooled pairs of the vesicle fractions obtained from either WT, *letA* mutant, or *dotA* mutant *L. pneumophila* or *E. coli* K-12 and then compared their ability to inhibit the delivery of beads to lysosomes that had been prelabeled with FDx. Macrophages efficiently delivered polysty-

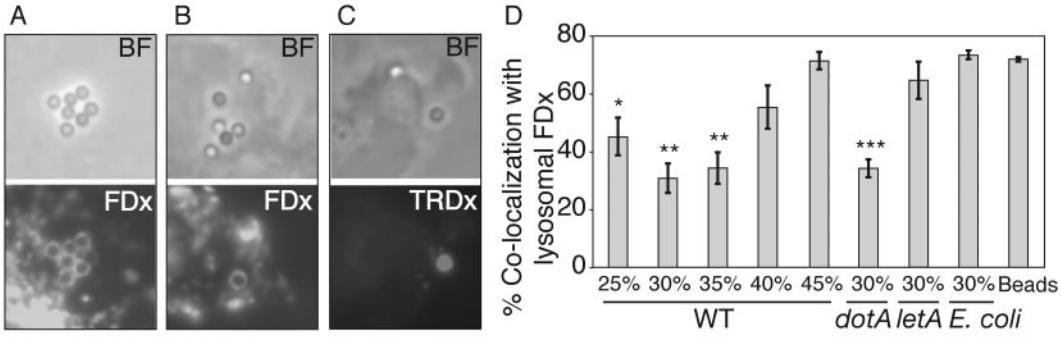

FIG. 6. *L. pneumophila* vesicles inhibit the delivery of phagocytosed particles to lysosomes. Beads were incubated with *letA* mutant vesicles (A) or with PE-phase WT vesicles (B), and then their colocalization with lysosomal FDx was analyzed by bright-field (BF) or fluorescence microscopy. Extracellular beads were stained with Texas Red-dextran (TRDx; C) and were not scored. The fraction of beads internalized with the populations of vesicles indicated that were delivered to lysosomes was scored by fluorescence microscopy (D) as shown in panels A to C. WT vesicles were collected from 25, 30, 35, 40, and 45% Optiprep fractions; *letA* mutant, *dotA* mutant, and K-12 *E. coli* vesicles from the 30% Optiprep fraction and untreated beads were analyzed in parallel. The percentage of beads that colocalize with lysosomal FDx was calculated as (the FDx-negative beads/the total number of TRDx-negative beads) × 100. Each experiment was performed more than four times; in each, >100 beads were scored in triplicate samples. The mean percent ± the standard deviation was calculated from four independent assays. Asterisks indicate statistically significant differences compared to beads at 2 h postinfection as determined by a two-tailed Student *t* test: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

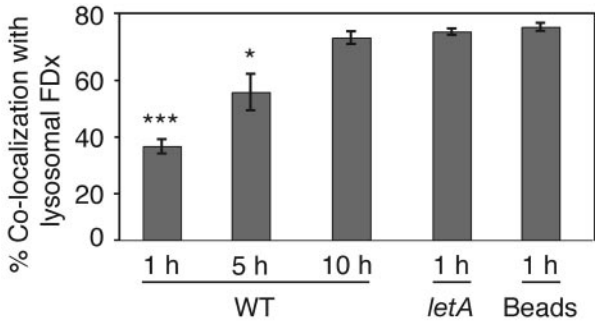

FIG. 7. *L. pneumophila* vesicles inhibit phagosome-lysosome fusion temporarily. Macrophages whose lysosomes were labeled fluorescently with FDx were incubated for 30 min with or without a suspension of WT or *letA* mutant vesicles, and then the macrophages were washed and incubated for an additional 1, 5, or 10 h before the addition of beads. After a 30-min period of phagocytosis, the fate of the beads was determined microscopically. Each experiment was performed more than three times, and >100 beads were scored in each experiment. Asterisks indicate statistically significant differences compared to beads at 1 h postinfection as determined by a two-tailed Student *t* test: ***, $P < 0.001$; and *, $P < 0.05$.

rene beads to the lysosomal compartment, since 72% of their phagosomes had acquired lysosomal FDx by 1 h (Fig. 6D). In contrast, when macrophages ingested beads in the presence of WT vesicles obtained from fractions 3 and 4 (30%) or 5 and 6 (35%), only 30 ± 5 and 34 ± 5 of the beads were delivered to lysosomes, respectively (Fig. 6B and D). The equivalent of 5.0 mM LPS from the WT 30% Optiprep fraction was sufficient to achieve maximal inhibition, based on the results of a dose-response experiment. When suspended with a 10.0, 5.0, 0.5, or 0.05 mM concentration of LPS equivalents of vesicles obtained from WT supernatants, 22, 24, 45, or 55% of the beads were delivered to lysosomes, respectively (data not shown).

The inhibitory activity appeared to be specific to *L. pneumophila*, developmentally regulated, and independent of type IV secretion. Vesicles obtained from the 30% Optiprep fraction of *E. coli* K-12 culture supernatants did not affect macrophage membrane traffic, since 73% ± 1% of the beads trafficked to phagolysosomes (Fig. 6D). Likewise, none of the five fractions obtained from *letA* mutant culture supernatants inhibited phagosome-lysosome fusion (Fig. 6A and D and data not shown). Furthermore, the *L. pneumophila* inhibitory activity did not require type IV secretion, since only 34% of the beads that were presented with vesicles from the *dotA* mutant 30% Optiprep fraction colocalized with lysosomes (Fig. 6D). Inhibition of phagosome-lysosome fusion by vesicle fractions was not due to toxicity. After a 1-h exposure to vesicles (equivalent to 20 mM LPS) obtained from either the 25, 30, 35, or 40% Optiprep fractions of either *L. pneumophila* or *E. coli* supernatants, macrophage viability was 100% in all cases, as judged by Alamar Blue reduction (data not shown).

Furthermore, inhibition of phagosome maturation was temporary, as judged by the results of a pulse-chase experiment (Fig. 7). Macrophages were incubated with 5 mM LPS equivalents from the WT 30% Optiprep fraction for 30 min and then cultured in fresh medium for 1, 5, or 10 h before being fed polystyrene beads. As expected, vesicles inhibited the maturation of phagosomes that formed 1 h after exposure to the inhibitory fraction, since only 31% ± 4% colocalized with the lysosomal marker. However, with time, macrophages gradually regained the ability to deliver beads to lysosomes. By 5 h after treatment with the vesicle preparation, 50% ± 17% of newly formed phagosomes matured into phagolysosomes; by 10 h, 71% ± 6% did so, an efficiency comparable to that of macrophages that were fed beads either alone (76% ± 0.5% colocalization with FDx) or beads suspended with vesicles shed by PE-phase *letA* mutants (74% ± 2% FDx positive; Fig. 7). Therefore, membrane vesicles released by transmissive *L. pneumophila* stall phagosome maturation; not until 5 to 10 h after phagocytosis do macrophages deliver the beads to the lysosomes.

When attached to beads, membrane vesicles inhibit phagosome-lysosome fusion. We next tested whether the inhibitory activity of membrane vesicles is sufficiently rapid and robust to inhibit fusion of its own phagosome with lysosomes, as occurs during natural *L. pneumophila* infections (35). For this purpose, vesicles obtained from the 30% Optiprep fraction were affixed to beads via a protein G- MAb 3/1 antibody linkage, and then the beads were fed to macrophages. Whereas 72% of beads that were untreated or decorated with *E. coli* K-12 vesicles colocalized with the lysosomal marker fluorescein-dextran, only 23.5% ± 1.3% or 27.2% ± 1.7% did so when bound to vesicles obtained from PE-phase WT or *dotA* mutant bacteria, respectively (Fig. 8A). Conversely, macrophages delivered 68% ± 2% of beads coated with PE-phase *letA* vesicles (30% Optiprep fraction) to the lysosomes as they localized with the FDx. Similar results were obtained when phagosome maturation was analyzed by using two other lysosomal markers, Lysotracker and cathepsin D. Whereas 75% ± 5% and 77% ± 3% of beads that were untreated or decorated with PE-phase *letA* mutant vesicles, respectively, colocalized with the lysosomal marker Lysotracker, only 28% ± 2% or 25% ± 5% did so when bound to vesicles obtained from PE-phase WT or *dotA* mutant bacteria, respectively (Fig. 8A). Consistent with this pattern, a cathepsin D-specific antibody stained 77% ± 2% and 76% ± 7% of beads that were untreated or decorated with PE-phase *letA* mutant vesicles, respectively, but only 24% ± 5% or 30% ± 5% did so when bound to vesicles obtained from PE-phase WT or *dotA* mutant bacteria, respectively (Fig. 8A).

To verify that MAb 3/1 was sufficient to coat beads with comparable amounts of WT and mutant membrane vesicles, the material attached to the beads was separated by SDS-PAGE and then silver stained by a protocol optimized for the visualization of carbohydrates (21). The concentration of WT and *letA* mutant material bound to the beads was similar, as judged by quantifying LPS by the purpald assay (data not shown) and by the comparable intensity of the silver-stained samples (Fig. 8B). Certain high-molecular-weight species consistently correlated with the ability to inhibit phagosome-lysosome fusion, since some bands that were present in the WT and *dotA* mutant membrane vesicle lanes were diminished in the *letA* vesicle samples (Fig. 8B). LetA/S-dependent high-molecular-weight species were more prominent when the vesicles were silver stained by using a protocol that is optimal for proteins (Fig. 4B). The identity and contribution to virulence of each of these species remains to be determined. Taken together, the data indicate that membrane vesicles released by

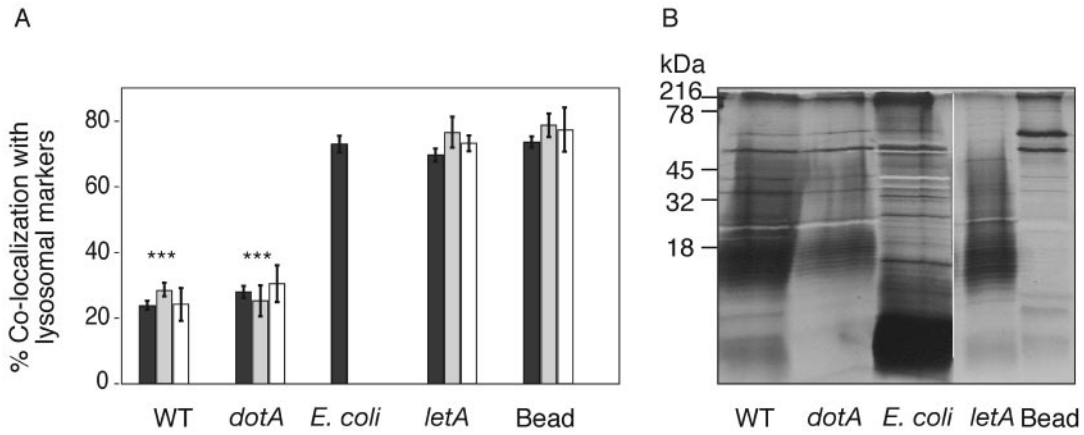

FIG. 8. The inhibitory activity and composition of *L. pneumophila* vesicles is coregulated. (A) Beads bound to MAb 3/1 were decorated with membrane vesicles from WT, *letA* mutant, or *dotA* mutant *L. pneumophila* or *E. coli* K-12 were assayed for the ability to inhibit phagosome-lysosome fusion. The delivery of beads to lysosomes was scored by fluorescence microscopy using three lysosomal markers: FDx (■), cathepsin D (▨), and Lysotracker (□). The experiment was performed three times, and >100 beads were scored in each trial. Differences in colocalization with the three lysosomal markers at 2 h postinfection were considered statistically significant when WT or *dotA* vesicles attached to beads were compared to untreated beads (two-tailed Student *t* test; ***, $P < 0.001$). (B) An aliquot of each sample analyzed in panel A was analyzed by SDS-PAGE and silver stained by a protocol optimal for carbohydrates. Each sample contained 3×10^6 beads, except the *letA* mutant, which contained 3×10^5 beads. As a reference for reagent-specific proteins, an excess of beads bound to protein G (Bead) was analyzed in parallel, and size standards are indicated.

transmissive *L. pneumophila* can inhibit the maturation of their surrounding phagosome by a mechanism that is independent of type IV secretion.

DISCUSSION

Here we provide evidence that membrane vesicles shed by *L. pneumophila* can inhibit phagosome-lysosome fusion in primary mouse macrophages. As it differentiates to the transmissive form, *L. pneumophila* gains the capacity to inhibit phagosome maturation and concomitantly alters the composition of the glycoconjugates on its surface (Fig. 1, 2, and 3). The composition of vesicles shed by *L. pneumophila* is also developmentally regulated (Fig. 3, 4, and 7), and their inhibitory activity is independent of type IV secretion (Fig. 5, 6, and 8). Therefore, membrane vesicles released by gram-negative pathogens can not only trigger an inflammatory response (10), deliver toxin to host cells (5, 32, 33), or carry quorum sensing signals (54) but also inhibit phagosome-lysosome fusion in macrophages.

Transmissive *L. pneumophila* have to meet two challenges: to inhibit their immediate delivery to lysosomes and to adapt to the vacuolar environment. Once transmissive *L. pneumophila* have established a protective vacuole removed from the lysosomal pathway, the CsrA repressor coordinately downregulates expression of transmission traits, generating a replicative-phase cell (57) with a distinct surface composition (Fig. 1 and 2). How long *L. pneumophila* remains isolated from the endosomal pathway varies in different host cells (64, 67, 77, 79). Multiple host parameters likely affect the potency of virulence factors, including their rate of endosomal traffic. Furthermore, *L. pneumophila* phagosome biogenesis is highly dynamic. As phagosomes mature, macrophage membrane proteins are sorted, rapidly generating a vacuole membrane whose composition is markedly different from that of the plasma membrane (15). Within minutes, the vacuole is apparently recognized as cargo by the autophagy machinery (2, 3). Numerous smooth vesicles derived from the endoplasmic reticulum attach to the cytoplasmic face of the membrane (34), which thins, indicating a change in the phospholipid composition (72). Concomitantly, *L. pneumophila* sheds LPS and other material into the vacuole (16). Presumably, in A/J macrophages, the dynamic interactions between the phagosomal and early secretory pathway membranes dilutes or inactivates the bacterial inhibitors of phagosome maturation, as the capacity of lysosomal membranes to merge with the vacuole is slowly restored (67). By

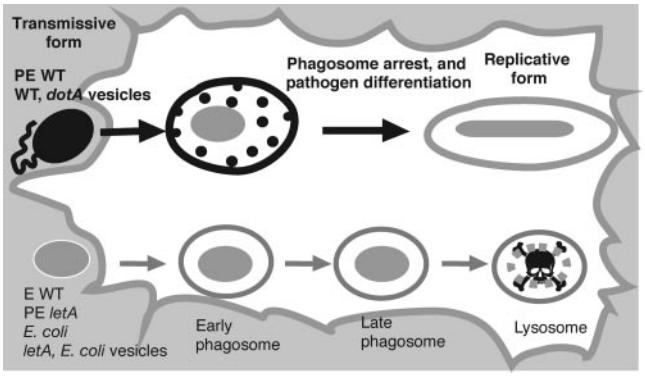

FIG. 9. Working model for the contribution of membrane vesicles to *L. pneumophila* growth in mouse macrophages. Transmissive *L. pneumophila* releases LPS-rich outer membrane vesicles that intercalate into the phagosomal membrane and inhibit its fusion with lysosomes. Within the stalled vacuole, *L. pneumophila* differentiates to the replicative form, downregulating expression of its transmission traits and inducing enzymes that modify its LPS. As a consequence, after several hours, the block to phagosome-lysosome fusion dissipates, and the surface properties of intracellular *L. pneumophila* change to promote replication within the harsh vacuolar compartment of its host.

analogy to *Salmonella* spp. (25), *Leishmania* spp. (20, 66), and mycobacteria (6, 24), we propose the following working model (Fig. 9). *L. pneumophila* sheds LPS-rich membrane vesicles that intercalate into its phagosomal membrane, altering its biophysical properties (Fig. 2A). By shedding vesicles into the phagosome, transmissive *L. pneumophila* would not only inhibit fusion with lysosomes but also promote remodeling of its surface to the intracellular replicative form.

During its coevolution with predatory amoebae, *L. pneumophila* likely acquired multiple mechanisms to survive in phagocyte vacuoles, some of which are redundant (53, 61). Although the concept that *L. pneumophila* exploits proteins to inhibit the host endocytic fusion machinery holds great appeal, a toxin with such biochemical activity has not yet been identified for this or any other intracellular pathogen. On the other hand, several studies indicate that glycoconjugates affixed to or shed from the microbial surface are sufficient to perturb membrane fusion. For example, *Leishmania* spp. (20, 66), mycobacteria (6, 24), or beads of a particular surface composition (18, 59) are not readily delivered to lysosomes. Likewise, formalin-killed WT *L. pneumophila*, live *dot/icm* mutants (7, 39), or membrane vesicles attached to beads (Fig. 8A) can inhibit phagosome-lysosome fusion independently of secretion machineries. Accordingly, we propose that, prior to inheritance of its type IV secretion machinery, ancestral *L. pneumophila* acquired a mechanism to exploit its surface glycoconjugates to inhibit lysosome degradation. By this model, one or more substrates delivered by type IV secretion is essential for *L. pneumophila* to sustain its interactions with the secretory pathway (19, 42, 72) and to inhibit acquisition of LAMP-1 (39), whereas developmentally regulated LPS species inhibit fusion with degradative lysosomes.

To date, direct tests of the contributions of particular LPS modifications to *L. pneumophila* pathogenesis have yielded negative results. Virulence, as judged by serum resistance and by replication in cultured monocytes or amoebae, is not affected by null mutations in five different LPS biosynthetic genes, including the *lag-1 O*-acetyltransferase (47, 49), and four contiguous genes of a large LPS biosynthetic operon that are predicted to encode methyltransferases and a sialic acid biosynthetic enzyme (45, 52). An LPS modification has been correlated to the virulence of one peculiar strain, but its molecular mechanism is an enigma. Mutant 811, the only avirulent *L. pneumophila* serogroup 1 strain known to express an LPS structural variant, harbors an unstable 30-kb locus of phage origin that alternates between an episomal and chromosomal state (46, 47). The status of the 30-kb element determines not only the LPS structure and the capacity to replicate in cultured macrophages and in guinea pigs but also other bacterial traits, including surface charge and motility. However, since neither the large mobile element nor its site of insertion contains LPS biosynthetic genes, its effect on other traits is indirect; most likely the phage element affects a global regulator of *L. pneumophila* differentiation (50).

It has long been appreciated that *L. pneumophila* serogroup 1 organisms are the most common cause of Legionnaires' disease (29), but how LPS composition affects the incidence of human disease is not known. Among serogroup 1 clinical isolates, 67% express an epitope recognized by MAb 3/1 (29). This MAby binds LPS that has been acetylated by the Lag-1 *O*-acetyltransferase, an enzyme encoded on an unstable genetic element that other serogroups lack (8, 31). The selective pressures exerted during transmission of *L. pneumophila* from water sources to the human lung that account for the prevalence of the MAb 3/1 epitope within clinical isolates have not been identified. The MAb 3/1 epitope did not correlate with the capacity to stall phagosome maturation, since inhibitory vesicles were abundant in PE-phase culture supernatants, but the epitope was not (Fig. 3). Furthermore, *L. pneumophila* cells that lack the MAb 3/1 epitope infect macrophages as efficiently as those that express this LPS motif (31, 49). Instead, the MAb 3/1 epitope may contribute to transmissibility in aerosols or to survival within human lungs.

The *L. pneumophila* genome encodes numerous enzymes predicted to assemble or modify LPS, including several acetylases and deacetylases (12, 13, 52). Biochemical studies indicate that the predominant modification to *L. pneumophila* LPS is acetylation of its O antigen, a homopolymer of legionaminic acid (80). Furthermore, the acetylases demonstrate substrate specificity, preferentially modifying LPS species of a particular size range (47). Compared to replicative-phase cells, transmissive-phase *L. pneumophila* express LPS species of higher molecular weight (Fig. 1), bind the lectin LPA more strongly (Fig. 2), and bind the hydrocarbon hexadecane less avidly (Fig. 2). Taking these observations into account, we speculate that, during the replicative phase, *L. pneumophila* increases the acetylation of its LPS to tolerate the harsh vacuolar compartments of amoebae and macrophages. In the transmissive phase, the pathogen deacetylates and elongates its LPS to forms that by some mechanism promote efficient inhibition of phagosome-lysosome fusion. Additional genetic and biochemical studies can test whether particular LPS or protein species associated with the outer membrane of *L. pneumophila* inhibit phagosome-lysosome fusion.

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